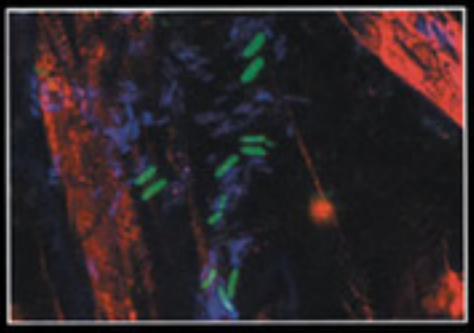
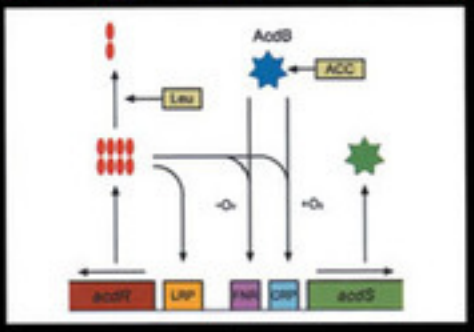
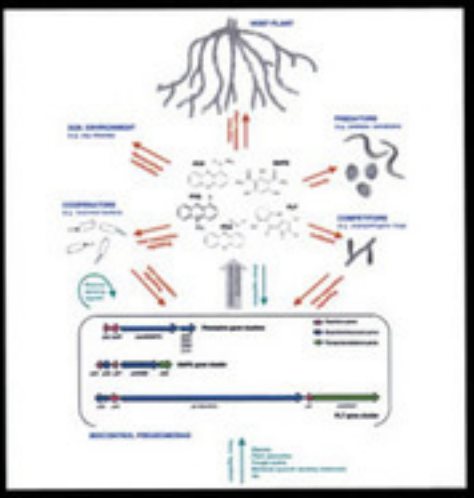
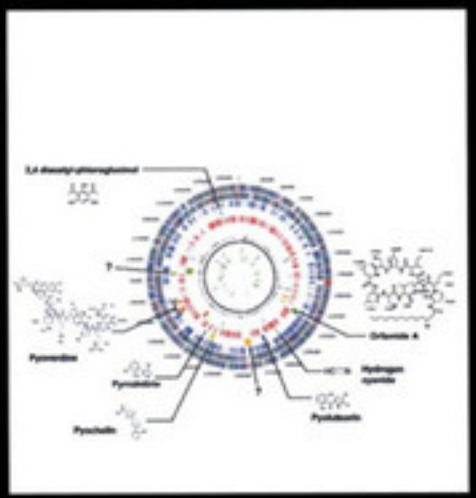
New Perspectives and Approaches in Plant Growth-Promoting Rhizobacteria Research









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From top to bottom: Confocal laser scanning microscopy analyses of colonies of *Pseudomonas fluorescens* WCS365 marked with green and cyan fluorescent proteins; Model of the transcriptional regulation of ACC deaminase expression in *Pseudomonas putida* UW4; Overview of interactions between biocontrol strains, plants, pathogens, predators, cooperators, and soil; Circular representation of the genome of *Pseudomonas fluorescens* Pf-5

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FOREWORD

Foreword

Peter A. H. M. Bakker · Jos M. Raaijmakers · Guido V. Bloemberg · Monica Höfte · Philippe Lemanceau · Mike Cooke

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New perspectives and approaches in plant growth-promoting rhizobacteria research

Plant growth-promoting rhizobacteria (PGPR) are defined as root-colonizing bacteria that exert beneficial effects on plant growth and development. Root colonization comprises the ability of bacteria to establish on or in the plant root, to propagate, survive and disperse along the growing root in the presence of the indigenous microflora. Rhizobacteria are considered as efficient microbial competitors in the root zone. Representatives of many different bacterial genera have been commercialized and/or introduced into soils, onto seeds, roots, tubers or other planting materials to improve crop growth. These bacterial genera include *Acinetobacter, Agrobacterium, Arthrobacter, Azospirillum, Bacillus, Bradyrhizobium, Frankia, Pseudomonas, Rhizobium, Serratia, Thiobacillus* and others. To date, probably the most widely used PGPR in agriculture are *Rhizobium* and *Bradyrhizobium* species for their nitrogen-fixing capacity in roots of Leguminosae. In addition to the promotion of plant growth, PGPR are also employed for controlling plant pathogens, enhancing efficiency of fertilizers, and degrading xenobiotic compounds (rhizoremediation). The application of PGPR is a growing market.

