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Mechanism of Action of the Plant Growth Promoting Bacterium *Paenibacillus polymyxa*

BY

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Abstract
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Paenibacillus polymyxa belongs to the group of plant growth promoting rhizobacteria (PGPR). Activities associated with *P. polymyxa*-treatment of plants in earlier experiments include, e.g., nitrogen fixation, soil phosphorus solubilization, production of antibiotics, auxin, chitinase, and hydrolytic enzymes, as well as promotion of increased soil porosity. My thesis work showed that, in stationary phase, *P. polymyxa* released the plant hormone cytokinin isopentenyladenine, in concentrations of about 1.5 nM.

In a gnotobiotic system with *Arabidopsis thaliana* as a model plant, it was shown that *P. polymyxa*-inoculation protects plants; challenge by either the pathogen *Erwinia carotovora* (biotic stress) or induction of drought (abiotic stress) showed that pre-inoculated plants were significantly more resistant than control plants. By RNA-differential display on RNA from *P. polymyxa*-treated or control plants, changes in gene expression were tested. One mRNA, encoding ERD15 (drought stress-responsive gene) showed a strong inoculation-dependent increase in abundance. In addition, several biotic stress-related genes were also activated by *P. polymyxa*.

Antagonism towards the fungal pathogens *Phytophthora palmivora* and *Pythium aphanidermatum* was studied. *P. polymyxa* counteracted the colonization of zoospores of both oomycetes on *A. thaliana* roots, and survival rates of plants treated with *P. polymyxa* were much higher when challenged by *P. aphanidermatum*.

Using a green fluorescent protein-tagged isolate of *P. polymyxa*, colonization of *A. thaliana* roots was investigated. Two main conclusions can be drawn. Firstly, the bacterium enters the root tissue (but not leaves) and is abundantly present in intercellular spaces. Secondly, the root becomes severely damaged, indicating that – under some conditions – *P. polymyxa* is a "deleterious bacterium", and in others it promotes growth. Based on work presented in my thesis, I argue that a balance between the activities of a PGPR, the genetic background and physiological state of a plant, and the environmental conditions employed in test systems, ultimately determines the resulting effect.

Keywords: Plant growth promotion, Induced resistance, Biocontrol, Deleterious rhizobacterium

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"It is the time you have spent on your rose that makes your rose so important".

Antoine de Saint-Exupery 'The Little Prince'

MAIN REFERENCES

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I **Timmusk, S.,** Nicander, B., Granhall, U., and E. Tillberg
Cytokinin production by *Paenibacillus polymyxa*.
Soil Biology and Biochemistry, 31 (1999): 1847-1852.

II **Timmusk, S.,** and E. G. H. Wagner
The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: A possible connection between biotic and abiotic stress responses.
*Molecular Plant-Microbe Interactions,*12 (1999): 951-959.

III **Timmusk, S.**, Grantcharova, N., Flärdh, K., and E. G. H. Wagner
Paenibacillus polymyxa colonization of *Arabidopsis thaliana* roots
Submitted

IV **Timmusk, S.,** van West, P., Gow, N. A. R., and E. G. H. Wagner
Antagonistic effects of *Paenibacillus polymyxa* towards the oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*
Manuscript.

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ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
DP	degree of polymerisation
ET	ethylene
GFP	green fluorescent protein
IAA	indole-3-acetic acid
ISR	induced systemic resistance
JA	jasmonic acid
OGAs	oligogalacturonides
PGPR	plant growth promoting rhizobacteria
PR	pathogenesis-related
RT-PCR	reverse transcription-polymerase chain reaction
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
TF	transcription factor

INTRODUCTION

PLANT RHIZOSPHERE

The term "rhizosphere" was already introduced by Hiltner in 1904, and is now defined as a volume of soil surrounding plant roots in which bacterial growth is stimulated (Sorensen, 1997). The rhizosphere has attracted much interest since it is a habitat in which several biologically important processes and interactions take place. The rhizosphere is populated by a diverse range of microorganisms, and the bacteria colonizing this habitat are called rhizobacteria (Schroth & Hancock, 1982).

PLANT ROOT COLONIZING BACTERIA

Root exudates are believed to determine which microorganisms colonize roots in the rhizosphere (Kunc & Macura, 1988). It is now known that plant roots also generate electrical signals; it has been shown that zoospores of oomycetic pathogens take advantage of these signals to guide their movements towards the root surface (Gow *et al.*, 1999).

It has been estimated that about 30% of plant photosynthate production is released via root exudation (Smith *et al.*, 1993). Both passive leakage and active secretion are involved (Rougier & Chaboud, 1989). Leakage involves low molecular weight compounds release while secretion usually involves high molecular weight compounds that are actively transported across the cell membranes (Rougier & Chaboud, 1989).Composition and extent of exudation are determined genetically (Bolton *et al.*, 1993). This involves a certain cost for the plant, and therefore must provide a selective advantage. It has often been suggested that root exudation evolved in plants as a means to stimulate active microflora (Bolton *et al.*, 1993). Exudation can provide both physical and chemical benefits to plants. E.g., root mucilages reduce friction between root tips and the soil and reduce root desiccation, improve the contact between the root and the soil, and contribute to soil structural stability (Rougier & Chaboud, 1989). Root exudates also attract microorganisms (Jaeger *et al.*, 1999; Lemanceau *et al.*, 1995). Conversely, rhizobacteria can also elicit root exudation (Bolton *et al.*, 1993) in a species-specific manner. E.g., metabolites

produced by *Pseudomonas aeruginosa* stimulated root exudates by perennial ryegrass twelve-fold (Merharg & Killham, 1995).

A variety of techniques have been used to study bacteria in their habitat, i.e. in mixed populations. Often but not always, cultivation and staining techniques can be employed. Since the rhizosphere harbours a great variety of bacteria, each with different nutritional requirements, it is impossible to apply cultivation techniques to all bacteria. It has also become clear that a significant fraction of bacteria belong to the group of viable but not culturable (VBNC) bacteria (Roszak & Colwell, 1987), thus resisting conventional cultivation attempts. Sometimes, the diversity in microbial communities bacteria in a given environment, such as the rhizosphere, can be approached by PCR-based techniques. For instance, an analysis of PCR-amplified rRNA/rDNA fragments by denaturating gradient gel electrophoresis, or temperature gradient gel electrophoresis, can resolve characteristic bands due to different GC-content. Such techniques have the potential to analyze the major constituents of microbial communities.

Often one needs to find out precisely where bacteria are located (e.g. during colonization), and/or what their physiological activity status is. In these cases, a number of marker genes have been developed for tagging of selected strains. Since the discovery of green fluorescent protein (GFP) it has been used extensively as a marker to detect microbes in environments, and to study microbial interactions with plants (e.g. Chalfie *et al*., Stoltzfus *et al*., 2000, Tombolini *et al*., 1999, Errampalli *et al*., 1999). The strong fluorescence of GFP allows rapid detection regardless of the energy status of the cells (Unge *et al.*, 1999), and provides a very stable marker in environmental samples (Elvang *et al.*, 2001; Unge *et al.*, 1999). Even VBNC bacteria have been monitored with GFP-methods (Lowder *et al.*, 2000). GFP fluorescence has been improved by mutations in the chromophore which changed excitation/emission spectra. Mutated versions of the *gfp* gene have given a range of derivatives that differ in emission wave length, e.g. enhanced cyan (ECFP), enhanced green (EGFP), and enhanced yellow (EYFP). Thus, different populations of tagged bacteria in the rhizosphere can be visualized simultaneously at the single cell level (Bloemberg *et al.*, 2000). To monitor also the bacterial energy status, combinations of *gfp* and other marker genes, e.g *luxAB* or *gusA*, have been used (Elvang *et al.*, 2001; Gau *et al.*, 2002; Unge *et al.*, 1999). Colonization sites for, e.g., *Azospirillum brasiliense* have been mapped using *gfp*- and *gusA*-tagging, simultaneously on emerging roots (Ramos *et al.*, 2002).

PLANT GROWTH PROMOTING RHIZOBACTERIA

Plant root colonizing bacteria can function as harmful, deleterious rhizobacteria (DRB) or beneficial, plant growth promoting rhizobacteria (PGPR). Rhizobacteria that inhibit plant growth have been described as deleterious rhizobacteria (Suslow & Schroth, 1982). Plant growth promotion by rhizobacteria can occur directly and indirectly (Glick, 1995; Persello-Cartieaux *et al.*, 2003). There are several ways by which plant growth promoting bacteria can affect plant growth directly, e.g. by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of siderophores that solubilize and sequester iron, or production of plant growth regulators (hormones) that enhance plant growth at various stages of development (Figure 1). Indirect growth promotion occurs when PGPR promote plant growth by improving growth restricting conditions (Glick *et al.*, 1999). This can happen directly by producing antagonistic substances, or indirectly by inducing resistance to pathogens (Glick, 1995). A bacterium can affect plant growth by one or

more of these mechanisms, and also use different abilities for growth promotion at various times during the life cycle of the plant (Glick *et al.*, 1999).

