12 Signalling in Rhizobacteria–Plant Interactions

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12.1 Introduction

Bacteria are by far the most abundant organisms in soil and they play a key role in nutrient cycling and soil fertility. The rhizosphere – the zone of 1–2 mm around plant roots – is rich in nutrients and provides niches different from those in bulk soil for bacteria to thrive. Microbial diversity in the soil and in the rhizosphere is huge. Multiple interactions occur between the bacteria and between bacteria and other microorganisms, involving competition, antibiosis, parasitism and predation. Various interactions also occur between bacteria and plant roots that can be beneficial, neutral or harmful to the plant. Deleterious effects comprise phytotoxic and pathogenic activities of the bacteria. Conversely, plants profit from bacterially induced growth promotion and protection against pathogens. Growth promotion can be the result of bacterial activities that increase the availability of water and mineral nutrients, as well as of symbiotic relationships such as the formation of root nodules in leguminous plants, in which atmospheric nitrogen is made available in reduced form. Nodulation of roots involves an intricate interplay of molecular signals between the bacterium and its host, and illustrates how such plant–rhizobacteria interactions proceed in an exquisitely controlled manner. In similar ways, suppression of disease-provoking microorganisms can occur through microbial antagonism in the rhizosphere as well as by specific interactions between the protective bacterium and its host. While antagonism involves mostly mechanisms that rhizobacteria likewise use to compete with other microorganisms in the root environment, interactions with plant roots may trigger an induced systemic resistance that enhances the defensive capacity of the plant to subsequent pathogen attack. In this way, the plant becomes better protected not only against soil-borne pathogenic fungi, but also to necrotrophic foliar pathogens. The chapter provides an overview of these beneficial relationships between rhizobacteria and their plant hosts with emphasis on the communicative signals that are involved in regulating the activities of both partners that lead to plant growth promotion and disease suppression.

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12.2 Plant Growth Promotion by Rhizobacteria

Substantially more microorganisms are present near plant root surfaces than in bulk soil. This "rhizosphere effect" is caused by the release of exudates from growing root tissues and the lysis of cells of older root parts (Lynch and Whipps 1991). Bacteria rapidly colonize growing root tips, using simple sugars, organic acids and amino acids as nutrients, whereas saprophytic fungi are more prevalent on older root parts, where cortical cells are being degraded. Numerous strains of bacteria can be isolated from plant roots with, in most cases, little specificity being apparent. However, release of selected nutrients from roots that are preferentially utilizable by specific bacterial strains favors selective colonization by the latter (Bowen 1991; Flores et al. 1999).

Root-colonizing bacteria are commonly referred to as "rhizobacteria". Most rhizobacteria remain confined to the root surface (rhizoplan), but some enter the root interior and behave as endophytes (Sturz et al. 2000). Several rhizobacterial strains have been found to increase plant growth after inoculation on to seeds and are therefore called "plant growth-promoting rhizobacteria" (PGPR*; Kloepper et al. 1980b). Such PGPR improved plant stand and increased yield, e.g. of potato (*Solanum tuberosum*), radish (*Raphanus sativus*), and sugar beet (*Beta vulgaris*; Kloepper et al. 1991), suggesting that plants benefit from rhizobacteria that live on the nutrients lost from the roots. The mechanisms of growth promotion by these PGPR are complex and appear to comprise both changes in the microbial balance in the rhizosphere and alterations in host plant physiology (Glick et al. 1999). By competition and production of antimicrobial compounds, PGPR can reduce populations of plant pathogens and deleterious rhizobacteria, which restrict plant growth. Some of these disease-suppressing activities, such as production of HCN, can reduce plant growth as well, but more often the net effect is improved plant development, resulting in more vigorous growth and increased yield of agricultural crops (Dowling and O'Gara 1994).

Growth promotion through direct stimulation of plant development is more difficult to demonstrate. In radish several rhizobacterial strains strongly increased average plant weight under non-sterile conditions, but failed to do so in a gnotobiotic system in which bacteria were introduced into sterilized soil (Kloepper and Schroth 1981). However, *Pseudomonas flu-*

* Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid; DAPG: 2,4-diacetylphloroglucinol; ENOD: early nodulin; EPS: extracellular polysaccharide; For: Fusarium oxysporum f. sp. raphani; IAA: indole-3-acetic acid; ISR: induced systemic resistance; JA: jasmonic acid; LPS: lipopolysaccharide; MeJA: methyl jasmonate; NPR1: non-expressor of pathogenesis-related proteins 1; OA: O-antigenic side chain; PCA: phenazine-1-carboxylic acid; PGPR: plant growth-promoting rhizobacteria; PRs: pathogenesis-related proteins; Pst: Pseudomonas syringae pv. tomato; SA: salicylic acid; SAR: systemic acquired resistance; VA: vesicular-arbuscular.

orescens strain WCS374 did increase radish leaf dry weight, but not tuber yield, in gnotobiotic culture, whereas, in non-sterile soil, the effect on leaf weight was non-significant and tuber fresh weight was increased (Table 12.1). During in vitro propagation of statice (*Limonium sinuatum*) the presence of an endophytic *Flavobacterium* sp. promoted growth and rooting (Van Zaayen et al. 1992). Such beneficial effects of microbial inoculants in in vitro cultures of plant tissue explants have been noted also in other species. For instance, in potato, bacterization increased stem length, shoot biomass and root biomass (Bensalim et al. 1998). In vitro culture of tomato (*Lycopersicon esculentum*) seedlings with the PGPR *Pseudomonas* sp. strain PsJN promoted shoot dry weight and increased resistance of transplants to verticillium wilt (Pillay and Nowak 1997; Sharma and Nowak 1998). Prevention of excessive moisture content and water soaking in oregano (*Origanum vulgare*) shoot cultures was sustained through multiple subcultures by selected polysaccharide-producing soil bacteria without re-inoculation (Ueno and Shetty 1998). These in vitro responses caused by the inoculants are referred to as "biotization" (Nowak 1998), and demonstrate that rhizobacteria can directly influence plant growth as well as enhance their tolerance to abiotic and biotic stresses.

The ways in which PGPR directly promote plant growth are not known with any certainty. When plants are grown in culture solution under gnotobiotic conditions, bacteria influence the uptake of ions. Under some conditions ion uptake by plant roots can be stimulated in the presence of bacteria, probably through the PGPR providing chelating agents or compounds promoting active ion transport. However, under other conditions, bacteria can be inhibitory, either by competing for nutrients or by producing phytotoxic compounds (Lynch 1982).

Most microorganisms produce siderophores when iron availability in the environment is low. These are low-molecular-weight metabolites with a high affinity for Fe^{3+} (Höfte 1993). They chelate Fe^{3+} from the environment and transport the iron into the microbial cells after being recognized by a specific siderophore receptor protein (Neilands 1981; De Weger et al. 1986; Leong 1986). Low availability of iron in soil for microorganisms is mainly due to the low solubility of ferric oxyhydroxy polymers. In well-oxidized soils the solubility of iron is largely controlled by $Fe(OH)_3$ (Lindsay and Schwab 1982). The solubility constant of this compound is extremely low ($K_{sol}=10^{-38}$), resulting in a concentration of 1.4×10^{-9} M Fe^{3+} at pH 7 or even lower in the presence of phosphate, whereas a concentration of 10^{-6} M is needed to support microbial growth (Neilands et al. 1987; Chipperfield and Ratledge 2000). Thus, the production of siderophores by microorganisms in slightly acidic, neutral and alkaline soils has to be considered a common phenomenon. The presence of microbially produced siderophores has indeed been demonstrated in a variety of soils (Powell et al. 1980; Akers 1981). Recently, the use of a reporter gene system has allowed monitoring of iron availability for microorganisms in the

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Table 12.1. Effect of treatment with a rhizobacterial strain on growth of radish under gnotobiotic and non-sterile conditions. (After M. Leeman, P.A.H.M. Bakker and B. Schippers, unpubl.)

Treatment (gnotobiotic)	Leaf dry weight (mg)	Tuber dry weight (mg)
Control	4.2 a	2.2 a
Pseudomonas fluorescens WCS374	5.7 b	2.3 a
Treatment (non-sterile)	**Leaf fresh weight (g)**	**Tuber fresh weight (g)**
Control	10.8 a	8.0 a
Pseudomonas fluorescens WCS374	12.1 a	10.2 b

rhizosphere, identifying situations that are conducive to the production of siderophores in this environment (Loper and Lindow 1994; Loper and Henkels 1997; Duijff et al. 1999).

