The regulation and ecological relevance of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp.

-Writing assessment -

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Contents

Abstract

2,4-diacetylphloroglucinol (2,4-DAPG)-producing fluorescent *Pseudomonas* species is a group of ubiquitous root colonizing gram-negative bacteria, which are well known for their biological control activity against many soilborne pathogens. Soilborne pathogens are responsible for a large loss of crop yield worldwide and they are difficult to control and application of pesticides are insufficient to control root diseases of important crop plants. Using 2,4-producing fluorescent *Pseudomonas* species as a biological control agent might contribute to the control of root diseases of important crop plants and increase crop production in a sustainable manner. However, the precise regulation and the mode of action of the antibiotic 2,4-DAPG is not fully known. Furthermore, the soil surrounding the plant's roots sustains a large microbial community with members that can negatively or positively affect 2,4-producing fluorescent *Pseudomonas* species. These largely unknown biotic interactions makes it difficult to use 2,4-producing fluorescent *Pseudomonas* species as a biocontrol agent. Elucidating the mechanism by which 2,4-DAPG produced by fluorescent *Pseudomonas* species can effectively suppress plant diseases and how the root microbiome affects 2,4-DAPG production will open new doors for the biocontrol of pathogens and increasing the crop quality and productivity.

Chapter 1. Introduction

The soil surrounding plant roots, the rhizosphere, supports large microbial populations due to the fact that the rhizosphere is rich in nutrients. Plant roots exudate photosynthates such as amino acids, sugars and proteins into the rhizosphere, where the microbial population can use these photosynthates for their own metabolism (Bais *et al.*, 2006). The microbial community present in the rhizosphere can contain 10^{11} microbial cells per gram root and more than 30.000 prokaryotic species (Egamberdieva *et al.*, 2008; Mendes *et al.*, 2011) and members of this community can have a beneficial, neutral or detrimental effect on plant health.Through the composition of the root exudates, plants are able to select for specific antagonistic soil organisms, such as plant growth-promoting rhizobacteria (PGPRs), as a defense against soilborne pathogens (Phillips *et al.*, 2004).

Soilborne pathogens are responsible for a large loss of crop yield worldwide. Soilborne pathogens are difficult to control and crop rotation, breeding for resistant plants varieties and application of pesticides are insufficient to control root diseases of important crop plants (Weller et al 2007; Haas and Défago, 2005). Several soils around the world have been described to be naturally disease suppressive against certain soilborne pathogens, and it has been revealed that this is caused by the presence of specific PGPRs (Weller *et al.*, 2002; Weller *et al.*, 2007). Using these bacteria as a biological control agent might contribute to the control of root diseases of important crop plants and increase crop production in a sustainable manner. However, biological control with PGPRs may work well in one location but not in the next due to a variety of factors, which will be discussed in this report (Weller *et al.*, 2007; Haas and Défago, 2005).

Examples of antagonistic PGPRs which are interesting from a biocontrol point of view are fluorescent *Pseudomonas* species. Fluorescent *Pseudomonas* species are beneficial bacteria that colonize plant roots and stimulate plant growth. They can protect plants against pathogens in a direct manner with for example the production and secretion of secondary metabolites such as the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin (PLT)(Weller *et al.,* 2002). Also they can outcompete pathogens for nutrients in the rhizosphere. Furthermore, certain fluorescent *Pseudomonas* species can induce a systemic resistance in plants that is effective against a broad spectrum of pathogens (Weller *et al.,* 2002; Weller *et al.,* 2012).
In the past decades al lot of research has been performed on fluorescent *Pseudomonas* species and their possible use as a biocontrol agents. Especially 2,4-DAPG-producing fluorescent *Pseudomonas* have been of interest due to their presence in naturally occurring disease suppressive soils however, many aspect are still unknown.

The focus of this review lies on 2,4-DAPG-producing fluorescent *Pseudomonas* species and their ability to suppress plant diseases in plants. The main goal of this review is to give a clear overview of what is currently known about 2,4-DAPG-producing fluorescent *Pseudomonas* species with a focus on the regulation of 2,4-DAPG production and the ecological relevance of 2,4-DAPG-producers on host plants and the microbial community. Therefore, a clear overview will be given of direct pathogen inhibition by different 2,4-DAPG-producing fluorescent *Pseudomonas* species, based on relevant literature. Also the effect of biological interactions (e.g. plant and microbial interactions) with 2,4-DAPG-producing fluorescent *Pseudomonas* species will be discussed. Finally the use of 2,4-DAPG producing fluorescent *Pseudomonas* species as biocontrol agents will be shortly discussed including future research suggestions to elucidate certain difficulties seen when using 2,4-DAPG-producing fluorescent *Pseudomonas* species as biocontrol agents

Chapter 2. Disease control by 2,4-DAPG-producing fluorescent *Pseudomonas* spp.

2,4-DAPG-producing fluorescent *Pseudomonas* species is a group of ubiquitous root colonizing gram-negative bacteria, which are well known for their biological control activity against many soilborne pathogens. 2,4-DAPG-producing fluorescent *Pseudomonas* spp. are also implicated in the natural suppressiveness of certain soils toward particular pathogens (Weller *et al.,* 2002). In this chapter, the state of knowledge on the mode of action of 2,4-DAPG on pathogens will be summarized as well as its role in the specific disease suppressiveness to certain plant diseases. Finally, a list of 2,4-DAPG-producing fluorescent *Pseudomonas* species and their associated biological control activities against specific pathogens will be presented.

Mode of action of 2,4-DAPG

2,4-DAPG is a polyketide antibiotic that is produced by certain fluorescent *Pseudomonas* species. 2,4-DAPG-producers can affect soilborne pathogens directly, due the antibacterial, antifungal, anthelmintic and phytotoxic properties of 2,4-DAPG (Dubuis *et al.,* 2007). Furthermore, 2,4-DAPG can also elicit induced systemic resistance (ISR) in host plants, and therefore affect pathogens indirectly. ISR is induced by beneficial micro-organisms that colonize the roots of plants and is effective against a broad spectrum of pathogens, including bacteria, fungi and insects (Van loon *et al.*, 2006; Van Wees *et al.*, 2008). ISR-expressing plants are primed for enhanced defense, which means that the defense responses are not activated directly but only upon a pathogen attack and in a faster and stronger manner. Several determinants have been described to elicit ISR, such asmicrobe-associated molecular patterns (MAMPs), volatile organic compounds (VOCs), and also DAPG is one of those determinants (Meziane *et al.,* 2005; Rye *et al.,* 2004; Weller *et al.,* 2012). Research by Weller and associates (2012) has shown that *Arabidopsis thaliana* plants that were pretreated with 2,4-DAPG showed an enhanced resistance against the pathogen *P. syringae* pv *tomato*.

The direct mode of action of 2,4-DAPG on pathogens has not been completely elucidated. In several studies, the yeast *Saccharomyces cerevisiae* has been used as a model to investigate the effect of 2,4-DAPG on fungal and eukaryotic cells. Gleeson and associates (2010) showed that 2,4-DAPG interferes with mitochondrial functions and research by Kwak and associates (2011) indicated that 2,4-DAPG disturbs cell membrane permeability, triggers a reactive oxygen burst and interrupts cell homeostasis in *S. cerevisiae.* Troppens and associates (2013) suggested that in yeast and probably in eukaryotes in general, 2,4-DAPG primarily affects mitochondrial functions, where as in bacteria it is likely that 2,4-DAPG affects the bacterial cell membrane. That 2,4-DAPG is likely to affect bacterial cell membranes is supported by the fact that the gram-negative bacteria *Vibrio parahaemolyticus* lyses more slowly and in response to higher 2,4-DAPG concentrations when compared to the gram-positive bacteria *Staphylococcus aureus* (Kamei and Isnansetyo, 2003). In the case of gram-negative bacteria, 2,4-DAPG has to affect two membranes in order to suppress the bacteria (since gram-negative bacteria have an inner plasma membrane,a thin layer of peptidoglycan and an outer membrane), while gram-positive bacteria have only a plasma membrane and a thick peptidoglycan layer. This difference in number of membranes might explain the differences seen between *Vibrio parahaemolyticus* and *Staphylococcus aureus* in their response to 2,4-DAPG.
The mode of action of 2,4-DAPG on the pathogenic oomycete *Pythium* has been investigated by De Souza and associates (2003). Their study showed that 2,4-DAPG inhibited zoospore swimming (which is necessary to colonize plant roots), cause alterations in the plasma membrane, vacuolization and cell content disintegration.