There is an active and growing group of scientists working on fundamental and applied aspects of PGPR. Since the late eighties,

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developments of PGPR research have been addressed at International Workshops on PGPR. The first meeting was held in 1987 in Orilla, Ontario, Canada, and since then in Interlaken, Switzerland (1990), Adelaide, Australia (1994), Sapporo, Japan (1997), Cordoba, Argentina (2000) and Calicut, India (2003). In 2006, the 7th workshop was organized in Noordwijkerhout, The Netherlands, where over 130 scientists from 17 countries worldwide participated and presented their results in 49 oral and 69 poster presentations.

Topics addressed during the PGPR workshops include:

- mechanisms of plant growth promotion and disease suppression
- traits involved in root colonization by PGPR
- the role of PGPR in microbial interactions
- the molecular and biochemical basis of disease suppression and root colonization
- the role of PGPR in disease-suppressive soils
- plant responses to PGPR
- discovery of novel PGPR strains and traits
- pathogen responses to PGPR
- risk assessment of PGPR
- production, formulation and delivery strategies of PGPR
- performance of PGPR in greenhouse trials and agricultural fields
- registration and commercialization of PGPR

In addition to these topics the 7th meeting focused on recent developments in genomics, proteomics and metabolomics of PGPR. The abstract book is available at http://www.bio.uu.nl/~fytopath/PDF%20files/abstract%20book%20PGPR%20final.pdf. Last but surely not least, this meeting was dedicated to the great efforts of several PGPR scientists. These are Jim Cook, Geneviève Défago, Ben Lugtenberg and Kees van Loon. In this special issue of the European Journal of Plant Pathology, key contributions are published that give an overview of the work presented at the workshop.

The attendance and excellent contributions by an ever-growing group of young scientists guarantees a healthy future for PGPR research. Our best wishes to David Weller and Joyce Loper who will organize the next workshop in the Pacific Northwest, USA.

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REVIEW PAPER

Plant responses to plant growth-promoting rhizobacteria

L. C. van Loon

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Abstract Non-pathogenic soilborne microorganisms can promote plant growth, as well as suppress diseases. Plant growth promotion is taken to result from improved nutrient acquisition or hormonal stimulation. Disease suppression can occur through microbial antagonism or induction of resistance in the plant. Several rhizobacterial strains have been shown to act as plant growth-promoting bacteria through both stimulation of growth and induced systemic resistance (ISR), but it is not clear in how far both mechanisms are connected. Induced resistance is manifested as a reduction of the number of diseased plants or in disease severity upon subsequent infection by a pathogen. Such reduced disease susceptibility can be local or systemic, result from developmental or environmental factors and depend on multiple mechanisms. The spectrum of diseases to which PGPR-elicited ISR confers enhanced resistance overlaps partly with that of pathogen-induced systemic acquired resistance (SAR). Both ISR and SAR represent a state of enhanced basal resistance of the plant that depends on the signalling compounds jasmonic acid and salicylic acid, respectively, and pathogens are differentially sensitive to the resistances activated by each of these signalling pathways. Root-colonizing *Pseudomonas* bacteria have been shown to alter plant gene expression in roots and leaves to different extents, indicative of recognition of one or more bacterial determinants by specific plant receptors. Conversely, plants can alter root exudation and secrete compounds that interfere with quorum sensing (QS) regulation in the bacteria. Such two-way signalling resembles the interaction of root-nodulating *Rhizobia* with legumes and between mycorrhizal fungi and roots of the majority of plant species. Although ISR-eliciting rhizobacteria can induce typical early defence-related responses in cell suspensions, in plants they do not necessarily activate defence-related gene expression. Instead, they appear to act through priming of effective resistance mechanisms, as reflected by earlier and stronger defence reactions once infection occurs.

