Mutualistic plant-microbe interactions in *Nicotiana attenuata* – from bacterial communities to single players

Dissertation

zur Erlangung des akademischen Grades

Doktor rerum naturalium
(Dr. rer. nat.)

vorgelegt

dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

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1 Introduction

1.1 Defining plant growth-promoting bacteria

Besides algae, terrestrial plants represent the main primary producers life depends on. As every living organism, plants have to cope and compete with diverse entities affecting plant and ecosystem productivity, some being so small that they have been overlooked for ages: microorganisms. Although Romans and Greeks were already aware of pathogens causing destructive plant diseases, their microbiological nature was elusive until the 19th century[1]. That a subset of microbes can affect plant growth positively came to light in the early 20th century: root-nodule forming bacteria, supplying nitrogen to the legume host, were described (Golding, 1905). The fact that the interior of plants can be colonized by fungi without causing symptoms was reported in 1902 by Freeman. This discovery led to the creation of the term "endophyte": microbes living asymptomatically for at least part of their life cycle inside plant tissues.

The rhizosphere is defined as the soil adjacent to roots, which is directly affected by root activities (e.g. root exudation of organic compounds) (Hiltner, 1904). It is known as a zone in which microbial life prospers and from which a subset of microbes selectively enters the roots to undergo an endophytic lifestyle. Many of these rhizosphere-dwelling microbes can affect plant growth positively, an observation that subsequently led to the definition of the term plant growth-promoting rhizobacteria (PGPR) (Kloepper & Schroth, 1979). These days the term "endophytes" is extended to bacteria; and PGPR include not only rhizosphere-inhabiting, but also endophytic organisms, creating the new term PGP bacteria (PGPB). The rhizo- and endospheres of all plant species investigated so far are colonized by PGPB (Rosenblueth & Martinez-Romero, 2006). Plants are lucrative hosts: their photoassimilates present versatile nutrient sources for heterotrophic microorganisms. Furthermore, plants offer diverse protective niches. In turn, there is a common consensus that plants depend on the presence of bacteria for healthy growth (DeCoste *et al.*, 2010; Partida-Martinez and Heil, 2011; but see also Aguilera *et al.*, 2011).

In my studies, I focused on plant growth-promotion (PGP) by root-associated PGPB, including endophytic and rhizosphere-dwelling bacteria.

1.2 How PGPB promote plant growth

Diverse mechanisms have been postulated for bacterial-driven PGP. The modes of action are commonly divided into either "direct" or "indirect". Direct mechanisms include the interference of microbes with plant hormone homeostasis, assistance with nutrient acquisition, or emission of volatile signaling molecules (Gamalero & Glick, 2011).

The production and excretion of plant-growth-stimulating hormones by microbes, e.g. indole-3-acetic acid analogs (IAA, auxins), cytokinins, or gibberellic acid leads to dose- and host genotype-dependent growth enhancement (Long *et al.*, 2008; Gamalero & Glick, 2011). Also, the

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[1] http://www.scribd.com/doc/11594622/Introduction-to-plant-pathology

bacterial enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCd) was found to cleave the ethylene (ET) precursor ACC, thereby lowering stress-associated plant ET production and in turn promoting plant growth (Glick *et al.*, 2007).

Second, supplying nutrients like nitrogen (N) to the host plant is a famous example of direct PGP. Not only symbiotic, nodule-forming rhizobia, but also free-living bacteria like *Azoarcus* sp. provide the often growth-limiting macronutrient N to the host (Hurek *et al.*, 2002; Franche *et al.*, 2009). Improving accessibility to other limiting nutrients via e.g. the excretion of iron-chelating siderophores by root-associated bacteria (Lemanceau *et al.*, 2009), or the solubilization of phosphates and organically bound phosphorous, has been reported to contribute to PGP as well (Rodriguez & Fraga, 1999).

Sulfur (S) is rated the 5th limiting macronutrient and is pivotal for plant health. It is a constituent of the amino acids methionine and cysteine, thioredoxins, enzymes and plant defensive compounds (e.g. glucosinolates) (Droux, 2004). In the last decades, reduced emissions of anthropogenic sulfur dioxide (SO_2) resulted in widespread S-deficiency in soils (McGrath & Zhao, 1995). Because plants cannot exploit S incorporated in soil organic matter, they depend on diverse bacterial strains, capable of oxidizing S, which provide easily accessible sulfate (SO_4^{-2}) to the plants (Kertesz & Mirleau, 2004; Anandham *et al.*, 2011).

A third direct method of microbe-caused plant growth modulation became apparent during the last decade: microbial volatiles. The exposure of plants to the microbial volatile bouquet resulted in growth effects ranging from dramatic growth enhancement to plant death. The production of dozens of compounds by diverse microbes has been demonstrated, as reviewed by Wenke *et al.* (2012). So far, only a few bioactive volatile organic compounds (VOCs), including 2,3-butanediol, acetoin, dimethyl disulfide (DMDS), 2-pentylfuran and dimethylhexadecylamine, have been identified (Ryu *et al.*, 2003; Kai *et al.*, 2010; Zou *et al.*, 2010; Velazquez-Becerra *et al.*, 2011). Additionally, "inorganic" bacterial volatiles, namely carbon dioxide (CO_2), hydrogen cyanide (HCN), ET and ammonia (NH_3) emission have been reported to shape the plant's growth (Weingart & Volksch, 1997; Weingart *et al.*, 2001; Kai & Piechulla, 2009; Kai *et al.*, 2010). Information on the action mode and signaling involved in plant growth regulation is limited (Bailly & Weisskopf, 2012; Wenke *et al.*, 2012).

Indirect mechanisms of PGP involve the induction of Induced Systemic Resistance (ISR) or Systemic Acquired Resistance (SAR). Both mechanisms implement complex plant signaling and arise either after an inoculation of non-pathogenic rhizobacteria (ISR) or after a local infection with fungal plant pathogens (SAR) (Goellner & Conrath, 2008; Vlot *et al.*, 2008). Additionally, microbes introduced or native to the rhizosphere can shape the plant-associated microbial community, thereby controlling plant diseases. So-called biocontrol agents are thought to inhibit plant pathogens by excretion of antimicrobial compounds (e.g. antibiotics, cell wall degrading enzymes, HCN or other toxins), competition for nutrients and space, degradation of pathogenic toxins, or hyper-colonization of fungal hyphae (Lugtenberg & Kamilova, 2009). On the other hand, introduced (inoculated) microbial species might pave the way for other beneficial interaction partners, a phenomenon called "microbial co-operation", which has been described for

symbiotic interaction partners like arbuscular mycorrhizal fungi (AMF) and rhizobia (Barea *et al.*, 2005).

1.3 Factors determining plant-PGPB interactions

A plethora of plant-PGPB interactions has been described and intensively studied. But not all bacteria, inhabiting the soil can associate with a plant. Complex communication between microbes and their future host is required for successful colonization (Hardoim *et al.*, 2008). Bacterial characteristics, summarized by the term "colonization traits", determine plant colonization and include attributes like motility, chemotactic responses, host recognition, root adherence, fast growth rates and several other genetic determinants as recently reviewed by Barret *et al.* (2011) and Compant *et al.* (2010). Endophytic colonization reflects an even more specific, long-lasting and intimate relationship between plants and bacteria, in which both partners have to maintain a healthy plant-microbe association. Usually, only a subgroup of rhizobacteria possessing specific traits is allowed to colonize the interior of the host. For example, the microbe has to overcome plant defense machinery, and usually PGPB, which provide services to the host, are thought to represent "competent endophytes" (Hardoim *et al.*, 2008; Taghavi *et al.*, 2009; Pineda *et al.*, 2012).

Next to stochastic processes, the soil the plant is cultivated in and from which bacteria are recruited is thought to determine the composition of plant microbial communities (Berg & Smalla, 2009; Lundberg *et al.*, 2012). Soil chemistry and physics in the rhizosphere, in turn, are shaped by root exudates, mucilage deposition and pH changes, and this zone is characterized by high microbial activity. It is estimated that plants exude huge amounts of their photoassimilates (5-40 % of the fixed carbon) into the rhizosphere, thereby secreting a plethora of carbon-rich compounds including amino acids, organic acids, fatty acids, and sugars, but also phenolics, putrescine, vitamins, and signaling substances (Jones *et al.*, 2009). Specific compounds have been shown to acidify the soil, thereby mobilizing nutrients (e.g. citrate, malic acid), or act allopathically against competing species (e.g. sorgoleone) (Shu *et al.*, 2005; Dayan *et al.*, 2009). Several substances attract or repel microbes (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one [DIMBOA], strigolactones, amino acids, sugars) or function as sporulation cues (Foo & Davies, 2011; Neal *et al.*, 2012) and hence are considered to shape rhizosphere microbial composition. Root exudate composition strongly depends on plant species and, as recent studies have shown, also on ecotype or cultivar within a species (Micallef *et al.*, 2009). Further, plant species or genetic make-up can provoke changes in the microbial community composition as shown by Grayston *et al.* (1998); Marschner *et al.* (2004); Manter *et al.* (2010) and Smalla *et al.* (2001). Very often, however, changes due to genetic modifications (e.g. the deletion or insertion of a gene and the resulting gene product) are limited and comparable to cultivar-specific differences (Kowalchuk *et al.*, 2003; Weinert *et al.*, 2009). Many publications contrasted the importance of soil and genotype effects – without clear consensus (Berg & Smalla, 2009).

Besides the aforementioned factors, plant developmental stage, biotic stresses and defense signaling, presence of microbial inoculants, climate, season, geography, land use, and agricultural

practices have all been shown to affect microbial colonization in the rhizo- and endosphere (Berg & Smalla, 2009; Bernard *et al.*, 2012).

1.4 Effects of plant hormone and defense signaling on mutualistic host-microbe interactions

How plant defenses against biotic stresses and plant hormone signaling affect the composition of root-associated microbes is not well understood. Commonly, plant defenses are directed against the establishment of pathogenic microbes or herbivores, but defense responses and excreted metabolites can also control the indigenous microflora as reviewed by Doornbos *et al.* (2012).