Disease suppressive soils and 2,4-DAPG

As mentioned before, 2,4-DAPG-producing fluorescent *Pseudomonas* species play a key role in many natural disease suppressive soils. Plant roots stimulate and support antagonistic soil organisms such as 2,4-DAPG-producing fluorescent *Pseudomonas* species as a defense against soilborne pathogens (Weller *et al.*, 2002). How plants stimulate and support 2,4-DAPG-producing fluorescent *Pseudomonas* species will be discussed in more detail in chapter 4.

Suppressive soils are defined as soils in which the pathogen does not establish or persist, establishes but cause little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil (Baker and Cook, 1974). Every natural soil has the capacity to suppress the growth or activity of soilborne pathogens to a limited extent and this phenomenon is called general disease suppression. General disease suppressive soils are also called conducive soils. No one specific microorganism is responsible for general disease suppression, it is caused by the total microbial activity in the soil where the total biomass competes with the pathogen for resources. General suppression is not transferable between soils, this in contrast to specific suppressive soils (Weller *et al.,* 2002; Berendsen *et al.,* 2012*)*.

Specific suppressive soils are caused by specific microorganisms that causes the soil to be suppressive to a specific plant disease. Specific suppressiveness is superimposed over the general suppressiveness of the soil and is highly effective against the specific pathogen. As mentioned before, specific suppressiveness of soil can be transferred to other conducive soils by addition of 0.1% to 10% of the suppressive soil (Mendes *et al.* 2011; Weller *et al.*, 2002).
Two different types of specific suppressive soils are described in the literature, namely “long-standing suppression” and “induced suppression”. In long-standing suppressive soils, the suppressiveness to a disease is naturally associated with the soil and its origin is unknown. Also the suppressiveness persist even in the absence of plants. In induced suppressive soils, the suppressiveness is initiated and sustained by crop monoculture, by growing crops that are susceptible to the disease or by addition of inoculum of the pathogen to the soil (Hornby, 1983; Hornby, 1998; Weller *et al.*, 2002). 2,4-DAPG-producing fluorescent *Pseudomonas* species are responsible for various suppressive soils in the world, which are effective against different plant diseases. These disease suppressive soils are for example effective against black root tot of tobacco, caused by *Thielaviopsis basicola* (Weller *et al.*, 2002), and against take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Weller *et al.*, 2007). Table 1 shows a list of examples of 2,4-DAPG-producing fluorescent *Pseudomonas* species which are known for their biocontrol activities against certain pathogens.

Table 1. Examples of 2,4-DAPG-producing fluorescent *Pseudomonas* species with associated biocontrol activities and origin.

Strain	Biocontrol activity	Origin	References
Strains from USA			
2-79	Wheat (Ggt)	Wheat	Weller and Cook (1983)
Q1-87, Q4-87, Q5-87,Q6-87, Q13-87, Q37-87, Q86-87, Q7-87, Q8-87, Q9-87, Q12-87, Q13-87, Q37-87, Q86-87 Q95-87, Q107-87, Q112-87, Q139-87	Wheat (Ggt)	Wheat	Keel *et al.* (1996))
Q2-87	Wheat (Ggt, Pu)	Wheat	Vincent *et al.* (1991)
Q65c-80, Q69c-80 , Q88-87, Q128-87	Wheat (Ggt)	Wheat	Harrison *et al.* (1993)
PF	Wheat (St)	Wheat	Levy *et al.* (1992)
Pf-5	Cotton (Pu, Rs), Cucumber (Pu)	Cotton	Howell *et al.* (1979)
Q8r1-96	Wheat (Ggt, Pu)	Wheat	Raaijmakers and Weller (1997)
Strains from Switzerland			
TM1'A4, TM1'A5	Cucumber(Pu, Ps), Cotton (Rs)	Tomato	Keel *et al.* (1996)
Pf1	Tobacco (Tb), cucumber (Pu)	Tobacco	Keel *et al.* (1996)
C6-2, C6-9, C6-11, C6-16 C6-23,C10-181, C10-186, C10-189, C10-190,C10-197, C10-204, C10-205, S7-29, S7-42, S7-46, S7-52, S8-62, S8-110, S8-130, S8-151	Tobacco (Tb)	Tobacco	Ramette *et al.* (2003)
CHA0	Tobacco (Tb), wheat (Ggt, Pu), cucumber (Pu), Pea (Pu)	Tobacco	Stutz *et al.* (1986)
CM1'A2, CΔ1'B2, C*1A1	Cucumber (Pu, Ps), cotton (Rs)	Cucumber	Fuchs and Défago (1991)
K93.2, K93.3	Cucumber (Pu), tomato (FORL)	Tobacco	Wang *et al.* (2001)
P12	Tobacco (Tb), cucumber (Pu)	Tobacco	Keel *et al.* (1996)
P97.38, P97.39	Cucumber (Pu), tomato (FORL)	Cucumber	Wang *et al.* (2001)
TM1A3, TM1B2	Cucumber (Pu, Ps), cotton (Rs)	Tomato	Fuchs and Défago (1991)
Strains from Italy			
PINR2, PINR3, PILH1	Cucumber (Pu), tomato (FORL)	Tobacco	Keel *et al.* (1996)
PITR2, PITR3	Cucumber (Pu), tomato (FORL)	Wheat	Keel *et al.* (1996)
K93.52	Cucumber (Pu), tomato (FORL)	Tomato	Wang *et al.* (2001
Strains from Ghana			
PGNR2, PGNR3, PGNR4	Cucumber (Pu), tomato (FORL)	Tobacco	Keel *et al.* (1996)
PGNR1, PGNL1	Cucumber (Pu), tomato (FORL)	Tobacco	Keel *et al.* (1996)
Strains from Ireland			
F113	Sugar beet (Pu), Potato (Pc)	Sugar beet	Fenton *et al.* (1992)
Strains from Slovakia			
K94.31, K94.37, K94.38	Tomato (FORL)	Cucumber	Wang *et al.* (2001)
P97.30	Cucumber (Pu), tomato (FORL)	Wheat	Wang *et al.* (2001)
K94.41	Cucumber (Pu), tomato (FORL)	Cucumber	Wang *et al.* (2001)
Strains from West Russia			
P96.25	Cucumber (Pu), tomato (FORL)	Wheat	Wang *et al.* (2001)
Strains from Estonia			
F96.27	Cucumber (Pu), tomato (FORL)	Cucumber	Wang *et al.* (2001)
Strains from Bhutan			
P97.1, P97.27	Cucumber (Pu), tomato (FORL)	Cucumber	Wang *et al.* (2001)
P97.6, P97.26	Cucumber (Pu), tomato (FORL)	Tomato	Wang *et al.* (2001)
Strains from Mexico			
F96.26	Tomato (FORL)	Cucumber	Wang *et al.* (2001)

Ggt, *Gaeumannomyces graminis* var. *tritici*; FORL, *Fusrium oxysporum* f. sp. *radices-lycopersici*; Pc, *Pectobacterium carotovorum* (formerly *Erwinia carotovora*); Ps, *Phomopsis sclerotioides*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; St, *Septoria tritici*; Tb, *Thielaviopsis basicola*.