Keywords Arabidopsis · Disease suppression · Induced systemic resistance · Plant growth promotion · Signal transduction · Systemic acquired resistance

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Plant growth promotion by rhizobacteria

Plant roots offer a niche for the proliferation of soil bacteria that thrive on root exudates and lysates. Population densities of bacteria in the rhizosphere may be up to 1,00-fold higher than in bulk soil and up

to 15% of the root surface may be covered by micro-colonies of a variety of bacterial strains. While these bacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Several of these metabolites can act as signalling compounds that are perceived by neighbouring cells within the same micro-colony, by cells of other bacteria that are present in the rhizosphere, or by root cells of the host plant (Van Loon and Bakker 2003; Bais et al. 2004; Gray and Smith 2005; Kiely et al. 2006).

The best-studied example of signal exchange is the *Rhizobium*—legume symbiosis, in which the plant releases flavonoid compounds that act as signals for the bacterium to secrete Nod factors. Nod factors are perceived by plant root hairs and function in a hormone-like fashion to induce root nodules in which the *Rhizobium* bacterium can fix atmospheric nitrogen. The bacterium grows at the expense of carbohydrates from the host, but provides fixed nitrogen for amino acid biosynthesis in return (Brencic and Winans 2005; Gray and Smith 2005). This symbiosis is a prime example of an intimate relationship between a soil bacterium and its host plant, and illustrates the concept behind the term 'plant growth-promoting rhizobacteria' (PGPR): in nitrogen-poor environments the *Rhizobium* bacterium promotes legume plant growth by providing a limiting nutrient.

Growth promotion by soil microorganisms is far from uncommon (Glick et al. 1999; Ryu et al. 2005) and can be considered part of a continuum in which interactions between plants and microorganisms range from deleterious (pathogens) to beneficial (PGPR). In the Netherlands, already 75 years ago observations were made by an assistant of Professor Johanna Westerdijk at the Phytopathological Laboratory 'Willie Commelin Scholten' in Baarn, about recovery from damping-off in turfgrass. The person, by the name of Van Luijk, identified several pathogenic *Pythium* species that were responsible for the disease, but he also observed that grass seeds germinated to a higher percentage in non-sterile than in sterilized soil (Van Luijk 1938). This was the first demonstration in the Netherlands that soil microorganisms can promote plant growth. The reason for this stimulatory effect of the biological agent present in the raw soil became clear only later. It turned out that non-pathogenic *Pythium* spp. were also present, took over and counteracted the actions of the pathogenic *Pythium* spp. and other deleterious soil microorganisms through microbial antagonism. These observations were the beginning of a research programme on antagonism between microorganisms that has been continuing to this day at Utrecht University.

The stimulation of seed germination and the recovery from damping-off of the turfgrass that were caused by the non-pathogenic *Pythium* spp. were apparent as a promotion of growth relative to appropriate control plants. However, in reality they were the result of disease suppression. Many bacteria in soil have similar properties (Compant et al. 2005; Haas and Défago 2005), but in a number of cases rhizobacteria can enhance plant growth in the absence of potentially pathogenic microorganisms, as has been shown in e.g. gnotobiotic systems (Van Loon and Bakker 2003). Over the years, several mechanisms of rhizobacterial growth promotion have been documented (Table 1). The ability to fix atmospheric nitrogen is present in various bacterial species that are either free-living in the soil, or associated with plant roots by growing endophytically (Dobbelaere et al. 2003). Poorly soluble inorganic nutrients that are rate-limiting for growth can be made available through the solubilizing action of bacterial siderophores or the secretion of organic acids (Vessey 2003). The high population densities of bacteria in the rhizosphere stimulate nutrient delivery and uptake by plant roots.

