Genotypic diversity and rhizosphere competence of antibiotic-producing *Pseudomonas* species

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Σα βγεις στον πηγαιμὸ για την Ιθὰκη,
να εὐχεσαι νὰναι μακρὺς ο δρὸμος,
γεμὰτος περιπὲτειες, γεμὰτος γνὼσεις.

Πὰντα στον νου σου νὰχεις την Ιθὰκη.
Το φθὰσιμον εκεὶ εἰναι ο προορισμὸς σου.
Αλλὰ μη βιὰζεις το ταξεὶδι διὸλου.
Καλλὶτερα χρὸνια πολλὰ να διαρκὲσει
και γὲρος πια ν'αρὰξεις στο νησὶ,
πλοὺσιος με ὸσα κὲρδισες στον δρὸμο.

Κ. Π. Καβὰφης

Στους γονεὶς μου Γιὰννη και Χριστὶνα.
Στον αδερφὸ μου Τὰκη

As you begin your journey to Ithaca
may you wish a long way to go
with adventures, with wisdom

Keep always Ithaca in your mind
Getting there is your destination
But do not force this journey at all
For many years lasting is desired
and old may you come in rest in this island
rich with all you got on your way.

K. P. Kavafis

To my parents Giannis and Christina
To my brother Takis

Contents

Chapter 1	General introduction and thesis outline	9
Chapter 2	Assessment of the genotypic diversity of antibiotic-producing *Pseudomonas* species in the rhizosphere by denaturing gradient gel electrophoresis	45
Chapter 3	Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* species	75
Chapter 4	Rhizosphere competence of genotypically different antibiotic-producing *Pseudomonas* species	101
Chapter 5	Biological control of multiple sugar beet pathogens by genotypically different antibiotic-producing *Pseudomonas* species	131
Chapter 6	General and summarizing discussion	159
Summary		173
Samenvating		177
Περìληψη		181
Nawoord		187
Curriculum vitae		190
Education Statement of the graduate school Production Ecology and Resource Conservation (PE&RC)		191

Chapter 1

General introduction and thesis outline

General Introduction

Introduction

Members of the genus *Pseudomonas* are rod-shaped Gram-negative bacteria that are characterized by their metabolic versatility, aerobic respiration, one or several polar flagella and a high genomic G+C content (59-68%) (Haas and Défago, 2005). This genus is heterogeneous and harbours plant, animal and human pathogenic species, including *P. aeruginosa*, *P. plecoglossicida, P. tolaasii,* and *P. syringae*. *Pseudomonas* species can be found in diverse environments and are an important component of the plant-associated microflora (Rainey, 1999). Among the variety of *Pseudomonas* species inhabiting the rhizosphere, certain strains of fluorescent pseudomonads have received particular attention because of their potential to control seed- and soilborne pathogenic fungi and oomycetes (Raaijmakers and Weller, 2001; Keel et al., 1992, 1996). These biocontrol strains, mostly classified as *P. fluorescens* and *P. putida*, are easy to culture *in vitro* and to manipulate genetically (Whipps, 2001). Their beneficial effects on plant health have been mainly attributed to active exclusion of pathogens from the rhizosphere through the secretion of a diverse array of antimicrobial metabolites (Thomashow and Weller, 1996; Handelsman and Stabb, 1996; Raaijmakers et al., 2002; Haas and Keel, 2003). Important antimicrobial compounds for which a major contribution to biocontrol has been demonstrated are 2,4-diacetylphloroglucinol, pyoluteorin, phenazines, pyrrolnitrin, cyclic lipopeptides, and the volatile hydrogen cyanide (Raaijmakers et al. 2002; Haas and Défago, 2005; Raaijmakers et al. 2006; Loper et al., 2007). In general, several of the effective biocontrol strains described to date produce at least one of these diffusible or volatile antibiotics. Some strains, such as *P. fluorescens* CHA0 and Pf-5, produce multiple antibiotics with overlapping or different degrees of activity against plant pathogens (Paulsen et al., 2005; Bottiglieri and Keel, 2006). In spite of numerous studies showing promising biocontrol activity in different host-pathogen systems, relatively few *Pseudomonas* strains have made it to the market as a commercial biocontrol product. To be effective in biocontrol of plant pathogens, *Pseudomonas* and other microbial inoculants have to meet several important criteria, including: i. effective and competitive colonization of the host plant (e.g. rhizosphere, spermosphere), ii. stimulation of plant defence by induced systemic resistance (ISR) and/or systemic acquired resistance (SAR), iii. direct antagonistic effects on the pathogen e.g., by antibiosis or by inactivation of virulence factors of the pathogen, and iv. expression and/or production of the antagonistic traits needs to occur at the right time and place (Lugtenberg and Bloemberg, 2004). Combining all of these traits into a single strain or mixtures of strains is likely to produce a more consistent and effective level of plant protection (Haas and Keel, 2003). In this context,

efficient exploitation of these bacteria in agriculture and horticulture requires more fundamental knowledge of traits that enhance their ecological performance (Rainey, 1999).

Pseudomonas and phloroglucinol antibiotics

Phloroglucinols are phenolic compounds produced by bacteria and plants (Bangera and Thomashow, 1996, 1999; Thomashow et al., 1997; Singh and Bharate, 2006). In *Pseudom*onas, at least three different derivatives have been identified, i.e. monoacetylphloroglucinol (MAPG), 2,4-diacetylphloroglucinol (DAPG) and triacetylphloroglucinol (TAPG) (**Figure 1**) (Shanahan et al., 1993, Bangera and Thomashow, 1999; Schnider-Keel et al., 2000; Raaijmakers et al., 2002).

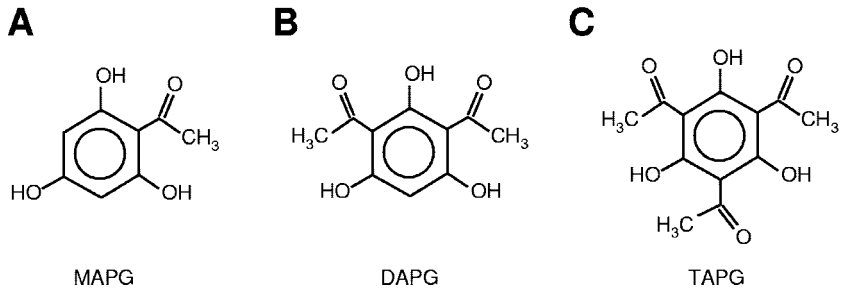

Figure 1: Structures of the three acylphloroglucinols produced by fluorescent pseudomonads. (A) Monoacetylphloroglucinol (MAPG), (B) 2,4-diacetylphloroglucinol (DAPG), (C) triacetylphloroglucinol (TAPG) (adapted from Bottiglieri and Keel, 2006).

Pseudomonas strains that produce DAPG also produce MAPG and, depending on the nutritional conditions, the ratio between MAPG and DAPG may change (Duffy and Defago, 1997). MAPG has been proposed to be the direct precursor and/or a degradation product of DAPG (**Figure 2**) (Bangera and Thomashow, 1999; Shanahan et al., 1993; Schnider-Keel et al., 2000). To date, DAPG has received most attention; it displays a remarkable activity spectrum as it inhibits the growth and/or activity of several bacteria, fungi, oomycetes, nematodes, and, at higher concentrations, also displays phytotoxic effects (Fuchs and Defago, 1991; Keel et al., 1992; Shanahan et al., 1992; Thomashow and Weller, 1995; Bangera and Thomashow, 1999; Isnansetyo et al., 2003; Isnansetyo and Kamei, 2003; de Souza et al., 2003; de la Fuente et al., 2004).

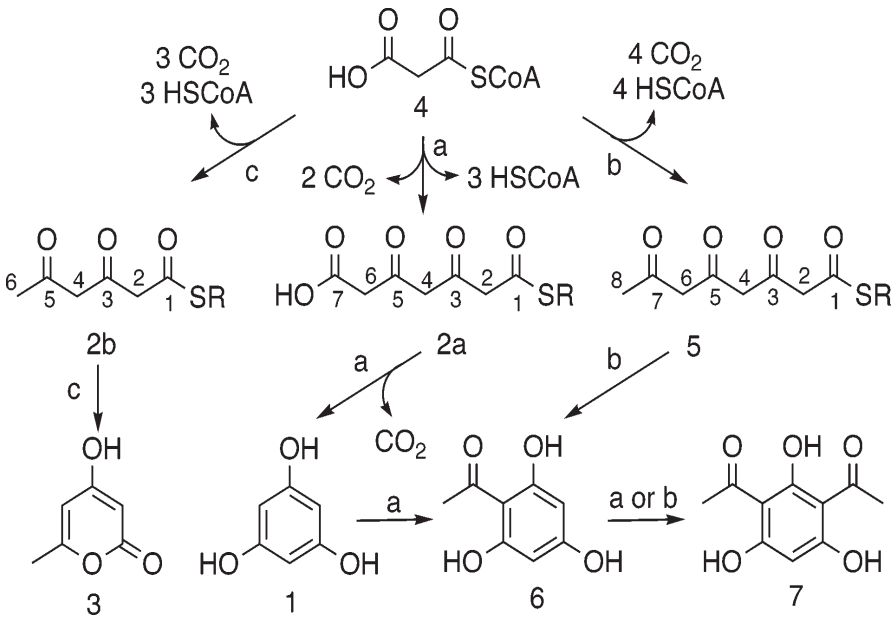

Figure 2: (a) Biosynthesis of acetylphloroglucinols **6** and **7** via phloroglucinol **1**. (b) Previously proposed biosynthesis of acetylphloroglucinols **6** and **7**. (c) Biosynthesis of triacetic acid lactone **3** (adapted from Achkar et al., 2005).

The differences in antimicrobial activity of the phloroglucinol derivatives are related to the level of acetylation: the higher the number of acetyl groups the greater the antimicrobial activity of phloroglucinol derivatives (de Souza et al., 2003). Given that in several *Pseudomonas* strains, the amounts and the ratio between MAPG and DAPG may vary (Duffy and Defago, 1999), one may postulate that also the biocontrol activity will change.

Although the effects of DAPG on pathogens are well documented, its mode of action remains unclear. Yoneyama et al. (1990) demonstrated that phloroglucinol derivatives, similar to phenol and quinone derivatives, can potentially inhibit photosystem II in plants. In experiments with the oomycete *Pythium,* de Souza et al. (2003) demonstrated that DAPG does not affect the synthesis and composition of its cell wall, but causes different stages of disorganization in hyphal tips including alteration (proliferation, retraction and disruption) of the plasma membrane, vacuolisation and cell content disintegration. Typical ultrastructural changes observed in fungi exposed to other phenolic fungicides such as quintozene, chloroneb and phenylphenol, include lysis of the inner mitochondrial membranes, vacuolisation of the nuclear envelop and increase in cell wall thickness (Lyr, 1995). Based on the structural similarities between DAPG and several herbicides, including members of

the phenol and quinine derivatives (Cohen et al., 1986), it has been suggested that DAPG may act as an inducer of defence mechanisms of plants against pathogens. Studies by Iavicoli et al. (2003) indeed showed a role of DAPG in induced systemic resistance (ISR): root inoculation of *Arabidopsis thaliana* with *P. fluorescens* strain CHA0 partially protected the leaves from infection by *Peronospora parasitica*; they proposed that DAPG produced by CHA0 leads to physiological changes in the roots that subsequently induce ISR. Last but not least, DAPG significantly enhanced the net efflux (i.e. exudation) of amino acids from roots of four plant species (alfalfa, maize, wheat and medicago) (Philips et al. 2004). Production of DAPG in the rhizosphere was considered to be a mechanism for manipulation of root exudation. In alfalfa, treating roots with 200μM DAPG increased the total net efflux of 16 amino acids by 1.600% in three hours.

DAPG and biocontrol

The role of DAPG in biological control of plant pathogens by *Pseudomonas* strains was evidenced in a series of experiments where (i) wild-type strains provided better control than DAPG-deficient mutant derivatives (Keel et al., 1992; Raaijmakers et al. 1998; de Souza et al., 2003), (ii) genetic complementation of DAPG-deficient mutants resulted in restoration of biocontrol activity (Vincent et al., 1991; Cronin et al., 1997), and (iii) expression of DAPG biosynthesis genes in heterologous, nonproducing strains resulted in enhanced biocontrol activity (Bangera and Thomashow, 1996). Indirect evidence for the implication of DAPG in biocontrol was obtained in studies with natural disease-suppressive soils, where DAPG-producing *Pseudomonas* populations were highly enriched compared to the disease-conducive counterpart (Raaijmakers et al. 1997; Raaijmakers and Weller, 1998; Weller et al., 2002). Since many *Pseudomonas* strains produce several other antibiotic compounds, the relative importance of DAPG in biocontrol is difficult to establish, because elimination of one biocontrol trait can modify the expression of other biocontrol traits (Haas and Keel, 2003; Baehler et al., 2006; Loper et al., 2007). More recently, Rezzonico et al. (2007) showed that the ability of 230 *Pseudomonas* strains (obtained from 63 soils world-wide) to produce DAPG and/or HCN was associated with a higher level of plant protection. Statistical analysis indicated that the ability to produce DAPG was associated with superior biocontrol activity in two pathosystems (i.e. *Pythium*-cucumber and *Fusarium*-tomato). Although this was also the case with the ability to produce HCN, this latter trait was found to have a less significant role than DAPG.

Biosynthesis and regulation of DAPG

DAPG is produced until the early stationary growth phase (Abbas et al., 2004; Baehler et al., 2005; Brodhagen et al., 2004; Pechy-Tarr et al., 2005; Schnider-Keel et al., 2000). Thereafter, it appears to be degraded by the producing bacterium, with MAPG temporarily accumulating as an intermediate product of the degradation process (Schnider-Keel et al., 2000). The genes required for the biosynthesis of DAPG have been identified in several pseudomonads, including *P. fluorescens* strains Q2-87, F113, CHA0 and Pf-5 (Bangera and Thomashow, 1999; Fenton et al., 1992; Delany et al., 2000; Schnider-Keel et al., 2000; Paulsen et al., 2005). The DAPG-biosynthetic locus includes the four biosynthetic genes *phl*ACBD (**Figure 3**) that are transcribed as a single operon and are required for the biosynthesis of both MAPG and DAPG (Bangera and Thomashow, 1999; Delany et al., 2000; Schnider-Keel et al., 2000; Mavrodi et al., 2001).

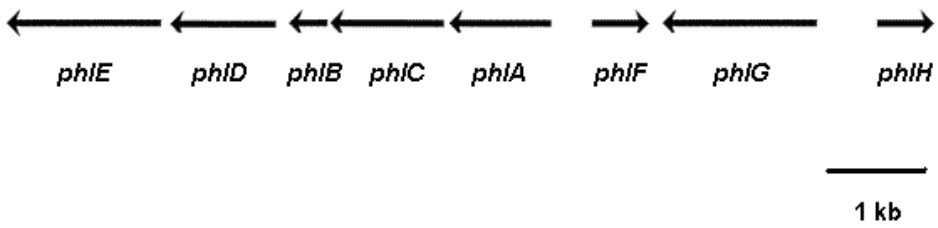

Figure 3: Organization of the 2,4-diacetylphloroglucinol (DAPG) gene cluster in *P. fluorescens* CHA0. Arrows indicate direction of transcription (adapted from Bottiglieri and Keel, 2006; Schnider-Keel et al., 2000; Bangera and Thomashow, 1996, 1999).

The products of the *phl*ACB genes also mediate the conversion of MAPG into DAPG (Bangera and Thomashow, 1999). *Phl*D encodes a polyketide synthase (PKS) with similarity to plant genes encoding chalcone or stilbene synthases (Bangera and Thomashow, 1999). PhlD plays an essential role in the two proposed routes for the biosynthesis of the DAPG: biosynthesis of DAPG from glucose (Achkar et al., 2005) and synthesis of MAPG (Bangera and Thomashow, 1999). Zha et al. (2006) demonstrated that PhlD belongs to the type III PKS family and exhibits a relatively broad substrate specificity compared to other members of this family. In addition to its ability to produce phloroglucinol it can produce a great diversity of products thereby expanding the existing reservoir of polyketides. The *phl*E gene located immediately downstream of the *phl*ACBD operon encodes a putative transmembrane permease (Bangera and Thomashow, 1999) which appears to be involved in DAPG resistance (Abbas et al., 2004). The divergently transcribed *phl*F gene is located adjacent to *phlA* gene and encodes a pathway-specific transcriptional repressor of the

DAPG biosynthetic operon (Bangera and Thomashow, 1999; Delany et al., 2000; Schnider-Keel et al., 2000). DAPG acts as the signal that dissociates PhlF from the *phl*A promoter, thereby auto-inducing its biosynthesis (Abbas et al., 2002; Haas and Keel, 2003; Schnider-Keel et al., 2000). The *phl*H gene is located, at least in *P. fluorescens* strain CHA0, downstream of *phl*F and is involved in pathway-specific control of DAPG biosynthesis. However, the precise role of PhlH remains to be elucidated. Also, the *phl*G gene was identified as an additional gene in the DAPG biosynthetic locus of *P. fluorescens* strain CHA0 (Schnider-Keel et al., 2000). The *phl*G gene has a size of 924-bp and is located between *phl*F and *phl*H (**Figure 3**). It has been demonstrated that *phl*G encodes a hydrolase (PhlG) that catalyzes the conversion of DAPG into MAPG and acetate. The enzymatic activity of PhlG appears to be highly specific for its substrate DAPG (Bottiglieri and Keel, 2006). The available genetic data from other DAPG-producing pseudomonads (Pf-5, Q2-87, F113) indicated that the *phl*G gene is not unique to strain CHA0, but appears to be commonly associated with the DAPG biosynthetic locus. At physiological concentrations, the antibiotic pyoluteorin was shown to have a negative effect on PhlG activity, reducing it by more than 50%. Expression of the *phl*G gene is negatively controlled by the pathway-specific regulators PhlF and PhlH and positively affected by the two-component regulatory system GacS/GacA (Bottiglieri and Keel, 2006).

The production of DAPG is subject to complex regulation. Besides the pathway-specific regulators PhlF and PhlH, a number of regulatory elements may directly or indirectly influence DAPG biosynthesis in response to environmental signals and to the physiological condition of the bacterial cell (Haas and Keel, 2003). In summary, regulation of DAPG production *in vitro* involves:

i. Transcriptional regulators, which are mostly pathway-specific, also called the sigma factors: the relative concentration of the sigma factors in the bacterial cell controls the production of DAPG in *P. fluorescens* (Bender et al., 1999; Schnider-Keel et al., 2000). In general, *Pseudomonas* has more than 20 sigma factors (Pechy-Tarr et al., 2005; Stover et al., 2000), including RpoD (sigma^{70}) for housekeeping, RpoS (sigma^{s} or sigma^{38}) for stress and stationary phase (Stockwell and Loper, 2005; Heeb et al., 2005), RpoN (sigma^{54}) for osmotic stress (Pechy-Tarr et al., 2005), RpoH (sigma^{32}) for heat stress, and RpoE (AlgU or sigma^{22}), a key determinant of extracellular polysaccharide production that is required for optimal survival of *P. fluorescens* CHA0 when exposed to desiccation and osmotic stress (Schnider-Keel et al., 2001).
ii. The GacS/GacA two-component system, which globally exerts a positive effect on the production of extracellular metabolites at a post-transcriptional level and is activated by

signal molecules which are produced by the bacterium at high cell population densities (Blumer et al., 1999; Kay et al., 2005). The chemical structure of the signal is not known, but it is not related to the structure of well-studied bacterial signals such as N-acyl-homoserine lactones (Heeb et al., 2002; Zuber et al., 2003). DAPG is produced only when the GacS/GacA two-component system is active in the various producer strains (Heeb and Haas, 2001). The deduced translational product of GacA is similar to response regulators in the FixJ family and GacS functions as the cognate sensor kinase (Bender et al., 1999). Small untranslated RNAs and RNA-binding proteins have important roles in the GacS/GacA signal transduction pathway and in DAPG production (Haas and Keel, 2003; Haas and Defago, 2005; Zuber et al., 2003). In strain CHA0, three small regulatory RNAs designated RsmX, RsmY and RsmZ are expressed under positive GacS/GacA control (Kay et al., 2005; Valverde et al., 2003).

iii. The two proteins MvaT and MvaV were recently established as novel global regulators of exoproduct formation, motility, and biocontrol activity in plant-associated *P. fluorescens* CHA0 (Baehler et al., 2006). The two proteins are members of an emerging family of MvaT-like regulators in pseudomonads that are structurally and functionally related to the DNA-binding protein H-NS. In *mvaT* and *mvaV* mutants of strain CHA0, DAPG production remained at wild-type levels, whereas DAPG biosynthesis was almost completely repressed in an *mvaT mvaV* double mutant (Baehler et al., 2006). Compared with the wild type, *mvaT* and *mvaV* mutants had reduced capacity to protect cucumber from root rot caused by *Pythium ultimum*. Biocontrol activity was nearly completely abolished in the double mutant. It was postulated that MvaT and MvaV act synergistically as higher level global regulatory elements in the complex network controlling expression of biocontrol traits in plant-beneficial pseudomonads.

iv. Quorum sensing (QS): the production of *N*-acyl homoserine lactone (AHL) signal molecules that, at a threshold concentration, bind to and activate a transcriptional regulator which then stimulates gene expression (Fuqua et al., 1994; Fuqua and Greenberg, 1998; Bloemberg and Lugtenberg, 2001; Parsek and Greenberg, 2005; Schuster and Greenberg, 2006). Examples of such signal synthases are CsaI-CsaR and PpuI-PpuR, both members of the LuxI-LuxR family, in the biocontrol strain *P. aureofaciens* 30-84 and in the bioremediation strain *P. putida* IsoF, respectively (Zhang and Pierson, 2001; Steidle et al., 2002). So far, however, QS has not been described in *P. fluorescens* strains CHA0 or Q2-87, in which DAPG is a key factor in biocontrol against plant diseases (Brodhagen et al., 2004; Maurhofer et al., 2004). In *P. fluorescens* CHA0, a QS-like behaviour reflecting the function of the GacS/GacA

system is positively autoregulated by CHA0 signals in densely grown cultures (Kay et al., 2005). The function of this QS circuit requires thiamine in *P. fluorescens* (Dubuis et al., 2006). In the biocontrol strain *P.fluorescens* F113, AHL signals are produced but the signal synthase, HdtS, is not a member of the LuxI-LuxR family and the phenotype regulated by this QS system is unknown (Laue et al., 2000). More recently, the QS system PcoI-PcoR in biocontrol strain *P. fluorescens* 2P24 was identified (Wei and Zhang, 2006). Their data indicated that QS is involved in regulation of biocontrol activity in this strain. However, it did not detectably affect the production of several metabolites, including DAPG and HCN that are important for its biocontrol capacity. Although the production of DAPG occurs in a population-dependent manner, it does not seem to be under the control of the classic QS regulation based upon N-acyl homoserine lactones (Loper et al., 2007), but perhaps is regulated by QS systems distinct from the classical LuxI-LuxR family.

Effects of abiotic and biotic factors on DAPG production

Microorganisms growing optimally in nutrient-rich media generally use their metabolic energy to synthesize cellular components and to grow. However, when growth is restricted, either because of nutrient limitation or at high cell density, *Pseudomonas* produces secondary metabolites, presumably as a strategy to remain competitive in their environment. When a single-cell organism such as *Pseudomonas* is grown in batch culture, production of DAPG is typically observed at the end of the exponential growth phase during the transition to the stationary phase (Haas and Keel, 2003). Whereas in well-shaken liquid media the bacteria experience a uniform environment, there is a dynamic but more structured environment in the rhizosphere with a heterogeneous spatial distribution of nutrients. Therefore, it is technically difficult to obtain a detailed picture of the exact location of individual nutrients in the rhizosphere and how biocontrol strains respond to the available nutrients. Nutrient content, oxygen tension, osmotic conditions, phosphate, carbon and nitrogen sources as well as fungal, bacterial and plant metabolites can all differentially influence the production of DAPG by *Pseudomonas*.

DAPG production can be modulated by a diverse array of abiotic and biotic factors, including carbon and nitrogen sources, metal ions and other minerals, and metabolites released by bacteria, fungi and plants (Duffy and Défago, 1999; Maurhofer et al., 2004; Notz et al., 2001). The impact environmental factors have on the production of DAPG has been studied both *in vitro* and *in situ* for a number of *Pseudomonas* strains (Duffy and Defago, 1999; Notz et al., 2001; Shanahan et al., 1992). These studies clearly showed that there is

high variation in antibiotic production among strains in response to different environmental conditions. For example, DAPG production was repressed in strain F113 by glucose, whereas in other strains of *P. fluorescens* glucose promoted DAPG production (Nowak-Thompson et al., 1994). Other carbon sources that promote DAPG production in *P. fluorescens* F113 were sucrose, fructose and mannitol, whereas sorbose repressed DAPG production (Shanahan et al., 1992). In a recent study (De La Fuente et al., 2007), the utilization of three carbon sources (trehalose, benzoate and valerate) by a collection of 55 DAPG-producing *P. fluorescens* strains was tested. Of the strains tested, 73%, 48% and 69% were able to utilize trehalose, benzoate and valerate as sole carbon sources, respectively. However, no correlation was found between a strains ability to utilize these carbon sources and superior rhizosphere competence on wheat and pea.

Among the biotic factors, the resident microflora (Raaijmakers et al., 1999) as well as the plant can strongly influence the expression of antibiotic biosynthetic genes in pseudomonads. It has been well demonstrated that *phlA* in strain CHA0 is expressed more strongly on maize and wheat roots than on bean and cucumber roots (Notz et al., 2001). This differential regulation is likely caused by differences in exudate composition between monocots and dicots, although the biochemical differences in the exudate constituents have not been elucidated yet. At a transcriptional level, DAPG positively controls the expression of its own biosynthesis genes via the pathway-specific regulator PhlF (Abbas et al. 2002; Baehler et al. 2005; Brodhagen et al. 2004; Maurhofer et al. 2004; Schnider-Keel et al. 2000). Remarkably, pyoluteorin strongly represses biosynthesis of DAPG in strains CHA0 and Pf-5 and vice versa, pointing to a mechanism of mutual feedback control that may help *P. fluorescens* to keep production of these compounds at balance. As exogenous signals, DAPG and pyoluteorin mediate both intra- and interpopulation communication. In the genetically distinct biocontrol strains CHA0 and Q2-87, DAPG produced by either strain on wheat roots is perceived as a positive signal boosting *in situ* expression of *phl* genes in the other strain (Maurhofer et al. 2004; Dubuis et al., 2007). The bacterial and plant metabolite salicylate as well as the fungal virulence factor fusaric acid inhibit DAPG production (Baehler et al., 2005; Schnider-Keel et al., 2000).

Detection and distribution of DAPG-producing *Pseudomonas*

A range of methods have been used to detect and characterize DAPG-producing *Pseudomonas* strains, including Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Keel et al. 1996; Mc Spadden et al., 2000; Picard et al. 2000), Random Amplified Polymorphic DNA (RAPD) analysis (Raaijmakers and Weller, 2001; Mavrodi et al. 2001),

whole-cell repetitive sequence-based PCR (rep-PCR) (McSpadden et al., 2000; Landa et al., 2002, 2005), restriction fragment length polymorphism (RFLP) analysis of the *phlD* gene (Mavrodi et al., 2001; McSpadden et al., 2001; Ramette et al., 2001; Wang et al., 2001; Landa et al., 2002, 2005), sequence analysis of *phlD* (Ramette et al., 2001; Mazzola et al., 2004) and BOX-PCR (McSpadden et al., 2001a). A notable difficulty of almost all of these methods is the requirement of isolation and cultivation of *phl*D$^+$ pseudomonads from soil and rhizosphere environments prior to their genotypic characterization. Isolation of *phl*D$^+$ pseudomonads can be achieved by plating on semi-selective media followed by colony hybridization (Raaijmakers et al., 1997), a time consuming method. Alternatively, direct characterization of *phl*D$^+$ *Pseudomonas* isolates in rhizosphere samples can be performed by a rapid PCR assay (McSpadden et al., 2001b). However, also the latter method requires cultivation of the rhizosphere sample in semi-selective nutrient broth prior to characterization of the *phlD*$^+$ genotype and may be biased toward detecting the most predominant genotype. More recently, the denaturing gradient gel electrophoresis (DGGE) methodology (Bergsma-Vlami et al., 2005a) **(chapter 2**) and the allele-specific primer-based technique (De la Fuente et al., 2006a) were developed and shown to be rapid and highly specific cultivation-independent methods for studies on DAPG-producers and on the interactions between genotypes of DAPG-producing *P. fluorescens* in natural environments. The most interesting application of both methods is the detection and quantification of two or more strains of different genotypes of DAPG producers competing in soil or on roots. In the same context, a real-time PCR assay was developed to quantify populations of DAPG-producing strains of *P.fluorescens* in soil and rhizosphere (Mavrodi et al., 2007). The detection limits of the optimized real-time PCR assay were 8 to 80 CFU for genomic DNA isolated from pure cultures of *P. fluorescens* and 80 to 800 CFU for bacterial DNA extracted from plant rhizosphere, corresponding to 10^4 and 10^5 CFU g^{-1} root. The greatest advantage of the developed real-time PCR is culture independence, which allows determination of population densities and the genotype composition of DAPG producers directly from the plant rhizospheres and soil.

Using one or a combination of these techniques indicated that diverse populations of DAPG-producing *P. fluorescens* inhabit diverse environments (Thomashow and Weller, 1995; Keel et al., 1996; McSpadden et al., 2000; Lee and Kim, 2001; Weller et al., 2002; Isnansetyo et al., 2003; De la Fuente et al., 2004). DAPG-producers were shown to be predominant constituents of the rhizosphere of wheat plants grown in soils that are naturally suppressive to take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Raaijmakers et al., 1997; McSpadden et al. 2000; Weller et al., 2002; Souza et al. 2003a).

They also have been isolated from soils that are naturally suppressive to black root rot of tobacco caused by *Thielaviopsis basicola* (Stutz et al., 1986; Keel et al. 1996; Ramette et al. 2003) or soil suppressive to pea wilt disease caused by *Fusarium oxysporum* (Landa et al. 2003). In a recent study, a collection of DAPG- and HCN-producing *Pseudomonas* isolates from Swiss soils suppressive or conducive to black root rot of tobacco were used to assess whether suppressiveness could be linked to soil-specific properties of individual pseudomonads (Ramette et al., 2006). The isolates were compared based on restriction analysis of the genes *phl*D and *hcn*BC, enterobacterial repetitive intergenic consensus (ERIC)-PCR profiling and their biocontrol ability. Restriction analyses of *phl*D and *hcn*BC indicated significant population differentiation occurring at the soil level, regardless of the level of soil suppressiveness. Additionally, no correlation was found between the origin of the isolates and their biocontrol ability *in vitro* and *in planta*. Thus, DAPG- and HCN-producing *Pseudomonas* isolates from suppressive soils were not different from those of nearby conducive soils (Ramette et al., 2006). It was therefore hypothesized that the suppressiveness of Swiss soils may rely on the differential effects of environmental factors on the expression of key biocontrol genes in pseudomonads rather than on differences in population structure of biocontrol Pseudomonas subcommunities or the biocontrol potential of individual DAPG- and HCN-producing *Pseudomonas* isolates.

Genotypic diversity of DAPG-producing *Pseudomonas*

The genotypic and phenotypic diversity that occurs in natural populations of biocontrol agents provides an enormous resource for improving biological control of plant diseases by identifying strains that are highly rhizosphere competent and superior in biocontrol (Handelsman and Stabb, 1996; Thomashow and Weller, 1996; Sharifi-Tehrani et al. 1998; Raaijmakers and Weller, 2001). This approach has been widely used to select for better biocontrol agents of insects and to improve the use of microorganisms in production of fermented foods and biodegradation of xenobiotic compounds, but not extensively for biocontrol agents of plant pathogens (reviewed in Stabb et al., 1994).

Different genotypes of DAPG-producing *Pseudomonas* spp. have been reported to differ in their ability to: (i) colonize the wheat rhizosphere and suppress take-all disease of wheat (de Souza et al., 2003, Raaijmakers and Weller 2001), (ii) colonize the rhizosphere of pea (Landa et al., 2002), (iii) colonize roots of two maize genotypes and their hybrids (Picard et al., 2008, 2004, 2000, Picard and Bosco, 2003, 2006), (iv) suppress Fusarium crown and root rot, and Pythium root rot (Sharifi-Tehrani et al. 1998), (v) utilize different carbon sources (Wang et al., 2001; de la Fuente et al., 2007) and catalyze the degradation of 1-

aminocyclopropane-1-carboxylate (Wang et al., 2001), and (vi) produce other antibiotics in addition to DAPG (Keel et al., 1996). To date, several studies have supported the hypothesis that certain indigenous *phl*D$^+$ genotypes preferentially colonize the roots of specific crop plants. In the first study, 16 genotypic groups were identified among 101 indigenous DAPG-producing isolates obtained from the rhizosphere of wheat grown in a take-all suppressive soil (Raaijmakers and Weller, 2001): one genotypic group, classified as BOX-PCR genotype D, explained almost 50% of the diversity and was shown to be highly rhizosphere-competent on wheat (McSpadden et al., 2000; Raaijmakers and Weller, 2001). In the second study, Landa et al. (2002) identified 17 whole-cell BOX-PCR groups among more than 300 DAPG-producing isolates obtained from the rhizosphere of pea; BOX-PCR genotypes D and P explained 47.3% and 48.0%, respectively, of the diversity and were significantly better colonizers of the pea rhizosphere than isolates representing other genotypes. Given the fact that rhizosphere competence is an essential prerequisite for successful biocontrol (Bull et al., 1991; Raaijmakers et al., 1995; Johnson, 1994; Raaijmakers and Weller, 1998; Lugtenberg et al., 2001), knowledge of the compatibility between host plant species and genotypes of antagonistic microorganisms is essential to further improve biological control.

Based on whole-cell repetitive sequence-based PCR (rep-PCR), 18 genotypes (A-Q and T) could be distinguished by BOX-PCR of a worldwide collection of over 200 *phl*D$^+$ isolates (McSpadden et al., 2000; Landa et al., 2002, 2005), and these correlated closely with groupings revealed by RFLP analysis of the *phlD* gene (Mavrodi et al., 2001; McSpadden et al., 2001b; Landa et al., 2005). Additional genotypes have been described recently by Mazzola et al. (2004) (genotypes PfY and PfZ), Bergsma-Vlami et al. (2005a) (genotypes PspC, PspD, PspF and PspZ) and by McSpadden et al. (2005) (genotypes R and S). Most genotypes distinguished by BOX-PCR produce similar quantities of DAPG *in vitro* (Mavrodi et al., 2001) and *in situ* (Raaijmakers and Weller, 2001), have similar substrate utilization profiles and do not considerably differ when compared by classical bacteriological tests (McSpadden et al., 2000; Raaijmakers and Wellller, 2001). However, they do 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 disease (Landa et al., 2003; Raaijmakers and Weller, 2001). Our current knowledge of the taxonomy, evolution and population structure of the DAPG-producing *Pseudomonas* spp. bacteria is mostly based on single-locus data (Ramette et al., 2001; Ramette et al., 2003; Rezzonico et al., 2004). The genetic and evolutionary relationship among DAPG-producing pseudomonads was recently investigated

in more detail by multilocus sequence typing (MLST) (Frapolli et al., 2007): a total of 65 pseudomonads consisting of 58 DAPG positive biocontrol strains of worldwide origin and seven DAPG negative representatives of *Pseudomonas* species were compared using 10 housekeeping genes (i.e. *rrs*, *dsbA*, *gyrB*, *rpoD*, *fdxA*, *recA*, *rpoB*, *fusA*, *rpsL* and *rpsG*). MLST differentiated 51 strains among 58 DAPG positive pseudomonads and was shown to be as discriminative as enterobacterial repetitive intergenic consensus (ERIC) PCR profiling. The topology derived from the phyllogenetic trees led to the identification of six main groups of DAPG-producing *Pseudomonas* spp., which taxonomically could correspond to at least six different species. The usefulness of this approach can be assessed at several levels, i.e. in terms of (i) strain discrimination, (ii) phylogenetic analysis and definition of homogenous strain clusters, (iii) taxonomy and clonality analysis (Frapolli et al., 2007).

Plant effect on DAPG-producing *Pseudomonas*

The effects of plant species on microbial communities (Azad et al., 1985; Miller et al., 1989; Caetano-Anolles and Gresshoff, 1991; Grayston et al., 1998; Smith and Goodman, 1999; Smith et al., 1999; Miethling et al., 2000; Spaink, 2000; Wieland et al., 2001; Kuske et al., 2002; Marschner et al., 2004) and on populations of specific bacterial genera have been well documented (Larkin et al., 1993; Lemanceau et al., 1995; Di Cello et al., 1997; Berg et al., 2002; Briones et al., 2002; Mazzola and Gu, 2002; Gu and Mazzola, 2003). For example, the composition of populations of fluorescent *Pseudomonas* spp. may vary significantly within the rhizosphere of different plant species cultivated in the same soil (Lemanceau et al., 1995), and may change dramatically over time in response to age-related physiological changes in plant species (Mazzola, 1999; Mazzola and Gu, 2000). In the same context, plant species variation was shown to significantly influence the community structure of *Pseudomonas* in the rhizosphere of two *Verticillium dahliae* host plants (Costa et al., 2006). In contrast, relatively few studies have addressed the effect of plant species on the dynamics, composition and activity of indigenous bacterial populations that share a specific antagonistic trait, such as the DAPG-producing *Pseudomonas* spp.

Traits of both the plant and the bacteria undoubtedly contribute to the affinity between some bacterial genotypes and certain host plant species (Landa et al., 2003). Identifying different genetic traits that have evolved in microorganisms to compete successfully in diverse rhizosphere environments may allow maximizing root colonization and disease suppression (Weller et al., 2002). Knowledge of such genetic traits involved in host preference of the antagonistic bacteria will help to identify strains that are adequately adapted to specific host-pathogen systems. Thus, it is not surprising that plant species and

cultivars differ in their response to rhizosphere colonization (Landa et al., 2002, 2003; Mazzola et al., 2004; Okubara et al., 2003) and disease suppression by introduced and indigenous rhizobacteria (Maurhoffer et al., 1995). Plants also initiate and maintain sophisticated mutualistic relationships with *phlD*$^+$ isolates (Landa et al., 2005), and certain *phlD*$^+$ genotypes have an affinity or preference for the roots of particular crops at the species and cultivar level (**chapter 3**) (Bergsma-Vlami et al., 2005b; Landa et al., 2002, 2003, 2005; Mazzola et al., 2004; Okubara et al., 2004). Isolates of at least four genotypes (B, D, E and L) occur in take-all decline (TAD) fields in Washington State (USA) (McSpadden et al., 2000; Raaijmakers and Weller, 2001), but D-genotype isolates are most abundant, owing to the exceptional ability of isolates of this genotype to colonize the rhizosphere of wheat and barley. In Dutch TAD fields, however, genotypes M and F are abundant (de Souza et al., 2003) but they are absent in Washington State TAD soils. Multiple genotypes of DAPG producers are also found on other field-grown crops (i.e. pea, flax, corn and soybean) and, again one or two genotypes dominate depending on the geographic location (de Souza et al., 2003; McSpadden et al., 2000; Raaijmakers and Weller, 2001) and the host plant (Landa et al., 2003, 2005; McSpadden et al., 2005).