In 2001, for example, Hamilton and Frank reported on the grazing-mediated stimulation of microbial activity in the rhizosphere of *Poa pratensis*. Furthermore, the infection of *Arabidopsis thaliana* with *Pseudomonas syringae* pv *tomato* (Pst DC3000) resulted in the recruitment of beneficial *Bacillus subtilis* strain FB17 into the plant's rhizosphere, an effect that could be correlated to the enhanced secretion of L-malic acid (Rudrappa *et al.*, 2008).

The activation of plant defenses involves complex signaling pathways comprising the phytohormones jasmonic acid (JA), salicylic acid (SA) and ET. To study the impact of single players, genetic tools, e.g. transgenic lines impaired in specific signaling components, are employed. The first study investigating the effects of JA and SA on epiphytic and endophytic culturable leaf bacterial communities was conducted by Kniskern *et al.* (2007). The use of *A. thaliana* mutants impaired in SA (*npr1* and *sid2*) and JA (*fad3/7/8*) signaling, as well as wild-type (WT, Col-0) plants with artificially elevated SA and JA levels revealed that the leaf endosphere of SA- induced plants yielded a lower bacterial diversity, whereas the composition and number of bacteria in SA-deficient mutants was similar to WT. On the other hand, JA-deficient plants supported a greater epiphytic bacterial diversity and community size than WT, and JA-treated plants tended to have reduced diversity and total number of bacteria. Variation in JA did not affect endophytic bacterial communities just as SA did not influence epiphytic bacterial composition. Generally, however, observed effects were only marginally significant.

In 2008, the effect of systemic acquired resistance (SAR) on rhizosphere microbial communities of *A. thaliana* was studied by Hein *et al*. SAR usually arises after a local infection with fungal plant pathogens and requires SA signaling. Incorporating mutants constitutive (*cpr1*) and non-inducible (*npr1-1*) for SAR, Hein *et al.* found visible changes in the indigenous rhizosphere community composition of SAR mutants; but effects on rhizosphere diversity due to constitutive SAR expression were not observed.

The prominent role of the gaseous plant hormone ET in plant-pathogen interactions has been intensively studied (van Loon *et al.*, 2006). Additionally, researchers have investigated the effect of ET signaling on specific mutualistic plant-microbe interactions, using selected microbial isolates as interaction partners. They could show that ET-insensitive tobacco plants (*tetr*) exhibited disease-like infection symptoms when challenged with otherwise non-pathogenic fungi (Knoester *et al.*, 1998). Also, an ET-insensitive *Medicago truncatula* line was found to be hypercolonized by rhizobia or *Klebsiella pneumoniae* 342, respectively (Penmetsa, 1997; Iniguez

et al., 2005). Recently, Camehl *et al.* (2010) reported on the dependence of *Piriformaspora indica* -mediated plant growth promotion on ethylene signaling to balance positive and negative outcomes of this symbiosis in *A. thaliana*. These findings point towards ET as a regulator for microbial colonization patterns.

1.5 The multifaceted roles of ethylene

The ambivalent hormone ET first attracted notice in 1901, when Dimitry Neljubov observed a swollen, stunted and negatively gravitropic phenotype in his pea seedlings – the so-called "triple response": a finding he could correlate to the ET emissions from coal gas[2]. The gaseous phytohormone ET functions in diverse developmental and physiological plant processes. Despite ET's simple nature (C_2H_4), its signaling pathways are complex and still not fully understood (Vandenbussche *et al.*, 2012). ET is produced by every plant and has been reported to participate in seed germination, root hair and lateral root development, root nodulation, senescence and abscission of plant organs (e.g. leaves, flowers and fruits), differential cell growth and pollination. It also modulates abiotic stress responses (e.g. to wounding, cold, drought), and, as mentioned above, resistance to pathogens and herbivores as well as colonization by non-pathogenic or mutualistic microbes (Nehring & Ecker, 2010; Schaller, 2012). Ethylene biosynthesis is stimulated after (a)biotic stresses through mitogen-activated protein kinase signaling and is synthesized through the Yang Cycle, also called the methionine cycle (Wang *et al.*, 2002). Nearly 80 % of cellular methionine is converted to S-adenosylmethionine (S-AdoMet), which serves as methyl donor for many molecules and also as substrate for ET synthesis. After the conversion of S-AdoMet to ACC, the direct precursor of ET, by the ACC synthase, S-AdoMet is recycled (Wang *et al.*, 2002). Ethylene is perceived by the plant via endoplasmic riticulum-integrated receptor proteins (e.g. ETHYLENE RESPONSE, ETR1 and 2) and at low ET concentrations, these receptors inhibit ET signaling by the activation of the downstream negative regulator CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1). High ET concentrations in turn lead to the release of the signal transduction suppression (Clark *et al.*, 1998). Ethylene signaling has been shown to interact with all other hormone pathways (i.e. IAA, gibberellic acid [GA], JA, SA, abscisic acid [ABA] and brassinosteroids [BR])(Yoo *et al.*, 2009; Kim *et al.*, 2012), leading to complex signaling networks.

Once the plant is confronted with a stress stimulus, ET production can be observed, resulting in an arrest of plant growth until the elicitor is removed. The benefit of ET for the plant is uncertain: on the one hand, ET serves as signaling molecule in the attenuation of stresses, on the other hand large amounts of ET favor growth stagnation and plant death (Stearns, 2003). To overcome these negative aspects for agricultural practices (e.g. horticulture), plants can be treated with different chemicals inhibiting either ET biosynthesis or signaling components (Serek *et al.*, 1995; Sisler & Serek, 1997). Advances in green biotechnology led to the creation of ET-insensitive plants, which, however, are hardly employed in agriculture due to many pleiotropic growth effects, like poor rooting and high pathogen susceptibility (Knoester *et al.*, 1998; Clark *et al.*, 1999; Geraats *et al.*, 2007).

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[2] http://xa.yimg.com/kq/groups/21666630/1470371657/name/22-Ethylene.pdf

1.6 The model system *Nicotiana attenuata*

Beneficial plant-microbe interactions have been most intensively studied in managed ecosystems in an agricultural context. To which extent associations with mutualistic microbes are formed by native plants and how plant fitness is influenced by these interactions remains elusive. It is thought that fitness benefits are most clearly seen when the host plants are under various realistic biotic and abiotic stresses. *Nicotiana attenuata*, a wild tobacco species native to the Great Basin Dessert, USA, germinates as a pioneer plant in post-fire, nitrogen-rich environments (Baldwin *et al.*, 1994). Often, monocultures establish, which are characterized by high intraspecific competition and genetic diversity, as well as strong herbivore pressure (Bahulikar *et al.*, 2004; Berg & Hallmann, 2006), rendering *N. attenuata* suitable as model plant for studying native plant-herbivore and mutualistic plant-microbe interactions.

1.7 Objectives

While the ecological, physiological and molecular mechanisms of the interaction between *N. attenuata* and its specialist herbivore *Manduca sexta* are well studied, knowledge of *N. attenuata*'s microbial interaction partners is sparse. *Nicotiana attenuata* was found to be colonized by Sebacinales fungi (*Piriformospora indica* and *Sebacina vermifera*) and these fungi were shown to benefit plant growth and fitness at the cost of herbivore resistance (Barazani *et al.*, 2005; Barazani *et al.*, 2007). Furthermore, *N. attenuata* is colonized by AMF in nature. The interplay with one AMF species, *Rhizophagus irregularis* (formerly known as *Glomus intraradices)*, however, represents a "negative symbiosis" resulting in diminished plant growth (Riedel *et al.*, 2008).

1.7.1 *Manuscript I*

Native bacterial communities associated with *N. attenuata* had not been investigated. To study the importance of mutualistic bacteria for the survival and fitness of the plant in a complex environment, information about the identity and occurrence of bacterial species colonizing the plant across different native soils was required. Furthermore, we were interested in plant genotype-specific cues controlling bacterial community assembly. Since the roles of the phytohormones JA and SA in shaping plant bacterial communities had been recently elucidated, we were interested in the function of ET signaling in this process. Therefore, WT and isogenic transformed plants deficient in ET biosynthesis (*ir-aco1*) or perception (*35S-etr1*) were grown in four different native soils, and culturable bacteria colonizing the root endosphere were determined.

We asked the following questions:
- Does ET biosynthesis and perception affect the composition of endophytic bacterial communities?
- Which role does the soil type play in this process?
- Do plant genotype-specific colonization patterns exist?
- How do the bacterial isolates affect plant growth?
- Do plant genotypes with impairments in ET signaling recruit more PGP isolates (e.g. isolates producing ACC deaminase and IAA) into their rhizosphere than WT does?

1.7.2 *Manuscript II*

Plant growth promotion effects induced by bacteria are common phenomena. While PGP traits and effects of hundreds microbial strains have been reported under *in vitro* conditions (Smyth *et al.*, 2011), the success rate of PGP isolates in the glasshouse and field is low. To test whether our observed *in vitro* PGP effects (**Manuscript I**) were consistent in a highly complex environment, we selected a promising bacterial candidate, *Bacillus* sp. B55, originally isolated from an ET-insensitive plant, and analyzed in the following aspects:

- Does the isolate B55 show a consistent PGP effect *in vitro* and in field conditions?
- Which PGP traits confer the observed growth modulation effects in nature?
- Does B55 inoculation affect the indigenous bacterial community?
- Is B55 PGP a general phenomenon, or does plant genotype affect the PGP outcome?

1.7.3 *Manuscript III*

B55 PGP effects on growth and survival of the ET-insensitive *N. attenuata* transformant (*35S-etr1*) in nature were substantial (**Manuscript II**). The mechanism behind the fitness increase, however, remained elusive. We therefore analyzed the role of bacterial VOCs in the interaction of B55 and *N. attenuata*, investigating the following features:

- How do B55 VOCs affect plant growth of WT and *35S-etr1* seedlings?
- Which VOCs are emitted by B55 and how do they singly influence plant growth?

Based on the finding that an S-containing VOC, DMDS, might be involved in VOC-mediated PGP, we were interested in the following questions:

- How does plant sulfate availability affect WT and *35S-etr1* plant growth?
- Can the bacterial DMDS compensate for sulfate deficiency in the plant's growth medium?

