Chapter 8
Role of Phenazine-1-Carboxamide Produced by *Pseudomonas chlororaphis* PCL1391 in the Control of Tomato Foot and Root Rot

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Abstract Tomato foot and root rot (TFRR) is a tomato root disease caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*). No chemicals are available which efficiently suppress TFRR. In this chapter we show that the bacterium *Pseudomonas chlororaphis* strain PCL1391 is able to suppress the disease. To this end it uses antibiosis as its (major) disease-suppressing mechanism. The produced antibiotic was identified as phenazine-1-carboxamide (PCN). In contrast to the PCN-producing bacterium, pseudomonads which produce the PCN biosynthetic precursor phenazine-1-carboxylic acid (PCA) as their major phenazine were not active in disease suppression. However, when PCA was converted to PCN by complementing these strains with the *phzH* gene, which encodes an amidotransferase, the complemented strains produced PCN and controled TFRR. In order to be effective in disease control, strain PCL1391 should be able to produce PCN under a variety of environmental conditions. We therefore studied the regulation of PCN production under various environmental factors, by regulatory genes, by the plant, and by the pathogenic fungus. Special attention was paid to the secondary metabolite fusaric acid secreted by the fungus. Fusaric acid is detected by the bacterium as a chemo-attractant to reach the fungus, to colonize its surface and to finally use it as a food source. Conversely, fusaric acid is used by the fungus to inhibit the production of PCN and to reduce the bacterial growth rate. It is clear that during disease control the PCN-producing bacterium wins this battle. The result of the evaluation of the described studies is that we can understand in quite some detail how *P. chlororaphis* strain PCL1391 acts as a disease control agent and also why it is not active under all environmental conditions.

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Abbreviations

CAS	Casamino acids
CLSM	Confocal laser scanning microscopy
C6-HSL	*N*-hexanoyl-L-homoserine lactone
Forl	*Fusarium oxysporum* f. sp. *radicis-lycopersici*
gfp	Gene encoding green fluorescent protein
PCA	Phenazine-1-carboxylic acid
PCN	Phenazine-1-carboxamide
TFRR	Tomato foot and root rot

8.1 Introduction

Tomato foot and root rot (TFRR) is a root disease caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*). TFRR is also called crown and root rot of tomato. TFRR is a serious problem for field and greenhouse crops (Jarvis 1988). Chemicals do not efficiently suppress TFRR (Benhamou et al. 1994). In contrast, some bacteria are fortunately able to reduce TFRR (Haas and Défago 2005; Lugtenberg and Kamilova 2009; Pliego et al. 2011). One of these bacteria is the phenazine-1-carboxamide (PCN) producing bacterium *Pseudomonas chlororaphis* strain PCL1391 (Chin-A-Woeng et al. 1998), which was isolated and studied extensively in our laboratory. In this chapter we describe the isolation of this strain, the requirement of PCN for disease control, the differential effect of strains producing PCN and PCA on disease suppression, the very complex regulation of PCN synthesis by both genetic and environmental factors, and finally draw conclusions on the role of PCN in various steps of the disease control process. Previously reviews have been published on the role of phenazines in biocontrol (Thomashow and Weller 1988; Chin-A-Woeng et al. 2003a, b) and on their biosynthesis and regulation (Mavrodi et al. 2006).

8.2 Isolation of *Pseudomonas chlororaphis* PCL1391 and Its Characterization as a Biocontrol Agent

P. chlororaphis strain PCL1391 was isolated from a tomato plant (provided by Prof. José Olivares) grown in a commercial field near Granada, Andalucia, Spain (Chin-A-Woeng et al. 1998). After removal of bulk soil, the root material and adhering rhizosphere soil particles were shaken with water, and dilutions of the suspension were spread on solid KB medium supplemented with carbenicillin, chloramphenicol and cycloheximide. After incubation at 28 °C, seventy of the colonies were tested for inhibition of *Forl* growth. To this end, a 0.5 × 0.5 cm

agar plug containing the fungus was stabbed in the middle of an LB agar plate, followed by inoculating bacterial strains (six per plate) at a distance of 3 cm from the fungus as described by Geels and Schippers (1983). Of these strains, *P. chlororaphis* strain PCL1391 appeared to form the largest fungal growth inhibition zone and was therefore chosen for further study.

