Characterizing and analyzing the ecological consequences of the plant-endophyte interactions in *Solanum nigrum* and *Nicotiana attenuata*

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Table of Contents

1. General introduction

1.1. What are endophytes?

The word "endophyte" means "in the plant" (derived from the Greek "endon" = "within", "phyton" = "plant"). This term can be used for a wide spectrum of potential hosts and inhabitants, e.g. bacteria (Kobayashi & Palumbo, 2000), fungi (Stone *et al.*, 2000), plants (Marler *et al.*, 1999), and insects inside plants (Feller, 1995), and algae within algae (Peters, 1991). "Endophyte" describes the organism's different life history strategies; these range from facultatively saprobic to parasitic to exploitive to mutualistic: pathogenic endophytic algae (Bouarab *et al.*, 1999), parasitic endophytic plants (Marler *et al.*, 1999), mutualistic endophytic bacteria (Chanway, 1996), mutualistic fungi (Schulz & Boyle, 2005), pathogenic bacteria and fungi in latent developmental phases (Sinclair & Cerkauskas, 1996), and commensalistic microorganisms (Sturz *et al.*, 2000).

Although there are diverse meanings for the term, endophytes are most commonly defined as those organisms whose "infections are inconspicuous, the infected host tissues are at least transiently symptomless, and the microbial colonization can be demonstrated to be internal" (Stone *et al.*, 2000). The authors use this definition to describe fungal endophytes only, but it is equally applicable to bacterial endophytes. Moreover, it is important to remember that the definition describes a momentary status. Thus it includes an assemblage of microorganisms which grow on dead or senescing tissues following an endophytic growth phase (Stone, 1987) and avirulent microorganisms and virulent pathogens in the early stages of infection (Sinclair & Cerkauskas, 1996; Kobayashi & Palumbo, 2000). Aware of the certain discrepancies, Schulz & Boyle (2005) came up with a more inclusive definition of endophyte to describe those bacteria and fungi that can be detected at a particular moment within the tissues of apparently healthy plant hosts. However, in practical terms, "bacterial endophytes" are defined as those bacteria that can be isolated from surface-disinfected plant tissue or extracted from within the plant and that do not visibly harm the plant (Hallmann *et al.*, 1997). I found this definition most applicable to my studies.

Bacterial endophytes can colonize any organ of the host plant (roots, shoots, leaves, seeds and ovules) (reviewed by Sturz *et al.*, 2000). Although colonization densities of bacterial endophytes are rarely as high as those of pathogenic bacteria, they are highest in the root tissue,

perhaps because this is the primary site of infection (Hallmann *et al.*, 1997; Kobayashi & Palumbo, 2000). Endophytic bacteria usually invade the roots passively, e.g. at opening sites on roots where lateral roots emerge or in wounds (Kobayashi & Palumbo, 2000), even achieving systemic colonization from a single site of entry (Hallmann *et al.*, 1997). They also can access intact plant tissue by invaginating the root hair cell, penetrating the junctions between root hairs and adjacent epidermal cells (Huang, 1986), or by producing cell wall hydrolyzing enzymes (Huang, 1986; Hallmann *et al.*, 1997). They primarily colonize intercellularly (Hinton & Bacon, 1995; Hallmann *et al.*, 1997), though they have also been found intracellularly, e.g. *Azoarcus* spp. (Hurek *et al.*, 1994).

1.2. Mechanisms of plant growth promotion by microorganisms

Diverse mechanisms are involved in plant-microbe interactions (Whipps, 2001; Compant *et al.*, 2005). A thorough understanding of the mechanisms that promote plant growth by microbes is fundamental to manipulating the rhizosphere and maximizing the processes that strongly influence plant productivity (Fig. 1).

In plant growth, phytohormones, e.g. indole-3-acetic acid (IAA), ethylene (ET), cytokinins, and gibberellins, play important roles. These hormones can be synthesized by plants and also by their associated microorganisms. Plant-associated bacteria can influence plant ET homeostasis by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme to degrade ACC, a precursor of ET biosynthesis, subsequently increasing root growth (Glick *et al.*, 2007) and reducing abiotic and biotic stresses caused by ET (reviewed by Saleem *et al.*, 2007). Moreover, volatile organic compounds (VOCs) of *Bacillus subtilis* GB03 regulate IAA homeostasis and cell expansion, providing a new paradigm for how rhizobacteria promote plant growth (Zhang *et al.*, 2007).

Improved nutrient acquisition is involved in direct plant growth promotion. Plant-associated microorganisms can supply macronutrients (nitrogen, phosphate, sulfate) and micronutrients (iron) for plants. The most prominent example of nutrient acquisition involves bacterial nitrogen (N) fixation (Dobbelaere *et al.*, 2003). Bacteria can oxidize sulfate (S) to provide it to plants (Banerjee & Yesmin, 2002). They can also liberate phosphorus (P) from organic compounds such as phytates (Unno *et al.*, 2005) or solubilize inorganic P by producing gluconic acid (de Werra *et al.*, 2009) and thus promote plant growth. By synthesizing

siderophores, a low molecular weight protein, bacteria can help plants to sequester iron from the environment (Katiyar & Goel, 2004). Moreover, VOCs of *Bacillus subtilis* GB03 activate the plant's own iron acquisition machinery to increase the assimilation of metal ions in *Arabidopsis* (Zhang *et al.*, 2009) and elevate photosynthesis levels by modulating endogenous sugar/ abscisic acid (ABA) signaling (Zhang *et al.*, 2008).

Indirectly, microorganisms promote plant growth by reducing the activity of microbial pathogens not only through microbial antagonism (reviewed by Raaijmakers *et al.*, 2009) but also by the activation of plant defenses, namely induced systemic resistance (ISR) (van Loon, 2007). Microbial antagonism includes inhibiting the growth of microbial pathogens by antibiotics, VOCs, toxins and biosurfactants; competing for colonization sites and nutrients; degrading the pathogenicity of pathogens such as toxins; and degrading fungal pathogen cell walls by introducing chitinases and β-1,3-glucanase.

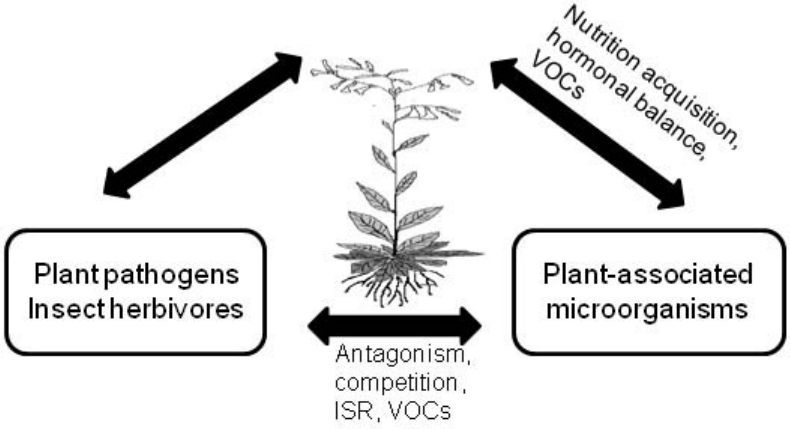

Figure 1. Plant-microbe interactions for promoting plant growth. Abbreviations: ISR: induced systemic resistance; VOCs: volatile organic compounds

1.3. Plant-driven selection of bacteria

Plants' photosynthates represent major sources of carbon, nitrogen and energy for plant-associated heterotrophic microorganisms, in particular, bacteria (Vandenkoornhuyse *et al.*, 2007), which in turn make plants very attractive as nutrient reservoirs for such bacteria. Plants require the presence of associated bacteria to growth and establish themselves in different ecosystems. For example, it is difficult to culture transplants of different species in the absence

of bacteria (Leifert *et al.*, 1989). Bacterial endophytes have been isolated from numerous plant species including both monocotyledons and dicotyledons (reviewed by Kobayashi & Palumbo, 2000). Microbes profit from plants because of the enhanced availability of nutrients, whereas plants can receive benefits from bacterial associates by growth enhancement or stress reduction. Therefore, mutualistic interactions between host plants and endophytes may be a result of the clear positive selection exerted on these associations (Thrall *et al.*, 2007).

The selective influence of plant species, cultivar, and genotype as well as plant developmental stages on bacteria associated with plants' roots is important for shaping microbial communities in the rhizosphere (Berg & Smalla, 2009; Micallef *et al.*, 2009). Mazzola *et al.*, (2004) showed that different wheat cultivars supported larger populations of specific *P. fluorescens* strains than do others. This phenomenon has been linked to the genetic make-up of plants (Neal *et al.*, 1973), possibly through differences in root exudation (Bais *et al.*, 2006). Specific exudate-dependent plant-bacterial interactions have been identified in transgenic plants engineered in exudate synthesis, in which the growth of *P. putida* was enhanced in direct response to the stimulated secretion of phenylpropanoid compounds (Narasimhan *et al.*, 2003). Other plant-specific factors such as root architecture and surface structure also influence rhizobial communities (Depret & Laguerre, 2008). There is still little evidence, however, to explain how different plant taxa mediate a differential rhizosphere influence to give rise to distinct bacterial endophyte communities.

1.4. Microbial cooperation in the rhizosphere

A variety of microbial forms can be found growing in rhizosphere micro-habitats. It is well accepted that members of any microbial group can develop important functions in an ecosystem (Giri *et al.*, 2005). Direct interactions between members of different microbial types often promote key processes that benefit plant growth and health. The cooperation between plant growth promoting rhizobacteria (PGPR) and *Rhizobium* improved nodulation and N_2-fixation in legume plants (Zhang *et al.*, 1996). Another type of interaction involves rhizosphere microbes and arbuscular mycorrhiza (AM) fungi and lead to establishing a functional mycorrhizosphere (Gryndler *et al.*, 2000). Mycorrhiza helper bacteria are known to stimulate mycelia growth and/or enhance mycorrhization (Garbaye, 1994). Furthermore, AM inoculation improves the establishment of PGPR (Barea *et al.*, 2002) as well as improving nodulation and rhizobial

activity within the nodules (Barea *et al.*, 1987). Multi-microbial interactions, including those between locally isolated AM fungi, phosphate-solubilizing bacteria, and *Azospirillum* have also reported, which indicate that microorganisms act synergistically when present simultaneously in the rhizosphere (Muthukumar *et al.*, 2001).

1.5. Multi-functional roles of ethylene in plant

ET is a small, readily diffusible hormone important for integrating developmental events with external stimuli. It is a critical component of such diverse developmental processes as seed germination, fruit ripening, abscission, and senescence (Abeles *et al.*, 1992). It is also an important stress hormone. Adverse biotic (pathogen and insect attack) and abiotic (wounding, salt, drought, cold, ozone and flooding) stimuli usually lead to ET synthesis. ET, in turn, slows down plant growth until the stress is removed. Furthermore, ET synthesis is regulated by developmental cues and other hormones, such as auxin, gibberellins (GA), cytokinins and brassinosteroids (BR) (reviewed by Yoo *et al.*, 2009). At the level of gene expression, ET induces the transcription of many genes in response to a multitude of environmental and developmental stimuli (reviewed by Klee, 2004). Thus, ET has a pivotal role in coordinating plants' internal growth, defense and survival mechanisms in response to environmental challenges (Fig. 2).

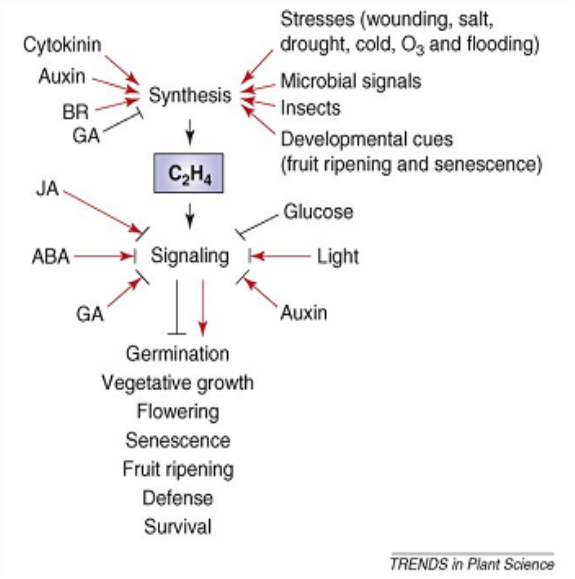

Figure 2. The ethylene regulatory network. Abbreviations: ABA, abscisic acid; BR, brassinosteroid; JA, jasmonic acid, GA: Gibberellic acid (Yoo *et al.*, 2009)

ET, however, has an alter ego. It has the ability to trigger exaggerated disease symptoms or to exacerbate an environmental pressure. It is unclear whether this reaction serves a useful purpose or is a malfunction in the plant's defense mechanism, but the generalization can be made that, except for fruit ripening, high levels of ET are usually deleterious to plant growth and health (reviewed by Stearns & Glick, 2003). Therefore, a range of methods is known to be able to prevent the inhibiting effect of ET on postharvest ornamental crops. Interfering with the plant's response to ET can in principle be achieved by: 1) inhibiting the plant's own ET production; 2) blocking ET's ability to bind to its receptor; or 3) blocking the plant's reaction to the binding of ET to the receptor. The most common methods of inhibiting ET biosynthesis and perception are summarized in Table 1 (reviewed by Serek *et al.*, 2006)).

Table 1. Strategies to inhibit ethylene effects by chemical, environmental and molecular genetic approaches (Serek *et al.*, 2006)

Process	Chemical/environmental inhibition	Molecular genetic inhibition
ACC synthase (ACS) ↓	AVG AOA High CO_2	Antisense ACS Co-suppression ACS
ACC ↓		Heterogenous expression of bacterial ACC deaminase
ACC oxidase (ACO) ↓	$CoCl_2$ Alpha-aminoisobutyric acid Low O_2 concentration High temperature	Antisense ACO Co-suppression ACO
Ethylene ↓	Removal by ventilation Chemical removal with $KMnO_4$ Absorption by zeolites	
Receptor ↓	STS; 2,5-NBD; DACP; 1-MCP; 3,3-DMCP 3-MCP; 1-DCP; 1-HCP	Expression of mutated ethylene receptor (*etr1-1*)
Responses	Low temperature Cytokinin	Expression of IPT gene from *Agrobacterium tumefaciens*

AVG: 1-aminoethoxyvinylglicine; AOA: aminooxyacetic acid; STS: silver thiosulfate; 2,5-NBD: 2,5 bicyclohepta-2,5-diene; DACP: diazocyclopentadiene; 1-MCP: 1-methylcyclopropene; 3,3-DMCP: 3,3-dimethylcyclopropene: 3-MCP: 3-methylcyclopropene; 1-DCP: 1-decylcyclopropene; 1-HCP: 1-hexylcyclopropene; ipt: cytokinin biosynthetic gene

A major advance in understanding how ET works was the discovery that it acts in a jasmonic acid (JA)-dependent pathway, namely induced systemic resistance (ISR), distinct from the salicylic acid (SA)-dependent systemic acquired resistance (SAR) pathway (Pieterse *et al.*, 1998). ISR results from the colonization of roots by non-pathogenic rhizobacteria, which enhances resistance to other pathogens. ET responsiveness is required for ISR to be induced and

expressed (Pieterse *et al.*, 1998). ET and JA cooperate in inducing ET response factor (ERF1), which drives the activation of defense-related genes such as *PR-4* and *Pdf1.2* and positively regulates the expression of JA-inducible genes involved in defense responses (Lorenzo *et al.*, 2003).

Great efforts have been made to show the role of ET in the SAR pathway (Verberne *et al.*, 2003). By carrying out reciprocal grafting between WT and transgenic salicylic acid-non-accumulating NahG or ET-insensitive Tetr tobacco plants, they could show that the SAR signal was dependent on ET. The burst of ET production that occurs during a hypersensitive reaction (HR) (De Laat & Van Loon, 1982) contributes substantially to the local induction of a subset of pathogenesis-related proteins (PRs) (Knoester *et al.*, 1998), while also enabling the plant to react systemically and develop SAR (Verberne *et al.*, 2003). These results indicate ET's important role in establishing SAR in tobacco against tobacco mosaic virus (TMV) (Ryals *et al.*, 1996).

ET also has a major function in the beneficial rhizobial symbiosis (Guinel & Geil, 2002). ET acts as a negative factor in the rhizobia's nodulation processes. However, recent discoveries suggest rhizobia use several strategies to reduce the amount of ET in order to decrease the negative effect of ET on nodulation. One strain of rhizobia produces rhizobitoxine, an inhibitor of ET synthesis (Yuhashi *et al.*, 2000). Active 1-aminocyclopropane-1-carboxylate (ACC) deaminase has been detected in a number of other rhizobial strains (Ma *et al.*, 2003). Whereas ET plays negative roles in rhizobial-plant interactions, it is indispensable for rhizobial nodulation processes via crack-entry (Ma *et al.*, 2003). ET mediates the phenotypic plasticity in root nodule development (Goormachtig *et al.*, 2004), determining the infection mechanisms of rhizobia as well as formation and positioning of nodule primordia (Guinel & Geil, 2002).

1.6. Native model plant species: Solanum nigrum *and* Nicotiana attenuata

Our group uses *Solanum nigrum* (black nightshade, Solanaceae) as an ecological model species (Schmidt *et al.*, 2004) (Fig. 3). Its phylogenetic proximity to tomato and potato allows us to use genetic tools and databases that have been established for these crops. Moreover, *S. nigrum* is a wild species that has not been under artificial selection for particular traits (e.g. traits that enhance yield). Its particular natural history makes it ideal for studying its interactions with other organisms. As an annual, it colonizes nitrogen-rich agricultural and disturbed habitats at a wide range of altitudes throughout its pan-arctic distribution.

Nicotiana attenuata Torr. ex Watson (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae) is a wild tobacco species native to the Great Basin Desert in western North America (Fig. 3). Its seed germination is regulated by stimulants from burned wood and inhibitors from litter (Baldwin *et al.*, 1994). As a consequence of the post-fire germination behavior, seeds germinate synchronously in nitrogen (N)-rich soils (Lynds & Baldwin, 1998) and hence plants rapidly grow when water availability is high. Because of its germination behavior, intense intraspecific competition, and highly variable herbivore interactions, *N. attenuata* is a particularly useful system to study herbivore resistance responses in nature (Baldwin, 2001).

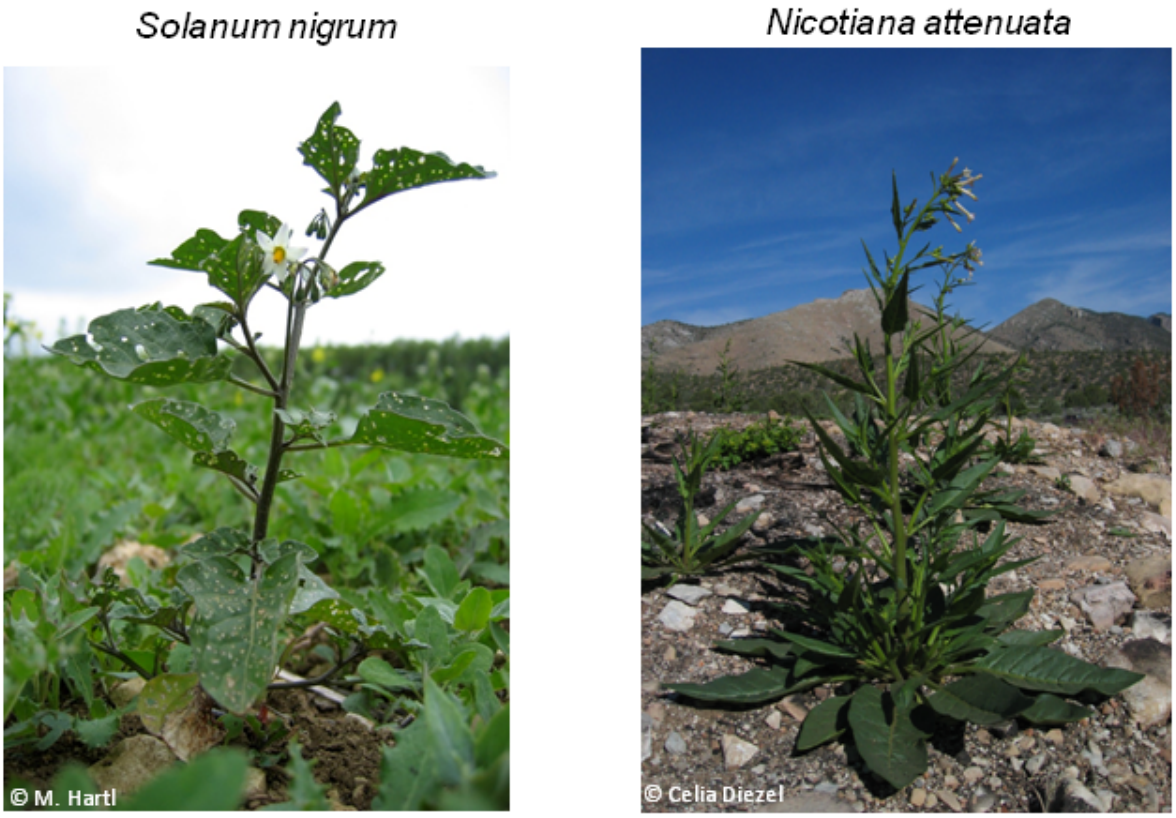

Figure 3. *Solanum nigrum* plant growing in the field in Jena, Germany (left) and *Nicotiana attenuata* plants growing in Utah, USA (right). Pictures were taken by Markus Hartl and Celia Diezel.

2. Objectives of the study

Although *S. nigrum* and *N. attenuata* are ideal ecological model systems for studying plant-herbivore interactions (Schmidt *et al.*, 2004; Baldwin, 2001), few reports detail how *N. attenuata* interacts with microorganisms. *N. attenuata* plants realize a fitness benefit from associating with Sebacinales fungi (*Piriformospora indica* and *Sebacina vermifera*), and their enhanced fitness comes at the expense of a reduction in resistance to herbivores (Barazani *et al.*, 2005; 2007). Studies on interactions between an arbuscular mycorrhizal species, *Glomus intraradices,* and *N. attenuata* have revealed a non-beneficial symbiosis (Riedel *et al.*, 2008). Nevertheless, no study has focused on native microbial communities and their effects on plant growth and fitness. Therefore, the objectives of this thesis are to characterize and analyze the ecological consequences of plant-endophyte interactions in *S. nigrum* and *N. attenuata*.

(I) Plant growth promoting (PGP) endophytic bacteria from roots and stems of wild type (WT) *S. nigrum* field-grown plants are isolated and identified, and the isolates exhibiting the clearest PGP traits (1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole-3-acetic acid (IAA) production) and exerting the strongest positive effects on root growth of *S. nigrum* are selected; WT plants of a closely related plant species, *N. attenuata,* are inoculated with these isolates. In addition, bacterial strains from a culture collection with known PGP traits are analyzed in order to address the question: Do the general PGP effects of bacterial endophytes translate to fitness benefits in *N. attenuata* and *S. nigrum*?

(II) In this study, wild type (WT) *N. attenuata* plants and isogenic transformed plants deficient in ethylene (ET) biosynthesis (ir-*aco1*) or perception (35S-*etr1*) are grown in four native soils collected in Utah, USA and the culturable bacterial endophytic communities of *N. attenuata*'s roots are characterized in order to address the following questions:

- Do plant ET signaling and the ability of plants to sense or produce ET play a role in the recruitment of endophytic bacteria?
- Do soil types influence the endophytic bacterial communities?
- Do bacterial isolates specifically colonize distinct host genotypes?
- How do the bacterial endophytes influence plant growth?

- Do plant genotypes with differential ET production affect the recruitment of ACC deaminase and IAA producing bacteria into the plant endosphere?

I focused on the culturable bacterial endophyte community in order to test hypotheses about the specificity of the association between plant and bacteria with re-colonization assays.

(III) A "native" *Bacillus* sp. strain isolated from the roots of 35S-*etr1* plants grown in native Utah soils is selected to address the following question: How does a native bacterial endophyte influence the growth and fitness of its host plants, *N. attenuata* (WT) and especially the highly susceptible 35S-*etr1* transgenic line in the native habitat, Utah, USA?

Chapter I. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses

1.1. Introduction

Symbiotic interactions are the driving force in ecosystems; symbiosis ranges from parasitism to mutualism and includes everything in between. The fitness outcomes for plants differ accordingly: if a plant is highly susceptible to pathogens, its fitness is likely to be low in pathogen-rich environments; if a plant cooperates with mutualists, it is likely to thrive even in adverse environments. Bacteria, which colonize the interface between living plant roots and soil, namely the rhizosphere, are abundant symbiotic partners of plants. These so-called rhizobacteria are said to be plant growth promoting (PGP). Those microbes able to colonize plant roots internally without negatively affecting the host are called endophytes (Schulz & Boyle, 2005). Although all of the approximately 300,000 plant species have been estimated to harbor one or more endophytes (Strobel *et al.*, 2004), few relationships between plants and these endophytes have been studied in detail; the legume-rhizobia symbiosis is an exception. The mutualistic interaction of legumes with rhizobia involves finely tuned recognition steps which ultimately lead to the production of root nodules in which the plants accommodate the bacteria (Oldroyd *et al.*, 2005). For other endophytic rhizobacteria, the processes of host-microbe signaling and colonization, and the mechanisms leading to mutual benefit are less well-characterized.

Bacterial endophytes can accelerate seedling emergence, promote plant establishment under adverse conditions and enhance plant growth (Chanway, 1997; Bent & Chanway, 1998). Endophytic bacteria are believed to elicit plant growth promotion in one of two ways: either (1) indirectly by helping plants acquire nutrients, e.g. via nitrogen fixation, phosphate solubilization (Wakelin *et al.*, 2004) or iron chelation (Costa & Loper, 1994), by preventing pathogen infections via antifungal or antibacterial agents, by outcompeting pathogens for nutrients by siderophore production, or by establishing the plant's systemic resistance (van Loon *et al.*, 1998); or (2) directly by producing phytohormones such as auxin or cytokinin (Madhaiyan *et al.*, 2006), or by producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which lowers plant ethylene (ET) levels (Glick, 1995). In addition to these plant-growth-promoting traits, endophytic bacteria must also be compatible with host plants and able to colonize the tissues of the host plants without being recognized as pathogens (Rosenblueth &

Martinez-Romero, 2006). A particular bacterium may affect plant growth and development using one or more of these mechanisms, and may use different ones at various times during the life cycle of the plant. While the mechanisms of growth promotion appear to be universal -- for example, by changing a plant's phytohormone metabolism -- it remains unclear how consistently bacterial endophytes elicit responses in host and non-host plant species.

Many studies have documented the interaction between PGP rhizobacteria and host plants. A mechanistic model was previously developed by Glick *et al.,* (1998) to explain the role of bacterial ACC deaminase and indole-3-acetic acid (IAA) in promoting plant growth. ET and IAA are implicated in virtually all aspects of plant growth and development, ranging from seed germination to shoot growth and leaf abscission (Woodward & Bartel, 2005). Therefore, production of ACC deaminase and IAA is likely an important and efficient way for endophytes to manipulate their plant hosts. Endophytic bacteria containing ACC deaminase promoting plant growth are usually located inside plant roots in the apoplast. The cleavage of ACC results in ammonia and α-ketobutyrate which are readily metabolized by the bacteria. In this way, these bacteria act as a sink for ACC. By lowering ET levels, the bacteria increase the growth of plant roots and shoots and reduce the inhibitory effects of ET synthesis. In addition to being produced by plants, IAA is also produced by root-associated bacteria such as *Enterobacter* spp., *Pseudomonas* spp., and *Azospirillium* spp. (Spaepen *et al.*, 2007). Lowering ET in plant roots also relieves the suppression of auxin response factor synthesis, and indirectly increases plant growth (Glick *et al.*, 2007).