The influence of microbial siderophores on plant iron nutrition depends on the ferric-chelating properties of the siderophores, as well as on the iron acquisition mechanism of the plant. Becker et al. (1985a) demonstrated negative effects of 10 μM of the pyoverdin siderophore of *Pseudomonas* sp. strain B10 on iron nutrition in pea (*Pisum sativum*). In contrast, the catechol siderophore of *Agrobacterium tumefaciens* stimulated chlorophyll synthesis in pea (Becker et al. 1985b). Pseudobactin 358, the pyoverdin siderophore of *Pseudomonas putida* strain WCS358, increased iron uptake and stimulated chlorophyll synthesis in barley (*Hordeum vulgare*; Duijff et al. 1994b), but had differential effects in the carnation (*Dianthus caryophyllus*) cultivars Lena and Pallas (Duijff et al. 1994a). The latter difference was attributed to iron-deficient plants of cv. Lena producing more and longer root hairs than iron-deficient plants of cv. Pallas, and the ferric-reducing activity of cv. Lena being higher than that of cv. Pallas.

Some bacteria solubilize organic phosphate by secreting phosphatase or inorganic phosphate from soil particles by releasing organic acids, and this could make phosphorus as well as micronutrients more readily available for plant growth in some soils (Kloepper et al. 1989). Free-living nitrogen-fixing bacteria, particularly of the genera *Azobacterium*, *Azospirillum* and *Clostridium*, are present in most soils and in plant rhizospheres. Also, some *Pseudomonas* spp. have the ability to fix nitrogen. However, it has been suggested that the contribution of bacterially fixed nitrogen to plants is minimal and that enhanced growth by an inoculated plant does not necessarily mean that the bacteria associated with the roots do fix nitrogen or pass the products of nitrogen fixation to the plant (James and Olivares 1997). Inoculation of young wheat (*Triticum aestivum*) plants with *Serratia rubidea* increased efflux of carbon compounds from roots and promoted nitrogen uptake and dry matter yield (Merbach and Ruppel 1992). However, it is not clear whether

this was due to a direct effect on nitrogen uptake or the result of other physiological changes in the plant caused by root bacterization.

By contrast, there is evidence linking nitrogen-reducing endophytes to biological nitrogen fixation in rice (*Oryza sativa*), sugar cane (*Saccharum officinale*) and sorghum (*Sorghum bicolor*; Reinhold-Hurek and Hurek 1998). Moreover, plant growth can be increased by dual inoculation with *Azospirillum* and phosphate-solubilizing bacteria. Combined inoculation of *A. brasilense* and the phosphate-solubilizing bacteria *Pseudomonas striata* or *Bacillus polymixa* significantly increased nitrogen and phosphorus content as well as grain yield of sorghum (Alagawadi and Gaur 1992). Similar increases in plant growth have been reported as a result of co-inoculation of diazotrophic PGPR with vesicular arbuscular (VA) mycorrhizas (Toro et al. 1998). Interactions between mycorrhizal fungi and rhizosphere bacteria relating to plant growth promotion are discussed more fully by Smith et al. elsewhere in this volume (Chap. 11).

Many bacteria have the ability to produce auxins, gibberellins, cytokinins and ethylene (Frankenberger and Arshad 1995). It has often been inferred that rhizobacterially produced auxins are responsible for growth promotion. However, compared with stem elongation, root growth is only slightly stimulated by auxin, and generally only at concentrations of 10^{-9} to 10^{-11} M, higher concentrations being strongly inhibitory (Thimann 1937). Indoleacetic acid (IAA) promotes ethylene production by stimulating the rate-limiting enzyme in the ethylene biosynthetic pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (Kende 1993), and the ethylene thus formed inhibits root elongation. If the auxin concentration reached in the root after uptake of bacterially produced IAA does not fall within the limits indicated above, no root growth promotion can be expected. Auxin is transported basipetally towards the root tip, but some might enter the phloem and be transported to the shoot. However, concentrations required for shoot growth are unlikely to be reached. On the other hand, auxin at 10^{-4} to 10^{-6} M promotes lateral root formation, and it cannot be excluded that locally bacterial microcolonies can produce auxin in amounts that would stimulate this process and, thereby, contribute to enhanced uptake of water and nutrients. For example, mutant strains of the PGPR *Azospirillum brasilense* that synthesized very low amounts of IAA compared with the wild-type strain no longer promoted the formation of lateral roots of wheat seedlings (Barbieri et al. 1986; Barbieri and Galli 1993). A mutant strain of the PGPR *P. fluorescens* strain BsP53a, that overproduced IAA, stimulated root development of blackcurrant cv. Shirjaevskaja softwood cuttings, but inhibited that of sour cherry cv. Vladimirskaja (Dubeikovsky et al. 1993). In potato plantlets grown in vitro, strain PsJN increased cytokinin content by inducing synthesis in the early stages of plant growth and development (Lazarovits and Nowak 1997). Thus, it appears that rhizobacteria also affect hormone metabolism and reactivity within the plant itself.

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Interestingly, growth promotion was linked recently not to the production of stimulatory hormones, but to reduction of the inhibitory hormone ethylene. Ethylene has been identified as a common component of the soil atmosphere and under certain conditions has been shown to reach concentrations high enough to influence plant growth and development (Smith 1976; Frankenberger and Arshad 1995). The PGPR *P. putida* strain GR12-2 was mutagenized to select for variants that were unable to utilize the ethylene precursor ACC as a sole nitrogen source. These mutants proved to be devoid of the ACC-deaminase activity that is present in wild-type GR12-2 cells. They had also lost the ability to promote root elongation of developing canola (*Brassica campestris*) seedlings under gnotobiotic conditions (Glick et al. 1994), and no longer promoted shoot growth of seedlings planted in soil (Glick et al. 1997).

Conversely, transforming *Escherichia coli* or *Pseudomonas* spp. strains with a cloned ACC deaminase gene enabled the bacteria to grow on ACC as a sole source of nitrogen and to promote the elongation of seedling roots (Shah et al. 1998). These results were interpreted in terms of a model in which the bacterial strains promote root elongation by binding to germinating seeds or developing roots and hydrolyzing ACC leaking from the plant tissues through deamination to ammonia and α-ketobutyrate (Fig. 12.1). By

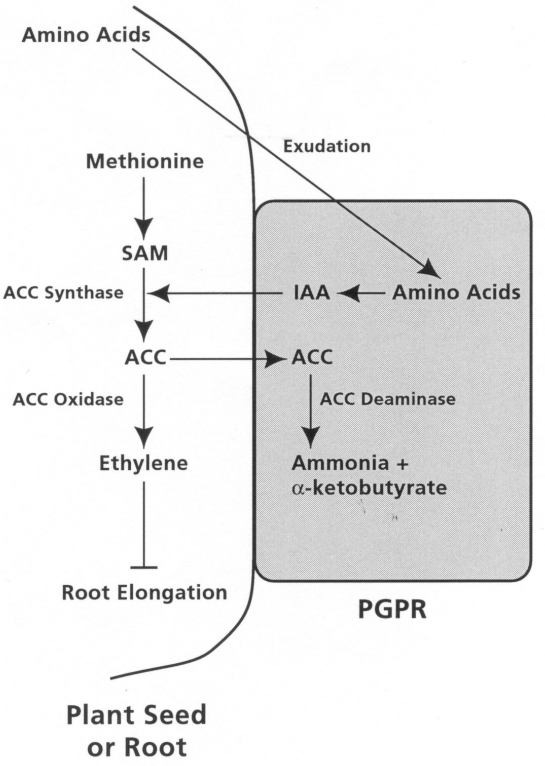

Fig. 12.1. Mechanism of the promotion of plant root elongation by rhizobacteria that possess ACC deaminase. *ACC* 1-Aminocyclopropane-1-carboxylate; *IAA* indole-3-acetic acid; *SAM* S-adenosylmethionine (adapted from Glick et al. 1999)

reducing the level of unbound ACC, re-uptake would be lowered, less ethylene would be produced and, consequently, roots grow longer (Glick et al. 1994, 1998). The ability to utilize ACC as a sole nitrogen source appeared to be limited to soil bacteria that are capable of stimulating plant growth (Glick et al. 1995), linking ACC-deaminating activity to growth promotion. Canola, lettuce (*Lactuca sativa*), tomato and wheat all responded with increased root length when seeds were treated with wild-type GR12-2 or with the chemical inhibitor of ethylene synthesis aminoethoxyvinylglycine, but not with the mutant strain. However, barley and oats (*Avena sativa*) did not respond to wild-type GR12-2, suggesting that promotion of plant growth by mechanisms that include hydrolyzing ACC could be limited largely to dicots (Hall et al. 1996).

