General discussion

Limitations for the isolation of endophytic bacteria

Endophytic bacteria can be found in virtually all parts of a plant, including roots and stems (Chapters 5 and 7) as well as reproductive and storage organs (Chapter 5). Since various plant tissues react differently to chemicals which are used for surface sterilization, each plant and plant organ require adjusted sterilization procedures. A harsh treatment of delicate plant samples can reduce quantitative and qualitative evaluation of bacterial endophytes, particularly of vegetative cells. On the other hand, a too mild surface sterilization may result in the isolation of epiphytic microorganisms which would be incorrectly identified as endophytes and this is why a proper control on sterility is required. Therefore, selecting an adequate sterilization protocol is a crucial step in endophytic research. We demonstrated that certain variations of established disinfection protocols (concentration of chemicals and incubation time) result in efficient sterilization of plant surfaces and allow the isolation of various phylogenetic groups of endophytic bacteria (Chapter 5 and 7).

From a technological point of view, cultivability and fast multiplication of microorganisms are important prerequisites for the production of effective bioproducts. In this context, use of the appropriate nutrient media and growth conditions can favor growth of some species over others and, as a result, increase the possibility of obtaining useful strains. For this reason, bacterial endophytes were isolated on general nutrient medium which supports the growth of fast-growing heterotrophic bacteria, such as those belonging to the phyla Firmicutes and Proteobacteria which harbor the majority of the species of agricultural importance (Chapters 5 and 7). To recover bacteria from a low-nutrient environment such as plant tissues, diluted synthetic media have been successfully used (Chapters 5 and 7; Adams and Kloepper, 2002). However, even after simulating the natural habitat, up to 99% of the bacteria cannot be isolated due to their (yet-) unknown growth requirements (Donachie et al., 2007). Recent progress in applying metagenomic tools and analysis of sequence information from the entire population might facilitate in finding an optimal cultivation strategy for (yet-) uncultured microorganisms and therefore result in increasing the number of biotechnologically promising strains (Handelsman, 2004).

Siblings, plant-beneficial strains and human pathogens

Isolation of bacteria in pure culture was followed by several initial screenings in order to discard siblings (Chapters 5 and 7). This procedure considerably decreased the number of strains for subsequent analysis and therefore made the remaining part of the screening process less laborious and time-consuming. Identification of the remaining strains showed the numerical prevalence of gram-negative Gammaproteobacteria (71%) over gram-positive Firmicutes (24%) and Actinobacteria (5%) (Table 3 in Chapter 5; Table 2 in Chapter 7). This finding confirms some of the earlier reports that members of the Gammaproteobacteria head the list of the most abundant culturable endophytes of many different plants (Kuklinsky-Sobral et al., 2004; Khan and Doty, 2009; Taghavi et al., 2009). As many as seven different genera were identified within these phyla, with *Bacillus* and *Pseudomonas* being the most frequently isolated ones (Chapters 5 and 7).

With regard to the plant-beneficial traits, 76% of the isolated strains had at least one of the tested beneficial properties indicating the occurrence of a large proportion of possible plant-beneficial strains among the isolated endophytic bacteria (Table 2 in Chapter 5; Table 1 in Chapter 7). The most common endophytic traits were (i) secretion of auxin (48%) and (ii) production of fungal cell-wall degrading enzymes (38%). The proportion of endophytic bacteria with antagonistic properties towards one or more fungal pathogens was 24% which is comparable with values reported by Berg et al. (2005) for endophytic bacteria of potato roots. Interestingly, the percentage of bacterial antagonists in the endosphere (21%) was always higher than found in the rhizosphere (14%). This fact, together with the notion that the root endosphere is the primary site attacked by most soilborne pathogens, allows the suggestion that plants can harbour specific endophytic bacterial groups in response to environmental stress. This suggestion is further supported by work of Siciliano et al (2001) who found that a number of bacterial genotypes containing catabolic genes for the degradation of petroleum hydrocarbons and nitrotoluenes increased in the interior of plant roots in response to soil pollution and that this response was contaminant-dependent. Understanding to which extent plants can regulate their bacterial inhabitants, remains an interesting research direction.