The central role of phytohormone signaling in plant-endophyte interactions suggests two scenarios: (1) Endophytic bacteria with general PGP traits, such as the ability to produce IAA and ACC deaminase, promote growth uniformly across plant species including non-hosts (Cakmakci *et al.*, 2007; Domenech *et al.*, 2007). Such endophytes are expected to be readily recruited by a novel host. (2) Once recruited by a particular host, endophytes undergo host-specific adaptations; the upshot is a highly specialized, finely tuned mutualism. Such mutualisms may make plants better able to tolerate the endophyte and the endophyte in turn more responsive to the plant's metabolism (Schulz & Boyle, 2005). Hence, non-host plants might recognize these endophytes as pathogens despite their plant-growth-promoting properties, either because they are pathogens for the non-host or because they elicit inappropriate responses in a non-host-plant species (Carroll, 1999).

In order to test these two hypotheses, I first isolated and identified plant-growth-promoting endophytic bacteria from black nightshade (*Solanum nigrum)*, a native plant that interacts with many partners in its habitat (Schmidt *et al.*, 2004). I then selected the isolates exhibiting the clearest plant-growth-promoting traits and exerting the strongest positive effects on root growth of *S. nigrum;* I inoculated a closely related plant species, *Nicotiana attenuata,* with these isolates. In addition, bacterial type strains from a culture collection with known PGP traits were analyzed to determine whether their general PGP effects translate to fitness benefits in *N. attenuata* and *S. nigrum*. I report markedly different growth and fitness responses of these plant species to the same bacterial strains. Our results are consistent with the scenario in which plant growth promotion by native endophytic bacteria is highly species-specific, regardless of whether or not they express general PGP traits.

1.2. Materials and methods

1.2.1. Plant materials

The following inbred lines were used in all experiments: *S. nigrum* Sn30 (Schmidt *et al.*, 2004); *N. attenuata* (synonymous with *N. torreyana*) genotype Utah (Baldwin, 1998). Seed germination procedures of *S. nigrum* and *N. attenuata* are described elsewhere (Krugel *et al.*, 2002; Schmidt *et al.*, 2004) and also detailed in General materials and methods.

1.2.2. Bacterial type strains

Six bacterial species were selected from the German culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen - DSMZ) *Pseudomonas brassicacearum* D13227, *Bacillus pumilus* D1794, *Pseudomonas putida* D50194, *Pseudomonas marginalis* D50276, *Methylobacterium fujisawaense* D5686 and *Pseudomonas fluorescens* D8568.

1.2.3. Isolation of culturable endophytic bacteria

S. nigrum plants were individually collected from field sites near Dornburg, Germany, or near the Max Planck Institute for Biogeochemistry, Jena, Germany. Roots were washed in tap water to remove soil; leaves, stems and roots were separated. Roots of *S. nigrum* plants growing in the margins of agricultural fields in the Dornburg and Saale valley were similarly collected.

Endophytic bacteria were isolated as described by Long *et al*., (2003) and also detailed in General materials and methods.

1.2.4. Plant culture

Seeds were surface-sterilized as described by Schmidt *et al*., (2004). Bacterial suspensions in sterile distilled water (10^8 cfu ml^{-1}) (colony forming unit-cfu) were used for seed inoculation; control seeds were treated with sterile distilled water only. The inoculated seeds (20-30 seeds) were incubated at room temperature overnight and transferred onto sterile filter papers (Whatman No.1) in Petri dishes. One week after bacterial inoculation, root and hypocotyl lengths were measured. Two independent experiments were carried out for all seedling assays.

1.2.5. Seedling vigor assay

Seventy-seven isolates were used for seed treatment. After surface disinfection, *S. nigrum* seeds were treated with pure cultures of these isolates (10^8 cfu ml^{-1}) in distilled water for 24h; control seeds were incubated in sterile distilled water for 24h.

Germination tests were carried out by the paper towel method (Wold, 1996). The germination paper was soaked in distilled water, 15-20 bacterially treated seeds and untreated seeds were placed on paper towels, rolled and wrapped with polythene to prevent drying, and incubated at 25 ± 2^{o}C for seven days, when the towels were unrolled and the number of seeds that had germinated was counted. On the same day, seedling vigor was analyzed using the method of Abdul Baki and Anderson (Abdulbak & Anderson, 1973). The lengths of roots and hypocotyls of all the individual seedlings were measured. The vigor index (VI) was calculated using the formula VI = (mean root length + mean hypocotyl length)*% germination. The experiment was repeated twice. The strains which gave high germination and vigor were selected for further experiments.

1.2.6. Transformation of bacteria with pDSK-GFPuv plasmid

Preparation of electro-competent cells was carried out as standard protocol for *E. coli* with some modifications. Briefly, 0.5 l Yeast Peptone Dextrose (YPD) broth (Sigma, Steinheim, Germany) was inoculated with 5 ml overnight, cultured and grown to an OD_{600} of 0.5 – 0.7 (0.5xYPD broth; 30°C ; 220 rpm). Cells were harvested by centrifugation (8000 x *g*, 4°C) and

washed 4 times in ice-cold 10% glycerol. Finally, the bacterial pellet was resuspended in 1.5ml 10% glycerol, divided into 40µl aliquots, and stored at -80°C. Transformation of bacteria with pDSK-GFPuv plasmid was done by electroporation as described by Wang *et al.,* (2007). Fluorescent transformants containing the plasmid pDSK-GFPuv were selected on Luria Broth (LB) agar plates supplemented with 50µg ml^{-1} kanamycin and identified under long-range UV light (365nm).

1.2.7. Confocal laser-scanning microscopy (CLSM)

Seeds were inoculated with green fluorescent protein (GFP)-labeled bacteria as described above. Seven days after inoculation, root colonization was observed with a CLS microscope LSM510 (Carl Zeiss, Jena, Germany) equipped with an Argon laser (458, 477, 488, 514nm) and detectors for monitoring GFP (495-590nm). Images were collected in a z-series from 30 to 130 optical sections ranging from 1.3 to 7.2µm in thickness. Optical sections, maximum intensity projections and overlays were generated, and single images were processed by selecting a subset from a z-series using the Zeiss LSM Image Browser, version 4.0 (Carl Zeiss).

1.2.8. Seedling ethylene measurement

ET emissions from seedlings were measured continuously and non-invasively in real-time with a photoacoustic spectrometer (INVIVO, Saint Ausgustin, Germany) as described by von Dahl *et al.,* (2007). Inoculated seeds that had germinated in 100ml three-neck flasks for 7 days at 25±2^{o}C were subjected to ET measurements. Five three-neck flasks were used for one treatment and empty flasks as well as flasks with seeds treated with sterile distilled water served as controls.

1.3. Results

1.3.1. Isolation and characterization of endophytic bacteria from S. nigrum

Seventy-seven endophytic bacterial isolates were isolated from roots, stems and leaves of black nightshade plants (*S. nigrum*) grown in two different native habitats in Jena, Germany. They were all characterized for their ability to 1) produce ACC deaminase; 2) synthesize the phytohormone IAA; 3) solubilize phosphate; and 4) colonize seedlings, since these traits are

associated with plant growth promotion (Sturz & Nowak, 2000). Twenty-three isolates were able to grow on the minimum medium DF salt supplemented with ACC as a sole N source, suggesting that they have ACC deaminase activity. One isolate was able to produce IAA without supplementation of Tryptophan (Trp) and 28 were able to produce IAA with supplementation of Trp. Six isolates were able to solubilize inorganic phosphate. Twenty-four isolates were able to colonize *S. nigrum* seedlings internally (Table 1).

Table 1. Biochemical characteristics of endophytic bacteria isolated from *S. nigrum*.

Origin*	No. isolates	Growth on DF salt with ACC†	*In vitro* IAA production‡		Phosphate solubilization	Seedling colonization
			-Trp	+Trp		
BGCR1	11	1	0	3	0	2
BGCSL1	4	0	0	1	0	0
BGCR2	13	5	0	5	2	5
BGCSL2	13	1	0	2	1	2
DR	9	2	1	5	1	3
DSL	8	5	0	5	0	4
DSR	12	2	0	2	1	2
SSR	7	6	0	5	1	6
Total	**77**	**23**	**1**	**28**	**6**	**24**

* Isolation of endophytic bacteria from roots/stem leaves from plants collected in 2 field plots of Max Planck Institute for Biogeochemistry (BGCR/SL), roots/stem leaves from plants collected in the Dornburg field (DR/SL), roots from plants grown in Dornburg field soil in greenhouse (DSR) and roots from plants grown in the greenhouse in soil from the Saale (SSR); † ACC: 1-Aminocyclopropane-1-carboxylate; ‡ IAA: Indole-3-acetic acid; Trp: DL-Tryptophan

1.3.2. Screening endophytic bacteria for plant growth promotion

A *S. nigrum* seedling vigor assay was used to screen the endophytic bacterial isolates for their PGP ability, using the isolates' effects on seed germination, root and hypocotyl growth; 37 of 77 isolates increased seedling vigor in the first assay and were screened a second time (Fig. 1). Of these 37 isolates, 22 significantly enhanced seed germination - up to 100% - compared with untreated controls (Fisher's PLSD test; $P<0.05$). One isolate, DSR3, inhibited seed germination. Twenty-seven isolates significantly increased the seedling root length compared with the control (Fisher's PLSD test; $P<0.05$). Eleven isolates significantly promoted the hypocotyl growth of seedlings (Fisher's PLSD test; $P<0.05$). Four isolates inhibited either root or hypocotyl growth (Fig. 1). Sixteen isolates were selected for further study because they had 1) one or more of the

PGP traits (Table 1) and 2) enhanced seedling growth in both screening trials. Isolate DSR10 strongly inhibited seedling growth and was used as a negative control in further experiments.

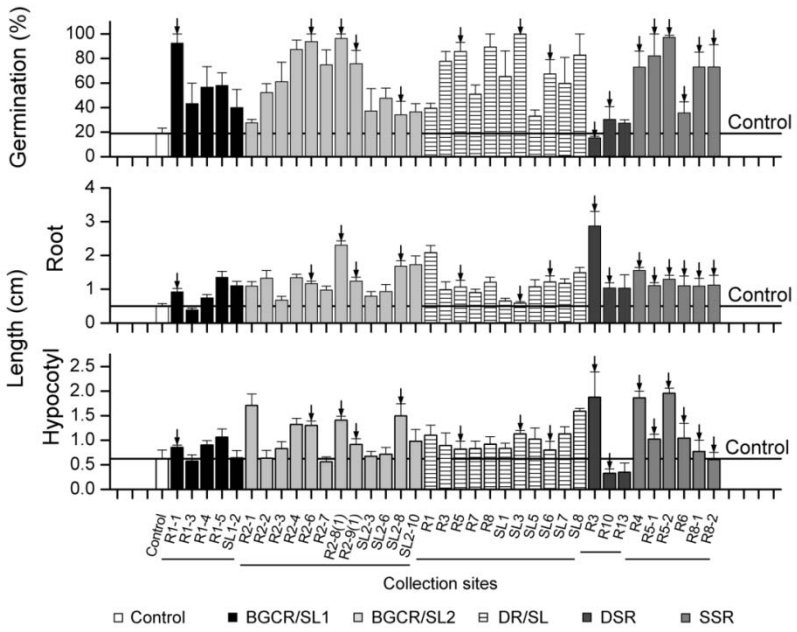

Figure 1. Effects of endophytic bacteria on seedling vigor. Mean (±SE; n = 30-40) percentage germination, root length (cm) and hypocotyl length (cm). Seeds treated with sterile distilled water served as controls (white bars). The different shadings of the bars indicate the origin of the isolate (roots/stem leaves from *S. nigrum* plants collected from 2 field plots of Max Planck Institute for Biogeochemistry (BGCR/SL), roots/stem leaves from *S. nigrum* plants collected in the Dornburg field (DR/SL), roots from *S. nigrum* plants grown in Dornburg field soil in the glasshouse (DSR) and roots from plants grown in the glasshouse in soil from the Saale valley (SSR)). Arrows identify the sixteen isolates that were selected for further study.

1.3.3. Identification of bacterial isolates.

Sixteen isolates were selected based on their PGP traits and seedling growth promotion. The 16S rRNA gene was amplified in these isolates using universal primers, and sequenced. The sequences were similar to those of 6 bacterial genera, namely *Pseudomonas*, *Acinetobacter*, *Pantoea* (formerly *Enterobacter*), *Agrobacterium*, and *Aeromonas* (Table 2) with high homology hits in the database ranging from 95 to 100% similarity. Ten isolates were identified to species. Isolate DSR10 was identified as *Agrobacterium tumefaciens*, a phytopathogen. The sequences are deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) under the accession numbers shown in Table 2.

Table 2. Identification of bacterial isolates using 16S rRNA gene sequences.

Bacterial isolates	GenBank accession number	Closest match according to the 16S rRNA gene sequence	No. of bases	Max. score	% match
BGCR1-1	EU434624	*Enterobacter agglomerans* strain A17	775	1400	99
BGCR2-6	EU434628	*Pseudomonas* sp. BSs20166	682	1205	98
BGCR2-8(1)	EU434629	*Pseudomonas* sp. S8-130	799	1476	100
BGCR2-9(1)	EU434630	*Pseudomonas brassicacearum* isolate MA250	868	1604	100
BGCSL2-8	EU434635	*Pseudomonas lutea* strain PSB2	806	1290	95
DR5	EU434637	*Pseudomonas thivervalensis* strain H2P3	506	922	99
DSL3	EU434639	*Enterobacter agglomerans* strain A17	888	1583	98
DSL6	EU434640	*Pantoea agglomerans* strain PTA-AF1	661	1216	99
DSR3	EU434642	*Aeromonas veronii* strain 211c	790	1448	99
DSR10	EU434641	*Agrobacterium tumefaciens* strain CCBAU 85035	636	994	95
SSR4	EU434643	*Pseudomonas* sp. S8-130	910	1676	99
SSR5-1	EU434644	*Pseudomonas* sp. S8-130	902	1642	99
SSR5-2	EU434645	*Pseudomonas* sp. S8-130	763	1410	100
SSR6	EU780008	*Acinetobacter calcoaceticus* strain M10	875	1616	100
SSR8-1	EU434646	*Pseudomonas fluorescens* 16S rRNA gene, strain F113	630	1164	100
SSR8-2	EU434647	*Pseudomonas* sp. OCY4	557	1022	99

1.3.4. Effects of ACC deaminase and IAA from endophytic bacteria on seedling root growth

In order to establish a link between bacterial and plant traits, I analyzed the correlation between physiological properties of the bacterial endophytes and their effects on inoculated *S. nigrum* seedlings. Two major bacterial characteristics were addressed, namely the abilities to degrade ACC through ACC deaminase and to synthesize IAA. Of 16 selected isolates, 7 possessed high levels of ACC deaminase ranging from 200 to 700 nmol mg protein^{-1} h^{-1} and significantly enhanced root growth compared with the control (Fisher's PLSD test, $P<0.05$). In order to confirm the correlation between ACC deaminase activity and seedling root growth, I performed a regression analysis of bacterial ACC deaminase activity and the root length of seedlings that had been inoculated with the corresponding isolate. A statistically significant, positive relationship ($r^2=0.534$; $P=0.0009$) was observed between ACC deaminase activity and root growth (Fig. 2A). In order to test whether reduced ACC levels in a plant affected ET metabolism, I determined the relation between bacterial ACC deaminase activity and plant ET emissions, using simple regression analysis. Although I found a significantly negative relationship ($r^2=0.679$ and $P=0.0063$) between these two factors (Fig. 2B), ACC deaminase activity and subsequent lower seedling ET emissions did not account for all positive effects on

root growth: another group of isolates with little ACC deaminase activity also promoted root growth (Fig. 2A). In addition to ACC deaminase, some isolates produce IAA (Table 1). Exogenously applying IAA to *S. nigrum* seeds has a dosage-dependent effect: IAA when added in the range of 100μg ml^{-1} to 10mg ml^{-1} to seeds inhibited seedling root growth, but not when added at two lower concentrations: 1 and 10μg ml^{-1} (Fig. S1). Applying IAA (1μg ml^{-1}) to seeds significantly increased the root growth of seedlings compared with the control. Inoculating seeds with 14 different IAA-producing isolates also modified root growth. Of these, two isolates SSR5-2 and BGCR2-9(1) increased root length in the range between 1.1 and 11μg ml^{-1} of IAA. In addition, three isolates (BGCR2-6, DSL6 and DSR10) whose IAA levels ranged from 93 to 154μg ml^{-1} inhibited root growth. The mean value of bacterial IAA in culture and root length of seedling inoculated with the respective IAA-producing isolates was analyzed using simple regression, and I found a statistically significant negative relationship (r^2=0.771 and P<0.0001) between bacterial IAA production and root growth (Fig. 2C).

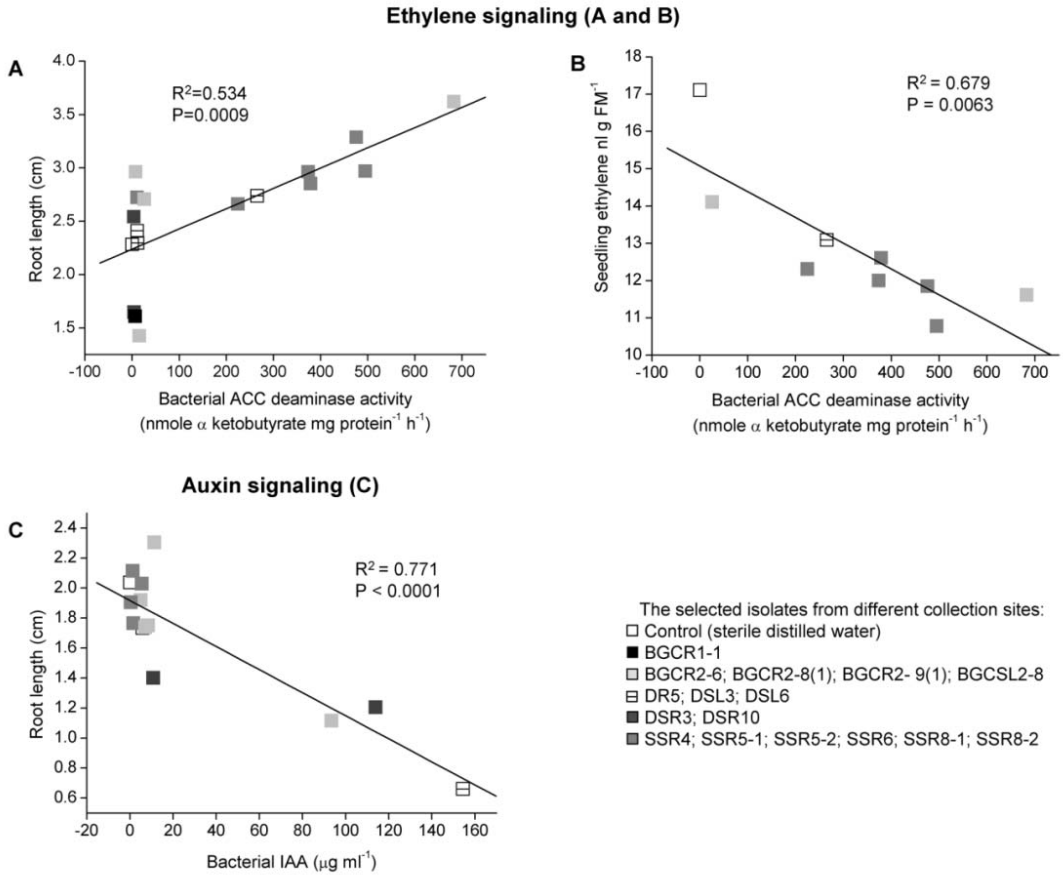

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Figure 2. Regression of bacterial traits that influence ethylene and auxin signaling against *S. nigrum* root growth as measured in the 16 isolates identified in Figure 1. **(A)** Regression of bacterial ACC deaminase activity and root lengths of seedlings inoculated with bacterial isolates. **(B)** Regression of bacterial ACC deaminase activity and ethylene emission from seedlings inoculated with bacterial isolates. **(C)** Regression of bacterial IAA and root lengths of seedlings inoculated with bacterial isolates.

1.3.5. Endophytic bacterial colonization in root

In order to quantify the colonization, I selected seven bacterial isolates with PGP effects. All were able to colonize the inner tissues of seedlings in concentrations of up to 10^6 cfu g^{-1} FM (Table S1). GFP-tagged strains, BGCR2-8(1) and DR5, revealed that they mainly colonize cortex cells and live intercellularly (Fig. 3).

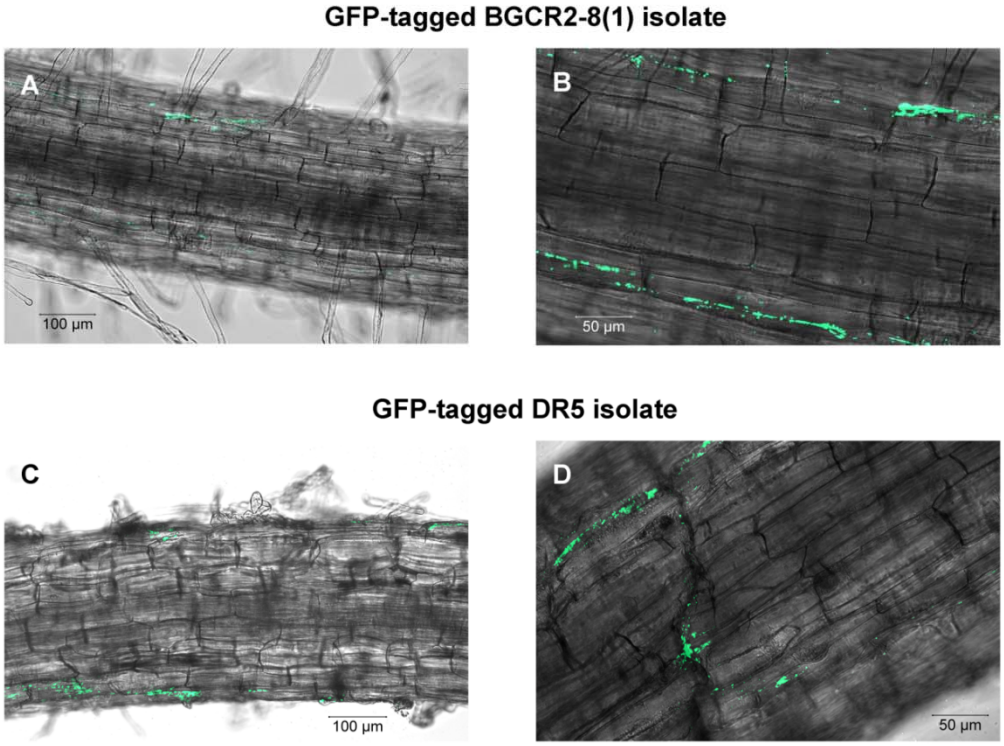


Figure 3. Confocal laser scanning microscopy of roots colonized by the GFP-tagged endophytic bacterial isolates. (A and B) Root colonization by GFP-tagged BGCR2-8(1) isolate at magnification of 100x **(A)** and 200x **(B)** and (C and D) root colonization by GFP-tagged DR5 isolate at magnification of 100x **(C)** and 200x **(D)**.

1.3.6. Growth response of S. nigrum *and* N. attenuata *to natural endophytic bacteria from* S. nigrum *and to type strains*

In order to determine the growth and fitness response of host and non-host plant species to these natural endophytic bacteria, I inoculated the seeds of *S. nigrum* and *N. attenuata* with the endophytic bacterial isolates from *S. nigrum* and measured length of seedling root and hypocotyl. Six isolates from the roots of *S. nigrum* with positive, neutral and negative effects on the root growth of *S. nigrum* were selected to determine the growth response of *N. attenuata* seedlings. These two solanaceous plant species responded differently to being inoculated with these isolates. Four (SSR5-1, SSR4, SSR8-1 and DR5) significantly promoted root growth of *S. nigrum* seedlings 7 days after inoculation (Fisher's PLSD test, $P<0.0001$, $P=0.002$, $P=0.004$ and $P=0.02$, respectively, Fig. 4A). However, none of the selected isolates promoted root growth in *N. attenuata* seedling and some of these isolates even inhibited root growth. These isolates had no effect on the hypocotyl growth of *S. nigrum* seedlings except for isolate SSR4, which significantly increased hypocotyl length 7 days after inoculation (Fisher's PLSD test, P=0.0451). Most of them promoted hypocotyl length of *N. attenuata* seedlings (Fig. 4B).

In order to test the specific response of these two Solanaceous species, I selected six bacterial species from the German culture collection (DSMZ) based on their ability to promote growth (Madhaiyan *et al.*, 2006). Of these six strains, four (*P. brassicacearum* D13227, *P. marginalis* D50276, *M. fujisawaense* D5686 and *P. fluorescens* D8568) exhibited ACC deaminase activity (data not shown). Three strains, *P. brassicacearum* D13227, *B. pumilis* D1794 and *P. marginalis* D50276, significantly promoted the shoot growth of *S. nigrum* 16 days after inoculation (Fisher's PLSD test, $P<0.0001$, $P<0.0001$ and $P=0.0044$, respectively, Fig. 5A). On the other hand, the three strains, *P. marginalis* D50276, *M. fujisawaense* D5686 and *P. fluorescens* D8568, promoted shoot growth of *N. attenuata* 17 days after inoculation (Fisher's PLSD test, $P<0.0001$, $P<0.0001$ and $P=0.0311$, respectively). Three strains, *P. brassicacearum* D13227, *B. pumilis* D1794 and *P. marginalis* D50276, significantly increased the fruit number of *S. nigrum* 48 days after inoculation (Fisher's PLSD test, $P=0.0485$, $P=0.0183$ and $P=0.0039$, respectively), but only one of these strains, *P. marginalis* D50276, significantly enhanced the capsule production in *N. attenuata* 68 days after inoculation (Fisher's PLSD test, $P=0.003$) (Fig. 5B). Finally, only one strain, *P. marginalis* D50276, positively affected the shoot growth and fitness of both plant species.

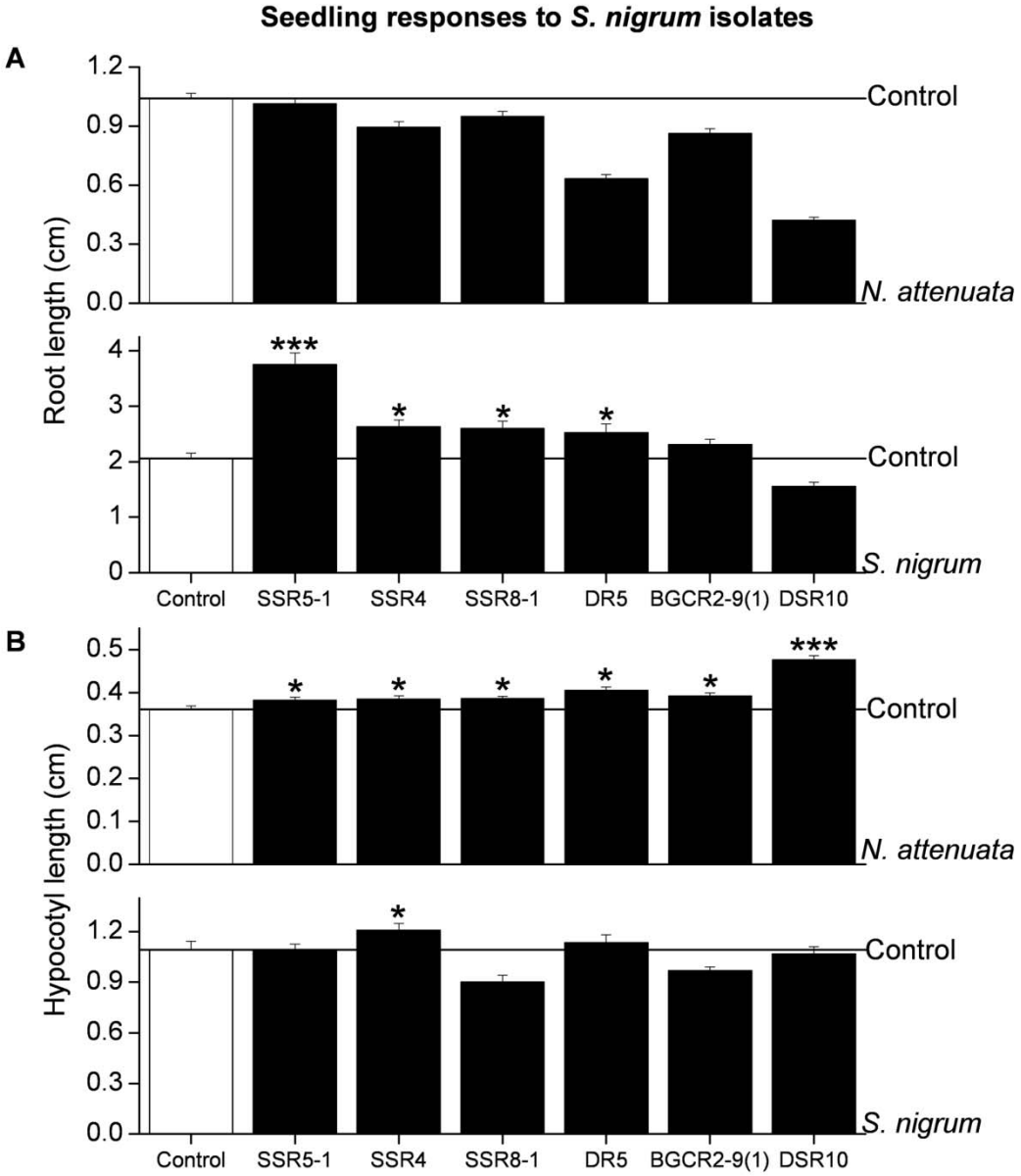


Figure 4. Comparison of of *S. nigrum* and *N. attenuata* seedling growth to bacterial colonization (A and B) of endophytic bacteria isolated from *S. nigrum*. Six isolates were selected based on their effects on *S. nigrum* seedling growth. **(A)** Mean root length (±SE) and **(B)** mean hypocotyl length (±SE) of *S. nigrum* and *N. attenuata* seedlings. Asterisks indicate significant differences in promotion of root and hypocotyl growth in *S. nigrum* and *N. attenuata* seedlings by the bacterial isolates compared to the control at $P<0.05$ (*); $P<0.001$ (**); and $P<0.0001$ (***).