In a recent study, the ability of nine crops (alfalfa, barley, bean, flax, lentil, lupine, oat, pea and wheat) to support indigenous populations of DAPG-producers was investigated (de la Fuente et al., 2006). DAPG-producers survive in bulk soil at densities below the detection level, but rapidly proliferate in the rhizosphere. Rhizosphere population densities differed among the various crops and cultivars, with lentil and oat supporting the highest and lowest densities of DAPG-producers, respectively. Additionally, in order to determine interactions among DAPG-producers in the rhizosphere, population dynamics of three genotypically different strains (Q8r1-96, F113 and MVP1-4) which were inoculated onto wheat or pea individually and in all possible combinations, were monitored. All three strains were similar in their ability to colonize the rhizosphere of wheat and pea when introduced alone, but when introduced together in equal densities, they differed according to the plant species. For example, in the wheat rhizosphere the population density of strain F113 was significantly higher than that of Q8r1-96 in the mix inoculation, but no significant differences were detected in the pea rhizosphere. In the same study, eight pea cultivars were grown in soil inoculated with strains Q8r1-96 or MVP1-4. The effect of the pea cultivar on rhizosphere colonization was dependent on the bacterial strain inoculated. Rhizosphere population densities of MVP!-4 did not differ significantly among pea cultivars, whereas population densities of Q8r1-96 did. Therefore, it was concluded that the host plant plays a key role in modulating both rhizosphere colonization by DAPG-producing *P. fluorescens* and the

interactions among different genotypes present in the same rhizosphere (De la Fuente et al., 2006). Two important questions still to be answered are why one genotype outcompetes another genotype on a specific crop and what mechanism(s) are involved. One possibility is that the niche overlap in the rhizosphere results in direct antagonism (Landa et al., 2002) involving, at least partly, the production of bacteriocins (Validov et al., 2005). A second possibility is that the composition and amount of the root exudates (Landa et al., 2005) or the physicochemical composition of the rhizosphere of a particular host favors the growth of one genotype over another. In a previous study, the superior root colonizer *P. fluorescens* Q8r1-96 (genotype D) was shown to utilize trehalose, benzoate and valerate as sole carbon sources whereas the average colonizers Q2-87 (genotype B) and 1M1-96 (genotype L) could not (Raaijmakers and Weller, 2001). In a recent study, however, no correlation was found between a strains ability to utilize these carbon sources and superior rhizosphere competence on wheat and pea. These results further suggest that in biocontrol studies, the failure of introduced rhizobacteria to establish and persist in the rhizosphere may result more from specific competition with indigenous, closely related strains with the same traits and overlapping niches than from competition with the total microflora.

Rhizosphere competence of DAPG-producing *Pseudomonas*

Rhizosphere competence is a dynamic process by which introduced bacterial inoculants make use of nutrients excreted by the seed and/or plant root, proliferate, efficiently colonize the root system, and survive over a considerable time period in the presence of indigenous microorganisms. Rhizosphere competence is a crucial element in beneficial plant-microbe interactions as (i) inadequate root colonization leads to decreased biocontrol activity (Schippers et al., 1987), and (ii) an inverse correlation exists between the population size of the biocontrol strain and the level of biocontrol (Bull et al., 1991; Johnson 1994; Raaijmakers et al., 1995, 1998). Root colonization has been the subject of extensive research during the past three decades mainly due to the fact that inconsistent colonization remains one of the major limitations to the widespread use of bacterial inoculants in agriculture. A major factor contributing to the inconsistent colonization by the bacterial inoculants remains their variable ecological performance (Weller, 1988). Numerous studies have been performed in order to identify traits and factors that contribute to successful establishment, spread and survival of bacterial inoculants in the rhizosphere. These include (i). biotic and abiotic soil factors (Howie et al., 1987; Notz et al., 2001), (ii). host genotype factors (Smith and Goodman, 1999; Weller and Thomashow, 1994), (ii) rhizosphere-induced (rhi) genes (Rainey 1999) and colonization genes (Lugtenberg et al., 2001).

Although strains of fluorescent *Pseudomonas* spp. that produce DAPG are phenotypically similar (McSpadden et al., 2000; Mavrodi et al., 2001), they differ considerably in their ability to colonize the rhizosphere of particular crop species (Landa et al., 2002, 2003; Raaijmakers and Weller, 2001). In particular, D-genotype isolates are highly aggressive colonizers of wheat and pea and have a stronger affinity for these crops as compared to most other genotypes. One approach to identifying genes that contribute to the exceptional rhizosphere competence of D-genotype strains was the application of genomic suppressive subtractive hybridization (SSH) (Mavrodi et al., 2002). DNA fragments present in the superior colonizer *P. fluorescens* Q8r1-96 but not in the less rhizosphere-competent strain Q2-87 were recovered, cloned, their sequences determined and analyzed, and their distribution among other DAPG-producing strains assessed. Several subtracted fragments were identified as candidates for further analysis. One of the open reading frames was similar to colicin M from *Escherichia coli*, three resembled known regulatory proteins, and 28 had no significant match with sequences of known function. More recently, in an effort to understand the molecular basis of the unique root-colonizing ability of D-genotype strain Q8r1-96, the role of other well-conserved genes common to saprophytic and pathogenic bacteria were investigated (Mavrodi et al., 2006b). The hypothesis was that such genes involved in active molecular dialogue between bacteria and their hosts might also provide insight into the unique affinity of D-genotype strains for their plant hosts. Among these genes is *dsbA*, which encodes a periplasmic protein that catalyses disulfide bond formation in exported proteins in Gram-negative bacteria. Among the effects of *dsbA* mutations are deficiencies in pathogenicity and competitiveness associated with loss of motility and the inability to produce fimbriae and secrete secondary metabolites. In this context, the *dsbA* orthologue in strain Q8r1-96 was characterized and evaluated for its contribution in root colonization and competitiveness in the wheat rhizosphere (Mavrodi et al., 2006b). It was shown that *dsbA* does not contribute to the exceptional rhizosphere competence of Q8r1-96.

Recent evidence of the presence of type III secretion genes in *P. fluorescens* strain SBW25 (Rainey, 1999; Preston et al., 2001) and the presence of related genes in many other antagonistic bacteria (Mazurier et al., 2004; Preston et al., 2001; Rezzonico et al., 2004) suggest that these conserved genes may also contribute to the unique rhizosphere competence of Q8r1-96 and related D-genotype strains (Mavrodi et al., 2006a) and may occur more frequently than was previously anticipated. In this context, the *ptsP* and *orfT* genes in Q8r1-96 were further investigated for their role in root colonization. The *ptsP* gene influence global processes, including organic nitrogen utilization, whereas the *orfT* gene contributes to the pathogenicity of *P. aeruginosa* in both plant and animal systems (Rahme

et al., 2000). Mutants of Q8r1-96 disrupted in either one of these two genes were characterized to determine their ability to colonize the rhizosphere of wheat grown in natural soil (Mavrodi et al., 2006a). The *ptsP* mutant was strongly impaired in wheat root colonization and its rhizosphere population densities were significant lower than those of the wild type, whereas the *orfT* mutant was not impaired. Collectively, these results suggest novel rhizosphere colonization functions for two genes, *ptsP* and *orfT*, that previously were linked with pathogenesis in *P. aeruginosa.*

Thesis outline

Pseudomonas species exert their beneficial effects on plant growth and health via several different mechanisms, including active exclusion of plant pathogens from the rhizosphere (Rainey, 1999). The phenolic antibiotic 2,4-diacetylphloroglucinol (DAPG) has been implicated in biological control of multiple plant pathogens by fluorescent *Pseudomonas* strains (Keel et al., 1990; Keel et al., 1992; Cronin et al., 1997a, b; Sharifi-Tehrani et al., 1998; Raaijmakers and Weller, 1998). Despite obvious benefits for agriculture, however, attempts to exploit DAPG-producing *Pseudomonas* strains as biocontrol inoculants have had limited success so far. Results obtained in field experiments are typically inconsistent and a major factor contributing to this inconsistency is their variable ecological performance (Weller, 1988). The ecological performance of DAPG-producing *Pseudomonas* strains is complex as it is affected by many different bacterial traits and a multitude of environmental factors. The **overall goal of this thesis project** was to unravel the genotypic and phenotypic characteristics underlying the ecological performance of DAPG-producing *Pseudomonas* strains in order to contribute to the improvement of their rhizosphere competence, survival and biocontrol efficacy.

The genotypic diversity among antibiotic-producing *Pseudomonas* species provides an enormous resource for identifying strains that are highly rhizosphere competent and superior in biological control of plant diseases. The aim in **chapter 2** was to develop a simple and rapid method to study the presence and genotypic diversity of indigenous DAPG-producing *Pseudomonas* strains directly in rhizosphere samples without their prior isolation or enrichment on nutrient media. New *phl*D-specific primers were developed and their specificity was tested on a range of different *phl*D$^+$ genotypes. Polymorphisms within the amplified 350-bp *phl*D fragments were assessed by Denaturing Gradient Gel Electrophoresis (DGGE) analysis, sequencing and phylogenetic analyses. The specificity and resolving capacity of the *phl*D-DGGE system was compared to currently used techniques, including *phl*D-RFLP (McSpadden et al. 2001a), RAPD analysis (Keel et al., 1996) and the rapid PCR assay (McSpadden et al. 2001b). Finally, the biological significance of this newly developed *phl*D-DGGE classification was tested in root colonization assays with sugar beet seedlings treated with eight genotypically different *phlD*$^+$ genotypes of *Pseudomonas*.

The aim of **chapter 3** was to study the influence of the host plant species on the population dynamics, genotypic diversity and activity of indigenous DAPG-producing *Pseudomonas* spp. Four different plant species were included in this study, i.e. wheat, sugar beet, potato and lily. Population dynamics of indigenous DAPG-producing *Pseudomonas*

spp. were monitored in the rhizosphere of the four plant species grown in two different agricultural soils. Secondly, the genotypic diversity of approximately 500 indigenous DAPG-producing *Pseudomonas* isolates associated with the roots of the four different plant species was determined by *phl*D-DGGE analysis, described in **chapter 2**. Finally, the effect of each of the four plant species on *in situ* DAPG production by the indigenous *phlD*$^+$ population was determined by High Pressure Liquid Chromatography (HPLC).

The objective of **chapter 4** was to determine the relationship between the genotype of a DAPG-producing *Pseudomonas* strain and its ability to colonize and survive in the plant rhizosphere. We hypothesized that particular genotypes (specialists), after being introduced into the plant rhizosphere, have evolved a preference for the colonization of specific crops, whereas others (generalists) can colonize multiple plant species equally well. In this way, the genotype can be considered predictive of the rhizosphere competence of DAPG-producing *Pseudomonas* spp. The population dynamics of the introduced DAPG-producing *Pseudomonas* strains was monitored in the rhizosphere of four plant species. Subsequently, we investigated the relation between the rhizosphere competence of a strain and the concentration of DAPG produced in the sugar beet rhizosphere.

In **chapter 5**, the biocontrol efficacy of genotypically different DAPG-producing *Pseudomonas* strains (isolated and identified in **chapters 3 and 4**) is tested against three major soil-borne pathogens of sugar beet, i.e. *Rhizoctonia solani*, *Pythium ultimum* and *Aphanomyces cochlioides*. The biocontrol efficacy was assessed in experiments performed under controlled greenhouse conditions and in field soil. The relationship between root colonization and biocontrol activity was investigated in detail.

The results obtained during the course of this PhD study are evaluated and placed in a broader perspective in the summarizing discussion in **chapter 6**. Special attention is given to the future perspectives of *Pseudomonas* strains as commercial biocontrol agents.

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Chapter 2

Assessment of the genotypic diversity of antibiotic-producing *Pseudomonas* species in the rhizosphere by denaturing gradient gel electrophoresis

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Assessment of the genotypic diversity of antibiotic-producing *Pseudomonas* species in the rhizosphere by denaturing gradient gel electrophoresis

M. Bergsma-Vlami, M.E. Prins, M. Staats, and Raaijmakers J.M.

Abstract

The genotypic diversity among antibiotic-producing *Pseudomonas* spp. provides an enormous resource for identifying strains that are highly rhizosphere competent and superior in biological control of plant diseases. In this study, a simple and rapid method was developed to determine the presence and genotypic diversity of 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas* strains in rhizosphere samples. Denaturing gradient gel electrophoresis (DGGE) of 350-bp fragments of *phlD*, a key gene involved in DAPG biosynthesis, allowed discrimination between genotypically different *phlD*$^+$ reference strains and indigenous isolates. DGGE analysis of the *phlD* fragments provided a higher level of discrimination between *phlD*$^+$ genotypes than obtained by currently used techniques and enabled detection of specific *phl*D$^+$ genotypes directly in rhizosphere samples with a detection limit of approximately 5x10^3 CFU/g root. DGGE also allowed simultaneous detection of multiple *phl*D$^+$ genotypes present in mixtures in rhizosphere samples. DGGE-analysis of 184 indigenous *phlD*$^+$ isolates obtained from the rhizosphere of wheat, sugar beet and potato plants, resulted in the identification of seven *phlD*$^+$ genotypes, five of which were not described previously based on sequence and phylogenetic analyses. Subsequent bioassays demonstrated that eight genotypically different *phlD*$^+$ genotypes differed substantially in their ability to colonize the rhizosphere of sugar beet seedlings. Collectively, these results demonstrated that DGGE analysis of the *phlD* gene allows for identification of new genotypic groups of specific antibiotic-producing *Pseudomonas* with a differential ability to colonize the rhizosphere of sugar beet seedlings.

Keywords: *Pseudomonas*, rhizosphere, *phl*D gene, DAPG, DGGE

Introduction

Antibiotic compounds produced by fluorescent *Pseudomonas* strains play key roles in the suppression of various soil-borne plant pathogens (Thomashow and Weller, 1996, Sharifi-Tehrani et al., 1998, Raaijmakers et al. 2002). 2,4-Diacetylphloroglucinol (DAPG) produced by *Pseudomonas fluorescens* has activity against a range of plant pathogens,

including bacteria, fungi and nematodes (reviewed in Raaijmakers et al. 2002). Recently, the broad-spectrum activity of DAPG also has drawn attention in the medical area due to its bacteriolytic activity against multidrug-resistant *Staphylococcus aureus* (Isnansetyo and Kamei 2003). DAPG-producing *Pseudomonas* spp. have been isolated from the rhizosphere of different crops grown in soils from diverse geographic regions (Keel et al., 1996), and are predominant constituents of the rhizosphere of wheat plants grown in soils that are naturally suppressive to take-all disease (Raaijmakers et al., 1997; McSpadden et al. 2000; Souza et al. 2003a). They also have been isolated from soils that are naturally suppressive to black root rot of tobacco (Keel et al. 1996; Ramette et al. 2003) or to Fusarium wilt disease (Landa et al. 2003).

Multiple genes are involved in biosynthesis and regulation of DAPG production in *Pseudomonas fluorescens* (reviewed in Haas and Keel, 2003). One of these genes, the polyketide synthase gene *phl*D, is essential for synthesis of the DAPG precursor monoacetylphloroglucinol (Bangera and Thomashow, 1999). It has been well documented that the *phl*D gene is conserved among DAPG-producing *Pseudomonas* strains of worldwide origin (Keel et al., 1996; Raaijmakers et al. 1997), but displays a certain degree of polymorphism (Mavrodi et al., 2001, Ramette et al., 2001; Landa et al. 2002). Given that the genotypic diversity among DAPG-producing *Pseudomonas* strains provides an enormous resource for identifying strains that are highly rhizosphere competent and superior in biological control of plant diseases (Sharifi-Tehrani et al. 1998; Raaijmakers and Weller, 2001), sequence heterogeneity of the *phl*D gene is now routinely used to assess the diversity of this group of antagonistic bacteria (McSpadden et al., 2001a, Mavrodi et al., 2001, Ramette et al., 2001; Wang et al., 2001; Landa et al. 2003). A range of other methods have been used to determine the genotypic diversity of DAPG-producing *Pseudomonas* strains, including Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Keel et al. 1996; Picard et al. 2000), Random Amplified Polymorphic DNA (RAPD) analysis (Raaijmakers and Weller, 2001; Mavrodi et al. 2001), and BOX-PCR (McSpadden et al., 2001a). A notable difficulty of all of these methods is the requirement of isolation and cultivation of *phl*D$^+$ pseudomonads from soil and rhizosphere environments prior to their genotypic characterization. Isolation of *phl*D$^+$ pseudomonads can be achieved by plating on semi-selective media followed by colony hybridization (Raaijmakers et al., 1997), a time consuming method. Alternatively, direct characterization of *phl*D$^+$ *Pseudomonas* isolates in rhizosphere samples can be performed by a rapid PCR assay (McSpadden et al., 2001b). However, also this latter method requires cultivation of the rhizosphere sample in semi-

selective nutrient broth prior to characterization of the *phlD*$^+$ genotype and may be biased toward detecting the most predominant genotype.

The aim of this work was to develop a simple and rapid method to study the presence and genotypic diversity of *phl*D$^+$ *Pseudomonas* strains directly in rhizosphere samples without their prior isolation or enrichment on nutrient media. New *phl*D specific primers were developed and their specificity was tested on a range of different *phl*D$^+$ genotypes, present alone and in mixtures. Polymorphisms within the amplified 350-bp *phl*D fragments were assessed by Denaturing Gradient Gel Electrophoresis (DGGE) analysis, sequencing and phylogenetic analysis. The specificity and resolving capacity of the PCR-DGGE system was compared to currently used techniques, including *phl*D-RFLP (McSpadden et al. 2001a), RAPD analysis (Keel et al., 1996) and the rapid PCR assay (McSpadden et al. 2001b). Finally, the biological significance of this newly developed PCR-DGGE classification was tested in root colonization assays with sugar beet seedlings treated with eight genotypically different *phlD*$^+$ genotypes of *Pseudomonas*.

Results and Discussion

Primer design and specificity

For detection of DAPG-producing *Pseudomonas* spp., a number of primers directed against sequences within the *phlD* gene have been developed previously (Raaijmakers et al. 1997; McSpadden et al. 2001b).

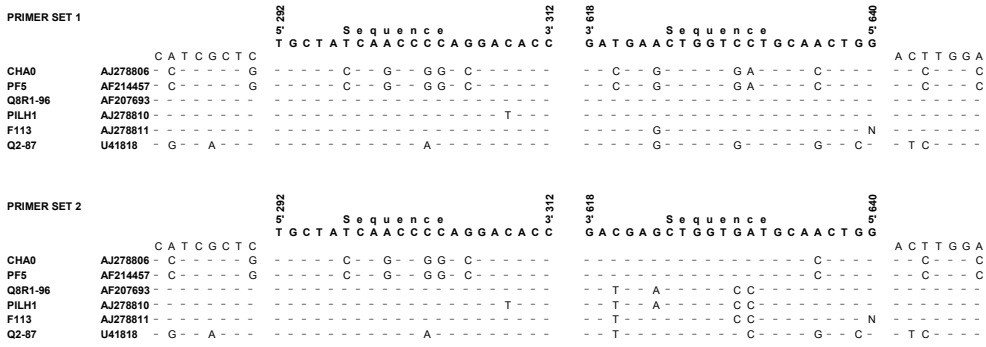

Figure 1: Comparison of partial *phl*D sequences from several *Pseudomonas* strains. Bases identical for all sequences are shown as dashes. The sequences and positioning of the two primer sets used in PCR-DGGE analysis are given at the top of each of the two alignments. The positions of the 5' and 3' ends of each of the primers correspond to the positions in the *phlD* sequence of PF-5 (AF214457).

The size of the amplification products of these primers ranges from approximately 600 to

750-bp, which is relatively large for further analysis by Denaturing Gradient Gel Electrophoresis (DGGE). Therefore, two new sets of oligonucleotide primers were developed against conserved sequences within the *phlD* gene of multiple reference strains (**Figure 1**). In both primer sets, the forward primer is identical (DGGE292for) whereas a 4-nucleotide difference exists between the two different reverse primers (DGGE618rev and 6DGGE618rev). Additionally, a 40-bp GC-clamp has been attached at the 5' end of the forward primer (**Table 2**).

Table 2: Properties of the oligonucleotide primers used in the PCR-DGGE analysis.

Primer	Sequence (5'-3')	G+C (%)	T_m (°C)
DGGE618rev	CCAGTTGCAGGACCAGTTCATC	55	67.9
6DGGE618rev	CCAGTTGCA**TC**ACCAG**C**TC**G**TC	59	67.9
DGGE292for	TGCTATCAACCCCAGGACACC	57	68.1
DGGE292forCG	CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGGC ACGGGGGGTGCTATCAACCCCAGGACACC	84	57.9

The first primer set resulted in amplification of fragments of the predicted size (approximately 350-bp) from DNA of four genotypically different *phlD*$^+$ reference strains (PILH1, F113, Q8R1-96 and Q2-87), and from DNA of each of 184 *phlD*$^+$ isolates obtained previously from the rhizosphere of three different plant species. No amplification product was obtained from DNA of *phl*D$^-$ strain R1SS101, but also not from DNA of *phl*D$^+$ reference strains CHA0 and Pf-5. At this stage, several attempts were made for further optimization of the first primer set, including adjustment of the primer concentrations, annealing temperature (temperatures tested ranged from 48°C to 70°C) and Mg^{2+} concentration. However, none of these attempts were successful. The second primer set resulted in amplification of the predicted 350-bp fragment from DNA of all six *phl*D$^+$ reference strains, including strains CHA0 and Pf-5, and from DNA of each of the 184 *phlD*$^+$ isolates (**Figure 2A, 2B and 2C**).

For successful amplification with the second primer set, a two-step PCR approach was required. In the first PCR step, no GC-clamp was present at the 5' end of the forward primer. After the first step, the PCR products were diluted 100 to 1000 times, after which the second PCR step was performed with the forward primer containing the GC-clamp. The PCR program in the one-step and two-step PCR amplifications was the same as described above.

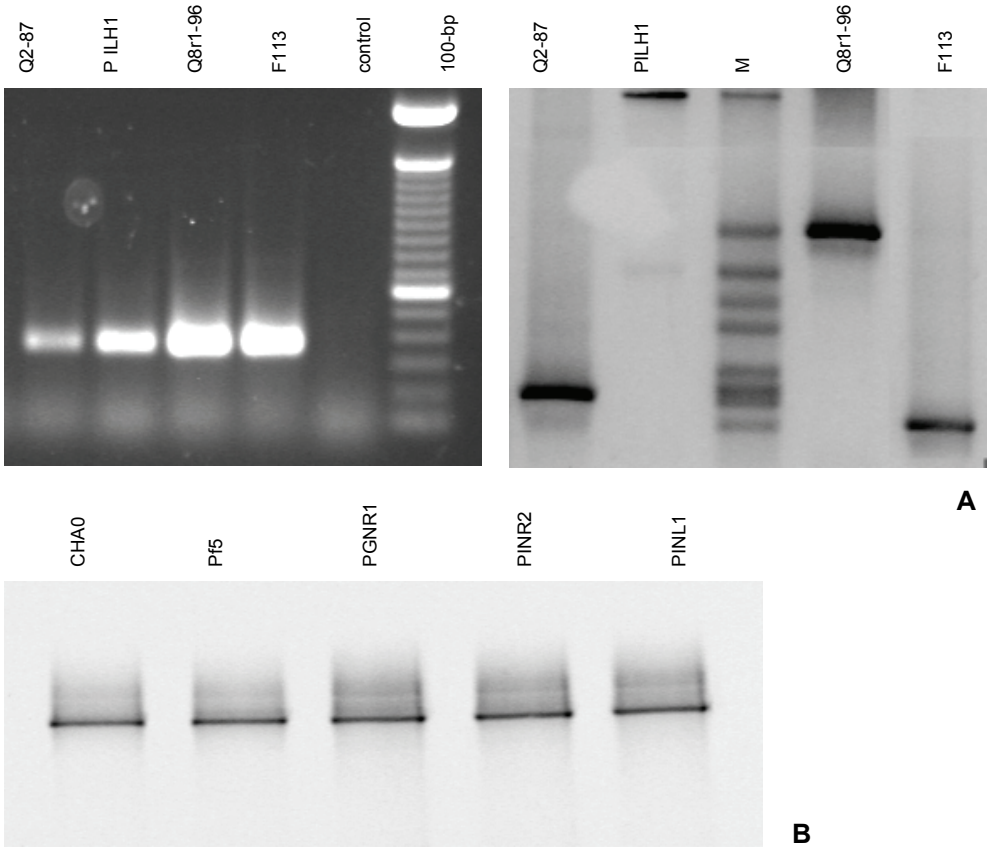

Figure 2: **A.** Agarose gel (left) and a 32-41% DGGE gel (right) of *phl*D fragments amplified from *phl*D$^+$ reference strains Q2-87, PILH1, Q8r1-96 and F113. M refers to the DGGE marker which includes independently amplified *phl*D fragments from nine reference and indigenous strains. **B.** A 40-51% DGGE gel of *phl*D fragments amplified from the *phl*D$^+$ reference strains CHA0, Pf-5, PGNR1, PINR2 and PINL1; **C.** A 32-41% DGGE gel of *phl*D fragments amplified from *phl*D$^+$ indigenous isolates obtained from the rhizosphere of multiple plant species. DGGE groups A (PWB152), B (PWB134), C (PSB516), D (PSB211), E (PSB113), F (PPB617) and Z (PWB5516).

DGGE-analysis

Denaturing and temperature gradient gel electrophoreses (DGGE or TGGE) are widely used to study the microbial diversity in environmental samples and to monitor changes in specific microbial groups or communities (Duineveld et al., 1998, Garbeva et al 2002a,b; Kowalchuk et al. 1997; Muyzer and Smalla, 1998, Salles et al. 2002, Van Elsas et al., 1998). DGGE allows analysis of a large number of samples which is essential for studying spatial and temporal variations in microbial populations (Heuer and Smalla, 1997, Muyzer et al., 1993, Muyzer et al., 1997, Muyzer and Smalla, 1998, Theron and Cloete, 2000, Nielsen et al., 1999). To date, most primers used in DGGE analysis target ribosomal DNA of different microbial genera. In this context, group-specific primers have been developed for a number of bacterial genera, including *Pseudomonas* and *Bacillus* (Garbeva et al., 2002a,b), *Burkholderia* (Salles et al., 2002), *Actinomycetes* (Hauer et al., 1997), and ammonium oxidizers and methanotrophs (Kowalchuk et al. 1997; Boon et al., 2002). Recently, primers targeting specific biosynthetic genes have been developed and when combined with DGGE fingerprinting have led to a better level of discrimination within a specific bacterial group. For example, primers directed against the *fli*C gene allowed for specific detection of the bacterial wilt pathogen *Ralstonia solanacearum* in soil and subsequent discrimination between strains obtained from various origins (Schonfeld et al., 2003). Rapid assessment of the diversity of methanogens was performed by DGGE analysis of the *nif*H gene (Wawer and Muyzer, 1995, Lovell et al., 2000). In the same line of research, terminal RFLP analysis (T-RFLP) of PCR-amplified *nif*H fragments was shown to be a rapid technique for profiling diazotrophic microbial communities (Tan et al., 2003). Additionally, the community structure of ammonia-oxidizing bacteria (Ibekwe et al., 2002) and bacteria of the marine environment (Dahllof et al., 2000) was explored by DGGE analysis of the *amo*A and the *rpo*B gene, respectively.

In the present study, DGGE analysis was performed on the *phlD* fragments amplified with the first and second primer sets described previously. A linear gradient from 32% to 41% denaturants allowed detection of the amplified fragments and gave optimal discrimination between genotypically different *phlD*$^+$ reference strains and isolates, except for strains CHA0 and Pf-5 (**Figure 2A and 2C**). For CHA0 and Pf-5, a gradient from 40% to 51% denaturants was required to detect the PCR-products amplified with the second primer set (**Figure 2B**). Attempts to design a DGGE gradient (32-51%) for all *phlD*$^+$ strains, including strains CHA0 and Pf-5, resulted in loss of discrimination between *phl*D$^+$ genotypes other than strains CHA0 and Pf-5. The difference in behavior of strains CHA0 and Pf-5 compared to other *phlD*$^+$ strains in both PCR and DGGE is supported by *phlD* sequence

data (see below) and has been described previously for PCR with other *phlD* primers (McSpadden et al. 2001b). For strains other than CHA0 and Pf-5, the migration of PCR fragments obtained with the first and second primer set differed, but DGGE-grouping of the *phlD*$^+$ genotypes was identical for both primer sets (data not shown). DGGE-analysis of PGNR1, PINR2 and PINL1, three other strains that are also very closely related to CHA0 and Pf-5 (Keel et al. 1996), showed that their amplified *phl*D-fragments migrated to the same position as the *phl*D fragments of strains CHA0 and Pf-5 (**Figure 2B**).

Table 3: Genotypic classification of 184 indigenous *phl*D$^+$ *Pseudomonas* isolates originating from the rhizospheres of multiple plant species by DGGE and RAPD analyses. N indicates the total number of isolates belonging to a specific RAPD group. Frequencies are expressed in percentage (%) of the total number of isolates included in this study.

DGGE-group	RAPD-group	N	Frequency DGGE	Frequency RAPD
A	A1	28	16.85	15.22
	A2	3		1.63
B	B1	19	10.33	10.33
C	C1	11	5.98	5.98
D	D1	2	1.09	1.09
E	E1	68	52.16	36.96
	E2	18		9.78
	E3	3		1.62
	E4	6		3.26
	E5	1		0.54
F	F1	5	2.72	2.72
Z	Z1	20	10.87	10.87
TOTAL	**12**	**184**	**100**	**100**
7				

DGGE-analysis of the 184 *phlD*$^+$ isolates obtained from the rhizospheres of wheat, sugar beet and potato, resulted in seven DGGE groups, designated A, B, C, D, E, F, and Z (**Figure 2C, Table 3**). Several of the 184 *phl*D$^+$ isolates were assigned to the same DGGE-group as reference strains PILH1 (DGGE-group A) and Q2-87 (DGGE-group B). None of the 184 *phl*D$^+$ isolates were assigned to DGGE-groups containing the reference strains Q8R1-96 (DGGE-group G), F113 (DGGE-group I) and CHA0 (DGGE-group M). Therefore, the number of isolates assigned to DGGE-groups C, D, E, F and Z may represent *phlD*$^+$ genotypes not described previously. DGGE-group E was the dominant *phl*D$^+$ genotype found among the collection of 184 indigenous *phl*D$^+$ isolates, representing approximately 52% of the diversity (**Table 3**).

Table 4: Genotypic classification of twelve indigenous *phl*D$^+$ *Pseudomonas* isolates originating from the rhizospheres of multiple plant species and five *phl*D$^+$ reference *Pseudomonas* strains, by DGGE and *phl*D-RFLP analyses. For the *phl*D-RFLP analysis, 629-bp fragments of the *phl*D gene were amplified with primers B2BF and BPR4 followed by restriction with *Hae*III, *Msp*I and *Taq*I (McSpadden et al., 2001b). NEWi indicates groups based on the RFLP analysis which do not correspond with any of the previously described 17 genotypes (Landa et al., 2002).

DGGE-genotype	RFLP-genotype	Representative
A	M	**D27B1**
A	M	PWB152
A	M	PSB459
B	B	**Q2-87**
B	NEW1	PWB134
C	NEW1	PSB516
D	NEW2	PSB211
E	D	PSB113
E	D	PPB433
E	D	PWB522
E	D	PPB239
E	D	PWB257
F	NEW3	PPB617
G	D	**Q8r1-96**
I	K	**F113**
M	A	**CHA0**
Z	NEW4	PWB5516

Comparison of PCR-DGGE, RAPD and *phlD*-RFLP analyses

To determine the resolving capacity of the classifications made by PCR-DGGE analysis of the *phlD* gene, RAPD analysis with three 10-mer primers and *phlD*-RFLP analysis were performed. RAPD analysis of the 184 indigenous *phlD*$^+$ isolates correlated well with the results obtained in PCR-DGGE but has a higher degree of discrimination than PCR-DGGE analysis (**Table 3**). RAPD analyses resulted in 12 different RAPD-groups, whereas PCR-DGGE resulted in seven different groups. Isolates belonging to DGGE-group E were assigned to 5 different RAPD groups (E1 thru E5) and isolates belonging to DGGE-group A were assigned to two RAPD groups (A1 and A2). A subset of 6 *phl*D$^+$ reference strains and 12 indigenous *phlD*$^+$ isolates, representing the twelve different RAPD groups, were analyzed by RFLP analysis of a 629-bp *phlD*-fragment with three restriction enzymes, a technique routinely used to determine the genotypic diversity among DAPG-producing *Pseudomonas* spp. (McSpadden et al. 2001b). Based on RFLP analysis, the two isolates (A1 and A2) belonging to DGGE-group A were identical to D27B1 a reference strain representing DGGE-genotype A (**Table 4**). Representative isolates from DGGE-groups D, F,

and Z could be distinguished on the basis of RFLP analysis. In contrast, however, RFLP profiles of reference strain Q8r1-96 (DGGE-group G) were identical to those of all five isolates from DGGE-group E (RAPD groups E1 thru E5). Similarly, RFLP profiles of isolates of DGGE-groups B and C were identical. These results indicated that PCR-DGGE provides a higher level of discrimination between *phlD*$^+$ genotypes than the currently used *phlD*-RFLP analysis.

Sequence and phylogenetic analyses

Sequence analyses of the 350-bp *phlD* fragments amplified from DNA of 12 isolates representing the different DGGE-genotypes (A, B, C, D, E, F, and Z) showed, in general, a good correlation between the GC-content of the amplified fragments and their migration patterns in the denaturing gel (**Figure 3**). GC-content ranged between 47.95% for DGGE genotype A positioned in the upper part of the 32-41% DGGE gradient and 51.72% for DGGE-genotype I positioned at the bottom. However, electrophoretic mobility of the amplified *phlD*-fragments was not determined by their GC-content alone. For example, fragments with the same GC-content (e.g. DGGE-groups F and D) migrated to different positions, whereas fragments with different GC-contents (e.g. DGGE-group A) migrated to the same position in the 32-41% DGGE gradient. These results illustrate that not only GC-content but also the positioning of the so-called melting domains (Muyzer and Smalla, 1998) within the amplified fragment determines their electrophoretic mobility. The relatively high GC-content of 59.6% of the *phl*D fragments of reference strains CHA0 and Pf-5 supported the requirement of a gradient with a higher percentage (40-51%) of denaturants as described previously. Phylogenetic analyses of the *phl*D sequences obtained in this study and present in the database (**Table 1**) showed in total six distinct clusters based on bootstrap values higher than 75% (**Figure 4**). Cluster I contained the reference strains Q8R1-96, Q65c80 and CM1A2, as well as isolates E1 through E5, five representatives of DGGE-genotype E. Representative isolates of DGGE-genotype B were clustered together (cluster II) with *phl*D$^+$ reference strains F113 and MI-96. Cluster III contained only representative isolates of DGGE-genotypes C, D, F and Z. Isolates A1 and A2, representing DGGE-genotype A, were classified in the same cluster (cluster IV) and together with reference strains PILH1 and PITR2, both classified by DGGE analysis as genotype A. Reference strain Q2-87 (DGGE-genotype B) formed a unique cluster (cluster V) including the recently described strain HR3-A13 (PfY; Mazzola et al. 2004). However, Q2-87 was not grouped close to PWB134, a strain also classified as DGGE-genotype B. These results are in accordance with results obtained by the RFLP analysis of strain Q2-87 and isolate

PWB134. Cluster VI was the most distant cluster obtained in the phylogenetic analyses containing reference strains CHA0 and Pf-5 and the recently described strain PR3-A52 (PfZ; Mazzola et al. 2004). Phylogenetic analyses of the *phl*D gene sequences by neighbor-joining or maximum likelihood yielded similar results and tree topologies.

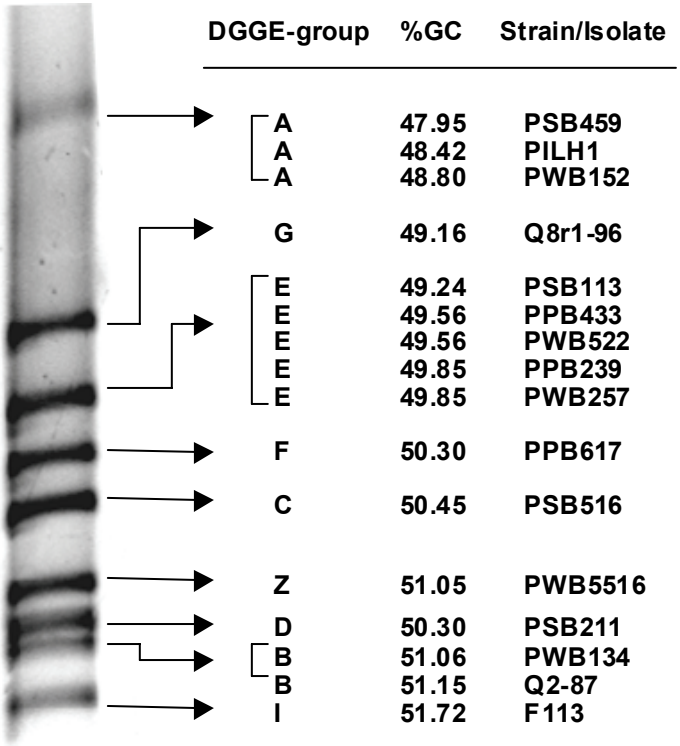

Figure 3: Relationship between the electrophoretic mobility of *phlD* fragments and their GC-content. For various strains and isolates representing nine different DGGE-groups, the electrophoretic mobility of 350-bp *phlD*-fragments in a denaturing gradient gel (32-41% denaturants) are shown. For three DGGE-groups (A, B and E), multiple isolates were included in the analysis.

