

# Chapter 3

## Life of Microbes in the Rhizosphere

**Ben Lugtenberg**

**Abstract** Life of microbes in the rhizosphere is best characterized as starvation for nutrients and attempts to survive. All microbes are hunting for food of which a substantial amount is supplied by the root in the form of exudate. The most successful microbes are attracted to food sources, such as to the root and to each other, by chemotaxis to specific exuded compounds. Subsequently they colonize the target organism. Specific exudate compounds can also initiate communication between organisms as the start of more specialized interactions, such as nodulation, pathogenesis, DNA transfer and the production of antibiotics. Some target organisms have developed defense reactions against such attacks which allows them to survive. All these processes are described in this chapter.

### 3.1 The Rhizosphere

The rhizosphere was defined by Lorentz Hiltner as “the soil compartment influenced by the roots of growing plants”. The rhizosphere is supposed to be no more than a few mm thick. It is 10- to 100-fold richer in microbes than the surrounding “bulk” soil because 6–21 % of the carbon fixed by the plant is secreted by the root. This phenomenon is called the rhizosphere effect. It is good to realize that the concentration of nutrients in the rhizosphere is still 100-fold lower than that in the usual laboratory media. The life style of microbes in the rhizosphere is therefore best characterized as starvation. Recent reviews on the rhizosphere are those of Haas and Défago (2005), Lugtenberg and Bloemberg (2004), Lugtenberg and Kamilova (2009), and Pinton et al. (2007).

Roots of many plants are colonized by mycorrhizal fungi which can function as fine extensions of the root and allow the plant to reach nutrients which cannot be reached by the thicker roots. The combination of root surface and attached mycorrhizal fungi is designated as the mycorrhizosphere (Chap. 25).

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Plant life is affected by both abiotic and biotic conditions. For the estimation of local conditions, bioreporters have been developed. These are bacterial derivatives harboring a promoter that reacts on the compound or condition of choice, and that is fused to a reporter gene encoding a protein which can easily be detected and quantified (for example *gfp*, *lux*, *lacZ* or *inaZ*). Reporter constructs respond for example to the presence of certain sugars, amino acids, or to conditions such as pH or the bioavailability of carbon, phosphate and oxygen (Chap. 10 in ref. Pinton et al. 2007).

**Abiotic Conditions** Abiotic conditions affecting plant growth include temperature, pH, soil type, water potential, and concentrations of bioavailable essential nutrients and salts. Soils can be rich or poor for plant growth. Rich soils contain sufficient water and nutrients. Drought is a major and increasing problem for plant growth (Chap. 28), and so is salination (Chap. 28). Nitrogen and phosphorous are the major nutrients whereas ions of potassium, iron and micronutrients are also required for plant life. Poor soils can be fertilized chemically, for example by adding N-P-K fertilizer. This can increase plant growth enormously but is not sustainable. Therefore the trend is to replace such chemicals by other means, for example by bacteria which generate nutrients in forms that can be used by the plant (Chap. 23 and 24). A neutral or high pH makes ferric iron ions insoluble and will therefore reduce plant growth. In contrast, at acid pH these ions will be soluble.

**Biotic Conditions** The rhizosphere contains microbes (bacteria and fungi) as well as small animals, such as amoebae, insect larvae, mites, nematodes and protozoa (Bonkowski et al. 2009). The microbes are collectively called the rhizosphere microbiome (Mendes et al. 2013) (Chap. 30 and 43). The main factors shaping the rhizosphere microbiome are the soil type and the plant genotype. The microbes can have a beneficial, neutral, or negative effect on plant growth. Some of the beneficial microbes have been cultured and formulated and are being sold as commercial products (Chaps. 32–34) and are applied as biopesticides or plant protection products (Chap. 18), biofertilizers (Chaps. 23 and 24), rhizoremediators (Chap. 29), phyto-stimulators (Chaps. 25 and 26), or stress controllers (Chap. 27). Beneficial microbes include strains of the bacteria *Bacillus* and *Pseudomonas*, of Arbuscular Mycorrhizal Fungi (AMF) (Chap. 25), and of the fungus *Trichoderma* (Chap. 36). Plant pathogens can be viruses (Chap. 13), bacteria (Chap. 9), fungi and oomycetes (Chap. 10), or nematodes (Chap. 11). Some insects cause pests (Chap. 12).

