Microbial control of plant diseases[1]

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Abstract

Most plant diseases are caused by fungi and oomycetes. Presently, the major method for controlling plant diseases is the use of agrochemicals. However, this practice raises health and environmental concerns among consumers and politicians. An alternative for chemicals is the application of products based on natural enemies of the pathogen. Several of such BCAs (Biological Control Agents) with bacteria or fungi as the active ingredient are already on the market. In this review we describe the discovery of such microbes as well as methods for their isolation. Using microscopy, we visualized biocontrol at the cellular level. Furthermore, we describe the role of root colonization by the BCA in biocontrol. Finally, mechanisms of biocontrol at the molecular level are described and the risk of resistance towards BCAs is discussed.

[1] To be published as a chapter in the book "Molecular Microbial Ecology of the Rhizosphere" (2013), Frans J. de Bruijn (ed), Wiley-Blackwell.

Introduction

Some practical aspects of biocontrol

For recent reviews on microbial control of plant root diseases the reader is referred to Berg (2009), Compant et al. (2005), Haas and Défago (2005), Lorito et al. (2010); Lugtenberg and Kamilova (2009), Pliego et al. (2011), and Raaijmakers et al. (2009). The concept of microbial control of plant root diseases originates from the discovery of disease-suppressive soils (Schroth and Hancock, 1982) and is explained in detail in excellent reviews by Weller et al. (2002) and Haas and Défago (2005). Briefly, disease-conducive soils contain pathogens and therefore cause plant disease. In contrast, there are soils which also contain pathogens but hardly cause disease. These so-called disease-suppressive soils contain microbes which suppress the action or growth of the pathogen or even kill the pathogen (Mendes et al., 2011). The disease-suppressive trait can be transferred to conducive soils by mixing the latter with a small amount of disease-suppressive soil. Details on factors influencing transfer of disease-suppressiveness can be found in Haas and Défago (2005).

Plant diseases are responsible for annual crop losses at a total value of more than 200 billion Euro (Agrios, 2005). Major root diseases are caused by fungi, oomycetes and nematodes. Also some bacteria are responsible for root diseases. The fungi include *Fusarium oxysporum*, *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani*, and *Thielaviopsis*. The major oomycetes are *Phytophthora* spp. and *Pythium* spp. The pathogenic bacteria include *Erwinia amylovora, Ralstonia solanacearum* and *Streptomyces scabies*, whereas *Meloidogyne incognita* is an example of a root-pathogenic nematode.

The major form of crop protection is the use of chemicals. However, this practice raises health and environmental concerns among public and politicians. As a result, many chemicals have been banned and more will follow. Also, some supermarket chains put pressure on fruit and vegetable producers by requiring zero tolerance.

An attractive alternative to chemical crop protection products, or more realistically, for the reduction of chemical input, is the use of disease-suppressing microbes. These are found among natural enemies of the pathogens. In principle, the use of these microbes is an environmentally friendly and safe way to replace or reduce chemicals. In case these microbes produce antibiotics, these molecules are produced in only minute amounts and only at the site where they are needed, i.e. on the plant surface. In contrast to this form of precision agriculture, most chemicals are applied in much higher amounts and a significant fraction of the applied molecules does not even reach

the plant surface. A disadvantage of biologicals is that they are often less efficient than chemicals and their action is less consistent than that of chemicals. Therefore a major challenge for biocontrol scientists and producers of microbial products is to create more efficient products. In order to sell a product, the producer should make clear that the product is safe and effective. Despite the strong public and political demand for biological alternatives for chemicals, there are no specific registration procedures for biologicals but they are regulated as general plant protection products which are designed for chemicals.

Although most crops are grown in soil, many greenhouse vegetables are nowadays grown on other substrates such as stonewool. New stonewool is practically sterile. This means that pathogens which invade the young plants can have a devastating effect on the whole plant population because the buffering capacity of indigenous microbes, which is strong in healthy soil, is absent in new stonewool. However, biocontrol microbes such as *Pseudomonas putida* strain PCL1760, added to new stonewool before planting, can protect the plantlets very efficiently against pathogens (Validov et al., 2009). Cells of this strain appeared to stick tightly to stonewool and remain the dominant microbe on the root for at least 3 weeks (Validov et al., 2007). This suggests that addition of such microbes to stonewool can replace indigenous microbes with respect to buffering capacity against pathogens.

Life style of microbes in the rhizosphere

The rhizosphere is defined as the soil area around the root which is influenced by the root (Hiltner, 1904). It is 10 to 1,000 times richer in microbes than bulk soil. This so-called rhizosphere effect is assumed to be caused by nutrients for microbes secreted by the root and by residues of dead roots or root cells. It has been estimated that 5 to 21 percent of the carbon fixed by the plant is secreted, mainly as exudate (Marschner, 1995).