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2 Manuscript overview

2.1 Manuscript I

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

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Summary

In this publication we report on the dominating effect of soil type on the assembly of the culturable microbial community of the coyote tobacco, *Nicotiana attenuata*, grown in native soils. We further show that impairments in the plant's ethylene signaling affects the diversity, but not the population size of microbes in the root's endosphere. Additionally we demonstrate that ethylene-insensitivity determines the root colonization of a specific bacterial isolate (*Pseudomonas thivirvalensis*), which is unable to colonize wild-type plants.

Author contributions

LHH and DGS contributed equally to the study.

LHH isolated bacteria, LHH and DGS identified them, carried out biochemical characterization, seedling growth promotion assays, re-inoculation and re-isolation assays. LHH, DGS and DDS designed experiments, grafted figures and wrote the manuscript. ITB designed the study, collected soil samples and revised the manuscript.

2.2   Manuscript II

A native plant growth promoting bacterium, Bacillus *sp. B55, rescues growth performance of an ethylene insensitive plant genotype in nature*

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Summary

In this study we report on the consistency of plant growth-promotion effects conferred by a native bacterial isolate, *Bacillus* sp. B55 on *Nicotiana attenuata* wild-type and the ethylene-insensitive line (*35S-etr1*) *in vitro*, in the glasshouse and in the field. We found that the impaired *35S-etr1* plants (the plant genotype from which B55 was isolated from), benefit more from this mutualistic interaction than WT; defining B55 as a part of the *35S-etr1* plant's "extended phenotype". The underlying mechanisms of plant growth promotion remained elusive.

Author contributions

DGM and LHH contributed equally to the study.

DGM and LHH designed experiments and carried out *in vitro* and glasshouse plant growth promotion assays. LHH did IAA and ET measurements, analyzed field data (2009) and re-isolated bacteria and revised the manuscript. DGM conducted late stage infection, transgenic line testing and analyzed field data (2010). DGM crafted the figures and wrote the manuscript. ITB designed the study, performed field experiments and revised the manuscript.

2.3   Manuscript III

Dimethyl disulfide, a bacterial volatile emitted by* Bacillus *sp. B55, promotes growth of* Nicotiana attenuata *and rescues the performance of an ethylene insensitive genotype

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Summary

In this manuscript we explored the plant growth–promotion effects of *Bacillus* sp. B55's volatiles, specifically the S-containing volatile dimethyl disulfide (DMDS), on *Nicotiana attenuata* wild-type and the ethylene-insensitive line (*35S-etr1*). Interestingly, *35S-etr1* seedlings realize a greater benefit of the exposure to the volatiles and DMDS than do WT plants. We demonstrate that *35S-etr1* plants suffer from an unbalanced S-metabolism and we propose that these plants benefit from their interaction with B55 by obtaining reduced S (in the form of bacterial DMDS) which helps to fulfill their S requirements.

Author contributions

DGM designed and conducted all sulfate and *35S-etr1* related experiments, grafted figures and wrote the manuscript. SM designed experiments and performed ^{35}S-radioactive labeling experiments and revised the manuscript. LHH carried out initial volatile-related plant growth promotion assays and revised the manuscript. SU helped with experiment performance. HW developed the method for glutathione measurements. ITB conceived the study and revised the manuscript.

3 Manuscript I

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

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3.1 Summary

• A plant's bacterial endophyte community is thought to be recruited from the rhizosphere, but how this recruitment is influenced by the plant's phytohormone signaling is unknown. Ethylene regulates plant–microbe interactions; here, we assess the role of ethylene in the recruitment of culturable endophytic bacteria from native soils.

• We grew wild-type *Nicotiana attenuata* plants and isogenic transformed plants deficient in ethylene 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 ethylene signaling. Plants grown in organic (vs mineral) soils harbored a more diverse community and plants impaired in ethylene homeostasis harbored a less diverse community than wild-type plants. Wild-type and ethylene 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.

• We propose that soil composition and ethylene homeostasis play central roles in structuring the bacterial endophyte community in *N. attenuata* roots.

3.2 Keywords

Bacterial diversity, bacterial endophytes, ethylene, native soils, *Nicotiana attenuata*

3.3 Introduction

Endophytic bacteria reside inside plants and have been extracted from surface-sterilized tissues of cultivated and native herbs and trees (Hallmann *et al.*, 1997; Ryan *et al.*, 2008). They are thought to be recruited from the surrounding soil (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 (nitrogen, phosphorus) 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).

Although plant–pathogen interactions and their underlying genetic mechanisms have been studied extensively, less is known about plant–rhizosphere or even endophyte–plant relationships. Numerous studies have characterized bacterial diversity in scores of plants, and have reported that the bacterial endophyte 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 A *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 into plant–pathogen interactions has demonstrated that three phytohormones play a central role in the mediation of 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 well colonized by bacterial endophytes and harbored lower species' diversity than plants of the Col ecotype, whereas JA signaling had no effect on the endophyte community (Kniskern *et al.*, 2007). To date, the influence of ET signaling on the composition of the endophyte community has not been examined thoroughly.

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 the senescence processes (Stearns & Glick, 2003). Other studies however, have highlighted the role of ET in pathogen resistance. The *Arabidopsis* mutant *ein2*, which is unable to perceive ET, is 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* is unaltered (Pieterse *et al.*, 1998). Reviewing the complicated and often contradictory reports on the role of ET, van Loon *et al.* (2006) recently concluded that the signaling function of ET depends on the nature of the pathogen.

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

The ET-related bacterial communities, 1-aminocyclopropane- 1-carboxylate deaminase (ACCd)- and indole-3-acetic acid (IAA)-producing bacteria, are known to increase plant growth by interfering with ET signaling (Glick *et al.*, 2007). Although the mechanisms of plant growth-promoting (PGP) rhizobacteria have been 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 these questions.

Endophytic bacterial populations can be regarded as a subset of the soil microbial community and are 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 signalling 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, nitrogen-rich soils (Lynds & Baldwin, 1998). So far, nothing is known about the diversity of endophytic bacterial communities associated with this plant in nature. Here, we characterize the culturable bacterial endophyte communities of *N. attenuata*'s roots and ask the following questions: Do soil types influence the bacterial communities? 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?

We 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. *Nicotiana attenuata ir-aco1* can sense ET, but is deficient in ET biosynthesis, and hence has 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 south-west Utah, USA, just before the germination of the native seed bank. We 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.

3.4 Materials and methods

Plant materials

The following inbred WT and transgenic lines of *Nicotiana attenuata* Torr. ex Wats. 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 have been fully characterized in von (von Dahl *et al.*, 2007). Seed germination procedures have been described elsewhere (Krugel *et al.*, 2002; Long *et al.*, 2008).

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.

Soil collection

Four types of soil were collected from *N. attenuata*'s native habitat in Utah, USA (Table 2) on 26 January 2008. All soils contained variable nitrogen levels consisting of NH_4^+ and NO_3^-, as described in Lynds and 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 2 months later. All soils were stored at 4°C for 3 d during shipment before being used in the experiments. Two organic soils, located at the base of burned (soil O1) or unburned (soil O2) juniper trees, were collected from the following location (37°04'02.6''N, 113°53'04.04''W). 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 (37°05'23.5''N, 113°50'42.4''W) and another (M2) from 8 km north on Rt. 91 near the TV tower that had burned in 2007 (37°06'01.9''N, 113°49'22.7''W).

Soil analysis

Analysis of total carbon and nitrogen contents in the soil samples was performed by the ChemLab of the Max Planck Institute for Biogeochemistry (Jena, Germany)[3]. 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 the soil pH.

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[3]http://www.bgc-jena.mpg.de/service/chem_lab/roma/elemental_analysis/elemental_analysis.shtml

Isolation of culturable endophytic bacteria

Ten days after germination, one seedling from each genotype (WT and transformed lines) was transferred individually to 7-cm pots containing the Utah soils. In total, three replicates per line and soil were cultivated. Plants and soils were cultured in the glasshouse (16 h : 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 d 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). Briefly, endophytic bacteria were isolated after removing epiphytes 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) and incubating the plates at 30°C for 10 d. After surface disinfection, root tissue was cut and titrated in distilled water; appropriate dilutions were plated onto half-strength YPDA and incubated at 30°C for 2–10 d. After incubation, distinct colonies were picked from the plates.

Pure cultures were inoculated on half-strength YPDA slant tubes, incubated at 30°C for 2 d and stored at 4°C. In addition, each culture was suspended in a 20% glycerol solution and stored at 80°C for long-term preservation.

Bacterial ACCd and IAA production assay

ACCd activity was determined as described by Glick (1995), by measuring the amount of α-ketobutyrate produced when the enzyme ACCd cleaves ACC. The number of nanomoles of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample with 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 colorimetric method. Briefly, DF salt agar medium supplemented with 5 mM L-tryptophan was inoculated with bacterial isolates, overlaid with a nitrocellulose membrane, and then incubated until bacterial colonies reached 1–2 mm in diameter. The membrane was moved to a filter paper saturated with Salkowski reagent and incubated until distinct red haloes formed around the colonies. Standard IAA was used as a positive control.

Amplified ribosomal DNA restriction analysis

Immediately after the establishment of pure bacterial cultures, genomic DNA was isolated from 1-d-old cultures grown on half-strength YPDA plates. Single colonies were suspended in water to obtain suspensions of *c.* 10^5 colony-forming units (CFU) ml^{-1}; 0.5 µl was mixed with 4.5 µl of 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 g for 5 min, the supernatant was used for PCR. Amplification of 16S rDNA was performed in a final volume of 10 µl containing 1 µl of genomic DNA, 10 µM of primers F27 (5'-AGAGTTTATCMTGGCTCAG- 3') (Edwards *et al.*, 1989) and R1492 (5'-GRTACCTTGTTACGACTT-3') (Lane, 1991), 10 mM of each dNTP, 5 mM of MgCl$_2$ and 0.05

U 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. Approximately 120 ng of DNA for each sample were digested in 20-µl reactions containing 2 µl of 10 x Buffer Tango (Fermentas)[4], 10–15 µl of sterile deionized water (depending on the DNA concentration) and 5 U of the restriction enzyme Hinf*I* or Msp*I* (Fermentas)[5]. The reaction was incubated at 37°C overnight. Band sizes were visualized in 2% agarose containing ethidium bromide under UV light.