Along with agriculturally important strains, the identified phyla are known to comprise a number of well-known human and plant pathogens. For this reason it is important to evaluate the biosafety risk of the isolated bacteria and to do this at an early stage of the screening for bioinoculant agents. Indeed, we found that all analyzed

plants, except *Heracleum sosnowskyi*, harbor close relatives of species associated with human and plant diseases (Chapters 5 and 7). Moreover, these species account for 43% of the diversity of the entire bacterial collection indicating a remarkably high incidence of potential pathogens among endophytes. This finding supports previous reports that pathogenic bacteria are widespread in many natural environments and that they use plants as alternative hosts and an important source of transmission (Berg et al., 2005; Tyler and Triplett, 2008). Although some of these potential pathogens have interesting plant-beneficial traits (Table 2 in Chapter 5 and Table 1 in Chapter 7) and might miss some of the virulence factors present in clinical isolates (Dong et al., 2003), they are prohibited for agricultural application due to their possible threat to human and environmental health. Therefore, they were excluded from further studies.

Endophytic bacteria promoting plant growth

Auxin production is the best documented mechanism of plant growth promotion used by various plant-associated bacteria, including endophytes (Chapters 2 and 3; Spaepen and Vanderleyden, 2011). Inoculation of plants with auxin-producing strains can result in increasing the total root absorption surface and subsequent nutrient uptake leading to enhanced plant growth and biomass production. However, we did not observe the expected phytostimulating effect of the auxin-producing endophytic bacteria after their application on radish, tomato or cucumber plants (Chapters 5 and 7). Moreover, the IAA-producing strain *P. fluorescens* WCS365 which shows plant growth promotion of these plants also failed to stimulate the plant biomass in our experiments (Malfanova et al., *unpublished*). Taking this fact into consideration, our results can possibly be explained by assuming increased plant sensitivity to exogenous auxins under certain growth conditions, particularly when P is limited. For example, it has been shown that when P is not limiting, even low concentrations of exogenous IAA (up to 100 nM) increase nodule numbers as well as dry shoot and root weight of common bean (Remans et al., 2007). However, when P is low, the same concentrations of IAA can have a negative effect on nodule formation. In agreement with this, the increase in nodulation induced by the well-known IAA-producing strain *Azospirillum brasilense* Sp245 detected under high P was not detected under low P (Remans et al., 2007).

Overall, these results indicate that soil fertility and plant nutrition status can determine the outcome of plant-microbial interactions. This suggestion is further supported by the fact that when NPK fertilizer was added to soil, two IAA-producing endophytic strains, namely *Pseudomonas putida* CR3 and *Rahnella aquatilis* HC2 were

able to stimulate growth of radish and some cereal plants (Zaplatkin et al., *unpublished*). Moreover, the phytostimulating effect of these strains was also recorded in salinated and heavy metal-contaminated soils (Fig. 1).