12.3 Rhizobium–Plant Interactions

Whether bacteria promote growth through production of plant hormones or through modulating hormone metabolism of the plant, it is clear that signalling between the bacteria and plant roots is of central importance. Apparently, upon colonization of the roots, bacteria start to produce signal molecules that are perceived and transduced within the cells of the plant, leading to a response resulting in increased growth of the whole plant. The *Rhizobium*–legume symbiosis is a special case in which the specific interaction between a rhizobacterium and a leguminous host leads to the formation of nitrogen-fixing root nodules as a result of a "two-way molecular conversation". This interaction can serve as a paradigm of how rhizobacteria and root cells can influence each other's activities.

The interaction of soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* (collectively referred to as "rhizobia") and legumes starts with attachment of the bacteria to developing root hairs through lectin binding (Gray et al. 1992; Kijne et al. 1992; Noel 1992). The root hairs then deform and curl at the tip, and the bacteria invade the root by a newly formed infection thread, which grows through the root hairs into the cortex. Simultaneously, cortical cells are mitotically activated, giving rise to the nodule primordium. Infection threads grow towards the primordium and the bacteria, surrounded by a plant-derived, enclosing peribacteroid membrane, are released into the cytoplasm of the host cells. The nodule primordium then develops into the nodule, while the bacteria differentiate into their endosymbiontic form, the bacteroids, able to fix gaseous nitrogen into ammonia through the action of the nitrogenase enzyme complex. Carbon skeletons provided by the plant are converted into amino acids and amides, which can be utilized by the plant for growth on nitrogen-poor soils (Heidstra and Bisseling 1996).

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Extensive signalling occurs in each of the steps leading to effective nodule formation. The initial interaction between the bacterium and its host is triggered by the perception by the bacterium of certain (iso)flavonoids that are secreted from the plant roots. Historically, the presence of flavonoid compounds in legumes has been associated most closely with pathogenic attack. Stimulation of isoflavonoid biosynthesis in plants is a common feature of their response to pathogens, irrespective of whether those are bacteria, fungi, viruses or nematodes. The general antimicrobial activity of these isoflavonoids appears to contribute to resistance to various infecting microorganisms and parasites (Dakora and Phillips 1996). Rhizobia are mutualistic symbionts, but the early events, such as root hair deformation and curling, infection thread formation and cortical cell division, suggest that these organisms evolved from a pathogenic ancestor (Djordjevic et al. 1987). In fact, plant defense reactions are evident in cases where the final stage of the symbiosis (e.g. nitrogen fixation) is genetically blocked in the microsymbiont (Parniske et al. 1991). Symbiotic VA mycorrhizas and rhizobia all enhance isoflavonoid production or exudation in their host legume by producing β-glucan elicitors of the types that induce defense reactions in plant cells in response to e.g. pathogenic fungi (Dakora and Phillips 1996).

Host specificity is a prominent aspect of root nodule formation (Long 1996; Broughton and Perret 1999). Most rhizobia have a narrow host range and only form nodules on a very limited set of legume species. (Iso)flavonoids are recognized in a rhizobacterial strain-specific manner by binding to the constitutively expressed bacterial nodulation protein NodD (Fig. 12.2), a product specified by the large *Sym* plasmid on which the genes for bacterial nodulation (*nod* genes) and nitrogen fixation (*nif* genes) are located. Binding to specific flavonoids turns NodD into a transcriptional activator of other *nod* genes that encode proteins involved in the synthesis of specific lipo-oligosaccharides (Nod factors) that, in turn, are recognized by host legumes (Fig. 12.2). Nod factors all have a chitin β-1,4-linked *N*-acetyl-D-glucosamine backbone, varying in length between three and six sugar units, and a fatty acyl chain on the C-2 position of the non-reducing sugar (Dénarié and Roche 1992). Host-specific *nod* genes in different rhizobial species are involved in the modification of the fatty acyl chain or the addition of strain-specific substitutions that confer host specificity. For instance, *R. meliloti* produces Nod factors that contain a sulfate group at the C-6 position of the glucosamine residue at the reducing end (Fig. 12.2). When this sulfate group is absent due to a mutation in the bacterium, the modified factors no longer allow nodulation of the normal host, alfalfa (*Medicago sativa*). Instead, the mutant bacteria have acquired the ability to nodulate common vetch (*Vicia sativa*) that is host to *R. leguminosarum* bv. *viciae*, a strain that itself produces Nod factors without a sulfate group substitution (Geurts and Franssen 1996; Heidstra and Bisseling 1996). It has been postulated that another level of recognition may exist, because Nod factors can be hydrolyzed by specific chitinases of plant or microbial ori-

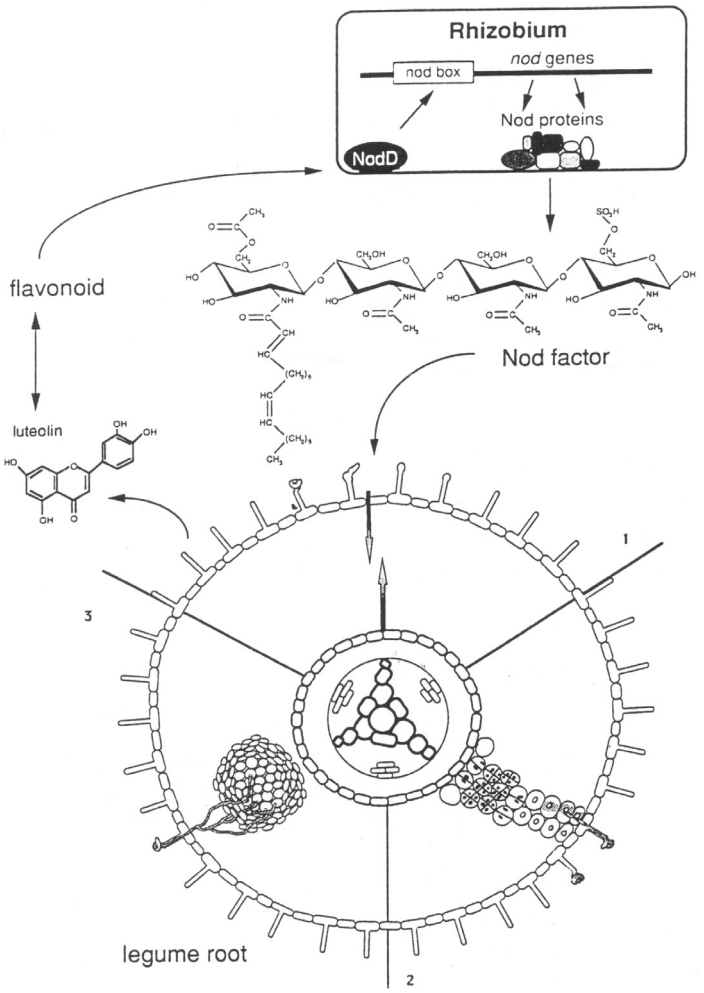

Fig. 12.2. Symbiotic interaction between rhizobia and leguminous plants. The plant exudes flavonoids, such as luteolin, that activate the NodD protein in the bacterium, leading to the production and secretion of Nod factors. The Nod factors induce root hair deformation (stage 1); rhizobia initiate infection and cortical cells start dividing (stage 2); the infection thread grows towards the developing nodule primordium where cells get infected (stage 3). Reproduced with permission from K. van de Sande and T. Bisseling, 1997. Essays in Biochemistry, vol. 32. Copyright The Biochemical Society

gin (Staehelin et al. 1994; Krishnan et al. 1999). Thus, effective nodulation could be prevented by the destruction of the rhizobial Nod factor by the plant itself or its rhizosphere microflora (Mellor and Collinge 1995).