Cluster analysis was performed to create dendrograms that grouped similar bacterial isolates from different plant genotypes and soil types together according to their banding profile. Unweighted pair-group moving average clustering and a Bray–Curtis similarity matrix were calculated for both restriction enzymes using the PAST[6] multivariate statistics program. Bray–Curtis clustering compares the presence or absence of restriction banding patterns in bacterial isolates from different plant genotypes and soil types. We refer to each banding pattern as an operational taxonomic unit (OTU). Given the fact that Hinf*I* restriction (Fig. S1a) yielded a more diverse banding profile than did Msp*I* digestion (Fig. S1b, see Supporting Information), further analysis was based on the Hinf*I* restriction.

16S rDNA gene sequencing

The same proportional number of bacterial isolates was selected for sequencing according to plant genotype, Hinf*I* OTU clustering and soil type. PCR products were purified using a QIAquick[TM] Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's manual. Direct sequencing using the primer R1492 with expected size *c.* 600 bp was conducted in Big Dye Mix (Applied Biosystems, Foster City, CA, USA), and purification of the sequencing reactions was performed using a NucleoSEQ Kit (Macherey-Nagel, Düren, Germany) and sequenced on an ABI310 sequencer[7] (Applied Biosystems). The editing of sequences was performed with EditSeq and SeqMan (DNAStar Lasergene 7, DNASTAR Inc., Madison, WI, USA). The analysis of sequences was carried out with the basic sequence alignment BLAST program (Altschul *et al.*, 1997) run against the database from the National Center for Biotechnology Information[8]. The sequences were deposited in GenBank[9] 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 neighbour-joining phylogenetic

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[4] http://www.fermentas.com
[5] http://www.fermentas.com
[6] http://folk.uio.no/ohammer/past/
[7] http://www.appliedbiosystems.com
[8] http://www.ncbi.nlm.nih.gov/BLAST
[9] http://www.ncbi.nlm.nih.gov/Genbank/

dendrogram (Saitou & Nei, 1987) was inferred, rooted and bootstrapped 1000 times (Felsenstein, 1985).

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 10 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 10 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 [optical density at 600 nm (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, three 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 h : 11 h day : night cycle, 155 µmol 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-d-old seedlings were planted in Teku pots (five replicates per genotype and bacterial inoculum) in a random design (16 h:8 h photoperiod at 200–300 µmol m^{-2} s^{-1}, 25 : 21°C and 45–55% relative humidity). The Teku pots were placed in separate trays to avoid cross-contamination and watered whenever needed with sterile water. One milliliter of single inoculum of the following bacterial isolates (Table 1) was applied to the rhizosphere: *P. brassicacearum* DSM13227, *Bacillus megaterium*, *Methylobacterium 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.

Seedling ET 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 h : 11 h day : night cycle, 155 µmol m^{-2} s^{-1}, 26 : 24°C). After 12 d, the 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.

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)

ACCd, 1-aminocyclopropane-1-carboxylate deaminase; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; IAA, indole-3-acetic acid; Trp, tryptophan.

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
M2	Mineral, burned	2.6	0.08	8.8

Microscopic analysis and root morphological investigations

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

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 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.

Data analysis

Analysis of the data was carried out using the StatView software package (SAS Institute Inc., Cary, NC, USA) with a completely randomized analysis of variance ($P < 0.05$). Fisher's protected least-significant difference test was used to compare means of log CFU g^{-1} fresh weight (FW) of fresh root or seedling, root length, number of root hairs per seedling and number of ACCd- and IAA-producing bacteria.

3.5   Results

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 and nitrogen, and pH (Table 2). The total carbon contents of the two organic soils (O1 and O2) were 18.7% and 17.7%, respectively, and their nitrogen contents were similar (0.73%). The carbon contents of the two mineral soils (M1 and M2) were 3.2% and 2.6% and their nitrogen contents were 0.07% and 0.08%, respectively. The pH of these soils was in the range pH 8.5–9.0. In order to estimate the degree of colonization of the three genotypes grown in the different soils, the 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, the colonization of WT plants was high, but variable, among the four soils, yet the 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

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.

revealed that the total endophytic bacterial community depended significantly on both plant genotype and soil type (P < 0.05, Table S1).

The diversity of culturable endophytic bacteria

In total, 139 bacterial isolates were picked from half-strength YPDA medium. However, 36 bacterial isolates were not viable after long-term preservation. Looking at bacterial colony morphology, we 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 Hinf*I*-digested 16S rDNA banding patterns. For 16S rDNA sequencing, 73 representative isolates were chosen on the basis of the plant genotype, soil type and OTU cluster from which they were originally isolated (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 the 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 organic soils.

We analyzed the culturable bacterial diversity of three plant genotypes that differed in their production and perception of ET (Table 3, Fig. 2). WT plants were found to harbor the highest diversity of endophytic bacteria sequenced, followed by *35S-etr1* and *ir-aco1* plants. Sequence analysis revealed that *Bacillus* sp. and *Pseudomonas* sp. were the most abundant genera isolated from WT and transgenic plants. Six bacterial genera were found in WT plants, three bacterial genera were found in *35S-etr1* plants and 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. *Pseudomonas thivervalensis* and *B. cereus* were found only in *35S-etr1* plant roots, and *M. extorquens* and *B. endophyticus*, were found exclusively in *ir-aco1* plant roots.

Bacterial diversity is also dependent on the soil type in which the different plant genotypes are grown. Plants grown in the two organic soils (O1 and O2) harbored a high endophytic bacterial diversity compared with the bacterial community of plants grown in the two mineral soils (M1 and M2); the latter community was represented by only *Pseudomonas* spp. The endophytic bacterial community of plants grown in the recently fire-affected soil, O1, was represented by two bacterial genera; by contrast, the endophytic bacterial community of *N. attenuata* plants grown in the unaffected soil, O2, was represented by six different genera (Table 4).

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 on single inoculations (Fig. 3b and Fig. S2). 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.*

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

Table 5 Number of bacterial endophytes influencing growth[1] of wild-type seedlings

Soil type	No. of total isolates	Beneficial[2]	Pathogenic[3]	Neutral[4]
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

[1], Parameters measured: root length, hypocotyl length, number of true leaves and lateral root formation.
[2], Bacterium enhanced at least one of the above parameters and without negative effect on the other parameters.
[3], Bacterium caused detrimental effects on at least one of the parameters.
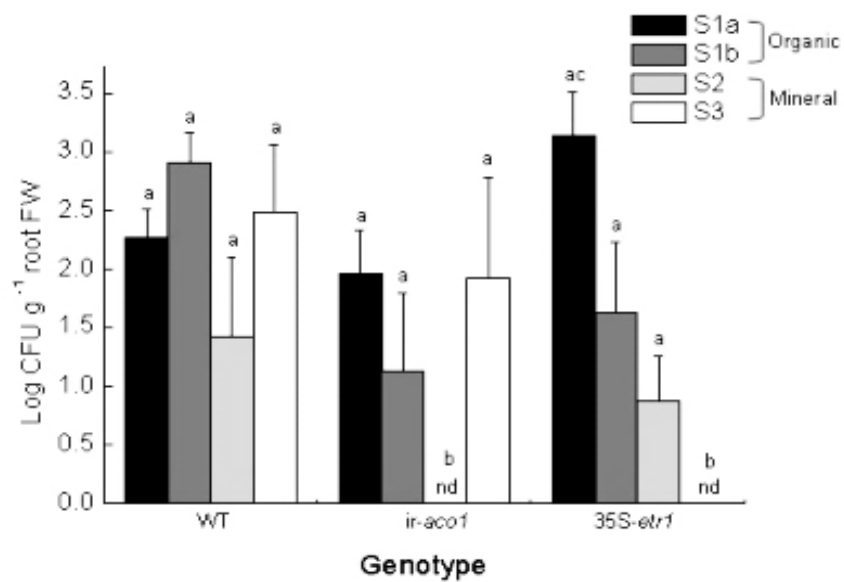[4], Bacterium did not influence any of the parameters.