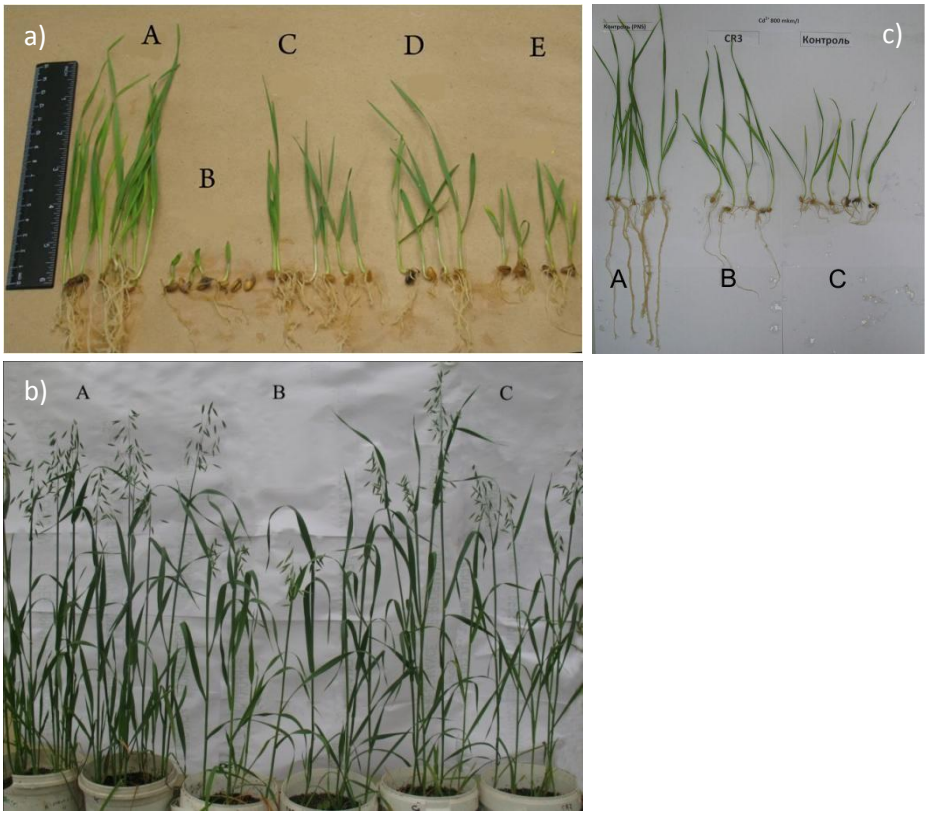

Fig. 1. The phytostimulating effect of endophytic bacteria on growth of cereal plants under high salt and heavy metal conditions.

a) Plant growth promotion of wheat by *Pseudomonas fluorescens* CR2 in the presence of 1% of salt. A – control without bacteria or salt added, B – control with 1% NaCl, C,D,E – three replicates of the experiment in which the bacterium and salt were present. A positive effect was also observed for the bacteria *Bacillus subtilis* HC8, *P. putida* CR3 and *Rahnella aquatilis* HC2.

b) Effect of *R. aquatilis* HC2 on growth of oat plants in the presence of 0.25% of salt.

A – control without salt or bacteria added; B – control with 0.25% NaCl added; C – plants inoculated with bacteria and salt.

c) Plant growth promotion of wheat by *P. putida* CR3 in the presence of 800 µM Cd^{2+}.

A – control without Cd^{2+}; B – plants inoculated with bacteria and Cd^{2+}; C – control with Cd^{2+}. A positive effect was also observed for CR2, HC2 and HC8.

All experiments and photos are from A. Zaplatkin, ARRIAM, Saint-Petersburg, Russia.

Along with auxins, other phytohormones are known to have a stimulatory effect on plant growth (Chapters 2 and 3). Since *Bacillus subtilis* HC8 is capable of promoting plant growth of both radish (Chapter 5) and wheat plants (Zaplatkin et al., *unpublished*) but does not produce detectable amounts of auxin, we tested this strain for its ability to produce cytokinins and gibberellins. While cytokinin production was not observed, a high amount of bioactive gibberellin was found to be secreted by *B. subtilis* HC8. Generally, GB-producing bacteria increase the endogenous content of these phytohormones and promote shoot and root growth of plants (Joo et al., 2005). However, since we did not detect GB in the tested plants, we cannot exclude an effect of other phytostimulatory compounds on growth promotion. Evidence for this notion was obtained by finding that *B. subtilis* HC8 was able to influence the growth of Arabidopsis seedlings in a split-plate assay when bacteria and plants were physically separated from each other (Malfanova et al., *unpublished*). This is likely due to the production of volatile compounds which have been shown to stimulate growth of various plants (Ryu et al., 2003).