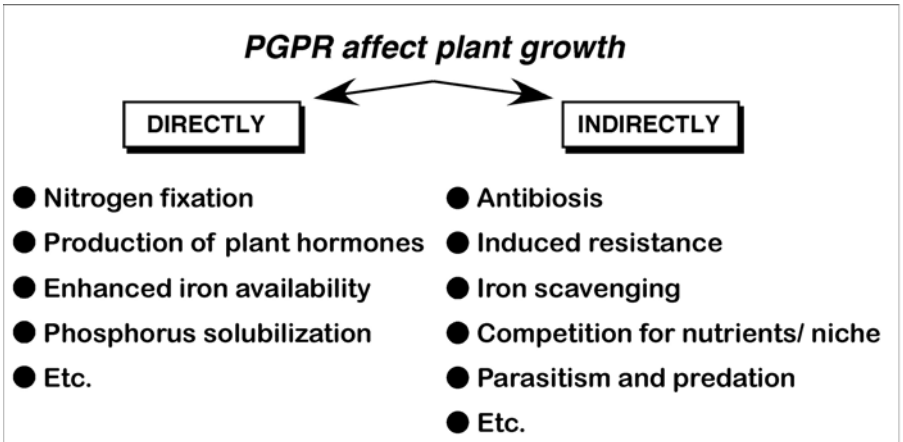

Fig. 1. Plant growth promoting bacteria affect plant growth directly and indirectly.

DIRECT PLANT GROWTH PROMOTION

The ways by which PGPR can influence plant growth directly may differ from species to species as well as from strain to strain. Symbiotic plant colonizers such as rhizobia mostly contribute to plant growth by nitrogen fixation. Free-living rhizobacteria usually do not rely on single mechanisms of promoting plant growth (Glick *et al.*, 1999). In addition to nitrogen fixation, several PGPR are also able to provide the plant with sufficient iron in iron-limited soils (Wang *et al.*, 1993), or other important minerals, e.g. phosphate (Singh & Singh, 1993).

Organic substances capable of regulating plant growth produced either endogenously or applied exogenously are called plant growth regulators. They regulate growth by affecting physiological and morphological processes at very low concentrations (Arshad & Frankenberger, 1998). Several microorganisms are capable of producing auxins, cytokinins, gibberellins, ethylene (ET), or abscisic acid (ABA). Auxins are produced by several rhizobacterial genera, e.g. *Azospirillum*, *Agrobacterium, Pseudomonas,* and *Erwinia* (Costacurta & Vanderleyden, 1995). In the case of *Azospirillium*, bacterial colonization takes place in the zone of lateral root emergence. *Azospirillum* inoculation increases the density and length of root hairs as well as the elongation rates of lateral roots, increasing the root surface area (Dobbelaere *et al.*, 2001; Fallik *et al.*, 1994). Using an *Azospirillum brasiliense* indole-pyruvate decarboxylase mutant producing only 10% of indoleacetic acid (IAA) compared to wild-type, a reduced ability to promote plant growth was demonstrated (Dobbelaere *et al.*, 1999).

Ethylene, a hormone produced in all plants, mediates several responses to developmental and environmental signals in plants. Its involvement in plant growth when excreted around the roots has also been shown (Arshad&Frankenberger,1998). The PGPR *Pseudomonas putida* GR12-2 stimulates plant root elongation (Glick *et al.*, 1994). Mutant strains lacking aminocyclopropane carboxylic acid (ACC) deaminase activity were unable to promote root elongation of canola seedlings; this enzyme hydrolyzes ACC, the immediate precursor of ET in plants

Cytokinins and gibberellins are produced in the rhizosphere by several bacteria, e.g. *Azospirillum, Agrobacterium,* and *Pseudomonas* genera (Gaudin *et al.*, 1994). Cytokinins promote root formation, but a minor overproduction of cytokinins instead leads to inhibition of root development, and severely deficient cytokinin mutant plants do not survive (Binns, 1994). Cytokinins are believed to be the signals involved in mediating of environmental stresses from roots to shoots. (Jackson, 1993). Nevertheless, more studies need to be conducted before cytokinin signalling can be fully understood.

The cytokinin balance is influenced by the levels of other growth regulators, e.g. auxins (Kaminek *et al.*, 1997), as well as by environmental cues. Inhibition of root growth by cytokinins is probably mediated by increasing auxin pools to inhibitory levels (Coenen & Lomax, 1998 and references therein), or ET pools (Cary *et al.*, 1995). Thus, PGPR can facilitate growth by altering the hormonal balance in the affected plant.

INDIRECT PLANT GROWTH PROMOTION

Several PGPR are known to reduce the effects of plant stresses by limiting phytopathogen-caused damage. This can occur, e.g., via local antagonism of soilborn pathogens, or by induction of systemic resistance against pathogens throughout the entire plant.

BIOCONTROL OF SOILBORN PATHOGENS

Over the last decades, a great diversity of rhizospheric microorganisms has been described, characterized, and - in many cases - tested for activity as biocontrol agents against soilborn pathogens. Such microorganisms can produce substances that may limit the damage caused by phytopathogens, e.g. by producing antibiotics, siderophores, and a variety of enzymes. These microorganisms can also function as competitors of pathogens for colonization sites and nutrients. Nevertheless, biocontrol has not yet become widely applied, for several reasons. E.g., the efficiency of a biocontrol strain under field conditions is likely to be affected by several environmental conditions: pH, temperature, water content, and interactions with other microorganisms. Also, some biocontrol agents that showed promising traits in initial experiments failed to be efficient rhizosphere colonizers under more complex biological conditions. This argues that it is worthwhile to address these limitations, and the genetic, biochemical, and physiological factors that contribute to the activity of biocontrol agents, by careful studies.

Antibiotic production

In many biocontrol systems, one or more antibiotics have been shown to play a role in disease suppression. Molecular tools have been effective here, because mutants defective in antibiotic production are easily obtained, and *in vitro* assays are useful tests. The most widely studied group of rhizospheric bacteria with respect to the production of antibiotics is that of the fluorescent pseudomonads. The first antibiotics described as being implicated in biocontrol were phenazine derivatives produced by fluorescent pseudomonads (Weller & Cook, 1983). Their role has been elucidated by transposon insertion mutations which result in a defect in production of phenazine-1-carboxylate, thus reducing disease suppressive activity (Pierson & Pierson, 1996). The genes encoding the enzymes responsible for synthesis of the metabolites have been isolated and their regulation studied (Bangera & Thomashow, 1996; Pierson *et al.*, 1995). Global regulatory elements have been shown to coordinate the production

of these metabolites (Pierson *et al.*, 1994). The presence of populations of other bacteria can influence phenazine production by *P. aureofaciens,* since mutants lacking the ability to produce an autoinducer signal required for induction of antibiotics synthesis can use autoinducers produced by other (related) rhizosphere inhabitants (Pierson & Pierson, 1996; Wood & Pierson, 1996). Also, other environmental sensors such as the regulatory proteins GacA and ApdA can influence the production of secondary metabolites involved in pseudomonad biocontrol (Corbell & Loper, 1995; Haas *et al.*, 2002). In addition, sigma factors are important for regulation of antibiotic production in fluorescent pseudomonads; housekeeping factor $sigma^{70}$ and the stress-related $sigma^{s}$ have critical roles in production of antibiotic metabolites in disease suppression (Schnider *et al.*, 1995).

Siderophores

Iron is abundant in the Earth's crust but most of it is in the highly insoluble form of ferric hydroxide, and thus unavailable to organisms in soil solution. Some bacteria have developed iron uptake systems (Neilands & Nakamura, 1991). These systems involve a siderophore – an iron binding ligand – and an uptake protein, needed to transport iron into the cell. It has been suggested that the ability to produce specific siderophores, and/or to utilize a broad spectrum of siderophores, may contribute to the root colonizing ability of *Pseudomonas* strains. The production of siderophores that chelate, and thereby scavenge, the ferric iron in the rhizosphere, may result in growth inhibition of other microorganism whose affinity for iron is lower (Kloepper *et al.*, 1988).