Take-all disease and take-all decline

Take-all is an important disease of wheat worldwide and is caused by the fungal pathogen *G. graminis* var. *tritici.* Barley, rye and triticale (a hybrid of wheat and rye) are also affected by this fungus, but to a lesser extent than wheat (Weller *et al.*, 2007; Cook, 2003). The methods for chemical control of take-all are limited and breeding for resistance has been unsuccessful (Berendsen *et al.*, 2012). Take-all can be controlled by crop rotation an tillage, because this greatly reduces the inoculum potential of *G. graminis* var. *tritici*. However, the trend currently seen in cropping systems are reduced tillage and several consecutive crops of wheat before a break, partially due to economic reasons. Reduced tillage and consecutive crops worsens take-all (Cook, 2003).

Take-all can also be controlled by growing wheat continuously in a field, which results in a spontaneous decline in disease severity and in a recovery of crop yield (Figure 1). This phenomenon is called take-all decline (TAD) and occurs globally. Furthermore, TAD has been associated with the build-up of 2,4-DAPG-producing fluorescent *Pseudomonas* species (Weller *et al.*, 2002 and references therein; Weller *et al.*, 2007). TAD is defined as the spontaneous decrease in the incidence and severity of take-all that occurs with the monoculture of wheat or other susceptible host crops after one or more severe disease outbreaks (Weller *et al.*, 2007; Cook and Weller, 1987; Hornby, 1979; Hornby, 1998).

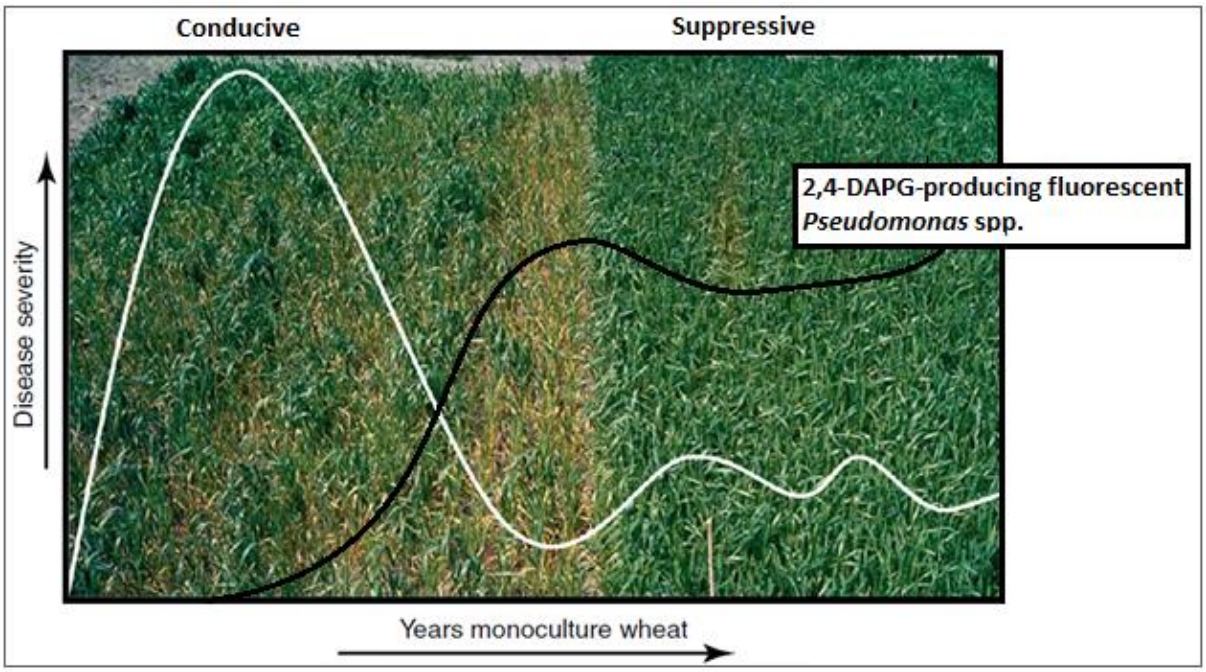

Figure 1. Model of the take-all decline and the role of 2,4-DAPG-producing fluorescent *Pseudomonas* species. During monoculture of wheat and after severe outbreaks of take-all, the disease severity declines over the course of several years (white line). The decline in disease severity is associated with an increase of 2,4-DAPG-producing fluorescent *Pseudomonas* species (black line). Adapted from Berendsen *et al.* (2012) and Weller *et al.* (2002).

Research by Raaijmaker and Weller (1998) revealed that the population density threshold required for suppression of take-all was in the order of 10^5 colony forming units of the 2,4-DAPG-producing *P. fluorescens* strain Q2-87 per gram or roots. There are many different genotypes within the group of fluorescent *Pseudomonas* species that produce 2,4-DAPG, which will be discussed in chapter 3. Often multiple genotypes of 2,4-DAPG-producers are present in the same soil, but usually only one or more genotypes are dominant in the rhizosphere of plants growing in that soil (Weller *et al.*, 2007). Genotypes of 2,4-DAPG-producers differ in rhizosphere competence (Weller *et al.*, 2002), and given the fact that plants can select for specific microorganisms in their rhizosphere (Phillips *et al.*, 2004), It is likely that there is a mutual "preference" or "affinity" between certain 2,4-DAPG-producers and plant species and cultivars (Weller *et al.* 2007).

Concluding, 2,4-DAPG is an antibiotic that is produced by certain fluorescent *Pseudomonas* species and due to this antibiotic function, 2,4-DAPG-producers can affect soilborne pathogens directly. Furthermore, 2,4-DAPG is also known to be a determinant for eliciting ISR in the host plant. 2,4-DAPG-producing fluorescent *Pseudomonas* species are responsible for various specific suppressive soils in the world, which are effective against different plant diseases. The direct mode of action of 2,4-DAPG on pathogens has not been completely elucidated, but it has been suggested that 2,4-DAPG primarily affects mitochondrial functions in eukaryotes, and affects the cell membrane in bacteria.

Chapter 3. Regulation of 2,4-DAPG biosynthesis and phylogenetic studies

The genetic capacity to produce 2,4-DAPG is conferred by the approximately 8 kb *phl* gene cluster that consists of eight genes, *phlHGFACBDE*. These genes are involved in the biosynthesis, regulation, export and degradation of 2,4-DAPG (Figure 2a; Moynilan *et al.*, 2009; Zhang *et al.*, 2014). The biosynthesis of 2,4-DAPG is highly regulated and a lot is still unknown. Furthermore, many abiotic an biotic factors have an effect on the biosynthesis of 2,4-DAPG and will be discussed in this chapter and in chapter 4.

2,4-DAPG biosynthesis and regulation

The biosynthetic locus of 2,4-DAPG contains four genes*, phlA, phlC*, *phlB* and *phlD*, which are transcribed as a single operon (*phlACBD*)(Zhang *et al.*, 2014). The *phl* biosynthetic genes are conserved among all known 2,4-DAPG-producing fluorescent *Pseudomonas* species and the key biosynthetic gene is *phlD*, which encodes a Type III polyketide synthase. PhlD catalyzes the synthesis of phloroglucinol (PG; a precursor of monoacylphloroglucinol (MAPG) and 2,4-DAPG) from three molecules of malonyl-coenzyme A (Figure 2b). The *phlACB* genes encode enzymes which are thought to form a complex that is required for the conversion of PG to DAPG (Troppens *et al*., 2013). *PhlA* encodes a β-ketoacyl-ACP synthase III, *phlC* encodes an condensing enzyme and *phlB* encodes a putative nucleic-acid binding enzyme (Zhang *et al.*, 2014). These enzymes together function as an acyltransferase which converts PG to MAPG and subsequently MAPG to 2,4-DAPG (Figure 2b; Troppens *et al.* 2013).