In a plate assay, *P. chlororaphis* strain PCL1391 also appeared to inhibit the in vitro growth of a range of fungi, including the phytopathogens *Alternaria dauci*, *Botrytis cinerea*, *Pythium ultimum*, *Rhizoctonia solani*, and *Verticillium albo-atrum*. The strain secretes a hydrophobic compound, identified as PCN, as well as HCN, chitinase(s), lipase(s), protease(s) and siderophore(s). It also appeared to colonize tomato roots as efficiently as the then best-known colonizer in our collection, *P. fluorescens* WCS365 (Lugtenberg and Dekkers 1999; Lugtenberg et al. 2001).

Disease control experiments were carried out using tomato seeds dipped in a suspension of bacteria (10^9/ml) in 1 % methylcellulose. After drying, the seeds were sown in potting soil containing *Forl* spores (3×10^6 spores per kg soil). Disease symptoms were scored after 3 weeks. Controls contained either no bacteria or no fungal spores. *P. chlororaphis* strain PCL1391 appeared to control TFRR efficiently, in contrast to *P. fluorescens* strains F113 (Shanahan et al. 1992) and WCS374 (Leemans et al. 1995), strains both known to control diseases caused by other fungi (Chin-A-Woeng et al. 1998).

Biocontrol of TFRR appeared to depend on at least two factors, namely PCN production and tomato root colonization. The need for PCN was concluded from the fact that a PCN biosynthetic mutant did not show significant disease control (Chin-A-Woeng et al. 1998). The need for root colonization was shown by testing competitive root colonization-negative mutants. Mutants impaired in each one of three known colonization traits, namely motility, prototrophy for amino acids, and the presence of a site-specific recombinase (Lugtenberg et al. 2001) all appeared to be negative in disease control (Chin-A-Woeng et al. 2000). *P. chlororaphis* strain PCL1391, like other pseudomonads, forms micro-colonies or biofilms on part of the root surface (Bloemberg et al. 1997; Bloemberg and Lugtenberg 2004).

The process of attachment of *Forl* to the root and subsequent invasion was visualized using (mutants of) the gene encoding green fluorescent protein (*gfp*) labelled *Forl* and confocal laser scanning microscopy (CLSM) (Fig. 8.1a–d). The tomato root is autofluorescent. The infection process starts with attachment of fungal hyphae to root hairs (Fig. 8.1a) followed by colonization of the grooves between the junctions of the epidermal cells (Fig. 8.1b), penetration of the root cells (Fig. 8.1c) and overgrowth of the internal root (Fig. 8.1d).

Pseudomonas bacteria were labelled in the same way. Their interaction with the root starts with colonizing the grooves between plant cells (Fig. 8.1e), the same sites as colonized by fungus. Subsequently the bacteria form micro-colonies or biofilms on part of the root (Fig. 8.1f). The bacteria in the biofilm are covered by a mucoid layer (see Fig. 8.1g, which is a detail of Fig. 8.1f, h, a scanning microscopy picture in which the mucoid layer is broken open). A biofilm creates ideal