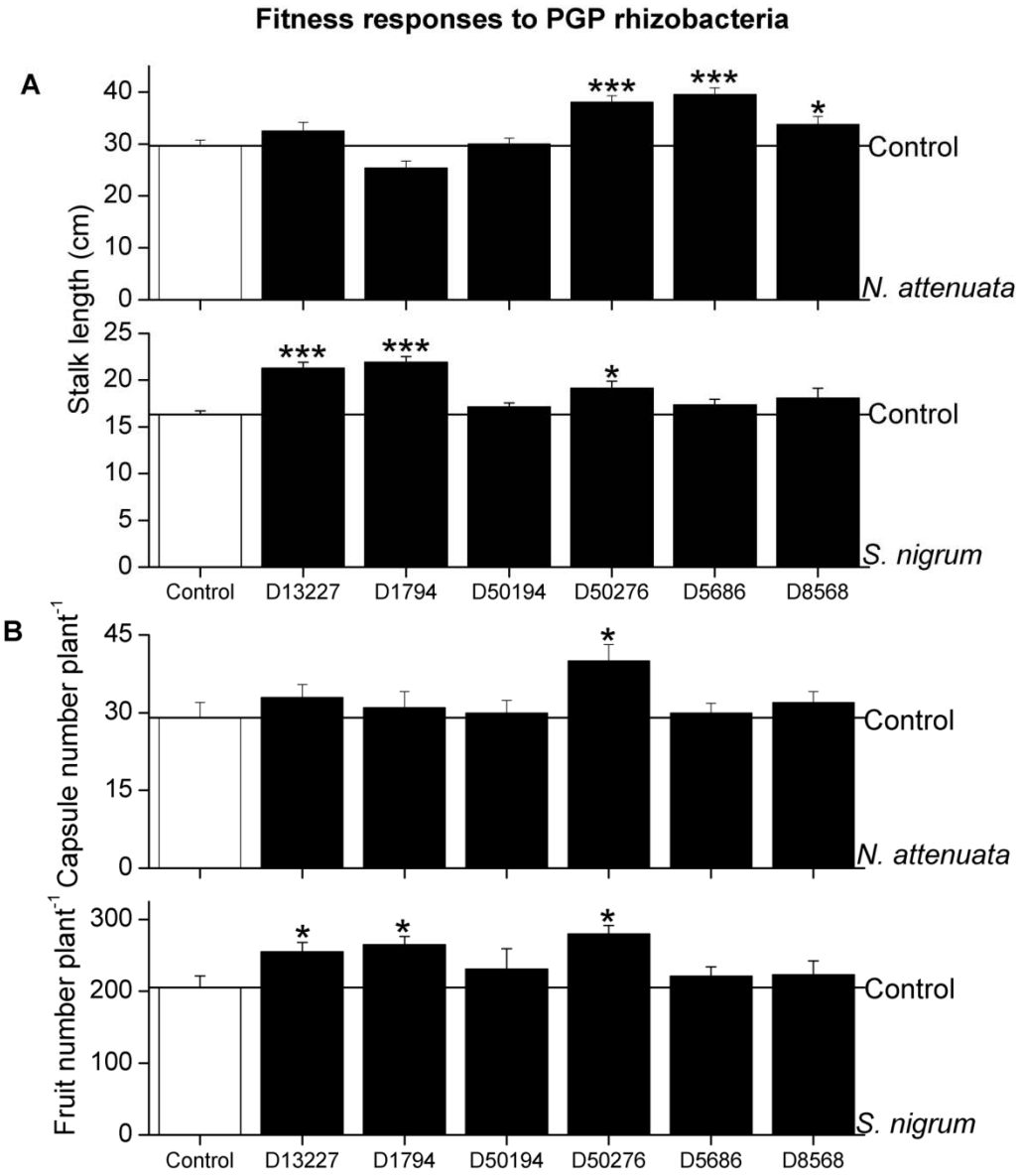


Figure 5. Reproductive growth and fitness responses of *S. nigrum* and *N. attenuata* plants to known mutualistic bacterial strains (*Pseudomonas brassicacearum* D13227, *Bacillus pumilis* D1794, *Pseudomonas putida* D50194, *Pseudomonas marginalis* D50276, *Methylobacterium fujisawaense* D5686 and *Pseudomonas fluorescens* D8568). **(A)** Mean stalk length (±SE) of *S. nigrum* and *N. attenuata*, **(B)** Mean (±SE) fruit number per plant (*S. nigrum*) and capsule number per plant (*N. attenuata*). Asterisks indicate significant differences (Fisher's PLSD test; $P<0.05$ (*); $P<0.001$ (**); and $P<0.0001$ (***)).

1.4. Discussion

The rhizosphere is where plant roots come in contact with soil-borne microbial communities. Plant-microbe interactions mostly involve microorganisms colonizing the roots of their hosts, namely growing plants. Colonization can take the form of many different interactions ranging from symbiosis to parasitism; each interaction affects plant fitness differently. Although plant-pathogen interactions are well studied, our understanding of the complex interactions of native endophytic bacteria with their plant hosts is rudimentary, at best. I isolated bacteria from field-grown *S. nigrum* and discovered a rich endophytic community with strong prevalence of *Pseudomonas* which is well-known for plant growth promotion (Mercado-Blanco & Bakker, 2007); many of the isolated bacteria promoted growth and fitness of their host by modulating ET and IAA homeostasis. Although these phytohormonal pathways are conserved across plant species, the effects on a related solanaceous non-host plant, *N. attenuata*, differed, notwithstanding the similar extent to which the bacteria colonized the roots. This raises the questions: How consistent are interactions between plants and endophytic bacteria? Which mechanisms underlie these interactions? And which factors determine the outcome of the interaction?

PGP mechanisms of endophytic bacteria are thought to be similar to those of PGP rhizobacteria; namely, they affect plant growth by producing phytohormones, such as cytokinins or auxins, or by degrading hormone precursors, such as ACC by ACC deaminase (Glick *et al.*, 1998; Madhaiyan *et al.*, 2006). This is largely supported by our findings. Changes in root growth of *S. nigrum* are clearly correlated to the production of IAA and ACC deaminase by a majority of endophytic bacteria I isolated (Fig. 2). Seedling ET emission were significantly lower after inoculation with ACC deaminase-producing isolates and subsequently, their roots grew longer than those of untreated seedlings (Fig. 2A & B). The relatively widespread production of IAA by plant-associated bacteria suggests that bacterial IAA stimulates root the development of host plants (Patten & Glick, 2002; Sergeeva *et al.*, 2007). I also observed that IAA-producing isolates stimulated root growth, but only when they released low quantities of IAA; high levels of bacterial or exogenously applied IAA repressed it (Fig. 2C; Fig. S1). The concentration of exogenous IAA apparently determines the outcome of the interaction with IAA-producing endophytes.

PGP rhizobacteria living in the rhizosphere are generally believed to be beneficial for all plant species they associate with because of their conserved influence of phytohormones on plant growth (Sturz & Nowak, 2000; Cakmakci *et al.*, 2007; Domenech *et al.*, 2007). Studies on conifers and PGP rhizobacteria suggest that bacteria isolated from the rhizosphere of spruce sometimes interact with only certain ecotypes and the outcome of the interaction depends largely on experimental conditions (Chanway *et al.*, 2000). For endophytic bacteria, even less is known. Zinniel *et al.,* (2002) studied the host range of 29 endophytic bacteria that had been isolated from sorghum or corn; 26 were able to colonize at least one other host plant in sufficient densities, leading to the conclusion that these interactions are largely unspecific. When *S. nigrum* interacts with PGP native endophytes, their influence on the homoeostasis of IAA and ET explains at least part of the observed phenotypes, including growth modulation. Given that hormonal regulation is conserved among plants, I had anticipated that these PGP effects of IAA and ET would be similar in *N. attenuata*. However, this was not the case; ACC deaminase and IAA apparently affect root growth in a highly host species-specific manner (Fig. 4A) and this specificity is determined by the bacteria.

One possible explanation for the discrepancy is the relationship between bacterial ACC deaminase and IAA, and these bacteria's mutual effects on root growth; some models describe how ACC deaminase counteracts ET-repressed auxin-response factors (ARFs) involved in root growth (Glick *et al.*, 2007). The presence of ACC deaminase-producing rhizobacteria in the rhizosphere can depress the expression of auxin response genes in the shoots (Glick *et al.*, 2007). Although it is well known that IAA can activate the transcription of ACC synthase (Kende & Zeevaart, 1997), it is less known whether ET inhibits IAA transport and signal transduction (Prayitno *et al.*, 2006). The feedback loop of ET inhibition of IAA synthesis may limit the amount of ACC synthase, ACC, and, ultimately, ET that is released in response to stressful events in the life of the plant. The cross-talk between ET and IAA is so tightly regulated that phytohormonal imbalances might disturb plant growth and plants are generally very sensitive to IAA. Another host's endophyte might thus produce too little or too much of it and, consequently, profoundly influence plant growth. Consistent with this scenario, is the observation that *N. attenuata* root growth decreased rather than increased when exposure to some PGP bacteria (Fig. 4A). Finally, it remains to be elucidated which additional compounds are important in mediating the interaction of beneficial endophytic bacteria with *S. nigrum*.

The different responses of host and non-host species to the natural endophytic bacteria may result from a combination of several factors. The colonization success of PGP rhizobacteria reportedly increases the growth and fitness of many host plant species (Chanway *et al.*, 2000; Benizri *et al.*, 2001). I found no significant differences in how successfully endophytic bacteria colonize the host, *S. nigrum*, and the non-host, *N. attenuata*. *Pseudomonas thivervalensis* DR5 colonized roots of both *N. attenuata* and *S. nigrum* ($1.4x10^9$ and $1.0x10^8$ cfu gFM^{-1}, respectively). However, *P. thivervalensis* DR5 significantly decreased root length of *N. attenuata*, while increasing root length in *S. nigrum*. In addition, endophytes may have evolved from parasites and may still have parasitic tendencies (Kogel *et al.*, 2006) potentially contributing to incompatible interactions with non-hosts. *N. attenuata* may recognize the endophytic bacteria from *S. nigrum* as pathogens regardless of their stimulatory or inhibitory effects on *S. nigrum*. Root growth diminishes when energy is allocated for defense or for saving storage above-ground. Our observations of increased hypocotyl growth of *N. attenuata* upon inoculation with the selected endophytic bacteria isolated from roots of *S. nigrum* are consistent with such a scenario (Fig. 4B). *S. nigrum*, however, has likely evolved to be able to discriminate between its specific endophytes and pathogens thanks to its long association with its natural endophytic bacterial communities. The way in which *N. attenuata* copes with the endophytic bacteria in its roots appears to be different but has not yet been analyzed. When the two plant species were inoculated with "generalistic" PGP rhizobacteria from the DSMZ culture collection, their growth and fitness differed (Fig. 5A&B). Clearly, the PGP effects of native/natural endophytic bacteria on their host and non-host plant species are not the same.

Different behaviour of endophytic bacteria in the host and non-host plant species might be linked to the different environmental conditions under which the host and non-host grow. Black nightshades occur throughout the world in pioneer communities on open, disturbed and nutrient-rich soils, such as riverbanks, and have invaded many agricultural habitats, such as fields, gardens, and wasteland (Schmidt *et al.*, 2004). In contrast, *N. attenuata* evolved to optimize its growth in the immediate post-fire environment of deserts in southwestern United States; seeds germinate synchronously into nitrogen (N)-rich soils and hence have selected to grow rapidly when water availability is high (Baldwin, 2001). Habitat-dependent co-evolution is likely to shape the particular endophytic bacterial communities that best fit a given habitat.

These findings demonstrate that native endophytic bacteria with PGP traits do not have general and predictable effects on the growth and fitness of all host plants, although the underlying mechanisms are conserved. Clearly much more can be learned from studying interactions between native endophytic bacteria and other native plant species in their ecological context.

Chapter II. The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception

2.1. Introduction

Endophytic bacteria reside inside plants and have been extracted from surface-sterilized tissues of cultivated and native herbs and trees (reviewed by Hallmann *et al.*, 1997; Ryan *et al.*, 2008). They are thought to be recruited from the surrounding soil (reviewed by Ryan *et al.*, 2008) and have a continuum of effects on their host plant, from advantageous to detrimental (Kobayashi & Palumbo, 2000). The beneficial effects, such as growth promotion and disease control (Sturz *et al.*, 1997; Sessitsch *et al.*, 2004; Long *et al.*, 2008) can result from indirect interactions during which endophytes may provide nutrients (N, P) to the plant or antagonize pathogens, as well as from direct interactions, such as when endophytes modify host phytohormone homeostasis (Sessitsch *et al.*, 2004; Berg *et al.*, 2005; Long *et al.*, 2008).

While plant-pathogen interactions and their underlying genetic mechanisms have been extensively studied, less is known about plant-rhizosphere or even endophyte-plant relationships. Numerous studies have characterized bacterial diversity in scores of plants and reported that the bacterial endophytic community can be specific to particular species of host plants and even cultivars (Sturz *et al.*, 1997; Adams & Kloepper, 2002; Araujo *et al.*, 2002; Zinniel *et al.*, 2002; Rasche *et al.*, 2006; Sun *et al.*, 2008). The composition of root exudates is thought to play a central role in recruiting bacteria from the bulk soil into the rhizosphere (Bais *et al.*, 2006; Hartmann *et al.*, 2008; Micallef. *et al.*, 2009) and finally into the endosphere (Balachandar *et al.*, 2006), but little is known about specific plant genes that facilitate the recognition and selection of endophytic bacteria.

Research in plant-pathogen interactions has demonstrated that three phytohormones play a central role in mediating resistance to plant pathogens: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Reymond & Farmer, 1998; De Vos *et al.*, 2006). Recently, the importance of JA and SA signaling in influencing bacterial endophyte communities was examined in *Arabidopsis thaliana*. *Arabidopsis* plants with elevated SA-regulated defense responses were less colonized by bacterial endophytes and harbored lower species diversity than plants of the Col ecotype, while JA signaling had no effect on the endophytic community (Kniskern *et al.*, 2007).

To date, the influence of ET signaling on the composition of the endophyte community has not been thoroughly examined.

ET mediates not only a diverse suite of metabolic and senescence processes (Bleecker & Kende, 2000) but also responses to abiotic and biotic stresses, including plant-pathogen interactions. During the early stages of these interactions, ET is thought to mediate the elicitation of defense reactions to pathogen attack (Piatti *et al.*, 1991), and in susceptible plant-pathogen interactions, an ET burst is commonly observed early in the interaction, accelerating senescence processes (Stearns & Glick, 2003). Other studies however, highlight the role of ET in pathogen resistance. The Arabidopsis mutants *ein2*, which are unable to perceive ET, are more susceptible to the necrotrophic fungus *Botrytis cinerea* than are WT plants (Thomma *et al.*, 1999); however, susceptibility to the biotrophic *Pseudomonas syringae* pv. *tomato* was not altered (Pieterse *et al.*, 1998). Reviewing the complicated and often contradictory reports on ET's role, van Loon *et al.*, (2006) recently concluded that ET's signaling function depends on the nature of the pathogen.

The role of ET in mediating plant-endophyte interactions remains largely unexplored. Iniguez *et al.*, (2005) reported the hypercolonization of an ET-insensitive *Medicago truncatula* line by the endophyte *Klebsiella pneumoniae* 342 (Kp342), and Cavalcante *et al.*, (2007) found that expression of an ET receptor and two ET transcription factors were differentially regulated in sugarcane in response to inoculation with diazotrophic endophytic bacteria.

The ET-related bacterial communities, ACC deaminase (ACCd) and IAA producing bacteria, are known to affect plant growth positively by interfering with ET signaling (Glick *et al.*, 2007). While the mechanisms of PGP are explored, the selective recruitment of these beneficial bacterial communities by the plant remains unknown (Hardoim *et al.*, 2008). Is the recruitment of ACCd and IAA producing bacteria into the plant endosphere more than a chance event? Is the colonization process mediated by a plant's ET production or perception? The use of plants with contrasting ET levels might help to elucidate this intriguing question.

Endophytic bacterial populations can be regarded as a subset of the soil microbial community and thus influenced by the soil's chemical and physical properties; however, in many cases, the rhizosphere communities of different plant species growing in the same soil are distinct (Marschner *et al.*, 2004; Garbeva *et al.*, 2008). That the same plant species can recruit a similar microbial community from different soils (Miethling *et al.*, 2000) points to a plant-driven

selection process, but the extent to which soil and host plant determine the composition of the endophyte community remains unresolved (Garbeva *et al.*, 2004).

In order to manipulate endophytic bacterial populations to benefit plants, a better understanding of how plant signaling systems (e.g. ET signaling) influence the recruitment of endophytic bacterial communities from different soil types is needed. The wild tobacco species *Nicotiana attenuata*, which is native to the Great Basin Desert, USA, has evolved the ability to germinate in post-fire N-rich soils (Lynds & Baldwin, 1998). So far, nothing is known about the diversity of endophytic bacterial communities associated with this plant in nature. Here I characterize the culturable bacterial endophyte communities of *N. attenuata*'s roots and ask the following questions: Do plant ET signaling and the ability of plants to sense or produce ET play a role in the recruitment of endophytic bacteria? Do bacterial isolates specifically colonize distinct host genotypes? How do the bacterial endophytes influence plant growth? Do plant genotype and differential ET production affect the recruitment of ACCd and IAA producing bacteria into the plant endosphere?

I used wild type (WT) and two isogenic transgenic lines of *N. attenuata* plants, ir-*aco1* and 35S-*etr1,* to assess the effects of ET signaling on the diversity of culturable endophytic bacteria in roots. *N. attenuata* ir-*aco1* can sense ET but is deficient in ET biosynthesis, and hence have enhanced sensitivity to ET; *N. attenuata* 35S-*etr1* plants are impaired in their ability to perceive ET and as a consequence tend to overproduce ET (von Dahl *et al.*, 2007). All genotypes were grown in four different soils, two organic and two mineral, collected from *N. attenuata*'s native habitat in SW Utah, USA, just before the germination of the native seed bank. I focused on the culturable bacterial endophyte community in order to test hypotheses about the specificity of the association between plant and bacteria with re-colonization assays. To the best of our knowledge, this is the first examination of ET signaling in the selection of bacterial endophytes.

2.2. Materials and Methods

2.2.1. Plant materials

The following inbred WT and transgenic lines of *N. attenuata* were used in all experiments: *N. attenuata* ecotype Utah inbred line 22, ET-biosynthesis-deficient transgenic line ir-*aco1* (A-03-321-10) and ET-insensitive line 35S-*etr1* (A-03-328-8), which are derived from

the same Utah inbred line and are therefore isogenic. The lines are fully characterized in von Dahl *et al.*, (2007). Seed germination procedures are described elsewhere (Krugel *et al.*, 2002; Long *et al.*, 2008) and also detailed in General materials and methods.

2.2.2. Bacterial strains

Two generalist bacterial species were selected from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), the German culture collection, *Pseudomonas brassicacearum* DSM13227 and *Pseudomonas fluorescens* DSM8568 for the experimental colonization assay. All other strains were isolated from plants grown in native Utah soils. The characteristics and origin of these isolates are presented in Table 1.

Table 1. Bacterial strains used in this study

Bacterial strains/isolates	**Origin**	**Characteristics**			**Reference**
		ACCd	**IAA-Trp**	**IAA+Trp**	
Sphingomonas sp.	WT plant root, soil O2	+	-	+	This study
Curtobacterium sp.	WT plant root, soil O2	+	-	+	This study
Bacillus endophyticus	ir-*aco1* plant root, soil O1	+	-	-	This study
Methylobacterium extorquens	ir-*aco1* plant root, soil O1	+	-	-	This study
Bacillus cereus	35S-*etr1* plant root, soil O2	+	+	+	This study
Pseudomonas thivervalensis	35S-*etr1* plant root, soil M1	+	+	+	This study
Bacillus megaterium	ir-*aco1* plant root, soil O1	+	+	+	This study
Bacillus simplex	ir-*aco1* plant root, soil O1	-	+	+	This study
Pseudomonas fluorescens DSM8568	DSMZ	+	+	+	Long *et al.* (2008)
Pseudomonas brassicacearum DSM13227	DSMZ	+	+	+	Long *et al.* (2008)

-, negative; +, positive

2.2.3. Soil collection

Four types of soils were collected from *N. attenuata*'s native habitat in Utah, USA (Table 2), on Jan. 26, 2008. All soils contained the variable N derived of NH_4^+ and NO_3^- described in (Lynds & Baldwin, 1998) and contained *N. attenuata* seeds from previous years of *N. attenuata* growth. The soils, therefore, contained the bacterial community from which the seeds would

recruit their endophyte community when they germinated two months later. All soils were stored at 4°C for three days during shipment before being used in experiments. Two organic soils, located at the base of burned (soil O1) or unburned (soil O2) juniper trees, were collected from the following location (N37° 04 02.6 W 113° 53 04.04). The juniper tree was burned by wildfires in 2004 and again in 2006. Two mineral soils were collected, one that had burned in 2006 and 2007 (M1) near Castle Cliffs along Rt. 91 (N37° 05 23.5 W 113° 50 42.4) and another (M2) from 8 km north on Rt. 91 near the TV tower that had burned in 2007 (N37° 06 01.9 W 113° 49 22.7).

2.2.4. Soil analysis

Analysis of total C and N contents in the soil samples was performed by the ChemLab of Max Planck Institute for Biogeochemistry (Jena, Germany) (http://www.bgc-jena.mpg.de/service/chem_lab/roma/elemental_analysis/elemental_analysis.shtml). For the pH measurement, 1 g of soil was suspended in 10 ml of distilled water and shaken for 3 min. The slurry was allowed to settle for 30 min at room temperature before a pH electrode (Schott) was dipped into the supernatant to determine soil pH.

2.2.5. Isolation of culturable endophytic bacteria

Ten days after germination, one seedling from each genotype (WT and transformed lines) was transferred individually to 7cm pots containing the Utah soils. In total, three replicates per line and soil were cultivated. Plants and soils were cultured in the glasshouse (16/8 h photoperiod at 200-300 µmol m^{-2} s^{-1}, 25/21°C, and 45-55 % relative humidity) and watered with sterile distilled water. Plants were harvested at the end of the rosette stage of growth (23 days after transplanting) and the soil was carefully removed from the roots under running tap water. Endophytic bacteria were isolated as described by Long *et al.*, (2008) and also detailed in General materials and methods.

2.2.6. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Approximately 120 ng of DNA (PCR product) for each sample was digested in 20 µl reactions containing 2 µl of 10X Buffer Tango (Fermentas, http://www.fermentas.com), 10-15 µl sterile deionized water (depending on the DNA concentration) and 5U of the restriction enzyme

HinfI or *MspI* (Fermentas, http://www.fermentas.com). The reaction was incubated at 37^{o}C overnight. Band sizes were visualized in 2% agarose containing ethidium bromide under UV light.

Cluster analysis was performed to create dendrograms that group similar bacterial isolates from different plant genotypes and soil types together according to their banding profile. The unweighted pair-group moving average (UPGMA) clustering and a Bray-Curtis similarity matrix were calculated for both restriction enzymes using PAST (http://folk.uio.no/ohammer/past/) multivariate statistics program. Bray-Curtis clustering compares the presence or absence of restriction banding patterns in bacterial isolates from different plant genotype and soil type. I refer to each banding pattern as an operational taxonomic unit (OTU). Given the fact that *HinfI* restriction yielded a more diverse banding profile than *MspI* digestion (Fig. S1b), further analysis was based on the *HinfI* restriction (Fig. S1a).

2.2.7. 16S rDNA gene sequencing

The same proportional number of bacterial isolates was selected for sequencing according to plant genotype, *HinfI*-OTU clustering and soil type. The sequencing was carried out as described in General materials and methods.

The sequences were deposited in GeneBank (http://www.ncbi.nlm.nih.gov/Genbank/) with the accession numbers FJ639178-FJ639250. Alignment with related sequences from type strains in GenBank, bootstrap calculations and phylogenetic tree construction were carried out with MEGA4 (Tamura *et al.*, 2007). Distances, including pair-wise deletions and insertions, were calculated according to Jukes and Cantor (Jukes & Cantor, 1969), whereupon the overall neighbor-joining (NJ) phylogenetic dendrogram (Saitou & Nei, 1987) was inferred, rooted and bootstrapped 1000 times (Felsenstein, 1985).

2.2.8. Bacterial colonization assays in vitro *and in the glasshouse*

In order to test the specificity of the observed pattern of bacterial endophyte colonization among the plant genotypes, seedlings of each genotype were individually inoculated with one of ten bacterial endophytes (Table 1), called “single inoculation”: two bacterial strains exclusively isolated from one plant genotype, called “specialists”; two bacterial strains isolated from all three plant genotypes, called “generalists”; and two type strains from the DSMZ culture collection

called “DSMZ generalists” known to be able to colonize an array of plant species, were included in the experiments as “positive controls”. An additional set of seedlings from each genotype was inoculated with a mixture of all ten bacterial endophytes, called “mixed inoculation”. The experimental set-up and the scheme for the cross-inoculation of bacterial isolates into host or non-host plants are described in Fig. 3A. Bacterial suspensions in sterile distilled water (OD_{600}=1.2) were used for seed inoculation; control seeds were treated with sterile distilled water only. For the *in vitro* colonization assays, the inoculated seeds (20 seeds per Petri dish, 3 dishes for each combination) were incubated at room temperature overnight and transferred to sterile filter papers (Whatman No.1) in Petri dishes maintained in Percival growth chambers (13/11h day/night cycle, 155µmol m^{-2} s^{-1}, 26/24°C). One week after inoculation, bacterial isolation was carried out as described above. Two independent experiments were carried out for all seedling assays. Bacterial identity was determined by 16S rDNA sequencing.

In the glasshouse, 12-day-old seedlings were planted in Teku pots (five replicates per genotype and bacterial inoculum) in a random design (16/8 h photoperiod at 200-300 µmol m^{-2} s^{-1}, 25/21°C, and 45-55 % r.h.). The Teku pots were placed in separate trays to avoid cross-contamination and watered whenever needed with sterile water. One ml of single inoculum of the following bacterial isolates (Table 1) was applied to the rhizosphere: *P. brassicacearum* DSM13227, *B. megaterium*, *M. extorquens*, *Curtobacterium* sp. and *P. thivervalensis*. Twelve days after inoculation, roots were collected and bacterial isolation was performed as described above. Bacterial identity was determined by 16S rDNA sequencing. One experiment was carried out.