Siderophore mechanisms will only be relevant under conditions of low iron availability. As soil pH decreases below 6, iron availabity increases and siderophores become less effective (Neilands & Nakamura, 1991). Optimal suppression of pathogens occurred at levels between 10^{-19} -10^{-24} M. The critical level of iron at which a siderophore-producing strain of *Pseudomonas putida* suppressed the growth of a fungal pathogen, *Fusarium oxysporium*, was found to be < 10^{-16} M (Neilands & Nakamura, 1991). Since the synthesis of each siderophore generally requires the activity of several gene products (Mercado-Blanco *et al.*, 2001), it is difficult to genetically engineer bacteria to produce modified siderophores. Complementation studies of siderophore-deficient mutants of *P. fluorescens* M114 indicated that at least five separate genetic loci are needed to encode the enzymes involved in the synthesis of the siderophore pseudobactin M114 (O'Sullivan *et al.*, 1990).

Parasitism

An additional mechanism by which biocontrol agents can reduce plant diseases is biocontrol agent parasitism on pathogens, mostly fungi. Digestion of the parasite cell wall is accomplished by several excreted enzymes including proteases, chitinases and glucanases. Individually, all these enzymes display antifungal activity, but they often act synergistically with antibiotics (Lorito *et al.*, 1993; Lorito *et al.*, 1994).

Competition for nutrients and niches

In addition to the above described and commonly reported antibiosis mechanisms there are other ways by which rhizobacteria can inhibit pathogens. One example concerns competition for nutrients and suitable colonization niches on the root surface. Such mechanisms are often overlooked, in part because they are difficult to study in biological systems. Competition for nutrients supplied by root exudates is probably a significant factor in most interactions between PGPR and pathogens.

Populations of bacteria established on a plant root could act as a sink for the nutrients in the rhizosphere, hence reducing the nutritional element availability for pathogen stimulation or subsequent colonization of the root. This mechanism is most probably often used by fluorescent pseudomonads due to their nutritional versatility, and because of their high growth rates in the rhizosphere (Sorensen, 1997).

BIOCONTROL BY INDUCING SYSTEMIC RESISTANCE

Systemic resistance refers to an increased level of resistance at sites within that plant distant to those at which induction had occurred. How bacteria trigger systemic resistance is still largely unknown. Several sequential events have however been envisaged. A bacterial component, most likely a metabolite, is perceived by the plant root/leaf through binding to a receptor. This recognition mediates the extracellular signal to an intracellular signal. Thereafter, the metabolite itself, or a signal generated by the plant cell, initiates a cascade of signal transduction. Eventually, the translocated signal is perceived by distant plant cells, triggering the activation of the defense arsenal of the challenged host plant.

Signalling in plants

Signal transduction pathways are activated upon microbial elicitor challenge leading in turn to activation of different sets of effector molecules. The application of molecular, genetic and biochemical techniques had led to the identification of key components of the signalling pathways that result in defense responses in *Arabidopsis*. Signalling molecules like salicylic acid (SA) (Metraux *et al.*, 1990), jasmonate (JA) (Penninckx *et al.*, 1996), and ethylene (ET) (Boller, 1991), when accumulating, coordinate the defense responses and, when applied exogenously, are even sufficient to induce resistance (Ryals, 1996). It has been shown that these signalling molecules activate specific sets of defense-related genes: SA induces genes encoding pathogenesis-related proteins (PRs) (Uknes *et al.*, 1992). These proteins have antimicrobial activity (Kombrink & Somssich, 1995). ET is involved in the expression of the genes encoding *Hel* (a heveine-like protein; (Potter *et al.*, 1993)), *ChiB* (basic chitinase; (Samac & Shah, 1994)), and *Pdf1.2* (a plant defensin; (Penninckx *et al.*, 1996)). Also JA has been shown to activate the genes encoding these three proteins (Penninckx *et al.*, 1996), all of which also possess antifungal activity. In addition, JA also activates the gene encoding a vegetative storage protein, *Atvsp* (Berger *et al.*, 1995). This protein accumulate in vacuoles, but its putative roll in defense activity has not been established.

It has been reported that, in *Arabidopsis*, two general defense pathways are induced, induced systemic resistance (ISR) and systemic acquired resistance (SAR). ISR is a rhizobacterially mediated systemic resistance that does not involve any damage to plant. By contrast, SAR is induced by foliar pathogens and results in activation of resistance mechanisms in uninfected parts (Figure 2). Thus, in SAR a first infection predisposes the plant to resist further attacks. SAR induction is dependent on the accumulation of SA and requires the regulatory (activator) protein NPR1**.** Beside SA accumulation, several JA- and ET-dependent resistance mechanisms that are independent of SA have also been reported (Thomma *et al.*, 1998; Thomma *et al.*, 2001a). JA and ET act synergistically in inducing genes for several PR proteins (Norman-Setterblad *et al.*, 2000). ET has been shown to *enhance* JA-dependent responses (Xu *et al.*, 1994), whereas SA *inhibits* the expression of JA–

dependent defense genes (Gupta *et al.*, 2000; Vidal *et al.*, 1997). Similarly, JA has also been shown to interfere with SA-dependent signalling (Niki *et al.*).

ISR can be triggered in plants which are unable to accumulate SA (*NahG* mutant plants). Based on this, one can conclude that SA is not required for ISR activation in *Arabidopsis*. Moreover, PR proteins do not usually accumulate in induced plants. However, the regulator NPR1 protein is required for expression of ISR (Pieterse *et al.*, 1996). *Arabidopsis* mutant plants in which either the ET or JA-responsive genes *etr1, ein2, ein7, or jar1* are defect, thus conferring a reduced sensitivity to ET and JA, are also affected in their expression of ISR. Application of either ACC or JA to wild-type plants induces a resistance that is not associated with the accumulation of PRs, but is dependent on a functional *npr1* gene. Treatment of the *jar1* mutant plant with ACC was effective in inducing resistance (Pieterse *et al.*, 1998). Application of JA to the *etr1* mutant, in contrast, did not elicit ISR (Pieterse *et al.*, 1998). Both compounds, ACC and JA, were ineffective in inducing resistance in the *npr1* mutant, thus supporting the requirement for this key regulator in the response. These results indicate that responsiveness to JA and ET are required sequentially, and before NPR1, in the ISR signal transduction pathway (Pieterse *et al.*, 1998).

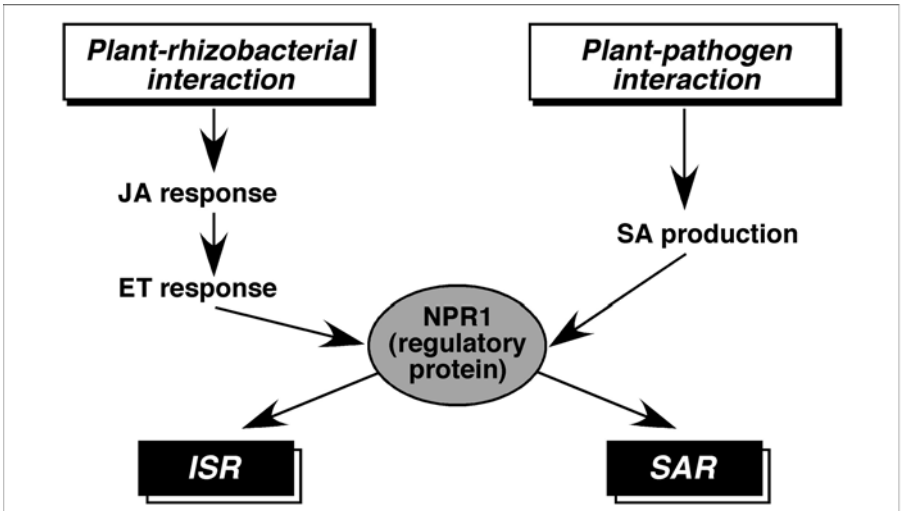

Fig 2. Signaling pathways in induced resistance (After Pieterse & Van Loon 1999).

Microbial signals involved in inducing resistance

Both types of induced resistance described above are initiated by microbial signals which are percieved by the plant.

Microbial signals in ISR

Rhizobacterially mediated systemically induced resistance is the result of a process that does not involve any obvious damage to the plant. For induction of ISR in radish, 10^5 colony forming units of the inducing *Pseudomonas sp*. strain per gram of root tissue was reported as a minimum threshold (Raaijmakers *et al.*, 1995). This level probably exceeds that found for individual bacterial strains in natural soils but can easily be obtained in more artificial systems.