The simplest nutrients in root exudate, which are the most attractive food sources for rhizosphere microbes, are organic acids, sugars, and amino acids. In addition, a large variety of compounds such as enzymes, fatty acids, nucleotides, osmoprotectants, putrescine, sterols and vitamins have been detected in root exudate, as well as signal molecules playing a major role in communication between different microbes and also between microbes and other organisms (see later on in this chapter). The exudate composition is the net result of secretion, conversion by soil

enzymes, and uptake by microbes and plant. For reviews on exudates, the reader is referred to Lugtenberg and Bloemberg (2004) and Uren (2007).

BCAs which are added to the soil have to compete for nutrients and niches on the plant root with indigenous microbes, such as bacteria and fungi, and with predators such as nematodes and protozoa. Microbes living in the rhizosphere usually live under nutrient-starvation conditions since the nutrient concentration is much lower than that in laboratory media (Lugtenberg and Kamilova, 2009). The doubling time of pseudomonads in the rhizosphere is 3 to 6 hours, i.e. ten times slower than in rich laboratory media (Haas and Défago, 2005). Also osmotic stress may play a role in the life of a rhizosphere microbe since the osmotic conditions may vary due to drought and rainfall. This is probably the reason why many rhizosphere microbes produce osmoprotectants (Berg et al., 2013).

BCAs may communicate with other organisms through a variety of signal molecules. We will restrict ourselves here to AHLs because they are relevant for biocontrol. AHLs are molecules secreted by many Gram-negative bacteria. They can sense the level of other bacteria of the same kind. When the concentration of these bacteria reaches a certain level (the quorum), as sensed by the extracellular concentration of AHLs, they start to produce many secondary metabolites and exo-enzymes (Uroz et al., 2009).

Visualisation of biocontrol

GFP can be visualized using CLSM. Since *gfp* mutants with different colors exist, several microbes labeled with GFP and derivatives can be visualized simultaneously in the same preparation against the autofluorescent plant root (Bloemberg et al., 2000). Using the combination of *gfp*-labeled microbes and CLSM, the process of biocontrol of TFRR was visualized, first by following the behavior of BCA and fungus on the root separately, later with all players present. After application on the seed and subsequent germination, the microbe starts to colonize the root collar, followed by colonization of the root, first as single cells and later as micro colonies or biofilms **(Fig. 1**) (Chin-A-Woeng et al., 1997; Bloemberg and Lugtenberg, 2004). The first step carried out by the causal agent of TFRR, *Forl,* is attachment of hyphae to root hairs (**Fig. 1a**). Subsequently the hyphae colonize preferentially the grooves along the junctions of the epidermal cells (**Fig 1b**), penetrate the epidermal cells (**Fig. 1c**) and overgrow the root completely (**Fig. 1d**) (Lagopodi et al., 2002). *Pseudomonas* BCAs, applied on the seed,