Endophytic *Bacillus* and *Pseudomonas* with biocontrol properties

Species of *Bacillus* have attracted considerable attention because they produce many potent antibiotics. Among them c-LPs present the structurally most diverse group due to the variation in the amino acid composition of the peptide ring as well as in the length, branching and saturation of the acyl chain (Chapter 4). We found that *B. subtilis* HC8 is able to produce all three major families of c-LPs, namely iturin A, fengycin A and B and surfactin (Table 2 in Chapter 6). Within each family, we detected a remarkable high number of different homologues – more than has been reported for other beneficial bacilli, including for the commercial strain *B. amyloliquefaciens* FZB42 (Koumautsi et al., 2004). Moreover, these homologues comprise variants with long fatty acyl chains which are assumed to be more bioactive (Ongena and Jacques, 2008; Raaijmakers et al., 2010). In case of fengycins, long C18 homologues of fengycin A and B secreted by HC8 have been described only for a limited number of strains (Ongena et al., 2005; Nihorimbere et al., 2012).

These results suggest that HC8 is a powerful producer of c-LPs, a trait which can favor broad biotechnological applications of this strain, for example in the control of phytopathogens. Indeed, HC8 shows biocontrol in both stonewool substrate (Chapter 5) as well as in soil (Malfanova et al., *unpublished*) and is able to reduce TFRR of tomato plants by almost 50 and 25%, respectively. It is interesting to note that the two

other c-LP-producing endophytic bacilli which lack some of the c-LP fractions present in HC8 (*unpublished*), failed to show significant biocontrol in any of the tested substrates (Chapter 5).

There are three main modes of action by which endophytic bacteria can exert biocontrol of phytopathogens. These are antibiosis, CNN and ISR (Chapter 4). We found that fengycin and iturin LPs are responsible for most, if not all, antifungal activity of HC8 in *in vitro* assays against *Forl* (Chapter 6). Whereas these metabolites can suppress growth of *Forl* in soil, we expect that antibiosis is not powerful in stonewool because of diffusion of c-LPs in PNS. In addition to antagonistic activity, c-LPs are known to trigger ISR. Particularly, fengycins and surfactins induced significant protection in bean and tomato leaves against *Botrytis cinerea* following root treatment (Ongena et al., 2007). c-LPs, and more specifically surfactins, can also stimulate motility (Bais et al., 2004) and solubilization of plant nutrients (Lindow and Brandl, 2003), the two processes important for CNN (Lugtenberg and Kamilova, 2009). These data suggest that production of c-LPs can be the molecular basis of at least three different disease control mechanisms.

Other examples of shared determinants of different biocontrol mechanisms include volatiles (Ryu et al., 2003) and hydrolytic enzymes (Connelly et al., 2004). These compounds are also produced by HC8 (Chapters 5 and 8) and could also be involved in the biocontrol of TFRR by this strain. Moreover, since c-LPs, hydrolytic enzymes and volatiles are involved in different modes of action, we can speculate that HC8 can use a combination of different disease control mechanisms. Therefore, it is not very likely for a pathogen to acquire resistance to HC8, which makes this strain a strong candidate for the development of a biofungicide. Also, its ability to interact with multiple plants (see the previous section) and to express a beneficial effect under different growth conditions can facilitate its use in multifaceted plant protection.

Three other promising biocontrol strains characterized in our study are *P. fluorescens* CS1 and CR2 and *P. putida* CR3 (Chapter 7). Unlike *B. subtilis* HC8, these strains do not produce any exo-enzymes or antifungal metabolites, except for CR3 which secretes protease and inhibits the oomycete *P. ultimum in vitro* (Table 1 in Chapter 7). Nevertheless, strains CR2 and CR3 appeared to be able to significantly reduce TFRR symptoms in soil and had a biocontrol effect comparable to HC8 (Fig. 1b in Chapter 7). Whereas CS1 failed to show biocontrol in soil, it was effective against TFRR in stonewool substrate where the other two pseudomonads were not active.