Fig. 1 Colony-forming units (CFU) isolated from roots of wild-type (WT) and transgenic *Nicotiana attenuata* plants grown in four different soils. FW, fresh weight; nd, not detected.
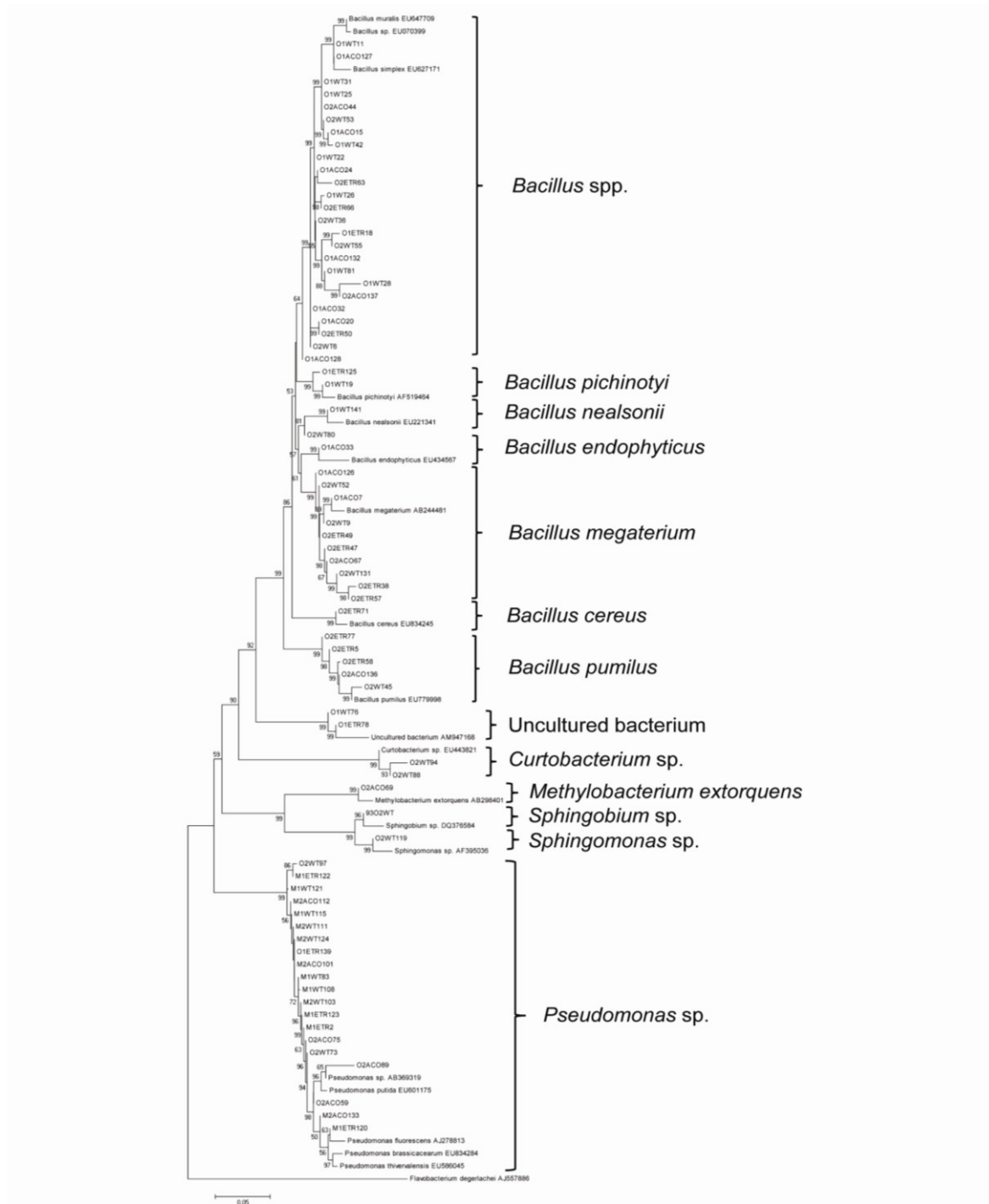
Fig. 2 Phylogenetic tree showing the relative positions of bacterial isolates as inferred from their 16S rDNA gene sequences using the neighbor-joining method. Bacterial species preceded by their GenBank accession numbers were used as standard strains. *Flavobacterium degerlachei* (AJ557886) was used as an outgroup organism. Bootstrap values above 50 are shown, representing the percentage of support for clusters out of 1000 replications. M, mineral; O, organic. The scale gives genetic distances. Seventy-three representative isolates were chosen based on the plant genotype, soil type and operational taxonomic unit (OUT) cluster from which they were isolated.
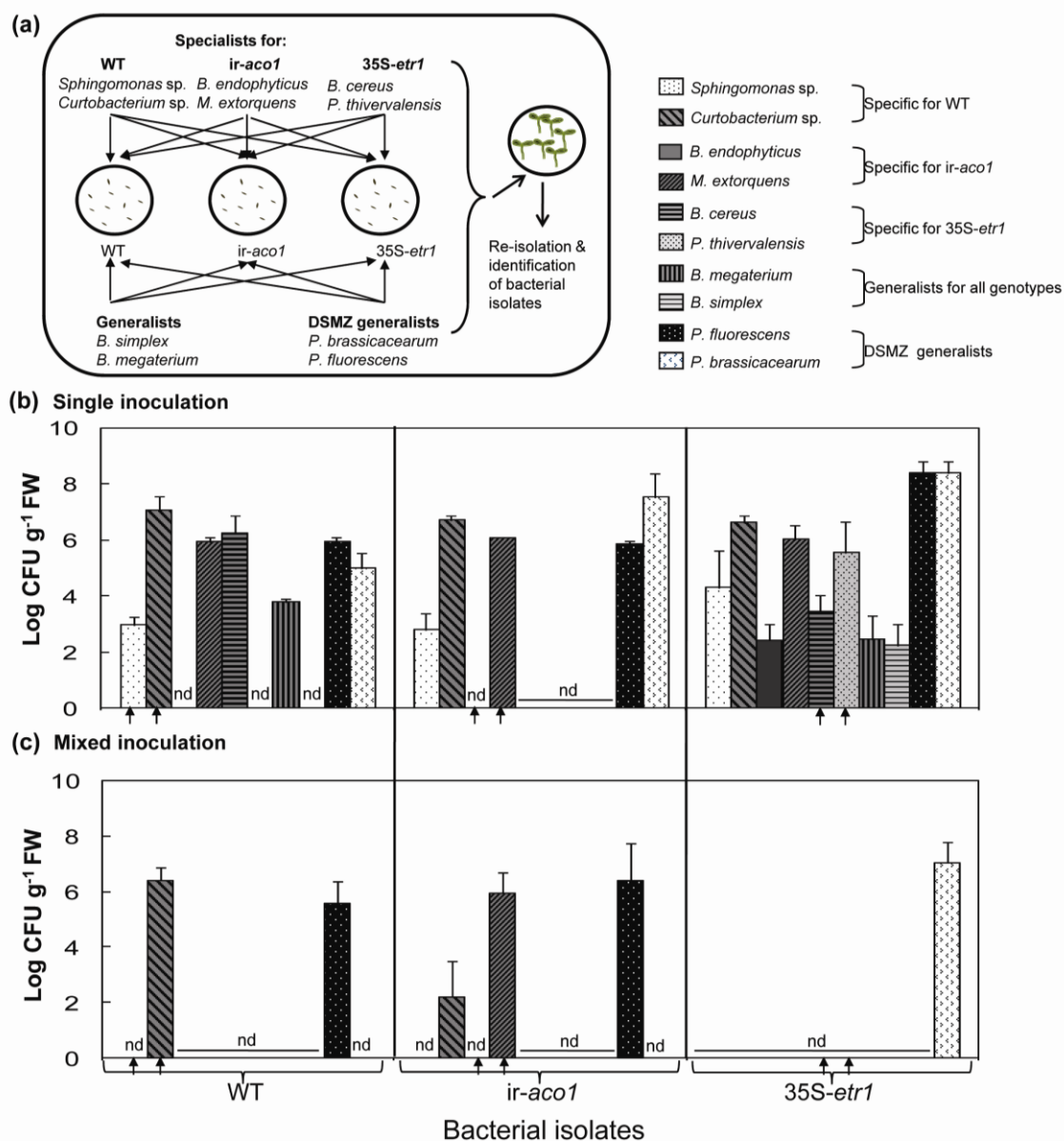
Fig. 3 *In vitro* colonization of different plant genotypes by individual bacterial isolates from treatment with single (b) and mixed (c) inoculum. The identity of bacterial isolates was confirmed by 16S rDNA sequencing. CFU, colony forming units; FW, fresh weight; nd, not detected; WT, wild-type. The arrows depict specific isolates for each plant genotype. Different shading patterns depict bacterial isolates. Schematic diagram **(a)** describes bacterial inoculation, re-isolation and identification.

fluorescens DSM8568 and *P. brassicacearum* DSM13227, colonized WT and the two transgenic seedlings. Interestingly, *35S-etr1* seedlings were colonized by all isolates on single inoculations. In the mixed inoculation treatments, the generalists, *P. fluorescens* DSM8568 and *P. brassicacearum* DSM13227, were 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.

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 reisolated 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.

Effects of bacterial endophytes on seedling growth

Single inoculation of 139 isolates into WT seeds affected seedling growth; the germination rate, however, was not influenced. Of these 139 isolates, 64 were beneficial with respect to seedling growth, 37 were pathogenic and 38 were neutral (Table 5). Among these 64 PGP 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. On the other hand, 13 isolates showing ACCd activity and 17 isolates producing IAA did not enhance seedling growth. Using plants impaired in either ET production (*ir-aco1*) or perception (*35S-etr1*), we 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 ($P > 0.05$, Tables S2, S3). However, the abundance of bacteria producing ACCd and IAA was greater in organic soils O1 and O2 (Fig. 5), whereas, in the mineral soil M1, plants harbored significantly fewer ACCd- and IAA-producing isolates ($P < 0.05$, Tables S2, S3), and no ACCd- and IAA-producing bacteria were detected in the plants grown in soil M2.