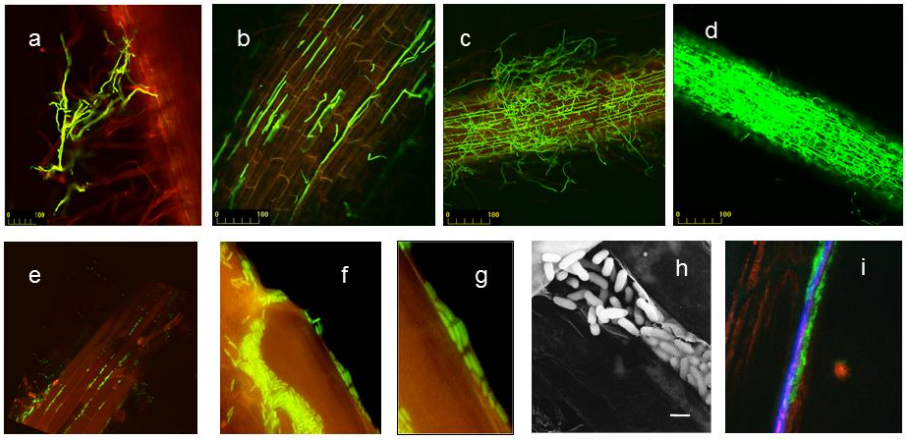

Fig. 1. Visualisation of biocontrol. CLSM (confocal laser scanning microscopy) (a-g and i) and scanning electron microscopy (h) were used to visualize control of TFRR caused by *Forl* (*Fusarium oxysporum* f. sp. *radicis-lycopersici*) by *Pseudomonas* biocontrol bacteria. For CLCM, bacteria and fungi were labeled using mutants of the *gfp* (green fluorescent protein) gene. The tomato root is autofluorescent. The infection process by the pathogen starts with attachment of hyphae to the root hairs (a) followed by colonization of the grooves between the junctions of the epidermal cells (b), penetration of the root cells (c) and overgrowth of the internal root (d). Upon seed germination, bacteria coated on the seed multiply, colonize the grooves between plant cells (e) and form biofilms on part of the root (f). Note that the bacteria in biofilms are covered by a mucoid layer (see g, which is a detail of f, and 1h in which the mucoid layer is broken open) which creates an ideal condition for quorum sensing and processes dependant on QS, such as F-mediated DNA transfer, and the syntheses of antibiotics and exo-enzymes. The bacteria also colonize the hyphae extensively (i).

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.

multiply extensively upon germination, start to colonize the root surface, first the grooves along the junctions between root cells (**Fig. 1e**), form biofilms **(Fig. 1f)** which are covered by a mucoid layer **(Fig. 1g, h)** and eventually reach the root tip. The bacteria also colonize the fungal hyphae **(Fig. 1i)** (Lagopodi et al., 2002; Bolwerk et al., 2003). The observation that the two microbes colonize the same niche on the root, initially suggested to us that they therefore have a fair chance to interact, which would be beneficial for biocontrol. However, it could be that even smaller micro-niches are required for BCA and pathogen to meet (see section f. Competition for nutrients and niches).

Under the tested biocontrol circumstances, i. e. BCA applied on the root and pathogen mixed through the sand, the BCA reaches the root first. In addition, it colonizes the hyphae extensively (Lagopodi et al., 2002; Bolwerk et al., 2003). The

metabolic basis of the initiation of the interactions during the colonization processes was unraveled: *Pseudomonas* is chemotactically attracted to the root by root exudate components, in particular malic acid and citric acid (De Weert et al., 2003), and chemotactically attracted to the fungus by fusaric acid (De Weert et al., 2002).

Detailed colonization studies suggest that each BCA is characterized by a more or less specific colonization pattern and mode of interaction with pathogens and plant hosts (Zachow et al., 2010; Compant et al., 2011).

Competitive root colonization by biocontrol microbes

Since root colonization is the delivery system of beneficial microbes and their products, effective biocontrol microbes should be rhizosphere competent. This has been proven for the mechanisms antibiosis (Chin-A-Woeng et al., 2000) and competition for nutrients and niches (Kamilova et al., 2005; Validov et al., 2007). For the mechanism ISR it seems to be sufficient that the microbe is present on part of the root although full root colonization provides better protection (Dekkers et al., 2000).

In order to identify traits involved in root colonization, a gnotobiotic competitive tomato root colonization system was developed in which bacteria from two different strains are applied on the seed and, upon germination, compete for nutrients by moving chemotactically towards the root tip. The ratio in which the microbes were found on the root tip was used as the criterion for effective competitive root colonization (Simons et al., 1996). This system was not only used for comparison of the competitive root colonization abilities of wild type strains, but also for the screening for competitive colonization mutants. After complementation analysis and after confirmation of the colonization defect of the putative mutants in a soil system, traits playing a role in competitive root colonization were identified. These traits include phase variation, motility, adhesion to the root, utilization of organic acids from exudate, the syntheses of amino acids, nucleotides, uracil, vitamin B1, and the LPS O-antigenic side chain, and the TTSS (Lugtenberg and Dekkers, 1999; Lugtenberg et al., 2001; Lugtenberg and Bloemberg, 2004; Lugtenberg and Kamilova, 2009). In the following we will discuss some important traits in detail, namely chemotaxis of the BCA towards the root, utilization of root exudate nutrients, and the role of TTSS in competitive colonization.

Not surprisingly, it turned out that not motility in general but chemotaxis towards specific root exudate components, especially malic acid and citric acid, is crucial for

effective competitive tomato root colonization by *Pseudomonas* (De Weert et al., 2003).

In an early stage of the colonization research fast growth on root exudate components was shown to be important. Consistent with this notion was the observation that mutants impaired in the utilization of the major group of exudate nutrients, organic acids, were impaired in competitive root colonization whereas mutants impaired in the utilization of sugars, which are present is lower amounts in tomato root exudate, showed practically normal behavior (Simons et al., 1997; Lugtenberg et al., 1999).