Other mechanisms of growth promotion involve modulation of plant regulatory mechanisms through the production of hormones or other compounds that influence plant development (Frankenberger and Arshad 1995). Many bacterial species are capable of producing auxin and/or ethylene, and synthesis of gibberellins and cytokinins has also been documented. Introduction of the rhizobacterial strain *Pseudomonas fluorescens* WCS417 in autoclaved soil promoted growth of *Arabidopsis* accession Col-0 by 33% (Pieterse and Van Loon 1999). A comparable growth promotion was seen when *Arabidopsis* seedlings were grown under gnotobiotic conditions on vertically oriented agar plates containing half-strength Hoagland nutrient medium. Compared to sterile grown control seedlings, WCS417-treated seedlings showed enhanced shoot and root development, enhanced greening and lateral root formation (S. van der Ent unpublished observation). Whether

Table 1 Mechanisms of plant growth promotion by rhizobacteria

Nitrogen fixation
Ion uptake
Iron, zinc, other essential micronutrients
Phosphate
Production of plant hormones
Auxins, gibberellins, cytokinins, ethylene
Modulation of plant development
ACC deaminase
'Elicitors'

WCS417 produces plant hormones is not known, but promotion of lateral root formation is a typical auxin effect (Tanimoto 2005). Obviously, enhanced lateral root formation increases the capacity to take up nutrients. For *Azospirillum brasilense* it has been shown that auxin is responsible for its growth-promoting action in wheat and pearl millet, as bacterial mutants that had lost 70% of their capacity to produce indole–acetic acid had lost their plant growth-promoting activity (Barbieri and Galli 1993).

Gibberellins and cytokinins both stimulate shoot development. Their effects on root growth are less well documented. Ethylene is usually considered an inhibitor of plant growth, but at low levels can actually promote growth in several plant species, including *Arabidopsis* (Pierik et al. 2006). At moderate levels it inhibits both root and shoot elongation, and at high levels it enhances senescence and organ abscission (Abeles et al. 1992). The direct precursor of ethylene in the plant biosynthetic pathway, 1-aminocyclopropane-1-carboxylic acid (ACC) is exuded from plant roots together with other amino acids. Rhizobacteria that possess the enzyme ACC deaminase can degrade ACC and utilize it as a carbon source. Under such conditions, re-uptake by the roots is prevented and the level of ACC in the roots is reduced. As a consequence, ethylene production by the roots is lowered, relieving inhibition of root growth. Thus, ACC deaminase-containing rhizobacteria can increase root growth by lowering endogenous ACC levels (Glick 2005). However, bacteria lacking ACC deaminase have also been shown to increase plant growth and such observations cannot be explained by known mechanisms. It is presumed that under such conditions bacterial cells possess certain surface components or secrete compounds that act as 'elicitors' of plant growth. Plant roots must be able to perceive and recognize such elicitors in ways similar to the recognition of elicitors from plant pathogens. In fact, plant pathogens might interfere with the action of PGPR by being perceived by similar receptors.

Plant-mediated disease suppression by rhizobacteria

When plants are growing naturally in soils, one cannot distinguish whether an apparent growth promotion is caused by bacterially stimulated plant growth or through suppression of deleterious soil microorganisms. Non-pathogenic rhizobacteria can antagonize pathogens through competition for nutrients, production of antibiotics and secretion of lytic enzymes (Handelsman and Stabb 1996; Van Loon and Bakker 2003). Such activities are particularly important in the rhizosphere where pathogenic fungi are attracted to plant roots. However, rhizobacteria can reduce the activity of pathogenic microorganisms not only through microbial antagonism, but also by activating the plant to better defend itself. This phenomenon, termed 'induced systemic resistance' (ISR) was first described by Van Peer et al. (1991) in carnation that was systemically protected against *Fusarium oxysporum* f.sp. *dianthi* upon treatment with strain WCS417, and by Wei et al. (1991) in cucumber, where six out of 94 rhizobacterial strains protected the leaves against anthracnose caused by *Colletotrichum orbiculare*. Protection as a result of microbial antagonism was excluded because the inducing rhizobacteria and the challenging pathogens were inoculated at, and remained confined to, spatially separated parts on the same plants. Hence, the protective effect was plant-mediated.