2.2.9. Seedling ethylene measurement

ET emissions from WT, ir-*aco1* and 35S-*etr1* seedlings were measured continuously and non-invasively in real-time with a photoacoustic spectrometer (INVIVO, Saint Augustin, Germany) as described by (von Dahl *et al.*, 2007). Thirty seeds were germinated in 100 ml three-neck flasks on filter paper and cultivated in a Percival growth chamber (13/11h day/night cycle, 155 µmol m^{-2} s^{-1}, 26/24°C). After 12 days, flasks containing the seedlings were subjected to ET measurements (five replicates per genotype). Five empty flasks with filter paper and sterile distilled water served as controls.

2.2.10. Microscopic analysis and root morphological investigations

Seeds of WT, ir-*aco1* and 35S-*etr1* were germinated for 12 days on filter paper as described above. For root hair count, the first 1mm section of the root was photographed under a Zeiss Image Z1 microscope (Zeiss, Germany, 100x magnifications). The pictures were printed and numbers of root hairs were counted. Observation of the whole seedling was carried out using an Olympus SZX10 microscope (Zeiss, Germany, 10x magnifications). Root length of seedlings was measured using a ruler.

2.2.11. Seedling response assay

Shortly after isolation, 139 bacterial isolates were singly inoculated into WT seeds on filter paper as described above. Seedling growth responses to bacterial inoculum were classified as beneficial, pathogenic and neutral (Table 5) based on their effects on root length, hypocotyl length, number of true leaves and lateral root formation. "Beneficial" means that the bacterium enhanced at least one of the above parameters and had no negative effect on the other parameters. "Pathogenic" means that the bacterium caused detrimental effects on at least one of the above parameters. "Neutral" means that the bacterium did not cause any effect on the above parameters in the inoculated seedlings.

2.3. Results

2.3.1. Both soil type and plant genotype influence the culturable endophytic bacterial community

The soils were characterized as organic (O1 and O2) and mineral (M1 and M2) based on the contents of carbon (C) and nitrogen (N) and pH (Table 2). The total C contents of the two organic soils (O1 and O2) are 18.7% and 17.7%, respectively, while their N contents are similar (0.73%). The C contents of the two mineral soils (M1 and M2) are 3.2% and 2.6% and their N contents are 0.07% and 0.08%, respectively. The pH of these soils ranges from 8.5 to 9.0. In order to estimate the degree of colonization of the three genotypes grown in the different soils, the colony-forming units (CFUs) of endophytic bacteria representing the total culturable endophytic bacterial community for each plant genotype by soil type combination were determined (Fig. 1). On average, colonization of WT plants was high but variable among the four soils, yet colonization of ir-*aco1* and 35S-*etr1* plants varied even more. Plants grown in the two

organic soils accommodated the most endophytic bacteria; those cultivated in the mineral soils M1 and M2 harbored significantly fewer ($P<0.05$). No bacterial endophytes were isolated from ir-*aco1* and 35S-*etr1* plants grown in the mineral soils, M1 and M2, respectively. A two-way ANOVA revealed that the total endophytic bacterial community depended significantly on both plant genotype and soil type ($P<0.05$, Table S1).

Table 2. Soil properties

Soil	Soil types	C content (%)	N content (%)	pH
O1	Organic, burned	18.72	0.73	8.5
O2	Organic, unburned	17.77	0.73	8.6
M1	Mineral, burned	3.16	0.07	9.0
M2	Mineral, burned	2.60	0.08	8.8

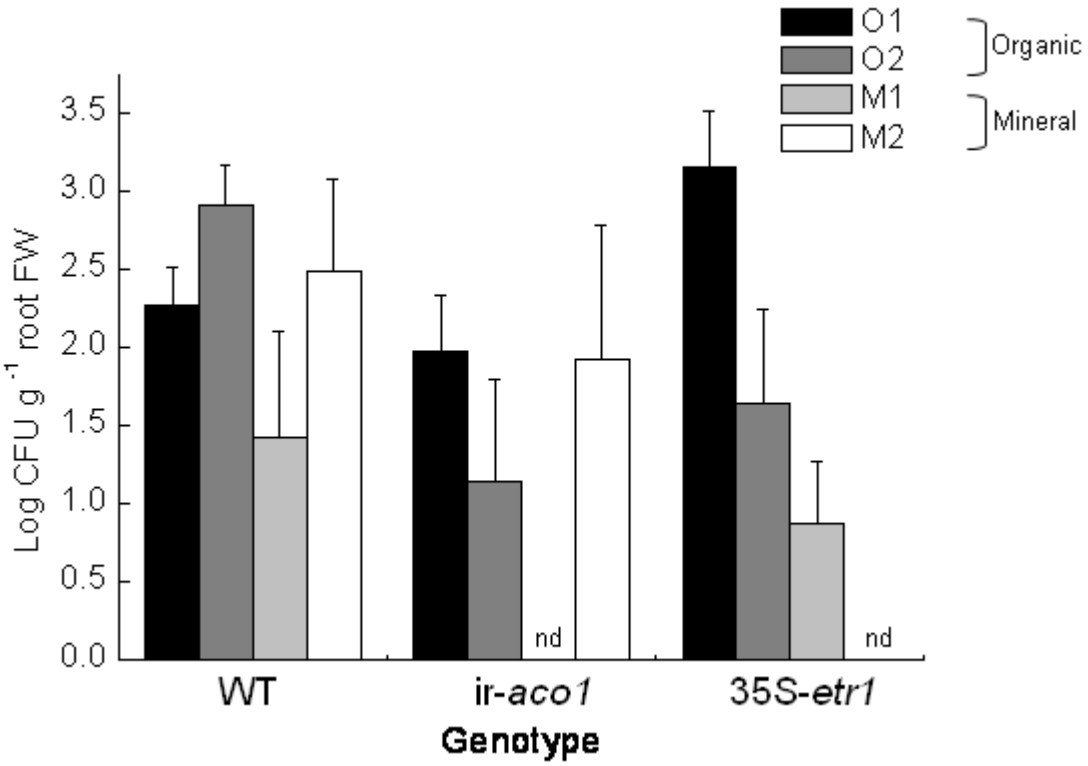


Figure 1. CFU isolated from roots of WT and transgenic plants grown in four different soils. nd: not detected.

2.3.2. The diversity of culturable endophytic bacteria

In total, 139 bacterial isolates were picked from half-strength YPDA media. However, 36 bacterial isolates were not viable after long-term preservation. Looking at bacterial colony morphology, I found that WT plants harbored the highest bacterial diversity (68 isolates), followed by ir-*aco1* (36 isolates) and 35S-*etr1* (35 isolates). All isolates were grouped into 28 OTUs according to similarities within *HinfI*-digested 16S rDNA banding patterns. For 16S rDNA sequencing, 73 representative isolates were chosen based on the plant genotype, soil type and OTU cluster that they were originally isolated from (Fig. 2).

Figure S1 shows the Bray-Curtis similarity of the OTU clusters in which the representative isolates with their origins (soil type and plant genotype) and number of isolates for each OTU cluster are presented. Most of the bacterial isolates from organic and mineral soils were found to separate into distinct OTU clusters. Bacterial isolates belonging to *Bacillus* sp. were isolated only from plants grown in the organic soils.

I analyzed the culturable bacterial diversity of three plant genotypes that differed in their production and perception of ET (Table 3 and Fig. 2). WT plants were found to harbor the highest diversity of endophytic bacteria sequenced, followed by 35S-*etr1* and ir-*aco1* plants. The sequence analysis revealed that *Bacillus* sp. and *Pseudomonas* sp. were the most abundant genera isolated from WT and transgenic plants. Fifteen bacterial species belonging to six bacterial genera were found in WT plants; eleven bacterial species belonging to three bacterial genera were found in 35S-*etr1* plants; and eight bacterial species belonging to three bacterial genera were found in ir-*aco1* plants. Interestingly, a small fraction of these isolates were found exclusively in either WT or transgenic plants. *Sphingobium* sp., *Sphingomonas* sp., *Curtobacterium* sp., *B. nealsonii*, and *P. brassicacearum* were found exclusively in WT plant roots. Two bacterial species, *P. thivervalensis* and *B. cereus,* were found only in 35S-*etr1* plant roots, and two bacterial species, *Methylobacterium extorquens* and *B. endophyticus,* were found exclusively in ir-*aco1* plant roots.

Bacterial diversity is also dependent on the soil type in which different plant genotypes were grown. Plants grown in the two organic soils (O1 and O2) harbored a high endophytic bacterial diversity compared to the bacterial community of plants grown in the two mineral soils (M1 and M2); this community was represented only by *Pseudomonas* spp. The endophytic bacterial community of plants grown in the recently fire-affected soil, O1, was represented by

two bacterial genera; in contrast, the endophytic bacterial community of *N. attenuata* plants grown in the unaffected soil O2 was represented by six different genera (Table 4).

Table 3. Number of culturable bacterial endophytes from different plant genotypes

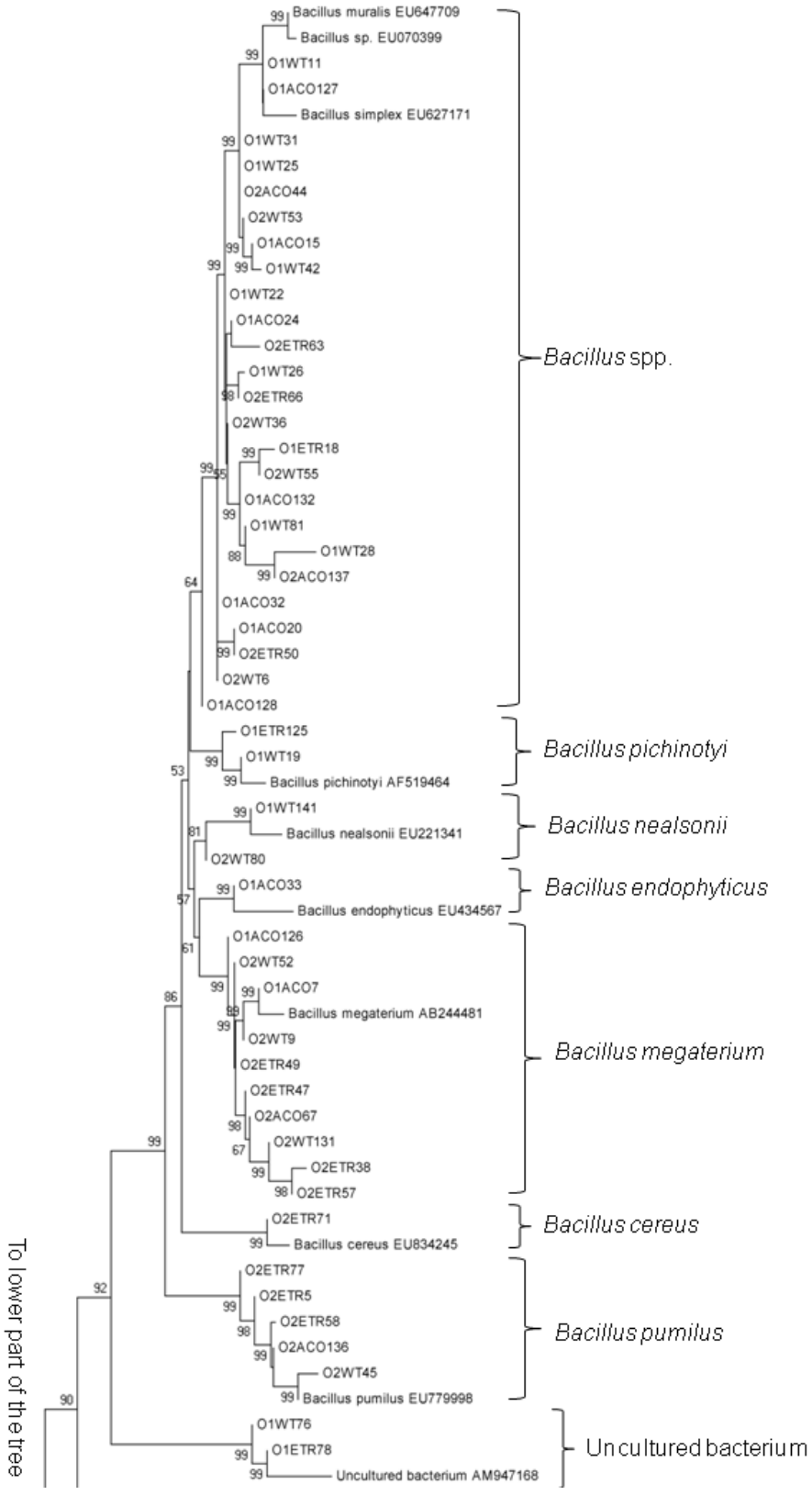
Bacterial genera/species	**WT**	**ir-*aco1***	**35S-*etr1***
Bacillus sp.	11	7	3
Bacillus simplex	2	2	1
B. pumilus	1	1	3
B. pichinotyi	1	-	1
B. nealsonii	1	-	-
B. muralis	1	1	1
B. megaterium	2	2	3
B. endophyticus	-	1	-
B. cereus	-	-	1
Curtobacterium sp.	2	-	-
Methylobacterium extorquens	-	1	-
Pseudomonas sp.	6	4	2
Pseudomonas thivervalensis	-	-	2
P. putida	1	-	1
P. fluorescens	1	2	-
P. brassicacearum	1	-	-
Sphingomonas sp.	1	-	-
Sphingobium sp.	1	-	-
Uncultured bacterium	1	-	1
Total	**33**	**21**	**19**

-, absence; the highlighted marks represent bacterial species specific for a particular genotype.

Table 4. Number of culturable bacterial endophytes from different soil types

Bacterial genera/species	**O1**	**O2**	**M1**	**M2**
Bacillus sp.	11	10	-	-
Bacillus simplex	3	2	-	-
B. pumilus	-	5	-	-
B. pichinotyi	2	-	-	-
B. nealsonii	1	-	-	-
B. muralis	2	1	-	-
B. megaterium	2	5	-	-
B. endophyticus	1	-	-	-
B. cereus	-	1	-	-
Curtobacterium sp.	-	2	-	-
Methylobacterium extorquens	-	1	-	-
Pseudomonas sp.	1	4	3	4
Pseudomonas thivervalensis	-	-	2	-
P. putida	-	-	2	-
P. fluorescens	-	1	1	1
P. brassicacearum	-	-	-	1
Sphingomonas sp.	-	1	-	-
Sphingobium sp.	-	1	-	-
Uncultured bacterium	2	-	-	-
Total	**25**	**34**	**8**	**6**

-, absence


Bacillus muralis EU647709
Bacillus sp. EU070399
O1WT11
O1ACO127
Bacillus simplex EU627171
O1WT31
O1WT25
O2ACO44
O2WT53
O1ACO15
O1WT42
O1WT22
O1ACO24
O2ETR63
O1WT26
O2ETR66
O2WT36
O1ETR18
O2WT55
O1ACO132
O1WT81
O1WT28
O2ACO137
O1ACO32
O1ACO20
O2ETR50
O2WT6
O1ACO128
Bacillus spp.
O1ETR125
O1WT19
Bacillus pichinotyi AF519464
Bacillus pichinotyi
O1WT141
Bacillus nealsonii EU221341
O2WT80
Bacillus nealsonii
O1ACO33
Bacillus endophyticus EU434567
Bacillus endophyticus
O1ACO126
O2WT52
O1ACO7
Bacillus megaterium AB244481
O2WT9
O2ETR49
O2ETR47
O2ACO67
O2WT131
O2ETR38
O2ETR57
Bacillus megaterium
O2ETR71
Bacillus cereus EU834245
Bacillus cereus
O2ETR77
O2ETR5
O2ETR58
O2ACO136
O2WT45
Bacillus pumilus EU779998
Bacillus pumilus
O1WT76
O1ETR78
Uncultured bacterium AM947168
Uncultured bacterium
To lower part of the tree

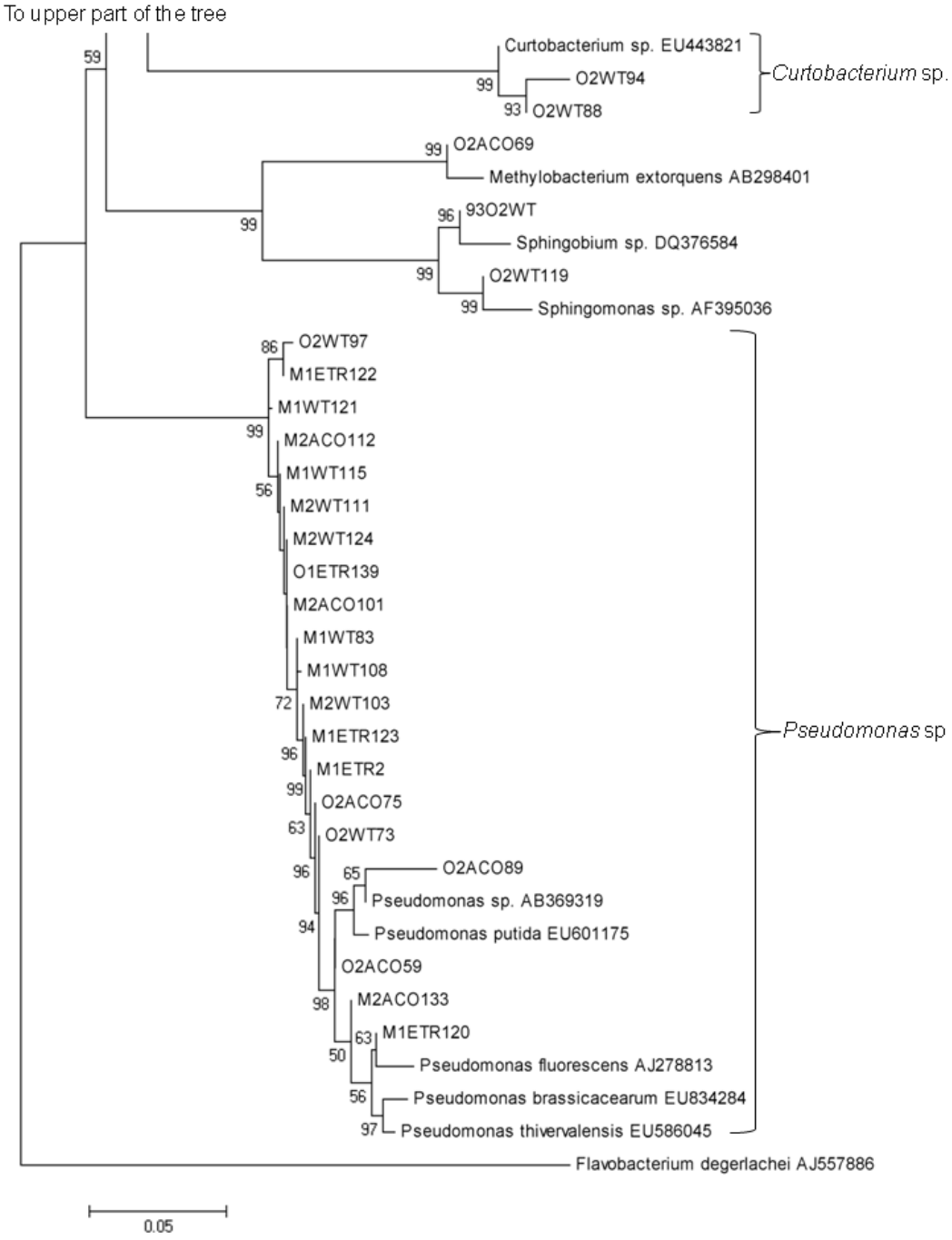


Figure 2. Phylogenetic tree showing the relative positions of bacterial isolates as inferred from their 16S rRNA gene sequences using the neighbor-joining method. Bacterial species preceded by their GenBank accession numbers were used as standard strains. *Flavobacterium degerlachei* (AJ557886) was used as an outgroup organism. Bootstrap values above 50 are shown, representing the percentage of support for clusters out of 1000 replications. O = organic, M = mineral. The scale gives genetic distances.

2.3.3. Specificity of bacterial endophyte colonization

All the seeds inoculated with bacteria germinated. Of the six specialist isolates, five were able to re-colonize their hosts and five were also found to colonize non-host seedlings upon single inoculations (Fig. 3B). The specialist for 35S-*etr1*, *P. thivervalensis*, colonized only 35S-*etr1* seedlings. The two generalists for all genotypes, *B. megaterium* and *B. simplex*, colonized 35S-*etr1* and WT seedlings, but not ir-*aco1* seedlings. The two DMSZ generalists, *P. fluorescens* DSM8568 and *P. brassicacearum* DSM13227, colonized WT and the two transgenic seedlings. Interestingly, 35S-*etr1* seedlings were colonized by all isolates upon single inoculations. In the mixed inoculation treatments, one of the generalists, either *P. fluorescens* DSM8568 or *P. brassicacearum* DSM13227, was able to infect all plant genotypes (Fig. 3C). Notably, the specialists *Curtobacterium* sp. and *M. extorquens* were able to re-colonize their particular hosts, WT and ir-*aco1*, respectively. The generalist *P. brassicacearum* DSM13227 fully colonized 35S-*etr1* seedlings.

2.3.4. Re-colonization and persistence in the glasshouse

The selected endophytic bacteria were able to re-colonize their hosts in a highly diverse and competitive environment in a non-sterile inoculation experiment under glasshouse conditions (Fig. 4). Of the five bacterial isolates (Table 1), *P. brassicacearum* DSM13227 and *B. megaterium* were re-isolated from the roots of all *N. attenuata* genotypes, whereas *M. extorquens* was found to re-colonize only the roots of ir-*aco1* and 35S-*etr1* plants. *Curtobacterium* sp. and *P. thivervalensis* did not colonize any *N. attenuata* genotype.

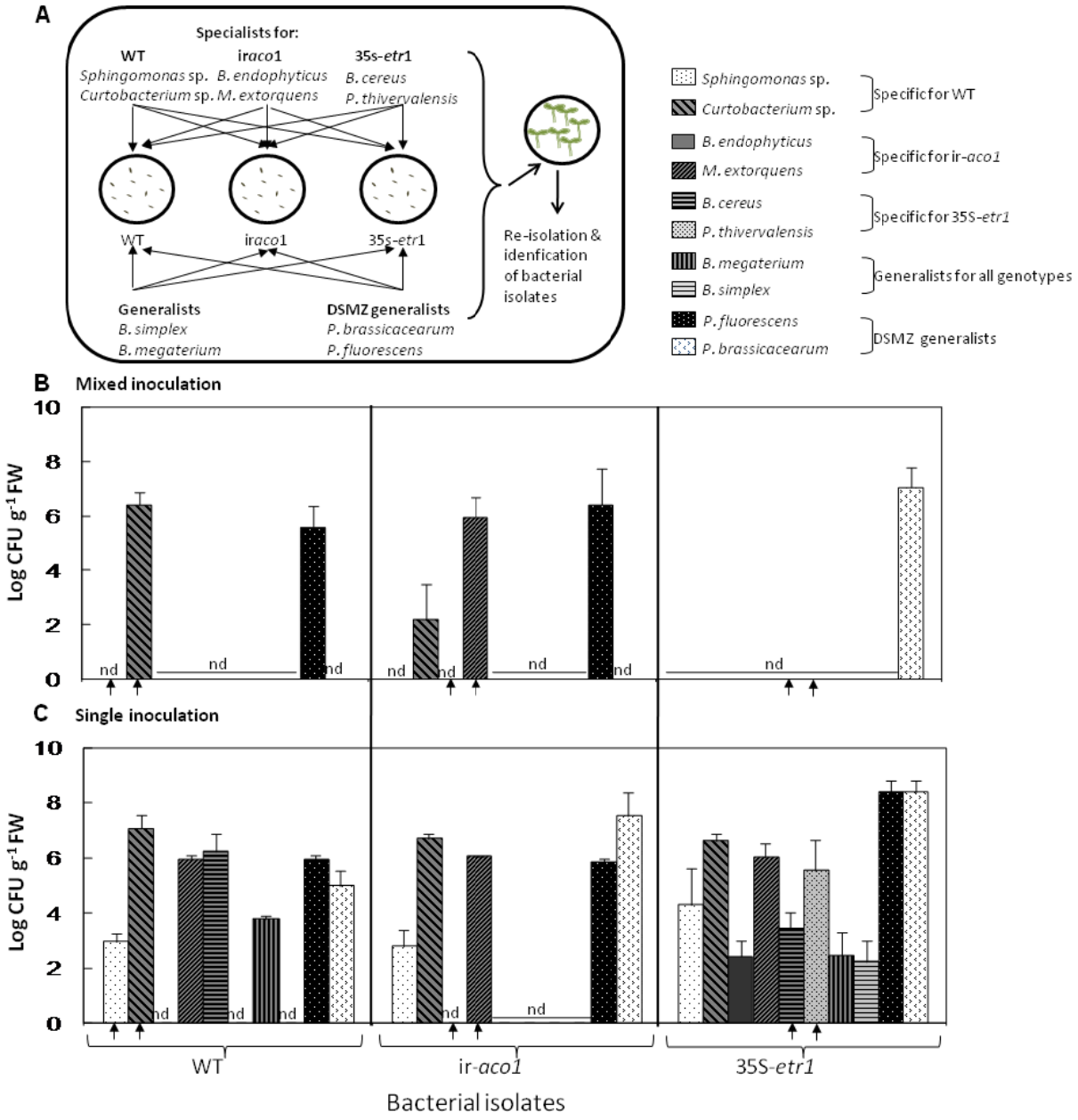


Figure 3. *In vitro* colonization of different plant genotypes by individual bacterial isolates from treatment with **(B)** single and **(C)** mixed inoculum. The identity of bacterial isolates was confirmed by 16S rDNA sequencing. nd: not detected. The arrows depict specific isolates for each plant genotype. Different shading patterns depict bacterial isolates. Schematic diagram **(A)** describes bacterial inoculation, re-isolation and identification.

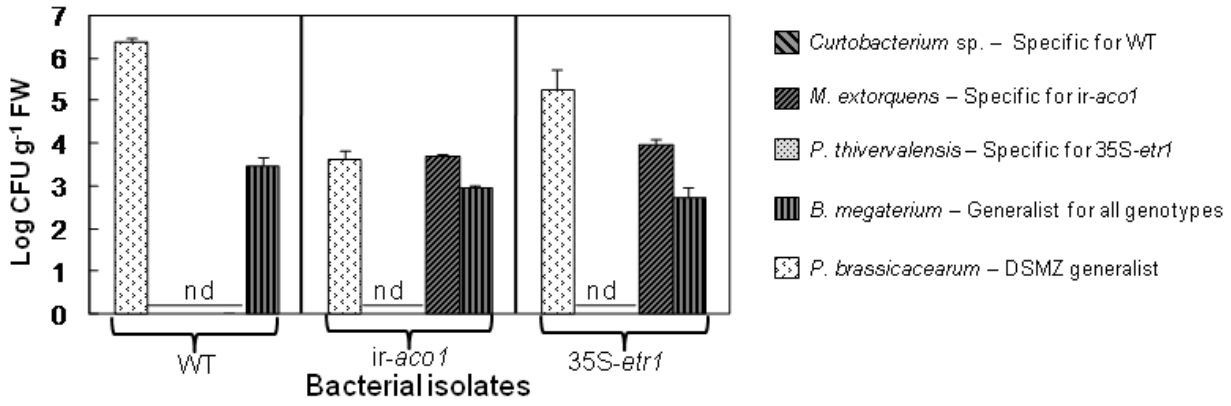


Figure 4. Colonization of different plant genotypes by individual bacterial isolates under glasshouse conditions. nd: not detected.