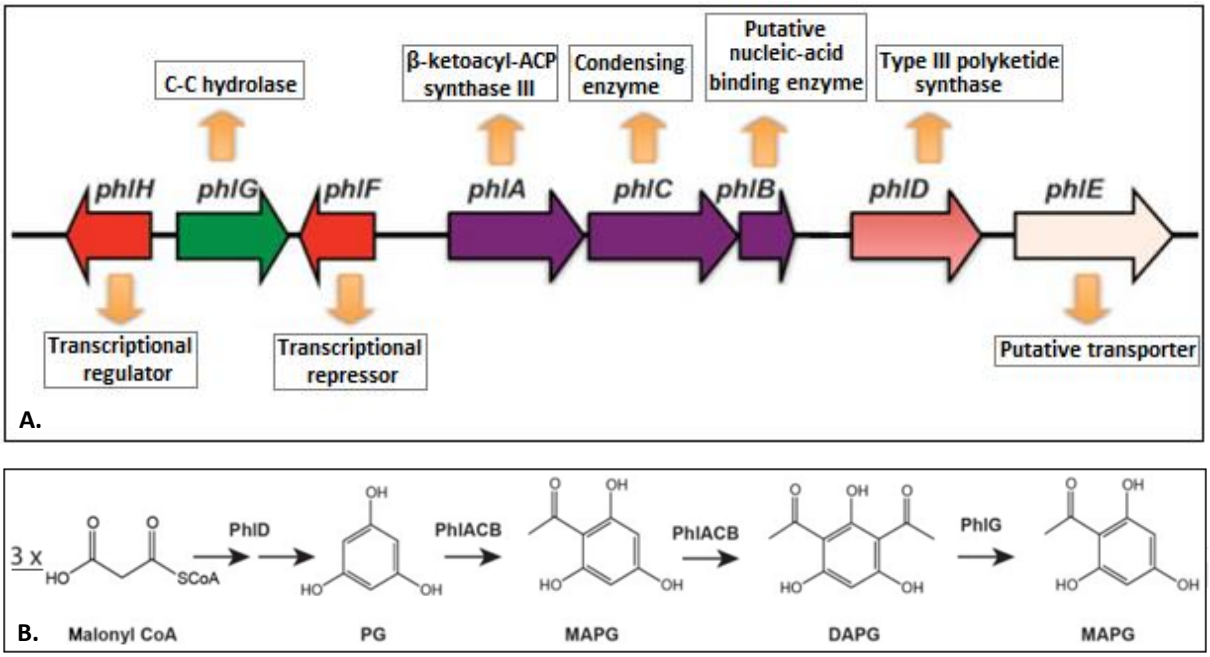

Figure 2. The *phl* gene cluster and 2,4-DAPG biosynthesis in 2,4-DAPG-producing fluorescent *Pseudomonas* species. (a) The *phl* gene cluster consists of eight genes, presented with their proposed function. (b). The biochemical pathway for 2,4-DAPG synthesis. PhlD catalyzes the synthesis of phloroglucinol (PG) from three molecules of malonyl-CoA. The *phlACB* gene products together form a complex, which converts PG to monoacylphloroglucinol (MAPG) and MAPG to 2,4-DAPG. 2,4-DAPG may be degraded to MAPG by the C-C hydrolase PhlG. Adapted from Troppens *et al.*, 2013.

The *phlACBD* operon is flanked downstream by the gene *phlE. PhlE* codes for a putative transport protein which has been suggested to export 2,4-DAPG to the outside of the cell (Abbas *et al.,* 2004). Upstream, the *phlACBD* operon is flanked by the genes *phlF*, *phlG* and *phlH*. *PhlF* encodes a transcriptional regulator of the TetR family that specifically represses the expression of the biosynthetic operon *phlACBD.* PhlF binds to the *phlO* operator located in the intergenic region between *phlF* and *phlA,* which causes the repression on the *phlACBD* operon and thus 2,4-DAPG production (Troppens *et al.,* 2013 and references therein). Mutational inactivation of the phlF gene causes constitutively derepressed 2,4-DAPG production (Abbas, 2002; Schnider-Keel *et al.,*, 2000). The transcription of *phlF* is growth phase-dependent with higher levels of transcription during the early to mid-exponential phase, the transcription of *phlA* during the early to mid-exponential phase is low. During the later exponential phase and early stationary phase, the level of *phlF* transcription falls en therefor the transcription of *phlA* rises. 2,4-DAPG itself acts as a derepressing signal by dissociating PhlF from the *phlO* operator. Therefore, 2,4-DAPG acts as an autoinducer of its own biosynthesis, which also means that 2,4-DAPG from one isolate can affect the production of 2,4-DAPG of other isolates (Maurhofer *et al.*, 2004).
The *PhlG* gene encodes a C-C hydrolase that degrades 2,4-DAPG to less toxic MAPG however, the biological role of PhlG is unknown and needs to be determined and also the fate of MAPG produced by PhlG needs to be elucidated (Bottiglieri and Keel 2006; Troppens *et al.,*2013). PhlH encodes for a second TetR-type transcriptional regulator that is thought to activate the expression of the *phlACBD* genes, either as an activator or as an antirepressor. Also, PhlH is thought to negatively regulate the expression of *phlG* (Schnider-Keel *et al.*, 2000). However, the precise mechanism still needs to be determined (Haas and Keel, 2003).

All 2,4-DAPG-producing fluorescent *Pseudomonas* species use the Gac/Rsm global-regulatory system to post-transcriptionally regulate the production of 2,4-DAPG. The key aspect of this system is that it enables the cell to respond to external stimuli as well as to intracellular changes however, the Gac/Rsm system is highly complex and not fully understood (Troppens *et al.*, 2013). As can be seen in Figure 3, upon interaction with unknown signals, the sensor histidine kinase GacS undergoes autophosphorylation. The signal is then relayed to the response regulator GacA by phosphotransfer, which in turn activates the transcription of the non-coding small RNAs RsmX, RsmY and RsmZ. The two translational repressors RsmA and RsmE are subsequently dissociated from the ribosome binding sites (RBS) of the target mRNA through the binding of RsmA and RsmE with the small RNAs RsmX, RsmY and RsmZ (Figure 3). The dissociation of RsmA and RsmE relieves the translational repression of the target mRNA and activates the biosynthesis of 2,4-DAPG.

Many other molecules involved in the regulation of 2,4-DAPG biosynthesis have been identified however, the relationship among these regulatory factors and there precise function has yet to be elucidated. The sigma factors RpoS and RpoD are examples of these regulatory factors. RpoS has been suggested to have an inducing effect on gene expression of the translational repressors RsmA. Mutations in the *rpoS* gene in *P. fluorescens* Pf-5 leads to an increased production of 2,4-DAPG, thought to be caused by a lack of induction of *rsmA* (Sarniquet *et al.*, 1995; Wu *et al.*, 2012). RpoD is thought to have an inducing effect on 2,4-DAPG production. Research by Schnider and associates (1995) showed that overexpression of the *rpoD* gene in *P. fluorescens* CHA0 enhances 2,4-DAPG production.
PsrA has been described to affect DAPG biosynthesis at the posttranscriptional level by activating *rpoS* gene expression, which in turn induces gene expression of the translational repressor RsmA (Haas and Keel, 2003; Wu *et al.*, 2012). Furthermore, PsrA negatively controls the transcription of *phlA* by binding to the promoter region (Wu *et al.*, 2012). Also the H-NS-related proteins MvaT and MvaV have been described to be involved in the regulation of 2,4-DAPG biosynthesis. It has been suggested by Baeler and associates (2006) that MvaT and MvaV act together in the regulation of DAPG biosynthesis. Their study showed that *mvaT* and *mvaV* mutants of CHA0 produced normal levels of 2,4-DAPG however, in *mvaT mvaV* double mutants 2,4-DAPG production was almost

completely abolished (Baehler *et al.*, 2006). Other examples of regulatory factors involved in 2,4-DAPG biosynthesis are the sigma factor RpoN, the oxidoreductase DsbA, the resistance-nodulation-division efflux pump EmhABC and the RNA chaperone Hfq, all with a largely unknown function (Wu *et al.,* 2012 and references therein). It likely that in the coming years the list of molecules involved in the regulation of 2,4-DAPG biosynthesis will grow and that the precise regulation of 2,4-DAPG biosynthesis will be elucidated.