The inducing bacteria are mostly saprophytic and can simultaneously induce resistance and promote plant growth (Pieterse & van Loon, 1999). In some cases, transposon mutagenesis experiments have indicated factors involved in induced resistance. E.g., it has been shown for three *Pseudomonas* strains that an outer membrane lipopolysaccharide (LPS) with strain specific O-antigenic carbohydrate side-chains is the elicitor (Leeman *et al.*, 1995). The purified siderophore of *P. fluorescens*, pseudobactin 374, could induce resistance in radish (Leeman *et al.*, 1996). It has also been shown that treatment of raddish plant roots even with nanogram quantities of SA was sufficient to induce systemic resistance against *Fusarium oxysporum* (Leeman *et al.*, 1996). *Arabidopsis* roots are capable of recognizing the presence of bacterial flagella. A receptor for bacterial flagellin was characterized in *Arabidopsis* roots (Gomez-Gomez & Boller, 2000); binding of flagellin leads to a reduction in root elongation (Gomez-Gomez *et al.*, 1999). As most resistance-inducing rhizobacteria promote growth and enhance root elongation (Glick *et al.*, 1999) the flagellin-induced growth inhibition must be compensated by other activities.

Microbial signals in SAR

Elicitors of diverse chemical nature from different plant pathogenic microbes have been shown to trigger plant defense responses. These can be (poly)peptides, glycoproteins, lipids, oligosaccharides (Nurnberger, 1999). **Pathogen** recognition can be a **specific** recognition event between an avirulence (*avr*) gene product of a pathogen and a corresponding, cognate, resistance (R) gene product of the host plant as in a gene–for-gene type of resistance (Flor, 1971; Keen, 1990). More than 30 bacterial avirulence genes have been cloned and proven to be determinants of incompatibility in the interactions between bacteria and the resistant plant (Leach & White, 1996). Most pathogen-derived elicitors are **non-specific** and induce defense responses in a great variety of plants (Kombrink & Somssich, 1995). Biotic elicitors originate either from host plants (endogenous elicitors) or from the plant pathogen (exogenous elicitors). **Exogenous** elicitors can be structural components of the pathogen surface or pathogen-derived metabolites. They can be released during pathogen growth or through the action of hydrolytic enzymes from the plant (Ebel & Cosio, 1994), and vary in their chemical nature (e.g., oligosaccharides, glycoproteins and peptides; (Nurnberger *et al.*, 1994)). Among the best characterized **endogenous** elicitors are the oligogalacturonides (OGAs) derived from the pectic components of cell walls. These are released upon cell damage and wounding, or through the action of pathogen-derived pectinases (Walton, 1994). The eliciting activity of the cell wall fragments is dependent on the degree of polymerisation (DP). OGAs with DP between 10 and 20 generally exhibit the strongest biological activity (Reymond *et al.*, 1996). The activation of plant defense responses by the elicitors is mediated by receptors that bind these molecules and initiate intracellular signal transduction. Some plasma membrane-associated receptors or elicitor binding proteins have been identified (Nurnberger *et al.*, 1995).

Several phytopathogenic bacteria produce harpins, a class of acidic, glycine-rich, heat stable proteinaceous elicitors of hypersensitive response (HR) in various plant-pathogen interactions (Wei *et al.*, 1992). Harpins have also been shown to trigger SAR (Strobel *et al.*, 1996). Their role in pathogenesis and defense signalling has been difficult to access since it may be masked by massive production of pectinolytic enzymes in pathogens such as *E. carotovora*.

Recently the role of harpins as elicitors of plant defense responses has gained strong support. Apparently, the elicitor harpin HrpN produced by *Erwinia* acts in concert, and cooperatively, with OGAs in plant defense signalling in *Arabidopsis* (Kariola *et al*., 2003).

To defend against microbial invaders, plants have to use a variety of defense systems to build up physical and chemical barriers. The efficiency of these barriers will decide whether ultimately the plant will be protected against the pathogen, or will become infected and harmed/ killed. With new experimental tools, such as for instance microarrays, it is now possible to analyze genome-wide changes in gene expression occurring in response to specific treatments. cDNA microarray analysis has, e.g., shown that *Arabidopsis* leaves inoculated with *Alternaria brassicola*, or treated with SA, JA, or ET, up or down-regulate hundreds of genes in response to one or more of these treatments (Schenk *et al*., 2000). The magnitude of the effect hints at a complex network of regulatory interactions and coordination.

PLANT ADAPTATION TO ABIOTIC STRESSES

Plants are constantly exposed to a variety of environmental stresses which limit plant productivity. Maximal productivity is rarely achieved since crop plants are often grown in environments to which they are not fully adapted. Over several centuries, breeding programmes have focused on generating crop species with enhanced productivity under suboptimal environmental conditions. Much work has been invested in understanding the mechanisms behind plant stress responses.

Drought, salt stress and freezing are abiotic stresses which all lead to cellular dehydration through different mechanisms. Even so, many plant species mount the same or similar molecular responses to all of these stresses. Moreover, many genes are induced by *all* these stresses, and the hormone abscisic acid (ABA) is involved in regulation. Control mechanisms for abiotic stress tolerance are based on activation and regulation of specific sets of stress-related genes involved in signalling, transcriptional control, protection of membranes and proteins, and free radical and toxic compound scavenging.

Heat-shock proteins (Hsp) and late embryogenesis abundant (LEA)-type proteins are two major types of stress-induced proteins that accumulate upon dehydration stress (Thomashow, 1998). The function of these proteins is largely unknown, but they often appear to act as molecular chaperones aiding in protein synthesis, targeting, maturation and degradation - a broad array of normal cellular processes (Török *et al*., 2001).

Recently it has been found that the dehydration-responsive transcription factors (TF) bind to corresponding cis-acting elements in promoters that contain the same motif as TFs (Thomashow *et al*., 2001) and activate their transcription. Several of those TFs are themselves stress-inducible. Some stress-responsive genes may share the same TF as indicated by the overlap of the gene expression profiles induced in response to different stresses (Chen *et al*., 2000). Significant improvement of stress tolerance was found when a single TF was overexpressed in *A. thaliana* plants (Thomashow, 1998).

Osmolytes are small organic molecules which are highly soluble and non-toxic to cells even at high concentrations. Accumulation of osmolytes lowers the water potential, and thus cells can maintain their water content and hence, their turgor. Recent studies indicate that osmolytes can also be free-radical scavengers or chemical chaperones by directly stabilizing membranes and proteins (Diamant *et al*., 2001). Osmolytes fall into three major groups: amino acids (e.g. proline), quaternary amines

(e.g. glycine betaine) and sugars (e.g. trehalose, mannitol). Recently, it has been shown that overexpression of osmolyte-producing genes can result in enhanced tolerance to dehydration stress (Garg *et al.*, 2002).

Abiotic stresses are usually accompanied by the formation of reactive oxygen species (ROS) (Mittler, 2002) which damage membranes and macromolecules. Plants have developed several antioxidation strategies to scavenge these toxic compounds. In addition to osmolytes, which also can act as antioxidants, enzymes such as catalase, superoxide dismutase, ascorbate peroxidase and glutathione reductase are produced. Transgenic tobacco plants overproducing some of these compounds showed reduced damage when exposed to oxidative stress (Oberschall *et al.*, 2000). Given a better understanding of these pathways, it may be possible to obtain plants with multiple stress tolerance (Bartels & Salamini, 2001).

AIM OF THE STUDY

The long term objective of my research was to understand the mechanism of action of the plant growth promoting bacterium *P. polymyxa* on host plants at a molecular level, and to study the prospects of enhancing plant fitness to biotic and abiotic stresses using rhizobacterial isolates.

More defined objectives of that project were to investigate the questions

1. Does *P. polymyxa* produce cytokinins under defined conditions?

2. Can *P. polymyxa* induce resistance against *E. carotovora* in *A. thaliana*?

3. Is the resistance of the SAR- or ISR-type?

4. Does *P. polymyxa* induce drought tolerance in *A. thaliana,* and - if so - what are the mechanisms involved?

5. Can *P. polymyxa* antagonize root pathogens such as *Phytophthora palmivora* and *Pythium aphanidermatum,* and - if so - what are the mechanisms involved?