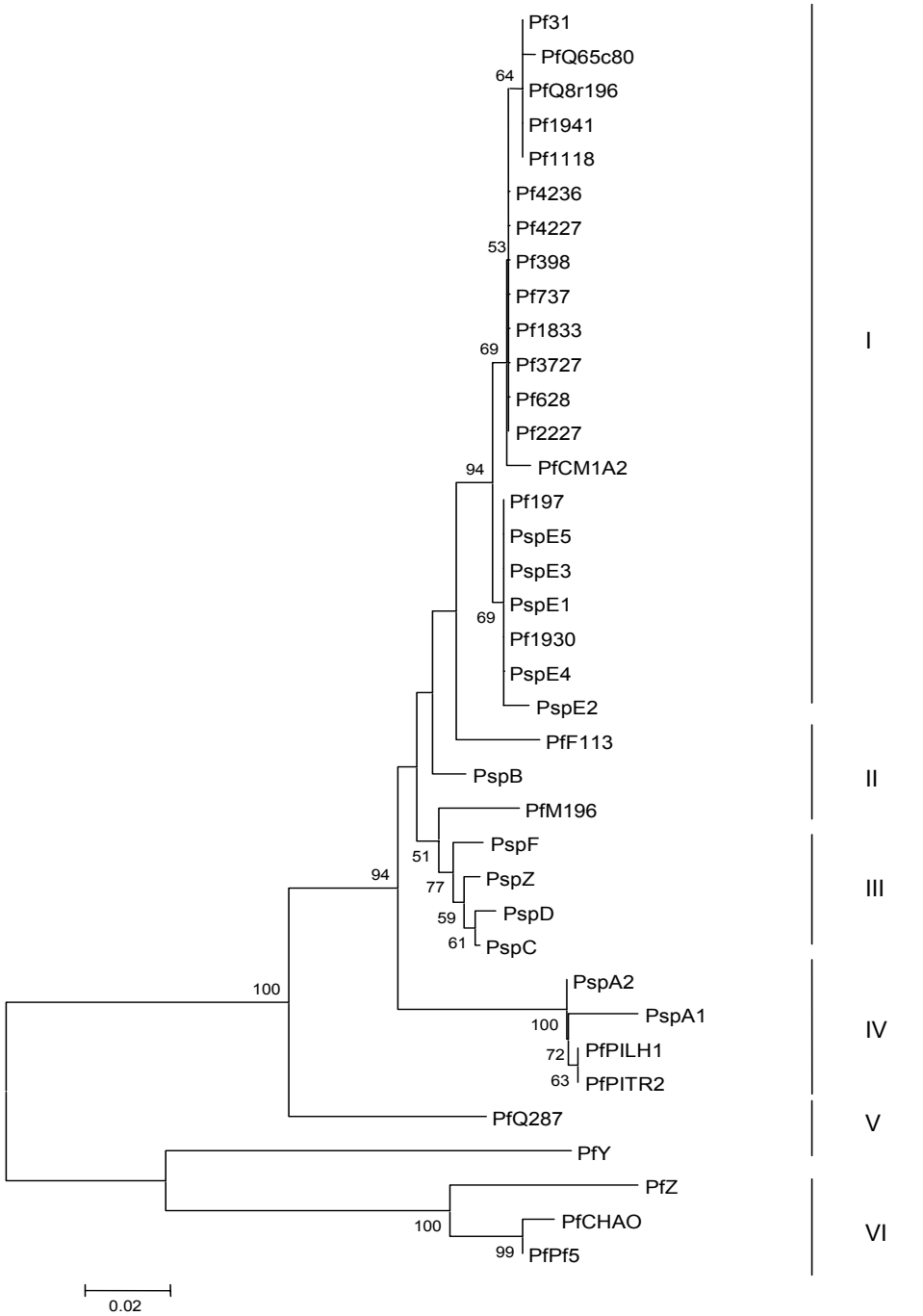

Figure 4: Phylogenetic tree (not rooted) of 325-bp *ph*lD fragments inferred by the neighbor-joining method. Bootstrap values higher than 50% are shown. Clusters (I through VI) are defined by bootstrap values > 75%.

PCR-DGGE of rhizosphere samples

A notable difficulty of *phlD*-RFLP and RAPD analyses is the requirement of isolation and cultivation of *phl*D$^+$ *Pseudomonas* isolates from soil and rhizosphere environments prior to their genotypic characterization. PCR-DGGE was performed on DNA extracted from rhizosphere samples obtained from roots of wheat plants grown for 10-12 days in soil treated with a spontaneous rifampicin-resistant derivative of isolate PWB532 (DGGE-group E). Isolate PWB532 was introduced in soil at initial densities ranging between 10 to 10^6 CFU/g. Rhizosphere samples were subjected to direct DNA extraction followed by PCR-DGGE, and were also dilution plated onto KMB supplemented with rifampicin for comparison purposes. Based on dilution plating, rhizosphere population densities of introduced isolate PWB532 ranged from $5x10^2$ to $5x10^6$ CFU/g root after 10-12 days of cultivation. PCR on DNA directly extracted from the wheat rhizosphere resulted in the amplification of the 350-bp *phlD* fragment when the density of PWB532 was equal and higher than $5x10^3$ CFU/g of root fresh weight. Subsequent DGGE analysis of the 350-bp fragments amplified from DNA extracted from the rhizosphere of wheat colonized by isolate PWB532 showed one single band corresponding to DGGE-group E (**Figure 5A**). No other DGGE-genotypes were detected. Additionally, no 350-bp amplification products were detected in the control treatments, which included rhizosphere samples from natural CB soil and from CB soil autoclaved twice prior to wheat cultivation (data not shown). The ramping PCR protocol applied on DNA extracted from rhizosphere samples was crucial as it considerably increased the sensitivity of the PCR amplification. Similar results were obtained in short-term experiments with sugar beet plants grown in soils treated with isolates PSC415 (DGGE group Z), Q8R1-96 (DGGE group G) and PPB3512 (DGGE group F) (**Figure 5B**). Collectively, these results indicate that PCR-DGGE can be used to detect specific *phl*D$^+$ genotypes directly in rhizosphere samples with a detection limit of approximately $5x10^3$ CFU/g root when using ethidium-bromide staining of the gel after electrophoresis. For ethidium-bromide stained gels, a detection limit of 10^5 CFU/g soil was reported previously for *R. solanacearum* (Schonfeld et al. 2003). When combined with Southern hybridization, however, cell densities of *R. solanacearum* of approximately 10^3 CFU/g soil could be detected (Schonfeld et al. 2003). The results of this latter study suggest that the detection limit for indigenous *phlD*$^+$ isolates may be increased further when combined with Southern hybridization or when using silver staining instead of ethidium bromide staining.

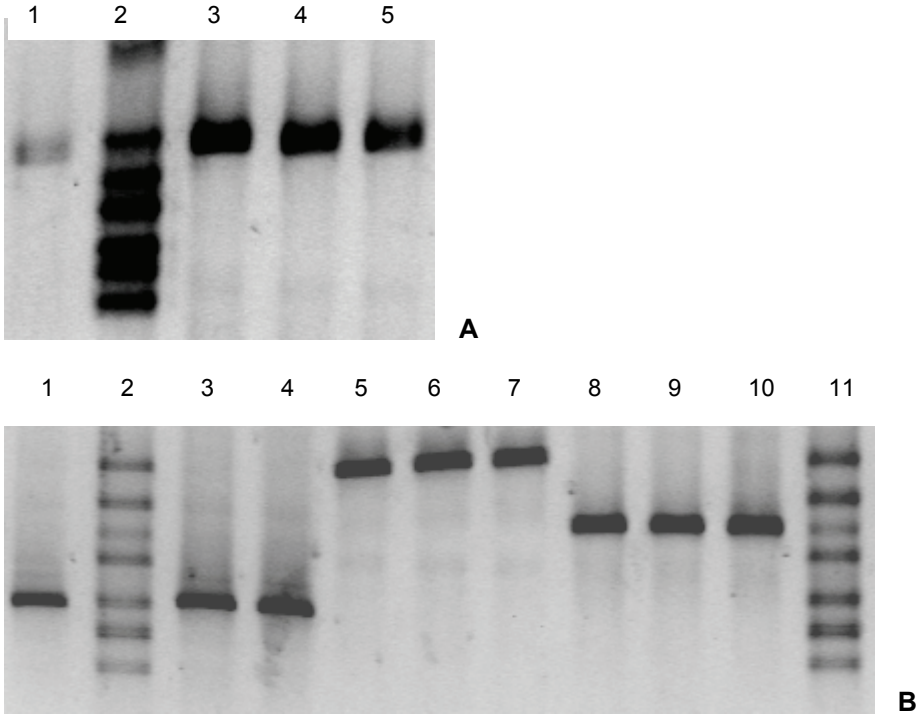

Figure 5: **A.** DGGE patterns (32-41%) of the 350-bp *phl*D fragment amplified from DNA extracted from the rhizosphere of wheat colonized by the rifampicin resistant derivative of *phl*D$^+$ isolate PWB532 representing DGGE group E. Based on dilution plating, isolate PWB532 was present at densities of approximately 5x10^3 (lane 1), 5x10^4 (lane 3), 5x10^5 (lane 4) and 5x10^6 (lane 5) CFU/g root. Lane 2 represents a marker composed of *phl*D fragments amplified from DNA of seven isolates corresponding to DGGE groups G, E, F, C, Z, B and I (from top to bottom).
B. DGGE patterns (32-41%) of 350-bp *phl*D fragment amplified from isolates PSC415 (DGGE Z), Q8R1-96 (DGGE G) and PPB3512 (DGGE F) introduced in the sugar beet rhizosphere. Lane 1: DGGE Z in density 5.3x10^5, lane 2: marker, lane 3: DGGE Z 2.7x10^5, lane 4: DGGE Z 1.5x10^5, lane 5: DGGE G in density 2.5x10^6, lane 6: DGGE G 7.6x10^5, lane 7: DGGE G 1.9x10^6, lane 8: DGGE F in density 8.5x10^3, lane 9: DGGE F 2.8X10^4, lane 10: DGGE F 2.4x10^4 CFU/g root and lane 11: marker. The marker consists of *phl*D fragments amplified from DNA of seven isolates corresponding to DGGE groups G, E, F, C, Z, B and I (from top to bottom).

A potential problem of PCR-DGGE on DNA extracted from environmental samples may be that, in mixed populations of *phlD*$^+$ isolates, certain *phlD* genes are preferentially amplified leading to an incorrect assessment of all the genotypes present. In the present study, a mixture of six genotypically different *phl*D$^+$ isolates (representatives of DGGE-genotypes B, C, E, F, G and Z) was introduced into wheat rhizosphere samples to a final density for each isolate of approximately 5x10^5 CFU/g root prior to DNA extraction. PCR-DGGE analysis showed that all six different genotypes were detectable in both replicates included (**Figure 6A**). In both mixed samples, one additional band was detected in the DGGE gel; this may be a heteroduplex between the different *phlD* sequence variants as

described previously by Kowalchuk et al. (1997) or it may represent another indigenous *phlD*$^+$ isolate present in the wheat rhizosphere sample. This latter aspect was not further pursued. Collectively these results indicated that PCR-DGGE of the *phlD* gene allows simultaneous detection of multiple genotypes present in a rhizosphere sample.

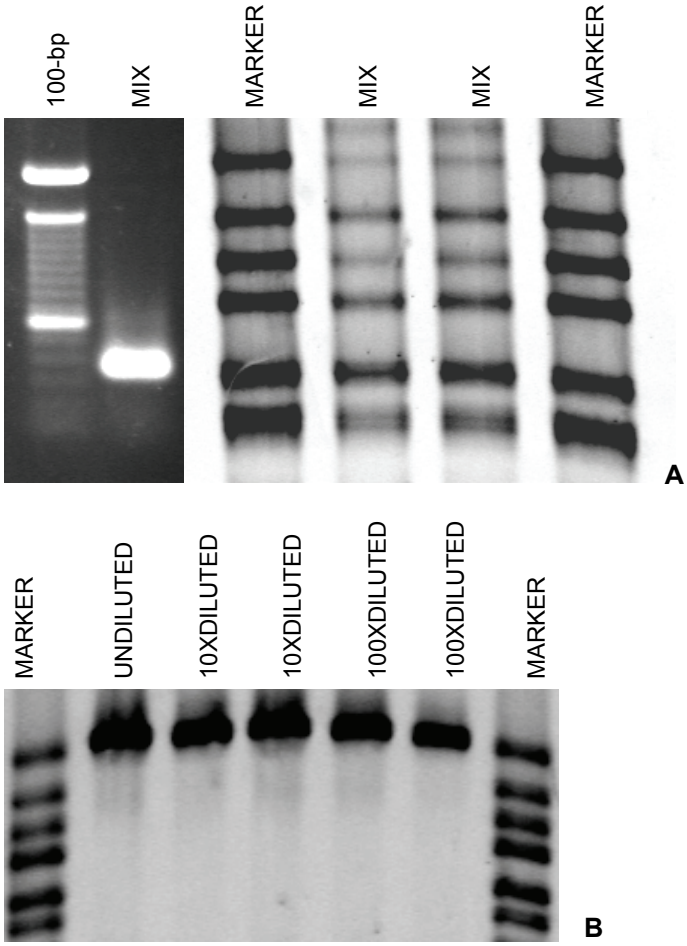

Figure 6: A. *phl*D fragment (350-bp) (left) and DGGE (32-41%) patterns (right) of the 350-bp *phl*D fragment obtained from the PCR amplification of a mixture of six distinct *phl*D$^+$ genotypes: PWB134 (DGGE B), PSB516 (DGGE C), PSB113 (DGGE E), PPB617 (DGGE F), PWB5516 (DGGE Z) and Q8R1-96 (DGGE G). All genotypes contributed equally in the mixture at a density of $5x10^5$ CFU/strain. The marker consists of *phl*D fragments amplified from DNA of six isolates corresponding to DGGE groups G, E, F, C, Z and B (from top to bottom).
B. DGGE pattern (32-41%) of the 350-bp *phl*D fragment obtained from the PCR amplification of the same mixture of the six distinct *phl*D$^+$ genotypes after an incubation step based on the method of McSpadden et al. (2001b). Serial dilutions (10x and 100x) of the sample were included. The marker consists of *phl*D fragments amplified from DNA of six isolates corresponding to DGGE groups G, E, F, C, Z and B (from top to bottom).

In this same experiment, the PCR-DGGE methodology was compared with the currently used rapid PCR-based protocol for rhizosphere samples (McSpadden et al., 2001b). For this latter rapid PCR-based protocol, different dilutions of the rhizosphere samples required incubation in nutrient broth for 48 h prior to PCR and genotypic characterization. PCR and subsequent genotypic characterization showed that the rapid PCR-based protocol resulted in detection of only DGGE group G (strain Q8r1-96) (**Figure 6B**), whereas PCR-DGGE resulted in detection of all six genotypes (**Figure 6A**). In conclusion, these results indicated that cultivation of a rhizosphere sample in nutrient broth prior to genotypic characterization introduces a bias toward detecting either the most dominant genotype or the genotype with higher growth rates or competitive abilities in the nutrient broth relative to the other genotypes present. This bias is circumvented by direct PCR-DGGE of the *phlD* gene.

Biological significance of PCR-DGGE classification of *phlD*$^+$ genotypes

In order to investigate the biological significance of the additional classifications of *phlD*$^+$ genotypes made by the PCR-DGGE methodology described in this study, the population dynamics of eight isolates representing different DGGE-groups was monitored during six successive growth cycles of sugar beet seedlings in soil obtained from an agricultural field (**Figure 7; Table 5**). Each of the 8 isolates was introduced only once (growth cycle 0) at initial densities of approximately $5X10^4$ CFU/g soil. After the first growth cycle of sugar beet, strains Q8r1-96 (DGGE-G) and PWB233 (DGGE-A) established the highest densities; population densities of strain Q8r1-96 (DGGE-G) further increased during growth cycles 2 to 4 to densities of approximately $3X10^7$ CFU/g root, whereas population densities of PWB233 (DGGE-A) leveled off at densities of approximately $5X10^6$ CFU/g root. After the first growth cycle, strains PWB532 (DGGE-E), PPB2310 (DGGE-C), PSC415 and (DGGE-Z) did not establish densities as high as strains Q8r1-96 (DGGE-G) and PWB233 (DGGE-A), but reached similar densities as PWB233 (DGGE-A) in growth cycles 2 to 4. After 6 growth cycles of sugar beet seedlings, population densities of the three genotypes A, E and G were significantly similar (**Table 5)**. Strains PSB211 (DGGE D), PSC2218 (DGGE B) and PPB3512 (DGGE-F) colonized the rhizosphere of sugar beet seedlings to a significantly lesser extent than the strains representing the other five DGGE-genotypes. The population densities for the three strains (DGGE groups D, B, and F) did not increase above 10^5 CFU/g root, but instead declined in the last growth cycle of sugar beet to densities of 10^4 (DGGE-D), $8X10^3$ (DGGE-B) and $4x10^3$ (DGGE-F) CFU/g root (**Figure 7**).

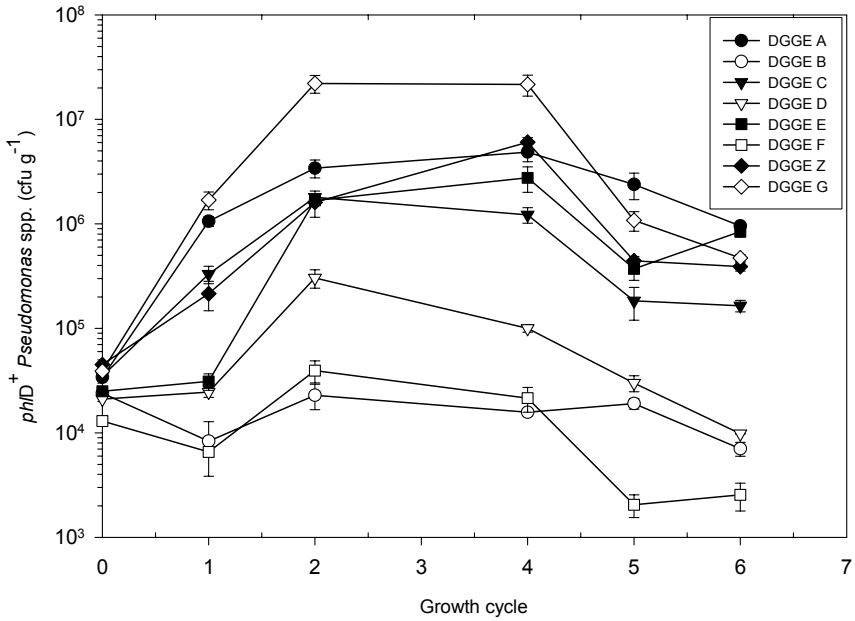

Figure 7: Population dynamics (expressed in CFU/g root) of representative isolates of eight different DGGE genotypes in the sugar beet rhizosphere. Isolates were introduced separately into soil only once (cycle 0) at a density of approximately $5x10^4$ CFU/g soil. The population dynamics of each of the isolates was monitored in time during six successive growth cycles of sugar beet of 10-12 days each. Cycle 3 was not included. For each growth cycle, mean values of 4 replicates are presented. Error bars represent the standard error. Statistical analysis is presented in detail in Table 5.

These results showed that considerable differences exist in the ability of different *phlD*$^+$ genotypes to colonize the rhizosphere of sugar beet seedlings, confirming and extending results obtained in previous studies (Raaijmakers and Weller, 2001; Landa et al. 2003). Strain Q8r1-96, representing DGGE-genotype G, was shown to be superior in colonization of the sugar beet rhizosphere, especially in the first four growth cycles. Similar observations were described for wheat (Raaijmakers and Weller, 2001) and pea (Landa et al. 2003). These results indicate that the ability of strain Q8r1-96 to rapidly establish and maintain high population densities in the rhizosphere is not linked to a specific plant species, but may be due to specific characteristics that enable this strain to be competitive in different rhizosphere environments. In this context, Mavrodi et al. (2002) recently identified possible new traits by subtractive hybridization that may contribute to the superior rhizosphere competence of strain Q8R1-96.

These traits include bacteriocin production, a trait that may be advantageous in intraspecific competition with other indigenous pseudomonads. Although strain Q8r1-96 (DGGE-genotype G) could not be distinguished from strain PWB532 (DGGE-genotype E) on

the basis of *phlD*-RFLP analysis, they differed considerably in their ability to colonize the rhizosphere of sugar beet seedlings. Similarly, strains PPB2310 (DGGE-C) and PSC2218 (DGGE-B), which could not be distinguished by *phlD*-RFLP analysis, differed significantly in their ability to colonize the sugar beet rhizosphere.

Table 5: Population dynamics (expressed in $\log_{10}$ CFU/g root) of representative isolates of eight different DGGE genotypes in the sugar beet rhizosphere. The population dynamics per DGGE genotype was followed in time during six successive growth cycles of sugar beet. Cycle 3 was not included. Mean values presented here are based on N=4 replicates.

		GROWTH CYCLE SUGAR BEET SEEDLINGS				
Strain	**DGGE-group**	**1**	**2**	**4**	**5**	**6**
PWB233	DGGE A	5.90 ab	6.52 b	6.68 b	6.38 a	5.96 a
PSC2218	DGGE B	3.89 ef	4.32 d	4.19 e	4.27 c	3.83 d
PPB2310	DGGE C	5.44 bc	6.23 b	6.01 c	5.25 b	5.19 c
PSB211	DGGE D	4.36 de	5.48 c	4.98 d	4.47 c	3.98 d
PWB532	DGGE E	4.48 d	6.22 b	6.43 bc	5.57 b	5.91 a
PPB3512	DGGE F	3.78 f	4.54 d	4.31 e	3.31 d	3.38 e
Q8r1-96	DGGE G	6.22 a	7.34 a	7.34 a	6.03 a	5.67 ab
PSC415	DGGE Z	5.29 c	6.16 b	6.59 b	5.53 b	5.50 bc

These results highlight that the additional classification of this widely distributed group of antibiotic-producing *Pseudomonas* spp. by PCR-DGGE analysis of the *phlD* gene also provides a biologically relevant discrimination. Given the level of polymorphism in specific genes involved in the regulation (*gac*A; Souza et al. 2003c) or biosynthesis of other antibiotic compounds, including pyrrolnitrin (Kirner et al., 1998, Souza et al. 2003b) and phenazine antibiotics (Raaijmakers et al. 1997; Mavrodi et al. 1998), this technique could easily be applied to provide an additional level of discrimination between isolates and strains producing other metabolites involved in rhizosphere competence and biological control of plant pathogens.

Conclusion

Establishing the presence of individual populations of antagonistic microorganisms in soil and rhizosphere environments is an important first step toward fully understanding their functional role in these natural environments. Additionally, the diversity within such indigenous populations of antagonistic microorganisms with a common biocontrol trait holds promise to further improve biological control, especially when this diversity reflects important interactions at the host-antagonist level. The technique described in this study allows direct

detection and assessment of the genotypic diversity of a specific group of bacteria that produce DAPG, a broad-spectrum antibiotic that has been implicated in biological control of multiple plant diseases and in the natural suppressiveness of soils. More specifically, our results indicated that the PCR-DGGE methodology can be used to detect specific *phl*D$^+$ genotypes directly in rhizosphere samples with a detection limit of approximately 5x10^3 CFU/g root and it allows simultaneous detection of multiple genotypes present in a rhizosphere sample. Subsequent bioassays have clearly shown that a differential ability of the genotypic groups exists with respect to colonization of the sugar beet rhizosphere, confirming the biological significance of this methodology.

Materials and methods

Pseudomonas strains and culture conditions

All *Pseudomonas* strains used in this study were cultured on King's medium B (KMB) agar (King et al., 1954) at 25^o C for 48 h. To determine the specificity and resolving capacity of the PCR-DGGE method developed in this study, multiple *phlD*$^+$ reference strains (**Table 1**) were tested as well as 184 indigenous *phlD*$^+$ *Pseudomonas* isolates obtained from the rhizospheres of three plant species (wheat, sugar beet and potato) by colony hybridization with a *phlD*-specific probe.

PCR-DGGE analysis.

PCR amplification was carried out in a 25 µl reaction mixture which contained 3 µl of a 40-fold diluted heat-lysed cell suspension (Raaijmakers et al. 1997), 1X GeneAmp ® PCR buffer (Perkin-Elmer Corp., Norwalk, Conn.), 500 µM of each deoxynucleoside triphosphate (Promega), 40 pmol of the reverse primer and 40 pmol of the forward primer (Amersham pharmacia biotech), 1.5 mM $MgCl_2$ and 1.0 U of AmpliTaq ® DNA polymerase (Perkin-Elmer). The PCR program consisted of an initial denaturation at 94 ^{o}C for 3 min, followed by 30 cycles of 94 ^{o}C for 30 sec, 67 ^{o}C for 30 sec and 72 ^{o}C for 60 sec. The reactions were performed on a Peltier Thermal Cycler-200 (Biozym, Landgraaf, the Netherlands). Fifteen µl of the PCR product was used for analysis by Denaturing Gradient Gel Electrophoresis (DGGE) with the Dcode universal mutation detection system (Bio-Rad Laboratories, Hercules, Calif.). DGGE analysis was based on the initial protocol of Muyzer et al. (1993) and performed using an 8% (w/v) acrylamide gel with a linear denaturing gradient (100% denaturant contained 7 M urea plus 40% (v/v) deionized formamide).

Table 1: *phl*D$^+$ *Pseudomonas* strains used in this study

Strain/Isolate	Code	Accession number *phl*D	Reference
Q2-87	PfQ287	U41818	Bangera and Thomashow, 1998
F113	PfF113	AJ278811	Fenton et al., 1992
CHA0	PfCHA0	AJ278806	Stutz et al., 1986
PF5	PfPf5	AF214457	Howell and Stipanovic, 1979
Q8R1-96	PfQ8R196	AF207693	Raaijmakers and Weller, 2001
Q65C-80	PfQ65c80	AJ278807	Harrison et al., 1993
CMIA2	PfCMIA2	AJ278808	Fuchs and Defago, 1991
MI-96	PfMI96	AF207692	Raaijmakers and Weller, 2001
PILH1	PfPILH1	AJ278810	Keel et al., 1996
PITR2	PfPITR2	AJ278809	Keel et al., 1996
HR3-A13	PfY	AY391780	Mazzola et al., 2004
PR3-A52	PfZ	AY391779	Mazzola et al., 2004
42-36	Pf4236	AF396857	Picard and Bosco, 2003
42-27	Pf4227	AF396856	Picard and Bosco, 2003
39-8	Pf398	AF396855	Picard and Bosco, 2003
37-27	Pf3727	AF396854	Picard and Bosco, 2003
22-27	Pf2227	AF396853	Picard and Bosco, 2003
19-41	Pf1941	AF396852	Picard and Bosco, 2003
19-30	Pf1930	AF396851	Picard and Bosco, 2003
19-7	Pf197	AF396850	Picard and Bosco, 2003
18-33	Pf1833	AF396849	Picard and Bosco, 2003
11-18	Pf1118	AF396848	Picard and Bosco, 2003
7-37	Pf737	AF396847	Picard and Bosco, 2003
6-28	Pf628	AF396846	Picard and Bosco, 2003
3-1	Pf31	AF396845	Picard and Bosco, 2003
D27B1	D27B1	na	Landa et al., 2002
PSB459	Psp A1	AY486314	this study
PWB152	Psp A2	AY486317	this study
PWB134	Psp B	AY486316	this study
PSB516	Psp C	AY486315	this study
PSB211	Psp D	AY486313	this study
PSB113	Psp E1	AY486312	this study
PPB433	Psp E2	AY486310	this study
PWB522	Psp E3	AY486319	this study
PPB239	Psp E4	AY486309	this study
PWB257	Psp E5	AY486318	this study
PPB617	Psp F	AY486311	this study
PWB5516	Psp Z	AY486320	this study

na : not available

A gradient from 32% (top) to 41% denaturants (bottom) gave, in almost all cases, optimal separation of the amplified products and was routinely used. Gels were run for 10 min at 200 V and subsequently for 16 h at 85 V (60^{o}C), stained with ethidium bromide (0.5 μg/ml 1 x TAE (pH 8.3)) for 30 min and visualized on an UV transilluminator.

DNA sequence analysis.

*Phl*D fragments from multiple representative strains were amplified using the Expand High Fidelity taq polymerase (Roche, Almere, Netherlands) and subsequently sequenced by BaseClear (Leiden, Netherlands). *Phl*D sequences obtained in this study were deposited with GenBank under the accession numbers presented in Table 1. Alignments of obtained *phl*D sequences and *phl*D sequences present in the databases were performed with Clustal W (Thompson et al., 1994). Distance matrices were computed in MEGA and phylogenetic trees were constructed using the neighbor-joining method (Saito and Nei, 1987) and their topology checked by bootstrap analysis (1000 data sets).

RFLP and RAPD analyses.

To determine the resolving capacity of the classifications assessed by PCR-DGGE analysis, multiple *phl*D^{+} strains were also subjected to *phl*D-Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) analyses, two techniques that are currently used to determine the genotypic diversity among DAPG-producing *Pseudomonas* spp. For *phl*D-RFLP analysis, 629-bp fragments of the *phl*D gene were amplified with primers B2BF and BPR4 followed by restriction with *Hae*III, *Msp*I or *Taq*I (McSpadden et al., 2001b). Restriction fragments were separated on a 2% agarose gel in 1xTAE for 2-3 h at 120 V. RAPD analysis with 10-mer primers M12, M13 and D7 was performed according to protocols described previously (Keel et al.,1996; Raaijmakers and Weller, 2001). The amplification products were separated on a 2% agarose gel in 1xTAE at 120 V for 3h. *phl*D-RFLP patterns and RAPD markers were visualized with a UV transilluminator and photographed using a digital camera. All PCR-RAPD amplifications were repeated at least two times and only the consistent RAPD markers were included in the evaluation. Sizes of the restriction fragments of the *phlD*-RFLP analysis and the RAPD markers were determined with the Phoretix 1D software (version 3.0, Phoretix International, England). Band positions were converted to Rf values (0 and 1) and profile similarities were calculated by determining the pairwise coefficients of similarity (Nei-Li distances) for the total number of lane patterns. Cluster analysis with neighbor-joining (Saito and Nei, 1987), and

the corresponding bootstrap analysis (1000 data sets), were performed with the Treecon (version 1.3b) software for Windows (Van de Peer and De Wachter, 1994).

Plant cultivation and DNA extraction from the rhizosphere.

Wheat plants (*Triticum aestivum* L. cv. Bussard) were grown in a soil consisting of the agricultural CB soil (Souza et al., 2003a) mixed in a 1:1 ratio (w/w) with quartz sand. Sixteen wheat seeds were sown in square PVC pots containing 250 g of soil dry weight. A spontaneous rifampicin resistant derivative of *phlD*$^+$ *Pseudomonas* isolate PWB532, representing DGGE group E, was introduced into soil to densities of 0, 10, 10^2, 10^3, 10^4, and 10^6 cells per gram and an initial water content of 20% (v/w). One additional control treatment consisted of soil autoclaved twice (with a 24 h interval between the two autoclave runs) to eliminate putative indigenous *phl*D$^+$ isolates. Each treatment consisted of three replicates. After 10-12 days of cultivation in a growth chamber at 20^{o}C with a 16-h photoperiod, wheat plants were harvested and rhizosphere samples were prepared for 1) enumeration of the introduced strain on selective agar plates, and 2) for direct DNA extraction followed by PCR-DGGE. For enumeration of the introduced strain, 0.5 g of roots with associated rhizosphere soil was suspended in 5.0 ml of sterile distilled water and shaken vigorously for 1 min on a Vortex mixer; samples were subsequently sonicated in an ultrasonic cleaner for 1 min and dilution plated onto KMB agar supplemented with delvocid (100 mg/l), chloroamphenicol (13 mg/l), ampicillin (40 mg/l) and rifampicin (100 mg/l) (Simon and Ridge, 1974). Plates were incubated for 3 days at 25^o C and colonies were enumerated. For direct DNA extraction from the rhizosphere, 0.5 g of roots with associated rhizosphere soil was suspended in 1.0 ml SPB (Saline Phosphate Buffer), shaken vigorously for 1 min on a Vortex mixer, and sonicated in an ultrasonic cleaner for 1 min. Roots were discarded and the suspension was centrifuged for 1 min at 10.000rpm. An additional amount of 0.5g rhizospheric soil of the same treatment was added and the sample was subsequently processed by beat beaten (3 times for 90 sec each). Cells were lysed following the protocol of the FastDNA^R SPIN Kit for Soil (BIO 101). The DNA pellet was dissolved in 50 μl Tris-EDTA (10 mM Tris, 0.1 mM EDTA, pH 8). PCR amplification of extracted DNA was performed in 50 μl reaction mixtures containing approximately 10-50 ng DNA. This amount of DNA was, in most cases, acquired after a 100-fold dilution of the DNA obtained with the FastDNA^R SPIN Kit. To enhance the specificity of the PCR reaction, a ramping-PCR was carried out as follows: the annealing temperature was initially 60^{o}C, and it was increased up to 72^{o}C proceeding in steps of 0.1^{o}C. The PCR programme consisted of an initial denaturation at 94^{o}C for 3 min, followed by 30 cycles of 94^{o}C for 30 sec, 60^{o}C for 30 sec, 0.1^{o}C/sec up to 72^{o}C, and 72^{o}C for 1 min.

PCR amplification was carried out as described above. The primer concentration used here was 20 pmol instead of 40 pmol per reaction.

Next to wheat, similar experiments were performed with sugar beet (*Beta vulgaris* cv. Auris). Twenty-eight sugar beet seeds were sown in small square pots containing 250 g dry weight of soil and cultivated in a climate room with controlled environment at 20^{o}C and a 16-h photoperiod. Genotypically different *phlD*$^{+}$ isolates were introduced separately into soil at a density of approximately 10^4 CFU/g soil. The *phlD*$^{+}$ isolates were spontaneous rifampicin-resistant derivatives of isolates PWB233 (DGGE group A), PSC2218 (DGGE group B), PPB2310 (DGGE group C), PSB211 (DGGE group D), PWB532 (DGGE group E), PPB3512 (DGGE group F), PSC415 (DGGE group Z) and Q8R1-96 (DGGE group G). For short-term colonization experiments, the sugar beet plants were harvested after 10-12 days cultivation. For long-term colonization studies, sugar beet plants were grown in the same pots for 6 successive cycles of 10-12 days each. Twice a week, the plants were treated with 1/3 Hoaglund's solution (macro-elements only). After 10-12 days of growth, plants were harvested and their root system with rhizosphere soil was collected. Excess of root material was mixed through the cultivated soil and represented approximately 0.125% (w/w) of the soil dry weight. The cultivated soil was subsequently returned to the same pot and replanted with sugar beet seeds. This process of plant growth and harvesting was repeated for six successive cycles. Four replicates were included per treatment. For both short-term and long-term colonization experiments, rhizosphere samples were plated onto selective media and subjected to direct DNA extraction as described earlier for the experiments with wheat.

Statistical analysis.

Population densities of the introduced *phlD*$^{+}$ fluorescent *Pseudomonas* were $\log_{10}$ transformed prior to statistical analysis. For the colonization assays with sugar beet seedlings, differences in population densities between the introduced strains were analyzed for each successive growth cycle by analysis of variance (ANOVA) followed by Tukey's Studentized Range Test (SAS Institute Inc., Cary, NC).

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Chapter 3

Influence of plant species on population dynamics, genotypic diversity and antibiotic production in the rhizosphere by indigenous *Pseudomonas* species

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Abstract

The population dynamics, genotypic diversity and activity of naturally-occurring 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas* spp. was investigated for four plant species (wheat, sugar beet, potato, lily) grown in two different soils. All four plant species tested, except lily and in some cases wheat, supported relatively high rhizosphere populations ($5*10^4$ to $1*10^6$ CFU g^{-1} root) of indigenous DAPG-producing *Pseudomonas* spp. during successive cultivation in both a take-all suppressive and a take-all conducive soil. Although lily supported on average the highest population densities of fluorescent *Pseudomonas* spp., it was the least supportive of DAPG-producing *Pseudomonas* spp. of all four plant species. The genotypic diversity of 492 DAPG-producing *Pseudomonas* isolates, assessed by Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the *phlD* gene, revealed a total of 7 genotypes. Some of the genotypes were found only in the rhizosphere of a specific plant, whereas the predominant genotypes were found at significantly higher frequencies in the rhizosphere of three plant species (wheat, sugar beet and potato). Statistical analysis of the *phl*D^+ genotype frequencies showed that the diversity of the *phl*D^+ isolates from lily was significantly lower than the diversity of *phlD*$^+$ isolates found on wheat, sugar beet or potato. Additionally, soil type had a significant effect on both the *phlD*$^+$ population density and the *phlD*$^+$ genotype frequencies, with the take-all suppressive soil being the most supportive. HPLC analysis further showed that the plant species had a significant effect on DAPG-production by the indigenous *phlD*$^+$ population: the wheat and potato rhizospheres supported significantly higher amounts of DAPG produced per cell basis than the rhizospheres of sugar beet and lily. Collectively, the results of this study showed that the host plant species has a significant influence on the dynamics, composition and activity of specific indigenous antagonistic *Pseudomonas* spp.

Keywords: *Pseudomonas*, 2,4-diacetylphloroglucinol (DAPG), host preference, genotypic diversity, antibiotic production in the rhizosphere

Introduction

During the last three decades it has been demonstrated that secondary metabolites produced by antagonistic bacteria play key roles in the suppression of various soil-borne plant pathogens (Thomashow and Weller, 1996; Raaijmakers et al., 2002). The antibiotic 2,4-diacetylphloroglucinol (DAPG) has received considerable attention and has been implicated in biological control of many plant pathogens by fluorescent *Pseudomonas* strains. The activity of DAPG against bacteria, fungi and nematodes has been well documented (Defago, 1993; Keel et al., 1992; Cronin et al., 1997a, 1997b; De Souza et al., 2003). DAPG-producing *Pseudomonas* spp. have been isolated from the rhizosphere of different crops grown in soils from diverse geographic regions (Keel et al., 1996). More specifically, DAPG-producing *Pseudomonas* spp. are predominant in soils that are naturally suppressive to take-all disease of wheat (Raaijmakers et al., 1997; McSpadden et al., 2000; De Souza et al., 2003) and have been isolated from soils that are suppressive to black root rot of tobacco (Keel et al., 1996; Ramette et al., 2003).

It has been postulated that the genotypic diversity within a group of microorganisms that share the same antagonistic trait provides a largely untapped resource for improving biological control of soil-borne pathogens (De Souza et al., 2003; Raaijmakers and Weller, 2001;Handelsman and Stabb, 1996). Different genotypes of DAPG-producing *Pseudomonas* spp. have been reported to differ in their ability to: 1) colonize the wheat rhizosphere and suppress take-all disease of wheat (De Souza et al., 2003; Raaijmakers and Weller, 2001), 2) colonize the rhizosphere of pea (Landa et al., 2002), 3) colonize roots of two maize inbred lines and their hybrid at different physiological stages (Picard et al., 2004; Picard et al., 2000), 4) suppress Fusarium crown and root rot and Pythium root rot (Sharifi-Tehrani et al., 1998), 5) utilize different carbon sources and catalyze the degradation of 1-aminocyclopropane-1-carboxylate (Wang et al., 2001), and 6) produce other antibiotics in addition to DAPG (Keel et al., 1996). To date, two studies have supported the hypothesis that certain indigenous *phlD*$^+$ genotypes preferentially colonize the roots of specific crop plants. In the first study, 16 genotypic groups were identified among 101 indigenous isolates obtained from the rhizosphere of wheat grown in a take-all suppressive soil (Raaijmakers and Weller, 2001): one genotypic group, classified as BOX-PCR genotype D, explained almost 50% of the diversity and was shown to be highly rhizosphere competent on wheat [10,13]. In the second study, Landa et al. (2002) identified 17 whole-cell BOX-PCR groups among more than 300 isolates obtained from the rhizosphere of pea; BOX-PCR genotypes D and P explained 47.3 and 48.0%, respectively, of the diversity and were significantly better colonizers of the pea rhizosphere than isolates representing other genotypes. Given

that rhizosphere competence is an essential prerequisite for successful biocontrol (Bull et al., 1991; Raaijmakers et al., 1995; Johnson, 1994; Raaijmakers and Weller, 1998; Lugtenberg et al., 2001), knowledge of the compatibility between host plant species and genotypes of antagonistic microorganisms is essential to further improve biological control. In addition to rhizosphere competence, *in situ* expression of the antagonistic trait is also crucial for successful biological control. To date, *in situ* DAPG production has been mainly demonstrated for specific model strains introduced into the rhizosphere at high densities (Thomashow et al., 1997; Notz et al., 2001; Maurhoffer et al., 1995). However, the effect of the host plant species on *in situ* DAPG production by indigenous *Pseudomonas* populations has not been addressed in detail and, to our knowledge, only been reported for the rhizosphere of wheat growing in a take-all suppressive soil (Raaijmakers et al., 1999).