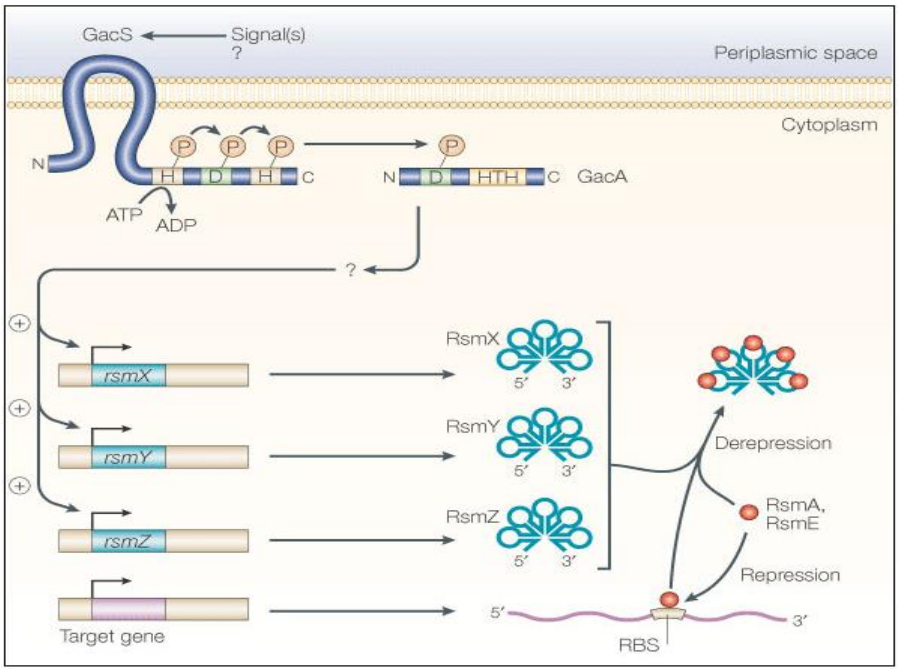

Figure 3. Model of the Gac/Rsm regulatory system in 2,4-DAPG producing fluorescent Pseudomonas species. Upon interaction with unknown signals, the sensor histidine kinase GacS is autophosphorylated and the signal is relayed to the response regulator GacA by phosphotransfer. GacA activates the transcription of the small RNAs RsmX, RsmY and RsmZ, which in turn dissociates the two translational repressors RsmA and RsmE from the ribosome binding sites (RBS) of the target mRNA. The dissociation of RsmA and RsmE relieves the translational repression of the target mRNA and activates the biosynthesis of 2,4-DAPG. Adapted from Haas and Defago (2005).

Several biotic factors have been described to have an effect on the production of 2,4-DAPG. For example, salicylates (produced by plants and some bacteria), pyoluteorin (PLT; produced by some bacteria)and fusaric acid (produced by *Fusarium oxysporum*) antagonize the derepression of the *phlACBD* operon by 2,4-DAPG, which leads to a PhlF-mediated repression of the *phlACBD* operon (Abbas *et al.*, 2002; Schnider-Keel *et al.*, 2000).
Also different types of carbon sources, exudated by plant roots, can influence 2,4-DAPG production. For example, sucrose and fructose as a carbon source for 2,4-DAPG-producing fluorescent *Pseudomonas* species results in a high 2,4-DAPG production, whereas glucose results in a very low 2,4-DAPG production (Shanahan *et al.*, 1992). More details of biotic effects on 2,4-DAPG-producing fluorescent *Pseudomonas* species will be discussed in chapter 4.

Phylogenic studies of 2,4-DAPG-producing fluorescent Pseudomonas species

In the last decade, a lot of studies on the phylogeny of 2,4-DAPG-producing fluorescent *Pseudomonas* species have been performed and as previously mentioned, there are various genotypes within this group of fluorescent *Pseudomonas* species. By understanding the phylogeny of 2,4-DAPG-producing fluorescent *Pseudomonas* species and the composition of genotypes in a soil, it might be possible to predict a soils' ability to suppress certain plant pathogens (Mavrodi *et al.*, 2007). Since it has been suggested that crop species and certain genotypes have a mutual "preference" or "affinity" for each other, matching the crop or cultivar with the most appropriate genotype might benefit effective biological control against plant pathogens in a sustainable manner (Weller *et al.*, 2007; Mavrodi et al 2001)

Several methods have been used to investigate the phylogeny of 2,4-DAPG-producing fluorescent *Pseudomonas* species. Amplified ribosomal DNA restriction analysis (ADRA) has revealed that there are three distinct lineages among 2,4-DAPG-producing fluorescent *Pseudomonas* species, designated as group 1,2 and 3 or A, B and C (McSpadden Garderner *et al.*, 2000; Weller *et al.* 2007). Several other methods have been used in various studies, and combining these results revealed a finer variation among 2,4-DAPG-producing fluorescent *Pseudomonas* species. At least 22 genotypes, designated as A to T, PfY and PfZ have been defined (Landa *et al.*, 2006; Mazolla *et al.*, 2004; Weller *et al.*, 2007). Examples of methods that have been used for the genotyping of 2,4-DAPG-producing fluorescent *Pseudomonas* species are denaturing gradient gel electrophoresis (DGGE) analysis, restriction fragment length polymorphism (RFLP) analysis, randomly amplified polymorphic DNA analysis and various other PCR variants (Mavrodi *et al.*, 2007 and references therein).

Strains from the same genotype could originate from very distant geographic locations, only group-C strains were all isolated from one single location, namely Quincy soil (Frapolli *et al.*, 2007). Washington state soils contain multiples genotypes however, strains belonging to the D genotype showed to be dominant. This dominance can be explained by the mutual "preference" between this genotype and wheat and pea, since Washington state soils have undergone continuous wheat or pea monoculture (Raaijmaker and Weller 2001; Mavrodi *et al.*, 2007).

To summarize, At least 22 genotypes of 2,4-DAPG-producing fluorescent *Pseudomonas* species have been defined, al containing the *phl* gene cluster that consists of eight genes, *phlHGFACBDE*. The biosynthetic locus of 2,4-DAPG contains four genes, *phlA, phlC*, *phlB* and *phlD*, which are transcribed as a single operon (*phlACBD*). All 2,4-DAPG-producers use the Gac/Rsm global-regulatory system to post-transcriptionally regulate the production of 2,4-DAPG and this regulatory system is highly complex and not fully understood.

Chapter 4. Biological interactions

The complete spectrum of effects conferred by 2,4-DAPG-producing fluorescent *Pseudomonas* species has not been deciphered yet. In this chapter, the current state of knowledge on biological interactions between 2,4-DAPG-producing fluorescent *Pseudomonas* species and plants and between 2,4-DAPG-producing fluorescent *Pseudomonas* species and other (micro)organisms will be discussed.

Interactions with the host plant

Plant roots allocate a large amount of their photosynthates to the roots and plant roots exudate up to 40% of these photosynthates into the rhizosphere. Therefore, the rhizosphere supports large microbial populations due to the fact that these photosynthates can be used for the metabolism of this microbial population (Bais *et al.*, 2006). Plants can adjust the composition of exudates and through this adjustment, plants can select for specific microorganisms in their rhizosphere by active secretion of compounds that specifically stimulate or inhibit these microorganisms (Figure 4; Doornbos *et al.*, 2012; Phillips *et al.*, 2004; Mark *et al.*,2005). As mentioned before, different types of carbon sources, exudated by plant roots, influences the production of 2,4-DAPG in fluorescent *Pseudomonas* species. When sucrose, fructose or mannitol is available as a carbon source, the production of 2,4-DAPG by fluorescent *Pseudomonas* species increases, whereas glucose and sorbose as a carbon source results in a very low 2,4-DAPG production (Figure 4; Shanahan *et al.*, 1992). 2,4-DAPG-producing fluorescent *Pseudomonas* species themselves also have an effect on the composition of root exudates. 2,4-DAPG can induce an increase in the amount of amino acids exudated by the hosts plant roots. 2,4-DAPG-producers can use these amino acids to increase their activity as well as the production of 2,4-DAPG and other compounds (Figure 4; Phillips et al 2004).