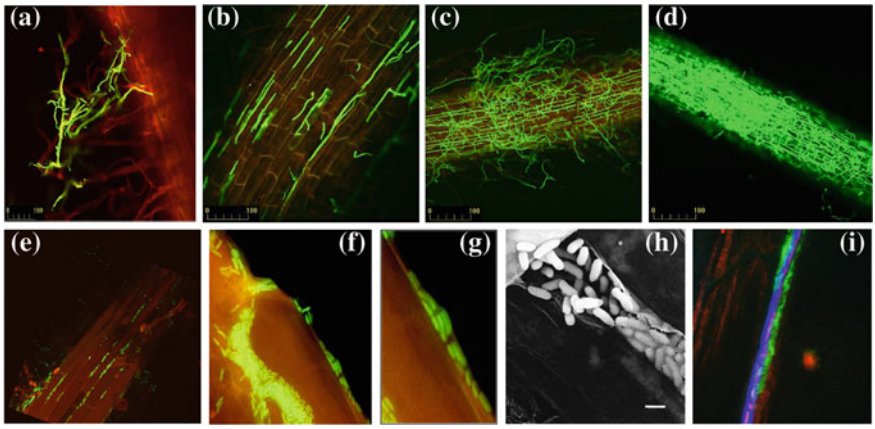

Fig. 8.1 Visualization of plant–microbe and microbe–microbe interactions during biocontrol. CLSM (**a**–**g** and **i**) and scanning electron microscopy (**h**) were used to visualize control of TFRR caused by Forl (*Fusarium oxysporum* fsp. *radicis-lycopersici*) by *Pseudomonas* biocontrol bacteria. For explanation, see text. Panels **a**, **c**, and **d** were reproduced from Lagopodi et al. 2002, panel **b** from Bolwerk et al. 2003, and panel **h** from Chin-A-Woeng et al. 1997. Panel **e** is from Bolwerk, Lagopodi and Bloemberg, *unpublished*. Panels **f** and **g** are from Bloemberg et al. 1997; Copyright © American Society for Microbiology

conditions for quorum sensing and processes dependent on quorum sensing, such as F-mediated DNA transfer, and the syntheses of antibiotics (e.g. PCN) and exoenzymes. The bacteria also attack the fungus directly by colonizing the hyphae extensively (Fig. 8.1i).

8.3 Comparison of the Roles of PCN and PCA in the Control of TFRR

Some biocontrol strains, e.g. *P. fluorescens* 2-79 (Thomashow and Weller 1988) and *P. aureofaciens* 30-84 (Pierson and Thomashow 1992), produce phenazine-1-carboxylic acid (PCA) but not PCN. In our hands, these strains were inactive in suppressing TFRR. In an attempt to understand this difference, the growth-inhibiting activities of equimolar amounts of PCN and PCA were compared as a function of the pH. At pH values of 5 and lower, both compounds were inhibitory but PCA was slightly more active. At pH values between 5.7 and 7.0, PCN was superior whereas PCA was even inactive at values between 5.9 and 7.0 (Chin-A-Woeng et al. 1998). It is likely that the difference in pH dependence of the antifungal activities of the two compounds is an important factor in the outcome of their disease control activities.

To determine the role of the amino group in biocontrol, the *phzH* gene, present in strain PCL1391 but not in the two PCA-producing strains, was identified and characterized. A *phzH* mutant appears to accumulate PCA instead of PCN. The

deduced PhzH protein shows homology with asparagine synthetases, which belong to class II glutamine amidotransferases. These results indicate that the conversion of PCA to PCN takes place via a transamidase reaction catalysed by PhzH. A *phzH* mutant of *P. chlororaphis* strain PCL1391 is unable to control TFRR. Transfer of the *phzH* gene to the PCA producing strains *P. fluorescens* 2-79 and to *P. aureofasciens* 30-84 enabled these strains to control TFRR (Chin-A-Woeng et al. 2001b). It must therefore be concluded that the amino group of PCN is crucial for the control of TFRR.

8.4 Regulation of PCN Synthesis by Quorum Sensing and Environmental Factors

The expression of the biosynthetic *phzABCDEFGH* operon (Chin-A-Woeng et al. 2001b) is regulated by quorum sensing (Bassler 1999). The *luxI* and *luxR* homologues of strain PCL1391, *phzI* and *phzR*, regulate the expression of the biosynthetic operon. PhzI produces *N*-hexanoyl-L-homoserine lactone (C_6-HSL) as the main autoinducer, whereas smaller amounts of C_4-HSL and C_8-HSL are also produced (Chin-A-Woeng et al. 2001a). The autoinducers supposedly activate the transcriptional activator PhzR by binding to it (Fig. 8.1). Activated PhzR is thought to turn on the biosynthetic *phz* genes. Quorum sensing is dependent on population density. Gene expression studies have shown that the culture supernatant confers positive regulation of *phzI*, not only by autoinducers but also by at least one unknown factor (Chin-A-Woeng et al. 2001b). Production of *N*-acyl homoserine lactones seems to be essential and necessary for PCN biosynthesis, as no condition (environmental or genetic change—except *phz* genes) has been found so far under which PCN is produced in the absence of autoinducer, and vice versa.