6. What is the pattern and mode of colonization of *P. polymyxa* on *A. thaliana* roots?

PRESENT INVESTIGATION

P. polymyxa (previously *Bacillus polymyxa* (Ash *et al.*, 1993), is a common soil bacterium that belongs to the group of PGPR (Figure 3). Activities that have been found to be associated with *P. polymyxa*-treatment of plants in earlier experiments include nitrogen fixation (Heulin *et al.*, 1994; Lindberg *et al.*, 1985), soil phosphorus solubilization (Singh & Singh, 1993), production of antibiotics ((Rosado & Seldin, 1993), and references therein), auxin (Lebuhn *et al.*, 1997), chitinase (Mavingui & Heulin, 1994), and hydrolytic enzymes (Nielsen & Sorensen, 1997), as well as promotion of increased soil porosity (Gouzou *et al.*, 1993). All these activities could account for/ contribute to plant growth promotion at various times and in various environments during the life cycle of a plant. Since cytokinin production by *P. polymyxa* had previously been reported (Holl *et al.*, 1988), but yeast extract (supposed

to contain cytokinins) had been present in the growth medium, we decided to reevalute cytokinin production in defined media.

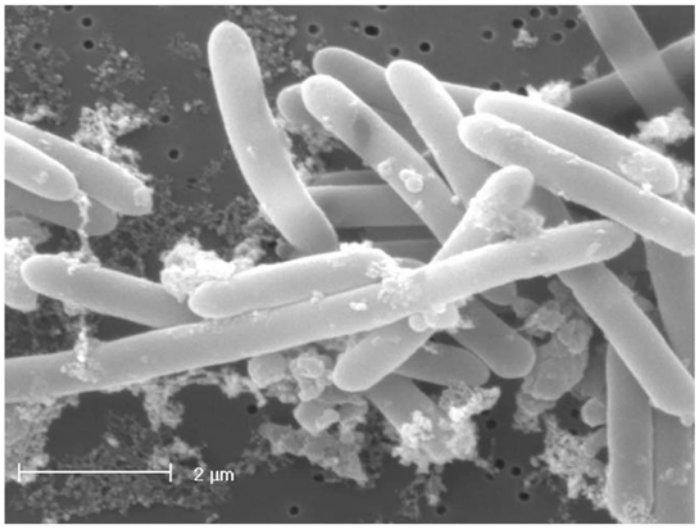

Fig. 3. Scanning electron micrograph of *P. polymyxa* isolate B2. Bacteria were grown overnight in LB medium and analyzed by Scanning electron microscopy.

CYTOKININ PRODUCTION BY *PAENIBACILLUS POLYMYXA* (paper I)

Cytokinins are a diverse group of labile compounds, present in small amounts in biological samples and often difficult to identify and quantify. We considered it of interest to investigate whether production of cytokinins could be a factor in the known plant growth promoting activity of certain *P. polymyxa* isolates. We used immunoaffinity chromatography to isolate, high performance liquid chromatography with on-line ultra violet spectrum detection to separate and characterize, and gas chromatography-mass spectrometry to identify cytokinins in culture media produced by *P. polymyxa*, grown in the presence or absence of yeast extract.

Using defined media, we could show that the cytokinin isopentenyladenine (iP) was indeed released by *P. polymyxa* during its stationary phase of growth. Its concentration, as estimated from UV absorbance data, was about 1.5 nM. It is known that even a limited increase in cytokinin production in transgenic plants can affect plant biomass and longevity (Gan & Amasino, 1995). It is clear however that the level of iP in our *in vitro* cultures did not adequately reflect the conditions in the rhizosphere - since plants/ plant cells were absent. E.g., *P. polymyxa* enters the intercellular spaces of roots (manuscript III). Some microenvironments - like intercellular spaces - into which bacterial cytokinins could be excreted, may be small in volume, thus resulting in disproportionally high local concentrations that might induce growth effects. Therefore, future studies should be conducted in the presence of plant roots, and the balance between different hormonal classes should be studied.

A GNOTOBIOTIC SYSTEM TO STUDY *P. POLYMYXA* EFFECTS IN PLANTS (papers II, III, and IV)

Interactions between plants and microorganisms are by their nature complex. The mechanisms involved in these interactions are particularly complicated because the interactions occur in a dynamic environment. Therefore, mechanistic studies can greatly benefit from reductionist approaches, provided that one is cautious with interpretations derived from such simplified experimental protocols.

We used a gnotobiotic system to study interactions between *P. polymyxa* and the model plant *A. thaliana*, in order to exclude the uncontrolled variations in experimental conditions associated with studies carried out on plants grown in soil. The PGPR *P. polymyxa* has a relatively wide host range. In addition to cereals, several dicots are susceptible to infection, including *A. thaliana*. *A. thaliana* was chosen as the model plant because it provides a good experimental system for genetic studies, many transposon-tagged mutants have been generated, and its entire genomic sequence is available in databases, thus facilitating the identification of genes scored in the approach employed.

E. carotovora ssp. *carotovora* (strain *Scc* 3193) was chosen as a pathogen to study induced resistance since it is a pathogen of very wide host range and therefore provides a system for the study of non-specific plant-pathogen interactions.

P. palmivora and *P aphanidermatum* were chosen as fungal pathogens that attack plant roots to study whether *P. polymyxa* could exert antagonism against them around roots. These pathogens belong to the most important soil-born pathogens and can be used as models to investigate host (*P. aphanidermatum*) and nonhost (*P. palmivora*) interactions to *Arabidopsis* plants.

P. POLYMYXA AS A BIOCONTROL AGENT

In addition to producing compounds favourable for plant growth, PGPR can also protect plants from various pathogens. This can occur by direct antagonism when protective bacteria and attacking organisms are in close proximity, in which case disease suppression is expected to be restricted to soil-born pathogens. On the other hand, PGPR may stimulate systemic defenses, inducing sustained changes in the plant which increase its tolerance to further infection by foliar or root pathogens.

INDUCED SYSTEMIC RESISTANCE BY *P. POLYMYXA* (papers II, and III)

Different strains of *P. polymyxa* isolated by Lindberg and Granhall (1984) were shown to be effective as PGPR on cereals. Since also the suppression of phytopathogens was observed, we wanted to study if induced resistance could be the mechanism of biocontrol observed in soil experiments.

P. polymyxa isolates protect *A. thaliana* against the pathogen *Erwinia carotovora*

We studied different strains of *P. polymyxa* isolated by Lindberg and Granhall (1984) that were shown to be effective as PGPR on cereals. A subset of these isolates, denoted *P. polymyxa* B2, B3, and B4, were tested in this study. *A. thaliana* plants were inoculated with a defined number of bacteria. After a period of 24 hrs, an antibiotic was added to both the inoculated and the control plants in order to reduce the number of free bacteria that had failed to enter plant roots.

A. thaliana seedlings were locally inoculated by *E. carotovora.* Inoculated plants incubated for 24 hrs after *E. carotovora* infection showed significant resistance to the pathogen. Limited maceration was generally observed around the infection site in the

plants that were pre-treated with *P. polymyxa* strain B2, but plants were still vital after two subsequent days of growth. In contrast, the leaves of control plants were almost completely macerated already after 12 hours. Viable count tests of *E. carotovora* extracted from leaves at half-day intervals after infection indicated decreasing numbers of the pathogen, compared to those in control plants. This clearly indicated that a systemic defense had been induced, since the sites of primary inoculation (*P. polymyxa*: roots) and pathogen infection (*E. carotovora*: leaves) were far apart.

***P. polymyxa* inoculation causes biotic stress**

P. polymyxa is known to be a PGPR. The induction of systemic resistance against *E. carotovora* via the root system would indicate the mechanism to be ISR. However, plants seemed to suffer from inoculation – stunted root systems were generally observed, and overall plant growth was reduced by about 30%. Because of the stress symptoms observed, we decided to study the expression of genes connected to biotic stress responses. It had been shown earlier that ET, JA and SA pathways were activated in biotic stress situations (Vidal *et al.*, 1998). Hence, we analyzed the expression of marker genes for each of these pathways: *HEL* (heveine – ET pathway; (Potter *et al.*, 1993)), *ATVSP* (vegetative storage protein acid phosphatase – JA pathway; (Berger *et al.*, 1995), and *PR-1* (SA pathway; (Uknes *et al.*, 1992)), were chosen. RT-PCR experiments showed that all three marker genes were overexpressed in plants previously treated with *P. polymyxa*, at induction levels varying from 2-6 fold. We inferred that this relatively small, but significant, increase is indicative of a "mild" biotic stress.

The observed reduction in growth rate/ biomass could represent a trade-off between maximal growth and induction of resistance. Even though *PR1* upregulation is not reported to be unambiguously characteristic of ISR, we still classified the phenomenon as ISR since it was induced by a rhizobacterium. Thus, in order to begin to understand the relationship between beneficial and harmful effects of *P. polymyxa* on *A. thaliana,* I considered it important to subsequently also characterize the colonization process in detail.