Experiments with split-root systems are very useful to investigate pathogen induced but plant-mediated effects on 2,4-DAPG-producing fluorescent *Pseudomonas* species and also to investigate *Pseudomonas*-induced but plant-mediated effects on pathogens.
In a study with barley plants growing in a split-root system, plants of which the roots were inoculated with *Pythium ultimum* on one side of the system induced *phlA* gene expression in *P. fluo*rescens CHA0 growing on the other side of the split root system (Figure 4; Jousset *et al.*, 2011). Induction of *phlA* gene expression suggests an increased production of 2,4-DAPG by CHA0 and due to the fact that there was no physical contact between *Pythium ultimum* and CHA0, the induction of *phlA* gene expression was mediated via systemic signalling of the plant. *Phl* gene expression in strain CHA0 is also enhanced in the rhizosphere of *Pythium* infected roots of wheat, cucumber and maize. *Phytium* itself does not enhance the *phl* gene expression, therefore it was suggested that the observed *phl* gene expression in strain CHA0 is enhanced due to alterations in root exudates caused by a pathogen attack (Figure 4; Notz *et al.*, 2001; Maurhofer *et al.* 2002)
A different study by Henkes and associates (2011) with barley plants growing in a split-root system showed that plants allocate shoot carbon away from roots infected with *Fusarium graminearum* and towards uninfected roots. Their study also showed that pre-inoculation of barley roots with CHA0 resulted in a maintained carbon delivery to *F. graminearum* infected roots, reduced infection symptoms for which no direct contact between *F. graminearum* and CHA0 was required and that CHA0 prevented a reduction in plant biomass. An explanation for the response of the plant to maintain carbon supply to infected roots might be that the plant invests in defence responses since CHA0 triggers systemic response that reduces *F. graminearum* infection. Maintaining the carbon supply ensures the plant that CHA0 can colonize the plant roots and induce a systemic defence response. Another explanation that is given in this study is that *Pseudomonads* may show selfish behaviour by redirecting the root carbon supply to themselves and risking that the pathogen might benefit from the carbon supply as well (Figure 4; Henkes *et al.*, 2011).

Besides direct inhibitory effects on pathogens and inducing ISR, fluorescent *Pseudomonas* species are also known for their ability to alter plant root architecture. A study by Brazelton and associates (2008; Figure 4) revealed that 2,4-DAPG inhibited primary root growth and stimulated lateral root formation in tomato seedlings by interacting with an auxin-dependent signalling pathway.
Varies compounds produced by plants as well as bacteria can have an effect on the production of 2,4-DAPG in fluorescent *Pseudomonas* species. It has been shown that indole-3-acetic acid (IAA) stimulates *phl* gene expression in strain CHA0 (Dubuis *et al.*, 2007). As mentioned before, salicilate downregulates the expression of the *phl* gene by antagonizing the derepression of the *phlACBD* operon by 2,4-DAPG. This antagonism leads to a PhlF-mediated repression of the *phlACBD* operon (Abbas *et al.*, 2002; Schnider-Keel *et al.*, 2000).

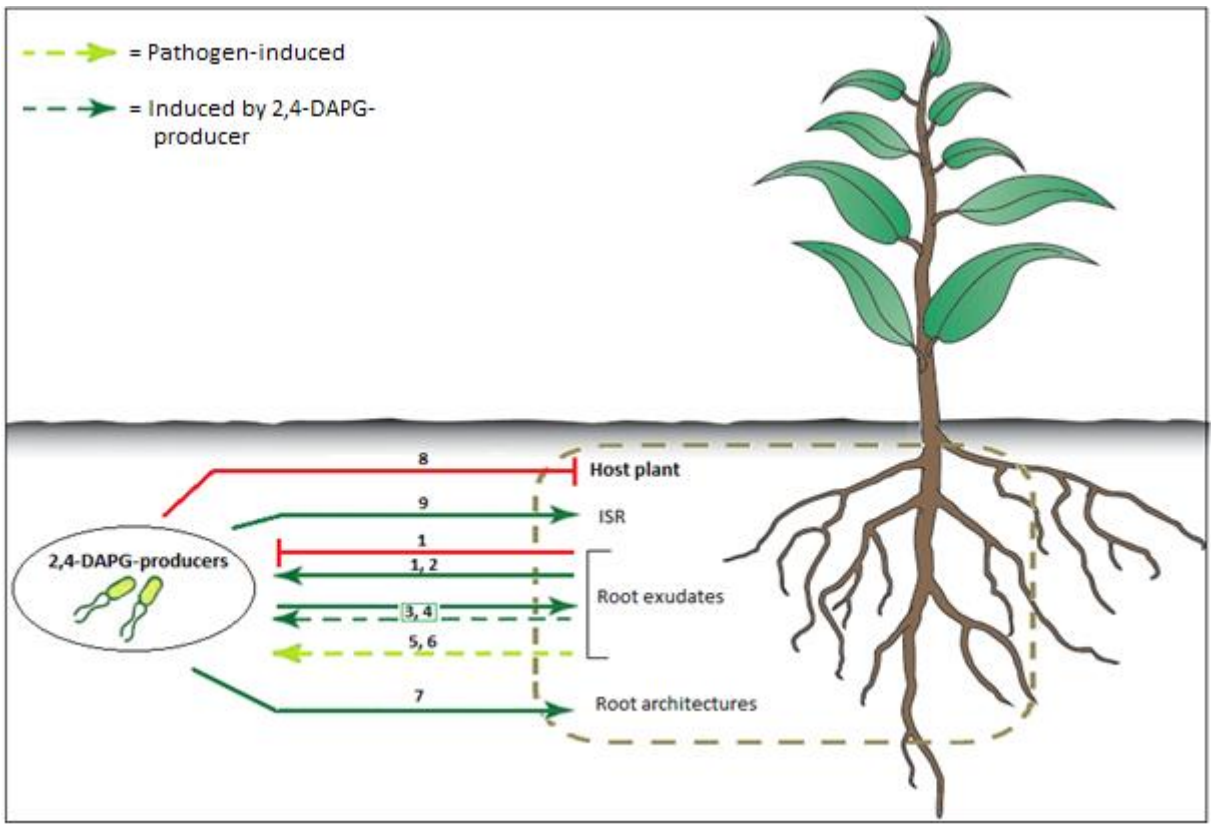

Figure 4. A simplified scheme of the biological interactions between 2,4-DAPG-producing fluorescent *Pseudomonas* species and host plants. (1) Doornbos *et al.*, 2012, (2) Shanahan *et al.*, 1992, (3) Phillips *et al.*, 2004, (4) Henkes *et al.*, 2011, (5) Jousset *et al.*, 2011, (6) Notz *et al.*, 2001, (7) Brazelton *et al.*, 2008, (8) Zhou *et al.* 2010, (9) Van Loon *et al.*, 2006.

Thus, the host plant can alter their root exudate composition to attract 2,4-DAPG-producers.
By producing 2,4-DAPG, fluorescent Pseudomonas species can influence the plant to alter its root exudate composition in a way that is beneficial for the 2,4-DAPG-producers.

Interactions with competing bacteria

In many bacterial genera, cells can communicateand influence each other by emitting and sensing chemical signals when population densities are high. Because the extracellular signal concentrations rises with increasing population densities, it has been suggested that bacteria can measure their population densities through these extracellular signal concentrations. This phenomenon has been termed quorum sensing, examples of quorum sensing signals are N-acyl-homoserine lactones (AHLs) and autoinducer 2 (AI-2; Miller and Bassler 2001; Dubuis and Haas, 2007).