Upon perception by epidermalroot cells, Nod factors induce the typical responses of root hair deformation, induction of plant "nodulin gene" expression, and formation of nodule primordia. Moreover, Nod factors promote the

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(iso)flavonoid biosynthesis that is stimulated by the bacterial elicitors. The earliest responses observed after application of Nod factors to legume roots are depolarization of root hair plasma membrane potential, spiking of cytoplasmic calcium levels in the root hairs, alkalinization of the root hair cytoplasm, rearrangement of the actin filaments and increased protoplasmic streaming, which all occur within minutes and prior to root hair deformation (Heidstra et al. 1994). After 3 h the root hairs are fully deformed and expression of plant early nodulin (ENOD) genes starts in anticipation of bacterial invasion. However, purified Nod factors alone are not sufficient to induce infection thread formation. Interaction of root hair cells with bacterial surface components, such as exopolysaccharide (EPS) or lipopolysaccharide (LPS), plays a further important role in the infection process (Gray et al. 1992; Noel 1992). These compounds are likely to act as additional signal molecules for eliciting infection thread formation (Hirsch 1992). Moreover, a concentration of Nod factor three orders of magnitude greater than for root hair deformation is required, suggesting that a different signalling pathway is involved.

Only root hairs just bulging out from the epidermal cells are sensitive to Nod factors: neither epidermal cells that have not yet formed root hairs, nor the old root hairs respond to Nod factors (Kurkdjian 1995). Root hair deformation is induced by picomolar concentrations of Nod factors, indicative of a hormone-like nature of the latter. Different Nod factor structural requirements of the various responses indicate that more than a single receptor is likely to be involved in Nod factor perception (e.g. Felle et al. 1996). Recent data suggest that lectin-like nucleotide phosphohydrolases (apyrases) possess the ability to bind Nod factors. Treatment of roots of *Dolichos biflorus* with antibodies against apyrase prevented nodulation, suggesting that apyrase is involved in the initiation of the root hair deformation (Etzler et al. 1999). In both soybean (*Glycine max*) and *Medicago trunculata*, apyrase mRNA is induced within 3 h after inoculation with rhizobia. Several mutants that are defective in nodulation lack the ability to express apyrase mRNA or to induce apyrase expression in response to rhizobial inoculation (Stacey 1999). Whether the apyrase protein really functions as a Nod signal receptor and how its enzymatic action may be coupled to signal transduction are questions that are currently attracting attention.

Purified Nod factors applied to the root surface not only induce responses in epidermal cells, but also in tissue inside the root, the pericycle and the cortex, that are not in direct physical contact with the medium containing the Nod factors. About 3 h after Nod factor addition and preceding the induction of cell divisions, a gene encoding a 10–13 amino acid long peptide (ENOD40) is induced in the pericycle (Vijn et al. 1995; Albrecht et al. 1999). After 16 h a substantial and prolonged inhibition of polar auxin transport was observed in vetch roots (Boot et al. 1999), preceding the first root cortical cell divisions that lead to the formation of nodule primordia or even nodule-like structures in the absence of bacteria. It seems unlikely that Nod factors themselves are

transported to the innermost layers. Rather, secondary signal molecules seem to be generated that are transported from the epidermis and interact with cortical and pericycle cells. However, no such signal has been identified so far.

The position in the root cortex where the nodule primordia are formed is almost exclusively opposite the protoxylem poles of the root vascular bundle. Positional information is specified by stele-derived uridine (Smit et al. 1995), which diffuses into the cortex in the protoxylem zones and positively stimulates cortical cell division. Localized production of ethylene in the phloem sectors of the root appears to act as a negative regulator by inhibiting cortical cell division (Heidstra et al. 1997). ACC oxidase, the enzyme catalyzing the last step in ethylene biosynthesis, is expressed specifically in the cell layers opposite the phloem in that part of the root where nodule primordia are induced upon inoculation with *Rhizobium*. This expression pattern, together with the inhibitory effect of ethylene on cell division, suggests that ethylene can locally suppress the formation of nodule primordia. That ethylene can act as a negative regulator of nodule formation is clearly seen in the ethylene-insensitive *M. trunculata* mutant *sickle*, which forms many more nodules than wild-type plants (Penmetsa and Cook 1997). Also, nodulation by low-nodulating pea *sym5* mutants is fully restored by application of Ag^+, a competitive inhibitor of ethylene perception (Fearn and LaRue 1991; Guinel and LaRue 1991). However, in soybean, varying effects of ethylene perception in the regulation of the numbers of nodules formed have been reported (Caba et al. 1999; Ligero et al. 1999; Schmidt et al. 1999). Nodule formation itself is taken to result from an increased cytokinin level in the root, which triggers cell division in conjunction with an increase in auxin resulting from inhibition of auxin transport due to flavonoids inhibiting this process (cf. Long 1996).

Although an overall picture of rhizobial root nodule formation is emerging, many details are still unknown of this complex but fascinating interaction between a rhizobacterium and its host. Thus, the ENOD40 peptide has homologs in non-leguminous plant species and appears to play a general role in regulating plant development by modulating sensitivity to auxin. It has been postulated that ENOD40 produced in the pericycle acts as a peptide hormone by diffusing into the inner cortex, changing the auxin/cytokinin balance and, thereby, triggering the onset of cell division (Van de Sande et al. 1996).

Root-exuded flavonoids that activate rhizobial *nod* genes can also stimulate growth of mycorrhizal fungi prior to infection, and plant genes encoding early nodulins are likewise activated in root tissues upon infection by VA mycorrhizal fungi (Van Rhijn et al. 1997). Genetic studies have demonstrated that some plant genes that regulate initial steps in the nodulation response of pea also control early stages of VA mycorrhizal development (Gianinazzi-Pearson 1996; Harrison 1997; Albrecht et al. 1999). In some mycorrhiza-resistant mutants of pea the mutant phenotype can be partially reverted by treatment with the auxin transport inhibitor triiodobenzoic acid (Muller 1999),

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indicative of a common regulation by alteration of the hormone balance of the root. Since both legumes and non-legumes are able to establish VA mycorrhizas with the same fungal species, one must assume that most vascular plants possess common symbiosis genes. In both bacterial and fungal symbiosis, the microsymbionts do not colonize root meristems or the central cylinder, but instead infect plants through the epidermis and multiply within the cortical parenchyma without triggering obvious defense reactions by the plant. Such findings suggest that the genetic program for nodulation may have arisen by adaptation of an ancestral mechanism regulating VA mycorrhizal symbiosis.

12.4 Disease Suppression by Rhizobacteria

Disease-suppressive properties are displayed by epiphytic, endophytic and symbiotic rhizobacteria. Extensive colonization of plant surfaces can prevent pathogens from establishing themselves on or in the plant. However, in addition both direct and indirect interactions between rhizobacteria and pathogens can reduce disease development or severity (Whipps 2001).

Soil-borne plant pathogens cause significant damage to crop production worldwide. Disease symptoms caused by these plant pathogens include damping-off, root rots, foot rots and wilting. For several soil-borne plant pathogens, including *Gaeumannomyces graminis* var. *tritici*, *Fusarium oxysporum*, *Fusarium solani*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, and *Sclerotium cepivorum*, disease-suppressive soils have been described (Cook and Baker 1983). In these soils expression of disease is limited despite the presence of a virulent pathogen, a susceptible crop and environmental conditions favorable for disease development. In several of these suppressive soils microbial populations that are antagonistic towards the pathogen play a key role in disease suppression. Selected strains from many genera of bacteria isolated from these suppressive soils have the potential to reduce plant diseases when applied to the plant root environment (Weller 1988). Using transposon mutagenesis, complementation studies, and reporter gene systems, the fluorescent pseudomonads in particular have received much attention with respect to the mechanisms involved in biocontrol. Mechanisms have been studied in detail not only to satisfy the curiosity of scientists, but also notably to improve the performance of biological control, either through selection of more effective strains, or through genetic modification of strains with traits desired. The modes of action that deal with a direct interference of the biological control agent with the pathogen include competition for substrates, siderophore-mediated competition for iron, antibiosis, and lytic activity.