Since mutants impaired in their TTSS are poor in competition with the parental strain for root colonization, it was concluded that type three secretion plays a role in competitive root colonization. Since the presence of the parental cells did not compensate the colonization defect of the mutants, it was suggested that the needle of the TTSS in wild type cells was not used to release nutrients from the plant cells into the environment because that would have resulted in phenotypic complementation. Rather, the presence of the needle gives the wild type a competitive growth advantage. Apparently, the needle was used to tap nutrients from the plant cell directly into the bacterium. Based on this result it was hypothesized that early in evolution the TTSS needle was developed to give the bacterial cell access to nutrients present in the plant cell and that the system later evolved to inject bacterial molecules into the plant cell (De Weert et al., 2007).

Antibiotics and biocontrol

Up to one third of rhizosphere bacteria produce AFMs and therefore may play a role in the control of diseases caused by fungi (Opelt et al., 2007). This has to be confirmed by mutational analysis followed by complementation studies. The best known antibiotics involved in biocontrol by Gram-negative bacteria are Phl, phenazines, pyoluteorin, pyrrolnitrin and the volatile HCN. The possible modes of action of several of these antibiotics are discussed by Haas and Défago (2005). Some bacilli can produce zwittermycin A (Emmert et al., 2004) and kanosamine (Milner et al., 1996). More recently, BCAs were discovered which produce the antibiotics D-gluconic acid (Kaur et al., 2006), 2-hexyl-5-propyl resorcinol (Cazorla et al., 2006) and the volatiles 2,3-butanediol (Ryu et al., 2003), 6-pentyl-α-pyrone (Lorito et al., 2010) and DMDS (Dandurishvili et al., 2011). The role of the volatile HCN in biocontrol has been known

for a long time (Haas and Défago, 2005) and it was recently discovered that also other volatiles can play a role in biocontrol (Ryu et al., 2003; Dandurishvili et al., 2011).

A class of antibiotics which was studied in great detail during the last decade is that of the c-LPs. These compounds are produced by several bacterial species, including *Bacillus* (Borriss, 2011; Chen et al., 2009; Ongena et al., 2007; Romero et al., 2007) and *Pseudomonas* (Raaijmakers et al., 2006; Raaijmakers et al., 2010). *Bacillus* c-LPs belong to three major families, the iturins (bacillomycins, iturins and mycosubtilins), the fengycins (plipastatins) and the surfactins (bamylocin A, esperins, lichenysins, pumilacidins and surfactins). These c-LPs are composed of seven (iturins and surfactins) or ten (fengycins) amino acids of both D- and L-configuration which form a ring linked to either a β-hydroxy (fengycins and surfactins) or a β-amino (iturins) fatty acid. Both the peptide moiety and the fatty acyl chain are essential for the biological functions of c-LPs (Jacques, 2011). All three major families of cLPs are key effector molecules of biological control. The mechanism of their beneficial action is based on direct antibiosis of phytopathogens and/or triggering ISR (Borriss 2011; Raaijmakers et al., 2010; Pérez-García et al., 2011). Iturins and fengycins are originally known for their strong antifungal activity against a wide range of phytopathogens while surfactins are mostly antibacterial (Ongena and Jacques, 2008). Recently Zeriouh et al. (2011) provided strong evidence for a major role of iturins in inhibition of the Gram-negative bacterial phytopathogens *Xanthomonas campestris* and *Pectobacterium carotovorum*. This is an interesting finding since the antibacterial activity of iturins was initially thought to be restricted to only a few Gram-positive species (Besson et al., 1978). Several mutational analysis studies have shown a role of iturins in biocontrol of both fungal and bacterial phytopathogens (Leclère et al., 2005; Arrebola et al., 2010; Zeriouh et al., 2011). Touré et al (2004) presented strong evidence for the involvement of fengycins in biocontrol of *Botrycis cinerea* on apple. They detected fengycins in infected tissues in inhibitory concentrations. Using mutational analysis, Yánez-Mendizábal et al (2011) showed a major role for fengycins in suppression of peach brown rot. Surfactins are very effective against *Pseudomonas syringae* on *Arabidopsis* plants (Bais et al. 2004). Fengycins and surfactins trigger defense pathways in bean and tomato plants (Ongena et al., 2007; Henry et al., 2011). Furthermore, when different families of c-LPs are co-produced they can interact in a synergistic manner resulting in more effective plant protection (Ongena et al., 2007; Romero et al., 2007). c-LPs and particularly surfactins are not only directly responsible for biocontrol, they are also

involved in motility and biofilm formation (Bais et al., 2004) and in cell differentiation and cannibalism (López et al., 2009).