In 2,4-DAPG-producing fluorescent *Pseudomonas* species activation of the Gac/Rsm cascade, which is essential for the production of 2,4-DAPG, occurs in a density dependent manner and this is a quorum sensing feature. As mentioned in chapter 3, it is not known what the signal is for activating the Gac/Rsm cascade. It has been shown that other *Pseudomonads* which do not produce 2,4-DAPG are able to induce 2,4-DAPG production in a neighbouring colony of strain CHA0 but the signal remained unknown (Figure 5; Dubuis and Haas, 2007).
Another way 2,4-DAPG-producing fluorescent *Pseudomonas* species can be influenced is by other competing bacteria during competition for resources during root colonization (Prieto *et al.*, 2011). When other bacteria are better in competing for resources, this may lead to a shortage of nutrients for the 2,4-DAPG-producers which can cause that 2,4-DAPG-producing fluorescent *Pseudomonas* species fail in colonizing the roots of plants (Figure 5; Berendsen *et al.*, 2012). 2,4-DAPG-producing fluorescent *Pseudomonas* species can also be influenced in their 2,4-DAPG production, for example by other fluorescent *Pseudomonas* strains that produce secondary metabolites such as 2,4-DAPG and PLT (Maurhofer *et al.*, 2004; Schnider-Keel *et al.*, 2000). As mentioned in chapter 3, 2,4-DAPG is an autoinducer of its own biosynthesis by dissociating the transcriptional repressor PhlF from the *phlO* operator. This means that 2,4-DAPG from one isolate can affect the production of 2,4-DAPG in other isolates (Figure 5; Maurhofer *et al.*, 2004). PLT represses *phlA* gene expression and 2,4-DAPG production with a probable mediator role for PhlF, since it was shown by Schnider-Keel and associates (2000; Figure 5) that an intact *phlF* gene was required for repression of *phlA* expression. Garbeva and associates (2011; Figure 5) demonstrated that *P. fluorescens* Pf0-1 shows a species-specific transcriptional and metabolic response to bacterial competitors. The bacterial competitors *Brevundimonas* sp. V52 and *Pedobacter* sp. V48, both induced the production of antimicrobial metabolites in strain Pf0-1, whereas *Bacillus* sp. V102 did not trigger such a response.

The rhizobacteria *Azospirillum brasilense* is an important plant-growth promoter, their plant-beneficial traits include nitrogen fixation and the production of phytohormones such as auxin (Couillerot *et al.*, 2011 and references therein). A study by Combes-Meynet and associates (2011; Figure 5) showed that 2,4-DAPG produced by P. fluorescens F113 upregulates gene expression of genes that are involved in several traits related to root colonization an growth promotion in *A. brasilense*. This indicates that F113 has a positive effect on the plant-growth promotion of *A.* brasilense however, research by Couillerot and associates (2011; Figure 5) showed that 2,4-DAPG has the potential to interfere with the root colonization by *A. brasilense* and their plant growth promotion capacity.
Thus, 2,4-DAPG production by fluorescent *Pseudomonas* species can be an advantage for these bacteria, since the antibacterial properties of 2,4-DAPG might give them an advantage during competition for resources. However, some bacteria are still able to outcompete 2,4-DAPG-producers. The previously described studies show that the interactions between bacteria are very complex and that the precise function of 2,4-DAPG-producing fluorescent *Pseudomonas* species within bacterial communities still needs to be determined.

Interactions with fungi

It has been clearly described by numerous reports that 2,4-DAPG is known to control pathogenic fungi, as discussed in chapter 2. Because of this antifungal properties of 2,4-DAPG, one might suspect that 2,4-DAPG also negatively effects mutualistic fungi. In a study where the biological interactions between the 2,4-DAPG-producing *P. fluorescens* CHA0 and *Trichoderma atrovirde* P1 (which has a broad spectrum of biocontrol activity) was investigated, results indicated that negative and positive effects on the expression of biocontrol genes may occur in both CHA0 and *T. atrovirde* P1 *in vitro* (Figure 5; Lutz *et al.*, 2006). In this study, 2,4-DAPG enhanced the expression of the gene *nag1*, which codes for a major biocontrol chitinase in *T. atrovirde* P1. However, an unidentified substance from CHA0 repressed the expression of this gene, as well as the expression of another gene that also encodes a chitinase. Furthermore, results from this study showed that *T. atrovirde* P1 enhanced *phlA* gene expression (Lutz *et al.*,2006).
Inoculation with the mycorrhizal fungus *Glomus intraradices* has shown to have a positive effect on *P. fluorescens* survival on maize roots however, it is not known whether this is a direct effect of *G. intraradices* or a plant mediated effect (Walker *et al.*, 2012). Mycorrhizal fungi might also have an indirect effect on 2,4-DAPG-producing fluorescent *Pseudomonas* species (Hol *et al.*, 2013). Mycorrhizal fungi may increase nutrient availability for plants and can increase plant growth (Jones *et al.*, 2004). This in turn can lead to a change in root exudate composition which can affect fluorescent *Pseudomonas* species in the rhizosphere (Jin *et al.*, 2010).

The pathogenic fungus *Fusarium oxysporum* produces fusaric acid and research has shown that fusaric acid strongly represses the expression of *phlA* and thus 2,4-DAPG production in CHA0. As was the case for the repressive actions of salicylate and PLT on *phlA* expression, an intact *phlF* gene was required for repression of *phlA* gene expression by fusaric acid, suggesting a mediator role for PhlF (Figure 5; Schnider-Keel *et al.*, 2000).

Interactions with bacterivores and decomposers

2,4-DAPG-producing *P. fluorescens* can use 2,4-DAPG and other metabolites as a self-defence against predatory protozoa and nematodes and the mechanism of this self-defence may depend on the predator (Figure 5; Jousset *et al.*, 2006; Neidig *et al.*, 2011). Exogenous 2,4-DAPG lyse cells of the amoeba *Vahlkampfia* sp. strain And12, whereas the ciliate *Colpoda steinii* and the flagellate *Neobodo designis* were almost not affected. However, ingestion of 2,4-DAPG-producing *Pseudomonas* strains very rapidly killed the flagellate *N. designis* (Jousset *et al.*, 2006).
Grazing of P. fluorescens Q2-87 by *Acanthamoeba castellanii* was found to increase 2,4-DAPG production by Q2-87 (Figure 5; Jousset and Bonkowski, 2010). An indirect manner by which bacterivores can have an effect on 2,4-DAPG-producing fluorescent *Pseudomonas* species is by selective grazing. When bacterivores avoid 2,4-DAPG-producing fluorescent *Pseudomonas* species and graze on other bacteria, it can give 2,4-DAPG-producers a competitive advantage (Pedersen *et al.*, 2009; Hol *et al.*, 2013).

It has been described that decomposers such as earthworms can have an indirect positive effect on *P. fluorescens* in the rhizosphere (Figure 5; Elmer, 2009; Hol *et al.*, 2013). Decomposers may increase nutrient availability for plants and can increase plant growth. As mentioned before, an increase plant growth can lead to a change in root exudate composition which can positively affect fluorescent pseudomonads in the rhizosphere (Jin *et al.*, 2010).