12.4.1 Competition for Substrate

Competition between pathogenic and saprophytic microorganisms for organic materials released from the roots can reduce growth and/or pathogenic activity of the pathogens. For this mode of action the classical approach of comparing biocontrol activities of specific catabolic mutants with wild-type strains is not simple, since any of a number of substrates could be utilized (Loper et al. 1997). The involvement of competition for nutrients in biological control by fluorescent *Pseudomonas* spp. was suggested in several studies. It was found that in vitro antagonistic activity is based on competition, and correlated with disease suppression. Moreover, addition of specific substrates to the plant pathogen system reduced biological control (Elad and Baker 1985; Elad and Chet 1987). For non-pathogenic *Fusarium oxysporum* isolate Fo47 the involvement of competition for carbon in the effective suppression of fusarium wilt on different crops has been studied in detail (Alabouvette et al. 1998).

Enterobacter cloacae can effectively suppress damping-off and root rot diseases caused by *Pythium* species (Nelson and Maloney 1992). It was demonstrated for *P. ultimum* that germination of sporangia is stimulated specifically by seed exudates (Nelson and Hsu 1994). *E. cloacae* is able to catabolize long-chain fatty acids, such as linoleic acid, a predominant stimulant of germination of *Pythium* sporangia in cotton (*Gossypium hirsutum*) seed exudate (Van Dijk and Nelson 1998). A mutant of *E. cloacae* not able to utilize linoleic acid showed a reduced suppression of *Pythium* seed rots, and restoration of linoleic acid utilization by complementation of this mutant also restored suppression of seed rot (Van Dijk and Nelson 1997). Thus, the importance of competition for this specific stimulatory compound in disease suppression was elegantly demonstrated.

12.4.2 Competition for Iron by Siderophores

As discussed above, rhizobacteria produce various types of siderophores to chelate the scarcely available Fe and, thereby, can deprive pathogens from acquiring iron (Fig. 12.3). Using Tn*5* transposon mutagenesis in plant growth-promoting *Pseudomonas putida* WCS358, mutants defective in siderophore biosynthesis were obtained (Marugg et al. 1985). Whereas the wild-type strain WCS358 increased potato root growth and tuber yield significantly in pot and field experiments, respectively, the mutants defective in siderophore biosynthesis had no such effect (Bakker et al. 1986, 1987). In these experiments with potato the increased plant growth was due to suppression of deleterious rhizosphere microorganisms (Schippers et al. 1987). The involvement of siderophore production in disease suppression by WCS358 was further stud-

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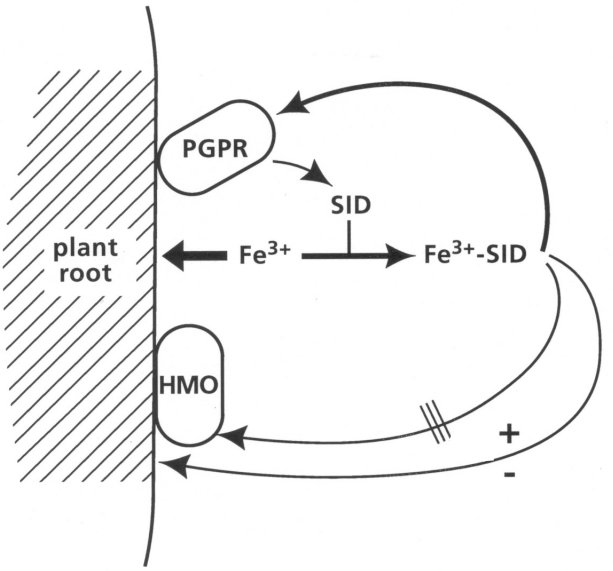

Fig. 12.3. Competition for iron between microorganisms in the rhizosphere: a plant growth-promoting rhizobacterium (*PGPR*) deprives a harmful microorganism (*HMO*) of iron by secreting siderophores (*SID*), which can (+) or cannot (–) also be used by plant roots. (After Bakker 1989)

ied on carnation, radish, and flax (*Linum usitatissimum*) using, respectively, *Fusarium oxysporum* f.sp. *dianthi*, *F. oxysporum* f.sp. *raphani* and *F. oxysporum* f.sp. *lini* as the pathogen. In all cases the siderophore mutant was less effective than the wild-type strain in suppression of disease (Duijff et al. 1993; Raaijmakers et al. 1995). Also in the combined effects of non-pathogenic *F. oxysporum* Fo47 and WCS358, the siderophore produced by the *Pseudomonas* strain plays a key role in suppression of fusarium wilt (Lemanceau et al. 1992, 1993; Leeman et al. 1996a; Duijff et al. 1999). Whereas the combination of Fo47 with the parental strain WCS358 suppressed fusarium wilt of carnation significantly better compared with the single treatments, a siderophore mutant of WCS358 had no such effect (Fig. 12.4). In this case the combined effects of siderophore-mediated competition for iron by WCS358 and effective competition for carbon by the non-pathogenic Fo47 explain the effective suppression of disease (Lemanceau et al. 1993). Siderophore production by fluorescent *Pseudomonas* spp. has been suggested or demonstrated to be similarly involved in the suppression of *Pythium* spp. (Becker and Cook 1988; Loper 1988), *G. graminis* var. *tritici* (Kloepper et al. 1980a), and *F. oxysporum* (Sneh et al. 1984; Elad and Baker 1985; Baker et al. 1986).

Siderophore mutants of fluorescent *Pseudomonas* spp. strains are comparable to the parental strains with regard to their abilities to colonize the rhizosphere (Bakker et al. 1990), in spite of their reduced competitiveness in acquiring iron. However, the mutants do produce a functional siderophore receptor and, thus, are still able to utilize the parental siderophore (Bitter et al. 1991). For different strains of fluorescent pseudomonads, including *P. putida* WCS358, utilization of siderophores is not restricted to the siderophore pro-

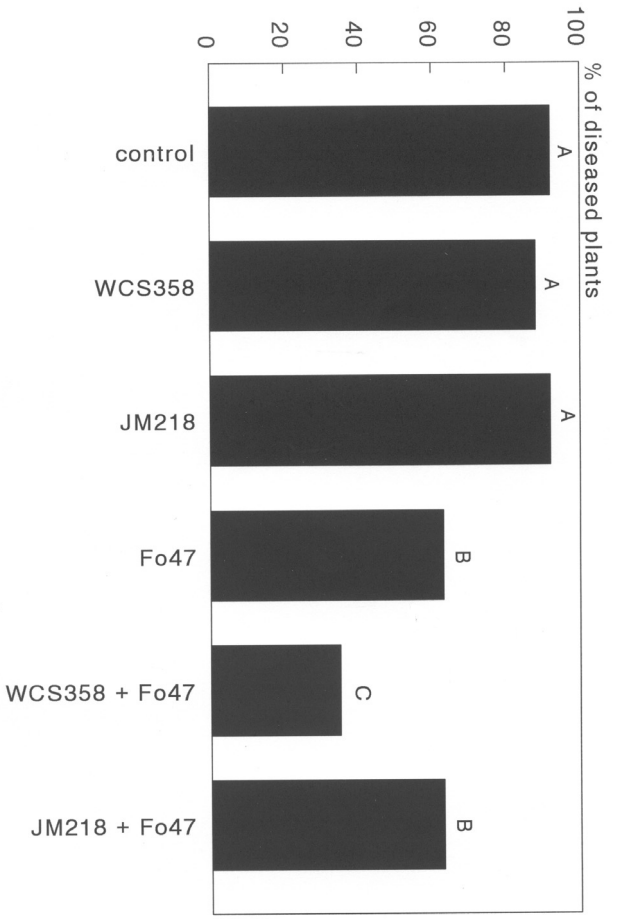

Fig. 12.4. Suppression of fusarium wilt of carnation by *Pseudomonas putida* WCS358 and a siderophore minus mutant of this strain (JM218), both applied either singly or in combination with non-pathogenic *Fusarium oxysporum* Fo47. *Different letterings* indicate significant differences. (Adapted from Lemanceau et al. 1992)

duced by the strain itself, but they can use those produced by many heterologous strains for their iron acquisition (Bakker et al. 1990; Raaijmakers et al. 1994; Koster et al. 1995). The latter observation can explain why siderophore mutants reach similar population densities as the wild type in rhizosphere colonization. Uptake of heterologous siderophores in WCS358 is regulated effectively. For instance, the *pupB* gene, encoding an outer membrane protein that recognizes the siderophores pseudobactin BN7 and pseudobactin BN8, is only expressed in the presence of the heterologous siderophores that are recognized (Koster et al. 1993).