ISR confers on the plant an enhanced defensive capacity (Van Loon et al. 1998; Van Loon and Bakker 2005). Upon infection with a challenging pathogen this enhanced defensive capacity is manifested as a reduction in the rate of disease development, resulting in fewer diseased plants or in lesser disease severity. The induced resistance is also evident locally and sometimes does not extend systemically (Van Loon 2000). When only local, it is difficult to prove, because the inducing bacterium and the challenging pathogen are not separated from

each other and direct antagonism is difficult to rule out. Only when specific eliciting components of the inducer are active in stimulating resistance in the plant but inactive in antagonizing the pathogen in vitro on different types of media, can locally induced resistance be inferred. Induction of resistance by live organisms always requires proof that the organisms cannot contact each other, a condition that can be met when an inducing rhizobacterium remains confined to the roots and the challenging pathogen colonizes only the leaves. Under such situations the inducing bacterium must trigger the roots to locally produce a signal that moves to the leaves to activate the enhanced defensive capacity systemically. The nature of this mobile signal has so far remained elusive.

Since its discovery, rhizobacteria-mediated ISR has been documented in at least 15 plant species (Van Loon and Bakker 2006). Its induction has been shown to share several characteristics (Table 2A), but its expression can involve different physiological mechanisms (Table 2B). ISR can be induced by various non-pathogenic microorganisms and by some types of stress that activate the same response in the plant. In contrast to *R*-gene-mediated resistance, it is not specific but active against all types of pathogens, as well as against several nematodes and insects. Once induced, plants may remain protected for a considerable part of their lifetime, indicating that when the state of ISR has been triggered in the plant, it is rather stable (Van Loon et al. 1998).

Upon challenge inoculation, ISR is expressed as a result of the altered physiological state of the plant. Expression may take different forms, depending on the activity of the inducing rhizobacterium and the nature of the interaction between the pathogen and the plant (Chester 1933). In fact, 'induced resistance' is an operational term to denote a condition in which a plant becomes less diseased compared to a control plant that was not induced. There are many ways in which developmental and environmental factors can influence plant-pathogen interactions. Damping-off due to infection by *Pythium*, *Fusarium* or *Rhizoctonia* is often confined to the seedling stage. Any condition that results in more rapid plant growth will shorten the vulnerable stage and be apparent as enhanced resistance. Rhizobacteria acting through growth promotion could protect plants through this mechanism. A similar type of ISR could occur in potato where accelerated development leads to enhanced adult plant resistance against late blight caused by *Phytophthora infestans* (Visker et al. 2003).

Some reports on ISR have indicated reduced symptom expression in the absence of a reduction in pathogen proliferation. This tolerance of the plant to the pathogen must have a physiological basis. Examples are the reduced damage of *Pythium ultimum*-infected cucumbers and lesser extent of soft rot of potato infected by *Erwinia carotovora* pv. *carotovora* upon prior treatment of the plants with ACC deaminase-containing rhizobacterial strains. By lowering the level of stress ethylene in the plant due to pathogenic attack, ACC deaminase acted synergistically with other mechanisms of biocontrol in reducing symptom development without having an effect on the population density of the pathogen (Wang et al. 2000).

Reduced disease can also be the outcome of alterations in the microbial populations in the rhizosphere as a result of altered host physiology. Numbers of resistance-inducing bacteria may be changed, or their expression of resistance-inducing traits may be altered (Mark et al. 2005). Plants commonly react to root colonization by rhizobacteria by increasing the release of exudates, and quantity and composition of root exudates vary with plant developmental stage (Phillips et al. 2004). Thus, plant growth promotion could alter root exudation. Moreover, rhizobacteria that act as minor pathogens or are perceived by the plant as a potential threat, are likely to change the rate and composition of exudates, and to increase the release of lysates.