We studied the influence of environmental conditions relevant for plant growth in detail, using growth at 28 °C in Vogel-Bonner medium amended with 0.05 % CAS (casamino acids) and 30 mM glucose as the basic medium, which was subsequently modified to study the effects of various factors (Van Rij et al. 2004). PCN production starts at the end of the exponential growth phase and continues to increase until the cells reach the stationary phase. Growth on the carbon sources glucose, L-pyroglutamic acid and glycerol results in the highest PCN levels (Van Rij et al. 2004). Omitting 0.05 % CAS from the growth medium dramatically reduces the levels of PCN production. Addition of extra CAS increases PCN levels. Testing of individual amino acids showed that all tested individual amino acids increase PCN levels at least 2-fold and that the largest increase is caused by phenylalanine (23 times) and tyrosine (13 times). A remarkable finding was that the addition of 1 mM phenylalanine causes the PCN production to start earlier in the growth curve (at an OD_{620} nm value of 1.0 instead of 2.0). Replacement of $(NH_4)_2SO_4$ by the same amounts of nitrogen from urea or $NaNO_3$ results in a decrease in PCN production. Testing of other ions showed that low Mg^{2+} increases

PCN levels and that salt stress (but not osmotic stress), and low concentrations of ammonium, ferric, phosphate, and sulphate ions reduce PCN levels (Van Rij et al. 2004).

At temperatures of 21, 28 and 31 °C, PCN production is similar but at 16 °C the level drops to practically zero. This may be related to the remarkable reduction in growth rate by 80 %. Reducing the O_2 level to 1 % results in a substantial increase in PCN production. The pH of the growth medium has a strong influence on the PCN level. Starting pH values of 7.0 and 8.0, resulting in final pH values of 6.5 and 7.1, respectively, result in normal PCN levels but a starting pH of 6.0, which results in a final pH of 4.2, abolishes PCN production (Van Rij et al. 2004).

Other relevant facts are the following: (1) attempts to find synergism between conditions that result in high PCN levels failed; (2) comparison of our results with literature data showed that some environmental factors have similar effects on other studied *Pseudomonas* strains but that other environmental factors have opposite effects in other strains; (3) analyses of autoinducer levels under conditions of high and low PCN production demonstrated that, under all tested conditions, PCN levels correlate with autoinducer levels, indicating that the regulation of PCN levels by environmental factors takes place at or upstream of autoinducer production (Van Rij et al. 2004).

8.5 Genetic Regulation of PCN Synthesis by the Bacterium

Considering the complex regulation of PCN production by environmental factors (see above), by the plant and by the fungus (see the following sections), it must be expected that many genes and regulatory cascades are involved in the regulation of PCN production. This indeed appears to be the case.

The previously mentioned quorum-sensing genes *phzI* and *phzR* are located at the end of a complex and not yet completely understood genetic regulatory cascade (Fig. 8.2). Every gene that has been shown to influence phenazine production by strain PCL1391 (except the *phz* genes themselves) was correlatively shown to affect autoinducer synthesis.

A two-component system including a membrane-associated sensor (GacS) and a response transcriptional regulator (GacA) activates the *phz* operon under all conditions tested so far. It responds to a yet-unknown environmental factor and is at the top of the PCN regulatory cascade. In *Pseudomonas* species, the *gac* genes are global regulators of secondary metabolism that are situated upstream of many regulatory cascades. They function as master regulators involved in the control of a substantial set of genes and traits such as the production of antibiotics, HCN and extracellular enzymes (Haas and Défago 2005).