2.3.5. Effects of bacterial endophytes on seedling growth

Single inoculation of 139 isolates into WT seeds affected seedling growth; germination rate, however, was not influenced. Of these 139 isolates, 64 were beneficial with respect to seedling growth, 37 pathogenic and 38 neutral (Table 5). Among these 64 plant-growth-promoting isolates, 35 showed ACCd activity and 37 produced IAA. However, 25 isolates lacking ACCd activity and 21 isolates unable to produce IAA also promoted seedling growth. Using plants impaired either in ET production (ir-*aco1*) or perception (35S-*etr1*), I investigated the influence of plant ET signaling on the recruitment of ACCd- and IAA-producing bacteria. The number of isolates producing ACCd and IAA was not significantly different among the plant genotypes ($P>0.05$, Table S2 and S3). However, the abundance of bacteria producing ACCd and IAA was greater in organic soils O1 and O2 (Fig. 5), while in mineral soil M1, plants harbored significantly fewer ACCd- and IAA-producing isolates ($P<0.05$, Table S2 and S3), and no ACCd- and IAA-producing bacteria were detected in the plants grown in soil M2.

2.3.6. ET production and root morphology of WT and transgenic plants

Constitutive ET emissions of 35S-*etr1* seedlings were significantly higher than those of WT and ir-*aco1* seedlings ($P<0.0001$) (Fig. 6A). WT seedlings produced a significantly larger number of root hairs in the investigated section than did seedlings of ir-*aco1* and 35S-*etr1* lines ($P<0.0001$) (Fig. 6C). Moreover, roots of 35S-*etr1* and ir-*aco1* seedlings are significantly longer

than those of WT seedlings (P=0.001) (Fig. 6B). The number of lateral roots was similar for the three genotypes (data not shown).

Table 5. Number of bacterial endophytes influencing growth* of wild-type seedlings

Soil type	No. of total isolates	Beneficial†	Pathogenic‡	Neutral*
O1	49	24	10	15
O2	55	28	10	17
M1	18	9	7	2
M2	17	3	10	4
Total	**139**	**64**	**37**	**38**

* Parameters measured: root length, hypocotyl length, number of true leaves and lateral root formation
† Bacterium enhanced at least one of the above parameters and without negative effect on the other parameters
‡ Bacterium caused detrimental effects on at least one of the parameters
* Bacterium did not influence any of the parameters

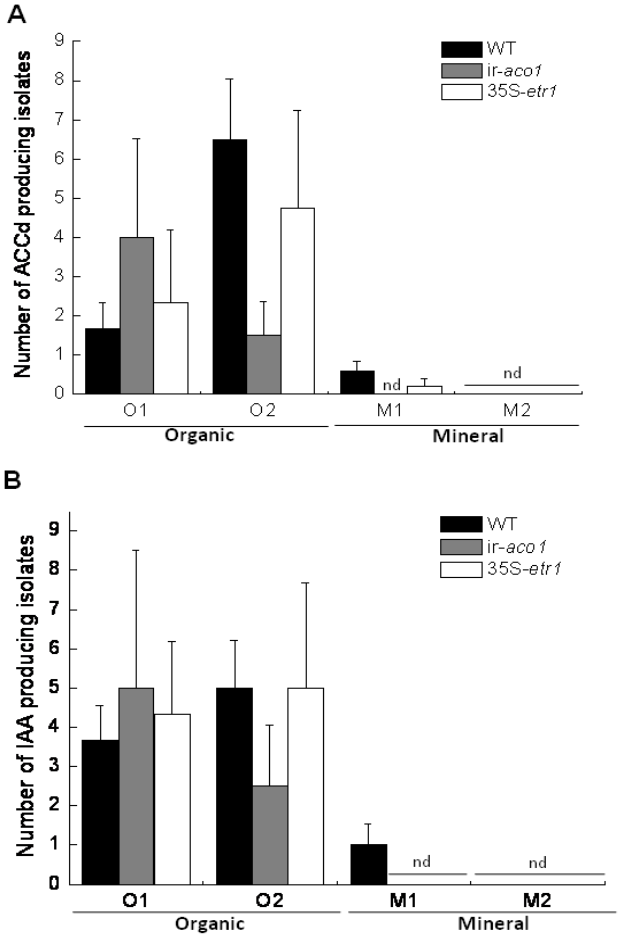


Figure 5. Diversity of endophytic bacteria producing **(A)** ACCd and **(B)** IAA. nd: not detected.

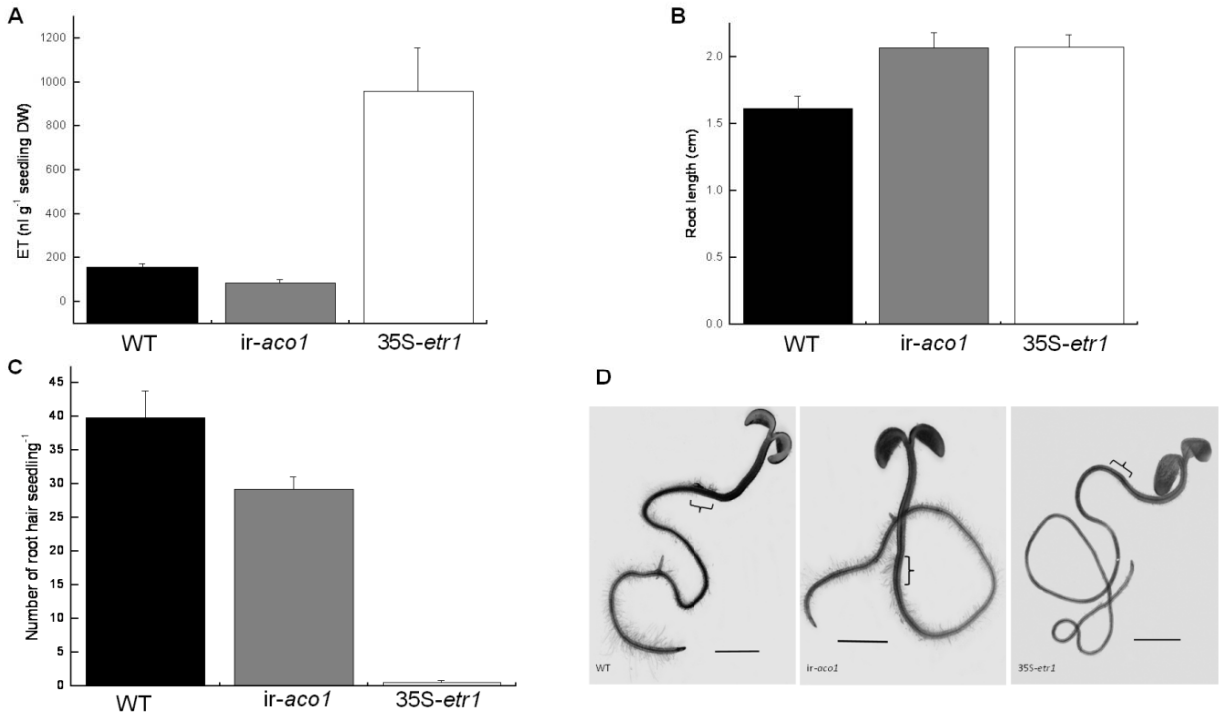


Figure 6. (A) ET production of WT, ir-*aco1* and 35S-*etr1* seedlings; **(B)** root length; **(C)** root hair number; **(D)** seedling morphology (10 x magnifications). The bar represents 2mm. The bracket depicts the zone of root hair counting.

2.4. Discussion

The composition of a plant's bacterial endophytic community is likely to be determined by many selective factors including soil type, plant genotype as well as stochastic sampling factors (Hardoim *et al.*, 2008), all of which influence the structure of bacterial endophyte populations. Soil type is known to be a major determinant of the composition of soil microbial community and in turn of the endophytic microbial communities, as the combination of soil texture and structure, organic matter, pH and the presence of key nutrients, i.e., C and N, determine the habitable niches for microbes in soil (Singh *et al.*, 2009). Here I demonstrate that soil type strongly influences the plant culturable bacterial endophyte community, as has been shown before by Rasche *et al.*, (2006). In addition, I report for the first time that plant ET signaling indirectly influences the recruitment of root bacterial endophytes in *N. attenuata*.

Soil is considered an endophyte bank, a "marketplace" where negotiations between plants and endophytes take place. Total culturable bacterial colonization as well as bacterial OTU

clusters were significantly higher in plants grown in the two organic soils with higher C and N contents (Table 2) compared with those in the plants grown in the two mineral soils (Figs. 1 & S1). It is widely accepted that organic matter promotes both plant and soil microbial growth due to higher C, P and N supply rates (Vaidya *et al.*, 2008) and enhanced nutrient availability may allow plants to support larger populations of endophytic bacteria. I found that the plants grown in the recently burned soil O1 and the unburned soil O2 harbored similar numbers of total culturable endophytic bacteria; however, they differed with respect to diversity. These findings concurred with the study of Hamman *et al.*, (2007), which found no significant difference in total microbial biomass between the unburned and burned soils, but that the communities in the burned sites differed in species composition from those of the unburned soils. The recent invasion of cheat grass into the Great Basin Desert has dramatically shortened the average fire cycle of this habitat, and conclusions about the importance of fire in structuring soil communities would require more detailed investigations.

ET signaling plays an important role in mediating different types of induced plant resistance to pathogens: induced systemic resistance (ISR) by rhizobacteria as reviewed by van Loon *et al.*, (2006) and systemic acquired resistance (SAR) by pathogens as reviewed by Sticher *et al.*, (1997). One may ask if these plant defense responses that require ET signaling also affect a plant's endophytic bacterial communities. Hallmann (2003) experimentally induced resistance in potato plants by applying *Rhizobium etli* G12 to one-half of a split potato root system; the endophytic bacterial spectrum was analyzed for the other "uninduced" half of the split root system and compared with that of a non-treated plant. Total bacterial diversity and number of bacterial species were significantly higher in elicited than in non-elicited roots. These results indicate that eliciting bacteria-mediated induced plant defense responses increases the density and spectrum of root bacterial endophytes. Using the *Arabidopsis* mutants *cpr1*and *npr1-1* which display either constitutive SAR or are unable to express SAR, Hein *et al.*, (2008) found visible differences in the rhizosphere community fingerprints of the mutant plants compared to WT; however, there was no clear decrease of rhizosphere species diversity associated with constitutive SAR expression. The study suggested that SAR can alter rhizosphere bacterial communities. Our study provided evidence that plant ET signaling influences the initial recruitment of bacterial communities from the soil. The two transgenic plants differed from WT plants with respect to the size of their total culturable endophytic bacterial community (Fig. 1) as

well as its diversity (Table 3). In their native soils, *N. attenuata* WT plants harbored a more diverse bacterial community compared to ir-*aco1* and 35S-*etr1* plants; a result opposite of the expectation that impairments of ET signaling would be associated with increases in bacterial endophyte population and diversity.

The smaller and less diverse community found in ET- signaling deficient plants suggests that many bacterial species may require the plant's ability to produce and/or perceive ET for them to become endophytic. The variation in the bacterial endophyte community of ir-*aco1* and 35S-*etr1* plants grown in native soils (Fig. 1) may also be explained by other biotic factors, e.g., endophytic fungi or unculturable bacteria that compete for the same niches in the plant (Singh *et al.*, 2009) and whose communities have not been investigated. On the other hand, the lack of ET signaling might facilitate plant-endophyte communication. For example, *P. thivervalensis* was originally isolated only from 35S-*etr1* plants, and even under stringent conditions (single *in vitro* inoculation) it was only able to colonize 35S-*etr1* plants, suggesting that high levels of ET coupled with an insensitivity to ET are required for colonization (Fig. 3B). This is consistent with the findings of Persello-Cartieaux *et al.*, (2001), who showed a similarly intimate relationship between the rhizosphere bacterium *P. thivervalensis* and *A. thaliana* mutant plants insensitive to IAA. Dong *et al.*, (2003) demonstrated the existence of a specific colonization pattern for the *Salmonella-Medicago truncatula* interaction. By testing four *Salmonella* strains and two *M. truncatula* lines (WT and the symbiosis mutant *dmi1*), they showed that the colonization of the host plant was an active process, determined by the characteristics of the specific bacterial strain and the plant. Hence, the recruitment of soil-dwelling bacteria into the endosphere is likely defined by host genotype and specific genes.

Since the ir-*aco1* and 35S-*etr1* plants have not yet been metabolically characterized beyond their differential ET production and perception, uncharacterized changes in root metabolomics (e.g. ACC accumulation) and exudates could explain the observed patterns of bacterial association. Buer *et al.*, (2006) demonstrated that ET signaling modulates flavonoid accumulation in *A. thaliana* roots and recent reviews by Bais *et al.*, (2006) and Hartmann *et al.*, (2008) highlight the importance of flavonoids and other root exudates in the plant-driven recruitment of rhizosphere bacteria. In addition, I hypothesize that root morphology might influence the endophytic bacterial community. For example, soil-dwelling bacteria probably enter roots via cracks in lateral root junctions and through root hairs as reviewed by Glick *et al.*,

(1999). While there is a consensus on possible effects of microbes on root morphology (Persello-Cartieaux *et al.*, 2001; Lopez-Bucio *et al.*, 2007), data on how plant root morphology itself initially affects the structure of an associated rhizosphere or endosphere bacterial community are scarce. Depret & Laguerre (2008) reported that modifications in host root and nodule development appear to influence the ability of particular rhizobial genotypes to colonize the host legume. I found that ET signaling affected root morphology of *N. attenuata* seedlings, specifically, root hair number and root length (Fig. 6). WT plants, which have significantly more root hairs, might provide microbes with a greater number of points of entry; however, stochastic sampling process may also account for the more diverse endophytic bacteria community (Hardoim *et al.*, 2008).

Our single inoculation experiments highlighted specific plant traits associated with deficiencies in ET signaling (Fig. 3B) that affected colonization patterns. For other genotype-endophyte associations (WT and ir-*aco1*), the interactions did not prove to be that stringent. However, these experiments with single inoculations of other specialists and generalists revealed that 35S-*etr1* plants were the most susceptible host plants tested. 35S-*etr1* plants were highly colonized by all bacterial endophytes tested, particularly in experiments lacking competition (Fig. 3B). This is consistent with the studies on an ET-insensitive mutant *sickle M. truncatula* in which *Klebsiella pneumoniae* 342 (Kp342), a bacterial endophyte enhancing plant growth and nutrition, hypercolonized the mutant compared to the WT plants (Iniguez *et al.*, 2005). Furthermore, the greater susceptibility of ET-insensitive plants with regard to pathogenic microbes has been demonstrated in various studies as reviewed by van Loon *et al.*, (2006). Thus, I propose that the similar resistance mechanisms might be involved in regulating colonization by endophytic and pathogenic bacteria under simplified conditions.

Not only do plants select particular bacterial communities, but interactions among the bacteria themselves influence colonization success. Complex dynamics in the bacterial communities such as facilitation and competition might play a role in the colonization process (Li & Alexander, 1986; Ramos *et al.*, 2003b; Verma *et al.*, 2004; Rosenblueth & Martinez-Romero, 2004). Li and Alexander (1986) showed that *Enterobacter aerogenes*, *Pseudomonas marginalis*, *Acinetobacter* sp., and *Klebsiella pneumoniae* suppressed the colonization by *Rhizobium meliloti* of roots grown on agar and reduced nodulation by *R. meliloti*. In the mixed inoculation studies, I found that 35S-*etr1* seedlings were fully colonized by the DSMZ generalist

P. brassicacearum DSM13227 (Fig. 3C), which apparently could exclude even the specialist *P. thivervalensis*. However, in WT and ir-*aco1* plants, the specialists successfully re-colonized their hosts in mixed inoculation experiments (Fig. 3C), suggesting that synergistic interactions occurred among the bacterial endophytes (Sturz *et al.*, 1997). In general, at least one of the two DSMZ generalists, *P. brassicacearum* DSM13227 and *P. fluorescens* DSM8568, was able to colonize the three *N. attenuata* lines upon single or mixed inoculation. These two strains belong to the genus *Pseudomonas,* which is known to be rapid and successful plant colonizer even under highly competitive situations (Lugtenberg *et al.*, 2001).

An additional trait of so-called "true" endophytes is their ability to re-infect their putative host (Rosenblueth & Martinez-Romero, 2006; Hardoim *et al.*, 2008). In our non-sterile glasshouse experiments, some of the inoculated endophytes were able to re-colonize their host, whereas some were not (Fig. 4). Since re-infection was successful for most host and also some non-host associations under *in vitro* conditions, these observation might be explained by other biotic factors mentioned above. I interpret these results as showing that the specific bacterial endophyte-host interactions depend not only on the host plant genotype but also on interspecific interactions of bacterial endophyte communities.

One of the advantages of studying culturable endophytic bacterial communities is the ability to investigate their effects on plant growth as reviewed by Barriuso *et al.*, (2008). I found that about half of the isolates were beneficial for WT plant growth (Table 5) under *in vitro* conditions. However, several "endophytic" isolates had pathogenic or neutral effects. How a particular bacterial endophyte community is selected by the host plant may reveal how plants tolerate harsh conditions. One example can be seen in the recruitment of beneficial ET-related bacterial communities by the host plant: the ACCd-producing bacteria, which are able to lower stress associated ET production in plant roots by cleaving the ET precursor, ACC, which results in enhanced plant growth (Glick *et al.*, 2007). IAA- and ACCd-producing bacteria were abundant in plants grown in the organic soils O1 and O2 (Fig. 3), which is in accordance with the total culturable bacterial community (Fig. 1). One might expect that plants whose ET signaling ability is impaired (35S-*etr1* and ir-*aco1*), which causes them to misread environmental signals and experience stress, might recruit such beneficial bacteria to greater extent than do WT plants. Interestingly, our results did not support this expectation: the different plant genotypes did not recruit ACCd/IAA-producing bacteria differently. It might be possible that cultivated plants such

as canola and tomato which rely on ACCd/IAA producing bacteria to ameliorate abiotic stresses, such as salinity or heavy metal, actively select for these bacterial communities under stress conditions (Glick *et al*., 2007). However, native plants like *N. attenuata* which have evolved to tolerate stressful environments might not recruit ACCd producing bacteria as PGPR, but merely let them in and they thrive as parasites. Thus I propose that plant's ability to produce or sense ET does not directly control the recruitment of these communities for native plants (Fig. 5 and Table S2 & S3). Furthermore, an association between plant growth and ACCd/IAA production was found in only half of the PGP isolates; hence, there are clearly many other ways by which bacterial endophytes can promote growth, and additional experiments will be required to elucidate these underlying mechanisms.

In conclusion, the results confirm the central role of soil type and highlight, though to a lesser extent, the role of ET signaling in shaping endophytic bacterial community structure. Specific interactions between endophytic bacteria and their host plants are regulated not only by plant ET signaling but also by the bacteria themselves. The host plants' ability to recruit ACCd/IAA-producing bacteria seems to be independent of ET signaling.

Chapter III. Native bacterial endophyte from *Nicotiana attenuata* enhances the growth and fitness of its host in the natural ecosystem

3.1. Introduction

Bacterial endophytes have been intensively studied in the context of agricultural practices rather than ecology or biodiversity (Hallmann *et al.*, 1997; Sturz *et al.*, 2000). Most studies have focused on bacterial endophytes and their ability to enhance the productivity of cultivated plants. Only a few studies have reported on the bacterial endophyte communities associated with native plant species and/or on assessing plant-growth-promoting (PGP) effects in nature (Hallmann *et al.*, 1997; Sturz *et al.*, 2000; Long *et al.*, 2008). Therefore, the endosphere of wild plant species represents a potentially promising source from which PGP endophytic bacteria can be isolated (Domenech *et al.*, 2007; Barriuso *et al.*, 2008).

Endophytic bacteria have been studied as possible inoculants for increasing plant productivity (Hallmann *et al.*, 1997). Several mechanisms have been postulated to explain how endophytes stimulate plant growth. These mechanisms are broadly categorized as direct or indirect (Glick, 1995). Direct mechanisms elicit growth promotion by bacterial determinants, including the interference of plant hormone homeostasis by the production of plant hormones. Examples of such hormones include indole-3-acetic acid (IAA), gibberellins (GA), cytokinin or the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCd), which degrades ACC, an ethylene (ET) precursor, subsequently enhancing root growth by reducing ET (Glick, 1995). Furthermore, the solubilization of inorganic phosphate (P) (Park *et al.*, 2009) as well as the release of volatile organic compounds (VOCs) is known to affect plant growth positively (Ryu *et al.*, 2003). Endophytic bacteria that promote plant growth indirectly by suppressing pathogens and eliciting induced systemic resistance (ISR) are well-known in biological control or defense against insect herbivores (Kloepper *et al.*, 1992; Dobbelaere *et al.*, 2003; Van Oosten *et al.*, 2008).

Bacterial endophytes qualify as plant-growth-promoting bacteria (PGPB) when they are able to colonize and elicit positive effects on the plant as reviewed by Hardoim *et al.*, (2008). Some bacterial endophytes are commercially available as inoculants for agriculture, but the inoculation of soil with these bacteria may affect the composition and structure of endosphere microbial communities as reviewed by Castro-Sowinski *et al.*, (2007). Synergistic effects

between the introduced bacterial endophytes and the endogenous microbial communities led to positive effects on plant growth (Ramos *et al.*, 2003a). However, the opposite might be true for antibiotic-producing bacterial endophytes that negatively affect resident microbial communities as reviewed by Castro-Sowinski *et al.*, (2007), again influencing plant growth. Therefore, knowledge of the structure of endosphere microbial communities and their diversity is the key to better understanding interactions with introduced bacterial endophytes and host plants in nature.

The plant hormone ET is known to regulate multiple physiological and developmental processes in plants, such as seedling emergence, leaf and flower senescence, and ripening organ abscission, and it is also involved in the reactions of plants to abiotic and biotic stresses (van Loon *et al.*, 2006). Blocking ET perception with ET response inhibitors such as 1-methylcyclopropene (1-MCP) helps to increase the longevity of flowers and ornamental plants (Serek *et al.*, 1995). However, the absence of ET perception was shown to weaken the ability of transgenic Tetr tobacco plants to withstand common, generally non-pathogenic, opportunistic soil-borne fungal organisms (Knoester *et al.*, 1998; Geraats *et al.*, 2002). Intensive research has been conducted using the mutated dominant ET resistance gene *etr1-1* from *Arabidopsis*. Early experiments with this gene were carried out on petunia (Wilkinson *et al.*, 1997). In these very thorough experiments, a CaMV35S::*etr1-1* construct was transferred to petunia, resulting in the constitutive expression of the *etr1-1* gene coupled with ET insensitivity. Nonetheless, constitutive gene expression gave rise to undesirable side effects such as the poor root development of cuttings, less efficient seed germination and rooting, and delayed seedling growth. Most of these effects can be explained by the role of ET plays at the different developmental stages (reviewed by Serek *et al.*, 2006).

Our *Nicotiana attenuata* mutant line 35S-*etr1* consistently shows a similar growth phenotype to the above-mentioned mutated plants and higher mortality compared to wild type (WT) plants, and thus is ideal for studying the recruitment of PGP bacterial endophytes and their effects on plant growth and survival in nature. In a previous study, many endophytic bacteria were isolated from the roots of WT, ir-*aco1* and 35S-*etr1 N. attenuata* plants grown in native Utah soils. A number of isolates were shown to be beneficial for seedling growth under *in vitro* conditions. For the present study, a native bacterial isolate, *Bacillus* sp., was selected among the most potential PGP isolates and used to address the following question: how does a native bacterial endophyte isolate influence the growth and fitness of its host plants, *N. attenuata* (WT),

and especially the highly susceptible 35S-*etr1* transgenic line in the native habitat, southwestern Utah, USA?

3.2. Materials and Methods

3.2.1. Plant materials and bacterial strain

The following inbred WT and transgenic lines of *N. attenuata* were used in all experiments: *N. attenuata* ecotype Utah inbred line 22 and ET-insensitive line 35S-*etr1* (A-03-328-8); these are derived from the same Utah inbred line and are therefore isogenic. The lines are fully characterized in von Dahl *et al.*, (2007). Seed germination procedures are described elsewhere (Krugel *et al.*, 2002; Long *et al.*, 2008) and also detailed in General materials and methods. The bacterial strain (*Bacillus* sp.) was isolated from 35S-*etr1* plants grown in native Utah soils.

3.2.2. Bacterial colonization assays in the glasshouse and in the field

Bacterial suspensions in sterile distilled water (OD_{600}=1.2) were used for seed inoculation; control seeds were treated with sterile distilled water only. For the *in vitro* colonization assays, the inoculated seeds of WT and 35S-*etr1* (20 seeds per Petri dish, 3 dishes for each combination) were incubated at room temperature overnight and transferred to GB5 medium in Petri dishes maintained in Percival growth chambers (13/11h day/night cycle, 155µmol m^{-2} s^{-1}, 26/24°C). Ten days after inoculation (DAI), bacterial isolation was carried out as described in General materials and methods. Two independent experiments were carried out for all seedling assays. Bacterial identity was determined by 16S rDNA sequencing.

For glasshouse experiments, 12-day-old seedlings were planted in Teku pots, and the inoculated and uninoculated seedlings (WT and 35S-*etr1*) were grown in separate Teku pots. These pots were maintained in Snijders growth chamber (16/8 h photoperiod at 200-300 µmol m^{-2} s^{-1}, 26/24°C, and 65-70 % r.h.). After 10 days, plants were transferred to 460ml pots containing a 1cm-thick layer of lecaton at the bottom filled up with sterile sand. Pots were placed on separate coasters to avoid cross-contamination. Each plant was fertilized with 50ml of $Ca(NO_3)_2.4H_2O$ and 0.4g l^{-1} and Flory B1 0.2 g l^{-1} every alternate day. Thirty days after inoculation, roots were collected and bacterial isolation was performed as described in General

materials and methods. Bacterial identity was determined by 16S rDNA sequencing. Two independent experiments were carried out for glasshouse conditions.

For the field experiment, 15-day-old seedlings (inoculated and uninoculated) were transferred to 50-mm peat pellets (Jiffy) 15 days after germination and hardened off to the environmental conditions of high sun exposure and low relative humidity over 2 weeks. Small, adapted, rosette-stage plants of equal size were transplanted into a natural population in Utah and watered every other day for 2 weeks until roots were established. Plants were grown in pairs of the inoculated and uninoculated WT and 35S-*etr1* plants and created a quadruplet planting formation (Fig. 1). Fifty-two DAI, 5 plants from each treatment were collected for bacterial isolation. At the end of the experiment (73DAI), all remaining plants were harvested and bacterial colonization was quantified. The experiment was carried out in the field season 2009 in southwestern Utah, USA. Bacterial identity was determined by 16S rDNA sequencing.

3.2.3. Ethylene measurement

ET emissions from WT and 35S-*etr1* seedlings (inoculated and uninoculated) were measured continuously and non-invasively in real-time with a photoacoustic spectrometer (INVIVO, Saint Augustin, Germany) as described by von Dahl *et al.*, (2007). Thirty seeds were germinated in 100 ml three-neck flasks on filter paper and cultivated in a Percival growth chamber (13/11h day/night cycle, 155 µmol m^{-2} s^{-1}, 26/24°C). After 12 days, flasks containing the seedlings were subjected to ET measurements (five replicates per treatment). Five empty flasks with filter paper and sterile distilled water served as blank controls. Two independent experiments were carried out.

ET emission from the leaves of inoculated and uninoculated WT/35S-*etr1* plants (36DAI) upon treatment with *M. sexta* oral secretions (OS) was measured. The fully expanded leaf (+1 leaf) was wounded with a fabric pattern wheel, and immediately 20µl of either water or *M. sexta* larvae OS (1/2 dilution) was applied to the puncture wounds. The leaf was cut and put into a 250ml three-neck flask. Five hours after accumulation, the flasks were subjected to ET measurement.