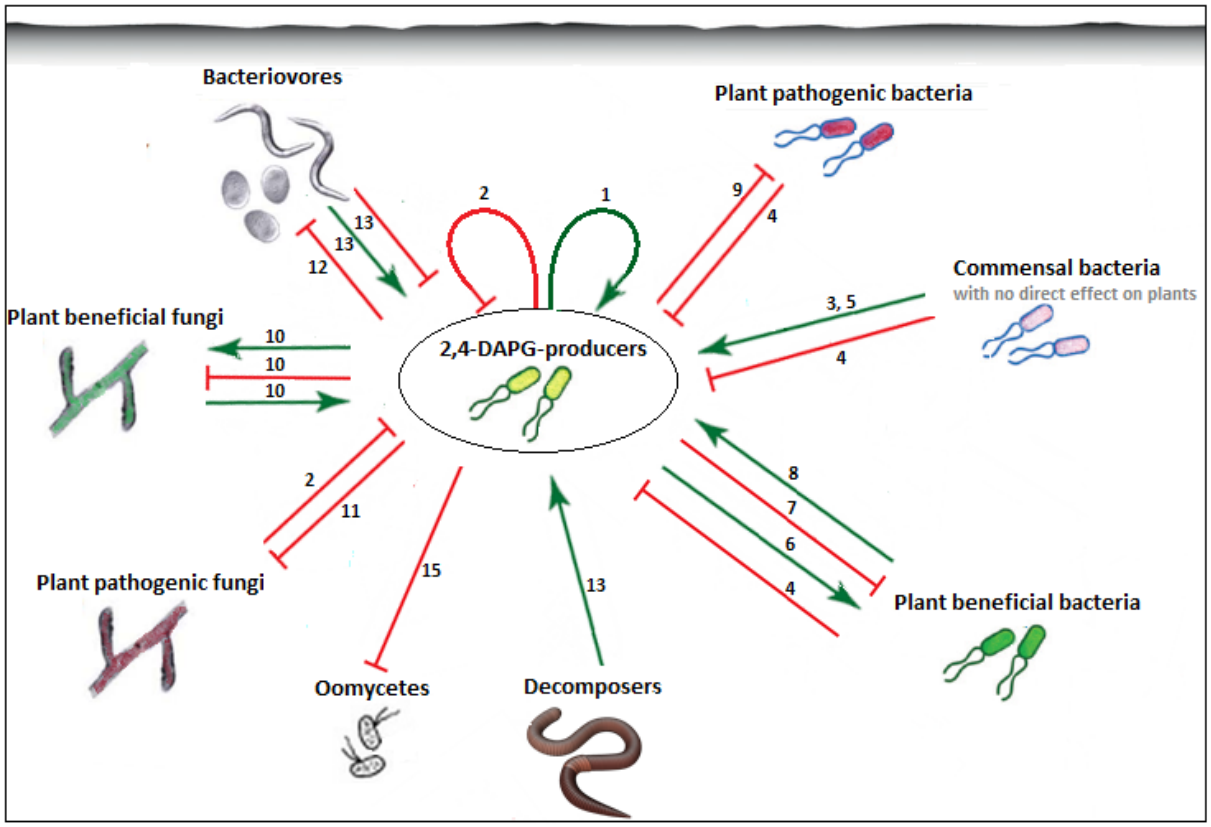

Figure 5. A simplified scheme of the biological interactions between 2,4-DAPG-producing fluorescent *Pseudomonas* species and other soil inhabitants. (1) Maurhofer *et al.*, 2004, (2) Schnider-Keel *et al.*,2000, (3) Dubuis and Haas, 2007, (4) Prieto *et al.*, 2011, (5) Garbeva *et al.*, 2011, (6) Combes-Meynet *et al.*,2011, (7) Couillerot *et al.*, 2011, (8) Berendsen *et al.*,2012, (9) Cronin *et al.*,1997, (10) Lutz *et al.*, 2006, (11) Weller *et al.*, 2002, (12) Jousset *et al.*, 2006, (13) Jousset and Bonkowski, 2010, (14) Elmer, 2009, (15) De Souza *et al.*, 2003.

Concluding, the biological interactions between 2,4-DAPG-producers and other soil inhabitants is very complex. Figure 4 and 5 are schematic schemes of these interactions. Because of the complexity of these biological interaction, the precise role of 2,4-DAPG-producers in a microbial community is largely unknown

Chapter 5. Discussion

With this review an overview has been given on the state of knowledge on the regulation of 2,4-DAPG production by 2,4-DAPG-producing fluorescent *Pseudomonas* species. Also direct en indirect pathogen inhibition by different 2,4-DAPG-producing fluorescent *Pseudomonas* species have been discussed, as well as the effect of biological interactions (e.g. plant and microbial interactions) with 2,4-DAPG-producing fluorescent *Pseudomonas* species. As can be deduced from the previous chapters, the regulation of 2,4-DAPG-producing fluorescent *Pseudomonas* species is highly complex, especially due to the various biological interactions between 2,4-DAPG-producers and other (micro)organisms in the rhizosphere.

Soilborne pathogens are difficult to control, crop rotation, breeding for resistant plants varieties and application of pesticides are insufficient to control root diseases of important crop plants. Biological control of soilborne pathogens by 2,4-DAPG-producing fluorescent *Pseudomonas* species might contribute to the control of root diseases of important crop plants and increase crop production in a sustainable manner. 2,4-DAPG-producing fluorescent *Pseudomonas* species occur worldwide and are effective against a broad range of plant pathogens (as described in chapter 2). Pathogen resistance against 2,4-DAPG is unlike since 2,4-DAPG attacks multiple basic cellular pathways (As described in chapter 3; Kwak *et al.*, 2011). However, due to the complexity of the regulation of 2,4-DAPG-producing fluorescent *Pseudomonas* species and the various biological interactions between 2,4-DAPG-producers and other (micro)organisms, the use 2,4-DAPG-producing fluorescent *Pseudomonas* species as biocontrol agents is complicated and more research is needed. Simply inoculating soils with 2,4-DAPG - producers will not aid to the biocontrol of certain soilborne pathogens. Also inoculating soils with mutant 2,4-DAPG -overproducers will not effectively aid to the plant's health because 2,4-DAPG can have phytotoxic effects (Zhou *et al.*, 2010).

Various direct and indirect (e.g. plant-mediated) effects of biological interactions with enhancing or repressing effects on 2,4-DAPG-producing fluorescent *Pseudomonas* species have been discussed in chapter 4. These interactions makes it very difficult to use 2,4-DAPG-producing fluorescent *Pseudomonas* species as biocontrol agents. For most soils, there is no knowledge of the microbial community present in the soil, which can contain 10^{11} microbial cells per gram root and more than 30.000 prokaryotic species (Egamberdieva *et al.*, 2008; Mendes *et al.*, 2011). Even when the microbial community is known, for most species it is unknown if and how they interact with 2,4-DAPG-producing fluorescent *Pseudomonas* species, so more research is needed on the effect of biological interactions between (micro)organisms and 2,4-DAPG-producers in the rhizosphere.

Plants can adjust the composition of exudates and therefore can select for specific microorganisms in their rhizosphere. It has been suggested that crop species and certain 2,4-DAPG-producing fluorescent *Pseudomonas* genotypes have a mutual "preference" for each other. Understanding the phylogeny of 2,4-DAPG-producers might aid in the use of these strain as biocontrol agents however, much more research is needed on for example which strain is the best suited strain for certain plant crops or cultivars.

Soil amendments can aid in the biocontrol of plant pathogens by 2,4-DAPG-producing fluorescent *Pseudomonas* species. Increasing the bioavailability of minerals and optimizing pH, humidity temperature en fertilizer input might al contribute to the effectiveness of the biocontrol of plant pathogens (Haas and Defago, 2005). Also cultivation of specific wheat varieties together with the desired crop can induce suppressiveness to specific pathogens. For example, cultivation of a specific wheat variety in orchard soils induced suppressiveness to *Rhizoctonia* root rot of apple, cause by *Rhizoctonia solani* AG5 (Mazolla *et al.*, 2002).

Concluding, much research is needed on different aspect of 2,4-DAPG-producing fluorescent *Pseudomonas* species. Elucidating the mechanism by which 2,4-DAPG produced by fluorescent *Pseudomonas* species can effectively suppress plant diseases and how the root microbiome affects 2,4-DAPG production will open new doors for the biocontrol of pathogens and increasing the crop quality and productivity.

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