Endophytic lifestyle

With regard to plant growth promotion and biocontrol, endophytism is in principle advantageous since endophytic biological agents can form a long-lasting association with plants in a relatively safe environment. Therefore, it is important to demonstrate the endophytic lifestyle of promising strains after production outside the plant and subsequent application. This can be done by visualization of labeled bacteria in inner plant tissues using confocal laser scanning microscopy (CLSM). Despite numerous attempts, we could not label *B. subtilis* HC8, neither by transforming it with reporter plasmids, nor by hybridizing it with fluorescent probes *in situ*. However we were able to show an endophytic lifestyle for *P. fluorescens* CS1 in gnotobiotic tomato plants using FISH followed by CLSM (Fig. 2). Microcolonies of CS1 have been detected in cortex tissues of seven days-old tomato roots as can be clearly observed in 3D projections (Fig. 2d-h).

Microscopic results of endophytic colonization of tomato plants by CS1 were further supported by the introduction of a rifampicin-resistant derivative of this strain inside tomato seeds followed by its re-isolation on antibiotic-containing medium (Malfanova et al., *unpublished*). Introduction of a biological agent inside a seed is generally referred to as seed biopriming and its aim is physiological enhancement of seed germination and plant vigor (Müller, 2006). Colonization of inner seed structures by bacterial cells is likely to be initiated by the process of water uptake by dormant seeds (imbibition) and release of growth substances which are readily used by bacteria (Bewley and Black, 1993). After four hours of incubation (imbibition time of tomato seeds) and two hours of air-drying, the population density of bacteria on the seed surface varies between 10^2-10^3 CFU per seed. Similar values were obtained for endophytic populations of rifampicin-resistant CS1^r following 36 h germination of seeds under gnotobiotic conditions. This value increases to 10^5 cfu for 7-days old tomato seedlings. These results indicate that CS1 is not only able to maintain itself, but can also thrive within a growing plant.

For successful endophytic colonization, bacteria should be capable of efficient utilization of carbon sources available inside a plant. In Chapter 7 we suggested that utilization of L-arabinose by endophytic pseudomonads might be important for their endophytic lifestyle in cucumber plants. It is unlikely that L-arabinose utilization is the only trait relevant for this complex life style. For example, other major xylem nutrients could also play a role, certainly in crops with a different xylem composition as well as for other endophytic bacterial species.

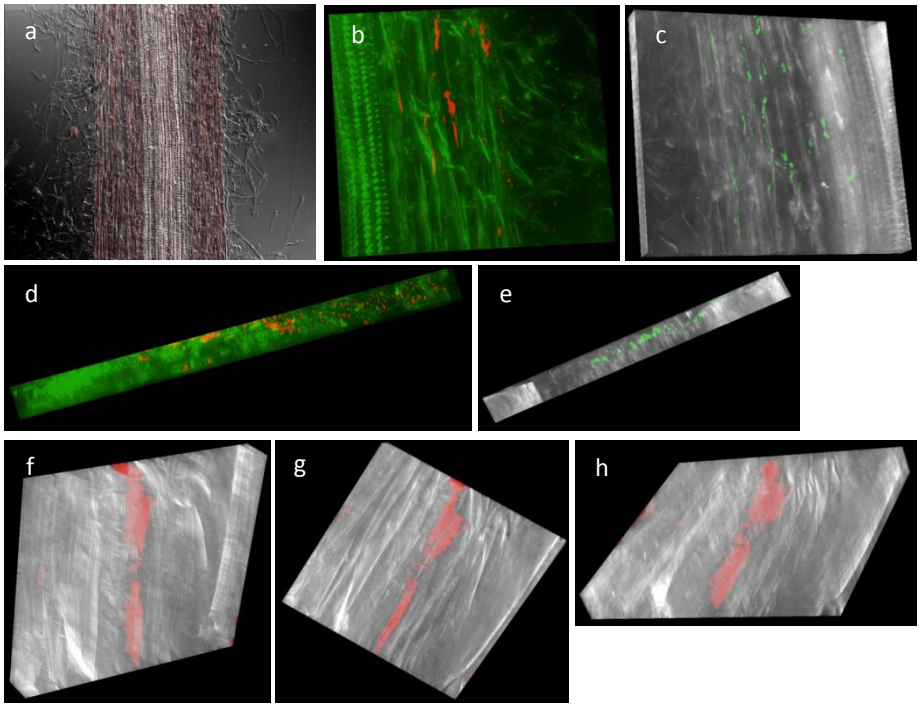

Fig. 2 Endophytic colonization of tomato roots by *Pseudomonas fluorescens* CS1 revealed by FISH followed by CLSM.