3.2.4. Seedling IAA quantification

The *Bacillus* sp.-inoculated and -uninoculated seedlings of WT and 35S-*etr1* were harvested 12 DAI and immediately frozen in liquid nitrogen and kept at -80°C until analyzed. Approximately 500mg of liquid nitrogen-ground leaf powders was extracted over night by diffusion in the dark at -20°C using 100% methanol containing 25mM diethylditho-carbamic acid (DECT, Sigma Aldrich: http://www.sigmaaldrich.com), and 50ng of internal standard $13C^6$ Indole-3-acetic acid (Cambridge Isotope Laboratories, Inc.: http://www.isotope.com). Samples were centrifuged at 3000 x g, 4°C for 30 minutes, and supernatants were collected before pellets were re-extracted with 100% methanol/25mM DECT on ice for 30 minutes. Samples were centrifuged as above and supernatants combined in single tubes. Water was added to adjust the final concentration of methanol in each supernatant to 50% methanol (v/v) and samples were passed through preconditioned Supelclean™ LC-18 SPE columns (Supelco: http://www.sigmaaldrich.com). Flow-through fractions were applied to an activated diethylaminoethyl Sephadex A25 (DEAE) column equilibrated with 50% methanol (Amersham Pharmacia Biotech: http://www1.gelifesciences.com) and samples were absorbed to the resin by gravity flow. After the samples were completely adsorbed, DEAE columns were rinsed with 50mL of 50% methanol (v/v). New Supelclean LC-18 SPE columns were coupled underneath the DEAE columns and IAA was eluted from DEAE using 6% formic acid (v/v) (Riedel-de Haen: http://www.sigmaalrich.com). IAA retentate was subsequently eluted from the Supelclean LC-18 SPE columns with 5mL of diethyl ether (Fluka: http://www.sigmaaldrich.com) after the SPE columns were briefly dried with air in the syringe. The remaining acidic water phase from ether fractions was removed by pipetting and samples were quickly evaporated under a stream of nitrogen. Dried samples were quantitatively dissolved in 1.5mL of 100% methanol and dried under vacuum in Eppendorf tubes (Eppendorf Concentrator 5301; http://www.eppendorf.com). Samples were finally re-dissolved in 150µL 70% (v/v) methanol, centrifuged at 16X1000g, 4°C for 30 minutes and 10 µL applied to 1200L LC/MS/MS/MS system (Varian, Palo Alto, CA, USA) for IAA measurement. An aliquot was transferred to HPLC vials and measured on a 1200 L liquid chromatography–triple quadrupole mass spectrometry system (Varian, Palo Alto, USA). 10 µl was injected onto a prodigy column (150x2 mm, 3µm diameter, Phenomenex, USA) attached to a pre-column (C18, 4 x 2 mm, Phenomenex, USA). A mobile phase composed of 0.05% acetic acid and acetonitrile was used in a gradient mode for the separation. The mobile

phase comprised of solvent A (0.05% acetic acid) and solvent B (acetonitrile) used in a gradient mode time/concentration for (min/%B): 1.5/20; 6'/97; 17'/97; 18'/20; 25'/20. Compound was detected as negative ions in a MRM mode. Molecular ions M-H (-) at m/z 174 generated from endogenous auxin and from its internal standards 180 were fragmented under 35V CE. The product ion of auxin and its internal standard is m/z 130 and 136, respectively. The ratio of ion intensities of the response of the product ions was used to quantify auxin content.

3.2.5. Plant growth performance and herbivore screen in the glasshouse and the field

In glasshouse, percentage of survivorship (%) of the inoculated and uninoculated plants was calculated 24DAI. Length of the longest leaf/rosette diameter and stalk length were measured from 25DAI. Total capsule number per plant was counted at the end of experiment (63DAI).

In the field, 53 replicates consisting of four WT and 35S-*etr1* (inoculated and uninoculated) plants were transplanted on 13 April 2009 (30DAI) into the field plot in a quadruplet planting formation as shown in Fig. 1. The distance between plants was 40cm.

Rosette diameter and stalk elongation were measured on 30 April, 16 May and 27 May (47, 63 and 73 DAI). The flower number was recorded for all plants on 27 May (73 DAI). The nectar volume and total sugar content were also measured on 27 May (73DAI) as described by (Kessler *et al.*, 2008).

Leaf area damage as a percentage of canopy damaged by herbivores and dryness, was estimated on 27 May (73 DAI). Characteristic damage caused by grasshoppers (*Trimerotropis* spp.), mirids (*Tupiocoris notatus* Distant), leaf miners, tree crickets and noctuidae was recorded separately.

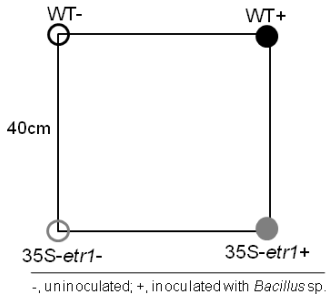


Figure 1. Experiment set up in the field plot in Utah, USA. WT and 35S-*etr1* plants were planted in pairs, uninoculated (-) and inoculated (+). Plants were monitored for growth, fitness and herbivore damage.

3.2.6. Manduca sexta *performance assay*

M. sexta eggs were supplied by North Carolina State University. Two *M. sexta* neonates were placed on the +1 leaf of each plant (inoculated and uninoculated WT and 35S-*etr1* plants) (n=10). Two days later, one larva was left on each plant. Larval mass was determined on days 4 (40DAI) and 8 (44DAI), by which time larvae were in the second or third instar. Due to mortality and to larval movement off the plants, the experiment was stopped after 8 days. Plants were randomized spatially in the glasshouse.

3.3. Results

3.3.1. PGP trait characterization of Bacillus *sp.*

Three PGP traits including production of IAA and ACCd and the ability of *Bacillus* sp. to solubilize inorganic P were characterized (Table 1). The *Bacillus* sp. isolate was able to produce IAA only in the presence of Trp (12.25 µg ml^{-1}). It also produced ACCd (0.347 µmole alpha-ketobutyrate mg^{-1} protein h^{-1}) and solubilized inorganic P 14 DAI.

Table 1. PGP trait characterization of *Bacillus* sp.

Bacterial isolate	IAA (µg/ml)†	ACCd*	Phosphate solubilization
Bacillus sp.	12.25±0.0073	0.347±0.001	+

Mean ± SE (n=5); †, IAA production (+Trp); *, µmole alpha-ketobutyrate mg^{-1} protein h^{-1}

3.3.2. Effects of Bacillus *sp. inoculation on plant growth and fitness*

Inoculating WT and 35S-*etr1* seeds with *Bacillus sp.* significantly enhanced seedling growth *in vitro* 10 DAI compared with the control seedlings. In the glasshouse, inoculation with *Bacillus* sp. increased survivorship of 35S-*etr1* plants compared to the uninoculated ones 24DAI (Fig. 2A). *Bacillus* sp.-inoculated plants showed a significant increase in rosette diameter and stalk length ($P<0.05$) 30 DAI (Fig. 2B and C) compared to the uninoculated plants. Fitness measurements represented by number of capsules per plant were carried out. A significant increase in total capsule number was found in the inoculated plants at the end of the glasshouse experiment (62 DAI) ($P<0.05$) (Fig. 2D).

An independent experiment in which *Bacillus* sp. was inoculated into the rhizosphere of 21-day-old WT seedlings instead of coating the seeds with the bacterium was carried out to investigate if the impacts of *Bacillus* sp. on plant growth could also obtain when plants recruit the bacterium at a later stage of development (Fig. 3). The inoculated WT plants had significantly bigger rosette diameters than did the uninoculated plants 12 DAI ($P<0.05$). *Bacillus sp*. colonized roots of the inoculated WT plants (10^3 cfu g^{-1} root FW) 44DAI.

In the field, *Bacillus* sp.-inoculated WT and 35S-*etr1* plants grew significantly bigger than did the uninoculated plants (rosette diameter and stalk length) ($P<0.05$) (Fig. 4A & B). Inoculation with *Bacillus* sp. also enhanced the survivorship of 35S-*etr1* plants (Fig. 4C), which have high mortality in the field. The number of flower was evaluated 62 DAI, and no significant difference was found between inoculated and uninoculated WT plants. Interestingly, the uninoculated 35S-*etr1* plants did not produce any flowers until the end of the experiment, while the inoculated 35S-*etr1* plants produced the same number of flowers as the WT uninoculated plants (Fig. 4D). Nectar production in flower, which is an important trait for fitness, was measured 73 DAI. No significant difference in nectar volume and total sugar content in nectar between the inoculated and uninoculated WT plants was found (Fig. 5B & 5C). A comparison of nectar volume between the uninoculated and inoculated 35S-*etr1* plants was not possible.

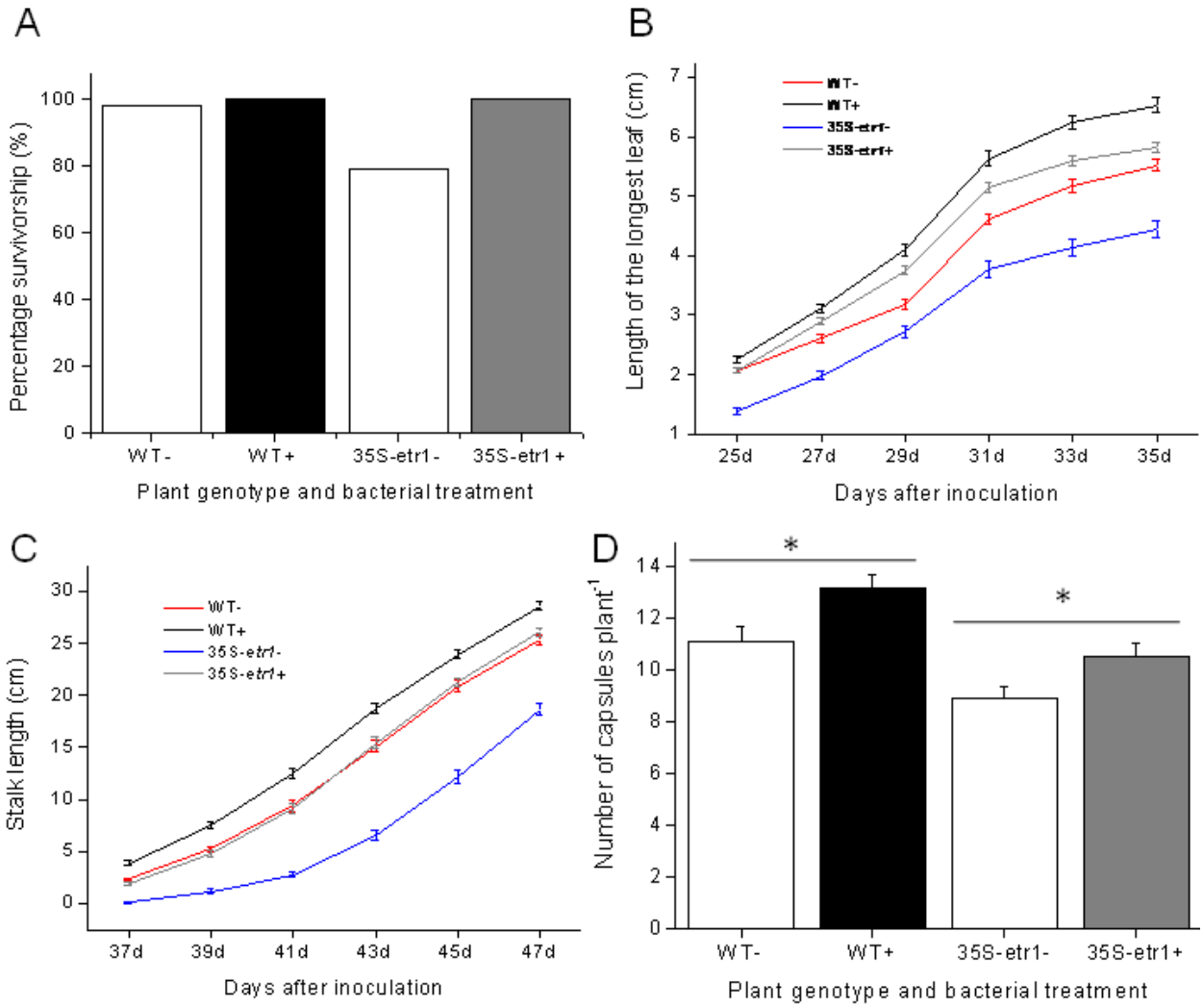


Figure 2. Performance of the uninoculated (-) and inoculated (+) WT and 35S-*etr1* plants in the glasshouse. (A) percentage survivorship (%) 24DAI; Mean ± SE of (B) length of the longest leaf (cm) 25, 27, 29, 31, 33 and 33 DAI, (C) stalk length (cm) 37, 39, 41, 43, 45 and 47 DAI, (D) total capsule number per plant 63 DAI. The asterisk (*) depicts a significant difference between the uninoculated (-) and inoculated (+) plants ($P<0.05$, ANOVA Fisher's PLSD test).

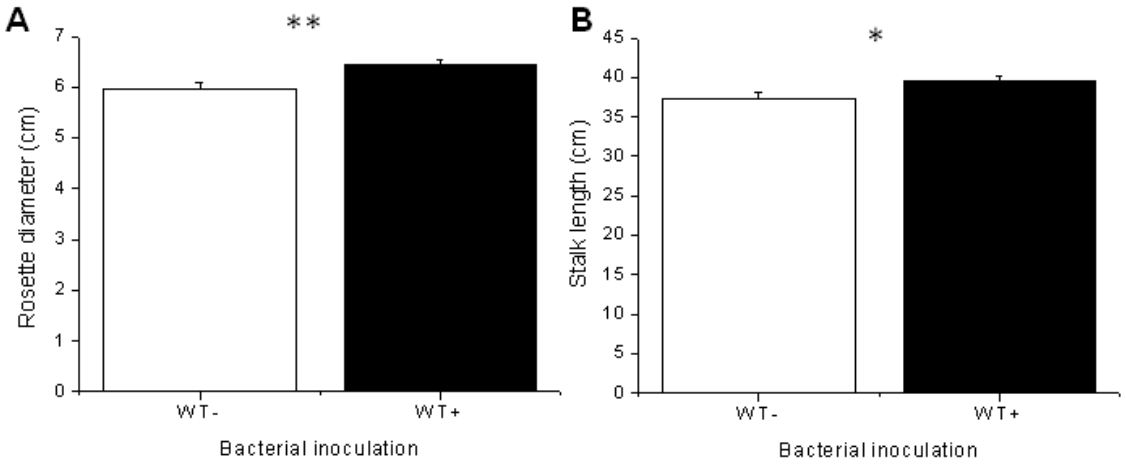


Figure 3. Growth of the uninoculated (-) and inoculated (+) WT plants upon inoculation of the rhizosphere with *Bacillus* sp. in the glasshouse. Mean ± SE of (A) rosette diameter 12DAI and (B) stalk length (cm) 30DAI. The asterisks (* and **) depicts significant difference between the uninoculated (-) and inoculated (+) plants (P<0.05, ANOVA Fisher's PLSD test).

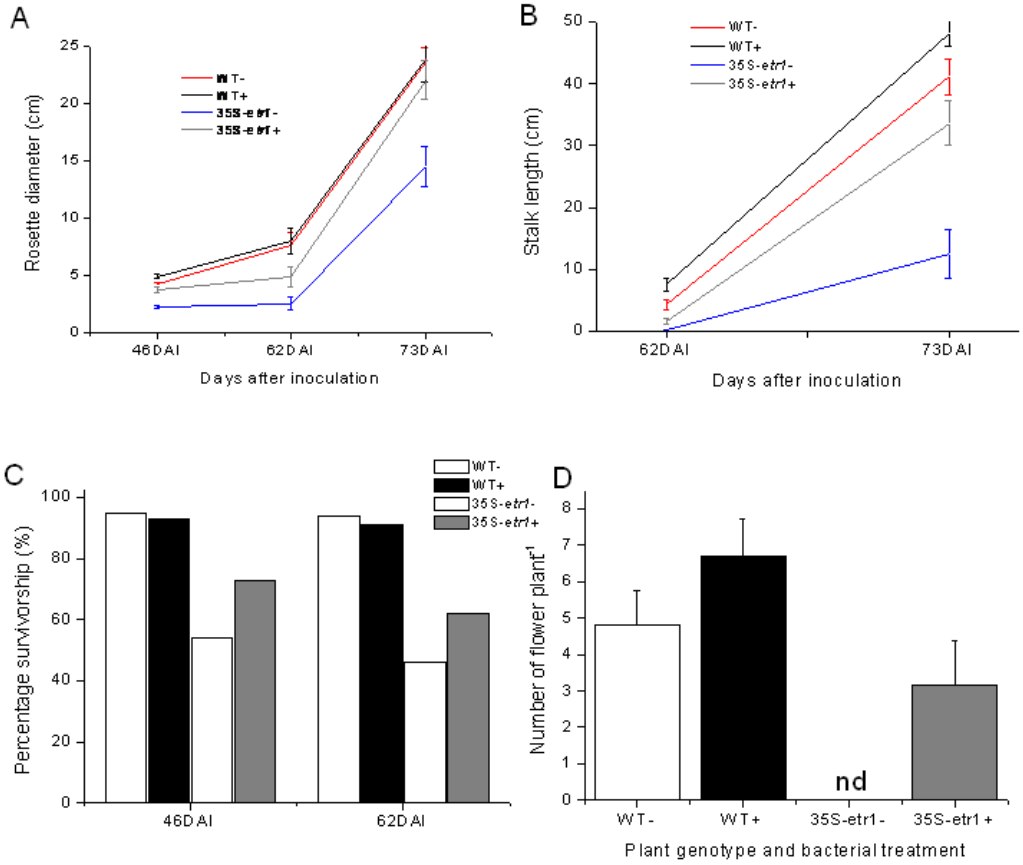


Figure 4. Performance of the uninoculated (-) and inoculated (+) WT and 35S-*etr1* in the field. Mean ± SE of (A) rosette diameter (cm) 46, 62 and 73 DAI (A); (B) stalk length (cm) 62 and 73 DAI; (C) survivorship (%) 46 and 62 DAI. and (D) number of flower 62 DAI. nd: not detected.

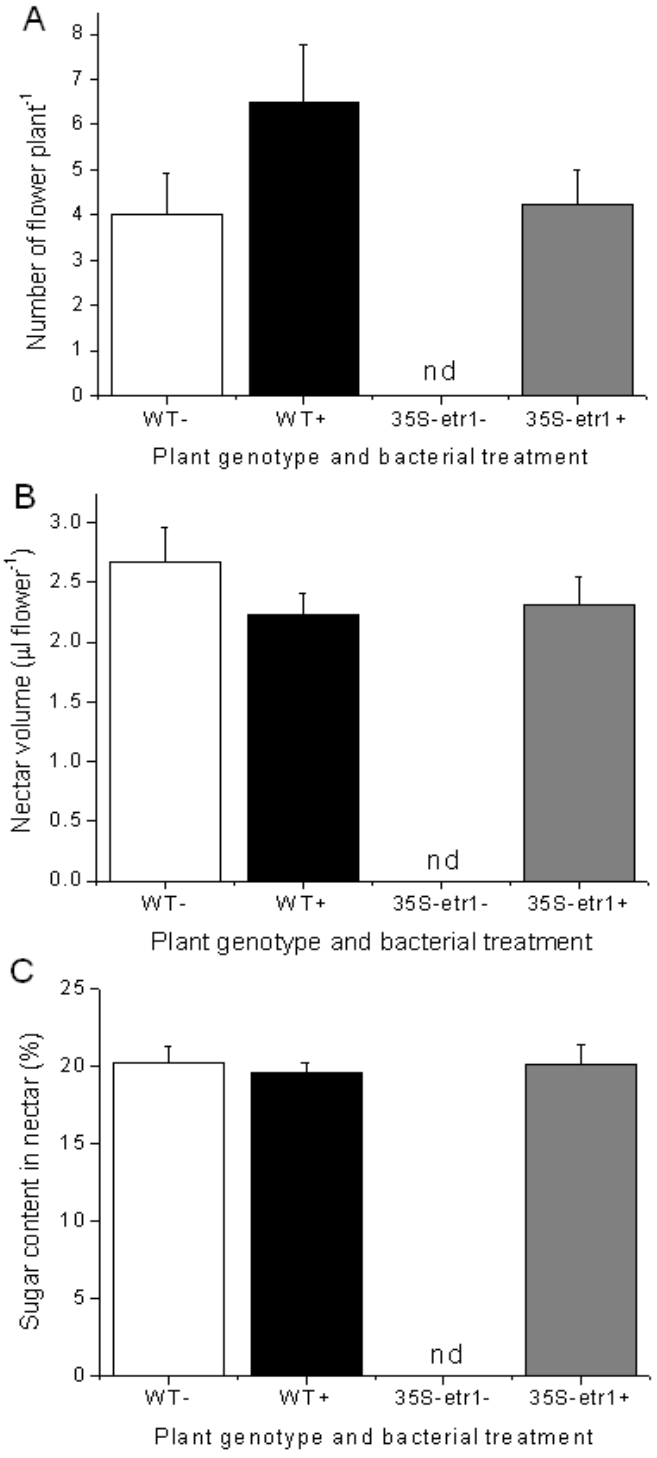


Figure 5. Mean ± SE of (A) flower number per plant; (B) nectar amount per plant (µl) and (C) nectar sugar content (%) of the uninoculated (-) and inoculated (+) WT and 35S-*etr1* plants 73DAI in the field. nd: not detected.

3.3.3. Effects of Bacillus *sp. inoculation on seedling IAA and ET production*

Seedlings of WT and 35S-*etr1* were inoculated with *Bacillus* sp. The inoculation resulted in a significantly higher number of later roots in the inoculated plants compared to in the uninoculated ones (Fig. 6). There was no significant difference in ET emission between the inoculated and uninoculated seedlings (Fig. 7A). No significant difference in free IAA content was found between inoculated and uninoculated WT seedlings (Fig. 7B). However, the inoculated 35S-*etr1* seedlings contained slightly more IAA than did the uninoculated 35S-*etr1* seedlings. There was no difference in IAA content between WT and 35S-*etr1* plants.

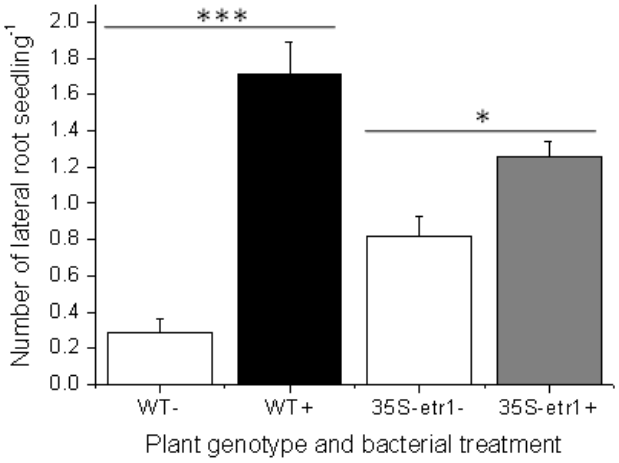


Figure 6. Mean ± SE of lateral root number of the uninoculated (-) and inoculated (+) WT and 35S-*etr1* seedlings 12DAI *in vitro*. The asterisks (* and ***) depicts significant difference between the uninoculated (-) and inoculated (+) seedlings (P<0.05, ANOVA Fisher's PLSD test).

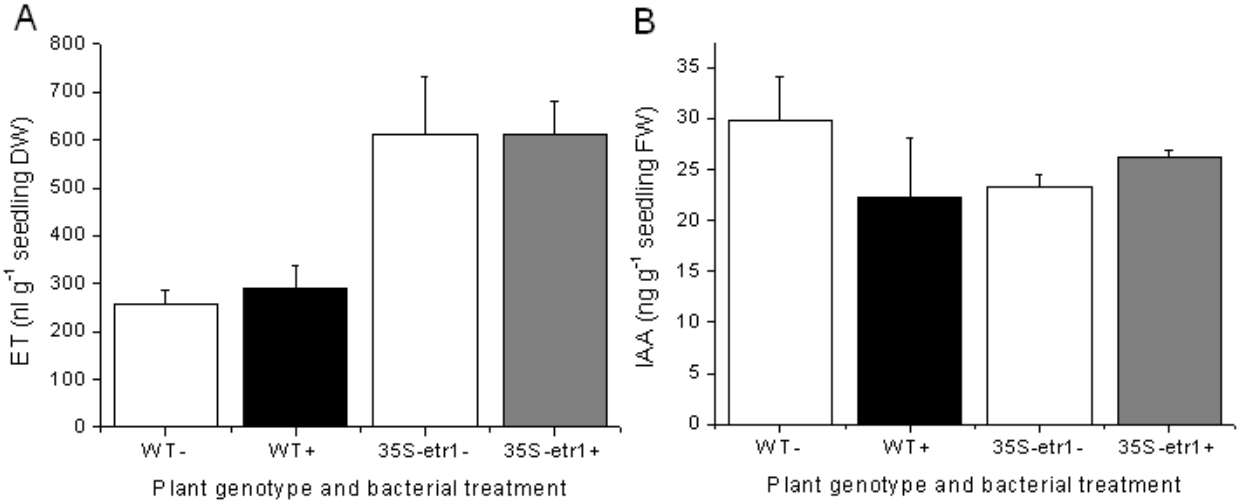


Figure 7. Mean ± SE of (A) ET emission (nl g^{-1} FW) and (B) total free IAA (ng g^{-1} FW) (B) in the uninoculated (-) and inoculated (+) WT and 35S-*etr1* seedlings 12DAI *in vitro*. DW: dry weight; FW: fresh weight

*3.3.4. Colonization of WT and 35S-*etr1 *plants by* Bacillus sp.

Bacillus sp. was able to colonize both WT and 35S-*etr1* seedlings at high levels (10^7 cfu g^{-1} root FW) 11 DAI (Fig. 8). The persistence of *Bacillus* sp. in glasshouse- and field-grown plants was observed till 30 DAI and 73 DAI, respectively. Although there was a continuous reduction in bacterial colonization in roots of the field plants over time, the colonization remained high with 10^5 and 10^4 cfu g^{-1} root FW, 47 DAI and 73 DAI, respectively. In the glasshouse, there was a strong correlation between the rate of bacterial colonization rate and the growth of rosette diameter 22 DAI (Fig. 9; $P<0.0001$ and $R^2=0.84$). However, no significant correlation between the extent of *Bacillus* sp. colonization and rosette diameter/stalk length in the field plants was observed at the end of the field experiment (73 DAI). Notably, *Bacillus* sp. could be re-isolated only from the roots of the inoculated plants.

3.3.5. Influence of Bacillus *sp. inoculation on the dominant resident bacterial endophyte communities*

The inoculation with *Bacillus* sp. strongly influenced the dominant bacterial endophyte communities of the inoculated plant roots. The "dominant bacterial endophyte" refers to the one colonized roots of the plants to the same extent as *Bacillus* sp did (Fig. 10). In *Bacillus* sp.-inoculated roots of the field-grown plants, several other species of bacterial endophytes had also become highly abundant while not being detectable in roots of the uninoculated plants at the end of the field experiment (73 DAI) (Fig. 10). Three different bacterial genera, namely, *Erwinia* sp., *Pseudomonas* sp., and *Pantoea* sp., dominated infected roots, while roots of uninoculated plants harbored only *Enterobacter cloacae* (Fig. 10). The inoculated WT plants were colonized by *Erwinia* sp. and *Pantoea* sp. Meanwhile, the inoculated 35S-*etr1* plants harbored *Erwinia* sp. and *Pseudomonas* sp. Furthermore, there was no difference in the extent of colonization by *E. cloacae* of the uninoculated WT and the 35S-*etr1* plants. Interestingly, these dominant bacterial isolates were able to colonize the roots as much as *Bacillus* sp. did (Fig. 10). Nevertheless, no difference in colonization by *Bacillus* sp. (10^5 cfu g^{-1} FW) of the inoculated plants was observed.