Mechanisms of biocontrol

For major reviews about biocontrol and its mechanisms the reader is referred to **Table 1**. An overview of the microbes most used for biocontrol of root diseases, their traits and mechanisms of action is presented in **Table 2**.

1. Antibiosis

Since antibiotic-producing bacteria occur frequently, are easy to isolate, and are interesting for molecular studies on biosynthesis and regulation, they are the best known class of BCAs. The production of antibiotics is very dependent on environmental conditions such as temperature, pH and the levels of various metal ions, particularly of Zn^{2+} (Duffy and Défago, 1999; van Rij et al., 2004). Tripartite interactions and signaling among plants, pathogens, and bacteria is involved in the regulation of antifungal traits of *Pseudomonas* (Jousset et al., 2011) Moreover, the effect of environmental conditions is strain-dependent (van Rij et al., 2004). Therefore, and because efficient colonization is required for antibiosis (Chin-A-Woeng et al., 2000; Dekkers et al., 2000), it is not surprising that some strains which show anti-fungal activity on plates, do not act as biocontrol agents *in vivo*. The identification and quantification of the antibiotics which are produced during biocontrol *in situ* is a challenge and has been shown only for a few cases (Tomashow and Weller, 1996).

The slow growth rate of bacteria in the rhizosphere favors the production of secondary metabolites (Haas and Défago, 2005). It is also very likely that the presence of bacterial biofilms under a mucoid layer (**Fig. 1 g,h**) is favorable for quorum sensing (Chin-A-Woeng et al., 1997), a prerequisite for the production of many antibiotics.

A risk of using an antibiotic-producing BCA in practice is that cross-resistance can occur with antibiotics used in human or animal practice. Another risk is that genes encoding the antibiotic production ability can be transferred to related strains (Zhang et al., 1993). This is a realistic possibility since some forms of conjugative transfer require quorum sensing which requires a high density of microbes. This is the case on the root where pseudomonads form micro colonies under a mucoid layer **(Fig 1g,h)** (Chin-A-Woeng et al., 1997). Indeed, it has been shown by van Elsas et al. (1988) that genetic material is exchanged at a high frequency in the rhizosphere. These facts form

Table 1. Major reviews about biocontrol and its mechanisms

Topic	References
Biocontrol general	Schroth and Hancock, 1982; Compant et al., 2005; Haas and Défago, 2005; Berg, 2009; Lugtenberg and Kamilova, 2009; Mendes et al., 2011
Biocontrol by Bacillus	Raaijmakers et al., 2006; Borriss, 2011; Pérez-Garcia et al., 2011
Biocontrol by Pseudomonas	Haas and Défago, 2005; Raaijmakers et al., 2006; Validov, 2007; Pliego et al., 2011
Biocontrol by Trichoderma	Harman et al., 2004 ; Lorito et al., 2010
Antibiosis	Thomashow and Weller, 1996; Opelt et al., 2007
CNN	Lugtenberg and Kamilova, 2009; Pliego et al., 2011
Ferric iron ion acquisition	Leong, 1986
Induced systemic resistance	Van Loon, 2007; Van Wees et al., 2008
Predation and parasitism	Harman et al., 2004; Lorito et al., 2010
Root colonization	Chin-A-Woeng et al., 1997; Lugtenberg and Dekkers, 1999; Lugtenberg et al., 2001; Bolwerk et al., 2003; De Weert et al., 2007

Table 2. Major microbes used for biocontrol, their traits and mechanisms of action

A. Bacillus	
Traits / mechanisms of action	**References**
Root colonization	Fan et al., 2011
Antibiosis	Romero et al., 2007 ; Ongena and Jacques, 2008 ; Chen et al., 2009 ; Raaijmakers et al., 2010; Borriss, 2011
Induced systemic resistance	Kloepper et al., 2004; Ongena et al., 2007
Signal interference	Dong et al., 2004
B. Trichoderma	
Root colonization	Harman et al., 2004; Harman, 2006
Antibiosis	Lorito et al., 2010
CNN	Lorito et al., 2010
Induced systemic resistance	Lorito et al., 2010
Predation and parasitism	Lorito et al., 2010
C. Pseudomonas and some other Gram-negatives	
Traits / mechanisms of action	**References**
Root colonization	Simons et al., 1996; Lugtenberg et al., 2001; Lagopodi et al., 2002; Berg, 2009; Lugtenberg and Kamilova, 2009
Antibiosis	Thomashow and Weller, 1996; Chin-A-Woeng et al., 1998; Haas and Défago, 2005; Compant et al., 2005; Cazorla et al., 2006; Raaijmakers et al., 2010; Egamberdieva et al., 2011
Predation and parasitism	Ordentlich et al., 1998
Induced systemic resistance	Audenaert et al., 2002; Iavicoli et al., 2003; Shuhegger et al., 2006; Van Wees et al., 2008
Competition for Nutrients and Niches	Kamilova et al., 2005; Pliego et al., 2007; Validov, 2007
Colonization of hyphae	Bolwerk et al., 2003; De Weert et al., 2003
Ferric iron ion acquisition	Kloepper et al., 1980; Leong, 1986

risks for human health and represent reasons why registration of products based on antibiotic-producing microbes is difficult.