The population densities and the diversity of the root microflora may affect the number and activity of resistance-inducing rhizobacteria. Quorum sensing (QS) within and between bacterial populations is a major regulatory mechanism in bacteria to adjust their metabolism to crowded conditions or other changes in the biotic and abiotic environment (Whitehead et al. 2001). Interference with bacterial QS by host plants has been documented. Plants can produce and secrete various compounds that mimic QS signals of bacteria and, thereby, alter bacterial activities in the rhizosphere (Bauer and Mathesius 2004). The ecological diversity and its consequences for metabolic activity of rhizosphere bacteria are only poorly known at present and deserve further investigation.

Table 2 The nature of systemically induced resistance in plants

(A) *Characteristics of induced systemic resistance*
The defensive capacity of the plant is enhanced through microbial stimulation or similar stresses
The enhanced defensive capacity is expressed systemically throughout the plant
Induced systemic resistance is active against fungi, bacteria, viruses and, sometimes, nematodes and insects
Once induced, systemic resistance is maintained for prolonged periods
(B) *Mechanisms of induced systemic resistance*
Developmental, escape: linked to growth promotion
Physiological, tolerance: reduced symptom expression
Environmental: associated with microbial antagonism in the rhizosphere; altered plant-insect interactions
Biochemical, resistance: induction of cell wall reinforcement,
Induction of phytoalexins
Induction of pathogenesis-related proteins
'Priming' of defence responses (resistance)

Rhizobacteria can also alter plant secondary metabolism, resulting in changed plant-insect relationships. Root colonization of cucumber by four different PGPR reduced the level of cucurbitacin, which acts as a feeding stimulant to cucumber beetles (Zehnder et al. 1997). Similar effects on insects that can transmit viruses, might reduce virus diseases through induced resistance against the insect vector rather than against the virus itself.

Finally, non-pathogenic rhizobacteria may activate inducible defence mechanisms in the plant in a similar way to pathogenic microorganisms. Such mechanisms can include reinforcement of plant cell walls, production of anti-microbial phytoalexins, synthesis of pathogenesis-related proteins (PRs) (Hammond-Kosack and Jones 1996), as well as an enhanced capacity to express these defence responses upon challenge inoculation with a pathogen, a mechanism known as 'priming' (Conrath et al. 2006). Activation of defence reactions suggests that even a beneficial rhizobacterium may be perceived by the plant as a potential threat, and that such perception involves production of resistance-eliciting compounds that act mechanistically similar to elicitors produced by plant pathogenic fungi and bacteria. Both nitrogen-fixing *Rhizobia* in legume root nodules and vesicular–arbuscular (VA) mycorrhizal fungi in roots have been shown to activate plant host defences when the symbiotic interaction becomes unproductive (Parniske et al. 1991; Hause and Fester 2005). Plants possess sensitive mechanisms to perceive both fungi and bacteria through conserved components that are specific to their kingdoms and act as general elicitors. These are commonly referred to as 'pathogen-associated molecular patterns' (PAMPs) (Nürnberger and Lipke 2005). During compatible plant-pathogen interactions and effective symbioses, the microorganisms actively suppress defensive activities in the host (Da Cunha et al. 2006). The relationship between root-colonizing, resistance-inducing PGPR and their hosts seems substantially less intimate than with either *Rhizobia* or mycorrhizal fungi, but the idea that PGPR may at the same time trigger and suppress defence reactions in the host, deserves consideration.

Expression of systemically induced resistance in the plant

Besides biochemical techniques, such as enzyme activity measurements and protein analysis, the development of molecular-biological techniques has allowed the reaction of plants to rhizobacteria to be determined at the transcriptional level by analyzing differential gene expression by a variety of techniques. Changes in a number of host plants in reaction to several resistance-inducing strains have been documented (Table 3). Many authors report increases in stress-related enzyme activities such as phenylalanine ammonia-lyase, peroxidase, polyphenoloxidase, ß-1,3-glucanase and chitinase, as well as induction of specific PRs in leaves of plants of which the roots were colonized by resistance-inducing PGPR (reviewed in Van Loon and Bakker 2005,