12.4.3 Antibiosis

It has been questioned for many years whether antibiotics are produced by soil microorganisms in quantities large enough to play a significant role in

microbial interactions (Williams and Vickers 1986). The introduction of genetic techniques and methods has provided clear evidence for the involvement of antibiotics in suppression of plant diseases by biological control agents (Fravel 1988; Loper et al. 1994). Antibiosis is now often implicated as an important mechanism of biological control, resulting from the fact that it is an attractive mechanism to study and can provide a highly effective mode of action (Handelsman and Stabb 1996). *P. fluorescens* strain 2-79 is suppressive to *G. graminis* var. *tritici*, the causal agent of take-all in wheat (Weller and Cook 1983). Using Tn*5* transposon mutants defective in the production of the antibiotic phenazine-1-carboxylic acid (PCA) and subsequent complementation of these mutants, Thomashow and Weller (1988) demonstrated the involvement of this antibiotic in control of take-all disease by strain 2-79 coated on wheat seeds. Using a similar approach, Keel et al. (1992) demonstrated the importance of 2,4-diacetylphloroglucinol (DAPG) in suppression of root diseases by *P. fluorescens* strain CHA0, that produces a wide variety of antifungal metabolites (Table 12.2). Other antibiotics described recently to be involved in disease suppression by fluorescent pseudomonads are phenazine-1-carboxamide (Chin-A-Woeng et al. 1998) and anthranilate (Anjaiah et al. 1998). For the biocontrol agent *Bacillus cereus* strain UW85 production of kanosamine and zwittermicin A was suggested to be important for its biocontrol activity (Silo-Suh et al. 1994; Milner et al. 1996). Another class of bacterial metabolites with antibiotic properties are rhamnolipid biosurfactants (Stanghellini and Miller 1997). Fungal zoospores lack a protective cell wall, leaving the plasma membrane exposed and vulnerable to influences from the

Table 12.2. Production of 2,4-diacetylphloroglucinol (DAPG) by *Pseudomonas fluorescens* strain CHA0 and its derivatives in the rhizosphere of wheat grown under gnotobiotic conditions and relationship between DAPG production and suppression of *Gaeumannomyces graminis* var. *tritici* induced take-all disease by the bacteria. (Data from Keel et al. 1992)

P. fluorescens	*G. graminis*	µg DAPG per g root	Root fresh weight (mg)	Disease rating
None	–	<0.01	320[a]	0[e]
	+	<0.01	156[c]	3.1[a]
CHA0 (DAPG$^+$)	–	0.94±0.48	332[a]	0[e]
	+	1.36±0.16	323[a]	0.7[d]
CHA625 (DAPG–)	–	<0.01	320[a]	0[e]
	+	<0.01	249[b]	1.9[b]
CHA625/pME3128 (DAPG$^+$)	–	0.26±0.14	335[a]	0[e]
	+	0.19±0.05	294[a]	1.3[c]

CHA625 is a mutant of CHA0 that lacks DAPG production; CHA625/pME3128 is a transformant of mutant CHA625 in which DAPG production is restored by complementation. Disease severity was rated on a 0–4 scale (0=no disease; 4=plants dead).
Different letterings indicate significant differences at the 5 % level.

environment. Strains of *Pseudomonas* spp. can rapidly kill zoospores by disrupting the plasma membrane by the rhamnolipid biosurfactant (Stanghellini and Miller 1997).

Antibiosis as a highly effective means of control of a soil-borne pathogen in natural soils was described recently by Raaijmakers and Weller (1998). They demonstrated that in take-all decline the build-up of populations of DAPG-producing *Pseudomonas* spp. plays a key role in the development of disease suppressiveness.

Production of antibiotics by rhizosphere bacteria is controlled by complex regulatory networks, in which plant, bacterial and environmental signals are involved. Cell density influences production of antibiotics by fluorescent pseudomonads in the rhizosphere (Pierson III et al. 1998). Diffusible *N*-acylhomoserine lactones are produced and utilized by *P. aureofaciens* strain 30-84 and they control the production of PCA (Pierson et al. 1998). These so-called autoinducers can diffuse freely across bacterial membranes. They accumulate in the environment, but also in the producing cells, as the density of the cells increases. When the autoinducer reaches a certain concentration within the cell, the transcription of specific genes is activated. It was recently demonstrated that *N*-acylhomoserine lactone mediated communication occurs between bacterial populations in complex consortia (Pierson et al. 1998; Eberl 1999), as well as between plants and bacteria (Teplitski et al. 2000). Thus, the production of antibiotics by introduced microorganisms can be influenced by the indigenous microflora. There is also evidence for signalling between the pathogen and the introduced biocontrol agent. A promoterless *lac*Z reporter gene was used to generate a transcriptional gene fusion library in *P. fluorescens* strain F113 in order to detect promotors whose activities are altered under specific environmental conditions. Using this library five gene clusters in F113 were identified that are repressed by the presence of *Pythium ultimum* and, interestingly, these gene clusters are important in colonization of the rhizosphere by this strain of *P. fluorescens* (Fedi et al. 1997).

Crown and root rot of tomato caused by *F. oxysporum* f.sp. *radicis-lycopersici* can be controlled by *P. fluorescens* CHA0. Duffy and Défago (1997) report that a metabolite of this fungal pathogen, fusaric acid, represses the production of DAPG and pyoluteorin, both metabolites of CHA0 with antifungal activity. Also, the plant can regulate promoter activity in pseudomonads. By using a library of transcriptional fusion mutants, Van Overbeek and Van Elsas (1995) identified a gene responding to the presence of wheat root exudate. This reporter gene was also induced in the presence of maize and grass roots, but not by roots of clover, suggesting crop-specific interactions. Finally, environmental factors have a significant influence on the production of specific metabolites by fluorescent pseudomonads. In the model strain *P. fluorescens* CHA0, it was recently demonstrated that production of antifungal metabolites can be differentially influenced by specific environmental factors (Duffy and Défago 1999).

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12.4.4 Lytic Activity

Certain biological control agents have been demonstrated to suppress disease by parasitizing the plant pathogen. In most cases the biocontrol agent is a fungus that parasitizes on a plant pathogenic fungus. Lytic activity has been demonstrated to be involved in this phenomenon and to comprise degradation of the chitin and glucans in the fungal cell wall and osmotic disruption of the cellular membrane. Transformants of *Trichoderma harzianum* that overexpress a chitinase are more effective in inhibition of growth of *Rhizoctonia solani* (Limon et al. 1999). More interestingly, transformants of *Trichoderma longibrachiatum* that overexpress the β-1,4-endoglucanase gene *egl1* were more effective in controlling effects of *P. ultimum* on cucumber plant emergence and health (Migheli et al. 1998). Woo et al. (1999) describe the genetic modification of *T. harzianum* strain P1, resulting in disruption of a single copy gene that encodes a 42-kDa endochitinase. The endochitinase mutant was compared with the wild-type strain with regard to its biocontrol activity against *Botrytis cinerea*, *R. solani* and *P. ultimum*. Whereas the mutant was as effective as the parental strain in controlling *P. ultimum* – an oomycete lacking chitin – it was less effective against the chitin-containing fungus *B. cinerea* and, surprisingly, it was more effective against *R. solani* (Woo et al. 1999). Thus, endochitinase activity is important in biocontrol of *B. cinerea* by *T. harzianum*, but for control of *Pythium* and *Rhizoctonia* other mechanisms appear to play a role.

For bacteria the role of lytic activity in biological control of plant pathogens is less clear. Many chitin-degrading soil bacteria have the ability to inhibit fungal growth. However, in many cases, bacterial antagonism was not associated with chitinase production (De Boer et al. 1998). On the other hand, it has been suggested that lysis of fungal cell walls of *F. oxysporum* f. sp. *cucumerinum* by *Paenibacillus* sp. 300 and *Streptomyces* sp. 385 is involved in biological control of fusarium wilt by these bacteria (Singh et al. 1999).

12.5 Rhizobacteria-Mediated Induced Systemic Resistance

Induced resistance results from perception of rhizobacteria by plant roots giving rise to an increased level of resistance that is expressed upon subsequent infection by a pathogen. Localized induction of resistance at the site where eliciting bacteria are present on the roots is difficult to demonstrate, because a challenging pathogen will also be subject to bacterial antagonism at this same location. In contrast, no direct interaction between inducing bacteria and a challenging pathogen is possible when each is present at spatially separated sites and no contact between the two is established. Under such

conditions, it was demonstrated that various non-pathogenic rhizobacterial strains can induce systemic resistance against fungi, bacteria, and viruses in Arabidopsis (*Arabidopsis thaliana*), bean (*Phaseolus vulgaris*), carnation, cucumber (*Cucumis sativus*), radish, tobacco (*Nicotiana tabacum*) and tomato (Van Loon et al. 1998). Relatively little is known about the "molecular conversation" between these bacteria and the plant compared with the *Rhizobium*–legume symbiosis. However, critical steps are being defined, in large part as a result of the analysis of Arabidopsis mutants that are impaired in resistance signalling pathways.