2. Signal interference

Several pathogens perform their action by hydrolyzing the cell walls of cells of their target plant. The production of many exo-enzymes is regulated by quorum sensing. One way to control exo-enzymes of pathogens is to inactivate the AHL molecule required for exo-enzyme production. This mechanism has been designated as signal interference (Dong et al., 2004). Two classes of AHL-inactivating enzymes have been identified, namely AHL-lactonases which hydrolyse the lactone ring, and AHL-acylases which break the amide linkage. For a review on these two enzymes, on AHL modifying enzymes, and on abiotic factors influencing the stability of AHLs, the reader is referred to Uroz et al. (2009).

In the pathosystem *Verticillium dahliae*-oilseed rape, the essential role of AHL-mediated signaling for disease suppression, including production of AFMs and VOCs, in *Serratia plymuthica* HRO C48 was demonstrated (Müller et al., 2009). Dandurishvili et al. (2011) reported that VOCs produced by rhizospheric strains *P.fluorescens* B-4117 and *S. plymuthica* IC1270 might be involved in the suppression of crown gall disease in tomato plants caused by *Agrobacterium*. Recently, Chernin et al. (2011) showed that VOCs emitted by cells of these strains, as well as the pure volatile DMDS, can cause significant suppression of transcription of AHL synthase genes *phzI* and *csaI*. Since AHLs play a role in conjugational transfer of *A. tumefaciens* Ti plasmids to the plant (Zhang et al., 1994), which is an essential step in crown gall formation, the volatile DMDS may control crown gall disease through signal interference.

3. Predation and parasitism

Since the cell walls of many fungi contain chitin, ß-1,3 glucan and protein, BCAs which produce exo-enzymes which degrade these compounds, alone or in combination, are often successful in killing the pathogen. This biocontrol mechanism is called P&P. It is used by some strains of *Trichoderma* (Harman et al., 2004) and *Serratia marscescens* (Ordentlich et al., 1998).

4. Induced systemic resistance

ISR is a broad spectrum plant immune response that is activated by some plant-beneficial bacteria that live on plant roots (Kloepper et al., 2004; van Wees et al., 2008;

Pieterse et al., 2009), such as *P. fluorescens* strains WCS417R (van Loon and Bakker, 2003; van Wees et al., 1997) and WCS365 (Kamilova et al., 2005). Immunized plants become potentiated to mobilize infection-induced defense responses faster and stronger after pathogen or insect attack, resulting in an enhanced level of protection. ISR microbes induce resistance systemically, i.e. also in distant plant parts such as leaves (Van Peer et al., 1991; Wei et al., 1991). The outcome of ISR can be a broad range of protection but it is also somewhat unpredictable. ISR can protect the plant against several pathogenic bacteria, fungi and viruses (van Loon et al., 1998; van Loon, 2007). The success of ISR-inducing strains depends on the plant species and cultivar (van Loon and Bakker, 2003; van Wees et al., 1997). The hormones jasmonic acid and ethylene are key regulators of ISR (van Wees et al., 2000). It was suggested that ISR resembles innate immunity and uses Toll like receptors (de Weert et al., 2007).

ISR does not require complete root colonization persé as was shown using competitive colonization mutants (Dekkers et al., 2000). In addition to live microbes, such as *Bacillus*, *Pseudomonas* and *Trichoderma*, ISR can be triggered by dead microbes and even by bacterial molecules and organelles such as siderophores, lipopolysaccharides, flagella, salicylic acid, the combination of pyocyanin and pyochelin (Audenaert et al., 2002), the volatile 2,3-butanediol (Ryu et al., 2003), the signal molecule AHL (Schuhegger et al., 2006), the antibiotic phloroglucinol (Iavicoli et al., 2003) and some c-LPs (Ongena et al., 2007; Pérez-García et al., 2011)