A healthy soil hardly contains pathogens. Soils harboring pathogens are called disease-conducive soils. However, in some cases, soils harbor pathogens but plants growing in this soil remain healthy. These disease-suppressive soils contain beneficial microbes which suppress the action of the pathogens (Chap. 38). Some soils contain human pathogens (Berg et al. 2005) which can be risky for agricultural workers as well as for consumers. It has been suggested that treatment of seeds of crop plants with products containing enhanced colonizing bacteria will reduce the number of pathogens on the root and, therefore, reduce this risk for humans (Egamberdieva et al. 2008).

**Nutrition of Rhizosphere Microbes** Between 6 and 21 % of the carbon fixed by the plant can be released by the plant root. This material consists of sloughed-off cells, macromolecules and small molecules. The latter are the favorite food sources of the rhizosphere microbes. Based on mutational studies it was concluded that the most important food sources exuded by tomato roots are organic acids, such as citric, malic, lactic, succinic, oxalic, and pyruvic acids. Other root exudate compounds include sugars (such as glucose, xylose, fructose, maltose, sucrose and ribose), amino acids, fatty acids, nucleotides, putrescine, and vitamins. A special group of exudate compounds are the signal molecules which are used for communication between the plant and microbes (see Sect. 3.4).

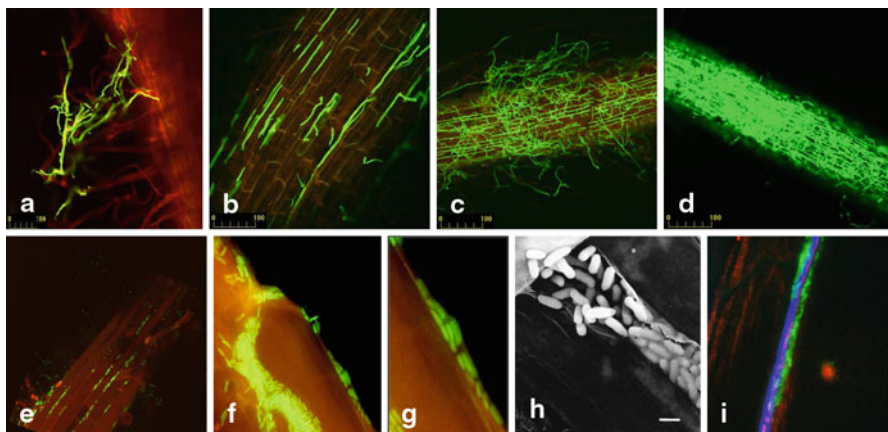
The qualitative and quantitative composition of root exudate is heavily influenced by the presence of microbes in the rhizosphere in a microbe species-dependant way. For example, the addition of the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* to a mono-axenic tomato system strongly decreased the amount of citric acid and increased the amount of succinic acid but did not influence the total amount of organic acids in exudate. In contrast, when the *Pseudomonas* biocontrol strain WCS365 was added in amounts sufficient for biocontrol, the total amount of organic acids—especially of citric acid—strongly increased whereas the level of succinic acid decreased dramatically (Kamilova et al. 2006).

## 3.2 Interactions Between Organisms in the Rhizosphere

Using sophisticated techniques (Chap. 31), interactions occurring between organisms in the rhizosphere can be visualized.

**Fungus—Plant Interactions** A fungal spore in the soil will germinate when it senses the presence of a plant root through certain chemicals secreted by the root (Lugtenberg and Kamilova 2009). Subsequently, the formed mycelium will grow into the direction of the root, attach to the root, colonize the root surface, and penetrate the root. This process is shown for the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* in Fig. 3.1a–3.1d.