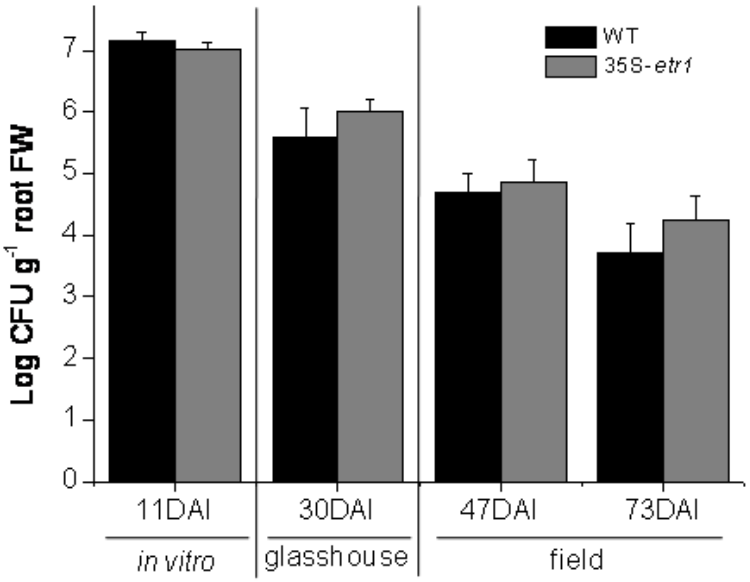


Figure 8. Mean ± SE of log CFU g^{-1} root FW of *Bacillus* sp. in WT (black bar) and 35S-*etr1* (gray bar) plants 11 DAI *in vitro*, 30DAI in the glasshouse, 47 and 73DAI in the field.

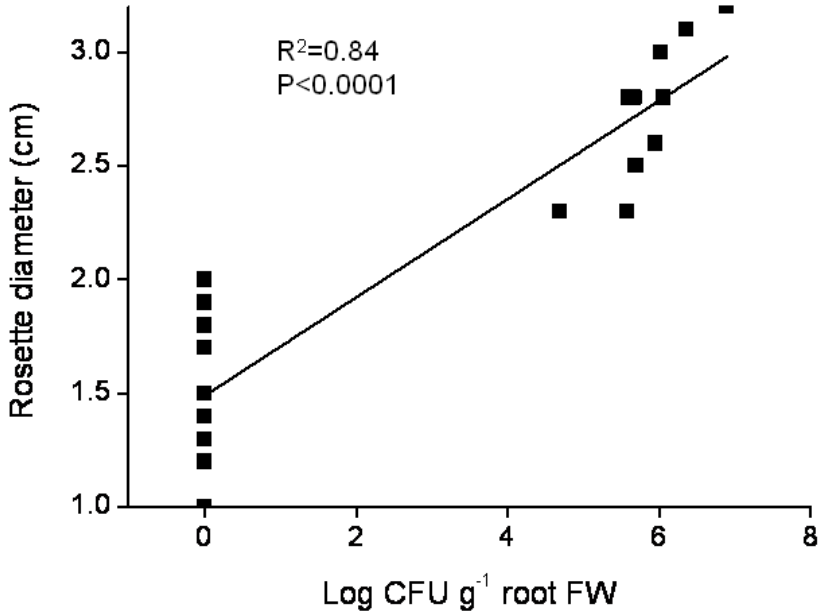


Figure 9. Correlation between *Bacillus* sp. colonization (log CFU g^{-1} root FW) and rosette diameter (cm) 22DAI. Significant correlation was determined by simple regression analysis ($P<0.0001$ and $R^2=0.84$).

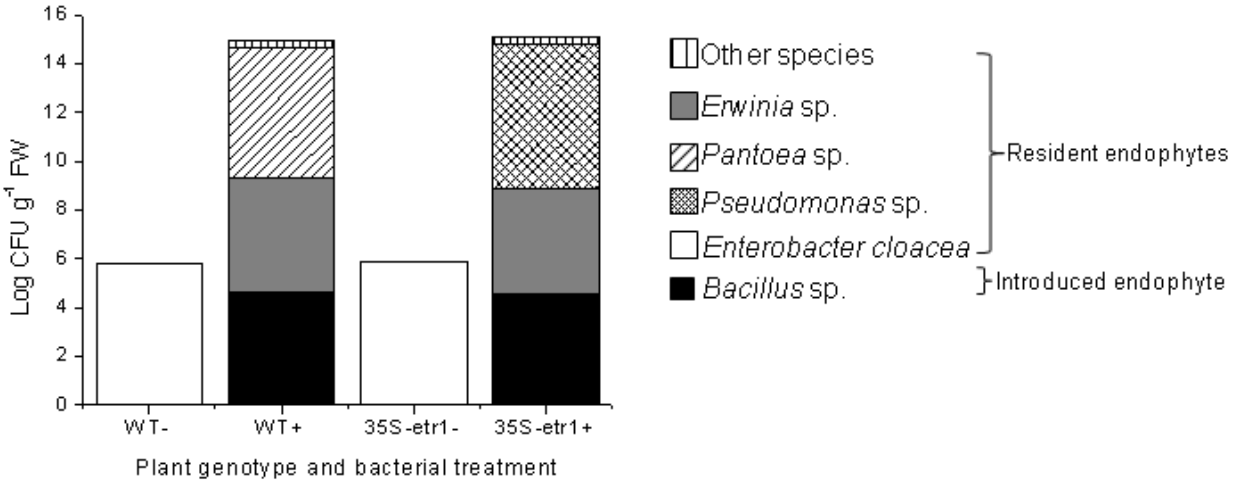


Figure 10. Effect of *Bacillus* sp. colonization (introduced endophyte-black bar) on the occurrence of dominant resident endophytes, namely *Erwinia* sp., *Pseudomonas* sp., and *Pantoea* sp., in the roots of the inoculated (+) WT and 35S-*etr1* plants and colonization of *Enterobacter cloacae* in the uninoculated (-) WT and 35S-*etr1* plants 73DAI in the field.

3.3.6. Effects of Bacillus *sp. inoculation on* M. sexta *performance and herbivore damage in the field*

In the glasshouse, *M. sexta* larvae were smaller on the inoculated WT plants than on the uninoculated ones 4 and 8 days after feeding (40 and 44DAI, respectively) ($P<0.05$). However, there was no difference in *M. sexta* performance between the inoculated and uninoculated 35S-*etr1* plants (Fig.11).

In the field, no significant difference was found in herbivore damage between the inoculated and uninoculated plants ($P>0.05$). However, the *Bacillus* sp. inoculated plants (both WT and 35S-*etr1*) tends to be more resistant to grasshoppers and tree crickets than were the uninoculated plants (Fig.12A & B). Conversely, the inoculated plants encountered more damage by mirids, noctuidae and leaf miners than did the uninoculated plants (Fig. 12C, D & F).

3.3.7. Inoculation of Bacillus *sp. stimulates induced ET burst*

In order to test if *Bacillus* sp. inoculation mediating plant defense against *M. sexta* is due to stimulation of ET production, *M. sexta* oral secretions (OS) were applied to the artificially wounded sites of leaves and ET emission was measured. The OS-induction of leaves from the inoculated plants (WT and 35S-*etr1*) resulted in significantly higher ET production ($P<0.05$)

compared to the OS-induction of leaves from the uninoculated plants (Fig.13). However, no significant difference in ET emission between the wounded leaves and wounded plus OS treated ones (P>0.05) was observed.

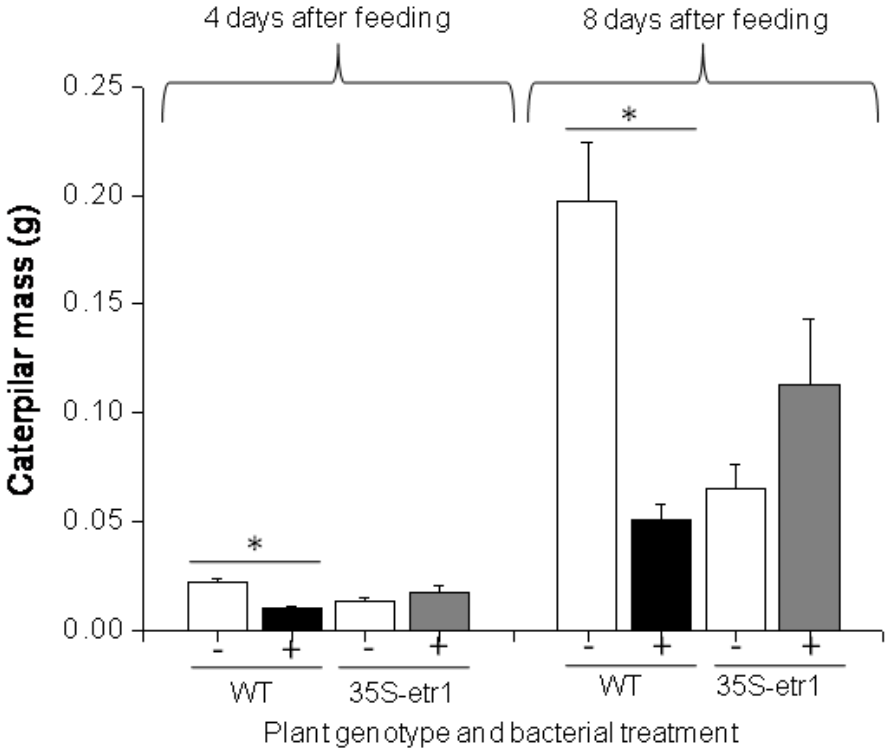


Figure 11. Larval mass of *Manduca sexta* 4 and 8 days after feeding on the uninoculated (-) and inoculated (+) WT and 35S-*etr1* plants 40 and 44DAI in the glasshouse. The asterisk (*) depicts the significant difference between the caterpillar mass on the uninoculated (-) and inoculated (+) WT plants (P<0.05, ANOVA Fisher's PLSD test).

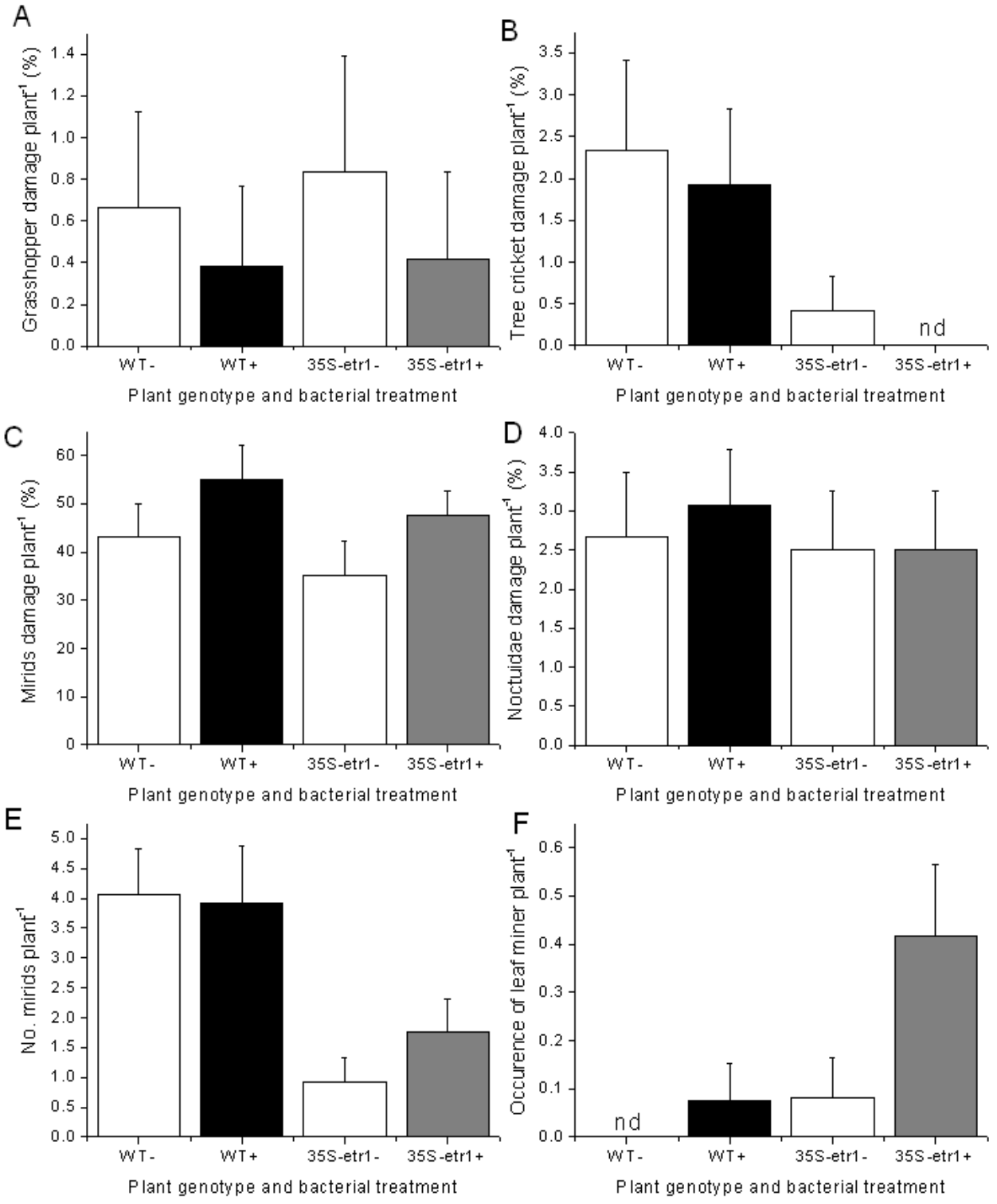


Figure 12. Herbivore damage to the uninoculated (-) and inoculated (+) WT and 35S-*etr1* plants in the field, Utah 73DAI. Percentage (%) of damage caused by (A) grasshoppers, (B) tree crickets, (C) mirids and (D) noctuidae; (E) number of mirids on each plant and (F) the occurrence of leaf miners on each plant. nd: not detected.

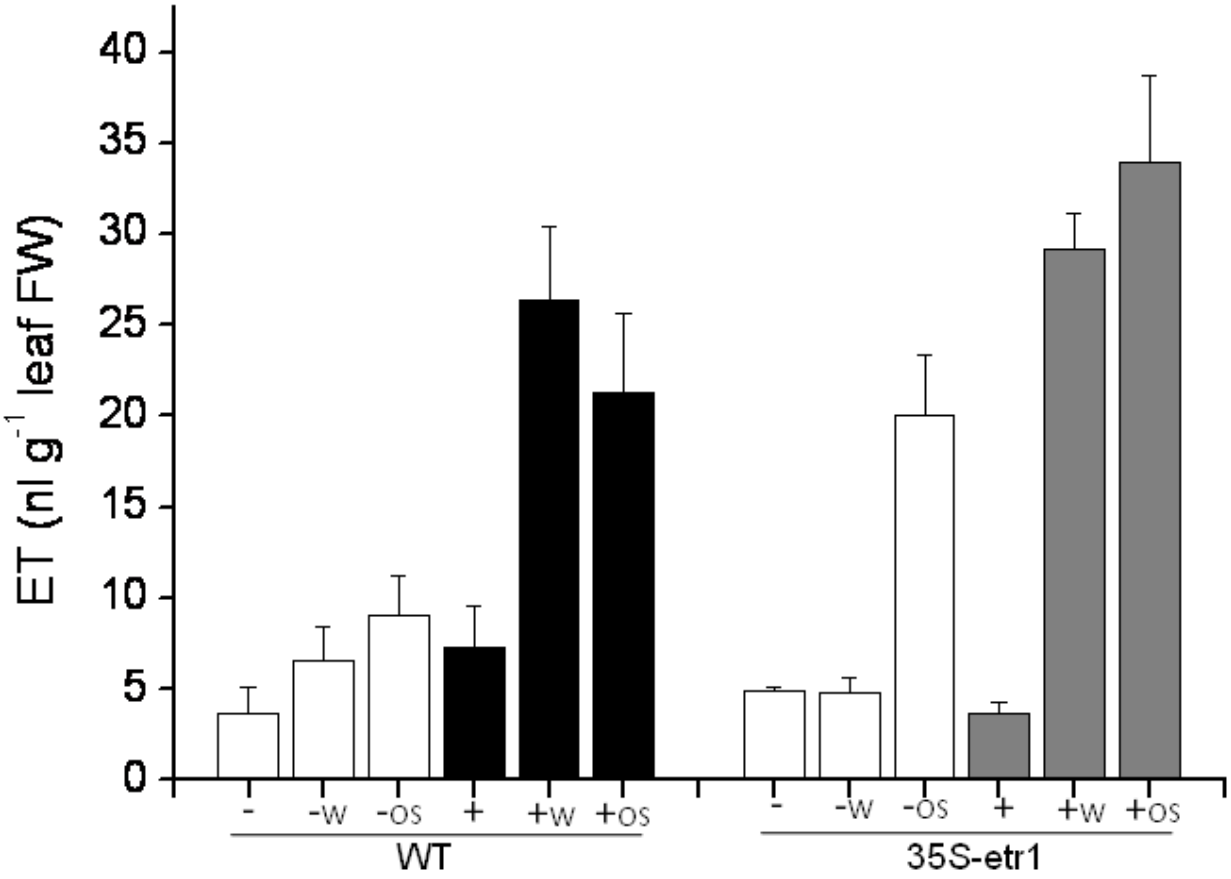


Figure 13. Mean ± SE of ET emission (nl g^{-1} leaf FW) from leaves of the uninoculated (-) and inoculated (+) WT and 35S-*etr1* plants upon mechanical wounding (W) or wounding plus *M. sexta* oral secretions (OS) application. FW: fresh weight.

3.4. Discussion

In a previous study, the success with which bacterial endophytes recruited from the native soils by WT and 35S-*etr1* plants was investigated. A number of endophytic bacterial isolates had beneficial effects on WT seedling growth. Deficient ET perception makes 35S-*etr1* plants highly mortal and susceptible to pathogen infection (Serek *et al.*, 2006; van Loon *et al.*, 2006). However, these plants might "select partners" which are beneficial for their growth and fitness in nature. Indeed, 35S-*etr1* plants which were reported to be unable to survive in the native habitats of *N. attenuata* (personal communication) performed better upon inoculation of a native *Bacillus* sp. isolate than did the uninoculated 35S-*etr1* plants.

Endophytic bacteria stimulate plant growth by producing IAA and ACCd and solubilizing inorganic P (Long *et al.*, 2008; Park *et al.*, 2009). Although *Bacillus* sp. isolate was

able to produce ACCd, there was no correlation between ACCd activity, ET production and root growth. The function of IAA appears to be prevalent in this isolate since the inoculated plants produced more lateral roots than the uninoculated ones (Fig. 6), reflecting the effects of IAA on plant roots (Celenza *et al.*, 1995). Root branching is one of the most important phenotypes plants can have to grow and survive in nature, because roots are involved in acquiring water and nutrients from the soils as reviewed by Wang *et al.*, (2006). No significant difference in free IAA levels between the inoculated and uninoculated WT and 35S-*etr1* plants was detected (Fig. 7). This suggests that inoculating plants with *Bacillus* sp. might alter plant IAA distribution and perception rather than only IAA levels which resulted in lateral root formation (Zhang *et al.*, 2007). Moreover, the synergistic effects of IAA and ACCd might be responsible for promoting plant growth (Glick *et al.*, 2007). A significant difference in the number of lateral roots per inoculated and uninoculated 35S-*etr1* seedling was found, though lateral root formation was reported to be dependent on ET response (Negi *et al.*, 2008). Bacterial IAA production has also been reported to be essential for rhizosphere competence and host colonization as reviewed by Spaepen *et al.*, (2007). *Bacillus* sp. turned out to be a strong root colonizer, probably due to high levels of IAA production, which might be augmented under field conditions where it has to compete with indigenous microflora for the same niches.

As the colonization by *Bacillus* sp. is only restricted to the roots, all the above-ground growth promotion is likely due to signaling transduction or nutrient transport occurring inside the plants, between roots and shoots as reviewed by Wang *et al.*, (2006). WT and 35S-*etr1* plants inoculated with *Bacillus sp.* showed a significant increase in plant growth under glasshouse and field conditions (Figs. 2 & 3). Interestingly, the inoculation also greatly enhanced plant fitness as measured by capsule production (glasshouse) (Fig. 2D) and flower number (field) (Fig. 4D). Although capsule number of the field plants could not be evaluated, the number of flowers suggests *Bacillus* sp. benefited the inoculated 35S-*etr1* plants (73DAI) (Fig. 5A). It is possible that the uninoculated 35S-*etr1* plants produced flowers after our experiment ended. However, delaying flower production will dramatically influence a plant's fitness in nature (Baker *et al.*, 1994; Baldwin, 2001).

Modifications in the plant-microorganism partnership bring about intricate reactions. What happens to the root bacterial endophyte communities when a bacterial endophyte is introduced into the roots? It has been reported that PGPB influenced resident microorganisms in

the rhizosphere as reviewed by Castro-Sowinski *et al.*, (2007). Consistent with these findings, I found that inoculation with *Bacillus* sp. strongly affected the root bacterial endophyte communities compared with those of the uninoculated plants in the field (Fig.10). The inoculated plants harbored a more diverse and dominant endophytic bacteria community. These dominant bacterial endophytes belong to *Erwinia* sp., *Pantoea* sp., and *Pseudomonas* sp., which are thought to be PGPB and biological control agents against pathogens (Montesinos *et al.*, 2002). In contrast, the uninoculated control plants (both WT and 35S-*etr1*) were dominated by only one bacterial species, *E. cloacae*. This bacterial species has been known to be antagonistic against other bacterial species as reviewed by Uddin & Viji, (2002). Thus, inter-populational interactions between *Bacillus* sp. and bacterial partners might contribute to enhancing plant growth and survival in the field (Ramos *et al.*, 2003b). Further investigations need to be done to verify if there are synergistic effects on plant growth and defense when these isolates are combined.

The ability to colonize the host plant is one of the most important traits of competent bacterial endophytes (reviewed by Hardoim *et al.*, 2008). Some bacterial endophytes significantly increased the colonization rate in the host plants over different stages of plant growth (reviewed by Kobayashi & Palumbo, 2000 and Hardoim *et al.*, 2008). However, I report a slight reduction in *Bacillus* sp. colonization from seedling (11 DAI) to rosette stage (30DAI) in the glasshouse as well as from rosette (47DAI) to flowering stage (73 DAI) in the field (Fig. 8). Micallef *et al.*, (2009) showed that as plants age, the slow-down in the active release of root exudates strongly influences the rhizobacterial and control bulk soil communities. It is also reasonable to presume that *Bacillus* sp. colonized the roots of the host plants at a high level in the early stages (seedling or rosette) when there was no competition with other microorganisms in the same niche. Furthermore, *N. attenuata* plants may regulate *Bacillus* sp. colonization, preventing this isolate from multiplying and becoming a biotic stressor (Rosenblueth & Martinez-Romero, 2006).

Since plants were inoculated with *Bacillus* sp. in the roots and none was detected in the leaf, the effects on herbivore performance are probably due to ISR. There have been very few studies on ISR induced by PGPR against herbivorous insects such as *Spodoptera exigua* (Van Oosten *et al.*, 2008) or reduced feeding by the cucumber beetles *Diabrotica undecimpunctata* and *Acalymma vittata* (Zehnder *et al.*, 2000). However, the effectiveness of ISR triggered by *Bacillus* sp. was proven against certain insect species, namely *M. sexta*, in the glasshouse only

(Fig. 11). No significant difference in damage caused by grasshoppers (*Trimerotropis* spp.), mirids (*Tupiocoris notatus* Distant), leaf miners, tree crickets and noctuidae between the inoculated and uninoculated plants was observed in the field (Fig. 12).

ET emission has been reported to be essential for establishing proper defense responses to *M. sexta* attack in *N. attenuata* (Kahl *et al.*, 2000; von Dahl *et al.*, 2007). The inoculated WT plants were primed to release more ET upon OS-induction or wound-induction while *Bacillus* sp. inoculation suppressed the growth of *M. sexta* larvae. However, the inoculated 35S-*etr1* plants did not influence *M. sexta*'s performance even though there was a significant ET emission after OS-induction in these plants. This suggests that the resistance to *M. sexta* induced by *Bacillus* sp. inoculation is dependent on ET perception. Herbivore-induced ET is known to modify the accumulation of direct defenses, including phenolics (Hudgins *et al.*, 2004), alkaloids (Kahl *et al.*, 2000) and protein-based defenses (Harfouche *et al.*, 2006), as well as the release of volatile organic compounds thought to function as indirect defenses (Schmelz *et al.*, 2003). Rather than being the principal elicitor of herbivore-induced defense responses, ET appears to play a more subtle role in modulating other defense signals, including JA (reviewed by von Dahl & Baldwin, 2007). Therefore, further studies of other phytohormones such as JA and SA as well as secondary metabolites and protease inhibitor activity induced upon treatment with *M. sexta* OS will help to address the underlying mechanisms of ISR elicited by *Bacillus* sp. against insect herbivory in *N. attenuata*.

In conclusion, ET perception is essential for plant growth and fitness in nature. *Bacillus* sp. isolate proved to be an ideal candidate for studying the ecological consequences of bacterial endophytes on the growth and fitness of *N. attenuata*. Because it shares common phenotypic traits of PGPB and has the ability to drive the root bacterial endophyte communities, *Bacillus* sp. isolate might be recruited by the host plant to help it grow and withstand abiotic and biotic stresses in nature. Moreover, native plants can function as true ‘filters’ of soil organisms, selecting those that are successful and competent endophytes. The complete cycle of bacterial endophyte selection and evaluation (trapping from native soils, inoculation of the host plants and tests of their efficiency by measuring the growth of host plants in native habitats) demonstrated the ecological relevance of the synergy between bacteria endophytes and *N. attenuata* in nature.

3. Summary

All plants in nature harbor a diverse community of endophytic bacteria which can positively affect host plant growth. Changes in plant growth frequently reflect alterations in phytohormone homoeostasis by plant-growth-promoting (PGP) rhizobacteria which can decrease ethylene (ET) levels enzymatically by 1-aminocyclopropane-1-carboxylate (ACC) deaminase or produce indole acetic acid (IAA). **(I)** Whether these common PGP mechanisms work similarly for different plant species has not been rigorously tested.

A plant's bacterial endophyte community is thought to be recruited from the rhizosphere, **(II)** but how this recruitment is influenced by the plant's phytohormone signaling is unknown. ET is known to regulate plant-microbe interactions; here I assess ET's role in the recruitment of culturable endophytic bacteria from native soils.

Endophytic bacteria have been well-studied to enhance plant growth of different importantly agricultural plant species. **(III)** However, little is known about how they influence growth and fitness of a native plant species in nature.

(I) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses.

I isolated bacterial endophytes from field-grown *Solanum nigrum*; characterized PGP traits (ACC deaminase activity, IAA production, phosphate solubilization and seedling colonization); and determined their effects on their host, *S. nigrum*, as well as on another Solanaceous native plant, *Nicotiana attenuata*. In *S. nigrum*, a majority of isolates that promoted root growth were associated with ACC deaminase activity and IAA production. However, in *N. attenuata*, IAA but not ACC deaminase activity was associated with root growth. Inoculating *N. attenuata* and *S. nigrum* with known PGP bacteria from a culture collection (DSMZ) reinforced the conclusion that the PGP effects are not highly conserved. I conclude that native/natural endophytic bacteria with PGP traits do not have general and predictable effects on the growth and fitness of all host plants, although the underlying mechanisms are conserved.

(II) The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception.

I grew wild type (WT) *Nicotiana attenuata* plants and isogenic transformed plants deficient in ET biosynthesis (ir-*aco1*) or perception (35S-*etr1*) in four native soils and quantified the extent of culturable bacterial endophyte colonization (by plate counting) and diversity (by amplified rDNA restriction analysis and 16S rDNA sequencing). The endophyte community composition was influenced by soil type and ET signaling. Plants grown in organic (vs. mineral) soils harbored a more diverse community and plants impaired in ET homeostasis harbored a less diverse community compared to WT plants. WT and ET-signaling-impaired plants fostered distinct bacteria in addition to common ones. *In vitro* re-colonization by common and genotype-specific isolates demonstrated the specificity of some associations and the susceptibility of 35S-*etr1* seedlings to all tested bacterial isolates, suggesting an active process of colonization driven by plant- and microbe-specific genes. In conclusion, the results confirm the central role of soil type and highlight, though to a lesser extent, the role of ET signaling in shaping endophytic bacterial community structure. Specific interactions between endophytic bacteria and their host plants are regulated not only by plant ET signaling but also by the bacteria themselves. The host plants' ability to recruit ACC deaminase/IAA-producing bacteria seems to be independent of ET signaling.