***P. polymyxa* colonization of *A. thaliana* root, and its endophytic mode of action**

I succeeded in tagging of the *P. polymyxa* isolate B1 by a plasmid-borne *gfp* (encoding green fluorescent protein) gene and used the resulting strain *P. polymyxa* B1::pCM20 to follow the colonization of *A. thaliana* roots over a time period of 24 hours. Bacterial colonization started at the root tip, invasion of the root occurred after 2 hours of infection, and population of the zone in the differentiation region was observed within 5 hours, at which time a significant invasion of root tissue was evident. By 24 hours, severe damage to the root was seen. Thus, *P. polymyxa* has two preferred zones of infection. The first one is located at the root tip in the zone of elongation, which sometimes results in the loss of the root cap. The other colonization region was observed in the differentiation zone. Similar regions of colonization had previously been reported for endophytes, a class of bacteria characterized by a root-invading but non-pathogenic life-style. e.g. *Azoarcus* (Hurek *et al.*, 1994), possibly because these root regions are rich nutrient sources (Jaeger *et al.*, 1999). Since some endophytic bacteria are able to enter roots and also move to aerial tissues, I analyzed the fate of *P. polymyxa* after invasion by a PCR approach. Up to 10^6 *P. polymyxa* cells were determined to be present in 1 g of surface-sterilized root tissue, but no bacteria were detected in leaf samples above background (<100 cells/ g of leaf tissue).

These colonization studies also told me that the abundant intercellular presence of bacteria caused damage to plants already within 5 hours. A slight degradation of the root tip was sometimes accompanied by a separation of the root cap. After 24 hours of inoculation, the root tips were often macerated. These observations, and the previously observed 30% reduction in the growth of plant, and the stunted root system, indicated that – under the gnotobiotic conditions used here – *P. polymyxa* behaves as a deleterious rhizobacterium.

P. POLYMYXA AS AN ANTAGONIST OF SOILBORN PATHOGENS **(papers III and IV)**

It is often reported that PGPR directly protect plants against harmful microorganisms; fungi, bacteria, viruses, nematodes. We studied *P. polymyxa* antagonism against the oomycete plant pathogens *P. palmivora* and *P. aphanidermatum.* These pathogens are some of the economically most devastating soil-born pathogens. Studies were conducted in three experimental systems: *in vitro* plate assays, observation of zoospore colonization under reversed microscope, and soil assays

***P. polymyxa* antagonism studied by plate assays and microscopy**

I tested whether co-cultivation of *P. polymyxa* with either *P. palmivora* or *P. aphanidermatum* on agar plates could indicate anti-oomycete activity. Upon incubation, radial growth of *P. palmivora* was severely inhibited, whereas *P. aphanidermatum* was not affected, and resulted in full spreading of the oomycete over the entire plate. Furthermore, we investigated whether attachment of *P. polymyxa* to roots of the model plant *A. thaliana* could interfere with the colonization process of fungal zoospores. We previously observed that *P. polymyxa* was predominantly associated with elongation and differentiation zones. In these experiments, *P. polymyxa* inoculation preceded infection by oomycete zoospores, and in control experiments bacteria were omitted. When roots were not pre-treated with bacteria, *P. aphanidermatum* zoospores were abundantly present around the tip of the main root and the adjacent root segments. When plant roots had been pre-inoculated by *P. polymyxa*, few zoospores were seen attached to this region of the root. We also conducted similar analyses of antagonism between *P. polymyxa* and *P. palmivora*. In this case, the zoospores attached almost exclusively to lateral roots, whereas the main root was left unaffected. When roots were pre-treated with *P. polymyxa*, zoospores were again occluded from binding to the same root segments. Thus, the results from this assay indicate that the PGPR is effective in counteracting attachment of zoospores to their preferred sites on the plant root. Interestingly, although the plate assay only showed an antagonistic effect against *P. palmivora*, the root attachment experiments suggested antagonism against both oomycetes.

***P. polymyxa* antagonism in soil assays**

As the conditions used in the previous experiments did not reflect a biologically relevant setting, we tested the effect of *P. polymyxa*-treatment in soil assays. Survival rates of plants infected by either one of the two pathogens were determined after seven days of growth. *P. polymyxa* significantly induced protection of plants; ten out of twelve plants survived when pre-treated with bacteria, whereas untreated plants showed a much lower viability (2/12 and 4/12, in separate experiments). Inoculation of plants with *P. polymyxa*, without subsequent *Pythium* infection, did not result in a significant loss of viability (11/12 and 12/12 survivors). In contrast to *P. aphanidermatum*, *P. palmivora* proved to be an inefficient pathogen on *A. thaliana*,

and hence it is not surprising that a protective effect of bacterial pre-treatment was not observed. Detailed microscopic investigations were performed to assess the results of the soil assay. In *P. polymyxa* pre-treated plants, the hyphal material located around the root tip and the adjacent zone was significantly less compare to that of plants cultivated without *P. polymyxa* pretreatment.

In a more detailed study, not related to the antagonism against oomycetes, GFP-tagged *P. polymyxa* B1::pCM20 was used to follow the colonization of *A. thaliana* roots (see above). There, it was observed that *P. polymyxa* formed an intensive biofilm. We followed biofilm formation in time course experiments. First, the bacterium formed a layer on the root surface. Subsequently, microcolonies appeared, which were dispersed throughout the layer, and then these microcolonies aggregated with each other.

When I followed the process of colonization over a period of about 30 minutes it was apparent that the zoospores of both fungal pathogens, *P. palmivora* and *P. aphanidermatum,* frequently approached the root surface but "bounced back". It seems likely that niche exclusion by *P.polymyxa* is the possible mechanism for the antagonism observed. *P. polymyxa* colonizes similar regions as the pathogens. The rhizobacterium is able to produce polysaccharides (e.g. levan; (Han, 1989)). The bacterial polysaccharide layer observed in our colonization experiments might simply prevent the pathogens from gaining access to the colonization niche on the root, but also limit the supply of available nutrients emerging from these regions of the root for stimulation of directed zoospore motility. The polysaccharides produced by *P. polymyxa* are highly complex, and only few organisms may possess the specific enzymatic machinery for their degradation, e.g. *P. polymyxa* itself (Bezzate *et al.*, 1994).

P. POLYMYXA INOCULATION ENHANCES PLANT DROUGHT TOLERANCE (papers II, and III)

It has been reported that *P. polymyxa* inoculation is most effective in relatively harsh and poor quality conditions (Chanway & Holl, 1994). These results are in agreement with findings by Granhall et al. and Timmusk et al. (unpublished data) using nutritional and drought stress conditions. E.g., drought-exposed barley plants tolerated this stress two weeks longer if they were previously inoculated with *P. polymyxa,* than control plants.

To study enhanced drought tolerance, *A. thaliana* plants were exposed to drought stress. Experiments were performed in the gnotobiotic system described earlier.We could observe that *P. polymyxa*-treated plants were more resistant and tolerated drought stress significantly better than control plants. The latter showed severe wilting symptoms after three days of exposure to drought stress.

We studied gene expression in plants associated with *P. polymyxa* treatments. From six gene sequences that scored positive, only one (*ERD15*) was proven to be differentially expressed by RT-PCR. *ERD15* is a drought responsive gene earlier isolated by Kiyosue et al. (Kiyosue *et al.*, 1994). Since the plants in these experiments were exposed to *P. polymyxa* but not to dehydration stress the differential expression of the *ERD15* gene was unexpected. We decided to test for expression of two other known drought-responsive genes, *RAB18* (Lang & Palva, 1992) and *LTI78* (Nordin *et al.*, 1993). In an RT-PCR-experiment, the expression of the abscisic acid (ABA)-responsive gene *RAB18* was found to be 4-fold higher in *P. polymyxa*-treated plants than in control plants, whereas the *LTI78* gene was not differentially expressed. Hence, we can conclude that *P. polymyxa*-induction of *ERD15* gene overexpression is

not unique, and that at least one additional drought responsive gene, *RAB18*, responds similarly.

It is possible that the *P. polymyxa*-induced biotic stress around the plant roots ultimately results in activation of a set of genes that carries out overlapping or complementary roles in stress defenses, thus – in this case – rendering plants more tolerant also to desiccation stress. In addition to that possibility we also studied the colonization pattern of *P. polymyxa* around the roots (see above). Biofilms are microbial colonies encased in an adhesive material, usually polysaccharides, attached to a surface. A variety of microorganisms produce extracellular polysaccharides as a capsule attached to the cell wall or as a slime secreted into the growth medium. Levan is the polysaccharide reported to be produced by *P. polymyxa* grown on sucrose (Han, 1989). Several soil bacteria can produce levan when grown on sucrose medium, e.g. several *Pseudomonas sp., Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Bacillus pumilus, Acetobacter sp., Serratia sp.,* and *P. polymyxa,* produce homopolysaccharides consisting of 90-100% levan (Han, 1989). These observations may suggest that the biofilm around the plant root, consisting of bacteria and bacterially produced polysaccharides, is instrumental in enhanced drought tolerance of plants.