**Bacterium—Plant Interactions** During treatment of seeds with microbes, use is made of the fact that microbes which are present on the seed are in the best position to colonize the roots of the emerging seedling. Therefore, the best way to colonize the root with a beneficial bacterium is to surface-sterilize the seed and coat it subsequently with cells of the beneficial bacterium of choice. After growth, the result is that the top of the seedling root is best colonized (with approx.  $10^6$  bacteria per cm root). A good colonizing bacterium can even reach the growing root tip. Coming from the seed, a bacterium colonizes the root, initially as individual cells (Fig. 3.1e) which subsequently multiply and grow out to micro-colonies (Fig. 3.1f), presently designated as biofilms (Chap. 7). Only approximately 15 % of the plant root surface can be covered by bacteria (Fig. 3.1f). Most bacteria are found on the junctions between epidermal cells (Fig. 3.1e). Mature biofilms usually consist of multiple layers of cells (Fig. 3.1f) and are covered with a mucous layer (Fig. 3.1g,h).



**Fig. 3.1** Visualization of plant-microbe and microbe-microbe interactions during biocontrol. Confocal Laser Scanning Microscopy (**a–g** and **i**) and scanning electron microscopy (**h**) were used to visualize control of tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *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. Reproduced from Lugtenberg and Girard (2013) by permission of the publisher

**Bacterium—Fungus Interactions** Some bacteria attach to fungal hyphae in order to use them as a food source (Fig. 3.1i).

### 3.3 Competitive Root Tip Colonization

In order to be successful in their beneficial action, cells of applied microbes have to reach the root in high numbers. Therefore, they have to win the competition with indigenous microbes for nutrients secreted by the root and for niches on the root. In order to identify bacterial traits important for competitive root colonization, Simons et al. (1996) screened individual random mutants of the efficient *P. fluorescens* root colonizing strain WCS365 in a mono-axenic sand system. Surface-sterilized tomato seeds were coated with a 1:1 mixture of one of the mutants and the wild type strain. After the root system had developed, the root tip was isolated and the ratio of wild type to mutant cells was determined. Competitive colonization mutants lose this competition and were found in lower numbers than the wild type. The mutated genes were characterized and the traits lost in the mutants were identified. It turned out that the number of traits involved in competitive root colonization is high. The best understandable traits can be grouped as follows. An excellent root tip colonizer should (i) be able to synthesize amino acids, vitamin B1, uracil, as well as the O-antigenic side chain of lipopolysaccharide; (ii) grow fast in root exudate, show a

chemotactic response towards the root, and adhere to the root; (iii) have a number of properties related to secretion (see Chap. 6), such as the *secB* gene involved in a protein secretion pathway, the type three secretion system, and an intact ColR/ColS two-component system. The latter property is supposed to be related to keeping protein pores in the outer membrane open. The most likely explanation for the need of the type three secretion system is that the needle of this system is required for tapping nutrients from the plant cell. For better understanding of these traits, see Chap. 6.

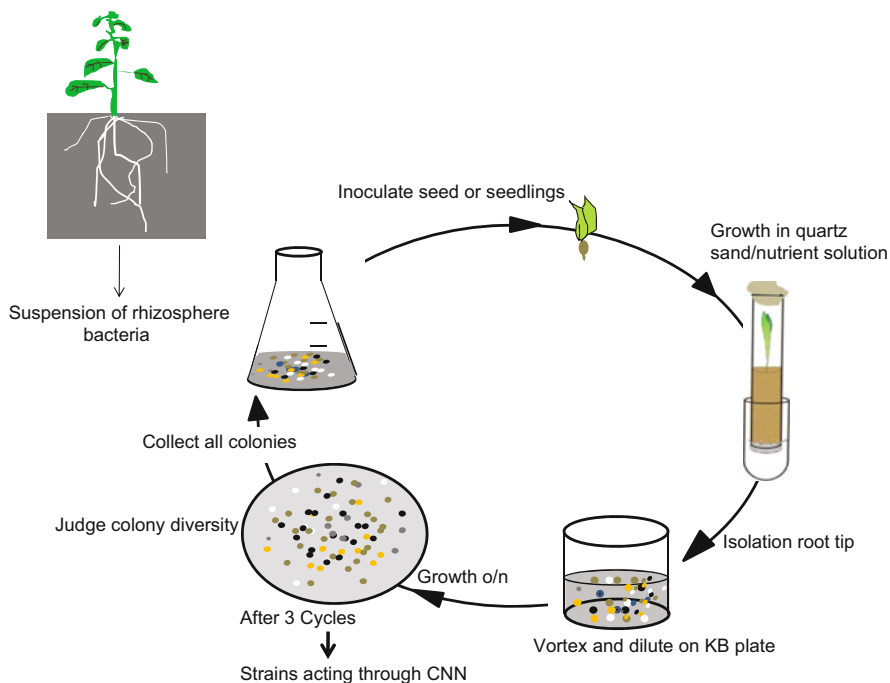
Good tomato root colonizers have also been tested on other crop plants. In contrast to general predictions, pseudomonads colonize roots of many other plants—such as cucumber and wheat—well, so there is hardly any host plant specificity involved in colonization. Moreover, mutants which are not colonizing tomato roots well, are also impaired in the colonization of wheat and cucumber roots, indicating that bacterial colonization traits are common for several plants.