Tomato seeds were surface-sterilized using twice a 4% sodium hypochlorite treatment for 10 min followed by 10 washes with sterile water. Sterile seeds were soaked for 30 min in a bacterial suspension of CS1 adjusted to 10^8 cfu/ml. The treated seeds were then placed in a quartz sand gnotobiotic system and allowed to germinate for seven days. Subsequently, plant roots were aseptically removed and treated according to the FISH protocol described by Cardinale et al., 2008.

a – 10× magnified (objective) section of a tomato root heavily colonized with CS1 (bacterial signal shown in red);

b,c – 20× magnified (objective) sections of a tomato root with fluorescent signal in the cortex (bacterial signal is in red (b) and green (c).

d,e – z-stack of sections b and c, respectively, viewed from the top

f,g,h – z-stack of 200× magnified (objective) section of tomato root with macrocolonies of CS1.

Experiment and photos are from A. Shcherbakov, ARRIAM, Saint-Petersburg, Russia.

Concluding remarks and future prospects

In this research project we succeeded in the isolation of many endophytic strains with plant-growth promoting and biocontrol abilities. Among them, *B. subtilis* HC8 isolated from giant hogweed proved to be the most versatile promising bioinoculant since it expressed its beneficial effect on diverse plants under different growth conditions. It was also shown that this strain, so far, is the only endophytic *Bacillus* – and one out of a few *Bacillus* strains – which is capable of producing excessively high levels of a large variety of c-LPs. The high and varied production of c-LPs and other metabolites by HC8 might partly explain its excellent plant growth-promoting and biocontrol properties and favor its broad biotechnological application. This and other beneficial endophytic bacteria are currently being tested in pilot trials under various environmental conditions to select the most effective strains with a wide spectrum of action. For final bioproduct development, a number of important aspects still need to be further investigated, including evaluation of toxicity and environmental impact, fermentation, preservation, storage and formulation.

Our results as well as literature data suggest that the plant response to endophytic bacteria is a very complex process which involves interplay of many known and (yet-) undefined biotic and abiotic factors. Therefore, understanding mechanisms of beneficial action of selected endophytes as well as of plant growth parameters, such as soil type and nutritional status, can help to develop a better formulation and application strategy. With regard to plant growth conditions, additional greenhouse and field experiments are required to provide more conclusive information about the potential of using auxin-producing strains for plant inoculation in soil with limited nutrient resources, for example in combination with other beneficial bacteria.

Attempts were made to shed light on the endophytic lifestyle of several isolated strains. For *P. fluorescens* CS1, we confirmed its endophytic nature by a combination of microscopy studies and re-isolation of the introduced bacterium. These results are promising for further studies, e.g. to use multiple time points to examine entry sites for bacterial cells and to follow bacterial spreading inside the plant. Whereas such colonization studies are normally conducted under gnotobiotic conditions, it would be more relevant to investigate whether the beneficial strains also have the endophytic lifestyle of under practical conditions.

We found that, in contrast to most rhizospheric *Pseudomonas* spp., endophytic pseudomonads isolated from cucumber plants were able to utilize L-arabinose, one of the most abundant available sugars in the xylem fluid of various plants. This and other

(yet-) unidentified traits could contribute to the complex interaction of endophytic bacteria and plants. To further test the role suggested for L-arabinose, it would be interesting to use an L-arabinose utilization-negative mutant of one of the isolated pseudomonads and compare the abilities of wild type and mutant to live endophytically.

References

Adams PD, Kloepper JW. 2002. Effect of host geno- type on indigenous bacterial endophytes of cotton (*Gossypium hirsutum* L.). Plant Soil 240: 181–189.