DISCUSSION

Several rhizobacterial strains actively promote plant growth and suppress disease. Microbial activities exerted in the rhizosphere influence plant growth, development and metabolism and can reduce the detrimental effects of various stresses. I have in this thesis work studied the ability of isolates of one of these PGPR, *P. polymyxa*, to enhance plant fitness to both biotic and abiotic stresses. In the following, I will discuss several observations with respect to their mechanistic implications.

P. POLYMYXA INDUCES SYSTEMIC RESISTANCE TO *E. CAROTOVORA*

We classified the observed resistance of *Arabidopsis* plants to *E. carotovora,* induced by *P. polymyxa*, tentatively as induced systemic resistance since it – at first glance – matches widely accepted criteria: resistance induced by plant growth promoting bacteria via the plant root system. However, studying the *P. polymyxa* mode of colonization on *Arabidopsis* roots (paper III) I could observe a severity of damage to the root which indicates that this bacterium rather should be regarded as a deleterious rhizobacterium (DB), at least under the experimental conditions investigated. In classical examples of ISR, the JA and ET signalling pathways are activated, whereas ISR is independent of the actions of SA. Since our results (paper II) indicate a small, but significant upregulation of marker genes in all three pathways in response to this plant root-degrading rhizobacterium, the experiments with *P. polymyxa* B2 are not easily reconciled with ISR (Persello-Cartieaux *et al.*, 2003). However, a signal-dependent assignment is not without problems: e.g., in the case of the *P. fluorescens* strain CHAO, JA levels are not elevated (Iavicoli *et al.*, 2003), and the interaction of this strain with tobacco plants had been shown to result in PR protein upregulation, thus indicating an involvement of the SA pathway in rhizobacterially induced systemic resistance (Maurhofer *et al.*, 1994).

The other type of induced resistance, SAR, is activated by pathogens, and constitutes a plant defense response activated in noninfected foliar tissue. SAR is known to be associated with an increase in endogenously synthesized SA (Durner *et al.*, 1997). Earlier, SA was suspected to be the only systemic signal for SAR. Indeed, the accumulation of this signal molecule was detected in phloem (Ryals, 1996). An

increase in SA concentration subsequently activates PR protein-encoding genes which are regarded as the marker genes for the SAR pathway (Uknes *et al.*, 1992). Later, SA-independent pathways, such as those involving JA and ET as the central signals, have been described (Thomma *et al.*, 1998) in connection with SAR.

More recently, it was demonstrated that different pathogens can trigger different defense pathways (Thomma *et al.*, 2001b), indicated by dinstinguishable sets of genes activated, and by different signal intensities. In studies involving the pathogens *A. brassicola, Plectosphaerella cucumerina, Botrytis cinera* and the *Pernospora parasitica* strain NOCO, both the SA- and the JA/ET-pathways were strongly activated (Thomma *et al.*, 2001b). Both *P. parasitica* strain EMWA and *E. carotovora* activate only the JA/ET-pathway. A minor activation of the SA-pathway seems to occur in case of *Pseudomonas syringae*, whereas the *P. syringae* strain Rpt2 activates through JA, ET and SA. This latter case is clearly reminiscent of the behavior of *P. polymyxa*-inoculated *Arabidopsis* plants (paper II), though – to our knowledge – our work provides one of only a few examples of resistance induced via the root system. Therefore, signals must necessarily travel/ be transmitted over long distances. Adding to the complexity of pathways, an *Arabidopsis* SA induction-deficient mutant has been described which does not accumulate SA yet expresses PR2 and PR5 (Nawrath & Metraux, 1999). In a study of nonhost resistance induced by *Phytophthora spp* in *A. thaliana*, induction appears not to follow any of the conventional signal transduction pathways (Roetschi *et al.*, 2001).

If we compare results from these studies to our observations, we can conclude that the two well-established types of induced resistance, pathogen-initiated SAR and plant growth promoting bacteria-initiated ISR, probably do not adequately reflect the complexity of signal transduction pathways involved in microbial-induced resistance. First of all, it is difficult to unambiguously define pathogens and beneficial bacteria, respectively. Whether a bacterial strain functions as PGPR or DR (i.e. a minor pathogen) certainly depends on the conditions applied. E.g., the *P. polymyxa* strain L6-16R promoted lodgepole pine growth in one location, inhibited growth at a second site, and had no effect at a third site (Chanway & Holl, 1994). The conclusions drawn by the authors suggested that inoculation may be useful when seedlings are outplanted on relatively harsh or poorer quality sites, but less so at higher quality sites where growth inhibition can be anticipated. We also observed deleterious effects of *P. polymyxa* under gnotobiotic conditions. *Arabidopsis* plants were dwarfed and had stunted roots, and damage to the root tips was seen in microscopic studies using GFP-tagged cells. However, the same treatment protocol also could induce resistance to *E. carotovora* and enhance plant drought tolerance – i.e. clearly beneficial effects. I conclude therefore that a designation of a bacterium as either growth promoting or deleterious is not without problems, since it will be the net result of positive and negative effects caused by the bacterium to a given plant under a given set of conditions.

A second important factor that has not generally been taken into account is the type and mode of action of microbial elicitors. Microorganisms that trigger variable defense responses (Thomma *et al.*, 2001b) span a wide taxonomical range, which suggests that they also should represent a broad range of infection modes (Thomma *et al.*, 2001b). The pathways activated in a plant might therefore largely be dependent on the types of elicitors that initiate the response.

What are the possible candidates for *P. polymyxa*-dependent elicitors? Several species of bacteria, and certain fungi, produce a variety of enzymes that degrade the plant cell wall. E.g., *P. polymyxa* has been reported to produce pectate lyase (PGL)

(Forrest & Lyon, 1990). I have studied pectinolytic enzyme production by the bacteria in plate assays and found that isolates B1, B2 and B4 produce pectate lyase, polygalacturonase and cellulase (unpublished). The degradation patterns observed in colonization studies can be reconciled with a role of these enzymes in the *P. polymyxa*/ *A. thaliana* interaction (paper III). Palva et al. (Palva *et al.*, 1993) showed that extracellular enzymes of *E. carotovora* induced resistance in Arabidopsis plant to *E. carotovora*, and that plant treatment with isolated pectinolytic enzymes systemically reduced pathogen growth in plant leaves. This induced systemic resistance correlates well with defense gene induction (Vidal *et al.*, 1998). Treatment with cell wall-degrading enzymes from *E. carotovora* also enhanced systemic resistance towards other pathogens, e.g. *Xanthomonas campestris*, the causative agent of black rot disease (Vidal *et al.*, 1998). These results suggest that the induced systemic resistance is not specific to *E. carotovora* but is effective against other plant pathogens as well. OGAs released from the plant cell wall can have a wide range of biological effects, some of which are exerted at low concentrations. OGAs have been shown to trigger JA/ET-dependent signalling (Kariola *et al.*, 2003).

Recently, a second type of elicitor which is cooperative with OGAs has been described in an *Arabidopsis*/ *Erwinia* pathosystem (Kariola *et al.*, 2003). Using various signal transduction mutants it was demonstrated that the *Erwina*-derived elicitors, harpin HrpN and polygalacturonase, trigger both SA-dependent and JA/ET-dependent pathways (Kariola *et al.*, 2003). The authors also showed that plant defense gene induction is strongly enhanced by the simultaneous presense of two types of elicitors In this context, it would be useful to investigate whether, in our system, the slight hypersensitive response observed around lesion sites may be due to harpins produced by *P. polymyxa*, and if so, whether they may be involved in eliciting defense responses. Similarly, it is still unclear whether OGAs released by pectinolytic enzymes produced by *P. polymyxa* ultimately contribute to induced systemic resistance against *E. carotovora.*

P. POLYMYXA ANTAGONIZES ROOT PATHOGENS

In paper IV, I studied *P. polymyxa*-mediated direct antagonism against the root pathogens *P. aphanidermatum* and *P. palmivora*. These experiments indicated that *P. polymyxa* is effective in protecting *A. thaliana* plants against *P. aphanidermatum,* and against *P. palmivora* zoospore colonization. The antagonistic effect towards *P. aphanidermatum* was further substantiated in soil experiments. However, the mechanism of pathogen occlusion is elusive. We observed clear antagonism against *P. aphanidermatum* in soil assays as well in the assays under reversed microscopy, but no antagonism in the plate assays. The production of secondary metabolites is often controlled by nutrients and growth rate. It is possible that antagonistic substances normally produced around plant roots are not synthesized on agar plates

Whereas direct antagonizing properties of rhizobacteria through production of antagonistic substances are well-known, niche exclusion and competition for nutrients from root exudates is less often reported. However, such mechanism are likely to play significant roles in antagonistic interactions. E.g., *P. polymyxa* colonizes the same regions on the plant roots that also serve as preferred colonization regions for the oomycetic pathogens. The biofilms observed (paper III) consist of bacteria and bacterially produced polysaccharides, the identity of which has not yet been investigated. We know, however, that when *P. polymyxa* is grown on glucose, it produces the homopolysaccharide levan (Han, 1989), a natural polymer of fructose, linked by fructofuranosidic bonds. The key biosynthetic enzyme is levansucrase.