(III) Native bacterial endophyte from *Nicotiana attenuata* enhances the growth and fitness of its host in natural ecosystem

In this study, I used wild type (WT) *Nicotiana attenuata*, a wild tobacco species native to the Great Basin Desert, Utah, USA, and a transformed line (35S-*etr1*) deficient in ethylene (ET) perception, usually prone to pathogen infection, and has high mortality. A *Bacillus* sp. strain isolated from roots of 35S-*etr1* plants grown in native Utah soils and its associated plant-growth-promoting (PGP) traits such as the ability to produce indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase as well as inorganic phosphate (P) solubilization were characterized. The effects of *Bacillus* sp. inoculation on the growth and fitness of *N. attenuata* wild-type (WT) and 35S-*etr1* plants were evaluated under glasshouse and

field conditions. In the glasshouse, measurements of rosette diameter, stalk length, and seed capsule number in the inoculated plants were significantly higher than in the uninoculated plants. These PGP effects were also observed under field conditions. Bacterial inoculation additionally resulted in a significant increase in the survivorship of 35S-*etr1* plants in glasshouse and in the field. The colonization of *N. attenuata* plants by *Bacillus* sp. persisted till the end of the experiment. Moreover, inoculation of this isolate strongly influenced the dominant bacterial endophyte communities in the roots of *N. attenuata* WT and 35S-*etr1* field plants. Although there was no significant difference in herbivore damage between the inoculated and uninoculated plants in the field, larvae of a specialist herbivore *Manduca sexta* performed worse on the inoculated plants than on the uninoculated plants in glasshouse. Taken together, it becomes clear that *Bacillus* sp. is beneficial for WT and 35S-*etr1* plant growth and fitness in the natural settings.

4. Zusammenfassung

Basierend auf der Feststellung, dass zahlreiche Arten endophytischer Bakterien in verschiedenen Pflanzen nachgewiesen wurden, wird vermutet, dass alle Pflanzen von endophytischen Bakterien besiedelt und durch diese im Wachstum beeinflusst werden können. Letzteres geschieht unter anderem durch das Eingreifen der Bakterien in die Hormonhomeostase der Wirtspflanzen. Zum Beispiel reduzieren wachstumsfördernde Rhizobakterien die Ethylenkonzentration in Pflanzen, indem sie dessen chemischen Vorläufer 1-aminocyclopropane-1-carboxylate (ACC) enzymatisch spalten. Der pflanzliche Hormonhaushalt kann auch direkt durch die Produktion von Hormonen, wie Indol-3-essigsäure (IAA), durch Bakterien beeinflusst werden.

Ob diese verbreiteten Mechanismen der Wachstumsförderung durch Mikroorganismen bei verschiedenen Pflanzen ähnlich Effekte auslösen, wurde noch nicht im Detail untersucht und ist Bestandteil dieser Arbeit **(I)**. Desweiteren besteht die Annahme, dass eine Selektion von Bakterien aus der Wurzelrhizosphere in die Endosphere durch den pflanzlichen Partner stattfindet. In wieweit die Signaltransduktion des Pflanzenhormons Ethylen dabei eine Rolle spielt, ist unbekannt **(II)**. Der landwirtschaftliche Aspekt wachstumsfördernder, endophytischer Bakterien wurde in zahlreichen Kulturpflanzen studiert. Über die Bedeutung dieser Bakterien für das Gedeihen und die Reproduktion wilder Pflanzen in ihrem natürlichen Umfeld erbringt dieses Manuskript neue Aufschlüsse **(III).**

(I) Natürlich vorkommende bakterielle Endophyten fördern das Pflanzenwachstum in Abhängigkeit von Pflanzenspezies; Beeinflussung der Hormonhomeostase führt nicht zu generellen Wachstumseffekten.

Zahlreiche kultivierbare, bakterielle Endophyten wurden von im Freiland gezogenen Nachtschatten (*Solanum nigrum*) isoliert und deren Pflanzenwachstums-fördernde Charakteristika wie ACC-Deaminase (ACCd) Aktivität, IAA Produktion, Phosphat-Solubilisation und Kolonisationsrate von Keimlingen bestimmt. Zudem wurden Wachstumseffekte dieser Bakterien auf ihre Herkunftspflanze, *S. nigrum*, und auf eine eng verwandte Solanaceae, *Nicotiana attenuata*, untersucht. Es zeigte sich, dass der Großteil der bakteriellen Isolate mit positiven Effekten auf das Wurzelwachstum von *S. nigrum* in Korrelation mit der Produktion von ACCd und IAA standen. Für *N. attenuata* wurde eine Verbindung von

Wurzelwachstum mit der Produktion von IAA, jedoch nicht mit ACCd, deutlich. Die Inokulation von *S. nigrum* und *N. attenuata* mit Stämmen Wachtsums-fördernder Bakterien einer Kulturensammlung (DSMZ) untermauerte die Schlussfolgerung, dass die selben bakteriellen wachstumsfördernden Eigenschaften nicht unbedingt die gleiche Wirkung auf unterschiedliche Pflanzenarten zeigen müssen.

(II) Die Zusammensetzung der kultivierbaren, endophytischen Bakteriengemeinschaft in *Nicotiana attenuata* Wurzeln unterliegt dem Einfluss von Bodencharakteristika und der Produktion sowie Wahrnehmung des Phytohormons Ethylen durch die Wirtspflanze

Wildtyp Pflanzen des wilden Tabaks, *Nicotina attenuta*, heimisch im Great Basin Dessert, Utah, USA, und transgene Linien, welche ein Defizit entweder in der Synthese von ET (ir-*aco*1) oder in der Wahrnehmung (35S-*etr*1) aufweisen, wurden in vier verschieden Böden aus Utah kultiviert. Die Böden wurden, basierend auf ihrem Stickstoff- und Kohlenstoffgehalt, als nährstoffreiche (organische) bzw. nährstoffarme (mineralische) Böden eingestuft. Die Kolonisationsrate der endophytischen Bakteriengemeinschaft wurde mittels Auszählen von Kulturplatten; die Diversität durch ARDRA (amplified rDNA restriction analysis) und Sequenzierung von repräsentativen Isolaten evaluiert. Die Versuche zeigten, dass der Bodentyp und Ethylen-Signaltransduktion die endophytische Bakteriengemeinschaft stark beeinflussten. Im Vergleich zu *N. attenuata* Pflanzen, die in den mineralischen Böden kultiviert wurden, wiesen Pflanzen, die in den organischen Böden gezogen wurden, eine höhere Diversität an kultivierbaren, endophytischen Bakterien auf. Desweiteren verfügten WT Pflanzen über eine vielfältigere bakterielle Endophytengemeinschaft als die transgenen Linien. Einige der endophytischen Spezies wurden nur in Verbindung mit bestimmten Genotypen gefunden. *In vitro* Kolonisationsstudien mit häufig bzw. nur spezifisch auftretenden Bakterienisolaten zeigten, dass manche der *N. attenuata*-Endophyten-Interaktionen in der Tat spezifisch sind und dass 35S-*etr*1 Keimlinge von allen getesteten bakteriellen Stämmen besiedelt werden konnte. Dies lässt auf einen aktiven Besiedlungsprozess schließen, d er durch pflanzliche und bakterielle Gene reguliert wird. Zusammenfassend lässt sich feststellen, dass Bodentyp und Ethylen-Signaltransduktion der Wirtpflanze auf die Struktur der endophytischen Bakteriengemeinschaft von *N. attenuata* starken Einfluss nehmen.

(III) Ökologische Auswirkungen von *Bacillus* sp. auf Wachstum und Fitness von *Nicotiana attenuata*

Aus Wurzeln, der in Utah-Böden kultivierten transgenen *N. attenuata* Linie 35S-*etr1*, welche eine hohe Anfälligkeit gegenüber Krankheiten und eine erhöhte Mortalitätsrate aufweist, wurde der bakterielle Endophyt *Bacillus* sp. isoliert. Pflanzenwachstums-fördernde Charakteristika wie die Produktion von IAA oder ACCd und die Solubilisierung von Phosphor wurden bestimmt und die Auswirkungen einer Inokulation von *Bacillus* sp. in *N. attenuata* WT und 35S-*etr1* unter Gewächshaus- und Freilandbedingungen untersucht. Die Inokulation im Gewächshaus führte zu signifikanter Zunahme in Rosettendurchmesser und Sprosslänge sowie der Anzahl der Samenkapseln bei beiden Genotypen gegenüber nicht-inokulierten Pflanzen. Diese Beobachtungen wurden unter Freilandbedingungen (Utah, USA) bestätigt. Zudem steigerte die Beimpfung der sonst sehr anfälligen 35S-*etr1*Pflanzen die Überlebensrate deutlich. Obwohl das Ausmaß der Besiedlung durch endophytische *Bacillus* sp. Bakterien während des Versuchs stetig abnahm, erwies sich die Kolonisation der Pflanzen bis zum Ende des Versuchs als persistent. Die künstliche Beimpfung von *N. attenuata* im Freiland beeinflusste die Zusammensetzung der bakteriellen Endophytengemeinschaft der Wurzeln von WT und 35S-*etr1*Pflanzen deutlich, jedoch zeigte die Inokulation keine Auswirkungen auf Insektenbefall. Im Gegensatz dazu führte die *Bacillus* sp Inokulation unter Gewächshausbedingungen bei WT Pflanzen zu einem verzögerten Wachstum der Raupen des Spezialisten *Manduca sexta*. Diese Ergebnisse demonstrieren, dass *Bacillus* sp. das Wachstum und die Fitness von WT und 35S-*etr1* Pflanzen in ihrer natürlichen Umgebung positiv beeinflussen kann.

5. General materials and methods

5.1. S. nigrum germination

- Prepare fresh sterilization solution: 0.1g of dichloroisocyanuric acid sodium salt (DCCS), 5ml water, 50µl of 0.5% Tween-20 stock
- Incubate seeds shaking for 5 minutes in the sterilization solution, then wash 3 times thoroughly with sterile water.
- Incubate seeds in 5ml 1M KNO_3 at 4ºC in darkness over night.
- Prepare germination plates: Dissolve 1x strength Gamborg's B5 (3.16g/L) in 980mL distilled water. Adjust pH to 6.80. Add 6g Phytagel (or 6g plant agar). Make sure that the stirrer is on before adding phytagel to prevent clumping. Autoclave for 121ºC for 20mins. Pour the media into 100x25mm Petri dishes in the sterile bench. Leave the lids of the Petri dishes open for faster cooling and also to prevent condensation water from settling onto the lids. Keep the media in a water bath set to 57ºC if you cannot pour the media immediately. Below this temperature, phytagel will become hard and it cannot be dissolved again in the microwave.
- Spill seeds together with the incubation solution into an empty sterile petri dish. Use a sterile forceps to plant 20 seeds well spaced into each Petri dish containing the germination media.
- Seal Petri dishes with parafilm and incubate for 7 days under the following conditions: 26ºC/16h 100% light, 24ºC/8h darkness in a Percival growth chamber.

5.2. N. attenuata germination

The following solutions and media should be prepared beforehand: Sterilization solution (to be prepared shortly before germination) containing 0.1g DCCS; 5ml distilled water; and 50µl of 0.5% Tween-20 detergent stock solution.

Stock solutions: 0.1M GA_3 (dissolves in ethanol) and sterile filtered; 50x diluted liquid smoke (House of Herbs, Passaic NJ) in distilled water. Autoclave and store at room temperature

Germination media: see above

Germination procedure:

- Sterilization of seeds: Pour some seeds into a plastic tube (e.g falcon tube) and incubate in the freshly prepared sterilization solution for 5mins. The seeds will turn lighter in color. After

5mins, decant the solution, along with any empty seeds that may float on the solution during the sterilization process, and rinse the seeds left in the tube at least 3 times in sterile water.

- Treatment with germination cues (Gibberellic acid and smoke): Add 50µl of the 0.1M GA_3 stock solution and 5ml of the diluted liquid smoke solution to the seeds. Incubate for 1 hour.
- Germination: After 1 hour, decant the GA_3/smoke solution and rinse the seeds in sterile water three times. Spill them onto a sterile Petri dish and use a sterile Pasteur pipette to transfer the seeds onto the germination media. It is important to note that the seeds should be placed gently on the media and not to be pressed into the media. Place 20-30 seeds per Petri dish and minimize the amount of water transferred to the dish. After finishing placing the seeds, seal the Petri dish with parafilm and incubate in a Percival chamber with the following regime set: 27ºC/16hrs 100% light, 24ºC/8hrs dark in a Percival growth chamber. Seedlings can be seen clearly after 6 days.

5.3. Bacterial endophyte isolation

Endophytic bacteria were isolated after removing epiphytes from root tissues by surface disinfection using serial washing in 70% ethanol for 1 min, sodium hypochlorite solution (1-1.5% available Cl^-) (Sigma, Steinheim, Germany) for 2 min and three rinses in sterilized distilled water. The success of disinfection was verified by plating aliquots of the sterile distilled water used in the final rinse onto half-strength Yeast Peptone Dextrose Agar (YPDA) (Sigma, Steinheim, Germany) and incubating the plates at 30^{o}C for 10 days. After surface disinfection, root tissue was cut and titrated in distilled water; appropriate dilutions were plated onto half-strength YPDA and incubated at 30^{o}C for 2-10 days. After incubation, distinct colonies were picked from the plates. Pure cultures were inoculated on half-strength YPDA slant tubes, incubated at 30^{o}C for 2 days and stored at 4^{o}C. Additionally, each culture was suspended in a 20% glycerol solution and stored at -80^{o}C for long-term preservation.

5.4. 16S rDNA sequencing

Immediately after the establishment of pure bacterial cultures, genomic DNA was isolated from one-day-old cultures grown on half-strength YPDA plates. Single colonies were suspended in water to obtain suspensions of approximately 10^5 cfu ml^{-1}. 0.5 µl of suspension was

mixed with 4.5 µl extraction buffer (10 mM Tris-HCl pH 7.6; 50 mM KCl; 0.1% Tween 20). Then the mixture was heated at 100°C for 10 min and immediately placed on ice. After centrifugation at 6000*xg* for 5 min, the supernatant was used for PCR. Amplification of 16S rDNA was performed in a 10 µl final volume containing 1 µl of genomic DNA, 10 µM of primer F27 (5'-AGAGTTTATCMTGGCTCAG-3') (Edwards *et al.*, 1989) and R1492 (5'-GRTACCTTGTTACGACTT-3') (Lane, 1991), 10 mM of each dNTP, 5 mM $MgCl_2$ and 0.05U of Taq DNA polymerase (Eppendorf, Hamburg, Germany). A negative control (PCR mixture without DNA template) was included in all PCR experiments. The reaction conditions were as follows: 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 20 s and primer extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The quality of the PCR reaction was examined by running an aliquot of the PCR mixture in 1.2% (w/v) agarose containing ethidium bromide.

PCR products were purified using QIAquick™ Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's manual. Direct sequencing using the primer R1492 with expected size approximately 600bp was conducted in Big Dye Mix (Applied Biosystems, Foster City, CA, USA) and purification of sequencing reactions was performed using NucleoSEQ Kit (Macherey-Nagel, Duren, Germany) and sequenced on a ABI310 sequencer (Applied Biosystems; http://www.appliedbiosystems.com). The editing of sequences was performed with EditSeq and SeqMan (DNAStar Lasergene 7, DNASTAR Inc.). Analysis of sequences was carried out with basic sequence alignment BLAST program (Altschul *et al.*, 1997) run against the database from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

5.5. Bacterial ACC deaminase, IAA and phosphate solubilization assay

ACC deaminase (ACCd) activity was determined as described by Glick, (1995) by measuring the amount of α-ketobutyrate produced when the enzyme ACCd cleaves ACC. The nmoles of α-ketobutyrate produced by this reaction were determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 nmol.

IAA production was determined as described by Bric *et al.*, (1991) using the colormetric method.

An inorganic phosphate solubilization assay was carried out as described by Verma *et al.*, (2001): inoculating bacterial isolates on Pikovskaya (PVK) medium containing (g/l): glucose, 10 g; $Ca_3(PO_4)2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4.H2O$, 0.002 g; and $FeSO_4.7H_2O$, 0.002 g supplemented with 1.5% Bacto-agar (Difco Laboratories, Detroit, MI, USA). Four plates were stabbed using sterile toothpicks. The halo and colony diameters were measured 14 days after the plates were incubated at 30^{o}C.

5.6. Data analysis

Analysis of the data was carried out using StatView software package (SAS Institute) with a completely randomized analysis of variance ($P<0.05$). One-way and two-way ANOVA followed by Fisher's PLSD test was used to compare significant differences among treatments. Correlation analysis was done with simple regression test.

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8. Declaration of independent work

I declare in accordance with the conferral of the doctoral degree from School of Biology and Pharmacy of Friedrich Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

The people who assisted in the experiments, data analysis and writing the manuscripts are listed as coauthors of the manuscript and acknowledged in the thesis. I was not assisted by any consultant for doctoral theses.

The thesis has not been previously submitted either to the Friedrich Schiller University or to any other University.

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Presentations

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10. Supplementary materials

10.1. Chapter I. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses.

Table S1. Seedling root colonization by endophytic bacterial isolates from *S. nigrum*. Bacterial re-isolation from seedling roots 7 days after inoculation with each bacterial isolate.

Sample	Seedling colonization (cfu g^{-1} FW)
SSR4	$2.3x10^6$
SSR5-1	$9.8x10^5$
SSR5-2	$4.0x10^6$
SSR8-1	$4.8x10^6$
SSR8-2	$3.2x10^6$
BGCR2-8(1)	$3.3x10^6$
DR5	$3.2x10^6$
BGCR2-6	$4.4x10^6$

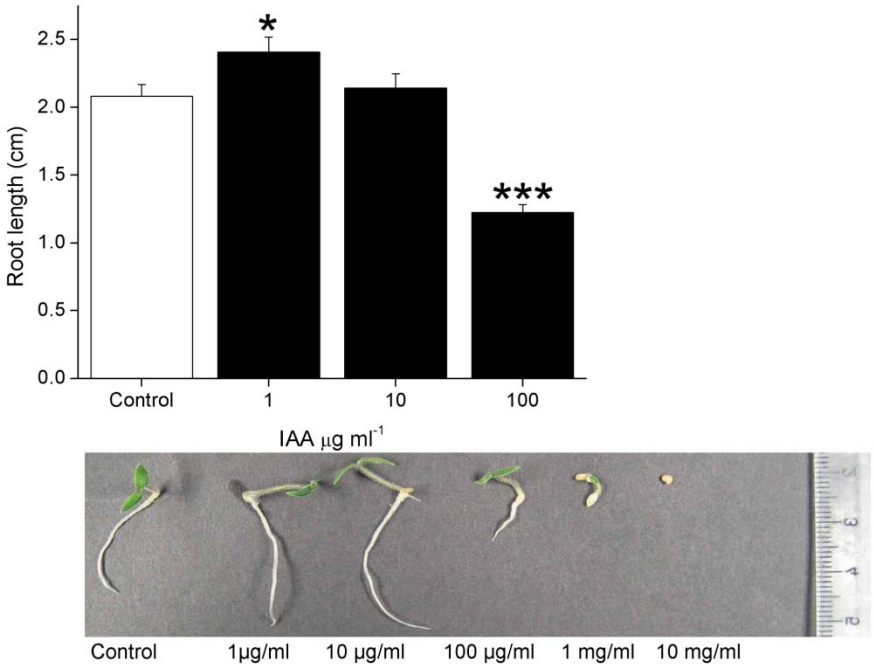

Figure S1. Effects of exogenous IAA application on root growth of *S. nigrum* seedlings. Asterisks indicate significant differences (Fisher's PLSD test; $P<0.05$ (*) and $P<0.0001$ (***)).

10.2. Chapter II. The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception.

Table S1. ANOVA table comparing effects of plant genotype and soil type on the total bacterial community

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Soil type	3	17.469	5.823	5.446	0.0035	16.337	0.918
Plant genotype	2	9.117	4.559	4.263	0.0220	8.526	0.708
Soil type* Plant genotype	6	16.382	2.730	2.553	0.0371	15.320	0.774
Residual	35	37.425	1.069				

Table S2. ANOVA table comparing effects of plant genotype and soil type on the number of ACCd producing bacteria

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lamda	Power
Soil type	3	140.503	46.834	8.511	0.0003	25.534	0.991
Plant genotype	2	4.681	2.341	0.425	0.6572	0.851	0.111
Soil type* Plant genotype	6	52.428	8.738	1.588	0.1828	9.528	0.519
Residual	32	176.083	5.503				

Table S3. ANOVA table comparing effects of plant genotype and soil type on the number of IAA producing bacteria

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lamda	Power
Soil type	3	178.995	59.665	8.011	0.0004	24.033	0.987
Plant genotype	2	2.369	1.184	0.159	0.8537	0.318	0.072
Soil type* Plant genotype	6	18.347	3.058	0.411	0.8665	2.463	0.148
Residual	32	238.333	7.448				

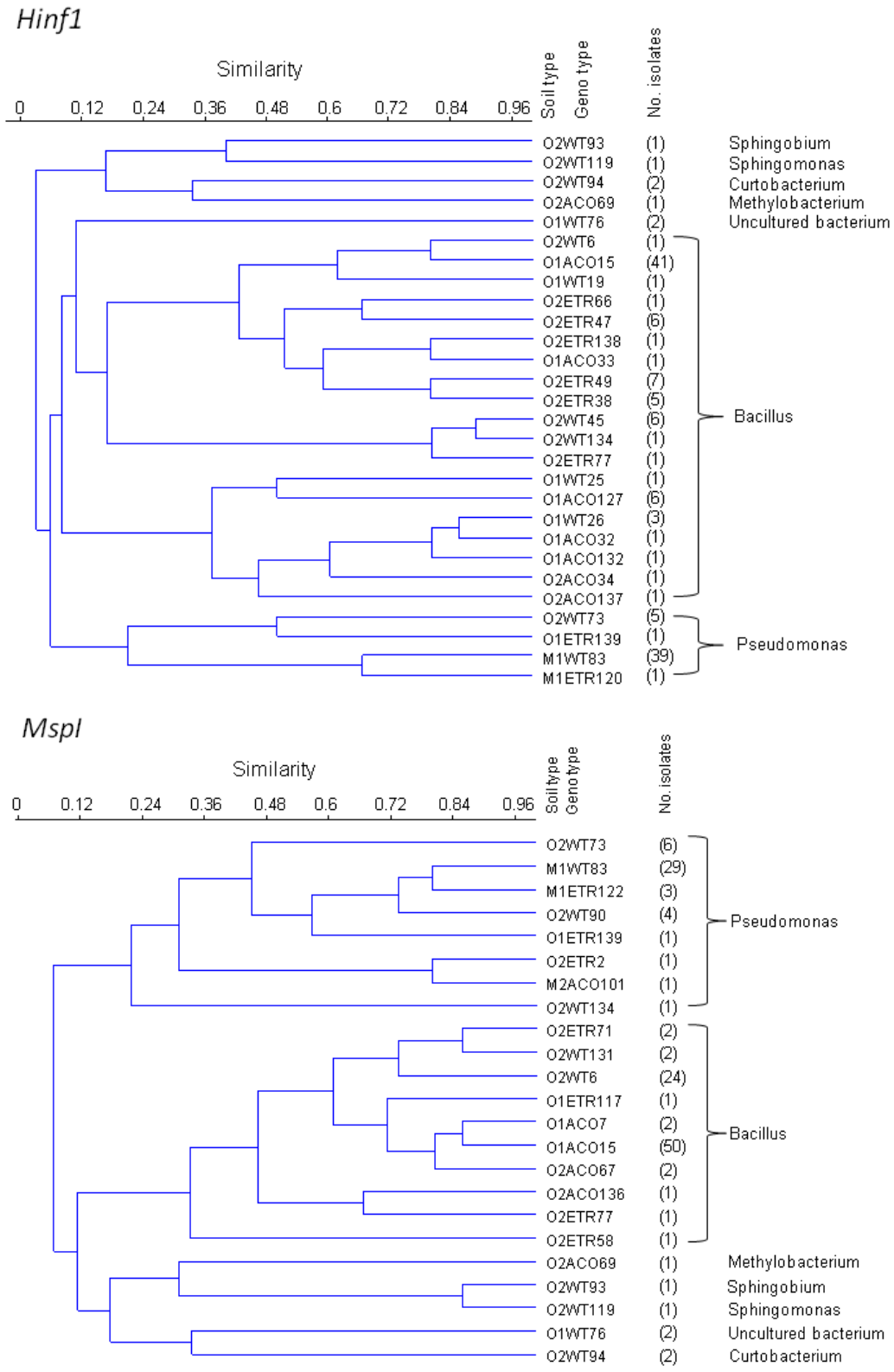

Figure S1. Dendrogram showing the OTUs (operational taxonomic units) of the culturable endophytic bacterial isolates with *HinfI* and *MspI*.

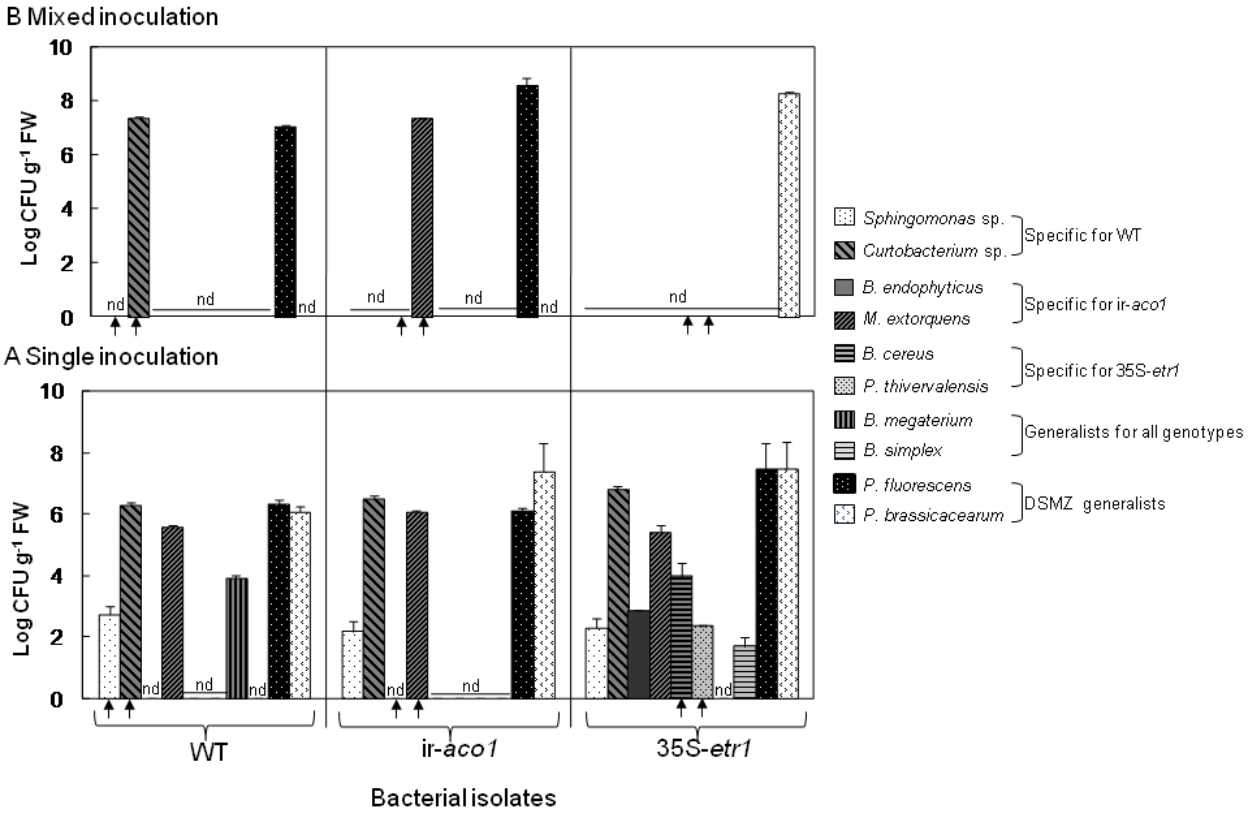

Figure S2. The second independent experiment of *in vitro* colonization of different genotypes by bacterial endophytes from **(A)** single inoculum and **(B)** mixed inoculum treatment.