Between GacS/GacA and the quorum-sensing system, at least three genes are responsible for modulating PCN synthesis. Firstly, *Pseudomonas* sigma regulator (*psrA*) regulates its own expression by negative feed-back (Chin-A-Woeng et al. 2005)

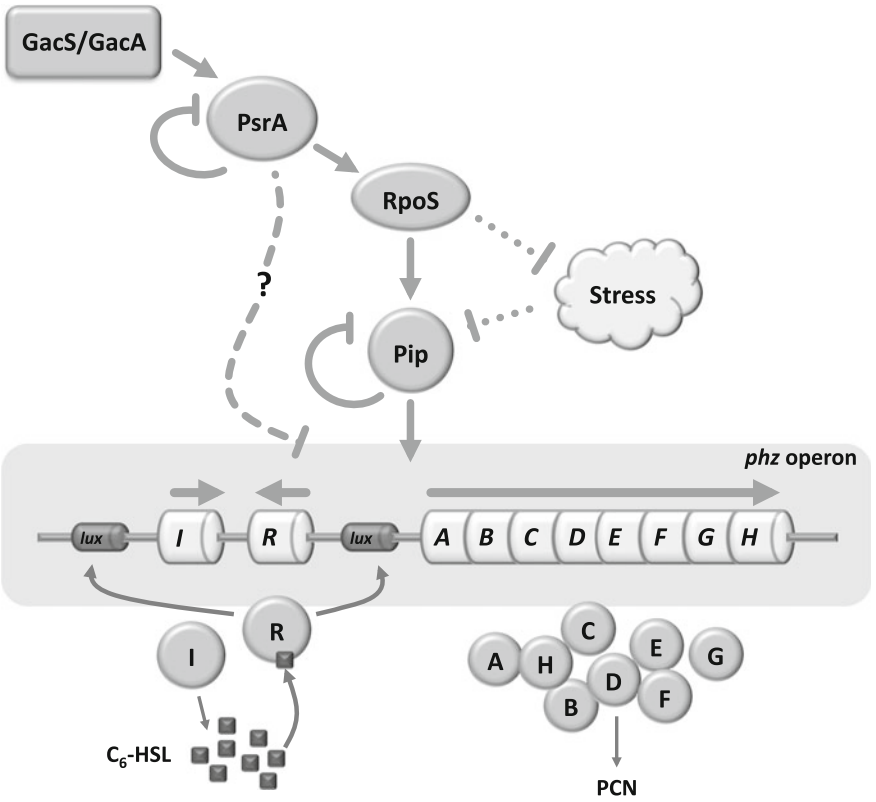

Fig. 8.2 Three main genetic pathways for the regulation of PCN synthesis. All pathways are downstream of the master regulatory system GacS/GacA and upstream of the quorum-sensing system PhzI/PhzR. *Dashed lines*: in rich medium, PsrA negatively regulates the *phz* operon via unknown genes. *Plain lines*: in minimum medium, PsrA positively regulates PCN production via RpoS and Pip. *Dotted lines*: in minimum medium, under several types of stress conditions (including the *Forl* toxin fusaric acid), PCN synthesis is switched off to give priority to another RpoS-regulated pathway: stress resistance. See Sect. 8.5 for more details

and stimulates expression of the second regulatory gene, *rpoS* (alternative sigma factor) (Girard et al. 2006a). It was shown that *psrA* negatively affects PCN production in rich medium (Chin-A-Woeng et al. 2005), but positively in poor medium (Girard et al. 2006a). In rich medium, *rpoS* does not have a significant influence on PCN production, but clearly stimulates it under poor conditions (Girard et al. 2006a). These differences reflect the complexity of the *phz* operon regulation by the environment and indicate that genes other than *rpoS* must be present downstream of *psrA* in the regulatory cascade. Thirdly, the *pip* gene—encoding the phenazine inducing protein Pip—was also found to have a negative auto-regulatory role and, just downstream of *rpoS*, a positive effect on PCN synthesis in poor medium (Girard et al. 2006b).

Interestingly, experiments involving sub-inhibitory concentrations of various stress factors in poor medium also link Pip to stress response by *P. chlororaphis*. We propose that Pip would be downstream of the stress sigma factor RpoS, a 'decision' point for attributing the use of energy to either PCN production (under favourable growth conditions) or stress resistance (Girard and Rigali 2011).

8.6 Regulation of PCN Synthesis by the Plant

Since *P. chlororaphis* strain PCL1391 is PCN-dependent for biocontrol (see Sect. 8.2), PCN is likely to be produced on the plant root. This turns out to be indeed the case. Using a derivative, designated as strain PCL1119, which harbours promoterless *luxAB* genes inserted in the *phzB* gene of the phenazine biosynthetic operon, Chin-A-Woeng et al. (1998) showed expression of the phenazine biosynthetic operon on the tomato root.