The aim of this work was to study the influence of the host plant species on the population dynamics, genotypic diversity and activity of indigenous DAPG-producing *Pseudomonas* spp. Four different plant species were included in this study, i.e. wheat, sugar beet, potato and lily. Population dynamics of indigenous DAPG-producing *Pseudomonas* spp. was monitored in the rhizosphere of the four plant species grown in two agricultural soils from The Netherlands. Secondly, the genotypic diversity of indigenous DAPG-producing *Pseudomonas* isolates associated with the roots of the four different plant species was determined by Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the *phl*D gene. Finally, the influence of each of the four plant species on *in situ* DAPG production by the indigenous *phlD*$^+$ population was determined by High Pressure Liquid Chromatography (HPLC).

Results

Effect of plant species on population dynamics of fluorescent *Pseudomonas* spp.

The four plant species were grown in the take-all suppressive SSB soil or the take-all conducive CB soil for five successive growth cycles. For growth cycles 1, 2, 3 and 5, the population density of total culturable fluorescent *Pseudomonas* was determined in the rhizosphere of each of the four plant species. For wheat, the population density of total fluorescent *Pseudomonas* spp. increased during successive cycling and ranged from $2*10^5$ to $6*10^6$ CFU/g root in CB soil and from $8*10^5$ to $4*10^6$ CFU/g root in SSB soil. For sugar beet, the total population of fluorescent pseudomonads ranged from $1*10^6$ to $5*10^6$ CFU/g root in both soils. For potato, the total population densities ranged from $2*10^6$ to $1*10^7$

CFU/g root in both soils. For lily, population densities of total fluorescent *Pseudomonas* spp. ranged during successive cycling from approximately $1*10^7$ to $4*10^6$ CFU/g root in CB soil and from $2*10^7$ to $3*10^6$ CFU/g root in SSB soil. To quantitatively determine the effect of the host plant species on the population dynamics of total fluorescent *Pseudomonas* spp., the AUCPC was calculated **(Figure 1).**

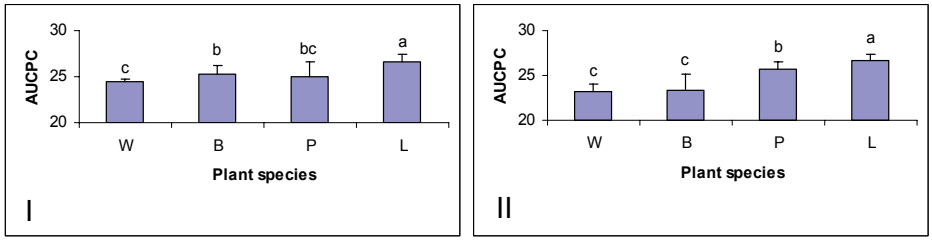

Figure 1: Area Under the Colonization Progress Curve (AUCPC) for the population of fluorescent *Pseudomonas* spp. in the rhizosphere of wheat (W), sugar beet (B), potato (P) and lily (L) grown in SSB (I) or CB soil (II). Mean values of 6 replicates are given. Error bars represent the standard error of the mean. For each soil, mean values with different letters are statistically different (P=0.05).

The results showed a significant effect of the plant species on the population densities of fluorescent *Pseudomonas* spp.: for the lily rhizosphere, the AUCPC in both SSB and CB soil was significantly higher than the AUCPC calculated for the other three plant species; the wheat rhizosphere presented the lowest AUCPC of all four plant species tested.

Effect of plant species on population dynamics of *phlD*$^+$ *Pseudomonas* spp.

Indigenous *Pseudomonas* spp. that harbor the *phlD* gene were isolated from the rhizosphere of all four plant species, although from the lily rhizosphere relatively few *phlD*$^+$ isolates were recovered. In the rhizospheres of sugar beet and potato grown in SSB or CB soil, *phlD*$^+$ population densities ranged from $4.7x10^4$ to $1.3x10^6$ CFU/g root during successive cycling (**Figures 2I and 2II).** The AUCPC for the *phlD*$^+$ pseudomonads showed a significant effect of the plant species, with lily being the least supportive of *phlD*$^+$ *Pseudomonas* spp. **(Figures 2III and 2IV)**. Soil also had a significant ($P<0.05$) effect on the *phlD*$^+$ population, with a higher AUCPC for the take-all suppressive SSB soil (data not shown). The frequency of the *phl*D$^+$ *Pseudomonas* population relative to the total fluorescent *Pseudomonas* population was significantly higher for the sugar beet rhizosphere than for the lily rhizosphere in both SSB and CB soils **(Table 2).**

Table 2: Relative frequencies of *phlD*$^+$ *Pseudomonas* spp. in the rhizosphere of wheat, sugar beet potato and lily plants grown in SSB or CB soil for five successive growth cycles. The relative frequency is the percentage (%) of *phlD*$^+$ pseudomonads relative to the total number of fluorescent pseudomonads in the rhizosphere of each of the four plant species. Mean numbers of 6 replicates are given. Means followed by a different letter are statistically different (P=0.05). Numbers between brackets refer to the standard error of the mean.

	Soil	
Crop	SSB	CB
Wheat	6.8 (2.2) ab	3.7 (1.2) ac
Sugar beet	8.7 (1.7) a	9.2 (1.8) a
Potato	6.5 (1.5) ab	5.9 (3.3) ac
Lily	2.7 (1.0) b	0.2 (0.1) c

Additionally, for the lily rhizosphere, the relative frequency of the *phl*D$^+$ *Pseudomonas* population was significantly higher in the SSB soil than in the CB soil. The relative frequency of the *phl*D$^+$ *Pseudomonas* population did not differ significantly between wheat, sugar beet and potato.

Effect of plant species on genotypic diversity of *phlD*$^+$ *Pseudomonas* spp.

From wheat, sugar beet, potato and lily, a total of 121, 193, 150 and 28 *phlD*$^+$ isolates, respectively, were randomly selected from both soils (SSB and CB) to determine the genotypic diversity by DGGE analysis of the *phlD* gene. This total number of 492 *phlD*$^+$ isolates represented approximately 52% of all the *phlD*-positive colonies detected by colony hybridization. For SSB soil, a total of 79, 110, and 87 *phlD*$^+$ isolates were selected for genotypic analysis from wheat, sugar beet, and potato, respectively, with an average number of approximately 16 isolates for each of the six replicates per crop. The 28 *phlD*$^+$ isolates selected from roots of lily grown in the SSB soil represented all the *phlD*$^+$ isolates detected during cycling, with most isolates (N=21) obtained in the first growth cycle. For CB soil, a total of 42, 83, and 63 *phlD*$^+$ isolates were selected for genotypic analysis from wheat, sugar beet, and potato, respectively, with an average number of approximately 10 isolates for each of the six replicates per crop. Seven different DGGE groups (designated DGGE-A, B, C, D, E, F and Z) were detected among these 492 indigenous *phlD*$^+$ *Pseudomonas* isolates. Certain *phlD*$^+$ genotypes were found at relatively low frequencies in the rhizosphere of only one host plant species, whereas other genotypic groups occurred at relatively high frequencies in the rhizosphere of multiple plant species **(Figure 3).**

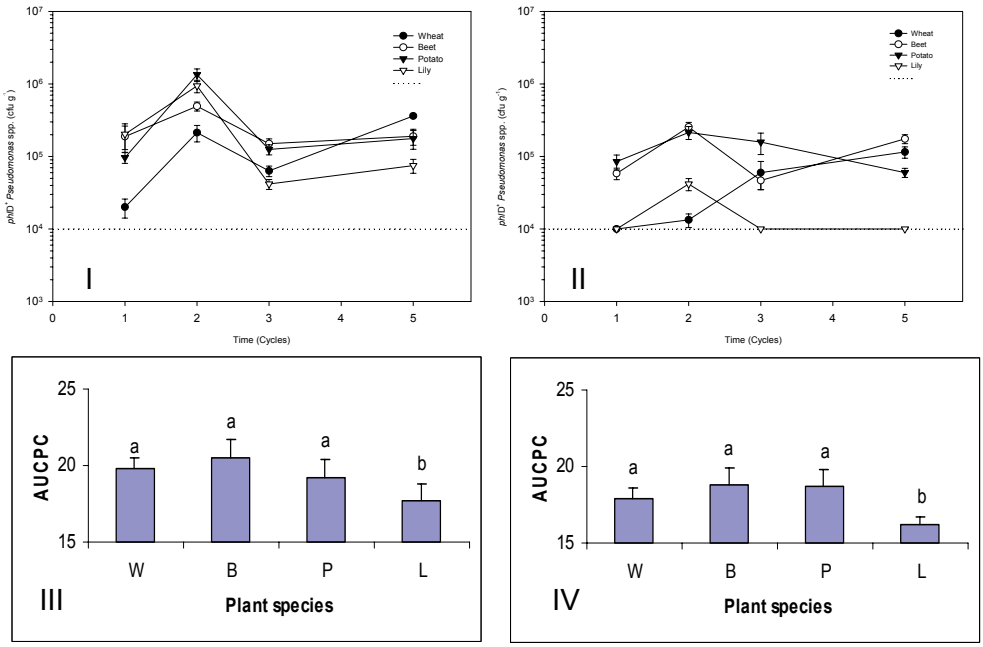

Figure 2: Population dynamics of *phl*D$^+$ *Pseudomonas* spp. in the rhizospheres of wheat, sugar beet, potato and lily grown for five successive cycles of 5 to 7 weeks each in SSB (I) or CB soil (II). Error bars represent the standard error of the mean. The dashed line represents the limit of detection (10^4 CFU/g root). In CB soil, *phlD*$^+$ populations were not detectable in cycles 3 and 5 and are represented as the detection limit of 10^4 CFU/g root. Panels III and IV present the Area Under the Colonization Progress Curve (AUCPC) for the population of *phlD*$^+$ *Pseudomonas* spp. in the rhizosphere of wheat (W), sugar beet (B), potato (P) and lily (L) grown in SSB soil (III) or CB soil (IV). Mean values of 6 replicates are given. Error bars represent the standard error of the mean. For the AUPC, mean values with different letters are statistically different (P=0.05).

For example, DGGE-genotype D was only found in the rhizosphere of sugar beet grown in SSB soil at a relatively low frequency of approximately 2%. In contrast, DGGE-genotype E was a dominant group in the rhizospheres of wheat, sugar beet and potato grown in the SSB soil and comprised on average 37, 47 and 41 %, respectively, of the *phlD*$^+$ isolates **(Figure 3I).** In addition to DGGE-group E, genotypes Z and B were major genotypic groups in the rhizosphere of wheat, sugar beet and potato grown in CB soil **(Figure 3II).** On roots of sugar beet seedlings cultivated in the SSB soil, all seven *phlD*$^+$ genotypes were found whereas on roots of wheat and potato 6 different genotypes were found **(Figure 3I).** In the rhizosphere of lily, only two *phlD*$^+$ genotypes were found. Also in the CB soil, different numbers of *phlD*$^+$ genotypes were detected on roots of the plant species tested: 3, 4 and 5 genotypes were detected in the rhizosphere of potato, wheat and sugar beet, respectively **(Figure 3II).**

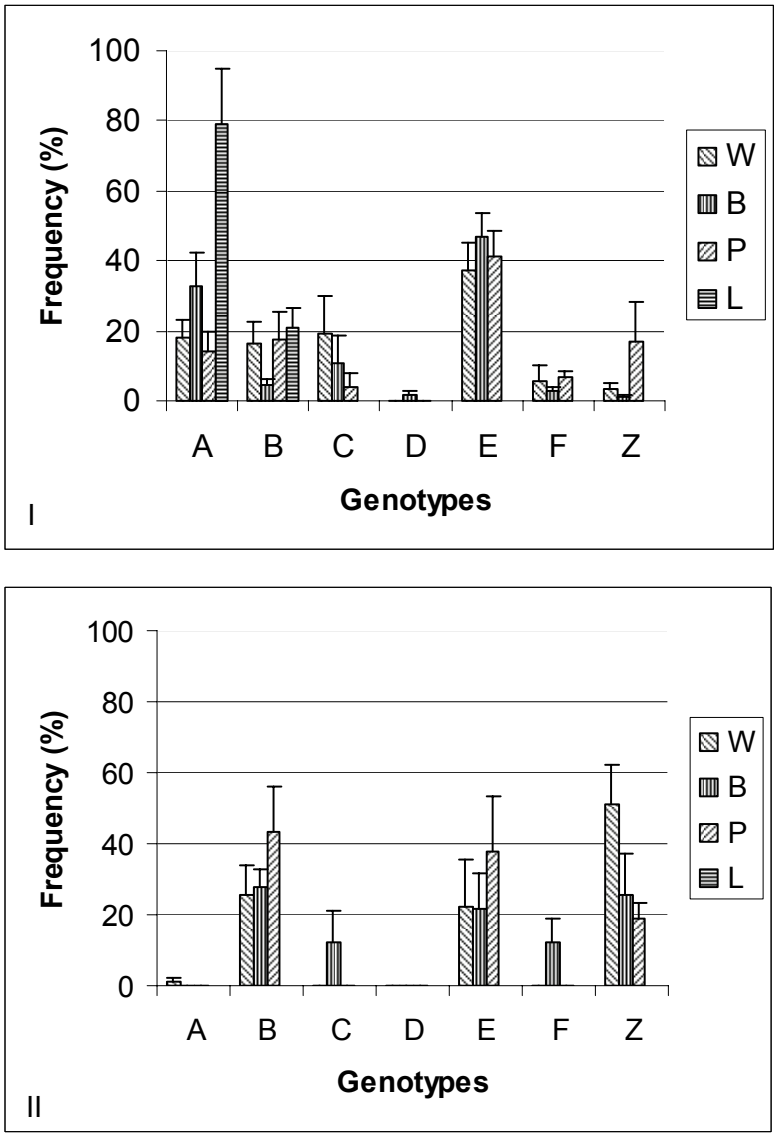

Figure 3: Genotypic diversity of *phl*D$^+$ pseudomonads isolated from the rhizospheres of wheat (W), sugar beet (B), potato (P) and lily (L) grown in SSB (I) or CB soil (II). Genotypic grouping is based on DGGE analysis of the *phlD* gene. For each soil, the frequencies of the *phl*D$^+$ genotypes are expressed as percentages (%) of the total number of *phl*D$^+$ isolates tested per replicate. Mean values of 6 replicates are given. Error bars represent the standard error of the mean.

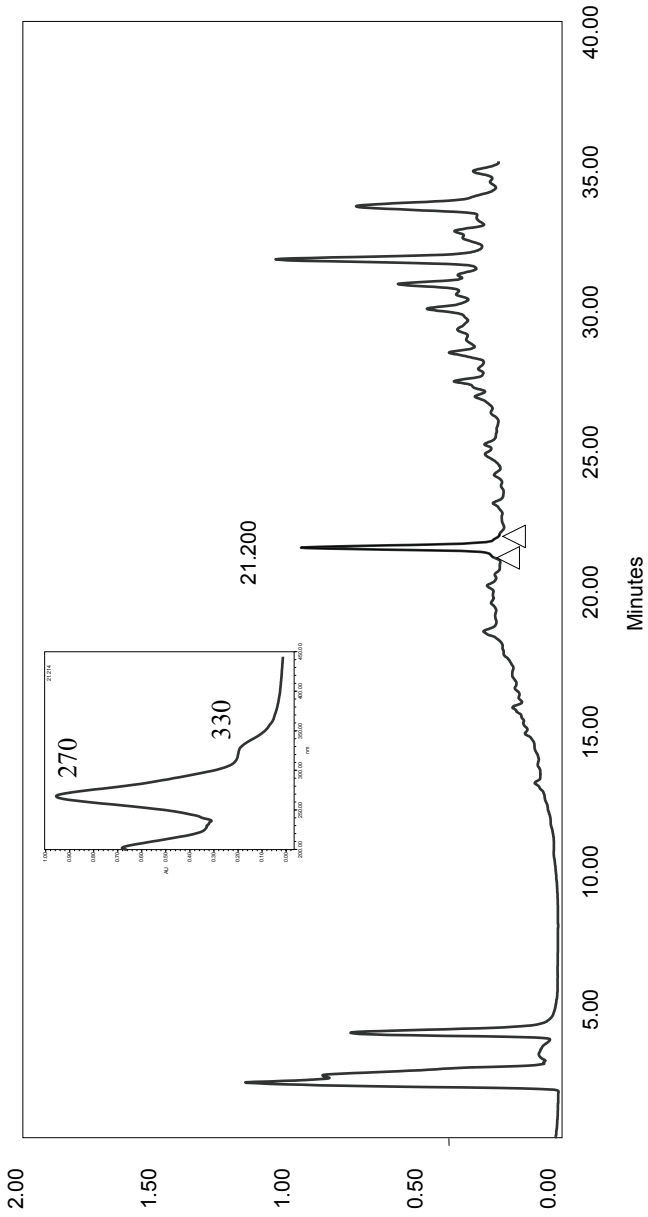

Figure 4: Typical chromatogram obtained by high pressure liquid chromatography (HPLC) of a sugar beet rhizosphere sample. 2,4-Diacetylphloroglucinol (DAPG) had a retention time of 21.2 min. Peak purity and spectral characteristics were determined by photodiode array spectroscopy and showed the expected spectrum of DAPG with peak maxima at 270nm and 330 nm (insert). Triangles (Δ) indicate the baseline borders used for the integration of the DAPG peak area.

The genotypic diversity of the *phlD*$^+$ isolates obtained from the lily rhizosphere was significantly different from the diversity of *phlD*$^+$ isolates obtained from roots of the other three plant species. Only DGGE-genotypes A and B were detected in the lily rhizosphere grown in the SSB soil at average frequencies of 79 and 21%, respectively. The Shannon-Weaver index, used as a measure of the genotypic diversity of the *phlD*$^+$ isolates, was 74.3 for the sugar beet rhizosphere, 69.5 for populations from the wheat rhizosphere, and 60.1 for the potato rhizosphere. Statistical analysis of the *phlD*$^+$ genotype frequencies, however, showed that the plant species had a significant ($P<0.05$) effect only in the case of the take-all suppressive SSB soil: the genotypic diversity of *phlD*$^+$ isolates from lily was significantly lower than the genotypic diversity of *phlD*$^+$ isolates found on the other three plant species. For the take-all conducive CB soil, no significant effects of the plant species on the *phlD*$^+$ genotype frequencies was found. Additionally, soil had a significant ($P<0.05$) effect on the *phlD*$^+$ genotype frequencies, with a higher frequency for the take-all suppressive SSB soil. This significant soil effect was supported by the Shannon-Weaver index: 64.1 for the SSB soil versus 59.8 for the CB soil.

Effect of plant species on *in situ* DAPG production

After five successive cycles, the SSB and CB soils were planted again with each of the four plant species. After five to seven weeks of plant growth, *phlD*$^+$ pseudomonads were enumerated, their genotypic diversity assessed, and DAPG concentrations determined by HPLC analysis. In the rhizospheres of sugar beet and potato grown in SSB or CB soil, *phlD*$^+$ population densities ranged from $2.6x10^5$ to $7.9x10^5$ CFU/g root. In the rhizosphere of wheat grown in SSB soil, similar *phlD*$^+$ population densities ($1.6x10^5$ CFU/g root) were found. On roots of wheat grown in the CB soil, *phlD*$^+$ population densities were relatively low ($2*10^4$ CFU/g root). In the rhizosphere of lily, *phlD*$^+$ pseudomonads were not detected for both CB and SSB soils. The frequencies of *phlD*$^+$ genotypes found in this experiment were very similar to the frequencies described previously **(Figure 3l).** For example, DGGE-genotype E was again the most dominant group in the rhizospheres of wheat, sugar beet and potato grown in the SSB soil and comprised 43, 51 and 48 %, respectively, of the *phlD*$^+$ isolates. DAPG had a retention time of approximately 21 min and its spectral characteristics matched with our spectral library **(Figure 4**). No DAPG was detected in the rhizosphere of lily grown in CB or SSB soil **(Figure 5).** For the other three plant species, significant differences were found for the *in situ* DAPG concentrations detected **(Figure 5**). For SSB soil, significantly higher amounts of DAPG were detected in the wheat and potato rhizosphere than in the rhizosphere of sugar beet.. When the amounts of DAPG detected were related to the

population densities of indigenous *phlD*$^+$ pseudomonads, determined in the same samples used for DAPG detection, the wheat rhizosphere supported a more than 7-fold higher DAPG concentration than the rhizosphere of sugar beet **(Figure 5III)**

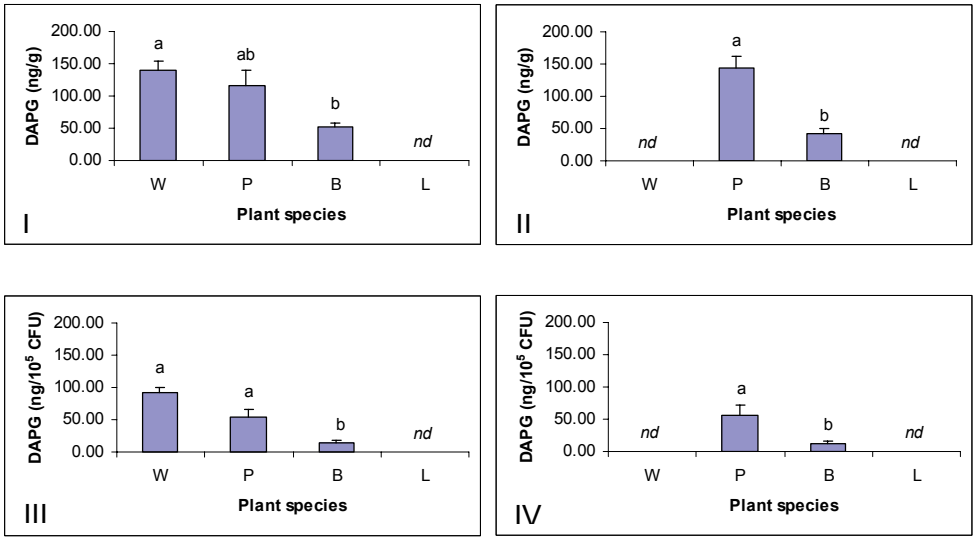

Figure 5: DAPG concentrations detected in the rhizospheres of wheat (W), sugar beet (B), potato (P) and lily (L) plants grown in SSB (panels I and III) or CB soil (panels II and IV). Panels I and II represent the total amount of DAPG detected per gram of root fresh weight of each of the four plant species; panels III and IV represent the total amount of DAPG per 10^5 CFU of *phl*D$^+$ pseudomonads detected in the rhizosphere of each of the four plant species. Mean values of 4 replicates are given. Error bars represent the standard error of the mean. For each of the panels, mean values with different letters indicate statistically significant differences (P=0.05).

In CB soil, DAPG was detected in the potato and sugar beet rhizospheres and not in the wheat and lily rhizospheres **(Figures 5II and 5IV).** As observed in the SSB soil, the potato rhizosphere supported significantly higher amounts of DAPG produced per population unit of *phlD*$^+$ pseudomonads than sugar beet **(Figure 5IV).**

Discussion

The effect of plant species on microbial communities (Grayston et al., 1998; Miethling et al., 2000; Wieland et al., 2001; Kuske et al., 2002) and populations of specific bacterial genera has been well documented (Lemanceau et al., 1995; DiCello et al., 1997; Berg et al., 2002; Briones et al., 2002; Mazzola and Gu, 2002; Gu and Mazzola, 2003; Da Mota et al., 2002). For example, the composition of populations of fluorescent *Pseudomonas* spp. may vary significantly within the rhizosphere of different plant species cultivated in the same soil (Lemanceau et al., 1995), and may change dramatically over time in response to

age-related physiological changes in plant species (Mazzola, 1999; Mazzola and Gu, 2000). In contrast, relatively few studies have addressed the effect of plant species on the dynamics, composition and activity of indigenous bacterial populations that share a specific antagonistic trait. The present study showed that all four plant species tested, except lily and in some cases wheat, supported relatively high rhizosphere populations ($5*10^4$ to $1*10^6$ CFU g^{-1} root) of indigenous DAPG-producing *Pseudomonas* spp. when grown in the same soils. Similar population densities of DAPG-producing *Pseudomonas* spp. have previously been reported for the rhizosphere of wheat (Raaijmakers et al., 1997; De Souza et al., 2003) and pea (Landa et al., 2002), grown in soils naturally suppressive to *Gaeumannomyces graminis* var. *tritici* and *Fusarium oxysporum* f.sp. *pisi*, respectively. Higher densities ($7*10^6$ CFU g^{-1} root) were reported for the rhizosphere of tobacco grown in a soil suppressive to *Thielaviopsis basicola* (Ramette et al., 2003). Although lily supported on average the highest population densities of fluorescent *Pseudomonas* spp. of all four crops, it was the least supportive of DAPG-producing pseudomonads. In a previous study, similar adverse effects on populations of DAPG-producing pseudomonads were observed for oats: population densities of DAPG-producing pseudomonads dropped within two successive growth cycles of oats to densities below the detection limit of 10^4 CFU g^{-1} root (Raaijmakers and Weller, 1998). Interesting in this context is the fact that both oats and members of the Liliaceae are well known for their production of steroid saponins in the rhizosphere (Mahato et al., 1982; Mimaki et al., 1999; Sang et al., 2003; Park et al., 2003). Saponins, including Avenacoside A and B from oats, exhibit activity against fungi, Gram-negative and Gram-positive bacteria (reviewed in Mahato et al., 1982). Studies on the chemical constituents of root exudates of lily, including saponins and other allelochemical compounds (Bertin et al., 2003), are being initiated to further unravel their role, if any, in the decline of indigenous DAPG-producing pseudomonads.

The population data also showed a relatively high density of DAPG-producing *Pseudomonas* spp. in the rhizosphere of sugar beet and potato grown successively in the take-all conducive CB soil and in the take-all suppressive SSB soil. Given that take-all suppressiveness of several soils, including the SSB soil (De Souza et al., 2003; Weller et al., 2002; Raaijmakers and Weller, 1998), functions in part through the enrichment of DAPG-producing fluorescent *Pseudomonas* spp. on roots of wheat, these results suggest that interruption of wheat monoculture with either sugar beet or potato will not upset the take-all decline phenomenon and that both crops may even have potential to induce suppressiveness of soils to take-all. Previous studies (Shipton, 1975) indicated that interruption of wheat monoculture with a rotation (break) crop may upset the take-all decline

phenomenon, so that upon return to wheat, the cycle of severe disease followed by decline will occur again. Rotation crops such as beans, alfalfa and oats made soil conducive to take-all, whereas grass mixtures maintained the suppressiveness (Raaijmakers and Weller, 1998; Cook, 1981). Suppressive soil grown to potatoes, however, showed some evidence of suppressiveness to take-all (Cook, 1981). This latter observation is in line with our results that showed that the potato rhizosphere supports both colonization and activity of indigenous DAPG-producing *Pseudomonas* spp. Additional experiments will be required to verify if rotation crops such as sugar beet and potato will not adversely affect take-all suppressiveness, or alternatively can be used to shorten the time period required to induce suppressiveness in soils to take-all.

A confounding factor in this context involves the genotypic composition of the DAPG-producing *Pseudomonas* population resident in the rhizospheres of other crop species. The genotypic diversity of 492 *phlD*$^+$ *Pseudomonas* isolates obtained in the present study, assessed by means of DGGE-analysis of the *phlD* gene, revealed a total of 7 genotypic groups. Some of the genotypic groups were found only in the rhizosphere of a specific plant species, whereas other genotypic groups were found in relatively high frequencies in the rhizosphere of multiple plant species. In general, no statistically significant effect of plant species on the *phl*D$^+$ genotype frequencies was found for both soils. Only lily had a significant effect on genotype frequencies with DGGE-genotype A being the most dominant. It should be emphasized that this may have been due, at least in part, to the smaller sample size of *phlD*$^+$ isolates obtained from lily. DGGE-genotype E represented almost 50% of the genotypic diversity present among the *phlD*$^+$ isolates originating from the other three plant species grown in the SSB soil. The widespread occurrence of DGGE-genotypes E and also B and Z in the rhizosphere of multiple crops opens up opportunities to apply specific strains or consortia of these three genotypically different DAPG-producing *Pseudomonas* strains for control of soil-borne pathogens on multiple crops, i.e. wheat, sugar beet and potato.

HPLC analysis showed that the plant species had a significant effect on DAPG-production by the indigenous *phlD*$^+$ population: the wheat and potato rhizospheres supported significantly higher amounts of DAPG produced per cell basis than the rhizospheres of sugar beet and lily. It should be noted that these studies were performed in small pots with a relatively high number of plants; whether these results can be extrapolated to field conditions remains to be determined. It is well known that the carbon composition of the growth medium significantly affects antibiotic production *in vitro* Nowak-Thompson et al., 1994; Shanahan et al., 1992). Therefore, variations in the carbon composition of root

exudates of different plant species may have contributed, at least in part, to the observed differences in DAPG production in the rhizosphere. The results obtained in this study support and extend previous results obtained in a gnotobiotic system with DAPG-producing *P. fluorescens* strain CHA0 introduced in the rhizosphere of wheat, maize, cucumber and bean (Notz et al., 2001). They showed that transcription of the *phlA* gene was significantly higher in the rhizospheres of the two monocots (wheat and maize) compared to the two dicots. In our study, DAPG was only detected by HPLC in rhizosphere samples when the population densities of the *phlD*$^+$ pseudomonads were higher than 10^5 CFU/g root. This is in agreement with results obtained previously (Raaijmakers et al., 1999), and may be due to technical limitations (detection limits) of the methodology, or confirm the observation that DAPG biosynthesis is dependent on cell density (Schnider-Keel et al., 2000). In addition to the rhizosphere, soil factors also can have a significant impact on the composition of rhizosphere microbial communities (Latour et al., 1996). In the present study, it was demonstrated that the diversity of *phlD*$^+$ *Pseudomonas* spp. was significantly higher in the take-all suppressive SSB soil than in the take-all conducive CB soil. Soil also appeared to be an important determinant of the genotypic composition of the *phlD*$^+$ *Pseudomonas* population. For example, DGGE-genotype Z was a dominant genotypic group in the CB soil, but only a minor group in the SSB soil.

In conclusion, the results of this study showed that host plant species and the soil have a significant influence on the dynamics, composition and activity of indigenous *phlD*$^+$ *Pseudomonas* spp. Traits of both the plant and the bacteria undoubtedly contribute to the affinity between some bacterial genotypes and certain host plant species (Landa et al., 2003). Identifying different genetic traits that have evolved in microorganisms to compete successfully in diverse soil and rhizosphere environments may allow maximizing root colonization and disease suppression (Weller et al., 2002). Knowledge of such genetic traits involved in host preference of the antagonistic bacteria obtained in the present study will help to identify strains that are adequately adapted to specific host-pathogen systems.

Material and methods

Bacterial strains and growth media

Pseudomonas strains were cultured routinely on King's medium B (KMB) agar [30] at 25^{o}C. Glycerol stocks of *Pseudomonas* isolates were made in liquid KMB containing 40% (v/v) glycerol and kept at –80^{o}C.

Soils

Two soils (CB and SSB) were obtained in December 1999 from agricultural fields at Woensdrecht, The Netherlands. Their physical-chemical characteristics were described previously (De Souza et al., 2003). In 1999, the CB soil was grown to wheat and sugar beet in a 1:2 year rotation scheme, whereas the SSB soil was cultivated continuously to wheat for 28 years. The CB soil is conducive to take-all disease of wheat, whereas the SSB soil is naturally suppressive to the take-all pathogen (De Souza et al., 2003). Both soils were collected from the upper 35-cm of the soil profile, air dried for approximately 10 days and subsequently dried at 20^{o}C for 24 hours. Prior to use, the soils were ground and passed through a 0.5-cm mesh screen. Soils were kept at room temperature prior to their use.

Plant cultivation

Sixteen wheat seeds (cv. Bussard) were sown in square plastic pots of 1.1 L, containing 800 g of soil, and covered with a 1-cm layer of soil. For cultivation of sugar beet, twenty-eight seeds (cv. Auris) were sown in square plastic pots of 1.1 L, containing 800 g of soil, and covered with a 1-cm layer of soil. Prior to sowing, sugar beet seeds were surface disinfected according to the protocol described by Keijer et al. (1997). For potato, two seed tubers (cv. Bintje) class S (virus free certified material) were planted at a depth of 6-8 cm in round plastic pots of 2.5 L containing 1200 g of soil. For lily, three bulbs (cv. Vivaldi) were planted at a depth of 6-8 cm in round plastic pots of 2.5 L containing 1500 g of soil. For long-term storage, the lily bulbs were kept at -2^{o}C in boxes with potting soil. Prior to planting, the bulbs were washed with tap water to remove the potting soil, then surface disinfected with a 0.5% solution of formalin and finally kept for one week at 4^{o}C in the dark. The four plant species were grown in a climate room with controlled environment at 15^{o}C and a 12-h photoperiod. Twice a week, the plants were treated with 1/3 Hoaglund's solution (macro-elements only). After four to five weeks of growth for wheat, sugar and potato and seven weeks of growth for lily, plants were harvested and their root system with rhizosphere soil was collected. Excess of root material was mixed through the cultivated soil and represented approximately 0.5%, 0.125%, 1% and 0.5% of the soil dry weight for wheat, sugar beet, potato and lily, respectively. The cultivated soil was subsequently returned to the same pot and replanted with the same plant species. This process of plant growth and harvesting was repeated for five successive cycles per soil. Six replicates were included per plant species and per soil type (CB and SSB).

Isolation of indigenous *phlD*$^+$ *Pseudomonas* spp. from the rhizosphere

At the end of each growth cycle, all plants were harvested and root samples with rhizosphere soil (0.5 g fresh weight) were dilution plated onto KMB agar supplemented with cyclohexamide (100 mg/l), chloroamphenicol (13 mg/l) and ampicillin (40 mg/l) (KMB$^+$) (Simon and Ridge, 1974). Plates were incubated for 48 hours at 25^{o}C and subsequently the total culturable pseudomonad populations were enumerated. Fluorescent colonies were enumerated under UV. The population density of indigenous fluorescent pseudomonads harbouring the *phlD* gene was determined by colony hybridization followed by PCR using a specific probe and primers (Raaijmakers et al., 1997).

Genotypic diversity of *phlD*$^+$ *Pseudomonas* spp.

Genotypic diversity of the indigenous *phlD*$^+$ *Pseudomonas* population was determined by Denaturing Gradient Gel Electrophoresis (DGGE) of a 350-bp fragment of the *phlD* gene. Based on sequence analyses of the *phlD* gene of multiple DAPG-producing *Pseudomonas* strains, and comparison with genotypic fingerprinting with neutral markers, DGGE analysis of the *phlD* gene was shown to be a rapid and reliable method to discriminate between different *phlD*$^+$ genotypes (Bergsma-Vlami et al., 2005). DNA from randomly selected colonies was amplified by PCR using primers DGGE292forCG and DGGE618rev (**Table 1**).

Table 1: Properties of the primers used for DGGE analysis of the *phlD* gene.

Primer	Sequence (5'-3')	G+C (%)	T_m (°C)
DGGE618rev	CCAGTTGCAGGACCAGTTCATC	55	67.9
DGGE292forCG	CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGGGGGGTGCTATCAACCCCAGGACACC	84	57.9

Prior to PCR, heat-lysed bacterial suspensions were prepared from pure cultures grown on KMB for 48 h at 25^{o}C. Two bacterial colonies were suspended in 100 μl of lysis solution (0.05 M NaOH, 0.25% sodium dodecyl sulfate [SDS]) and incubated for 15 min at 100^{o}C. Suspensions were centrifuged for 1 min at 7.000xg and diluted 40-fold in sterile distilled water. The PCR amplification was carried out in a 25-μl reaction mixture which contained 3 μl of a 40-fold diluted heat-lysed cell suspension, 1X GeneAmp ® PCR buffer (Applied Biosystems), 0.5 mM of each dATP, dCTP, dGTP, dTTP (Promega), 40 pmol of each primer

(Amersham Pharmacia Biotech, Roosendaal, Netherlands), 1.5 mM $MgCl_2$ and 1.0 U of AmpliTaq ® DNA polymerase (Applied Biosystems). The reactions were performed on a PTC-200 thermocycler (Biozym, Landgraaf, The Netherlands). The following conditions were used: 94°C for 3 min (1 cycle); 94°C for 30 s, 67°C for 30 s and 72°C for 60 s (30 cycles); and 72°C for 10 min (1 cycle). The PCR products were separated on a 1% agarose-Tris-acetic-EDTA (1XTAE) gel and visualised under UV with a 100-bp DNA Ladder (GibcoBRL) as marker. DGGE separation of the amplified *phl*D fragments was performed with 15 μl of the PCR products using the Dcode universal mutation detection system (Bio-Rad Laboratories, Hercules, Calif.). DGGE was performed with 8% (wt/vol) acrylamide gels containing a linear denaturing gradient ranging from 32% at the top of the gel to 41% at the bottom; 100% denaturant contained 7 M urea plus 40% (v/v) deionized formamide. *PhlD* fragments of nine genotypically different *Pseudomonas* strains were used as markers. Electrophoresis consisted of 10 min at 200 V and then 16 h at 85 V. After electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml) in 1X TAE (pH=8.3) and photographed under a UV transilluminator.

In situ antibiotic production

SSB and CB soils grown to the corresponding host plant species for 5 successive cycles (as described above) were kept at 4°C for two weeks and subsequently mixed with non-cycled soil of the same type in a 1:4 ratio (w/w) prior to initiation of the experiments. Pots containing 400 and 800 g soil were used for wheat or sugar beet and potato or lily, respectively. In all cases, plants were grown in a climate room with controlled environment at 15°C with a 12-h photoperiod. Twice a week the plants were treated with 1/3 Hoaglund's solution (macro-elements only). After five to seven weeks of growth, plants were harvested and their root system with rhizosphere soil was collected. Four replicates were included per soil type (CB and SSB). Total populations of culturable *Pseudomonas* spp. and *phlD*$^+$ *Pseudomonas* spp. were monitored according to the protocols described above.

DAPG was isolated from roots of wheat, sugar beet, potato and lily according to the method described by Bonsall et al. (1997). For the extraction of DAPG from the rhizosphere of wheat, potato, lily and sugar beet, approximately 44, 25, 18 and 12 g of roots with adhering soil, respectively, was used. Samples were transferred to 250-ml flasks with 50 ml of 80% acetone and acidified to pH 2.0 with 10% trifluoroacetic acid (TFA) and then shaken at 200 rpm for 2 h at room temperature. Samples were subsequently filtered through a Buchner funnel and the filtrate was then centrifuged at 19000xg for 30 min at 4°C. The supernatant was evaporated to a volume of approximately 10 ml, acidified to pH 2.0 with

10% TFA, extracted twice with 10 ml ethyl acetate (HPLC grade), and evaporated to dryness. Extracts were resuspended in 1 ml methanol (HPLC grade), and then centrifuged in an Eppendorf 5415 centrifuge (Eppendorf, Hamburg, Germany) at 9600xg for 4 min at room temperature prior to HPLC analysis. The HPLC system (Waters) consisted of a 717 autosampler, a 600E solvent delivery system, a 600 controller, and a 996 photodiode array detector. Root extracts were fractionated by C_{18} reverse phase HPLC (Waters symmetry column, 3.9 by 150 mm) with 100 µl sample injections. Solvent conditions included a flow rate of 0.5 ml/min with a 2 min initialization at 10% acetonitril (ACN) plus 0.1% TFA followed by a 28 min gradient to 100% ACN plus 0.1% TFA. HPLC gradient profiles were monitored at 270 and 330 nm which represent the peak maxima of DAPG in the designated solvent system. DAPG concentrations were calculated on the basis of the area of the DAPG peak using the 6-point standard curve [DAPG] = 8.3 X 10^{-5} X A, where A represents the peak area and [DAPG] represents the concentration of DAPG in ng. The retention time of DAPG in the conditions specified above was approximately 21 minutes. To make a correct comparison between the *in situ* DAPG production levels found in the rhizosphere of the different plants species, the extraction efficiencies of DAPG were determined by spiking a known amount (1-5 µg) of DAPG into rhizosphere samples of each of the four plant species. For each plant species, three replicates were used. For wheat, sugar beet, potato, and lily, the extraction efficiencies were 56 (± 0.64), 54 (± 5.70), 39 (± 1.98) and 34 (± 1.25) %, respectively. Consequently, *in situ* DAPG production levels detected in the rhizosphere of each of the plant species were corrected according to these extraction efficiencies.