5. Competition for ferric iron ions

All organisms need Fe^{3+} for growth. Under conditions of Fe^{3+}-limitation, many bacteria secrete Fe^{3+}-chelating compounds, called siderophores. The siderophore-Fe^{3+} complex is subsequently bound to Fe^{3+}-limitation-inducible outer membrane protein receptors and the Fe^{3+} ion is transported into the bacterial cell, in which it becomes biologically active as Fe^{2+}. An example of a siderophore is pyoverdin or pseudobactin, the pigment responsible for the fluorescence of fluorescent pseudomonads. Fe^{3+} is poorly soluble under aerobic conditions at neutral and alkaline pH. Some bacteria produce siderophores which are sufficiently strong to bind Fe^{3+} to the extent that fungi in their neighbourhood cannot grow anymore under iron limitation and siderophore-producing bacteria can then act as biocontrol agents (Leong, 1986), as examplified by the control of *Erwinia carotovora* by *P. fluorescens* strains (Kloepper et al., 1980).

6. Competition for nutrients and niches

Kamilova et al. (2005) showed that CNN is a mechanism for biocontrol. They selected enhanced root tip colonizers from a crude mixture of rhizosphere bacteria. Approximately half of the selected enhanced colonizers appeared to be able to control TFRR caused by *Forl*. They showed that such strains out compete other microbes in competition for exudate nutrients and in competition for niches on the root (Kamilova et al., 2005; Validov et al., 2007). The observation that not all enhanced colonizers are BCAs can be explained by a discovery of Pliego et al. (2008) who found that two very similar *Pseudomonas* strains, selected for their efficient colonizing abilities, colonized different micro-niches on the root. This difference was used as an explanation why one strain is able to control the disease avocado white root rot whereas the other strain could not.

Bacteria controlling disease using CNN as a mechanism have several advantages. (i). CNN is the only mechanism for which strains can be selected. So, such strains can be isolated from a soil, a plant and a climate of preference. (ii). Most CNN strains do not produce antibiotics which is an advantage for registration since regulatory authorities do not like the introduction of antibiotic-producing strains in the environment. (iii). In case antibiotic production is considered to be an advantage, strains can be selected which use a combination of CNN and antibiosis as mechanisms (Pliego et al., 2007). (iv). Resistance against BCAs using CNN as their biocontrol mechanism is hard to imagine. The same applies for biocontrol strains which use both CNN and antibiosis as mechanisms since pathogens resistant to one mechanism can be controlled by the other mechanism.

7. Interference with activity, survival, multiplication, germination, sporulation and spreading of the pathogen

Studies with biocontrol strain *P. fluorescens* WCS365 have shown that this strain shows a series of activities which contribute to control of TFRR. (i). Cells of the strain are attracted to FA secreted by the hyphae. Subsequently they colonize the hyphal surface of the pathogen extensively, resulting in the formation of micro colonies or biofilms **(Fig. 1i)** (de Weert et al. 2003). This is probably the first step in an attempt to use the fungus as a food source. It is likely that colonization of hyphae makes the fungus less virulent, inhibits its activity and is detrimental for its survival and multiplication**.** (ii). Microconidia of *Forl* germinate in tomato root exudate. Germination is inhibited by biocontrol strain *P. fluorescens* WCS365, presumably because of nutrient deprivation.

(iii). When hyphae are grown in tomato root exudate, microconidia are formed. These are spores that can spread the pathogen through the environment. The presence of WCS365 reduces spore formation and therefore reduces pathogen spread (Kamilova et al., 2008). In conclusion, *P. fluorescens* WCS365 bacteria inhibit activity, survival, multiplication, germination, sporulation and spreading of the pathogen. We have not studied other bacteria or BCAs on these traits, which therefore may not be unique for *P. fluorescens* WCS365.

Resistance towards biocontrol microbes

Several mechanisms of resistance towards BCAs have been discovered in fungi (Duffy et al., 2003) which resemble resistance mechanisms used by bacteria against antibiotics. (i). Inhibition of antibiotic production. The secondary metabolite FA, secreted by many Fusarium strains (Notz et al., 2002), previously shown to be a chemoattractant for biocontrol strain *P. fluorescens* WCS365 (De Weert et al., 2003), inhibits the synthesis of Phl in the biocontrol bacterium *P. fluorescens* CHA0 by repression of the *phlA* promoter (Duffy and Défago, 1997). FA also inhibits the synthesis of another antibiotic, PCN, in another biocontrol bacterium, namely *P. chlororaphis* PCL1391. In this case a different inhibition mechanism is used, namely at or before the level of AHL production (van Rij et al., 2005). Note that AHL is required for the synthesis of PCN but not for that of Phl. (ii). Detoxification of the antibiotic. Between 18 and 25 percent of the isolated *Fusarium* strains were tolerant to Phl. Deacetylation of the antibiotic to the mono-acetyl form is the major mechanism of action (Schouten et al., 2004). Another form of detoxification is acetylation, which is used by biocontrol strain *Bacillus subtilis* strain UW85, the producer of the antibiotic zwittermycin A (Milner et al., 1996). (iii). The presence of phenazine induces an efflux pump for this compound in *Botrytis cinerea.* Mutants lacking the pump are more sensitive to the antibiotic (Schoonbeek et al., 2002).