Growth on the carbon sources glucose, L-pyroglutamic acid and glycerol results in high PCN levels (7.9 μM PCN produced per OD_{620} value for glucose), whereas growth on the five most common carbon sources found in tomato root exudate (citric acid, malic acid, lactic acid, succinic acid and pyruvic acid; Lugtenberg and Bloemberg 2004) results in limited PCN levels (only 1.0, 0.05, 1.2, 0.13, and 0.17 μM PCN per OD_{620} value, respectively). From these results it was concluded that the tomato root exudate composition is far from optimal for PCN production by *P. chlororaphis* strain PCL1391 (Van Rij et al. 2004).

Some exudate components indirectly contribute to PCN production by functioning as chemo-attractants for *Pseudomonas* cells. De Weert et al. (2002) reported the results of assays measuring chemotaxis of biocontrol strain *P. fluorescens* WCS365 towards individual tomato root exudate components. They found positive chemotaxis towards some organic acids and some amino acids but not towards sugars. Comparison of the minimal active concentrations with the concentrations estimated to be present in exudate led to the conclusion that malic acid and citric acid are the major chemo-attractants in the tomato rhizosphere (De Weert et al. 2002).

8.7 Regulation of PCN Synthesis by the Pathogen

Killing of the pathogenic fungus *Forl* by the PCN-producing *P. chlororaphis* strain PCL1391 is not as easy as it may look since it was found that the fungus has developed a smart defence strategy. It appears that the secondary metabolite fusaric acid, secreted by *Fusarium*, plays a crucial role in the interaction between fungus and bacterium. *Forl* and many other *Fusarium* strains produce the fungal

toxin fusaric acid (Notz et al. 2002). At a fusaric acid concentration of 0.3 mM, PCN production levels of the bacterium start to decrease, while 1.5 mM fusaric acid decreases PCN production by as much as 97 %. This fusaric acid concentration also decreases the growth rate of the bacterium by 25 % (Van Rij et al. 2004). It is conceivable that such conditions can be reached on the root. Since *P. fluorescens* strain WCS365 is chemotactically attracted towards fusaric acid secreted by *Forl* (De Weert et al. 2003), we consider this likely to also be the case for *P. chlororaphis* strain PCL1391. These observations suggest a dual role of fusaric acid in biocontrol: on the one hand it is used by the bacterium as a guide towards hyphae to use them as a food source, whereas on the other hand it disarms the biocontrol bacterium by inhibiting the production of PCN, its major weapon against the fungus, as well as by inhibiting growth. Successful biocontrol apparently depends on the ratio of the activities of the bacterial and fungal metabolites PCN and fusaric acid, respectively.

In attempts to understand the role of fusaric acid in more detail, we performed a number of genetic studies. Since fusaric acid also represses the production of the quorum-sensing signal C_6-HSL, it is clear that inhibition of PCN synthesis by fusaric acid occurs at or before the level of C_6-HSL synthesis (Van Rij et al. 2004). Further studies indicated that PCN repression by fusaric acid is maintained even during PCN production-stimulating growth conditions such as the presence of additional phenylalanine, additional ferric iron ions, and a low Mg^{2+} concentration. In contrast, constitutive expression of *phzI* or *phzR* increases C_6-HSL levels and stops the repression of PCN production by fusaric acid (Van Rij et al. 2005). Transcriptome analysis confirmed that fusaric acid represses expression of the biosynthetic *phz* operon as well as of the quorum sensing regulatory genes *phzI* and *phzR*. Fusaric acid does not affect the expression of *gacS*, *rpoS* and *psrA*, genes which have been shown to regulate the synthesis of PCN (Girard et al. 2006a; Chin-A-Woeng et al. 2005). These results show that reduction of PCN synthesis by fusaric acid is the result of a direct or indirect repression of *phzR* and *phzI*. An interesting observation is that fusaric acid not only represses the production of the antibiotic PCN but also of another antibiotic involved in biocontrol of plant diseases, namely 2,4-diacetylphloroglucinol, produced by biocontrol strain *P.fluorescens* CHA0 (Duffy and Défago 1999). Therefore, and because this strain does not produce acyl-homoserine lactones, it is likely that fusaric acid interferes with PCN synthesis by indirect repression of *phzR* and *phzI*, at least partially via the Pip regulator (see Fig. 8.2, Girard et al. 2011).