Interestingly, it is possible to increase the colonization ability of bacteria or to select strongly enhanced root tip colonizers. (i) When cells of a transposon mutant library of *P. fluorescens* strain WCS365 were put on a seedling and selected for enhanced root tip colonizers, the best colonizing mutant was characterized as a strain carrying a mutation in the gene encoding MutY, an enzyme which repairs mutations in the DNA. We assume that this enhanced colonizing *mutY* derivative has collected a combination of mutations which have fine-tuned the strain in such a way that it is better adapted to the conditions on the root (De Weert et al. 2004a). It would be interesting to compare the nucleotide sequences of these strains in order to evaluate which traits contribute to enhanced colonization. (ii) Using the same monoaxenic system and a mixture of numerous rhizosphere strains, it appeared possible to select strains which are enhanced colonizers (Fig. 3.2). Half of these strains are even able to control the disease tomato foot and root rot by a mechanism designated as Competition for Nutrients and Niches (CNN) (Kamilova et al. 2005; Fig. 3.2; Chap. 18).

### 3.4 Communication Between Organisms by Chemical Signaling

Many compounds secreted by plant roots and microbes function as signals that play a role in the communication between plant and microbe or between microbes. A selection of examples is given below.

**Chemoattraction** Bacteria in the rhizosphere usually live under conditions of starvation for nutrients and, consequently, are hunting for food. They have developed sensing systems which guide them towards compounds secreted by plant roots and by fungal hyphae. *Pseudomonas* cells from soil find the tomato root because they are attracted by root exudate. In order to identify the exudate component which is most active in the tomato rhizosphere, the chemo attractant activity of the individual representatives of the major groups of exudate components of tomato, namely organic acids, sugars, and amino acids, was analyzed. Experiments showed that



**Fig. 3.2** Enrichment of bacteria which compete efficiently for nutrients and niches. Starting from a seed on which a crude mixture of rhizosphere bacteria is applied, enhanced competing bacteria are enriched for by repeatedly selecting for those cells which—after application to a sterile seed—reach the root tip first. Reproduced from Pliego et al. (2011) by permission of the publisher), after modification by Clara Pliego

sugars are inactive, that dicarboxylic acids are active, and that amino acids (especially L-leucine) are the most active chemoattractants. However, when this data is corrected for the levels of the individual components estimated to be present in the tomato rhizosphere, it was concluded that malic acid and citric acid are the major chemoattractants for *Pseudomonas* cells in the rhizosphere (De Weert et al. 2002). After reaching the root surface, the bacteria colonize it (Fig. 3.1e,f).

After reaching the fungal hyphae, the bacteria colonize the surface (Fig. 3.1i). Bacterial cells also try to find fungal hyphae because they can be used as food sources. They have developed a system to detect specific fungal products. This process has been extensively studied in the case of *Pseudomonas* cells trying to find hyphae of the fungus *Fusarium oxysporum* fsp. *radicis-lycopersici*. Using a series of *Fusarium oxysporum* f. sp. *radicis-lycopersici* strains which differ in the levels of secreted fusaric acid, it was found that the more fusaric acid is secreted, the stronger bacteria are attracted to the fungus. Finally, using synthesized chemically pure fusaric acid, the notion that fusaric acid is the major chemo attractant for *Pseudomonas* cells, was confirmed (De Weert et al. 2004b).