Statistical analysis

Population densities of total and *phlD*$^+$ fluorescent *Pseudomonas* were $\log_{10}$ transformed prior to statistical analysis. The Area Under the Colonization Progress Curve (AUCPC), which is a measure to assess rhizosphere colonization over time (i.e. for all five successive growth cycles), was calculated for each of the four plant species grown in CB and SSB soils using the trapezoidal integration method described by Landa et al. (2002). Population densities and AUCPC values were subjected to analysis of variance (ANOVA) followed by Tukey's Studentized Range Test, after certifying normal distribution and homogeneity of variances (SAS Institute Inc., Cary, NC). The genotypic diversity (*H*) of *phlD*$^+$ *Pseudomonas* isolates was assessed by the Shannon-Weaver index, which is described by the equation: $H = \Sigma(pi*logpi)$, where pi = ni/Ni (n_i = number of isolates per genotype and Ni = total number of isolates). The Shannon-Weaver index included similar numbers of individuals (Ni) from the wheat, sugar beet and potato rhizospheres. The

number of *phl*D$^+$ isolates from roots of lily plants was relatively low and different from the other three plant species and therefore not included in these calculations. For each soil and each plant species, isolates classified as a specific *phlD*$^+$ genotype were expressed as percentages of the total number of *phl*D$^+$ isolates obtained per replicate. These genotype frequencies (%) were arcsine transformed prior to statistical analysis. Statistical differences in *phlD*$^+$ genotype frequencies between plant species were determined by the non-parametric Kruskal-Wallis test followed by Tukey's Studentized Range Test. For each plant species, 6 replicates were used per soil. Differences in amounts of DAPG detected in the rhizospheres of the plant species were determined by ANOVA followed by Tukey's Studentized Range Test. For each plant species, 4 replicates were used per soil.

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Chapter 4

Rhizosphere competence of genotypically different antibiotic-producing *Pseudomonas* species

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In preparation

Rhizosphere competence of genotypically different antibiotic-producing *Pseudomonas* species

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Abstract

Genotypically different strains of 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas* spp. were evaluated for their ability to colonize the rhizosphere of four different host plants: wheat, sugar beet, potato and lily. We hypothesized that certain *Pseudomonas* genotypes have evolved a preference for colonization of specific crops, whereas others can colonize equally well multiple plant species. Three genotypes (A, Z and G) were shown to be superior in long-term colonization of roots of wheat, sugar beet and potato plants. These results suggest that the rhizosphere competence of these three genotypes is due to specific characteristics that enable these generalist strains to be competitive in different rhizosphere environments. On the other hand, successful establishment and survival of genotypes E, C and F was dependent on the plant species and, therefore, these strains are considered to be specialists. When introduced into the sugar beet rhizosphere, the genotypes differed significantly in their ability to produce DAPG *in situ*: genotype E produced much more DAPG per cell basis (143.6 ng per 10^5 cells) than the other genotypes with DAPG concentrations ranging from 1.00 to 57.3 ng per 10^5 cells. Dose-response experiments showed significant correlations between the rhizosphere competence of a genotype and *in situ* DAPG production levels. In general, these correlations suggest that genotypes that produce high amounts of DAPG *in situ* establish lower population densities in the sugar beet rhizosphere than genotypes that produce small amounts of DAPG per cell basis. To our knowledge, this is the first study that shows an inverse correlation between rhizosphere competence of fluorescent *Pseudomonas* strains and *in situ* antibiotic production.

Keywords: root colonization, *Pseudomonas fluorescens*, 2,4-diacetylphloroglucinol

Introduction

The rhizosphere forms a nutrient-rich niche for microorganisms due to exudation of organic acids, sugars, amino acids and other compounds (Berg et al., 2006, Lugtenberg et al., 1999, Raaijmakers et al., 2008). *Pseudomonas* species are important constituents of the rhizosphere (Rainey, 1999) and involved in promoting plant growth and health. They exert their beneficial effects on plant health via several different mechanisms, including active exclusion of plant pathogens from the rhizosphere (Weller, 1988, Cook et al., 1995; Thomashow and Weller, 1996, Handelsman and Stabb, 1996).

Strains of *Pseudomonas fluorescens* that produce the broad-spectrum antibiotic 2,4-diacetylphloroglucinol (DAPG) exhibit antifungal, antibacterial, and anthelminthic activity and have been implicated in biological control of multiple plant pathogens (reviewed in Haas and Defago, 2005). Most of the genotypes of DAPG-producing *Pseudomonas* described to date produce similar quantities of DAPG *in vitro* (Mavrodi et al., 2001) and *in situ* (Raaijmakers and Weller, 2001), have similar substrate utilization profiles (McSpadden et al., 2000, Raaijmakers and Weller, 2001) and do not differ markedly when compared by classical bacteriological tests. However, different genotypes may differ considerably in their rhizosphere competence and biocontrol activity (Sharifi-Tehrani et al. 1998; De Souza et al., 2003, Raaijmakers and Weller 2001, Landa et al., 2002, 2003, Picard et al., 2004, Bergsma-Vlami et al. 2005, De La Fuente et al. 2006, Mavrodi et al., 2006, Picard et al., 2008).

Rhizosphere competence is a dynamic process by which introduced bacterial inoculants establish on the seed and/or plant root, proliferate, colonize the root system and survive over a considerable time period in the presence of indigenous microorganisms (Weller, 1988). Rhizosphere competence is a crucial element in beneficial plant-microbe interactions and has been the subject of intense research during the past three decades. This is due to the fact that inconsistent colonization remains one of the major limitations to the widespread use of bacterial inoculants in agriculture (Schippers et al., 1987; Bull et al. 1991; Johnson, 1994; Raaijmakers et al. 1995; Raaijmakers and Weller, 1998; Lugtenberg et al. 2001). Despite obvious benefits for agriculture, attempts to exploit DAPG-producing *Pseudomonas* strains as biocontrol inoculants have had limited success so far. Only a handful of the biopesticides registered to date contain fluorescent *Pseudomonas* strains as the active ingredient (Fravel, 2005; Haas and Defago, 2005). The rhizosphere competence and the ecological performance of DAPG-producing *Pseudomonas* strains is complex and affected by many different bacterial traits and a multitude of environmental factors: it involves interactions among the introduced strain, the pathogen and the indigenous rhizosphere microflora (Weller and Thomashow, 1994, Smith and Goodman, 1999, Rainey

1999, Notz et al., 2001, Lugtenberg et al., 2001, Bergsma-Vlami et al., 2005). Furthermore, the discovery of type III secretion genes in many non-pathogenic *Pseudomonas* strains (Preston et al., 2001, Rainey, 1999, Mazurier et al., 2004, Rezzonico et al., 2004, Dubuis et al., 2007) suggests that at least some rhizobacteria may have a more sophisticated relationship with host tissues than previously recognized.

Most root colonization studies have focused only on one plant species and the compatibility between different host plant species and genotypes of antagonistic microorganisms has not been investigated extensively. A better understanding of the rhizosphere competence of DAPG-producing *Pseudomonas* strains on different plant species is essential to improve biological control and to establish compatible antagonist-plant combinations. The objective of this study was to determine the relationship between the genotype of DAPG-producing *Pseudomonas* strains and their ability to colonize the rhizosphere of different plant species. We hypothesize that certain genotypes of DAPG-producing *Pseudomonas* have evolved a preference for the colonization of specific crops (specialists), whereas others can colonize equally well the rhizosphere of multiple plant species (generalists). Four different and agriculturally important plant species were included in this study, i.e., wheat, sugar beet, potato and lily. The population dynamics of eight genotypically different DAPG-producing *Pseudomonas* strains were monitored in the rhizosphere of each of the four plant species. For sugar beet seedlings, the relationship between *in situ* DAPG production and rhizosphere competence of the introduced *Pseudomonas* strains was investigated.

Results

Rhizosphere competence of genotypically different *Pseudomonas* strains

Short-term experiments with different plant species

Data obtained in the dose-response experiments were analyzed by the equation $Y=\alpha*X/(\beta+X)$, which is based on the Michaelis-Menten kinetics of substrate-limited growth (Raaijmakers and Weller, 2001). X the initial density of the introduced strain and Y the final population density in the rhizosphere. The α-value is a measure of the maximum density (log CFU g^{-1} root) that can be achieved, and the β–value is the initial density (log CFU g^{-1} soil) necessary to reach half of the maximum density (**Figure 1**).

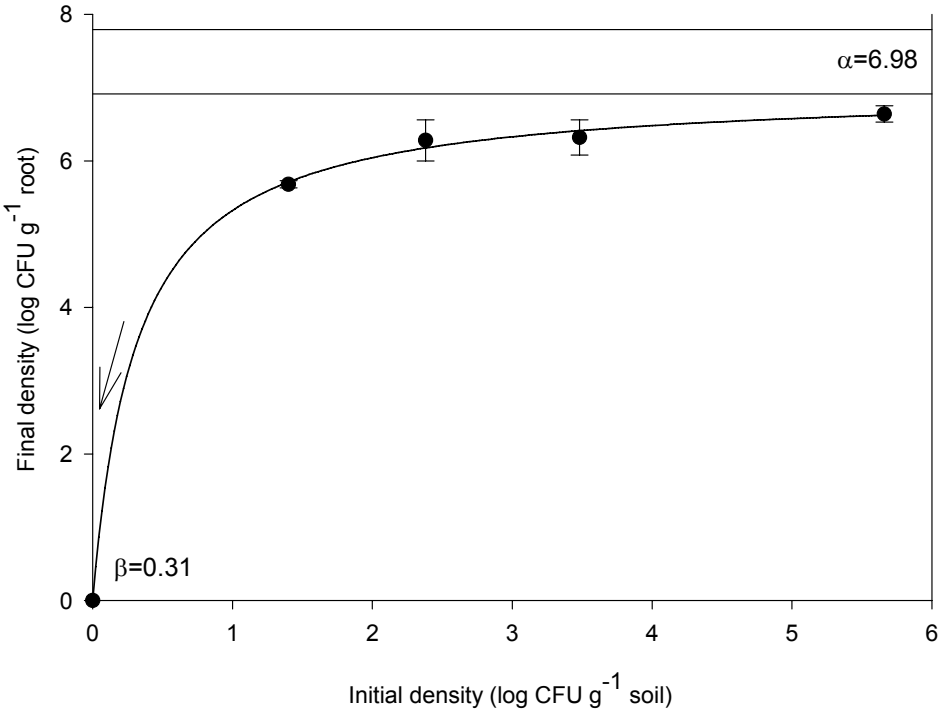


Figure 1: Typical dose-response curve describing the relationship between initial and final population density of introduced *Pseudomonas* strains. The equation used in the regression analysis was $Y=\alpha*X/(X+\beta)$, where Y is the final density (log CFU g^{-1} root) and X the initial density (log CFU g^{-1} soil). The α -value is a measure of the maximum density (log CFU g^{-1} root) that can be achieved and the β–value is the initial density (log CFU g^{-1} soil) necessary to reach half of the maximum density. In the example presented here, the introduced *Pseudomonas* strain has an α value of 6.98 log CFU g^{-1} root and a β value of 0.31 log CFU g^{-1} soil. Parameters α and β provide a quantitative measure for the rhizosphere competence of a particular strain: strains exhibiting high α and low β values are highly rhizosphere competent.

Consequently, parameters α and β provide a quantitative measure for the rhizosphere competence: strains exhibiting high α and low β values are highly rhizosphere competent **(Figure 1)**. The results of the non-linear regression analysis showed significant differences in the rhizosphere competence of the eight genotypically different *Pseudomonas* strains for each of the four plant species but also among the different plant species (**Figure 2**). Relative to the others, genotypes A and G were highly rhizosphere competent on wheat, genotypes A, C, Z and G on sugar beet, genotypes A, C, F and G on potato, and genotype A on lily.

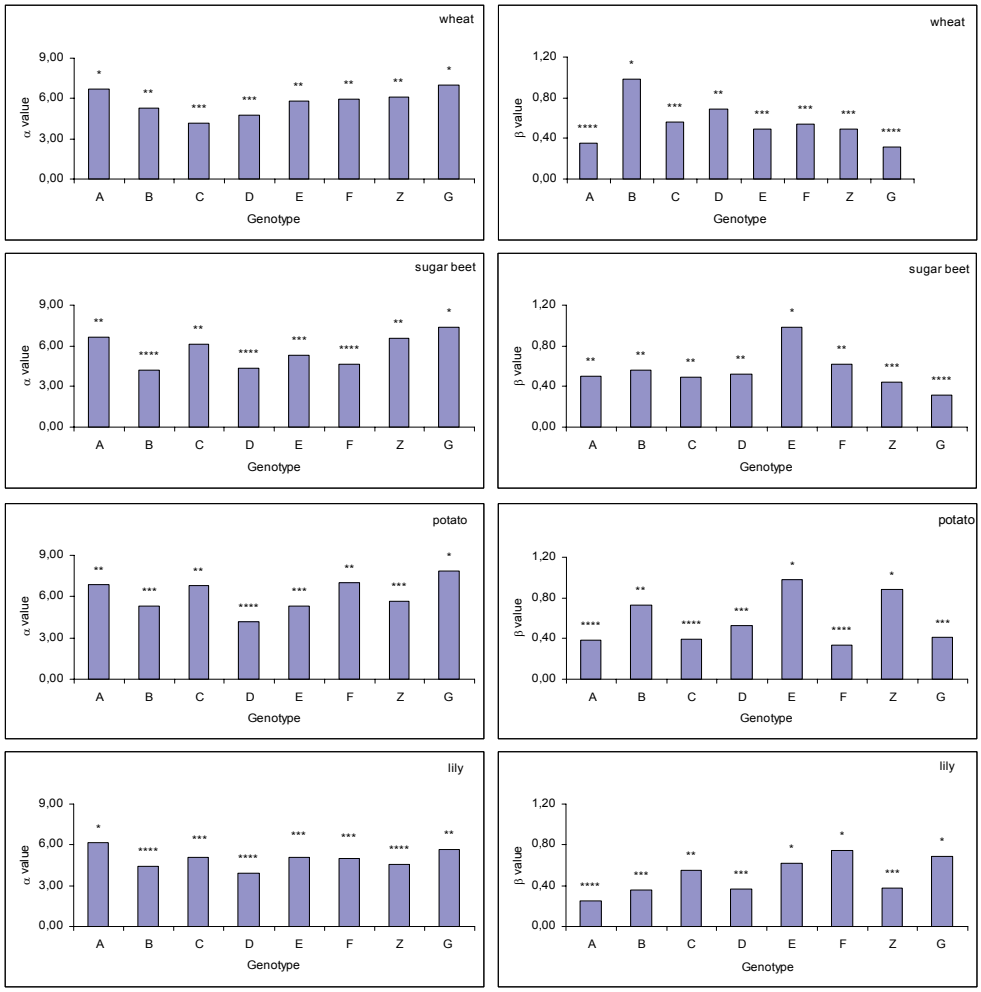


Figure 2: Rhizosphere competence of eight genotypically different DAPG-producing *Pseudomonas* strains (A-G, and Z) on wheat, sugar beet, potato and lily. Strains were introduced into soil at initial population densities of approximately 10^2, 10^3, 10^4 and 10^6 cells g^{-1} soil fresh weight, prior to sowing and/or planting. Non-linear regression was performed to determine the relationship between the initial (log CFU g^{-1} soil) and the final densities (log CFU g^{-1} root) of the introduced strains. The equation used was $Y=\alpha*X/(X+\beta)$, where Y is the final density (log CFU g^{-1} root), X the initial density (log CFU g^{-1} soil), α the maximum final density (log CFU g^{-1} root) and β the initial density (log CFU g^{-1} soil) necessary to reach half of the maximum final density. The experiment was repeated twice and representative results are shown. Based on this non-linear regression analysis, 95% Confidence Intervals (CI) were calculated for the α and β values based on the formula: $95\%CI=\alpha+/-t(p/2;df*se)$ and $95\%CI=\beta+/-t(p/2;df*se)$, where "df" are the degrees of freedom of the experiment and "se" is the standard error of the α or β values. For each of the panels, genotypes with the same number of asterisks are not significantly different (*P*=0.05). Mean values of 4 replicates are given. The experiment was repeated twice and representative results are shown.

The maximum final density (α-value) achieved by these rhizosphere competent genotypes was above 6 log CFU g^{-1} root and the corresponding β values below 0.5 log CFU g^{-1} soil (**Figure 2**). Especially genotype A exhibits a high level of rhizosphere competence for all four different plant hosts. Additionally, genotype Z presents similar levels of rhizosphere competence on wheat and sugar beet, but not on potato and lily (**Figure 2**). Although the maximum final density achieved by genotype E was above 5 log CFU g^{-1} root for all plant species, it required an initial density (β value) above 1 log CFU g^{-1} soil for some plant species to reach half of the maximum final density. Genotypes B, C, D and F presented, in general, a lower rhizosphere competence on all four different host plants because they established lower final densities (α-values) and required higher initial densities (β values) to reach half of the maximum population densities (**Figure 2**). Genotype G was more rhizosphere competent on multiple plant species than most of the other genotypes tested, except on lily where genotype A exhibited a significantly higher rhizosphere competence.

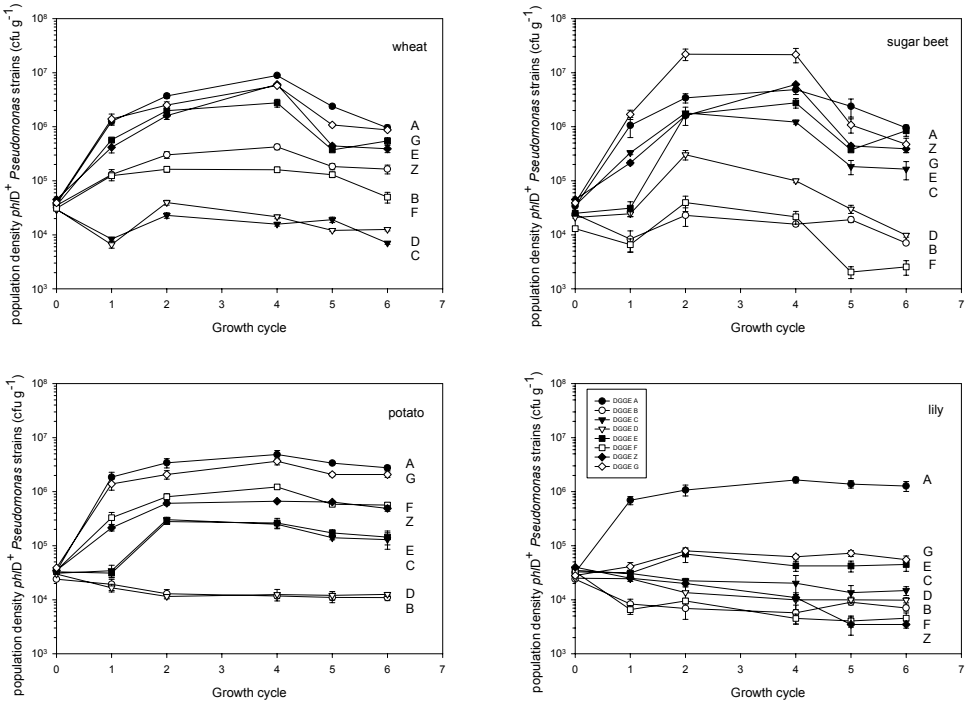


Figure 3: Rhizosphere competence (CFU g^{-1} root) of eight genotypically different DAPG-producing *Pseudomonas* strains (A-G, and Z) on wheat, sugar beet, potato and lily. Strains were introduced separately into soil only once (cycle 0) at a density of approximately $5x10^4$ CFU g^{-1} soil. The population dynamics of each strain was monitored during six successive growth cycles. For each growth cycle, mean values of 4 replicates are presented. Error bars represent the standard error of the mean. The experiment was repeated twice and representative results are shown.

Long-term experiments with different plant species

The population dynamics of the eight genotypically different *Pseudomonas* strains was monitored during six successive growth cycles of each of the four different plant species grown in agricultural soil; the eight strains were introduced separately and only once (growth cycle 0) at initial densities of approximately 5×10^4 CFU g^{-1} soil. The results of the statistical analyses showed that time (t), genotype (i) and their interaction (t*I) had a significant effect (P<0.001) on the population dynamics of the eight strains (**Figure 3**). Genotypes A, Z and G were shown to be superior in long-term colonization of roots of wheat, sugar beet and potato plants. In the case of lily, however, rhizosphere competence of genotypes Z and G was low and their population densities remained below 10^5 CFU g^{-1} root. These results (with the exception of lily) suggest that the rhizosphere competence of genotypes A, Z and G is not linked to a specific plant species, but may be due to specific characteristics that enable these generalist strains to be competitive in different rhizosphere environments.

For wheat, genotypes G, A, E and Z established the highest densities after two growth cycles; the population densities of genotypes G and A further increased to densities between $1.1X10^6$ and $8.9X10^6$ CFU g^{-1} root in growth cycles 4 and 5, whereas population densities of E and Z levelled off at densities of approximately $4X10^5$ and $6X10^6$ CFU g^{-1} root (**Figure 3**). After 6 growth cycles of the wheat seedlings, population densities of the four genotypes A, E, Z and G were similar. Between the first and the fifth growth cycle, genotypes B and F did not establish densities as high as genotypes G, A, E and Z, and their populations densities consistently remained below 10^6 CFU g^{-1} root. Genotypes C and D colonized the rhizosphere of wheat seedlings to a significantly lesser extent than the other six genotypes.

After the first growth cycle of sugar beet, genotypes G and A established the highest densities; population densities of genotype G further increased during growth cycles 2-4 to densities of approximately $3X10^7$ CFU g^{-1} root, whereas population densities of genotype A levelled off at densities of approximately $5X10^6$ CFU g^{-1} root. After the first growth cycle, genotypes E, C, Z did not establish densities as high as G and A, but reached similar densities as A in growth cycles 2 to 4 (**Figure 3**). After six growth cycles of sugar beet seedlings, population densities of the three genotypes A, E, Z and G were similar. Genotypes D, B and F colonized the rhizosphere of sugar beet seedlings to a significantly lesser extent than the other five genotypes. The population densities of the three genotypes D, B, and F did not increase above 10^5 CFU g^{-1} root, but instead declined in the last growth cycle of sugar beet to densities of 10^4, $8X10^3$ and $4x10^3$ CFU g^{-1} root, respectively.

For potato, genotypes A and G established the highest densities after the first

growth cycle; population densities of these strains further increased during growth cycles 2 to 4 to densities of approximately $4.9X10^6$ and $3.6X10^6$ CFU g^{-1} root, respectively. After six growth cycles, genotypes Z and F acquired relatively high population densities in the potato rhizosphere of $4.9X10^5$ and $5.6X10^5$ CFU g^{-1} root, respectively (**Figure 3**). Genotypes D, B, C and E colonized the rhizosphere of potato plants to a significantly lesser extent than the other four genotypes. After the first growth cycle of lily, only genotype A was able to establish high densities; population densities of this genotype further increased during growth cycles 2 to 4 to densities of approximately $1.5X10^6$ CFU g^{-1} root (**Figure 3**). Genotypes G, E, C and D colonized the rhizosphere of lily to a significantly lesser extent than A; after six growth cycles, population densities were $5.5X10^4$, $4.5X10^4$, $1.5X10^4$ and $1.0X10^4$ CFU g^{-1} root, respectively. Genotypes B and F colonized the rhizosphere of lily to a significantly lesser extent than the other six genotypes. The population densities of these latter two genotypes did not increase above 10^4 CFU g^{-1} root, but instead declined in the last growth cycle of lily to densities of $7X10^3$ and $4.5x10^3$ CFU g^{-1} root, respectively.

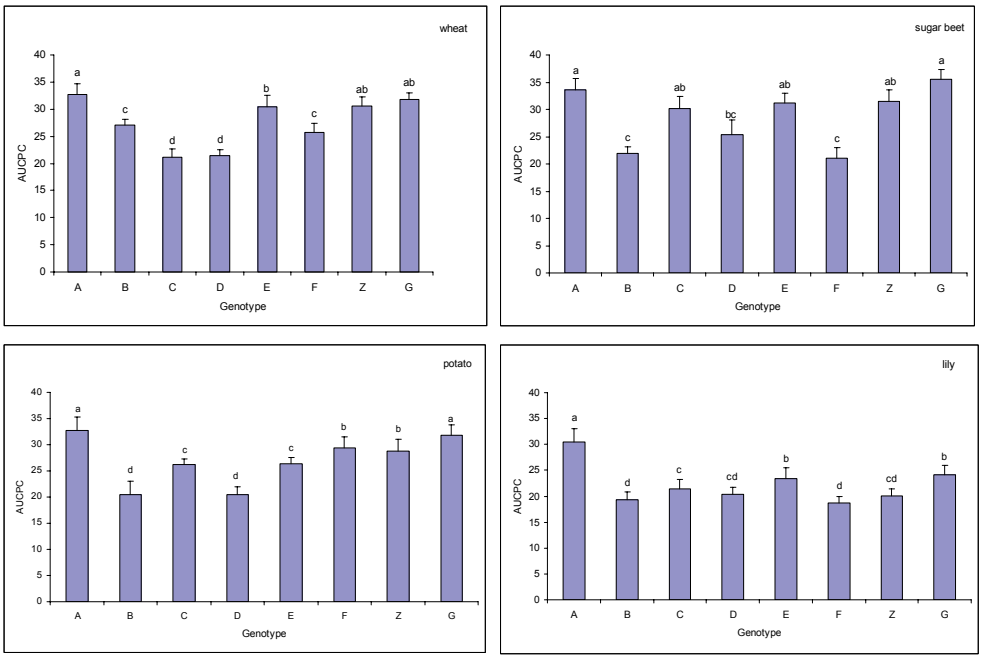


Figure 4: Area Under the Colonization Progress Curve (AUCPC) of eight DAPG-producing *Pseudomonas* strains (A-G, and Z) introduced into the rhizosphere of wheat, sugar beet, potato and lily. Strains were introduced separately into soil only once (cycle 0) at a density of approximately $5x10^4$ CFU g^{-1} soil and their population densities were monitored during six successive cycles. AUCPC was calculated using the trapezoidal integration method described by Landa et al. (2002). Mean values of 4 replicates are presented. Error bars represent the standard error of the mean. For each of the panels, mean values with different letters indicate statistically significant differences (P=0.05). The experiment was repeated twice and representative results are shown.

To quantitatively assess the rhizosphere colonization of the different introduced strains during the 6 growth cycles of the four plant species, the area under the colonization progress curve (AUCPC) was calculated (**Figure 4**). Results showed a significant (P<.0001) effect of the plant species (p), the genotype (i) and of their interaction (p*i). In general, high AUCPC values were found for genotypes A, G and Z, whereas lower AUCPC values were found for genotypes B and D (**Figure 4**). For genotypes E, C and F intermediate AUCPC values were found; successful establishment and survival of these three strains was dependent on the plant species, suggesting that these genotypes may be regarded as specialists.

Physiological characteristics

Based on the Biolog GN2 microplate analysis, a method for phenotypic characterization of a wide range of Gram-negative bacteria, 95 discrete substrates were tested and resulted in metabolic fingerprints for all eight genotypes. In total, the *Pseudomonas* strains could utilize 59 of the 95 single substrates after incubation for 48 h. All eight *Pseudomonas* strains could utilize 25 of these 59 substrates. Differentiation of the strains was based on the remaining 34 substrates. For example only genotypes A and G were able to utilize D-sorbitol as a sole carbon source and only genotype E could utilize succinamic acid. Genotypes A, E and G could utilize arginine, whereas genotypes A and E could utilize putrescin as a sole carbon source. Additionally, only genotypes G and Z were able to utilize 2-aminoethanol as a sole carbon source and only genotypes A, E, G and Z could utilize D-trehalose. Based on fatty acid methyl ester (FAME) analysis, all eight genotypes were classified in RNA-group I of the genus *Pseudomonas*. The FAME and Biolog analyses did not allow characterization of the strains to the species level. Based on additional substrate utilization tests (API-20NE, Biomerieux), genotypes A, E, G and Z were classified as *P. fluorescens* with similarity indices ranging from 92.3% to 99.9%.

None of the eight *Pseudomonas* strains harboured biosynthetic genes for phenazine (phz), pyrrolnitrin (prn) and pyoluteorin (plt) antibiotics. Protease production was found in five of the eight strains and phospholipase C activity was only detected in strains A and Z. Results obtained from cultivation of the *Pseudomonas* strains on SW medium (Siegmund and Wagner, 1991), as well as drop-collapse assays and surface tension measurements, indicated that none of the eight strains produced detectable amounts of biosurfactants.

DAPG production *in vitro*

In vitro DAPG production of the eight *Pseudomonas* strains was monitored in liquid KMB after incubation for 2, 5, 11, 14, 18, 24, 36 and 48 hours at 25°C. All strains reached population densities between 10^{10}-10^{11} CFU ml^{-1} culture after 48 hours of cultivation. Additionally, growth patterns *in vitro* were very similar for all genotypes except for genotype E: the exponential growth phase took more time for E (14 h) than for the other genotypes (11 h).

T**able 2**: *In vitro* production of 2,4-diacetylphloroglucinol (DAPG) by eight *Pseudomonas* strains. Mean values of three replicates are presented. The standard errors (SE) of the means are given between brackets.

Strain	Genotype*	DAPG production (μg ml^{-1}) (SE)	
		after 11h	after 36h
PWB233	A	1.7 (0.7)	133.7 (28.9)
PSC2218	B	22.0 (6.9)	214.9 (42.1)
PPB2310	C	2.7 (0.9)	237.5 (38.6)
PSB211	D	9.7 (5.6)	225.4 (29.9)
PWB532	E	482.0 (58.2)	177.9 (29.3)
PPB3512	F	4.3 (1.3)	17.0 (5.8)
PSC415	Z	22.0 (7.3)	98.3 (25.8)
Q8r1-96	G	274.9 (39.6)	8.5 (4.8)
Q4C5	G-	*nd***	*nd***

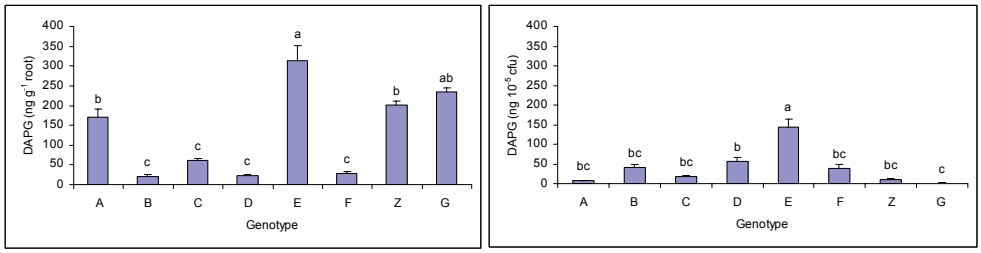
*Genotype classification is based on DGGE analysis of the *phl*D gene (Bergsma-Vlami et al., 2005a).
***nd*: no DAPG was detected.

DAPG-production increased in time for most genotypes except for genotypes E and G: for these latter two genotypes, DAPG production decreased after 11 hours of cultivation presumably due to PhlG-mediated conversion of DAPG to MAPG (Bottiglieri and Keel, 2006) (**Table 2**).

DAPG production on roots of sugar beet plants

Bacterial strains were introduced on sugar beet seeds to final densities ranging from $5x10^6$ and 10^7 CFU $seed^{-1}$. After four weeks of plant growth, genotypes A, Z and G were shown to be superior in colonization of roots of sugar beet, with population densities above 10^6 CFU g^{-1} root, as shown previously (**Figure 3**). Genotypes D, B and F colonized

the rhizosphere of sugar beet seedlings to a significantly lesser extent than the other five genotypes. The population densities for the three genotypes D, B, and F did not increase above 10^5 CFU g^{-1} root. *In situ* DAPG production in the sugar beet rhizosphere (expressed as ng DAPG g^{-1} root fresh weight or as ng DAPG per 10^5 cells) showed substantial and significant differences (P<.0001) between the genotypes (**Figure 5A and 5B**).



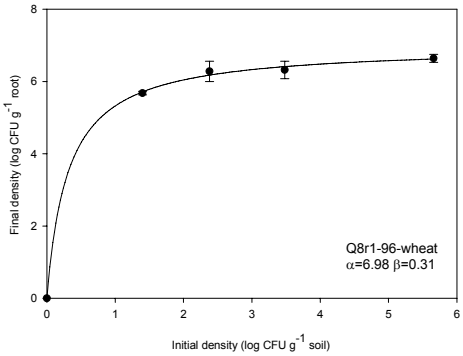
A B

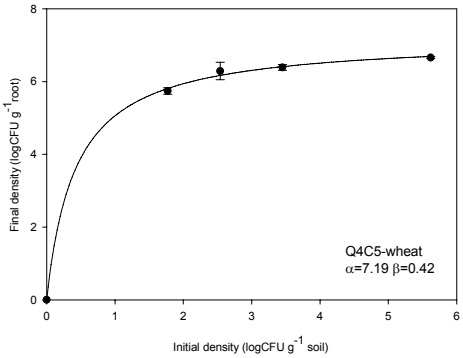
Figure 5: DAPG concentrations detected in the rhizosphere of sugar beet plants after introduction of a *Pseudomonas* strain (A-G, and Z) on the sugar beet seed at a density of approximately $5x10^6$ cfu $seed^{-1}$. Panel A represents the total amount of DAPG detected per g root fresh weight, whereas panel B represents the total amount of DAPG per 10^5 CFU of the introduced strain. Mean values of five replicates are given. Error bars represent the standard error of the mean. For each of the panels, mean values with different letters indicate statistically significant differences (P=0.05).

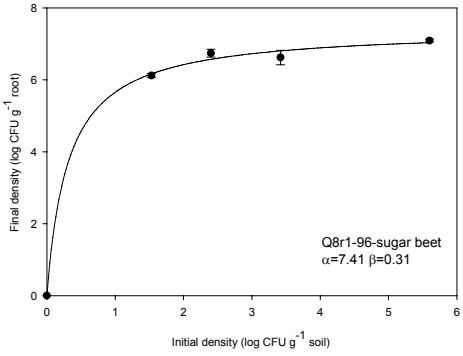
Significantly higher amounts of DAPG were detected in the rhizosphere of sugar beet for genotypes E (312.8 ng g^{-1} root), G (233.4 ng g^{-1} root), Z (201.8 ng g^{-1} root) and A (171.6 ng g^{-1} root), than detected for genotypes C (62.2 ng g^{-1} root) , F (28.8 ng g^{-1} root), D (23.0 ng g^{-1} root) and B (21.2 ng g^{-1} root). When the amounts of DAPG were related to the population densities of the introduced strains, determined in the same samples as used for DAPG detection, genotype E produced the highest amounts of DAPG per cell basis (143.6 ng per 10^5 cells) compared to the other genotypes with DAPG concentrations ranging from 1.0 to 57.3 ng per 10^5 cells.

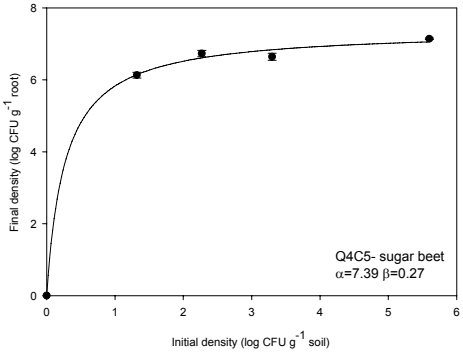
Role of DAPG production in rhizosphere competence

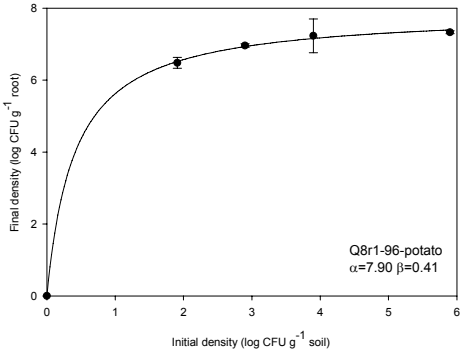
To determine the role of DAPG-production in rhizosphere competence, two approaches were taken. The first involved comparison of the rhizosphere competence of genotype G with that of its DAPG-deficient mutant Q4C5 (G-). The second approach involved analysis of the relation between rhzosphere competence parameters (α, β) and *in situ* DAPG production levels.

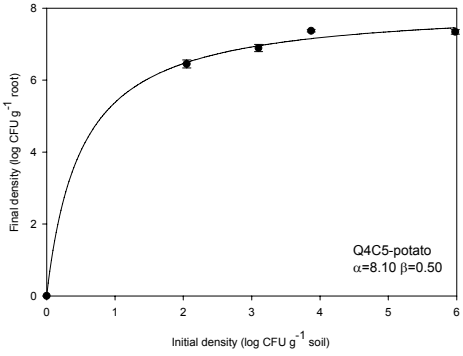

Final density (log CFU g-1 root)
Initial density (log CFU g-1 soil)
Q8r1-96-wheat
α=6.98 β=0.31

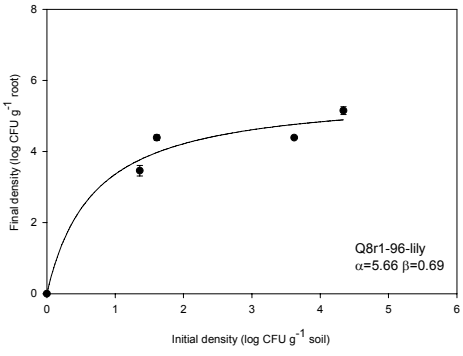

Final density (logCFU g-1 root)
Initial density (logCFU g-1 soil)
Q4C5-wheat
α=7.19 β=0.42


Final density (log CFU g-1 root)
Initial density (log CFU g-1 soil)
Q8r1-96-sugar beet
α=7.41 β=0.31


Final density (log CFU g-1 root)
Initial density (log CFU g-1 soil)
Q4C5- sugar beet
α=7.39 β=0.27


Final density (log CFU g-1 root)
Initial density (log CFU g-1 soil)
Q8r1-96-potato
α=7.90 β=0.41


Final density (log CFU g-1 root)
Initial density (log CFU g-1 soil)
Q4C5-potato
α=8.10 β=0.50


Final density (log CFU g-1 root)
Initial density (log CFU g-1 soil)
Q8r1-96-lily
α=5.66 β=0.69

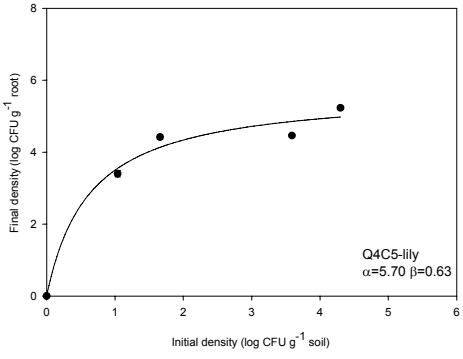

Final density (log CFU g-1 root)
Initial density (log CFU g-1 soil)
Q4C5-lily
α=5.70 β=0.63

Figure 6: Relationship between the initial density (log CFU per g soil) and the final densities (log CFU g^{-1} root) on roots of wheat, sugar beet, potato and lily, respectively, of *Pseudomonas fluorescens* strain Q8r1-96 and its respective DAPG-defective mutant Q4C5. Prior to sowing and/or planting, soil was treated with each of the strains, at densities of approximately 0, 10^2, 10^3, 10^4 and 10^6 CFU g^{-1} soil. Means of four replicates are shown. Error bars represent the standard error of the mean. Non-linear regression was performed to determine the relationship between the initial and the final densities of the introduced strain. The equation used was $Y=\alpha*X/(X+\beta)$, where Y is the final density (log CFU g^{-1} root), X is the initial density (log CFU g^{-1} soil), α is the maximum final density (log CFU g^{-1} root) and β is the initial density (log CFU g^{-1} soil) necessary to reach half of the maximum final density.