Transcriptome analysis also showed that genes that are highly up-regulated by fusaric acid also are up-regulated by iron starvation in *P. aeruginosa* (Ochsner et al. 2002; Palma et al. 2003; Ghysels et al. 2004), suggesting an overlapping stress response to fusaric acid and iron starvation (Van Rij et al. 2005).

8.8 Conclusions on the Role of PCN in Various Steps of the Biocontrol Process

In biocontrol experiments, spores of the fungal pathogen *Forl* are mixed with the soil while the beneficial PCN-producing bacterium *P. chlororaphis* strain PCL1391 is coated on the tomato seeds. Upon germination of the seeds, the bacterium is attracted to the root by root-exudate compounds, which are also utilized for multiplication. The bacterium colonizes the root, first as single cells, later as microcolonies or biofilms (Fig. 8.1; Bloemberg et al. 1997). The majority of the bacteria are found at the grooves along the junctions of the epidermal cells. The bacterium reaches the plant root earlier than the fungal hyphae (Bolwerk et al. 2003).

Roots of seedlings secrete components that allow the fungal spores to germinate (Kamilova et al. 2005; Steinkellner et al. 2005) and attract the hyphae to the root. Using *gfp*-labelled *Forl*, the process of tomato root infection by *Forl* was analysed. The first step is attachment of hyphae to the root hairs. This is followed by root colonization of the grooves along the junctions of the epidermal cells. Finally, the hyphae penetrate the plant cells, overgrow the root and cause the death of the plant (Lagopodi et al. 2002).

In the case of disease control, the bacterium has reached the root first and controls fungal growth. It out-competes the fungus for growth on exudate components and does not allow the hyphae to penetrate the plant root. Instead, the bacterium colonizes the fungal hyphae, weakens them, and eventually uses them as food (Bolwerk et al. 2003; De Weert et al. 2003).

Analyses of molecular details of the plant-bacterium-fungal interaction have revealed the following. (1) Exudate compounds (especially malic acid and citric acid; De Weert et al. 2002) are used as chemo-attractant to guide the bacterium to the root where it uses major exudate compounds (including citric, malic, lactic, oxalic, pyruvic and succinic acids; Kamilova et al. 2006; Lugtenberg et al. 2001; Lugtenberg and Bloemberg 2004; Lugtenberg and Kamilova 2009) for multiplication. (2) In vitro interference contrast microscopy experiments show that PCN negatively affects hyphal growth and branching of the fungus, which presumably negatively affects the colonization and infection abilities of the fungus (Bolwerk et al. 2003). (3) The hyphae secrete fusaric acid, which is used by the bacterium as a chemo-attractant to find the hyphae, colonise them, and use them as food. Fusaric acid is used by the fungus to inhibit the biosynthesis of PCN, the major weapon of the bacterium. One can predict that in the case of successful disease control the PCN-producing bacterium has been more successful than the fusaric acid-producing fungus. It is likely that the following factors contribute to the success of disease control. (a) The fact that bacterium and fungus colonize the same niche, which allows the bacterium to optimally attack the fungus. (b) The timing by the bacterium, which reaches the grooves on the plant root first and builds up high numbers of cells and a high PCN concentration before the fungus arrives. (c) The relative concentrations and efficiencies of the weapons PCN and fusaric acid on the

battlefield. (d) Some of the observations reported in this chapter negatively impact application of phenazine-producing strains in biocontrol. Firstly, the pH of some soils has a strong effect on the *efficacy* by which PCA, but much less PCN, inhibits fungal growth. Secondly, many environmental factors influence the level of PCN *production.* Therefore it can be predicted that biocontrol by *P. chlororaphis* strain PCL1391 will not be effective in all soils and not under all environmental conditions.

The result of the evaluation of the studies described here is that we can understand in quite some detail how the PCN-producing *Pseudomonas chlororaphis* strain PCL1391 acts as a disease control agent and also why it is not active under all environmental conditions.

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