In order to avoid resistance in biocontrol, it is preferable to use a BCA which uses more than one mechanism. Alternatively, a combination of BCAs with different mechanisms of action can be used. If a pathogen is resistant to one mechanism it can still be inactivated by a second one. Suitable microbes would for example be *Trichoderma* spp. (Lorito et al., 2010), which use at least mechanisms a, c and d (see section Mechanisms of biocontrol), and *P. fluorescens* WCS365, which uses at least mechanisms d, e, f and g, and some bacilli, which use mechanisms a and d. A summary of processes in which signal molecules and nutrients play a role in the rhizosphere

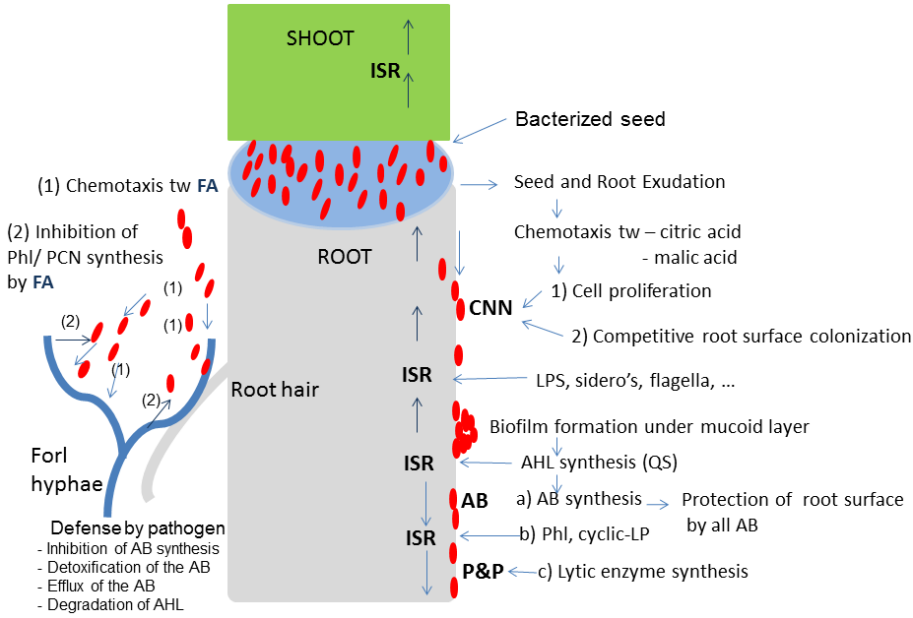

Fig. 2. Nutrients and molecules involved in biocontrol of TFRR. Cells of a hypothetical biocontrol bacterium applied on the seed proliferate on nutrients from seed exudate. Subsequently they are attracted to the root by citric acid and malic acid from root exudate and successfully compete for root exudate nutrients and niches in case the mechanism is CNN. Specific cell surface components and secondary metabolites of the BCA can induce the mechanism ISR. Upon formation of biofilms, the resulting quorum results in AHL synthesis. AHL in turn leads to synthesis of antibiotics, some of which also cause ISR, and of exo-enzymes which are required for the mechanism P&P. When the pathogen *Forl* arrives close to the root, cells of a BCA can be chemo-attracted to FA secreted by the hyphae, and subsequently colonize the hyphae. Whether syntheses of the AFFs phenazine and Phl are inhibited by FA or whether the cells of the BCA damage or kill the hyphae will depend on timing and concentrations of the metabolites and organisms and on whether the fungus is resistant and, if so, by which mechanism. Additional abbreviations: AB, antibiotic; LP, lipopeptide.

during biocontrol is shown in **Fig. 2**.

Conclusions

Phytopathogenic fungi and oomycetes cause enormous crop losses. Presently, chemical agents are the major way of disease control but they have the disadvantages that i) many of them are detrimental for health and environment, and ii) that resistance occurs rather fast. In this review, we discuss that products which contain natural microbial enemies of these pathogens are a realistic alternative and addition to chemical pesticides. The quality of these BCAs can be further increased by using fundamental knowledge to improve methods for their production and to increase their shelf life. In addition, the fast development of very advanced techniques in microbial

ecology and a focus on mechanisms of actions make improvement of strain selection feasible.

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