**Initiation of the Nodulation Process** Certain flavonoids secreted by roots of leguminous plants signal to (*Brady*)*rhizobium* bacteria that a root of their leguminous host plant is close by and that the process of nodule formation can be initiated. These flavonoids induce the nodulation (*nod*) genes in the bacterium which encode the enzymes for the synthesis of Nod-metabolites or lipochitin oligosaccharides (LCOs). These LCOs initiate the formation of nodules in which bacteria, in the form of bacteroides, fix atmospheric nitrogen for the plant.

**Induction of *vir* gene Expression in *Agrobacterium*** Phenolic compounds released from plant wound sites, such as 3',5' dimethoxy-4'-hydroxy acetophenone (commonly known as acetosyringone) and  $\alpha$ -hydroxy acetosyringone, are the key inducers of bacterial virulence genes. A pH of around 5.5 and a temperature below 30 °C are also required. Particular sugars enhance the level of induction (Chap. 37).

**N-Acyl Homoserine Lactones (AHLs) and Quorum Sensing** (Chap. 7) AHLs are signal molecules secreted by many Gram-negative bacteria. They are used to recognize bacteria of their own kind. These molecules are supposed to diffuse more or less freely over the membranes. If the bacterial cell concentration reaches a certain density, the quorum, the intracellular AHL concentration reaches a level sufficiently high to initiate the synthesis of increased amounts of AHL, which in turn initiates the production of several proteins, including those involved in the synthesis of many antibiotics (Chap. 18) as well as virulence factors. AHLs also play a role in exchanging DNA by inducing F-pilus-mediated conjugation .

**AMF-Plant Interaction** Flavonoids present in root exudates can initiate interactions of plant roots with AMF such as stimulation of spore germination and hyphal branching. More recently, it was reported that secreted representatives of a group of plant hormones, the strigolactones, can also cause branching of neighboring AMF spores, thereby increasing their chance to encounter a plant root. In addition, the AMF releases signal molecules, identified as lipochito-oligosaccharides or Myc factors which stimulate root growth and branching (Chap. 25).

**Volatiles** Both plants and microbes can produce a range of volatile organic compounds, briefly designated as volatiles. Whereas volatile plant hormones such as ethylene, methyl jasmonate, and methyl salicylate function as airborne signals in mediating plant communication, several bacterial volatiles play a role in biocontrol (HCN) or induce systemic resistance in plants (such as 2,3-butanediol and its precursor acetoin) (Chap. 8).

**War in the Rhizosphere** Microbes in the rhizosphere can be attacked by their colleagues but they are not always simple victims. Like bacteria, some fungi have developed resistance mechanisms against antibiotics. The following mechanisms of fungal and bacterial tolerance or resistance have been shown. (i) Detoxification of the antibiotic. Some biocontrol strains produce the antibiotic 2,4-diacetyl phloroglucinol, for example to kill *Fusarium* strains. Some *Fusarium* strains produce an enzyme that deacetylates the antibiotic to mono-acetyl phloroglucinol which is a lot less fungitoxic. (ii) Efflux of the antibiotic. Upon exposure to phenazine antibiotics, some

strains of the fungus *Botrytis cinerea* induces an efflux pump for the antibiotic. (iii) Repression of the synthesis of an antibiotic. The biocontrol fungus *Trichoderma atroviride* P1 produces chitinase enzymes to attack the cell wall of fungi (Chap. 6). The *Fusarium* mycotoxin deoxynivalenol inhibits the expression of the chitinase genes *ech42* and *nag1* which contribute to the biocontrol activity. Another example of repression of antibiotic synthesis is the *Fusarium* metabolite fusaric acid which inhibits the syntheses of the antibiotics 2,4-diacetyl phloroglucinol and phenazine-1-carboxamide produced by some *Pseudomonas* biocontrol strains. Finally, AHLs are often required for the synthesis of antibiotics and virulence factors. Some bacteria protect themselves by enzymatic inactivation of AHL.

**Gene Transfer in the Rhizosphere** A well known form of gene transfer between bacteria, namely F-pilus mediated conjugation, requires AHL. In biofilms, bacteria are very close to each other and covered by a mucous layer (Fig. 3.1f–h). These conditions seem ideal for keeping the intracellular AHL concentration high and therefore for stimulating conjugation. Indeed, it has been reported that gene transfer between bacteria in the rhizosphere is very efficient (van Elsas et al. 1988).

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