Rhizobacterially mediated induced systemic resistance (ISR) is phenotypically similar to the better-known systemic acquired resistance (SAR), the induced state that develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue (Ryals et al. 1996; Sticher et al. 1997; Fig. 12.5). SAR is a generally occurring phenomenon that confers an enhanced defensive capacity against all types of pathogens. Under the influence of the primary infection, a signal – the nature of which is still unclear – is generated and transported throughout the plant, thereby establishing the induced state. SAR is characterized by a requirement for salicylic acid (SA) as a signal and by the SA-mediated accumulation of several families of pathogenesis-related proteins (PRs), among which are chitinases and glucanases with potential antifungal activity (Durner et al. 1997; Kombrink and Somssich 1997).

ISR resembles SAR in that it is effective against different types of pathogens, but it differs from SAR in that the inducing rhizobacterium does

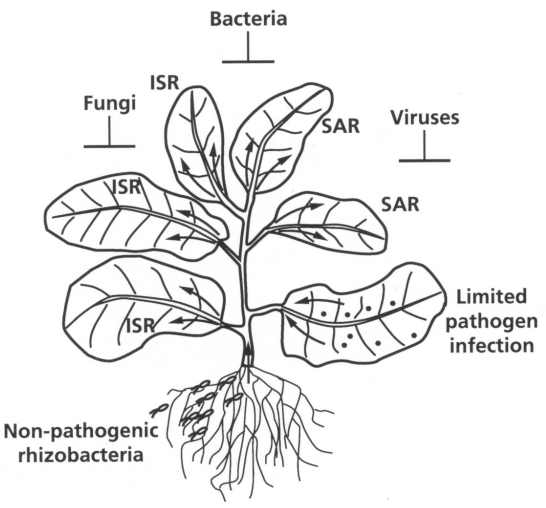

Fig. 12.5. Diagrammatic representation of systemic resistance against fungi, bacteria and viruses induced locally by root colonization by non-pathogenic rhizobacteria (*left* ISR) or by limited pathogen infection (*right* SAR). *ISR* Induced systemic resistance; *SAR* systemic acquired resistance

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not cause any visible symptoms in the host. At least in *Arabidopsis*, SA is not involved as a signal and no PRs accumulate (Pieterse et al. 1996). ISR thus constitutes a mechanistically different type of induced resistance and has so far been found to be triggered only by selected rhizobacterial strains (Van Loon 1997). Several *Pseudomonas* spp. isolates have the ability to elicit ISR, but do so differentially in different plant species. For instance, when using *F. oxysporum* as the challenging pathogen, strain WCS358 elicits ISR in *Arabidopsis* but not in radish, strain WCS374 elicits ISR in radish but not in *Arabidopsis*, and *P. fluorescens* strain WCS417 elicits ISR in both *Arabidopsis* and radish. Such species specificity implies that *Arabidopsis* and radish – although both crucifers – either do not offer an environment in which bacterial determinants for resistance induction are expressed in a similar manner, or the same bacterial signals are differentially perceived or transduced.

A prerequisite for resistance induction in general is that the rhizobacteria are able to colonize the roots to a sufficient level. In radish the minimal number of bacteria required was determined to be 10^5 colony-forming units (cfu) per g root (Raaijmakers et al. 1995). Upon isolation of fluorescent pseudomonads from roots of different crop plants growing in a silty loam soil, a high diversity of isolates was recovered (Glandorf et al. 1993), suggesting that in nature no single strain is likely to exceed the threshold level for eliciting ISR. This can explain why plants with levels of up to 10^6 cfu of pseudomonads and 10^9 cfu of total bacteria per g root are not usually found to be induced already. Species specificity cannot be readily explained by differential root colonization. Growing plants in autoclaved soil mixed with individual bacterial strains always led to similar levels of root colonization well above the threshold concentration (Van Wees et al. 1997).

The question of which bacterial determinants are involved in the elicitation of ISR has been addressed by investigating effects of the purified factors and comparing levels of resistance induced by wild-type strains and by selected mutants. It was established that in carnation and radish the O-antigenic side chain of the bacterial outer membrane lipopolysaccharide (LPS) acts as the main determinant (Van Peer and Schippers 1992; Leeman et al. 1995). Treatment of roots with purified bacterial LPS was as effective as living bacteria in eliciting ISR. In radish, bacterial mutants lacking the O-antigenic side chain of the LPS (OA–) did not trigger ISR (Leeman et al. 1995). Thus, cell surface components present in the LPS appear to be the inducing factor. Probably, the carbohydrate side chain of the LPS is recognized by a receptor at the root surface. However, neither the detailed structure of the LPS of the inducing strains, nor a binding entity on roots of carnation or radish, has been identified.

The situation in *Arabidopsis* is more complex in that LPS-containing cell wall preparations of strain WCS417 elicit ISR in this plant species, but an OA– mutant still induced levels of protection similar to wild-type WCS417. This indicates that ISR-inducing bacteria produce more than a single factor triggering ISR in Arabidopsis (Van Wees et al. 1997). Siderophores have also been

implicated in the induction of resistance in Arabidopsis (Van Loon et al. 1998), as well as in tobacco (Maurhofer et al. 1994) and radish (Leeman et al. 1996b). However, their contribution to the elicitation of ISR by bacteria in the rhizosphere is uncertain.

The picture is further complicated by the capacity of certain bacterial strains to produce SA under the iron-limiting conditions that are likely to occur in the rhizosphere. For *Pseudomonas aeruginosa* strain 7NSK2 it has been demonstrated that induction of resistance in tobacco against tobacco mosaic virus (De Meyer and Höfte 1998) and in bean against gray mold caused by *Botrytis cinerea* (De Meyer and Höfte 1997) is dependent on the production of SA, because bacterial mutants impaired in SA biosynthesis were no longer inducive. Such experiments have yet to be performed for WCS374 and WCS417. Both these strains can produce SA and induce a resistance in radish under iron-limiting conditions that is not abolished in OA– mutants (Leeman et al. 1996b). Bacterially produced SA can be readily taken up by plant roots and be transported to distant plant parts. The type of induced resistance resulting resembles SAR in its requirement for SA and differs from that induced by non-SA-producing strains. Thus, it is clear that different bacterial components can act as determinants and that these are differentially recognized by different plant species.

The systemic resistance induced in Arabidopsis by WCS358 or WCS417 is equally effective against the fungal root pathogen *F. oxysporum* f. sp. *raphani* (For) and the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (Pst). When using Pst as the challenging pathogen, it was observed that most Arabidopsis ecotypes reacted to induction by the rhizobacteria with a reduction in the proportion of leaves with symptoms of bacterial speck disease. However, ecotypes RLD and Ws-O were non-responsive to the rhizobacteria and became as diseased as non-bacterized control plants infected with Pst (Ton et al. 1999). Non-responsiveness was not caused by poor root colonization or inability to perceive inducing determinants. Rather, the ecotypes appeared to be impaired in a step in the signal-transduction pathway leading to ISR. Different crossings established that responsiveness was inherited as a monogenic, dominant trait, and was correlated with basal resistance against Pst, i.e. RLD and Ws-O are more sensitive to Pst than other ecotypes. These observations suggest that ISR makes use of a signal-transduction pathway that is likewise involved in plant defense against primary infection (Ton et al. 1999).

Testing of known resistance-signalling mutants in Arabidopsis revealed that development of ISR does indeed require components that are also implicated in genetically determined primary resistance to pathogens. Using the jasmonate (JA) response mutant *jar1*, the ethylene response mutant *etr1*, and the SAR signalling mutant *npr1*, it became clear that ISR requires responsiveness to both plant hormones, and shares with SAR a dependency on the regulatory protein NPR1. NPR1 is an ankyrin repeat-containing protein with homology to the mammalian transcription inhibitory regulatory factor IκBα,

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which plays a role in disease resistance responses in a wide range of higher organisms (Cao et al. 1997; Ryals et al. 1997). NPR1 has been shown to interact with transcription factors involved in the expression of PR-mRNAs (Zhang et al. 1999). Because PRs are not induced during ISR, it is surprising that NPR1 is required not only for SAR, but also for ISR. This dual requirement demonstrates that both signal-transduction pathways share at least one component (Fig. 12.6), pointing to various pathogens and non-pathogenic rhizobacteria stimulating partly overlapping signalling pathways leading to the induced resistant state.