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. 6b). Moreover, the roots of *35S-etr1* and *ir-aco1* seedlings were significantly longer than those of WT seedlings ($P = 0.001$) (Fig. 6c). The number of lateral roots was similar for the three genotypes (data not shown).
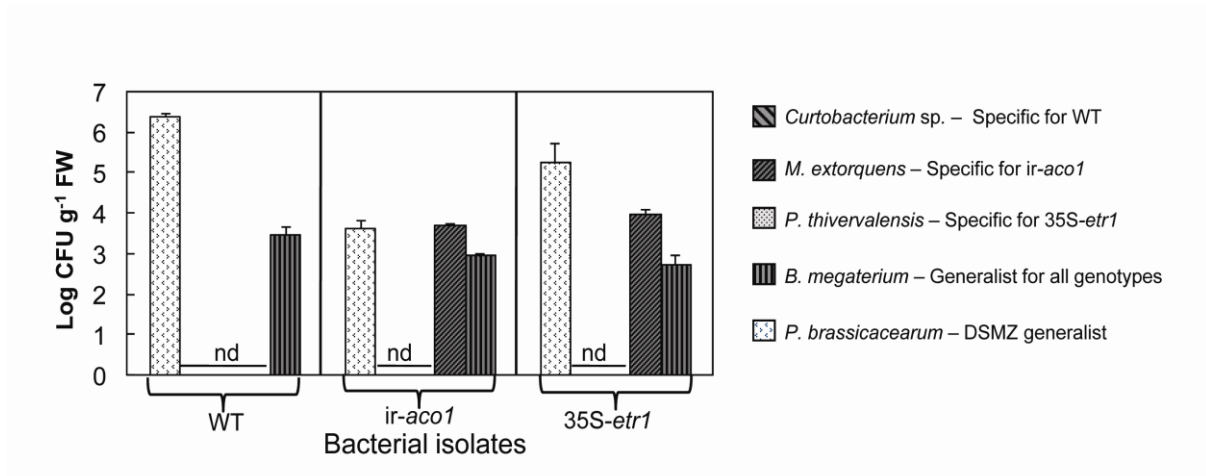
Fig. 4 Colonization of different plant genotypes by individual bacterial isolates under glasshouse conditions. CFU, colony forming units; FW, fresh weight; nd, not detected.
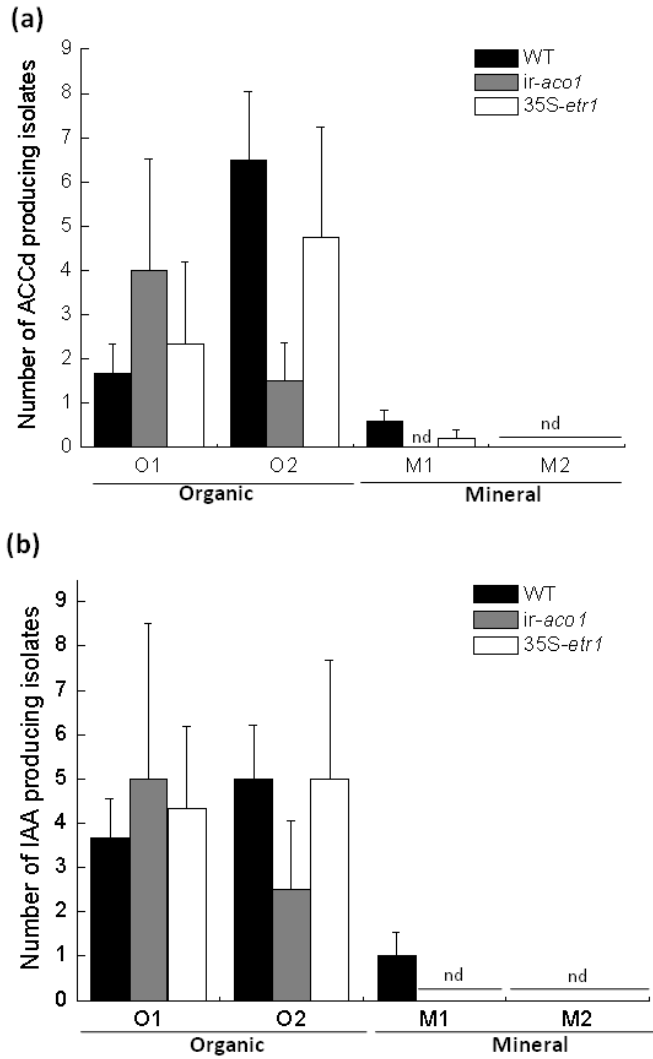
Fig. 5 Abundance of endophytic bacteria producing 1-aminocyclopropane-1-carboxylate deaminase (ACCd) (a) and indole-3-acetic acid (IAA) (b). *Nicotiana attenuata* wild-type (WT) (black bars), *ir-aco1* (grey bars) and *35S-etr1* (white bars). nd, not detected.
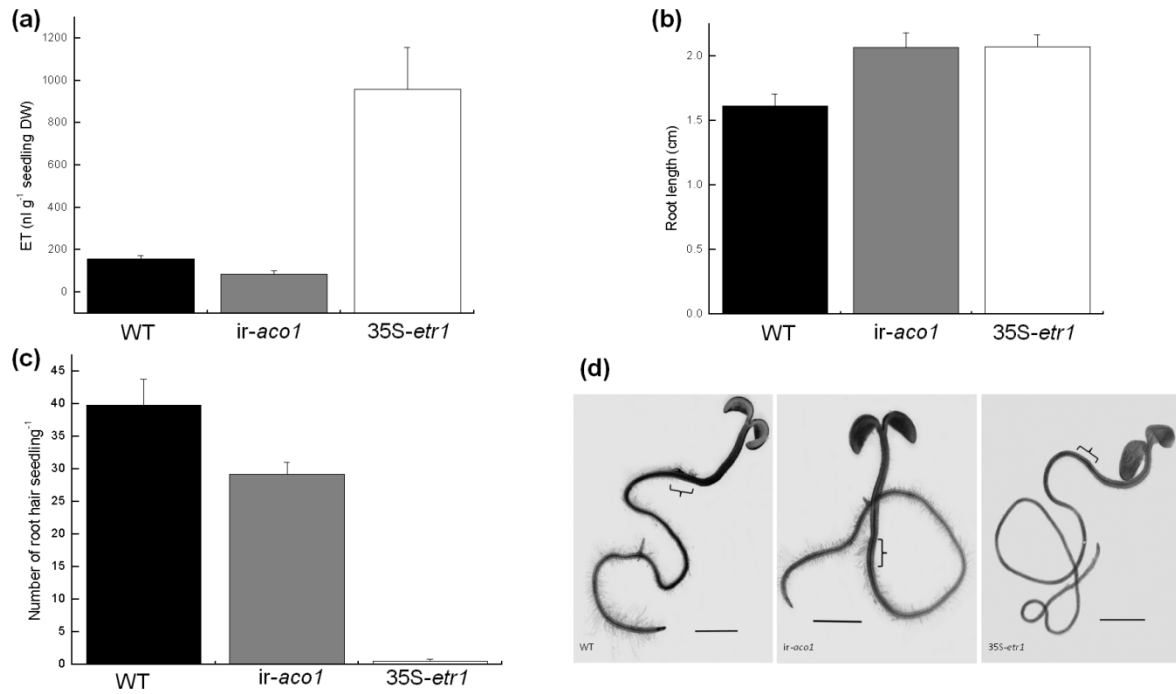
Fig. 6 Ethylene (ET) production (a), root length (b), root hair number (c) and seedling morphology (d) (x 10 magnifications) of wild-type (WT), *ir-aco1* and *35S-etr1 Nicotiana attenuata* seedlings; bars, 2mm; the brackets depict the zone of root hair counting. DW, dry weight.

3.6 Discussion

The composition of a plant's bacterial endophyte community is likely to be determined by many selective factors, including soil type and 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 the soil microbial community and, in turn, of the endophytic microbial communities (Singh *et al.*, 2009). In this study, we have demonstrated that the soil type strongly influences the plant culturable bacterial endophyte community, as shown by Rasche *et al.* (2006). In addition, we report for the first time that plant ET signaling indirectly influences the recruitment of root bacterial endophytes in *N. attenuata*.

Soil is considered as an endophyte bank, a 'marketplace' in which 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 carbon and nitrogen contents (Table 2) than in plants grown in the two mineral soils (Fig. 1, and Fig. S1). It is widely accepted that organic matter promotes both plant and soil microbial growth as a result of higher carbon, phosphorus and nitrogen supply rates (Vaidya *et al.*, 2008), and enhanced nutrient availability may allow plants to support larger populations of endophytic bacteria. We 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), who found no significant difference in total microbial biomass between unburned and burned soils, but a difference in community diversity. 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 will require more detailed investigations.

ET signaling plays an important role in mediating different types of induced plant resistance to pathogens: induced systemic resistance by rhizobacteria (van Loon *et al.*, 2006) and systemic acquired resistance (SAR) by pathogens (Sticher *et al.*, 1997). One may ask whether these plant defense responses that require ET signaling also affect a plant's endophytic bacterial community. Hallmann (2003) experimentally induced resistance in potato plants by applying *Rhizobium etli* G12 to one-half of a split potato root system, and showed that the total bacterial diversity and number of bacterial species were significantly higher in elicited than in non-elicited roots. Using the *Arabidopsis* SAR mutants *cpr1* and *npr1-1*, Hein *et al.* (2008) found differences in the rhizosphere community fingerprints of the mutant plants compared with WT; however, there was no clear decrease in rhizosphere species' diversity associated with constitutive SAR expression. Our study provided evidence that plant ET signaling influences the initial recruitment of bacterial communities from the soil. In native soils, *N. attenuata* WT plants harbored a more diverse bacterial community than did *ir-aco1* and *35S-etr1* plants, a result opposite to 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.

However, 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–Md. truncatula* interaction. By testing four *Salmonella* strains and two *Md. truncatula* lines (WT and the symbiosis mutant *dmi1*), they showed that the colonization of the host plant was an active process. Hence, the recruitment of soil-dwelling bacteria into the endosphere is probably defined by host genotype and specific genes.

As 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 (Bais *et al.*, 2006; Buer *et al.*, 2006; Hartmann A *et al.*, 2008). In addition, we hypothesize that root morphology might influence the endophytic bacterial community, as soil-dwelling bacteria probably enter roots via cracks in lateral root junctions and through root hairs (Glick *et al.*, 1999). Further, Depret and 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. We found that ET signaling affected the root morphology of *N. attenuata* seedlings (Fig. 6d). WT plants, which have significantly more root hairs, might provide microbes with a greater number of points of entry; however, the stochastic sampling process may also account for the more diverse endophytic bacterial community (Hardoim *et al.*, 2008).

Not only do plants select particular bacterial communities, but interactions among bacteria themselves and with other microbes 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.*, 2003; Rosenblueth & Martinez-Romero, 2004; Verma *et al.*, 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, we 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 was able to colonize the three *N. attenuata* lines on single or mixed inoculation. These two strains belong to the genus *Pseudomonas*, which is known to be a rapid and successful plant colonizer, even under highly competitive situations (Lugtenberg *et al.*, 2001).

One of the advantages of studying culturable endophytic bacterial communities is the ability to investigate their effects on plant growth (Barriuso *et al.*, 2008). We found that about

one-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- and IAA-producing bacteria. These bacteria were abundant in plants grown in the organic soils O1 and O2 (Fig. 5). 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 a 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 which rely on ACCd/ IAA-producing bacteria to ameliorate abiotic stresses actively select for these bacterial communities under stress conditions (Glick *et al.*, 2007). However, native plants, such as *N. attenuata*, which have evolved to tolerate stressful environments, might not recruit ACCd-producing bacteria as PGP rhizobacteria, but merely let them in, where they thrive as parasites. Thus, we propose that a plant's ability to produce or sense ET does not directly control the recruitment of these communities for native plants (Fig. 5, Tables S2, S3). Furthermore, an association between plant growth and ACCd/ IAA production was found in only one-half of the PGP isolates; hence, there are clearly many other ways by which bacterial endophytes can promote growth.

In conclusion, the results confirm the central role of soil type, and highlight, to a lesser extent, the role of ET signalling 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 plant's ability to recruit ACCd/ IAA-producing bacteria seems to be independent of ET signaling.