Berg G, Eberl L, Hartmann A. 2005a. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environ. Microbiol. 71: 4203–4213.

Berg G, Krechel A, Ditz M, Sikora RA, Ulrich A, Hallmann J. 2005b. Endophytic and ectophytic potato-associated bacterial communi- ties differ in structure and antagonistic function against plant patho- genic fungi. FEMS Microbiol. Ecol. 51: 215-229.

Bewley J, Black M. 1993. Seeds: Physiology of development and germination. Plenum Press, New York.

Cardinale M, Vieira de Castro J, Müller H, Berg G, and Grube M. 2008*. In situ* analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of Alphaproteobacteria. FEMS Microbiol. Ecol. 66: 63–71.

Connelly MB, Young GM, Sloma A. 2004. Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. J. Bacteriol. 186: 4159-4167.

Donachie SP, Foster JS, Brown MV. 2007 Culture clash: challenging the dogma of microbial diversity. ISME 1: 97-102.

Dong Y, Chelius MK, Brisse S, Kozyrovska N, Podschun R, Triplett EW. 2003. Comparisons between two Klebsiella: the plant endophyte *K. pneumoniae* 342 and a clinical isolate *K. pneumoniae* MGH78578. Symbiosis 35: 247-259.

Handelsman J. 2004. Metagenomics: application of genomics to uncultured microorganisms. Microbiol. Mol. Biol. Rev 68: 669–685

Joo GJ, Kim Y.M, Kim JT, Rhee IK, Kim JH, Lee IJ. 2005. Gibberellins-producing rhizobacteria increase endogenous gibberellins content and promote growth of red peppers. J. Microbiol. 43: 510–515.

Khan Z, Doty SL. 2009. Characterization of bacterial endophytes of sweet potato plants. Plant Soil 322: 197–207.

Koumoutsi A, Chen X-H, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J. Bacteriol. 186: 1084–1096.

Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AP, Azevedo JL. 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ. Microbiol. 6: 1244-1251.

Lindow SE, Brandl MT. 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69: 1875–1883.

Lugtenberg B, Kamilova F. 2009. Plant-growth-promoting-rhizobacteria. Annu. Rev. Microbiol. 63: 541-556.

Müller H. 2007. Novel concepts in biological plant protection on the basis of the biological control agent *Serratia plymuthica* HRO-C48. PhD thesis, University of Rostock, Germany.

Nihorimbere V, Cawoy H, Seyer A, Brunelle A, Thonart P, Ongena M. 2012. Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S499. FEMS Microbiol. Ecol. 79: 176–191.

Ongena M, Adam A, Jourdan E, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P. 2007. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ. Microbiol. 9: 1084-1090.

Ongena M, Jacques P. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. 16: 115-125.

Ongena M, Jacques P, Touré Y, Destain J, Jabrane A, Thonart P. 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. Appl. Microbiol. Biotechnol. 69: 29–38.

Raaijmakers JM, de Bruijn I, Nybroe O, Ongena M. 2010. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol. Rev. 34: 1037-1062.

Remans R, Croonenborghs A, Gutierrez RT, Michiels J, Vanderleyden J. 2007. Effects of plant growth-promoting rhizobacteria on nodulation of *Phaseolus vulgaris* L. are dependent on plant P nutrition. Eur. J. Plant Pathol. 119: 341–351.

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW. 2003. Bacterial volatiles promote growth in Arabidopsis. Proc. Natl. Acad. Sci. (USA) 100: 4927-4932.

Siciliano S, Fortin N, Himoc N et al. 2001. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl. Environ. Microbiol. 67: 2469–2475.

Spaepen S, Vanderleyden J. 2011. Auxin and plant-microbe interactions. Cold Spring Harbor perspectives in biology 3: 1-13.

Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J, van der Lelie D. 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl. Environ. Microbiol. 75: 748-757.

Tyler HL, Triplett EW. 2008. Plants as a habitat for beneficial and/or human pathogenic bacteria. Annu. Rev. Phytopathol. 46: 53–73.