JA and ethylene are produced together with SA during pathogen-induced necrotizing reactions giving rise to SAR, but, in contrast to SA, they are not involved in the establishment of SAR (Pieterse et al. 1998). Application of either methyl jasmonate (MeJA) or the ethylene precursor ACC induces a resistance in *Arabidopsis* against Pst that fully mimics the effect of root colonization with WCS417. However, no increases in endogenous JA or ethylene production were apparent in *Arabidopsis* treated with WCS417 (Pieterse et al. 2000). Yet, JA and ethylene must be involved, because, if responsiveness to either is lost, no ISR develops. Treatment with MeJA still induced ISR in the ethylene non-responsive mutant *etr1*, whereas treatment with ACC did not elicit ISR in the JA response-mutant *jar1*. These data demonstrate that the JA and ethylene responses are engaged in this order in triggering ISR. Because JA can increase sensitivity to ethylene (Tsai et al. 1996), it may be envisaged that

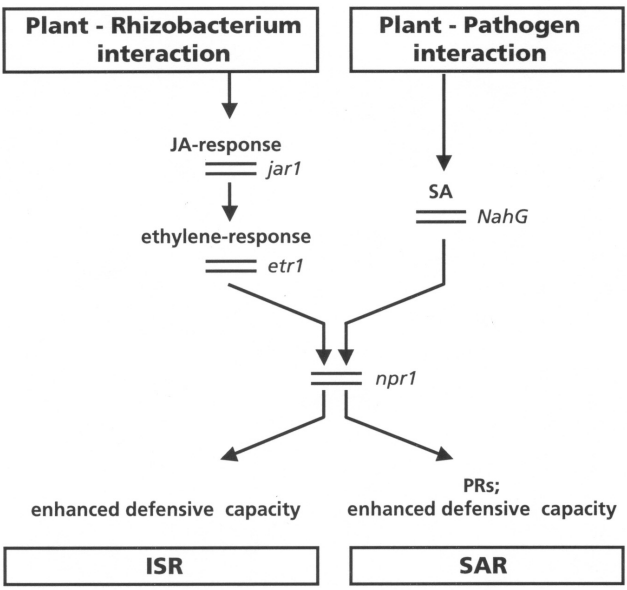

Fig. 12.6. Signalling in *Arabidopsis thaliana* leading to rhizobacteria-mediated induced systemic resistance (*ISR*) or to pathogen-induced systemic acquired resistance (*SAR*). *JA* Jasmonate; *PRs* pathogenesis-related proteins; *SA* salicylate. (Van Loon et al. 1998)

the JA response leads to an enhanced sensitivity to ethylene. Whether the requirement of the JA response in the absence of an increase in endogenous JA also comprises an increase in sensitivity to JA remains an open question. Nevertheless, it must be concluded that, in the induction of ISR, recognition of specific bacterial determinants at the root surface modulates JA and ethylene signalling in the plant and, through the action of the protein NPR1, results in systemically enhanced resistance (Fig. 12.6).

By taking advantage of an *Arabidopsis* mutant that is impaired in ethylene perception in the roots but not in the shoots, it was investigated whether the requirement for ethylene signalling occurs during induction of ISR in the roots or during expression of ISR in the leaves. This *eir1* mutant did not express ISR upon application of WCS417 to the roots, but did exhibit ISR when the inducing bacteria were infiltrated into the leaves. These results demonstrate that, for the induction of ISR, ethylene responsiveness is required at the site of application of the inducing rhizobacteria (Knoester et al. 1999). Because JA perception is required prior to ethylene perception, by inference it can be concluded that also JA-dependent signalling must occur in the root cells that are contacted by the inducing bacteria. How bacterial determinants activate the JA signalling pathway in root cells is totally unclear at present. Equally unclear are the nature of the signal that is transported from the root to other plant parts, and how the induced state becomes established. So far no consistent changes in antimicrobial compounds, enzyme activities, protein patterns or mRNA abundance have been observed upon induction; only after challenge inoculation is an enhanced defensive capacity expressed in the plant.

The same rhizobacterial strains may suppress disease by both microbial antagonism and eliciting ISR, as well as promote plant growth. Thus, WCS417 not only induces resistance in *Arabidopsis*, but was also found to increase fresh weight by 32 % (Pieterse and Van Loon 1999). Such results bear testimony to the intricate interactions between root-colonizing bacteria and their hosts and indicate that not only rhizobia, but also free-living rhizobacteria have intimate relationships with plant roots. Signals are continuously being exchanged, which influence the physiology of both the plant and the microbial partner. Recently, it has become clear that plants have a sensitive perception system for the most conserved domain of bacterial flagellin and react by activating an early defense response. However, *Rhizobium* and some plant pathogenic bacteria exhibit divergence in the N-terminal conserved domain of flagellin, suggesting that this difference enables them to evade plant defenses and to invade the host (Felix et al. 1999). The presence of substantial numbers of a wide variety of microorganisms on plant roots also allows extensive signalling between microbes (Pierson III et al. 1998), resulting in antagonistic or synergistic effects on root colonization, plant growth, and suppression of disease. For example, certain strains of PGPR increased growth and development, nodulation and nitrogen fixation by *Rhizobium* in

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bean (Srinivasan et al. 1996, 1997) and soybean (Shabayev et al. 1996; Dashti et al. 1997). Similarly, ectomycorrhizal formation on eucalypt (*Eucalyptus diversicolor*) seedlings was significantly increased upon inoculation with specific PGPR (Dunstan et al. 1998). A combination of two *Pseudomonas* strains, antagonising For by competition for iron and inducing resistance, respectively, reduced fusarium wilt in radish more than each strain by itself (De Boer et al. 1999).

12.6 Summary and Prospects

In view of the multiple and dynamic interactions between microorganisms and plant roots, the rhizosphere must be considered a signalling network between many partners, the details of which form a challenge for scientific research, as well as a promise for environmentally friendly agronomic practices. Although some of the mechanisms involved are being elucidated in increasing detail at the molecular level, the complexities appear far greater than anticipated. Growth promotion by rhizobacteria has been associated with deamination of root-derived ACC, but many PGPR with plant growth-promoting properties do not possess ACC deaminase. Clearly, their stimulatory activity must be based on other mechanisms. None of these have been unequivocally established, nor is it evident to what extent increases in nutrient availability as a result of bacterial action in the rhizosphere contribute quantitatively to plant nutrition. Root nodule formation in legumes by symbiotic rhizobia is far better understood and can serve as a model for unraveling the complex interactions between bacteria and plant roots. However, it is still unknown how rhizobial Nod factors are perceived by the roots and how nodule initiation and differentiation are controlled. There are similarities between these symbiotic and mycorrhizal and pathogenic host–microbe interactions, and a more profound understanding of the mechanisms involved may lead to the development of new agricultural applications that can increase sustainability by making use of already evolved and functioning mechanisms for nutrient supply and biological disease control.

Many rhizobacteria can antagonize pathogens either directly through competition for nutrients, production of antimicrobial compounds or secretion of lytic enzymes, or indirectly by stimulating plant host defenses. Many details about what factors are involved in the expression of these mechanisms in the rhizosphere are still lacking, and the molecular basis of rhizobacterially mediated induced systemic resistance has yet to be clarified. However, bacteria are easily transformable and the use of well-defined mutants together with complementation analysis allows the significance of individual genes to be assessed. To provide direct evidence of the functioning of relevant mechanisms in the rhizosphere, various reporter genes are available to monitor gene

expression in situ in time and space by using suitable transformants carrying promoter-reporter constructs. Gene expression profiling through the use of microarrays will be the method of choice to identify novel genes that are expressed in both rhizobacteria and plant hosts during their interactions. These approaches will lead to a far fuller understanding of the many physiological processes involved, their regulation at the molecular level, and their ecological and evolutionary implications for the functioning of plant roots.

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