When comparing the dose-response kinetics of genotype G (Q8r1-96) with those of its DAPG-deficient mutant G- (Q4C5), no significant differences in rhizosphere competence were found for each of the four plant species (**Figure 6**). These results suggest that, at least for genotype G, DAPG production does not signicantly contribute to its rhizosphere competence.

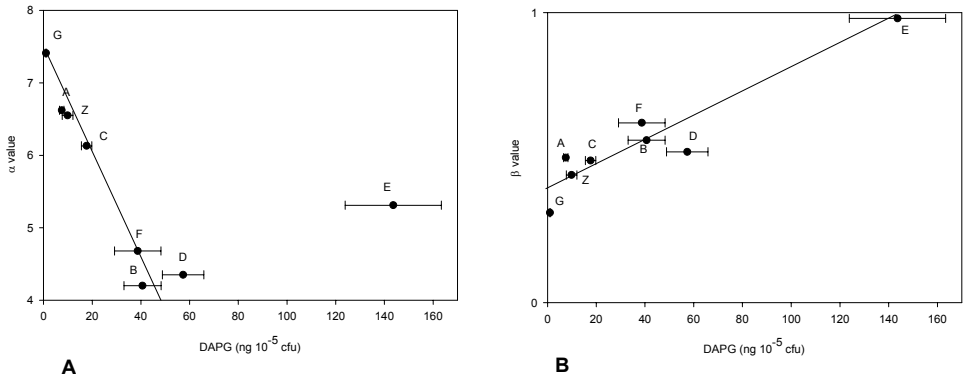


Figure 7: Panels A and B represent the relationship between the DAPG concentration produced by each of the eight introduced *Pseudomonas* strains (A-G, and Z) in the sugar beet rhizosphere and its corresponding α and β values, acquired by the non-linear regression described in **Figure 2**. The relationship between the DAPG concentration produced per cell basis and its corresponding α or β values was analyzed by a linear regression. The equation used to analyse this relationship was $Q=Q_0+\alpha*C$, where Q represents the α or β value, C the DAPG concentration produced *in situ*, and Q_0 the minimum value of Q. Genotype E was excluded from the regression analysis with the α values due to significant disturbance of the analysis (outlyer).

To determine the relationship, if any, between *in situ* DAPG production and rhizosphere competence for all eight *Pseudomonas* strains, correlation analysis was performed on DAPG concentrations produced in the sugar beet rhizosphere by an introduced strain and its corresponding rhizosphere competence parameters (α, β) (**Figure 7A and 7B**).

The equation used to analyse this relationship was $Q=Q_0+\alpha*C$, where Q represents the α or β value, C the DAPG concentration produced *in situ*, and Q_0 the minimum value of Q. Significant correlations were found between *in situ* DAPG production levels (ng per 10^5 cells) of the introduced *Pseudomonas* strains and the corresponding α values (**Figure 7A;** r^2=0.92, P<0.0001, negative correlation) and β values (**Figure 7B;** r^2=0.88, P<0.0001, positive correlation). Genotype E was excluded from the regression analysis with the α values due to significant disturbance of the analysis (outlyer). The outcome of the correlation analyses suggests that the higher the *in situ* DAPG production per cell basis, the lower the rhizosphere competence of the strain is.

Discussion

The compatibility between different host plant species and genotypes of antagonistic microorganisms has not been investigated extensively and is essential to further improve the efficacy and consistency of biological control. In the present study, we determined the relationship between the genotype of DAPG-producing *Pseudomonas* strains and their ability to colonize the rhizosphere of four different plant species: wheat, sugar beet, potato and lily. Dose-response experiments strongly indicated a significant difference in the rhizosphere competence of the eight different *Pseudomonas* genotypes for one plant species but also for different plant species. Genotypes A, Z and G performed superiorly on wheat and sugar beet, genotypes A, C, F and G on potato, and genotype A on lily. The fact that genotypes A, Z and G were highly rhizosphere competent on multiple plant species and did not present a preference for a specific plant species indicates that these genotypes are generalists. These results confirm and extend previous results obtained by Raaijmakers and Weller (2001), who showed that *P. fluorescens* Q8r1-96 (genotype G) was more rhizosphere competent on wheat than two other genotypes.

Recent studies have demonstrated that the plant species modulates the population size and composition of indigenous or introduced DAPG-producers (Bergsma-Vlami et al., 2005, Landa et al., 2003, McSpadden et al., 2005, Picard and Bosco, 2003, Bertin et al., 2003, Lynch and Whipps, 1990, Raaijmakers et al. 2008, Picard and Bosco, 2008). Also our study shows that successful establishment and survival of some *Pseudomonas* genotypes (E, C, and F) depends on the plant species. However, our results also indicate that the rhizosphere competence of genotypes A, Z and G is not linked to a plant species, but may be due to specific characteristics that enable these genotypes to be competitive in different rhizosphere environments. Previous studies showed that the exceptional rhizosphere competence of *P. fluorescens* strain Q8r1-96 (genotype G) could not be attributed to several

colonization traits and genes described for bacterial pathogens (Validov et al., 2005; Mavrodi et al., 2006b). In the same context, the *ptsP* and *orfT* genes in strain Q8r1-96 were investigated for their role in root colonization (Mavrodi et al., 2006a). The *ptsP* gene encodes for a protein of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), whereas *orfT* encodes for a protein related to the superfamily of transmembrane facilitators associated with transport. The rhizosphere competence of the *ptsP* and *orfT* mutants was negatively impaired in mixed inoculations with the wildtype strain Q8r1-96. Results further suggested that the *ptsP* and *orfT* genes were linked to motility and production of DAPG: the *ptsP* mutant produced significantly less DAPG, whereas the *orfT* mutant produced larger amounts of DAPG than wild type strain Q8r1-96 (Mavrodi et al., 2006a). Biochemical analyses further indicated that the superior rhizosphere competence of Q8r1-96 was not related to elevated *in situ* DAPG production levels but possibly to its ability to utilize specific substrates, including trehalose, valerate and/or benzoate (Raaijmakers and Weller, 2001). In a recent study, however, no correlation was found between the ability of strain Q8r1-96 and 54 other strains to utilize these carbon sources and their superior rhizosphere competence on wheat and pea (De La Fuente, 2007).

Results of the present study show that a total of 59 out of the 95 single carbon sources were utilized by the eight *Pseudomonas* strains tested, of which 25 substrates could be utilized by all the strains. More specifically, 18 carbon sources could be utilized by genotype A, 25 by B, 22 by C, 5 by D, 18 by E, 11 by F, 21 by Z and 19 by G. Only the highly rhizosphere competent genotypes A and G were able to utilize D-sorbitol as a sole carbon source. D-sorbitol has been identified as component of seed exudates of the sugar beet cultivar Rex (Casey et al., 1998). Its role in rhizosphere competence remains elusive. Genotypes A, E and G could utilize arginine which is an important amino acid present in root exudates (Bertin et al., 2003): arginine was detected among other amino acids (1.1 μM) in tomato root exudates, and when a plant nutrient solution was supplemented with arginine the growth of biocontrol strain WCS365 was significantly enhanced (Simons et al., 1997). Only genotypes A and E could utilize putrescine, which is a component of the seed exudates of the sugar beet cultivar Rex (Casey et al., 1998). Previously Kuiper et al. (2001) demonstrated that in *Pseudomonas* strain WCS365 a mutation in the promoter region of the putrescine uptake genes (*pot* operon) results in increased uptake of putrescine and decreased competitive colonization ability. Whether the ability of genotypes A and E to utilize putrescine as a sole carbon source is related to their high rhizosphere competence needs further investigation. Additionally, only genotypes G and Z were able to utilize 2-aminoethanol as a sole carbon source and only genotypes A, E, G and Z could utilize D-

trehalose. Trehalose has been implicated in osmotolerance and as an inducer of antagonism toward *Pythium debaryanum* in *P. fluorescens* ATCC 17400 (Gaballa et al., 1997). Whether the high rhizosphere competence and survival of genotypes A, E, G and Z in sugar beet is related to their ability to utilize D-trehalose is yet unclear. So far, no correlation was found between the ability 55 strains to utilize this carbon source and their superior rhizosphere competence on wheat and pea (De La Fuente, 2007).

Although it is tempting to speculate on the possible functions of utilization of specific substrates in root colonization and survival, studies with specific mutants will be necessary to more conclusively identify the role of these and other substrates in the rhizosphere competence of DAPG-producing *Pseudomonas*. Preliminary results obtained in the phenotypic screening of approximately 2000 mutants of genotype A, resulted in isolation of 44 interesting mutants lacking the ability to grow in the presence of a single or multiple substrates (data not shown). Twelve substrates (sugars and/or amino acids) were initially taken into consideration based on the composition of seed exudates of sugar beet (Casey et al., 1998). Preliminary results revealed significant variation in the sugar beet seed colonization by several of these mutants. Further investigation is ongoing to identify the genes disrupted in these mutants.

Results of the present study clearly demonstrated a significant difference in DAPG production among the eight strains. Genotype E produced higher amounts of DAPG per cell basis (143.6 ng per 10^5 cells) than the other genotypes in the rhizosphere of sugar beet. Genotype G produced the lowest amounts of DAPG per cell basis (1.00 ng per 10^5 cells) in the sugar beet rhizosphere, which is of the same order as the DAPG concentration (0.3 ng per 10^5 cells) detected for genotype G in the wheat rhizosphere (Raaijmakers and Weller, 2001). Two approaches were taken to determine the role of DAPG-production in rhizosphere competence. The first involved comparison of the rhizosphere competence of genotype G with that of its DAPG-deficient mutant G- which showed no significant differences in colonization between wildtype and mutant for four plant species. These results suggest that DAPG production does not have a major effect on the rhizosphere competence of genotype G. Previous results demonstrated that DAPG production is also not an important trait for genotype G in colonization of the wheat rhizosphere (Raaijmakers and Weller, 2001). Similarly, loss of DAPG production did not adversely affect the ecological fitness of *P. fluorescens* F113 in the rhizosphere of sugar beet (Carroll et al., 1995).

To further study the possible involvement of DAPG production in root colonization, the relation between rhizosphere competence parameters (α, β) and *in situ* DAPG production levels were analysed for all eight *Pseudomonas* strains. Significant inverse

correlations were found between *in situ* DAPG production levels and these rhizosphere competence parameters. In general, these correlations suggest that strains producing high amounts of DAPG per cell basis are less rhizosphere competent. Production of DAPG in the rhizosphere was recently considered to be a mechanism by which bacteria can manipulate root exudation as DAPG significantly enhanced the exudation of amino acids from roots of four plant species (Philips et al. 2004). Based on these results, we expected that higher DAPG production levels would lead to more rhizosphere competence. However, our results suggest the opposite. One possible explanation may be that higher exudation of amino acids promotes the growth and activity of the indigenous microflora, especially when the released amino acids cannot be utilized by the introduced DAPG-producing *Pseudomonas* strains. In conclusion, this is to our knowledge the first time that antibiotic production by fluorescent *Pseudomonas* strains appears to have an adverse effect on rhizosphere competence. To further support this hypothesis, root colonization experiments should be conducted in which the rhizosphere competence of strains/genotypes that produce relatively high amounts of DAPG per cell basis is compared to that of their DAPG-deficient mutants.

Material and Methods

Bacterial strains and growth media

Characteristics of the *Pseudomonas* strains producing the antibiotic 2,4-diacetylphloroglucinol (DAPG) used in this study are given in **Table 1**.

Table 1: Strains of 2,4-diacetylphloroglucinol-producing *Pseudomonas* used in the present study.

Strain	Genotype*	Plant/Origin	Reference
PWB233	A	WHEAT/NL	Bergsma-Vlami et al., 2005
PSC2218	B	SUGAR BEET/NL	Bergsma-Vlami et al., 2005
PPB2310	C	POTATO/NL	Bergsma-Vlami et al., 2005
PSB211	D	SUGAR BEET/NL	Bergsma-Vlami et al., 2005
PWB532	E	WHEAT/NL	Bergsma-Vlami et al., 2005
PPB3512	F	POTATO/NL	Bergsma-Vlami et al., 2005
PSC415	Z	SUGAR BEET/NL	Bergsma-Vlami et al., 2005
Q8R1-96	G	WHEAT/USA	Raaijmakers and Weller, 2001
Q4C5**	G-	WHEAT/NL	De Souza et al., 2003

*Genotype classification is based on DGGE analysis of the *phl*D gene (Bergsma-Vlami et al., 2005a).

**Q4C5 is the DAPG-deficient mutant of strain Q8r1-96 (De Souza et al., 2003).

Based on sequence analysis of *phlD*, a key gene in DAPG biosynthesis, these strains are representative of eight genotypically distinct groups (Bergsma-Vlami et al. 2005a). For strain Q8r1-96 (genotype G), DAPG-deficient mutant Q4C5 (De Souza et al. 2003) was included. The *Pseudomonas* strains were cultured routinely on King's medium B (KMB) agar at 25^{o}C and stored in 40% (v/v) glycerol at -80^{o}C. For each strain, a spontaneous rifampin-resistant derivative was used in the rhizosphere competence experiments. The population densities of the strains in the rhizosphere were enumerated by plating rhizosphere samples on King's medium B (KMB) agar supplemented with delvocid (100μg ml^{-1}), chloroamphenicol (13 μg ml^{-1}), ampicillin (40 μg ml^{-1}) and rifampin (100μg ml^{-1}) (Raaijmakers et al. 1997).

Physiological characteristics

The *Pseudomonas* strains were characterized by a range of physiological tests, including fatty acid methyl ester analysis (FAME) and substrate utilization profile (Biolog). FAME and Biolog analysis was performed by DSMZ-GmbH (Germany). Additionally, the strains were tested for their ability to produce antibiotic compounds other than DAPG, including: phenazines (phz), pyrrolnitrin (prn) and pyoluteorin (plt). PCR-amplifications using primers PHZ1 and PHZ2, PRND1 and PRND2, and PLTC1 and PLTC2, respectively were performed (Mavrodi et al., 1998; de Souza et al., 2003) and the predicted amplified DNA fragment should have a length of 1408 bp, 786 bp and 438 bp, respectively. In addition, all *Pseudomonas* strains were tested for their ability to produce biosurfactant compounds. Several tests related to biosurfactant production in *Pseudomonas* were conducted including: halo formation on medium containing CTAB-methylene blue (SW) (Siegmund and Wagner, 1991), drop-collapse test, foam formation and surface tension measurements (de Souza et al., 2003). The protease and phospholipase C activity of the *Pseudomonas* strains was tested on skim milk medium and egg yolk plates, respectively (Sacherer et al., 1994).

Rhizosphere competence experiments

Short-term experiments with different plant species (dose-response analysis)

Fifteen wheat seeds (cv. Bussard) and thirty sugar beet seeds (cv. Auris) were sown in square plastic pots containing 250 g of soil, and covered with a 1-cm layer of soil. For potato, two seed tubers (cv. Bintje) class S (virus free certified material) were planted at a depth of 6-8 cm in round plastic pots containing 500 g of soil. For lily, one bulb (cv. Vivaldi) was planted at a depth of 6-8 cm in round plastic pots containing 300 g of soil. The soil used in these experiments was a mixture of CB soil, SSB soil and river sand mixed in a ratio of 1:1:2 (w/w/w). Characteristics of the CB and SSB agricultural soils have been previously

described (De Souza et al., 2003, Bergsma-Vlami et al. 2005b). Washed cell suspensions of each of the *Pseudomonas* strains were incorporated into the soil to obtain initial population densities of approximately 10^2, 10^3, 10^4 and 10^6 cells g^{-1} soil fresh weight, prior to sowing and/or planting. For each strain and each initial population density, four replicates were used per plant species. The four plant species were grown in the greenhouse under controlled conditions at 20°C and a 16-h photoperiod. Twice a week, the plants were treated with 1/3 Hoaglund's solution (macro-elements only). After four weeks of growth for wheat and sugar beet, and five weeks of growth for potato and lily, plants were harvested and their root system with tightly adhering rhizosphere soil collected. Root fragments representative of the entire root system were pooled (approximately 0.5 g of root fresh weight) to determine the rhizosphere populations densities of the introduced strains.

Long-term experiments with different plant species

Cell suspensions of each of the *Pseudomonas* strains were incorporated into the soil only once at an initial density of approximately $5x10^4$ cells g^{-1} soil fresh weight. Plants were grown for six successive cycles as described previously (Bergsma-Vlami et al. 2005a). Briefly, after four weeks of growth for wheat and sugar beet and five weeks of growth for potato and lily, plants were harvested and their root system with adhered rhizosphere soil was collected. Excess of root material was mixed through the cultivated soil and represented approximately 0.5% of the soil dry weight. The cultivated soil was subsequently returned to the same pot and replanted with the same plant species. During every growth cycle, the introduced rifampin-resistant *Pseudomonas* strains were isolated from the rhizospheres of the four different plant species as described above. Four replicates were included per plant species.

DAPG production

In vitro DAPG production by the *Pseudomonas* strains was assessed in liquid KMB and quantified by C_{18}-reverse phase high performance liquid chromatography (RP-HPLC) followed by photodiode array spectroscopy. DAPG production was assessed at 2, 5, 11, 14, 18, 24, 36 and 48 hours of growth at 25°C. Three replicates per *Pseudomonas* strains were included. For quantification of DAPG production, a 6-point standard curve was used (Bergsma-Vlami et al. 2005b). Growth was assessed at the given time points by measuring the optical density of the bacterial cultures at 600nm.

For determining *in situ* DAPG production, thirty sugar beet seeds (cv. Auris) treated with cell suspensions of each of the *Pseudomonas* strains were sown in pots containing 250

g soil. Prior to sowing, seeds were soaked into washed cell suspensions (10^9 cells ml^{-1}) for two hours at room temperature and under continuous agitation. The treated seeds were subsequently air-dried for 45 min in a laminar flow cabinet. Final densities of the introduced bacterial strains on the seeds were determined by dilution plating onto selective KMB and ranged between $5x10^6$ and 10^7 cfu $seed^{-1}$. Sugar beet plants were grown in a climate room under controlled conditions at 20°C with a 16-h photoperiod. Twice a week the plants received 1/3 Hoaglund's nutrient solution (macro-elements only). After four weeks of growth, sugar beet plants were harvested and their root system with adhering rhizosphere soil was collected. Five replicates, each consisting of three combined pots, were used per bacterial treatment. Population densities of the introduced *Pseudomonas* strains were determined according to the protocols described above. DAPG was isolated from roots of sugar beet according to the method described by Bonsall et al. (1997) and Raaijmakers et al. (1999). For the extraction of DAPG from the rhizosphere of sugar beet, approximately 25 g of roots with adhering soil was used. Extracts were resuspended in 1 ml methanol (HPLC grade) prior to RP-HPLC analysis. The HPLC system (Waters) consisted of a 717 autosampler, a 600E solvent delivery system, a 600 controller, and a 996 photodiode array detector. HPLC gradient profiles were monitored at 270 and 330 nm which represent the peak maxima of DAPG in the designated solvent system. DAPG concentrations were calculated on the basis of the area of the DAPG peak using the 6-point standard curve [DAPG] = 8.3 X 10^{-5} X A, where A represents the peak area and [DAPG] represents the concentration of DAPG in ng (Bergsma-Vlami et al., 2005). The retention time of DAPG in the conditions specified above was approximately 27 minutes. To make a correct comparison between the *in situ* DAPG production levels found in the rhizosphere of sugar beet, the extraction efficiency of DAPG was determined by spiking a known amount (1-5 µg) of DAPG into rhizosphere samples of sugar beet; 3 replicate samples were used for this. The extraction efficiency was 54% (± 5.70). Consequently, *in situ* DAPG production levels detected in the rhizosphere of sugar beet were corrected according to this extraction efficiency.

Statistical analysis

Data from the rhizosphere competence assays were analyzed by non-linear regression to determine the relationship between the initial density of each of the introduced *Pseudomonas* strains and their final density on roots of each of the four different host plants. The equation used was $Y=\alpha*X/(\beta+X)$, where Y represents the final density (log CFU g^{-1} root), X the initial density (log CFU g^{-1} soil), α the maximum final density (log CFU g^{-1} root), and β the initial density (log CFU g^{-1} soil) necessary to reach half of the maximum final

density; this equation is based on the Michaelis-Menten kinetics (saturation function) used to describe substrate limited growth of bacteria (Raaijmakers and Weller, 2001). Statistically significant differences between the different *Pseudomonas* strains based on the α and β values were determined by analysis of the 95% confidence intervals CI given by the formula 95%CI=α+/–t{p/2;df*se(α)} and 95%CI=β+/–t{p/2;df*se(β)}, where df are the degrees of freedom, se(α) is the standard error of the α, and se(β) is the standard error of the β as calculated by the non-linear regression analysis described above. The Area Under the Colonization Progress Curve (AUCPC), which is a measure to assess rhizosphere colonization over time (i.e. for all six successive growth cycles), was calculated for each combination of *Pseudomonas* strain and host plant species using the trapezoidal integration method described previously (Landa et al., 2002, Bergsma-Vlami et al., 2005b). Population densities of introduced *Pseudomonas* strains were $\log_{10}$ transformed prior to statistical analysis. Population densities and AUCPC values were subjected to analysis of variance (ANOVA) followed by Tukey's Studentized Range Test, after certifying normal distribution and homogeneity of variances (SAS Institute Inc., Cary, NC). Four replicates were used. Differences in amounts of DAPG detected in the rhizosphere of sugar beet plants treated with different *Pseudomonas* strains were determined by ANOVA followed by Tukey's Studentized Range Test. Five replicates were used. All experiments were performed at least twice and representative results are shown. The relationship between the DAPG concentration produced by a strain introduced in the sugar beet rhizosphere and its corresponding alfa (α) or beta (β) value acquired by the non-linear regression was analyzed using a linear regression. The equation used was $Q=Q_0+\alpha*C$, where Q represents the alfa (α) or beta (β) values acquired by the non-linear regression described above, C is the DAPG concentration produced and Q_0 gives the minimum value of the Q.

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Chapter 5

Biological control of multiple sugar beet pathogens by genotypically different antibiotic-producing *Pseudomonas* species

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In preparation

Biological control of multiple sugar beet pathogens by genotypically different antibiotic-producing *Pseudomonas* species

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Abstract

The broad-spectrum antibiotic 2,4-diacetylphloroglucinol (DAPG) plays an important role in the suppression of plant pathogens by several *Pseudomonas fluorescens* strains. In the present study, eight genotypically different DAPG-producing *Pseudomonas* strains (genotypes A–G and Z) were evaluated for their efficacy to control three different pathogens of sugar beet, i.e. *Pythium ultimum*, *Rhizoctonia solani* and *Aphanomyces cochlioides*. Results showed significant variation in sensitivity of *P. ultimum*, *R. solani* and *A. cochlioides* to either pure DAPG or to antagonism by the DAPG-producing *Pseudomonas* strains, with *R. solani* being the least sensitive. These results are in parallel with results of the plant assays which showed that *R. solani* damping-off could not be controlled by any of the eight DAPG-producing *Pseudomonas* strains. In contrast, *P. ultimum* was effectively controlled by all eight strains and differential effects were observed in biocontrol activity against *A. cochlioides*. *Pseudomonas* genotype G was the most effective in biocontrol of *Pythium* and *Aphanomyces* damping-off, and its biocontrol activity was due, at least in part, to DAPG production as the DAPG-deficient mutant was significantly less effective. When the bacterial strains were applied directly to the sugar beet seeds, all eight *Pseudomonas* strains were more effective in biocontrol of *Aphanomyces* damping-off disease than when applied as a cell suspension. Also in agricultural soil, four *Pseudomonas* strains effectively controlled *Aphanomyces* damping-off disease. Comparative analysis of the rhizosphere competence of the eight DAPG-producing *Pseudomonas* strains and their efficacy to control *A. cochlioides* revealed a highly significant correlation between their ability to colonize the sugar beet rhizosphere and the level of disease control. These results suggest that the more rhizosphere competent DAPG-producing *Pseudomonas* strains are, the higher their efficacy is to control *A. cochlioides* in sugar beet.

Keywords: 2,4-diacetylphloroglucinol, *Pythium ultimum, Aphanomyces cochlioides, Rhizoctonia solani,* biological control

Introduction

Sugar beet (*Beta vulgaris* L.) is susceptible to numerous diseases, including seed and root rot, and damping-off (Leach, 1986; Duffus and Ruppel, 1993). Pre- and post-emergence damping-off result in poor stand establishment and are caused principally by the oomycetes *Pythium* and *Aphanomyces cochlioides* Drechs., and the basidiomycete *Rhizoctonia solani* Kühn (Kuznia et al., 1992, Dick, 2001, West et al., 2003, Agrios, 2005). Several *Pythium* species have been reported as pathogens on sugar beet (Van der Plaats-Niterink, 1981), but *P. ultimum* and *P. aphanidermatum* are the most common (Leach, 1986). Additionally, root rot of adult plants caused by *A. cochlioides* and *R. solani* can significantly reduce sugar beet sucrose accumulation and tonnage (Duffus and Ruppel, 1993).

A. cochlioides is a serious pathogen of sugar beet in the United States, Canada, Chile, Japan, and Europe (Duffus and Ruppel, 1993, Leach, 1986, Papavizas and Ayers, 1974). When soil conditions are moist and warm (22 to 28°C), disease can occur in two phases: an acute seedling blight and a chronic root rot of older plants, also called black root (Leach, 1986). *Aphanomyces* disease symptoms vary in intensity from mild to severe and affected areas can range from a meter in diameter to as large as an entire field (Windels and Lamey, 1998). This spatial variation may result from variable densities of inoculum or abiotic factors that influence disease development throughout the growing season (Pfender and Hagedorn, 1983). Because there is no direct method yet to quantify populations of *A. cochlioides* in soil, the root rot index value derived from a greenhouse seedling assay serves as an indirect estimate of the relative activity and inoculum density of the pathogen in soil (Beale et al., 2002). *A. cochlioides* alone can result in significant losses wherever sugar beet is grown (Smith, 1997). It produces short-lived, motile, biflagellate zoospores and long-lived dormant oospores. Oospores of *A. cochlioides* can persist in soil for years in the absence of a sugar beet crop (Windels and Lamey, 1998). Once liberated from the sporangia, the zoospores of *A. cochlioides* locate host roots via perception of cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone), a host-specific flavonoid signal released from the roots (Horio et al., 1992; Islam et al., 2005). When they arrive at the host surface, zoospores become immobilized by shedding flagella and are transformed into cystospores (encysted zoospores). The cystospores then germinate to form germ tubes and invade the root tissues directly or via appressoria (Islam et al., 2001, 2002, 2003). This sequence of events is extremely rapid and leads to infection within 30 to 40 min after the zoospores arrive at the host surface (Islam and Tahara, 2001). Thus, zoospore taxis is an essential part of the pre-infection process and is a potential target for controlling diseases caused by *A. cochlioides*

and other soilborne zoosporogenic phytopathogens. Control measures for *Aphanomyces* root rot include the use of moderately resistant cultivars treated with the fungicide hymexazol (Tachigaren) and cultural practices such as management of soil moisture (drainage, cultivation), weed control, soil fertilization, and early planting to avoid soil conditions that favour disease (Windels and Lamey, 1998).

Rhizoctonia crown and root rot, caused by *R. solani* anastomosis group (AG) 2-2, is one of the most damaging sugar beet diseases worldwide. Economic losses were estimated to average 2% in the United States; however, damage can vary greatly (0 to 50%) from field to field depending on cropping history and environment (Whitney and Duffus, 1986). In addition to root and crown rot of adult sugar beet roots, the fungus also can cause post-emergence damping-off. Aboveground symptoms include yellowing, sudden wilting of leaves, and blackening of petioles (Whitney and Duffus, 1986). Belowground, a dark brown-gray rot starts near the crown and spreads over the root surface. *R. solani* AG2-2 prefers temperatures of 25 to 28°C and moist conditions for optimal growth (Rush and Winter, 1990, Windels and Brantner, 2000). The pathogen is endemic in all sugar beet growing areas and is a soil inhabitant; thus crop rotations are of minor value in disease control (Whitney and Duffus, 1986, Rush and Winter, 1990). Early planting in spring, preventing movement of infested soil into the crown during cultivation, maintenance of adequate fertility for good crop growth, and good soil drainage are all measures to reduce damping-off and crop losses.

Pythium ultimum is the most significant causal agent of damping- off (Martin and Loper, 1999). In sugar beet, the taxonomic group *P. ultimum* var. *ultimum* has been recognized that does not produce motile zoospores (Van Der Plaats-Niterink, 1981, Saunders and Hancock, 1994). *Pythium ultimum* mediates damping-off of sugar beet seedlings under conditions of cool temperature and high soil water content. Despite their virulence, *Pythium* spp. are generally considered to be poor microbial competitors in the presence of other plant-associated bacteria (Elad and Chet, 1987, Tedla and Stanghellini, 1992). To compensate for this, *Pythium* spp. rely on the production of survival propagules such as oospores and sporangia (Stanghellini, 1974), which germinate rapidly in response to fatty acids present in the exudates of germinating seeds (Ruttledge and Nelson 1997). This allows *Pythium* species to infect seeds at a time when microbial activities, and thus competitive interactions, are low around the seed surface. *P. ultimum* is currently controlled by the incorporation of metalaxyl-based fungicides in the seed pellet (Brantner et al, 1998).

In this study, we investigated the activity and biocontrol efficacy of antagonistic bacteria to protect seeds and seedlings of sugar beet against the three pathogens *P. ultimum, A. cochlioides* and *R. solani*. We specifically focused on fluorescent *Pseudomonas*

strains that produce the antibiotic 2,4-diacetylphloroglucinol (DAPG). A large number of DAPG-producing *Pseudomonas* strains has been identified as effective biocontrol agents of diverse root pathogens (Raaijmakers et al., 2002; Haas and Defago, 2005; Weller et al. 2002, 2007). The enormous genotypic diversity among DAPG-producing *Pseudomonas* strains has received considerable attention and several studies have shown that different genotypes of DAPG-producers can differ considerably in their ability to colonize host plants and to suppress specific diseases (Mavrodi et al., 2001, McSpadden et al., 2000, 2001b, Landa et al., 2002, 2006, Picard et al., 2008, de Souza et al., 2003, Raaijmakers and Weller 2001, Sharifi-Tehrani et al. 1998, Mazzola et al.,2004, Bergsma-Vlami et al., 2005a, Dubuis et al., 2007). However, most biocontrol studies conducted with DAPG-producing *Pseudomonas* strains only focus on one pathogen of a specific crop, in spite of the fact that most crops suffer from the simultaneous attack by multiple pathogens during specific growth stages. In the present work, the efficacy of eight genotypically different DAPG-producing *Pseudomonas* strains to control the three pathogens of sugar beet was investigated. In the bioassays with *A. cochlioides*, different inoculum sources, i.e. swimming zoospores, encysted zoospores or mycelium, were taken into consideration when the biocontrol efficacy of these strains was assessed. The relationship between the biocontrol efficacy against *A. cochlioides* and rhizosphere competence of the introduced strains was addressed in more detail.

Results

Sensitivity of sugar beet pathogens to DAPG and DAPG-producing *Pseudomonas*

The results of the *in vitro* assay show that the three sugar beet pathogens, *P. ultimum*, *R. solani* and *A. cochlioides*, differ considerably in their sensitivity to purified DAPG (**Figure 1A, 1B and 1C**). *P. ultimum* grew well up to DAPG concentrations of 16 μg ml^{-1}, but at concentrations of 32 μg ml^{-1} and higher, mycelial growth was significantly restricted (**Figure 1A).** On the other hand, *R. solani* could still grow at the highest DAPG concentration of 256 μg ml^{-1}. Growth of *R. solani* mycelium was not adversely affected up to a DAPG concentration of 64 μg ml^{-1} (**Figure 1B)**. At DAPG concentrations of 128 μg ml^{-1} and 256 μg ml^{-1}, growth of *R. solani* was restricted and colony morphology was altered. Especially at a DAPG concentration of 256 μg ml^{-1}, the mycelium grew upwards into the air instead of growing over the agar surface. Similar to *P. ultimum*, mycelium of *A. cochlioides* (**Figure 1C)** could grow optimally up to DAPG concentrations of 32 μg ml^{-1}. At DAPG concentration of 64

μg ml^{-1}, mycelial growth was significantly restricted and at a concentration of 128 μg ml^{-1} mycelial growth was very poor. No growth was observed at a DAPG concentration of 256 μg ml^{-1}. The DAPG-sensitivity of the three sugar beet pathogens was reflected in their EC_{50} and EC_{95} values: based on a probit transformation, mycelial growth of *P. ultimum, R. solani* and *A. cochlioides* is inhibited by 50% at DAPG concentrations of 11.8μg ml^{-1}, 33.8 μg ml^{-1} and 64.0 μg ml^{-1}, respectively, and by 95% at DAPG concentrations of 71.6 μg ml^{-1}, 369.0 μg ml^{-1} and 128.0 μg ml^{-1}, respectively.

The results of the dual-culture assay on 1/5th-strength potato dextrose agar (PDA) demonstrated that the sensitivity of each of the three pathogens to *in vitro* antagonism by the DAPG-producing *Pseudomonas* strains differed considerably with *R. solani* being the least sensitive (**Figure 2**). All *Pseudomonas* strains significantly inhibited mycelial growth of *P. ultimum* and *A. cochlioides*.

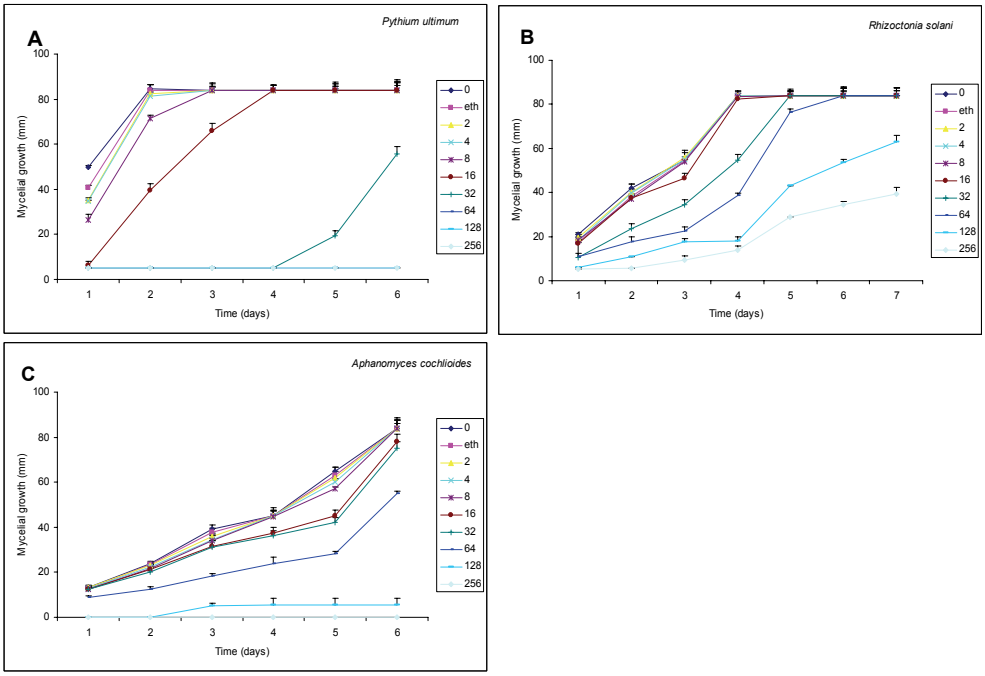

Figure 1: Activity of DAPG against mycelial growth (mm) of *Pythium ultimum* (A), *Rhizoctonia solani* (B) and *Aphanomyces cochlioides* (C). Growth inhibition of mycelium was assayed *in vitro* on PDA-medium supplemented with different concentrations of purified DAPG ranging between 0 μg/ml and 256 μg/ml. Because DAPG was suspended in ethanol, an ethanol control (eth) was also included. Three replications were included per DAPG concentration. Growth inhibition was registered between 1 and 7 days by measuring the diameter of the growing mycelium. Error bars represent the standard error of the mean.

Five strains (genotypes B, C, D, E and G) displayed significant antagonistic activity against *R. solani in vitro*, whereas genotypes A, F and Z exhibited no significant activity towards *R. solani*. Stereoscopic observations of *R. solani* hyphae growing close to *Pseudomonas* colonies revealed alterations in the hyphal morphology: especially in interactions with genotypes E and G, *R. solani* responded by the formation of aerial mycelium. For genotype G (strain Q8r1-96), the role of DAPG production in growth inhibition of the three pathogens was tested by including its DAPG-deficient mutant Q4C5 (G^-). The results show that growth inhibition of *R. solani* and *A. cochlioides* by mutant G^- was significantly less than the growth inhibition by its parental strain (**Figure 2**). For *P. ultimum*, the difference in activity between wildtype Q8R1-96 and its mutant Q4C5 was minor (**Figure 2**), suggesting that other mechanisms play a more predominant role in growth inhibition.

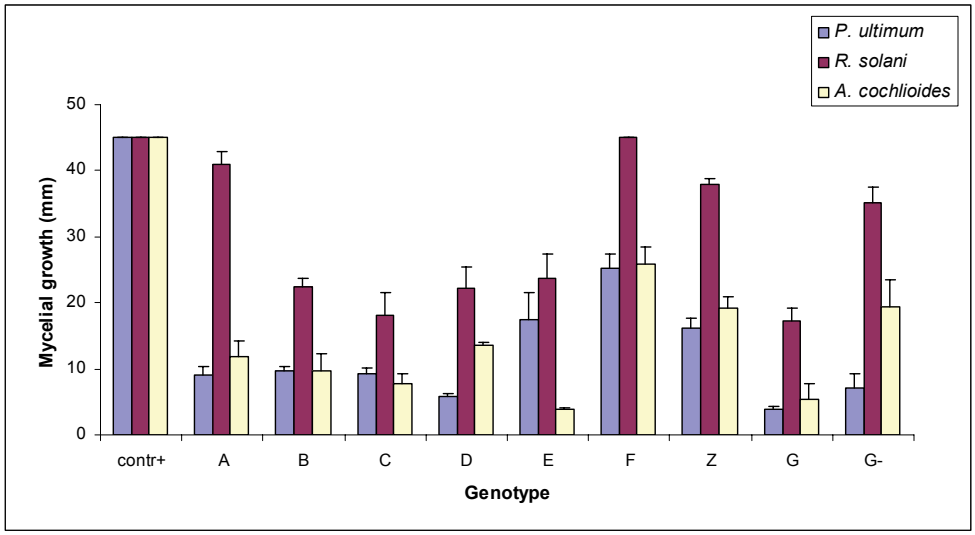

Figure 2: Activity of eight genotypically different DAPG-producing *Pseudomonas* strains (A-G, and Z) against *Pythium ultimum*, *Rhizoctonia solani* and *Aphanomyces cochlioides*, respectively (dual culture assays). Mycelial growth (mm) was assessed after 7 days of growth. For each pathogen – *Pseudomonas* genotype combination, 4 replicates were included. G- represents the DAPG-deficient mutant (Q4C5) of genotype G (Q8r1-96). Error bars give the standard error of the mean.