3.7 Acknowledgements

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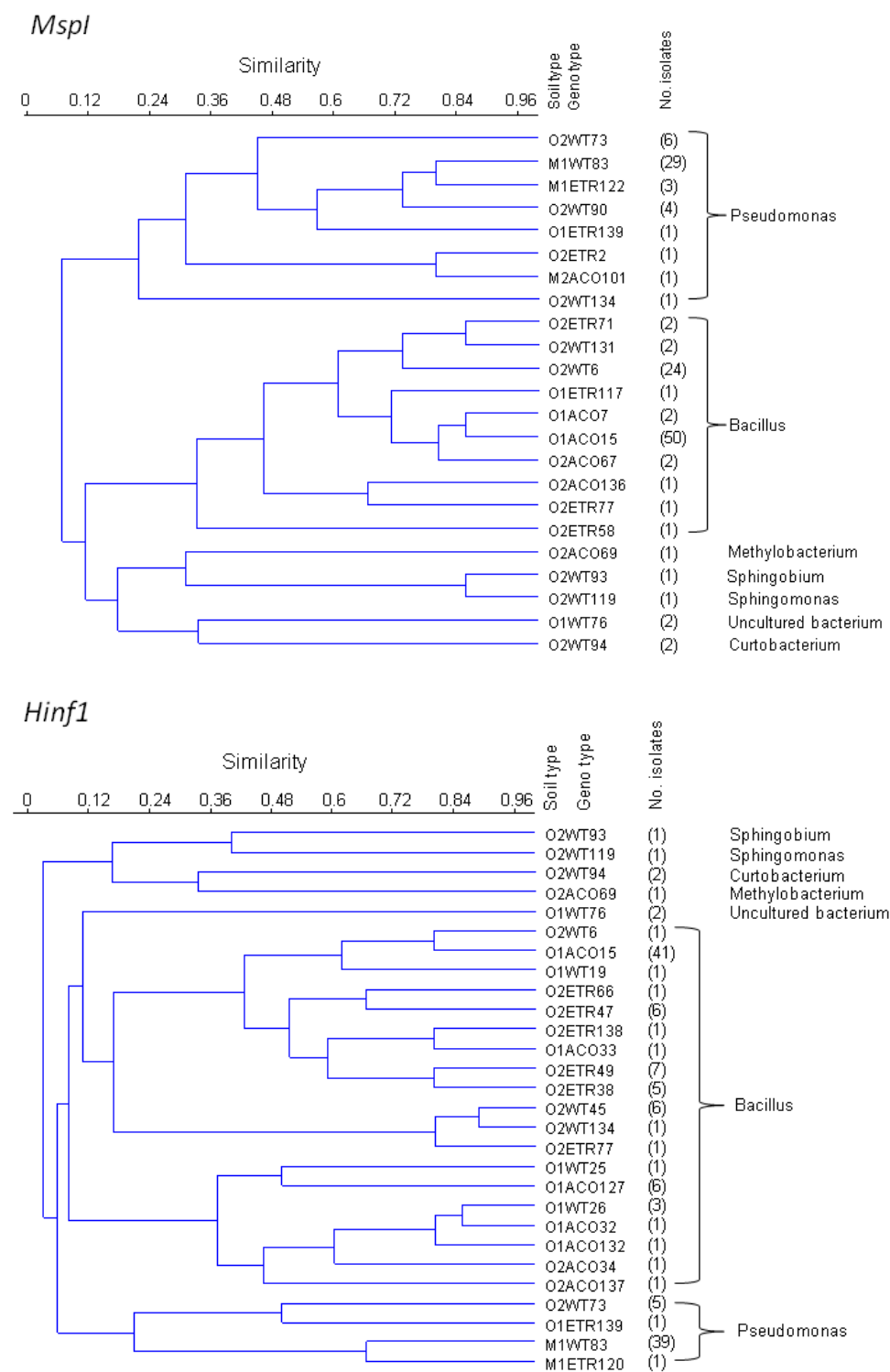
3.9 Supplemental Figures and Tables

Fig. S1 Dendrograms showing the operational taxonomic units (OTUs) of the culturable endophytic bacterial isolates (*HinfI* (a) and *MspI* (b))
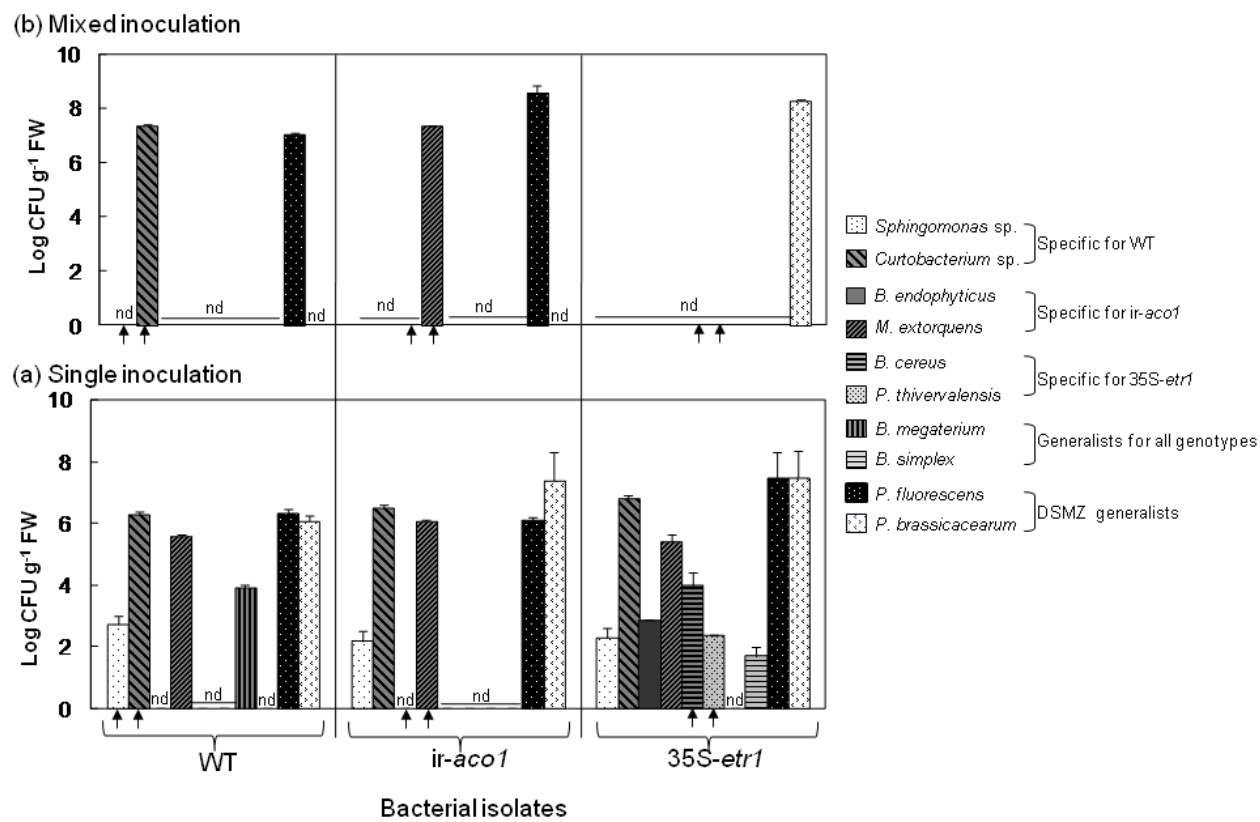
Fig. S2 The second independent experiment of *in vitro* colonization of different genotypes by bacterial endophytes from single inoculum (a) and mixed inoculum (b) treatment.

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				

4 Manuscript II

A native plant growth promoting bacterium,* Bacillus *sp. B55, rescues growth performance of an ethylene insensitive plant genotype in nature

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4.1 Abstract

Many plants have intimate relationships with soil microbes, which improve the plant's growth and fitness through a variety of mechanisms. *Bacillus* sp. isolates are natural root associated bacteria, isolated from *Nicotiana attenuata* plant roots growing in native soils. A particular isolate B55, was found to have dramatic plant growth promoting (PGP) effects on wild type (WT) and transgenic plants impaired in ethylene (ET) perception (*35S-etr1*), the genotype from which this bacterium was first isolated. B55 not only improves *N. attenuata* growth under *in vitro*, glasshouse and field conditions, but it also "rescues" many of the deleterious phenotypes associated with ET insensitivity. Most notably, B55 dramatically increases the growth and survival of *35S-etr1* plants under field conditions. To our knowledge, this is the first demonstration of a PGP effect in a native plant-microbe association under natural conditions. Our study demonstrates that this facultative mutualistic plant-microbe interaction should be viewed as part of the plant's extended phenotype. Possible modalities of recruitment and mechanisms of PGP are discussed.

4.2 Keywords

Nicotiana attenuata, *Bacillus* sp., ethylene insensitive, plant growth promotion, microbial community, nature

4.3   Introduction

In addition to the well-studied mutualistic associations that plants have evolved with nitrogen-fixing microbes and mycorrhizae (Franche *et al.*, 2009; Kiers *et al.*, 2011), plants also associate with plant growth promoting bacteria (PGPB), which refer to rhizobacteria and endophytes enhancing their host's growth and productivity. PGPB have been intensively studied in the context of agricultural practices as means of increasing the productivity of cultivated plants (Maheshwari, 2011). Much less is known about the role of PGPB in an ecological context and whether they increase the growth or fitness of native plants remains unknown. Only a few studies have reported on the root-associated bacterial communities of native plants (Zinniel *et al.*, 2002; Long *et al.*, 2008; Long *et al.*, 2010), and it is not known if the plant-growth-promoting (PGP) effects of PGPB occur in nature.

Several mechanisms have been postulated to explain how PGPB stimulate plant growth; broadly categorized as being either direct or indirect (Glick, 1995). Direct mechanisms include the interference with plant hormone homeostasis and increasing nutrient availability to the host by solubilizing inorganic phosphate, or fixing of atmospheric nitrogen (Gamalero & Glick, 2011). Furthermore, *in vitro* studies have suggested that PGP effects can be mediated by the release of volatile organic compounds (Ryu *et al.*, 2003) which by still unknown mechanisms have PGP effects. PGPB 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 (Pineda *et al.*, 2010; Gamalero & Glick, 2011). Bacteria qualify as PGPBs when they are able to colonize and elicit positive effects for the plant (Compant *et al.*, 2010). Some bacterial formulations are commercially available for agriculture, even though PGPB often lose their PGP effects when applied under field conditions (reviewed in Kloepper *et al.*, 1989). The inoculation of soil with these microbes may affect the composition and structure of microbial communities, which can result in positive effects on plant growth (Ramos *et al.*, 2003; Jha *et al.*, 2010), but opposite effects have also been reported (Castro-Sowinski *et al.*, 2007; Berg & Zachow, 2011).