Empirical observations link polysaccharides to improved structure in agricultural soils (e.g. (Gouzou *et al.*, 1993)). *P. polymyxa*-produced polysaccharides have been shown to improve soil aggregation and, via promotion of porous structures, to improve aeration (Gouzou *et al.*, 1993). Also an increase in soil adhesion to plant roots was observed (Gouzou *et al.*, 1993). In one study, a *P. polymyxa* strain carrying a disrupted levansucrase gene was shown to have lost its soil-improving characteristics (Bezzate *et al.*, 2000), suggesting that levan synthesis is the main mechanism by which *P. polymyxa* promotes soil adhesion to roots. I suggest that it is possible that the production of levan by *P. polymyxa* is a major factor in colonization of *Arabidopsis* roots, and in protection from colonization by the zoospores of the oomycete pathogens. The layer of bacteria and mycoidal polysaccharides around the preferred colonization regions could tentatively explain the exclusion of niche as well as a reduced nutritional stimulation for these zoospores.

Almost 25% of the worldwide annual expenditure for fungicides is aimed at control of late blight of potato caused by *Phytophthor*a *infestans*. An emerging problem, manifest in the last years, is the dramatic rise in pathogen resistance against fungicides. Therefore, studies of nonhost resistance has attracted an increased interest, and experimental *Arabidopsis-Phytophthora* pathosystems have been developed (Kamoun, 2001; Roetschi *et al*., 2001) . In principle, such studies might address the questions of which resistance genes are at play, but also to what extent induced defense responses contribute to nonhost resistance in a plant. It is likely that redundant signal perception and plant defense mechanisms constitute the molecular basis of nonhost resistance

Several fungal strains have been used to study nonhost resistance in the *Arabidopsis-Phytophthora* pathosystem. Different isolates of *P. porri* were analyzed for compatible or incompatible interaction with *Arabidopsis* plants. Visible symptoms of infection ranged from callose accumulation to plant tissue colonization by a dense network of intra- and intercellular hyphae. In paper IV, I observed zoospore accumulation which was however restricted to lateral roots. It is conceivable that *Phytophthora* zoospores accumulate around the roots simply because they have to cope with a limited lifetime/ activity period of 24-48 hours (Erwin & Ribeiro, 1996). Thus, one could speculate that they might benefit from attempting to settle down on *any* root encountered within this time span. It is also possible that, in the early stage of infection, the plant defence mechanisms prevent entry of the pathogen into the plant. It would be of interest to investigate the involvement of phytoalexin (Roetschi *et al.*, 2001) as well as SA, JA and ET synthesis in this interaction. Since the studies carried out with *P. porri* isolates (Roetschi *et al.*, 2001) used leaf infections, a study in which Arabidopsis roots were infected instead. might provide a useful comparison with respect to the symptoms obtained, and the mechanisms at work.

P. POLYMYXA ENHANCES PLANT DROUGHT TOLERANCE

Paper I showed that *P. polymyxa* inoculation resulted in upregulation of the drought-responsive *Arabidopsis* genes *ERD15* and *RAB18*, which is congruent with plant tolerance to drought stress. However, previous work had indicated that overexpression of *single* drought-responsive proteins do not necessarily confer plant stress tolerance. Tobacco plants transformed with three resurrection plant *Craterostigma plantagineum* cDNAs (*pcC6-19:* homolog of *rab16*, *pcC3-06*: homolog of *leaD29*, *pcC27-45*: homolog of *lea14*) did not show increased drought tolerance (Iturriaga *et al.*, 1992). However, coordinately activated expression of drought responsive proteins by the same transcription factor(s) increased stress

tolerance in transgenic plants (Kasuga *et al.*, 1999). Thus, TFs acting on regulatory elements shared by several promoters might account for induction in response to *P. polymyxa* stress in induction of drought response genes. It is not unexpected that different plant stress pathways could be activated in concert. E.g., damage on plant tissues, whether due to freezing, drought or salt stress, can be thought to facilitate pathogen access. Several abiotic and biotic stress situations might entail similar physiological effects and thus, a communication or co-regulation between different pathways may be evolutionarily selected for. The roles of TFs in plant stress tolerance acquisition have been demonstrated and this knowledge should soon contribute to agricultural practice. Thus, ectopic expression of selected TF genes permits overexpression of downstream stress-associated genes. On the downside, such transgenic plants tend to display growth retardation (Kasuga *et al.*, 1999), and activation of non-stress genes that negatively affect agronomic characteristics of the crop must be considered.

Several soil bacteria produce osmolytes to protect themselves against frequent fluctuations in osmotic conditions. A close relative to *P. polymyxa*, *B. subtilis*, produces glycine betaine (Lucht & Bremer, 1994). This compound lowers the water potential outside the cell wall. If we assume that *P. polymyxa* also produces osmolytes, and inhabits intercellular spaces (see paper III), the increased concentration of these compounds could be sensed by the plant as a local dehydration. Consequently, dehydration genes such as *ERD15* and *RAB18* (paper II) would be activated.

A final scenario that may account for drought tolerance induced by *P. polymyxa* involves the bacterial biofilm (paper III). Its formation around the plant root may create a mechanical protection layer which could prevent water loss. *P. polymyxa* is most effective in relatively poor and harsh conditions (Chanway & Holl, 1994). This is in agreement with results that showed the bacterium to protect plants in nutritional and drought stress conditions (Timmusk and Granhall, unpublished). Since a major fraction of root exudates consists of sucrose, I hypothesize that the bacterial levan layer produced around roots could at least partly protect *A. thaliana* roots from drought stress, and explain plant growth promotion by *P. polymyxa* especially under poor quality conditions.

FUTURE PERSPECTIVES

P. polymyxa is a PGPR. Since *P. polymyxa* is able to produce several antibiotics, it is expected to have a competitive advantage in rhizosphere colonization by outcompeting other rhizobacterial colonizers. Even though, until now, bacilli have received less attention as biocontrol agents than, e.g., pseudomonads, they should be particularly attractive since they produce stable endospores which can survive harsh environmental conditions that could cause problems for other biocontrol agents.

In our studies we have shown that *P. polymyxa* can enhance plant fitness by protection from several biotic and abiotic stresses. From these studies, it has become clear that future work must be directed towards mechanistic questions. In particular, I advocate investigations of the role of abundantly produced polysaccharides in antagonistic interactions and in enhanced drought tolerance. Polysaccharide production could be beneficial for the bacterium for several reasons. It would provide a nutritition and could protect against environmental stresses. If it can be shown that levan is also produced by *P. polymyxa* when in contact with the plant root, this might

also protect the host plant against abiotic (drought) and biotic (pathogen) stress. Disruption of the *P. polymyxa* levansucrase gene (responsible for levan production) would permit us to ask whether levan indeed is required for colonization of *Arabidopsis* roots, and protection against pathogens and drought stress. It might also be useful to genetically engineer levan production of *P. polymyxa* and other rhizospheric bacteria, testing for improved biocontrol.

Another topic which, in my view, deserves to be covered in future work, concerns the implications of the observed "endophytic" life-style of *P. polymyxa*. Here, it would be of interest to investigate whether the *Arabidopsis* experiments are indicative of a property of *P. polymyxa* which also can be supported on other host plants, and under more relevant environmental conditions. Most biocontrol agents selected from the rhizosphere do not fulfill their initial promise. Failures are often due to poor rhizosphere competence, and to difficulties associated with the instability of bacterial biocontrol agents in long term culture (Schroth & Hancock, 1982). The intimate relationship between endophytic bacteria and their hosts could make them good candidates for successful biocontrol agents. This would, in part, circumvent the problem of selecting bacteria that display high rhizosphere competence which is often considered obligatory for successful seed or root bacterization treatment.

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