Biocontrol of damping-off diseases of sugar beet

To minimize the variation in biotic and abiotic conditions, the first series of biocontrol assays were performed in rockwool (**Figure 3**). Bacterial inoculants were applied as cell suspensions directly into rockwool plugs. Initial bacterial concentrations ranged from $6x10^8$ cfu ml^{-1} (genotype E) to $3x10^9$ cfu ml^{-1} (genotype F). Root colonization assays with the DAPG-producers introduced into the rockwool plugs showed that, in absence of the pathogens, each of the eight tested strains reached a density of approximately 10^6 cfu g^{-1}

root after 8 days of plant growth (data not shown). Mycelial plugs of *P. ultimum* or *R. solani,* or 100 zoospores of *A. cochlioides* were added into the rock wool system. The results of the bioassays with *P. ultimum* showed that all DAPG-producing *Pseudomonas* strains significantly reduced pre- and post-emergence damping-off of sugar beet (**Figure 3A1 and 3A2**).

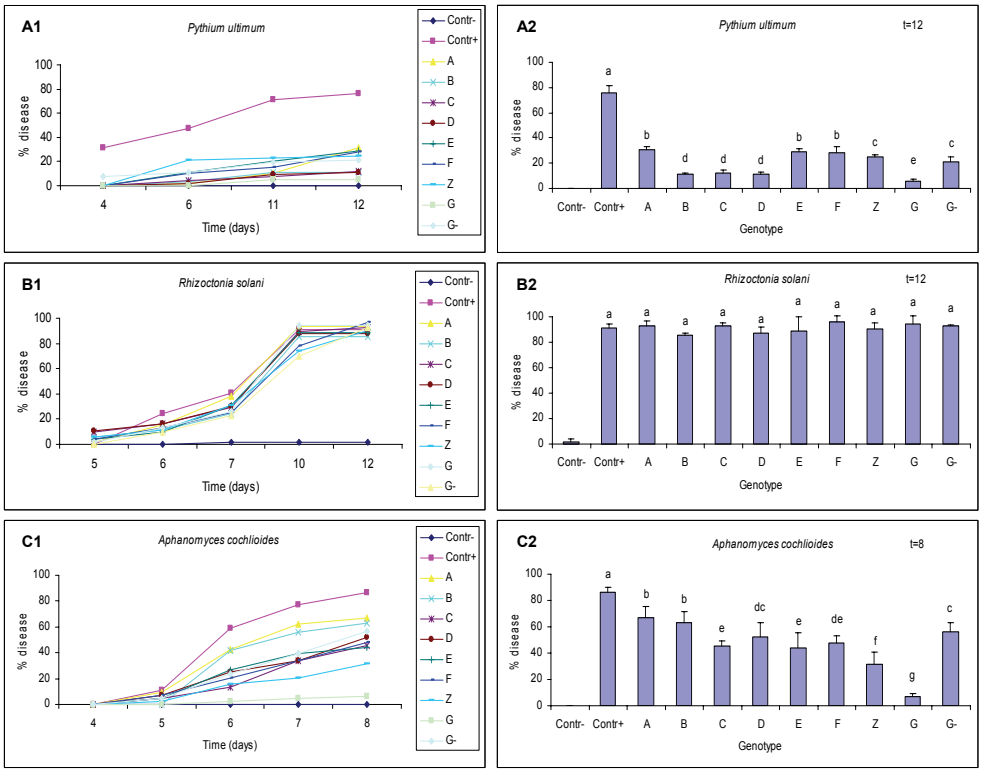

Figure 3: Suppression of pre- and post-emergence damping-off disease of sugar beet caused by *Pythium ultimum* (Panels A1 and A2), *Rhizoctonia solani* (Panels B1 and B2) and *Aphanomyces cochlioides* (Panels C1 and C2), by eight genotypically different DAPG-producing *Pseudomonas* strains (A-G, and Z). Sugar beet seeds were sown in small pots containing rockwool and bacteria were applied as cell suspensions (10^9 cells/rockwool pot). Pre- and post-emergence damping-off was scored at different time points after sowing. The disease incidence (%) was calculated by dividing the number of plants suffering from damping-off disease by the total number of seedlings. Each treatment had six replicates with nine plants per replicate. Per rockwool pot, one mycelial plug of *P. ultimum* or *R. solani,* or 100 zoospores of *A. cochlioides* were introduced. G- represents the DAPG-deficient mutant (Q4C5) of genotype G (Q8r1-96). Error bars represent the standard error of the mean. For each of the Panels A2, B2 and C2, mean values with different letters indicate statistically significant differences (P=0.05).

Genotype G (strain Q8R1-96) was the most effective of all strains tested and also its DAPG-deficient mutant G^- (Q4C5) significantly reduced *Pythium* damping-off, but to a lesser extent than its parental strain. In general, disease ranged between 5% (genotype G) and 31% (genotype A). These results correlate well with the results obtained in the *in vitro*

assays (**Figure 2**). The results of the bioassays with *R. solani* showed that none of the DAPG-producing strains were able to protect sugar beet seedlings (**Figure 3B1 and 3B2**). At 12 days after inoculation, almost all plants were infected by *R. solani* (90%-100%) (**Figure 3B2**). These results are in parallel to results acquired in the *in vitro* sensitivity assay (**Figure 1**) and partly to results obtained in the dual culture assays (**Figure 2**). For *A. cochlioides*, substantial variation in biocontrol efficacy was observed for the genotypically different DAPG-producing strains (**Figure 3C1 and 3C2**): disease incidence ranged between 7% (genotype G) and 67% (genotype A). Genotype G was again the most effective and genotypes A and B were the least effective. For genotype G, the role of DAPG in biocontrol of *Aphanomyces* damping-off was evident since DAPG-deficient mutant G$^-$ was substantially less effective in disease control. These results are in parallel to results acquired in the dual culture *in vitro* assay (**Figure 2**).

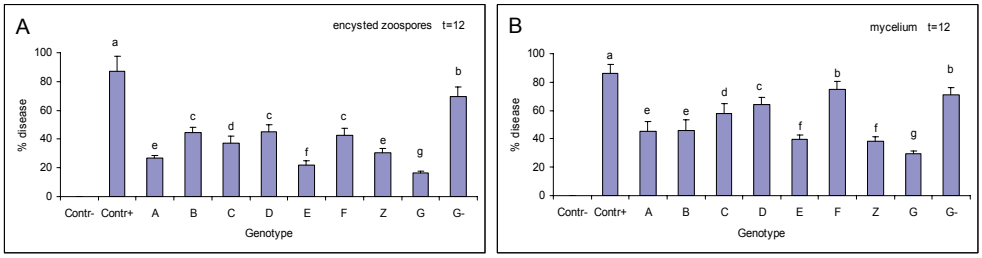

Figure 4: Suppression of *Aphanomyces cochlioides* damping-off disease of sugar beet by eight genotypically different DAPG-producing *Pseudomonas* strains (A-G, and Z). Seeds were sown in small pots containing rockwool and bacteria were applied as suspensions (10^9 cells per rockwool pot). Per rockwool pot, 500 encysted zoospores (Panel A), or a mycelium plug from a young culture (Panel B) of *A. cochlioides* were included. The disease incidence (%) was calculated by dividing the number of plants suffering from damping-off disease by the total number of seedlings. Each treatment had six replicates with nine plants per replicate. Damping-off disease was assessed after 12 days of incubation. G- represents the DAPG-deficient mutant (Q4C5) of genotype G (Q8r1-96). Error bars represent the standard error of the mean. For each panel, mean values with different letters indicate statistically significant differences (P=0.05).

Given that *A. cochlioides* can infect sugar beet seeds and seedlings also from mycelium or encysted zoospores, additional biocontrol assays were conducted to evaluate the biocontrol efficacy of the different *Pseudomonas* strains. The results show that most *Pseudomonas* strains are also effective in controlling *Aphanomyces* damping-off of sugar beet when encysted zoospores or mycelium serve as pathogen inoculum (**Figure 4**). Also in these assays, the DAPG-deficient mutant G$^-$ was less efficient in biocontrol than its parental strain (**Figure 4**) suggesting that DAPG production is an important trait in the biocontrol activity of genotype G against *A. cochlioides*. In an effort to further improve the biocontrol ability of the DAPG-producing *Pseudomonas* strains against *A. cochlioides*, bacterial inoculants were applied directly on seed (**Figure 5**). Bacterial concentrations ranged

between 1.2x10^7 cfu seed^{-1} (genotype A) and 6.3x10^7 cfu seed^{-1} (genotype F). When the bacterial strains were applied directly to the sugar beet seeds (**Figure 5A1 and 5A2**), all strains were more effective in biocontrol of *Aphanomyces* damping-off disease than when they were applied directly to the rockwool as a cell suspension.

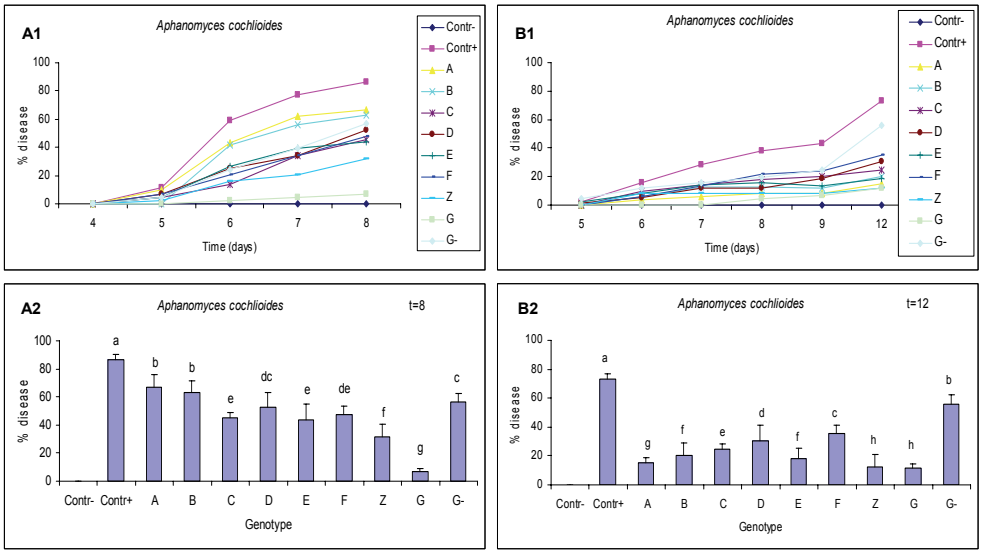

Figure 5: Suppression of *Aphanomyces cochlioides* damping-off disease of sugar beet (cv. Auris) by eight genotypically different DAPG-producing *Pseudomonas* strains (A-G, and Z). Seeds were sown in small pots containing rockwool and bacteria were applied as suspensions (10^9 cells/rock wool pot) (panels A1 and A2) or directly on the seed (10^7 cells seed^{-1}) (panels B1 and B2). Per rock wool pot, 100 zoospores of *A. cochlioides* were included. Damping-off disease was scored at different time points after sowing. The disease incidence (%) was calculated by dividing the number of plants suffering from damping-off disease by the total number of seedlings. Each treatment had six replicates with nine plants per replicate. Panel A2 represents the post-emergence damping-off after 8 days of incubation and panel B2 after 12 days of incubation. G- represents the DAPG-deficient mutant (Q4C5) of genotype G (Q8r1-96). Error bars represent the standard error of the mean. For each panel, mean values with different letters indicate statistically significant differences (*P*=0.05).

Biocontrol of *Aphanomyces* damping-off disease of sugar beet in soil

To increase the abiotic and biotic complexity, biocontrol and root colonization assays were subsequently performed in an agricultural soil obtained from fields grown to wheat and sugar beet. For the biocontrol assays performed in this soil (**Figure 6**), seeds were sown in small pots and bacteria were applied directly on the seed (10^7 cells seed^{-1}): 1000 zoospores of *A. cochlioides* were included as the pathogen inoculum. The results show that after 28 days of plant growth, genotypes G, Z, A and E significantly reduced the disease incidence, whereas genotypes B, C, D and F did not provide a significant level of disease suppression compared to the control (**Figure 6).** Like in the rockwool bioassays,

DAPG-deficient mutant G$^-$ was significantly less effective in biocontrol than its parental strain (**Figure 6**).

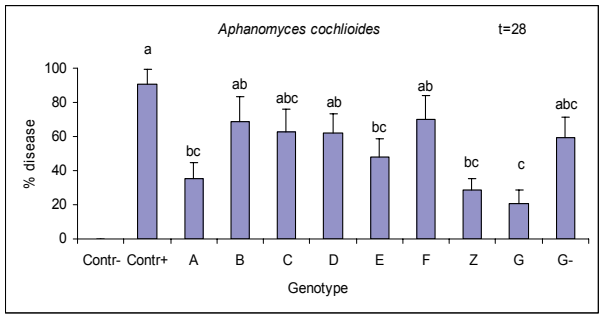

Figure 6: Suppression of *Aphanomyces cochlioides* damping-off disease of sugar beet by eight genotypically different DAPG-producing *Pseudomonas* strains (A-G, and Z). Seeds were sown in small pots containing agricultural soil and bacteria were applied directly on the seed (10^7 cells seed^{-1}). Per pot, 1000 zoospores of *A. cochlioides* were introduced. Damping-off disease was scored at twenty-eight days after zoospore inoculation. Disease incidence (%) was calculated by dividing the number of plants suffering from damping-off disease by the total number of seedlings. Each treatment had four replicates with ten plants per replicate. G- represents the DAPG-deficient mutant (Q4C5) of genotype G (Q8r1-96). Error bars represent the standard error of the mean. For each treatment, mean values with different letters indicate statistically significant differences (*P*=0.05).

Relationship root colonization and biocontrol efficacy

Rhizosphere colonization data obtained from the biocontrol assay (**Figure 6**) showed that after 28 days of plant growth, population densities ranged from $6.9x10^3$ cfu g^{-1} (F) to $3.4x10^6$ cfu g^{-1} root (G). For genotypes G, A, Z and E, significantly higher population densities were detected in the sugar beet rhizosphere than for the other genotypes. Population densities for G, A, Z and E were $3.4x10^6$, $1.8x10^6$, $6.7x10^5$ and $6.5x10^5$ cfu g^{-1} root, respectively. The DAPG-deficient mutant G- colonized the rhizosphere of sugar beet equally well as its parental strain. These results indicate that genotypes G, Z, A and E establish and maintain high population densities in the sugar beet rhizosphere in the presence of the pathogen *A. cochlioides.* Identical rhizosphere competence levels had been previously acquired for each of the *Pseudomonas* genotypes in a separate experiment with the same agricultural soil (**Figure 7A**) (Chapter 4). The results show that genotypes G, Z, A, E and also C are better colonizers of the sugar beet rhizosphere than the other genotypes (**Figure 7A**).

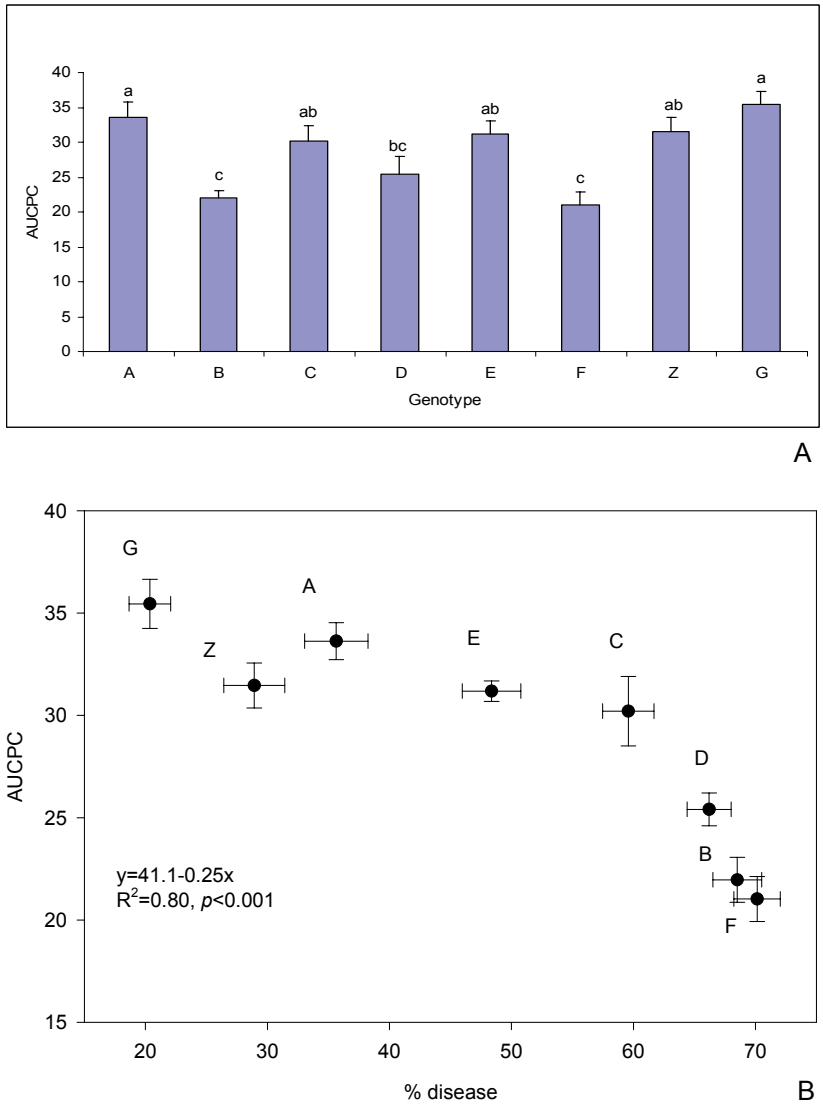

Figure 7: (A) Area Under the Colonization Progress Curve (AUCPC) of each DAPG-producing *Pseudomonas* genotype (A-G, and Z) introduced into the rhizosphere of sugar beet. Mean values of 4 replicates are presented. Error bars represent the standard error of the mean. Mean values with different letters indicate statistically significant differences (P=0.05). (B) Relationship between disease incidence (%) caused by *A. cochlioides* on sugar beet seedlings and the AUCPC of the eight DAPG-producing *Pseudomonas* genotypes. The relationship was analyzed by linear regression.

To establish a correlation between the biocontrol efficacy of the DAPG-producing strains and their rhizosphere competence, disease incidence caused by *A. cochlioides* on sugar beet seedlings and the AUCPC of each of the DAPG-producing genotypes were plotted

(**Figure 7B**) and their relationship analyzed by regression. The equation acquired from the regression analysis was Y=41.1-0.25*X (R^2=0.80, P<0.001), where Y represents the AUCPC and X the disease incidence (%). These results indicate that the more rhizosphere competent DAPG-producing strains are, the higher their efficacy is to control *A. cochlioides* in sugar beet.

Discussion

The production of antifungal metabolites, such as DAPG, is recognized as an important trait in the biocontrol of plant diseases by antagonistic microorganisms (Bangera and Thomashow, 1996, 1999; Keel et al., 1992, Handelsman and Stabb, 1996). Numerous studies have demonstrated that DAPG-producing *Pseudomonas* spp. can suppress a wide variety of plant pathogens, including fungi, bacteria and nematodes (Stutz et al., 1986; Keel et al., 1990; Vincent et al., 1991; Fenton et al., 1992; Keel et al., 1992; Cronin et al., 1997a, b; Duffy and Defago, 1997; Sharifi-Tehrani et al., 1998). In this study, we examined the potential of DAPG-producing *Pseudomonas* strains for biological control of *Pythium ultimum, Rhizoctonia solani* and *Aphanomyces cochlioides,* three economically important pathogens of sugar beet. The results showed that these three sugar beet pathogens differ considerably in their sensitivity to DAPG, with *R. solani* being the least sensitive. These results confirm and extend results obtained previously for other soil-borne pathogens (Keel et al., 1992).

Among *Pythium* species there can be considerable variation in sensitivity to DAPG: mycelial growth of fourteen *Pythium* isolates, obtained from multiple host plants and representing eight different species, was completely inhibited at DAPG concentrations ranging from 8 to more than 300 μg ml^{-1}, with *P. volutum* as the most sensitive and *Pythium deliense* as the most insensitive (De Souza et al., 2003). Also work by Mazzola et al. (1995) showed that various isolates of the take-all fungus *Gaeumannomyces graminis* var. *tritici* can differ substantially in their sensitivity toward DAPG; isolates that were relatively insensitive to DAPG *in vitro* could no longer be suppressed *in situ* by a DAPG-producing *Pseudomonas* strain. This variation in sensitivity within target pathogen populations to specific metabolites produced by antagonistic microorganisms can be an important factor in the inconsistency of biological control encountered under field conditions.

The mechanisms underlying the observed variation in sensitivity between pathogens or between isolates within a pathogen species are largely undetermined. Similarly, the exact mechanism of action of DAPG is unknown. For *P. ultimum* it was demonstrated that DAPG, at very low concentrations of 0.4 ng ml^{-1}, could lyse zoospores and cause extreme cellular disorganization in hyphal tips (De Souza et al., 2003). Recent

studies also showed that substantial variation in sensitivity to DAPG exists among pathogenic and nonpathogenic *Fusarium oxysporum* (Schouten et al., 2004). In a collection of 70 pathogenic *F. oxysporum*, representing 13 formae speciales and 27 nonpathogenic *F. oxysporum* isolates, 18% and 25% of the isolates, respectively, were insensitive to DAPG. No clear relationship between DAPG insensitivity and geographical origin or formae speciales of *F. oxysporum* was observed, suggesting that the traits responsible for DAPG insensitivity have developed independently, or are easily transferred within and between populations. For two DAPG-tolerant *F. oxysporum* strains, analysis by mass spectrometry indicated that de-acetylation of DAPG to the less fungitoxic derivatives MAPG and phloroglucinol is among the initial defence mechanisms of DAPG degradation. Similar levels of insensitivity to DAPG were found among natural populations of *B. cinerea* (Schouten et al., 2008). The ABC transporter BcAtrB was shown to provide the first line of defence, preventing accumulation of DAPG in the cell to toxic concentrations, whereas the extracellular laccase BcLCC2 mediates subsequent degradation of DAPG.

Similar to the results obtained with pure DAPG, also the sensitivity of *P. ultimum*, *R. solani* and *A. cochlioides* to *in vitro* antagonism by the DAPG-producing *Pseudomonas* strains differed considerably, with *R. solani* being again the least sensitive. Microscopic observations of *R. solani* hyphae growing close to the *Pseudomonas* colonies revealed alterations in the hyphal morphology: in interactions with genotypes E and G, *R. solani* responded by the formation of aerial mycelium. Genotypes E and G have been demonstrated to produce higher amounts of DAPG *in vitro* than the other genotypes included in this study (Chapter 4). The role of DAPG production in *in vitro* growth inhibition of the three pathogens was confirmed for genotype G, since the growth inhibition by DAPG-deficient mutant Q4C5 was significantly less than that provided by its wildtype strain; for *P. ultimum*, the difference in activity between genotype G and its mutant G- was minor, suggesting that additional mechanisms play a more predominant role in growth inhibition.

The results of the rockwool bioassays showed that all DAPG-producing *Pseudomonas* strains significantly reduced pre- and post-emergence damping-off of sugar beet caused by *P. ultimum,* whereas none of the DAPG-producing strains were able to protect sugar beet seedlings against *R. solani.* Additionally, substantial variation in biocontrol efficacy was observed for the different DAPG-producing strains against *A. cochlioides*. The lack of correlation between *in vitro* inhibition of pathogens and the ability to suppress disease caused by those pathogens in plants has been documented previously, and attributed mainly to the differential expression of genes *in situ* and *in vitro* (Reddy et al., 1993). The results of the rockwool bioassays, however, are in parallel with results acquired

in the *in vitro* assays. This might be attributed to minimal variation in biotic and abiotic conditions that prevail in the rockwool system. A similar correlation between *in vitro* and *in vivo* effects was found for the effect DAPG-producing strain F113 on hatching of the nematode *G. rostochiensis* (Cronin et al., 1997a). Both under *in vitro* conditions and in soil microcosms, exposure to DAPG-producing *P. fluorescens* F113 increased the ability of *G. rostochiensis* to hatch and reduced the percentage of mobile juveniles of the potato cyst nematode. In contrast to F113, the DAPG-deficient mutant F113G22 had no effect on juvenile mobility and hatch ability of *G. rostochiensis* under *in vitro* conditions or in soil microcosms (Cronin et al., 1997a). This is in accordance with our results of genotype G, where the role of DAPG in biocontrol of *Aphanomyces* damping-off was evident since DAPG-deficient mutant G^- was substantially less effective in disease control.

Studies on the effects of antimicrobial compounds produced by antagonistic microorganisms on plant pathogens often consider only one specific stage in the life cycle of the pathogen. Most pathogen life cycles, however, are diverse and comprise numerous structures that allow pathogens to respond adequately to selection pressure exerted by competing microorganisms (Raaijmakers et al., 2008). For example, oomycete pathogens can infect host tissues by means of mycelium, zoosporangia, zoospores and oospores. Understanding the variation in sensitivity of different phases of the life cycle of a pathogen to a specific biocontrol trait will give more insight into the potential efficacy of biocontrol agents harbouring that particular trait. Given that *A. cochlioides* can infect sugar beet seeds and seedlings also from mycelium or encysted zoospores, additional biocontrol assays were performed to evaluate the biocontrol efficacy of the different *Pseudomonas* strains. Our results demonstrated that most *Pseudomonas* strains are also effective in controlling *Aphanomyces* damping-off of sugar beet when encysted zoospores or mycelium serve as pathogen inoculum. For several of the *Pseudomonas* strains, biological control of *Aphanomyces* damping-off was more enhanced when encysted zoospores were used as inoculum propagules. In the same context, variation in sensitivity to DAPG was observed within *Pythium* infection propagules (De Souza et al., 2003a). Mycelium was the most resistant structure, followed by zoospore cysts and zoospores. DAPG adversely affected the behaviour of zoospores of *Pythium ultimum* var. *sporangiferum* at concentrations of 0.8 ng ml^{-1} and higher. At a concentration 0.8 ng ml^{-1}, all zoospores stopped swimming and approximately 50% of the zoospores disintegrated. This high sensitivity of zoospores to DAPG may be explained by the lack of a protective cell wall (Deacon and Donaldson, 1993). This is supported by the observation that encysted zoospores, which have a cell wall, were significantly more resistant to DAPG than non-encysted zoospores (De Souza et al., 2003).

In an effort to further improve the biocontrol ability of the DAPG-producing *Pseudomonas* spp. strains against *A. cochlioides*, bacterial inoculants were applied directly on the sugar beet seed. When applied to the seeds, all strains were significantly more effective in biocontrol of *Aphanomyces* damping-off disease than when applied as a cell suspension. It has been earlier reported that the application of Gram-negative bacteria as biological seed treatments on sugar beet during seed priming results in prior colonization (Callan et al. 1990, 1991, Paternoster and Burns 1996, Paternoster 1997) due to acclimatization to the seed coat environment and therefore improve subsequent survival. Additionally, bacteria capable of producing protective exopolysaccharides *in situ*, such as pseudomonads, may do so during seed priming (Callan et al. 1997).

As the regulation and expression of bacterial genes will be different under diverse conditions, such as the rockwool system and complex soil environments, all potential biocontrol agents must be screened for their activity in a soil system and under field conditions (Ellis et al., 2000). In our greenhouse bioassays with agricultural soil, it was shown that genotypes G, Z, A and E significantly reduced Aphanomyces damping-off disease, whereas genotypes B, C, D and F did not provide a significant level of disease suppression compared to the control. Preliminary results of a small-scale field experiment showed significant reductions in *Aphanomyces* disease incidence by introduction of *Pseudomonas* genotypes A, E and Z. Their efficacy (an average of 38% disease reduction relative to the control) was promising, but did not yet reach the level of disease control obtained by application of the fungicide hymexazol (Tachigaren), which gave 61% disease reduction relative to the control. Whether the higher biocontrol efficacy of genotypes A, E and Z under field conditions was due to a better rhizosphere competence was not investigated and will be subject of future field experiments.

The positive relationship between root colonization and biocontrol activity by introduced rhizobacteria is well established, especially for *Pseudomonas* (Raaijmakers et al., 1995, Raaijmakers and Weller, 1998, 2001; Lugtenberg et al. 2001). Also in this study, a highly significant correlation (R^2=0.80, P<0.001) was found between the damping-off disease incidence (%) caused by *A. cochlioides* on sugar beet seedlings and the rhizosphere competence of the DAPG-producing *Pseudomonas* genotypes. These results indicate that highly rhizosphere competent *Pseudomonas* strains are very good biocontrol agents of *A. cochlioides* in sugar beet.

In conclusion, the results of this study show that different genotypes of DAPG-producing *Pseudomonas* strains show substantial variation in their ability to: 1) colonize the rhizosphere of sugar beet plants, and 2) control multiple seed- and soilborne pathogens of

sugar beet. These results exemplify the importance of establishing compatible host-antagonist combinations to effectively control plant pathogens.

Materials and methods

Bacterial strains and growth media

Eight DAPG-producing *Pseudomonas* strains were used in this study (**Table 1**). The *Pseudomonas* strains were cultured routinely on King's medium B (KMB) agar or on Pseudomonas agar (PSA, Difco) at 25^{o}C. Glycerol stocks of *Pseudomonas* isolates were made in liquid KMB containing 40% (v/v) glycerol and kept at –80^{o}C. Previous work showed that these eight strains belong in genotypically distinct groups based on DGGE analysis of the *phl*D gene (Bergsma-Vlami et al., 2005b).

Table 1: *Pseudomonas* spp. strains included in the present study.

Strain	Genotype*	DAPG	Origin	Reference
PWB233	A	+	NL	Bergsma-Vlami et al., 2005
PSC2218	B	+	NL	Bergsma-Vlami et al., 2005
PPB2310	C	+	NL	Bergsma-Vlami et al., 2005
PSB211	D	+	NL	Bergsma-Vlami et al., 2005
PWB532	E	+	NL	Bergsma-Vlami et al., 2005
PPB3512	F	+	NL	Bergsma-Vlami et al., 2005
PSC415	Z	+	NL	Bergsma-Vlami et al., 2005
Q8R1-96	G	+	USA	Raaijmakers and Weller, 2001
Q4C5**	G-	-	NL	De Souza et al., 2003

*Genotype classification is based on DGGE analysis of the *phl*D gene (Bergsma-Vlami et al., 2005a)
**Q4C5 is the DAPG-deficient mutant of *Pseudomonas fluorescens* strain Q8r1-96

Additionally, the DAPG-deficient mutant Q4C5 of strain Q8R1-96 was included to determine the role of DAPG production in biological control (De Souza et al., 2003). For all experiments described in this study, spontaneous rifampin-resistant derivatives of the strains were used. The introduced *Pseudomonas* strains were isolated from seeds, soil and rhizosphere environments on KMB agar supplemented with delvocid (100μg ml^{-1}), chloroamphenicol (13 μg ml^{-1}), ampicillin (40 μg ml^{-1}) and rifampin (100μg ml^{-1}).

Seed coating

Cells of the DAPG-producing *Pseudomonas* strains, grown for 48 h on *Pseudomonas* agar (PSA) at 25°C, were collected and washed by centrifugation for 10 min at 4000 x *g* at 4°C. Cell pellets were resuspended in sterile water and bacterial densities were measured spectrophotometrically (OD_{600}). In all experiments, naked seeds of the sugar beet cultivar Auris RS1105 were soaked in bacterial suspensions (10^9 cells ml^{-1}) for 2 h under continuous agitation and thereafter dried for 45 min in the flow cabinet, unless differently stated. The density of the bacteria on seeds was approximately 10^7 cells $seed^{-1}$. Treated seeds were kept at 4 °C for 1-2 days before being used in the bioassays.

Plant pathogens

The sugar beet pathogens included in this study were *Rhizoctonia solani* AG2.25, *Aphanomyces cochlioides* (isolate MV-A3 or B19) and *Pythium ultimum* (IRS). All three were isolated from the sugar beet rhizosphere and acquired from Dr. Hans Schneider (IRS, Bergen op Zoom, the Netherlands). The pathogens were routinely grown on potato dextrose agar (PDA) and incubated at 25 ° C. Pathogens were stored for short term on PDA plates at 15 °C in the dark. For long term storage, mycelial plugs were immersed in sterile mineral oil or sterile distilled water, and stored at 15 °C. Inoculum used in the bioassays was prepared as follows. For *Aphanomyces cochlioides*, zoospores of isolate B19 were acquired after placing mycelial plugs of young cultures in the dark at 20 ° C in a Petri dish with sterile distilled water; after 24 h, zoospores were collected and enumerated. In the rock wool bioassays, 100 zoospores were added per rock wool plug (20-mm diameter and 25-mm height). In the assays performed with agricultural soil (CB soil + SSB soil + river sand), 1000 zoospores per pot were added. To obtain encysted zoospores, a mechanical stimulus resulting from vigorous mixing for 25 to 30 s was used resulting in 100% encystment. For *Rhizoctonia solani* and *Pythium ultimum*: rock wool plugs were inoculated with 1 mycelial plug (0.5-cm-diameter) of a 3-day-old culture, at the side of the rock wool plug.

In vitro activity of DAPG and DAPG-producing *Pseudomonas* strains

Sensitivity of the three pathogens to DAPG was tested on potato dextrose agar (PDA) medium buffered with 0.0015 M citric acid to a pH 6.5. DAPG was added at concentrations ranging from 2 to 256 μg/ml of PDA medium; for each concentration, three replicate plates were used. A 0.5-cm-diameter agar plug covered with actively growing mycelium was placed in the centre of the plate and incubated at 25 ° C for 7 days; every day, the diameter of the growing fungal colony was measured. In parallel, the growth-

inhibitory activity of the eight *Pseudomonas* strains against the three pathogens was tested in dual culture assays on 1/5th strength PDA plates. Washed cell suspensions of the bacterial strains (10 µl 10^6 cells/ml) were applied at the perimeter of the plates one day prior to pathogen inoculation in the centre of the plates. Plates were incubated at 25 ^oC for 7 days, and four replicates were used per *Pseudomonas* strain.

Rock wool bioassays

Biocontrol activity of each of the bacterial strains was tested in trays with rock wool (Grodan) plugs (20-mm diameter and 25-mm height). Bacterial inoculum was prepared from cell cultures grown on PSA plates at 25 ^{o} C for 72 h. Bacterial cells were collected and pelleted by centrifugation at 4000 X *g* for 10 min at 4^o C followed by washing in sterile destilled water and resuspending to a final density of 10^9 CFU ml^{-1}. Four ml of the bacterial suspension was added to each rockwool plug; in the control 4 ml of sterile water was added. Next, one ml of a zoospore suspension (100 zoospores ml^{-1}) or one mycelial plug of each pathogen was added, and one sugar beet seed was sown per rock wool plug and covered with a 1-cm layer of river sand. The rock wool trays were placed in humid boxes with a transparent lid and incubated in a climate chamber (20 ^{o}C, 16-h photoperiod). Germination of the sugar beet seeds was scored at 4-5 days after sowing, and post-emergence damping-off was scored between 5 and 12 days after sowing. The disease incidence was calculated by dividing the number of plants with typical symptoms of damping-off disease by the total number of seedlings. Each treatment had six replicates with nine plants per replicate. Root colonization assays with the DAPG-producers introduced into the rock wool plugs showed that after 8 days of incubation and plant growth each of the eight tested strains reached a density of approximately 10^6 cfu g^{-1} root (data not shown).

Soil bioassays

For the soil bioassays, sugar beet seeds were sown in small plastic pots containing 25 g of a mixture of CB soil, SSB soil and river sand (1:1:2 v/v/v ratio) as previously described (de Souza et al., 2003, Bergsma-Vlami et al., 2005a). The *Pseudomonas* strains were introduced into the soil at initial densities ranging from 1 - 5 x 10^9 cells g^{-1} soil dry weight one day prior to pathogen inoculation. For *A. cochlioides,* zoospores were introduced at a density of 1000 zoospores pot^{-1}. Plants were grown in the climate room under controlled conditions (20^{o}C, 16-h photoperiod). After twenty eight days of plant growth, disease incidence (%) was assessed. For each bacterial strain, six replicates of ten plants were included.

Rhizosphere competence on sugar beet

To determine the relationship between rhizosphere competence and biocontrol activity, each of the *Pseudomonas* strains were introduced into agricultural soil (mixture of CB soil, SSB soil and river sand 1:1:2 v/v/v ratio) at an initial density of 10^4 cells g^{-1} soil fresh weight. Sugar beet plants were grown for six successive cycles as described previously (Bergsma-Vlami et al., 2005a). After four weeks of growth, plants were harvested and their root system with rhizosphere soil was collected. Excess of root material was mixed through the cultivated soil and represented approximately 0.5% of the soil dry weight. The cultivated soil was subsequently returned to the same pot and replanted with sugar beet. In each growth cycle, rhizosphere population densities of the introduced *Pseudomonas* strains were determined by plating rhizosphere samples onto selective medium. For each strain, four replicates were included.

Statistical analysis

Differences in sensitivity of *Aphanomyces cochlioides, Rhizoctonia solani* and *Pythium ultimum* to DAPG and DAPG-producing *Pseudomonas* strains were determined by Analysis of Variance (ANOVA) followed by Tukey's Studentized Range Test. Additionally, the Effective Concentrations (EC_{50} and EC_{95}) at which 50% and 95% of mycelial growth per pathogen is inhibited were calculated using a probit transformation. Differences in disease incidence caused by *A. cochlioides*, *R. solani* and *P. ultimum* were determined by Analysis of Variance (ANOVA) followed by Tukey's Studentized Range Test. Values of disease incidence (%) were arcsin transformed prior to statistical analysis, when necessary to acquire normalized data. The Area Under the Colonization Progress Curve (AUCPC), which is a measure to assess rhizosphere colonization over time, was calculated for each *Pseudomonas* genotype using the trapezoidal integration method described previously (Landa et al., 2002, Bergsma-Vlami et al., 2005a). Population densities of introduced *Pseudomonas* strains were $\log_{10}$ transformed prior to statistical analysis. Population densities and AUCPC values were subjected to analysis of variance (ANOVA) followed by Tukey's Studentized Range Test, after certifying normal distribution and homogeneity of variances (SAS Institute Inc., Cary, NC). The relationship between the percentage (%) of disease incidence caused by *Aphanomyces cochlioides* on sugar beet seedlings and the AUCPC for each *Pseudomonas* strain was analyzed using a linear regression (polynomial). The equation used was $Y=Y_0+\alpha*X$, where Y represents the AUCPC, X is the % of disease

and Y_0 gives the maximum value of Y. All experiments were performed at least twice and representative results are shown.

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Chapter 6

General and summarizing discussion

General and summarizing discussion

Pseudomonas species exert their beneficial effect on plant growth and health via several different mechanisms, including active exclusion of plant pathogens from the rhizosphere (Rainey, 1999, Haas and Keel, 2003, Haas and Defago, 2005). The antibiotic 2,4-diacetylphloroglucinol (DAPG) has been implicated in biological control of multiple plant pathogens and a large number of DAPG-producing Pseudomonas strains are effective biocontrol agents (reviewes in Raaijmakers et al., 2002; Haas and Defago, 2005; Weller et al. 2002, 2007). Despite obvious benefits for agriculture and horticulture, attempts to exploit DAPG-producing Pseudomonas strains as biocontrol inoculants have had limited success so far (Haas and Defago, 2005; Fravel, 2005). Results obtained in field experiments are typically inconsistent and a major factor contributing to this inconsistency is their variable ecological performance (Weller, 1988). The ecological performance of DAPG-producing Pseudomonas strains is complex and affected by many different bacterial traits and a multitude of environmental factors. In this thesis, several genotypic and phenotypic characteristics underlying the ecological performance of DAPG-producing Pseudomonas strains were investigated with the ultimate goal to improve their rhizosphere competence, survival and biocontrol efficacy.

Genotypic diversity of DAPG-producing *Pseudomonas*

Most of the existing methods to assess the genotypic diversity of DAPG-producing *Pseudomonas* strains require the isolation and cultivation of *Pseudomonas* strains from soil and rhizosphere environments prior to their genotypic characterization (Keel et al. 1996; Picard et al. 2000, McSpadden et al., 2000, 2001a, 2001b, Landa et al., 2002, 2006, Raaijmakers and Weller, 2001; Mavrodi et al. 2001, Ramette et al., 2001, Wang et al., 2001, Mazzola et al., 2004). To discriminate between genotypically different DAPG-producing *Pseudomonas* strains directly in rhizosphere samples, a simple and rapid method was developed (**chapter 2**) based on polymorphisms in the polyketide synthase gene *phl*D. Denaturing Gradient Gel Electrophoresis (DGGE) analysis, sequencing and phylogenetic analyses of indigenous *phlD*$^+$ isolates obtained from the rhizosphere of wheat, sugar beet and potato plants, resulted in the identification of seven *phlD*$^+$ genotypes, designated A, B, C, D, E, F, and Z, five of which were not described previously (C, D, E, F and Z). None of the *phl*D$^+$ isolates identified in this study were assigned to groups of well-known DAPG-producers, including DGGE-groups G (reference strain Q8R1-96), I (strain F113) and M (strain CHA0).

The *phlD*-DGGE technique allowed direct detection and assessment of the genotypic diversity of DAPG-producing *Pseudomonas* in the rhizosphere (**chapter 2**). A single strain introduced in the rhizosphere of wheat was detectable at densities equal or higher than $5x10^3$ CFU g^{-1} of root fresh weight. Additionally, *phlD*-DGGE analysis allowed simultaneous detection of *phl*D^+ isolates present in mixtures in the wheat rhizosphere at a final density for each isolate of approximately $5x10^5$ CFU g^{-1} root. Compared to cultivation-based approaches, the *phlD*-DGGE technique does not have the bias toward detecting either the most dominant genotype or the genotype with higher growth rates or competitive abilities during cultivation. More recently, also the allele-specific primer-based technique and real-time PCR assay were shown to be rapid and highly specific methods for studies on the population dynamics of DAPG-producers and interactions between different genotypes in natural environments (De La Fuente et al., 2006, Mavrodi et al., 2007).

Influence of plant species on the dynamics and diversity of DAPG-producing *Pseudomonas*

Plant species can have a profound effect on the dynamics and diversity of the root-associated microflora. The results described in this thesis showed that four different plant species (wheat, sugar beet, potato, lily) supported relatively high rhizosphere populations ($5*10^4$ to $1*10^6$ CFU g^{-1} root) of indigenous DAPG-producing *Pseudomonas* spp. Similar population densities of DAPG-producers were previously reported for the rhizosphere of wheat (Raaijmakers et al., 1997, De Souza et al., 2003) and pea (Landa et al., 2002), grown in soils naturally suppressive to *Gaeumannomyces graminis* var. *tritici* or *Fusarium oxysporum* f.sp. *pisi*, respectively. Higher densities ($7*10^6$ CFU g^{-1} root) were reported for the rhizosphere of tobacco grown in a soil suppressive to *Thielaviopsis basicola* (Ramette et al., 2003). Although lily supported on average the highest population densities of fluorescent *Pseudomonas* spp. of all four crops, it was the least supportive of DAPG-producing pseudomonads (**chapter 3**). In a previous study, similar adverse effects on populations of DAPG-producing pseudomonads were observed for oats (Raaijmakers and Weller, 1998). Interesting in this context is the fact that both oats and Liliaceae are well known for the secretion of steroid saponins into the rhizosphere (Mimaki et al., 1999, Sang et al., 2003, Park et al., 2003). Saponins, including avenacoside A and B from oats, exhibit activity against fungi, Gram-negative and Gram-positive bacteria (Mahato et al., 1982). Whether DAPG-producing pseudomonads are more sensitive to saponins than other bacterial genera or species has not yet been investigated.

Analysis of the genotypic diversity of 492 DAPG-producing *Pseudomonas* isolates, revealed a total of 7 genotypic groups (**chapter 3**). Some of the genotypic groups were found only in the rhizosphere of a specific plant species, whereas other genotypic groups were found in relatively high frequencies in the rhizosphere of three plant species (wheat, sugar beet and potato). Statistical analysis of the *phl*D$^+$ genotype frequencies showed that the diversity of the *phl*D$^+$ isolates from lily was significantly lower than the diversity of *phlD*$^+$ isolates found on wheat, sugar beet or potato. For lily, DGGE-genotype A was the most dominant. For the other three plant species, no statistically significant effects on *phl*D$^+$ genotype frequencies were found (**chapter 3**). DGGE-genotype E represented almost 50% of the genotypic diversity present among the *phlD*$^+$ isolates originating from three plant species. The widespread occurrence of DGGE-genotypes E and also B and Z in the rhizospheres of multiple crops opens up opportunities to apply specific strains or consortia of these genotypically different DAPG-producing *Pseudomonas* strains for control of soil-borne pathogens on multiple crops, i.e. wheat, sugar beet and potato.

Subsequent HPLC analysis showed that the plant species had a significant effect on DAPG-production by the indigenous *phlD*$^+$ population: the wheat and potato rhizospheres supported significantly higher amounts of DAPG produced per cell basis than the rhizospheres of sugar beet and lily (**chapter 3**). It is well known that the carbon composition of the growth medium significantly affects antibiotic production *in vitro* (Nowak-Thompson et al., 1994, Shanahan et al., 1992). Therefore, variations in the carbon composition of root exudates of different plant species may have contributed, at least in part, to the observed differences in DAPG production in the rhizosphere. The results obtained in this study support and extend previous results obtained in a gnotobiotic system with DAPG-producing *P. fluorescens* strain CHA0 (Notz et al., 2001). They showed that transcription of the *phlA* gene was significantly higher in the rhizospheres of the two monocots (wheat and maize) compared to two dicots (bean and cucumber). In our study (**chapter 3**), DAPG was only detected in rhizosphere samples when the population densities of the *phlD*$^+$ pseudomonads were higher than 10^5 CFU g^{-1} root. This is in agreement with results obtained previously (Raaijmakers et al., 1999), and may have been due to technical limitations of the methodology, or confirm the observation that DAPG biosynthesis is dependent on cell density (Schnider-Keel et al., 2000).

Rhizosphere competence of DAPG-producing *Pseudomonas*

The plant species may modulate the population size and composition of indigenous or introduced DAPG-producers (Lynch and Whipps, 1990, Bergsma-Vlami et al., 2005, Landa et al., 2003, McSpadden et al., 2005, Picard and Bosco, 2003, Bertin et al., 2003, Raaijmakers et al. 2008, Picard et al., 2008). Results described in this thesis showed significant differences in rhizosphere competence and survival of DAPG-producing strains for one and multiple plant species (**chapter 4**)**.** Three genotypes of DAPG-producing *Pseudomonas* (A-G, and Z) were shown to be superior in long-term colonization of roots of wheat, sugar beet and potato plants. These results suggest that the ability of these three genotypes to rapidly establish and maintain high population densities in the rhizosphere is not linked to a specific plant species, but is due to specific characteristics that enable these generalist strains to be competitive in different rhizosphere environments. On the other hand, successful establishment and survival of genotypes E, C and F was dependent on the plant species and, therefore, these strains are considered to be specialists.

When introduced into the sugar beet rhizosphere, the genotypes differed significantly in their ability to produce DAPG *in situ*: genotype E produced much more DAPG per cell basis (143.6 ng per 10^5 cells) than the other genotypes with DAPG concentrations ranging from 1.0 and 57.3 ng per 10^5 cells (**chapter 4**)**.** To determine the role of DAPG-production in rhizosphere competence, two approaches were taken. The first involved comparison of the rhizosphere competence of genotype G with that of its DAPG-deficient mutant G-. The results showed that DAPG production does not contribute to the rhizosphere competence of genotype G. Previous results also demonstrated that DAPG production is not an important trait for genotype G in colonization of the wheat rhizosphere (Raaijmakers and Weller, 2001). Similarly, loss of DAPG production did not reduce the ecological fitness of *P. fluorescens* F113 in the rhizosphere of sugar beet (Carroll et al., 1995).

In the second approach, dose-response experiments were performed and showed significant correlations between the rhizosphere competence of a genotype and *in situ* DAPG production levels (**chapter 4**)**.** In general, these correlations suggest that genotypes that produce high amounts of DAPG per cell basis *in situ* establish lower population densities in the sugar beet rhizosphere than genotypes that produce small amounts of DAPG. To our knowledge, this is the first study that shows an inverse correlation between rhizosphere competence of fluorescent *Pseudomonas* strains and *in situ* antibiotic production. To further support this hypothesis, root colonization experiments should be conducted where the rhizosphere competence of strains/genotypes that produce relatively high amounts of DAPG *in situ* is compared to that of their DAPG-deficient mutants.

Biocontrol ability of DAPG-producing *Pseudomonas*

DAPG plays an important role in the suppression of plant pathogens by several *Pseudomonas fluorescens* strains (reviewed in Raaijmakers et al., 2002; Haas and Defago, 2005; Weller et al. 2002, 2007). In the present study, eight genotypically different DAPG-producing *Pseudomonas* strains (genotypes A–G and Z) were evaluated for their efficacy to control three different pathogens of sugar beet, i.e. *Pythium ultimum*, *Rhizoctonia solani* and *Aphanomyces cochlioides*. Results showed significant variation in sensitivity of *P. ultimum*, *R. solani* and *A. cochlioides* to either pure DAPG or to antagonism by the DAPG-producing *Pseudomonas* strains, with *R. solani* being the least sensitive (**chapter 5**)**.** These results are in parallel to results of the *in planta* assays which showed that *R. solani* damping-off could not be controlled by any of the eight DAPG-producing *Pseudomonas* strains. In contrast, *P. ultimum* was effectively controlled by all eight strains and differential effects were observed in biocontrol activity against *A. cochlioides* (**chapter 5**). *Pseudomonas* genotype G was the most effective in biocontrol of *Pythium* and *Aphanomyces* damping-off, and its biocontrol activity was due, at least in part, to DAPG production (**chapter 5**).

Also in agricultural soil, four genotypes (G, Z, A and E) effectively controlled *Aphanomyces* damping-off disease and rhizosphere competence was shown to be an important determinant of the efficacy to control *A. cochlioides* in sugar beet (**chapter 5**). Preliminary results of a small-scale field experiment also showed significant reductions in *Aphanomyces* disease incidence by introduction of *Pseudomonas* genotypes A, E and Z. Their biocontrol efficacy (on average 38% disease reduction relative to the control) was promising, but did not yet reach the level of disease control (61% disease reduction) obtained by application of the fungicide hymexazol (Tachigaren). Whether the higher biocontrol efficacy of genotypes A, E and Z under field conditions was due to a better rhizosphere competence than observed for the other genotypes was not investigated and will be addressed in future field experiments.

Future perspectives

Given that rhizosphere competence is an essential prerequisite for successful control of seed and soil-borne pathogens, knowledge of the compatibility between host plant species and antagonistic microorganisms is essential to improve biological control. The results of this thesis revealed that several DAPG-producing *Pseudomonas* strains exhibit a high rhizosphere competence on multiple plant species, whereas others have a preference for single plant species. However, little is known about the molecular and biochemical mechanisms underlying the colonization preference of DAPG-producing biocontrol bacteria.

Additionally, the natural functions of DAPG for the producing strains are largely unclear. Production of DAPG in the rhizosphere was recently proposed to be a mechanism by which bacteria manipulate the nutritional status in the rhizosphere as DAPG significantly enhances the exudation of amino acids from plant roots (Philips et al. 2004). Based on these results, we expected that strains that produce higher levels of DAPG would be more rhizosphere competent. However, our results show that strains that produce high amounts of DAPG per cell basis establish lower population densities in the rhizosphere. One possible explanation may be that higher exudation of amino acids promotes the growth and activity of the indigenous microflora, especially when DAPG leads to the release of amino acids that cannot or less efficiently be utilized by the introduced *Pseudomonas* strains.

Recent studies with maize showed that it may be possible to increase plant health by a breeding strategy directed towards plant genotypes that favour the early establishment of high population densities of DAPG-producing *Pseudomonas* species (Picard et al., 2008). Identifying plant traits that support or promote the growth and activity of introduced biocontrol strains is a potential alternative or supplementary strategy towards higher sustainability in agriculture. The identification of genetic traits in host preference of beneficial microorganisms will therefore be of great importance, because it may allow maximizing root colonization and enhancing the efficacy and consistency of biological control.

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Summary

The phenolic antibiotic 2,4-diacetylphloroglucinol (DAPG) has been implicated in biological control of multiple plant pathogens by fluorescent *Pseudomonas* species. DAPG-producing *Pseudomonas* strains are effective biocontrol agents, however, their ecological performance is often highly variable resulting in inconsistent disease suppression. The ecological performance is complex and determined by many bacterial traits and environmental factors, including the host plant. In this thesis, several genotypic and phenotypic characteristics underlying the ecological performance of DAPG-producing *Pseudomonas* were investigated.

To discriminate between genotypically different DAPG-producing *Pseudomonas* strains directly in rhizosphere samples without their prior isolation or enrichment on nutrient media, a simple and rapid method was developed based on polymorphisms in the polyketide synthase gene *phl*D. Denaturing Gradient Gel Electrophoresis (DGGE) analysis, sequencing and phylogenetic analyses of indigenous *phlD*$^+$ isolates obtained from the rhizosphere of wheat, sugar beet and potato plants, resulted in the identification of seven *phlD*$^+$ genotypes, designated A, B, C, D, E, F, and Z, five of which were not described previously (C, D, E, F and Z). The *phlD*-DGGE analysis allowed simultaneous detection of multiple *phl*D$^+$ isolates in the rhizosphere and, compared to cultivation-based approaches, this technique does not have the bias toward detecting either the most dominant genotype or the genotype with higher growth rates or competitive abilities during cultivation.

Subsequent studies with representative strains of each of the *Pseudomonas* genotypes showed that three genotypes (A, Z and G) were superior in long-term colonization of roots of wheat, sugar beet and potato plants. These results suggest that their rhizosphere competence is not linked to a specific plant species, but is due to yet unknown characteristics that enable these strains to be competitive in different rhizosphere environments. In contrast, the rhizosphere competence of *Pseudomonas* genotypes E, C and F was dependent on the plant species and, therefore, these strains are considered to be specialists instead of generalists.

Results of this thesis further showed that the host plant species also have a significant effect on DAPG-production by indigenous *phlD*$^+$ *Pseudomonas*: the wheat and potato rhizospheres supported significantly higher amounts of DAPG produced per cell basis than the rhizospheres of sugar beet and lily. In the same context, the eight *Pseudomonas* genotypes differed significantly in their ability to produce DAPG in the rhizosphere of sugar beet plants with *in situ* DAPG concentrations ranging from 1 to 144 ng per 10^5 cells. Based on these data, significant correlations were established between the rhizosphere competence of a genotype and *in situ* DAPG production levels. In general, these

correlations suggest that *Pseudomonas* genotypes that produce high amounts of DAPG per cell basis *in situ* establish lower population densities in the sugar beet rhizosphere than genotypes that produce small amounts of DAPG. To our knowledge, this is the first study that shows an inverse correlation between rhizosphere competence of *Pseudomonas* strains and *in situ* antibiotic production.

Biocontrol assays showed that *P. ultimum* was effectively controlled by all eight *Pseudomonas* strains and differential effects were observed in biocontrol activity against *A. cochlioides*. *Pseudomonas* genotype G was the most effective in biocontrol of *Pythium* and *Aphanomyces* damping-off, and its biocontrol activity was due, at least in part, to DAPG production as its DAPG-deficient mutant was significantly less effective. Comparative analysis of the eight DAPG-producing *Pseudomonas* genotypes revealed a highly significant correlation between their rhizosphere competence and efficacy to control Aphanomyces damping-off of sugar beet. These results indicate that the more rhizosphere competent DAPG-producing *Pseudomonas* strains are, the higher their efficacy is to control *A. cochlioides* in sugar beet. The promising results obtained with genotypes A, Z and G in the sugar beet bioassays provide a strong basis for their implementation in the current integrated disease management strategies in sugar beet.

The results acquired in this thesis have shown that the identification of the genotypic diversity and rhizosphere competence of antibiotic-producing Pseudomonas species is of great value, because it may allow maximizing root colonization and disease suppression. Knowledge of genetic traits involved in host preference of these antagonistic bacteria will help to identify strains that are adequately adapted to specific host-pathogen systems. Similarly, looking into plant traits that promote the growth and activity of introduced biocontrol strains can be highly complementary and further contribute to sustainability in agriculture.

Samenvatting

Samenvatting

Het antibioticum 2,4-diacetylphloroglucinol (DAPG) speelt een belangrijke rol in biologische bestrijding door fluorescerende *Pseudomonas* soorten van verschillende pathogenen die plantenziekten veroorzaken. DAPG-producerende *Pseudomonas* stammen zijn effectief in biologische bestrijding, maar hun saprofytisch vermogen is vaak variabel met als gevolg dat ook de mate van ziekteonderdrukking niet consistent is. Het saprofytisch vermogen van DAPG-producerende *Pseudomonas* stammen is complex en wordt bepaald door diverse bacteriële eigenschappen en omgevingsfactoren waaronder de verschillende waardplanten die gekoloniseerd en beschermd moeten worden. In dit proefschrift zijn een aantal genotypische en phenotypische eigenschappen van DAPG-producerende *Pseudomonas* stammen onderzocht en is bepaald in welke mate deze bijdragen aan het saprofytisch vermogen en de effectiviteit van biologische bestrijding.

Om de genotypische diversiteit van DAPG-producerende *Pseudomonas* isolaten direct in de rhizosfeer te kunnen bepalen zonder ze eerst te moeten isoleren, is er een snelle en eenvoudige methode ontwikkeld die gebasseerd is op polymorphismen in *phl*D, een essentieel gen in de biosynthese van DAPG. Op basis van DGGE (Denaturing Gradient Gel Electrophoresis) analyse van het *phlD* gen, sequentie en fylogenetische analyses van DAPG producerende bacteriën in de rhizosfeer van tarwe, suikerbiet en aardappel konden zeven *phlD*$^+$ genotypen (A, B, C, D, E, F en Z) worden onderscheiden, waarvan vijf (C, D, E, F en Z) nog nooit eerder waren beschreven. De *phlD*-DGGE analyse maakte het tevens mogelijk om meerdere genotypen van DAPG-producerende *Pseudomonas* stammen tegelijkertijd in de rhizosfeer te detecteren en te onderscheiden.

Vervolgexperimenten met *Pseudomonas* stammen die representatief zijn voor elk genotype toonden aan dat drie genotypen (A, Z en G) zeer goed de rhizosfeer van tarwe, suikerbiet en aardappel kunnen koloniseren. Dit resultaat suggereert dat het saprofytisch vermogen van deze drie genotypen niet zozeer bepaald wordt door één specifieke plantensoort, maar het gevolg is van nog onbekende eigenschappen die deze *Pseudomonas* stammen een competitief voordeel geven onder verschillende rhizosfeer condities. In tegenstelling tot de drie genotypen A, Z en G, bleek het saprofytisch vermogen van de *Pseudomonas* genotypen E, C en F wel afhankelijk te zijn van de plantensoort. Deze stammen werden daarom aangemerkt als specialisten in plaats van generalisten.

Resultaten beschreven in dit proefschrift tonen tevens aan dat de plantensoort een significant effect heeft op de productie van DAPG door natuurlijke *phlD*$^+$ *Pseudomonas* populaties: in de rhizosfeer van tarwe en van aardappel werden significant hogere DAPG-concentraties (per cel) gemeten dan in de rhizosfeer van suikerbiet en lelie. Ook wanneer

verschillende *Pseudomonas* genotypen geïntroduceerd werden in de rhizosfeer van suikerbietplanten werden grote verschillen in DAPG-productie gemeten, met concentraties variërend van 1 to 144 ng per 10^5 cellen. Op basis van deze resultaten werden significante correlaties gevonden tussen het saprofytisch vermogen in de rhizosfeer en de DAPG-concentratie. In het algemeen suggereren deze correlaties dat *Pseudomonas* stammen die grote hoeveelheden DAPG produceren in de rhizosfeer van suikerbiet een lagere populatiedichtheid bereiken, terwijl stammen die kleine hoeveelheden DAPG produceren juist hogere populatiedichtheden realiseren. Dit is de eerste keer dat er een reciproke relatie aangetoond wordt tussen het saprofytisch vermogen van *Pseudomonas* stammen en de *in situ* DAPG productie.

Studies naar het vermogen van de verschillende *Pseudomonas* stammen om drie verschillende suikerbietpathogenen te onderdrukken laten zien dat *Pythium ultimum* effectief werd onderdrukt door alle acht DAPG-producenten. In biologische bestrijding van *Aphanomyces cochlioides* werden differentiële effecten waargenomen, terwijl geen van de acht onderzochte *Pseudomonas* stammen in staat was om *Rhizoctonia solani* te onderdrukken. *Pseudomonas* genotype G was het meest effectief in de biologische bestrijding van *Pythium* en *Aphanomyces*; het ziekteonderdrukkend vermogen van genotype G was deels gebaseerd op DAPG-productie, omdat de DAPG-mutant minder effectief was. De resultaten van deze biotoetsen met acht DAPG-producerende *Pseudomonas* genotypen toonden een significante correlatie aan tussen het koloniserend vermogen van deze stammen en hun vermogen om *A. cochlioides* te onderdrukken. De veelbelovende biologische activiteit van met name genotypen A, Z en G vormen een ideaal uitgangspunt om de effectiviteit van deze stammen nader te toetsen in veldexperimenten met als doel deze biologische agentia te implementeren in de bestaande beheersmaatregelen in de suikerbietenteelt.

Περὶληψη

Περὶληψη

Βακτὴρια του γένους *Pseudomonas* τα οποὶα παράγουν την αντιβιοτικὴ ουσὶα 2,4-δι-ακετυλο-φλορογλουσινόλη της ομάδας των φαινολών, ἐχουν συχνὰ χαρακτηριστεὶ ικανὰ για την βιολογικὴ καταπολέμηση πολλαπλών ασθενειών των φυτών. Παρόλα αυτὰ, η οικολογικὴ τους δράση εἰναι συχνὰ ασταθὴς με αποτέλεσμα να βοηθοὺν στην μείωση ασθενειών στα φυτὰ με μὴ συνεπὴ τρόπο. Η οικολογικὴ τους δράση εἰναι ἐνας πολὺπλοκος παράγοντας που καθορὶζεται από διαφορετικὰ χαρακτηριστικὰ των βακτηρὶων καθὼς και από περιβαντολλογικοὺς παράγοντες, συμπεριλαμβανομένου του ἰδιου του φυτοὺ. Στην παροὺσα διδακτορικὴ μελέτη, διαφορετικὰ γονοτυπικὰ και φαινοτυπικὰ χαρακτηριστικὰ αναφερὸμενα στην οικολογικὴ δράση των βακτηρὶων του γένους *Pseudomonas* που παράγουν την αντιβιοτικὴ ουσὶα 2,4-δι-ακετυλο-φλορογλουσινόλη ἐχουν λεπτομερὼς μελετηθεὶ.

Με σκοπὸ τον ἀμεσο χαρακτηρισμὸ, κατευθεὶαν στο ριζικὸ σὺστημα του φυτοὺ, ποικὶλων βακτηριακὼν γονοτὺπων του γένους *Pseudomonas* που παράγουν την αντιβιοτικὴ ουσὶα 2,4-δι-ακετυλο-φλορογλουσινόλη, χωρὶς την προκαταβολικὴ απομὸνωσὴ τους ὴ τον προκαταβολικὸ εμπλουτισμὸ τους σε θρεπτικὰ υποστρὼματα, σχεδιὰσαμε μια απλὴ και γρὴγορη μὲθοδο βασιζὸμενη σε πολυμορφισμοὺς του γονιδὶου *phl*D. Η ανὰλυση με βὰση την μὲθοδο DGGE (Denaturing Gradient Gel Electropheresis) καθὼς και φυλογενετικὲς αναλὺσεις *phl*D$^+$ βακτηρὶων ενδημικὼν στο ριζικὸ σὺστημα του σιταριοὺ, του ζαχαρὸτευτλου και της πατὰτας, εἰχε ως αποτέλεσμα τον χαρακτηρισμὸ επτὰ *phl*D$^+$ γονοτὺπων, συγκεκριμένα A, B, C, D, E, F και Z. Οι γονὸτυποι C, D, E, F και Z δεν ἐχουν περιγραφτεὶ προηγουμένως. Με βὰση την ανὰλυση *phl*D-DGGE, ταυτὸχρονη ανὶχνευση πολλαπλὼν *phl*D$^+$ βακτηρὶων πραγματοποιὴθηκε στο ριζικὸ σὺστημα. Συγκρὶνοντας την ανὰλυση *phl*D-DGGE με ἀλλες αναλὺσεις που βασὶζονται σε προκαταβολικὴ καλλιὲργεια των απομονωμένων *phl*D$^+$ βακτηρὶων σε θρεπτικὰ υποστρὼματα, καταλὴγουμε στο συμπὲρασμα ὸτι η ανὰλυση *phl*D-DGGE δεν παρουσιὰζει προκατὰληψη ως προς την ανὶχνευση εἰτε του περισσὸτερου κυρὶαρχου γονοτὺπου, ὴ του γονοτὺπου με τους υψηλὸτερους ρυθμοὺς ανὰπτυξης ὴ με τις υψηλὸτερες ανταγωνιστικὲς δραστηριὸτητες κατὰ την διὰρκεια της καλλιὲργειας τους. Μελὲτες με αντιπροσωπευτικὰ βακτὴρια από οκτὼ γονοτὺπους ἐδειξαν ὸτι τρεις γονὸτυποι (A, Z και G) επιβὶωσαν στο ριζικὸ σὺστημα του σιταριοὺ, του ζαχαρὸτευτλου και της πατὰτας για μεγὰλο χρονικὸ διὰστημα. Αυτὰ τα αποτελὲσματα υποδεικνὺουν ὸτι η ικανὸτητα αυτὼν των γονοτὺπων για να αποικὶσουν και να επιβιὼσουν στο ριζικὸ σὺστημα δεν εξαρτὰται από το εἰδος του φυτοὺ που

χρησιμοποιήθηκε, αλλά οφείλεται σε άλλους παράγοντες που δεν έχουν ακόμη καθοριστεί και οι οποίοι επιτρέπουν αυτά τα βακτήρια να είναι ανταγωνιστικά στο ριζικό σύστημα διαφορετικών φυτών. Σε αντίθεση, η ικανότητα των γονοτύπων E, C και F να αποικίσουν και να επιβιώσουν στο ριζικό σύστημα, εξαρτάται από το είδος του φυτού που χρησιμοποιήθηκε

Αποτελέσματα αυτής της διδακτορικής μελέτης επείσης έδειξαν ότι το είδος του φυτού παίζει έναν σημαντικό ρόλο στην παραγωγή της αντιβιοτικής ουσίας 2,4-δι-ακετυλο-φλορογλουσινόλη από έναν ενδημικό *phl*D$^+$ πληθυσμό βακτηρίων του γένους *Pseudomonas* Το ριζικό σύστημα του σιταριού και της πατάτας παρουσίασαν σημαντικά μεγαλύτερες συγκεντρώσεις της ουσίας αυτής από ότι το ριζικό σύστημα του ζαχαρότευτλου και του *Lilium*. Παράλληλα, οι οκτώ βακτηριακοί γονότυποι διαφοροποιήθηκαν σημαντικά όσον αφορά την ικανότητά τους να παράγουν την αντιβιοτική ουσία στο ριζικό σύστημα του ζαχαρότευτλου, με συγκεντρώσεις, *in situ,* κυμαινόμενες απο 1 ως 144 ng ανα 10^5 κύτταρα. Με βάση τα αποτελέσματα αυτά, σημαντικές συσχετίσεις καθορίστηκαν ανάμεσα στην ικανότητα ενός γονοτύπου να αποικίσει και να επιβιώσει στο ριζικό σύστημα και στην παραγωγή της αντιβιοτικής ουσίας 2,4-δι-ακετυλο-φλορογλουσινόλη. Οι συσχετίσεις αυτές υποδεικνύουν ότι γονότυποι για τους οποίους υψηλά ποσά της αντιβιοτικής ουσίας 2,4-δι-ακετυλο-φλορογλουσινόλη παράγονται, *in situ,* ανά βακτηριακό κύτταρο, καθιερώνουν χαμηλές πληθυσμιακές συγκεντρώσεις στο ριζικό σύστημα του ζαχαρότευτλου από ότι γονότυποι που παράγουν χαμηλά ποσά της ίδιας αντιβιοτικής ουσίας. Αυτή είναι η πρώτη μελέτη που δείχνει μια αντιστρόφως ανάλογη σχέση μεταξύ της ικανότητας ενός *phl*D$^+$ βακτηρίου του γένους *Pseudomonas* να αποικίσει και να επιβιώσει στο ριζικό σύστημα και της παραγωγής της αντιβιοτικής ουσίας, *in situ*.

Πειράματα όσον αφορά την βιολογική καταπολέμηση του *P. ultimum,* έδειξαν ότι όλοι οι οκτώ βακτηριακοί γονότυποι καταπολέμησαν αποτελεσματικά το παθογόνο αυτό. Διαφορετικός βαθμός καταπολέμησης παρατηρήθηκε για τους οκτώ γονοτύπους εναντίον του παθογόνου *A. cochlioides*. Ο γονότυπος G ήταν ο πιο αποτελεσματικός στην καταπολέμηση του *P. ultimum* και *A. cochlioides* στο ζαχαρότευτλο και η βιολογική δράση του γονοτύπου G οφειλόταν, εν μέρει τουλάχιστον, στην παραγωγή της αντιβιοτικής ουσίας, 2,4-δι-ακετυλο-φλορογλουσινόλη καθώς ο μεταλλαγμένος γονότυπος (*phl*D$^-$) στο γονίδιο *phl*D ήταν σημαντικά λιγότερο αποτελεσματικός. Συγκριτική ανάλυση των οκτώ βακτηριακών γονοτύπων ανέδειξε μια πολύ σημαντική συσχέτιση μεταξύ της ικανότητάς τους να αποκίσουν και να επιβιώσουν στο ριζικό σύστημα και της αποτελεσματικότητας τους ενάντια του παθογόνου *A. cochlioides* στο ζαχαρότευτλο. Αυτά τα αποτελέσματα υποδεικνύουν ότι όσο πιο ικανά είναι τα βακτήρια αυτά να αποικίσουν και να επιβιώσουν στο ριζικό σύστημα,

τὸσο πιο αποτελεσματικὰ εἰναι στην καταπολὲμηση του *A. cochlioides* στο ζαχαρὸτευτλο Τα αποτελὲσματα για τους γονοτὺπους A, Z και G στο ζαχαρὸτευτλο αποτελοὺν μια ισχυρὴ βὰση για την συμμετοχὴ τους στο επικρατοὺμενο ολοκληρωμὲνο πρὸγραμμα καταπολὲμησης παθογὸνων στο ζαχαρὸτευτλο.

Τα αποτελὲσματα αυτὴς της διδακτορικὴς μελὲτης ὲδειξαν ὸτι ο χαρακτηρισμὸς της γονοτυπικὴς παραλλακτικὸτητας και της ικανὸτητας των βακτηρὶων του γὲνους *Pseudomonas* να αποικὶσουν και να επιβιὼσουν στο ριζικὸ σὺστημα εἰναι ανεκτὶμητης αξὶας, επειδὴ μπορεὶ να επιτρὲψει αὺξηση της ικανὸτητας για καταπολὲμηση παθογὸνων. Η γνὼση γενετικὼν χαρακτηριστικὼν των ανταγωνιστικὼν αυτὼν βακτηρὶων που σχετὶζονται με την προτὶμησὴ τους για το ἑνα ἠ το ἀλλο εἰδος φυτοὺ βοηθὰει στην αναγνὼριση συγκεκριμὲνων βακτηρὶων τα οποὶα εἰναι σε ικανοποιητικὸ βαθμὸ προσαρμοσμὲνα σε ἑνα συγκεκριμὲνο σὺστημα φυτοὺ - παθογὸνου. Παρὰλληλα ερευνὼντας χαρακτηριστικὰ των φυτὼν τα οποὶα προωθοὺν την ανὰπτυξη και την ενεργὸ δρὰση των ανταγωνιστικὼν αυτὼν βακτηρὶων μπορεὶ να συμβὰλει επεὶσης στην αποτελεσματικὸτητὰ τους.

Nawoord

Nu is het me eindelijk gelukt, het proefschrift is af! Dit *boekje* mag dan wel mijn proefschrift zijn, toch zijn er heel veel mensen die direct of indirect hun steentje hebben bijgedragen.

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Dit OIO project was zeer breed en ambitieus opgezet en had als doel om veel fundamenteel maar ook veel toegepaste aspecten van de DAPG-producerende *Pseudomonas* te belichten. Daarom werd een paar maanden na mijn aanstelling een analisten functie vrijgegeven. Eerste werd Wim binnen het STW project aangesteld voor bijna een jaar. Samen hebben we een groot deel van de eerste isolaties van de *Pseudomonas* stammen uitgevoerd. Wim, bedankt voor al je hulp bij het bewaren van die alle eerste reusachtige berg isolaten in the vriezer. Na Wim kwam Mieke het project versterken. Mieke, ik zal onze alle eerste ontmoeting tijdens je kraamvisite van Yonah samen met andere Fyto mensen nooit vergeten. En ik wil jou Mieke in het bijzonder bedanken voor je betrokkenheid bij mijn OIO project en voor je grote bijdrage aan dit proefschrift! Jouw gezelligheid en behulpzaamheid zijn erg gewaardeerd, niet alleen binnen ons project, maar ook binnen het hele Fyto clubje! Tijdens de laatste periode van mijn OIO project, heeft Corrie enorm veel werk vericht aan de verschillende suikerbiet biotoetsen. Corrie bedankt voor je hulp, kennis en deskundigheid bij de biotoetsen. Ik kon altijd op jou rekenen! Alleen zou ik het nooit gedaan kunnen hebben. En ook bedankt voor de gezelligheid en discussies tijdens onze reisjes naar IRS en naar Scientia Terrae. Niet alleen Corrie kwam het project versterken tijdens de laatste periode. Kiona heeft, als

studentassistent, ook een belangrijk steentje op de substraat utilizatie assays bijgedragen. Kiona, dank je wel en succes met je studie.

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Maria Bergsma-Vlami
Wageningen, The Netherlands
May 2008

Curriculum vitae

Maria Bergsma-Vlami werd op 31 mei 1969 geboren in Athene, Griekenland. Ze behaalde in 1988 haar diploma aan het 3de Stedelijk Lyceum in Chania, Kreta, Griekenland. Het jaar erna begon ze haar studie Plant Productie Systemen aan de Landbouwuniversiteit van Athene met specialisaties in Plantenveredeling en Gewasbescherming. Haar onderzoeksstage heeft zij verricht in 1993 bij het Nederlands Instituut voor Koolhydraat Onderzoek (NIKO-TNO) in Groningen aan regulatie van glycoalkaloiden in *Solanum tuberosum.* In 1994 deed zij een afstudeerstudie naar de somaclonale variatie van *Vicia faba* L. embryos aan het Laboratorium voor Celgenetica van de Rijksuniversiteit Groningen. Begin 1996 sloot zij haar studie aan de Landbouwuniversiteit van Athene af met het behalen van haar doctoraaldiploma. Vervolgens is zij verhuisd naar Wageningen waar zij in 1997 met de MSc studie "Integrated Pest Management" aan de Wageningen Universiteit begon. Bij het Laboratorium voor Fytopathologie (sectie Ecologie) bewerkte zij een afstudeervak naar inactiveringsmechanismen van persistente bodempathogenen bij biologische bodemontsmetting. In january 1999 behaalde zij haar MSc diploma (*cum laude)*. Zij vervolgde haar werkzaamheden bij het Laboratorium voor Fytopathologie als junior-onderzoeker. Van october 1999 to augustus 2005 deed zij promotieonderzoek bij het Laboratorium voor Fytopathologie Wageningen Universiteit, in deeltijdaanstelling. Tijdens deze periode werden haar twee kinderen, Yonah en Zetta, geboren. Sinds maart 2008 is zij werkzaam bij het Nationale Referentie Laboratorium, discipline Moleculaire Biologie van de Plantenziektekundige Dienst in Wageningen.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of Literature (5.6 ECTS)
- 2,4-diacetylphloroglucinol-producing *Pseudomonas* species (2000)

Laboratory Training and Working Visits (3.7 ECTS)
- Denaturing Gradient Gel Electrophoresis (DGGE) technique (2000)
- Expertise on sugar beet (2004)

Post-Graduate Courses (4.2 ECTS)
- PhD Autumn School (1999)
- PhD Autumn School: Interactions between plants and attacking organisms: mechanisms, genetics, ecology and evolution (2000)
- PhD Winter School: Functional Biodiversity for sustainable crop protection (2001)

Deficiency, Refresh, Brush-up Courses (1.4 ECTS)
- Molecular phylogenies: reconstruction and interpretation (2003)

Competence Strengthening / Skills Courses (ECTS)
Note: term 2c was not compulsory when the candidate submitted her TSP.

Discussion Groups / Local Seminars and Other Meetings (7 ECTS)
- PE&RC discussion group 3 (2001-2002)
- Weekly Phytopathology group discussion (2000-2005)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (3 ECTS)
- National *Pseudomonas* meeting, Baarn, The Netherlands (2000)
- PE&RC Day (2000 and 2001)
- Biometris Conference: Quantitative methods for life and earth, WICC, Wageningen (2001)
- Gewasbeschermingsmanifestatie, Ede (2002)
- Willie Commelin Scholtendag, Utrecht University, Utrecht (2001, 2002)
- CBS/Wageningen Phytopathology symposium (2003)

International Symposia, Workshops and Conferences (7.6 ECTS)
- 9th International Symposium on Microbial Ecology (ISME-9), RAI, Amsterdam, The Netherlands (2001)
- IOBC/OILB Biocontrol meeting: Influence of abiotic and biotic factors on biocontrol agents, Kusadasi, Turkey (2002)
- International Congress on Plant Pathology (ICPP), Christchurch, New Zealand (2003)
- Rhizosphere congress: Perspectives and Challenges – A tribute to Lorenz Hilterner, Munich, Germany (2004)
- IOBC-meeting: Multitrophic Interactions in soil, Wageningen, The Netherlands (2005)

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