The plant hormone ethylene (ET) is known to regulate many different physiological and developmental processes in plants, such as seedling emergence, leaf and flower senescence and organ abscission, and it is also known to mediate plant responses to abiotic and biotic stresses (Bleecker & Kende, 2000; van Loon *et al.*, 2006). Blocking ET perception with inhibitors such as 1-methylcyclopropene (1-MCP) helps to increase the longevity of flowers, fruits and ornamental plants (Serek *et al.*, 1995). Ectopically expressing a mutant ET receptor from *Arabidopsis* (*etr1-1*) renders plants constitutively insensitive to ET and has revealed the many roles of ET in negotiating (a)biotic stresses (Chang *et al.*, 1993). Transgenic *Tetr* tobacco plants are unable to withstand attack from common, generally non-pathogenic, opportunistic soil-borne fungal organisms (Knoester *et al.*, 1998; Geraats *et al.*, 2002). Transgenic petunia had poor root development of cuttings, less efficient seed germination and rooting, and delayed seedling growth (Wilkinson *et al.*, 1997; Clark *et al.*, 1999). Several studies found that ET signaling also plays important roles in the communication between plants and mutualistic microbes (Penmetsa, 1997; Iniguez *et al.*, 2005; Camehl *et al.*, 2010; Long *et al.*, 2010). For example, *Pseudomonas thivervalensis* was originally isolated from *35S-etr1* transformed *Nicotiana attenuata* plants, and

even under stringent conditions (single *in vitro* inoculation) was only able to colonize these *35S-etr1* plants, suggesting that high levels of ET production (a trait associated with ET insensitivity (von Dahl *et al.*, 2007)) coupled with ET insensitivity and its associated changes in metabolism are required for this specific colonization process (Long *et al.*, 2010).

We have developed *N. attenuata* (coyote tobacco) as a model plant to identify the traits required for the survival of plants in the rough and tumble of their natural environments. To this end we have developed a molecular tool box and a field station so that we transform plants (silenced in the expression of specific genes), fully characterize them under glasshouse conditions and release them into their natural habitat at the field station for detailed characterizations of their ecological performance (Baldwin, 2001). In a previous study (Long *et al.*, 2010), we planted wild type (WT) and isogenic lines transformed to be defective in ET production and perception (*ir-aco1* and *35S-etr1*) in native Utah soils collected from areas with natural seed banks, to identify the culturable endophytic bacterial community that colonizes *N. attenuata* seedlings when they germinate from their long-lived seed banks. Isolates of *Bacillus* sp. strains were found in the roots of the three lines and one particular isolate, dubbed B55, isolated from the roots of *35S-etr1* plants appeared to be beneficial for *in vitro* seedling growth. Here we explore the PGP potential of this native bacterial isolate, B55, in WT and *35S-etr1* hosts grown *in vitro*, in the glasshouse and finally at the field station in Utah.

We had planted *35S-etr1* plants into native habitats of the Great Basin Desert in Utah for 4 field seasons, and learned that these ET insensitive plants have many of the same difficulties described above for transformed cultivated tobacco and petunia. The plants have problems establishing strong root growth at the seedling stage, in resisting pathogen attack during rosette stage growth, and herbivore attack during the flowering stage (Paschold *et al.* 2007; IT Baldwin unpublished results), difficulties which strongly reduce their survivorship compared to WT plants in the field. Hence these plants were ideal for conducting the first study of the potential PGP effects of a native bacterium (B55) on its host plant in nature. We had fully expected, given what is known about the mechanisms by which PGP comes about (e.g. alterations in ET homeostasis) and the speed with which microbial communities evolve, not to see any PGP effects in nature. However, B55 inoculation not only improved *N. attenuata* WT's growth under *in vitro*, glasshouse and field conditions, but it also "rescued" many of the deleterious phenotypes of *35S-etr1* plants and dramatically increased their survival under field conditions.

4.4   Material & Methods

Plant materials and bacterial strain

The 30th selfed WT line of *Nicotiana attenuata*, originally collected from a native population 35km upstream from the field plantation, and an isogenic ET insensitive transgenic line (*35S-etr1*; A-03-328-8), fully characterized in von Dahl *et al.* (2007) were used in all experiments. The other transgenic *N. attenuata* lines used for plant growth promotion assays (Figure 8) were also fully characterized and these lines and their associated references are summarized in Table 2. Seed germination procedures have been described elsewhere (Long *et al.*, 2010). *Bacillus* sp. B55 (Genebank accession number: JX101913) was isolated from an *35S-etr1*

plant grown in native Utah soil (Long *et al.*, 2010). Unless noted otherwise, B55 was routinely cultured on half-strength yeast peptone dextrose agar (YPDA; Sigma, Steinheim, Germany) at 30°C.

Bacterial ACCd, IAA production and P solubilization assays

1-aminocyclopropane-1-carboxylate (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.

Indole-3-acetic acid (IAA) production was determined as described by Bric *et al.* (1991) using the colormetric method. Briefly, DF salt medium supplemented with 5 mM l-tryptophan was inoculated with bacterial isolates and incubated at 30°C. After an incubation period of 48 h on a rotary shaker (200 rpm, 30°C), bacterial cells were removed by centrifugation (4000 x g, 10 min). One mL of bacterial supernatant was mixed vigorously with 2 mL of Salkowski's reagent. The mixture was incubated at room temperature for 20 min and the absorbance was measured at 540 nm. Synthetic IAA was used as a positive control.

An inorganic phosphate (P) solubilization assay was carried out after Verma *et al*. (2001) by inoculating bacterial isolates on Pikovskaya medium. Plates were stabbed using sterile toothpicks. The halo and colony diameters were measured 14 days after the plates were incubated at 30°C.

Inoculation procedures

Surface sterilized seeds were incubated over night at room temperature in 3 mL of bacterial suspension in sterile water (OD_{600}=1.0); controls were treated with sterile water only. If not otherwise stated, seeds were transferred to Petri dishes containing GB5 medium (Gamborg's B5 media, Duchefa, Haarlem, The Netherlands) and maintained in a Percival growth chamber (13/11 h day/night cycle, 155 µmol m^{-2} s^{-1}, 30/28°C). For "late stage" inoculations, 10-day-old seedlings were transferred to Teku pots (Poeppelmann, Lohne, Germany) filled with sand (0.7-1.2 mm grain size, Raiffeisen, Germany). At day 20, plants were carefully removed from the sand and roots dipped for 2 min into a B55 suspension (OD_{600}=1.0). Afterwards, plants were placed in 10 cm round pots containing lecaton (Easy Green, Eschborn, Germany & Fibo ExClay, Lamstedt, Germany) and sand (Figure 4A). Three and six days after transfer, plants were once again inoculated by pouring 50 mL of a B55 suspension (OD_{600}=1.0) over the roots.

Plant ET, IAA and P content quantification

ET emissions from B55-inoculated and non-inoculated WT and *35S-etr1* seedlings were measured non-invasively with a photoacoustic spectrometer (INVIVO, Sankt 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 (13/11 h day/night cycle, 155 µmol m^{-2} s^{-1}, 30/28°C). After 12 days, flasks containing the seedlings were subjected to ET

measurements (5 replicates per treatment). Five empty flasks with filter paper and sterile water served as controls. Two independent experiments were carried out.

For IAA quantification, 12-day-old B55-inoculated and non-inoculated WT and *35S-etr1* seedlings were harvested and immediately frozen in liquid nitrogen and stored at -80°C until analysis. Approximately 500 mg ground seedling powder was extracted according to the method by Onkokesung *et al.* (2010) and analyzed on a 1200L quadrupole tandem mass spectrometry system (Varian[10]).

Dried rosette material of B55-inoculated or control WT (38-day-old) plants was used for total P analysis. Analysis was conducted using a microwave-assisted digestion. Briefly, about 100 mg of sample were dissolved in 3 mL suprapur 65% HNO_3 (Merck, Darmstadt, Germany) and digested in a Multiwave® (Anton Paar, Graz, Austria). Subsequently, samples were transferred to 50 mL glass vessels and diluted with ultrapure water (Millipore) and submitted to analyses by ICP-OES (OptimaTM 3300 DV, PerkinElmer, Shelton, CT, USA). P was detected at $\lambda = 177.4$ nm. SRM 1573a tomato leaves and SRM 1575a pine needles (NIST, Gaithersburg, USA) were used as reference material. Total P analysis was carried out at the Max Planck Institute for Biogeochemistry, Jena, Germany.

In vitro seedling growth measurements

Length of the primary root and number of lateral roots of vertically (Figure 1A, upper panels) grown B55-inoculated or non-inoculated WT and *35S-etr1* seedlings were determined after 10 days of growth. After 12 days of horizontal growth (Figure 1A, lower panels), secondary leaves were counted and leaf surface area was analyzed (according to the video tutorial by Zach Jarou[11]) using Adobe Photoshop C5. Chlorophyll *a* and *b* contents of 12-day-old seedlings were analyzed spectrophotometrically from an 80 % acetone extract using a TECAN plate reader (Tecan, Crailsheim, Germany). Two independent experiments with four replicate Petri dishes containing at least seven (vertical placement) or 20 seeds (horizontal placement) were carried out. *In vitro* B55 colonization was determined for seedlings grown for 8 days on Whatman No. 1 filter paper amended with 1.5 mL of sterile fertilizer (0.6 g $Ca(NO_3)_2.4H_2O$ and 0.3 g Flory Basisdünger 1 [Euflor, Munich, Germany] per L). Two independent experiments were carried out.

Bacterial re-isolation

Rhizoplane bacteria were isolated by vigorously vortexing roots in sterile water for 2 min; appropriate dilutions were mounted on half-strength YPDA and incubated at 30°C. Endophytic bacteria were isolated following the procedure of Long *et al.* (2010) by removing root epiphytes by surface disinfection. After appropriate surface disinfection, root tissue was cut and titrated in distilled water; dilutions were plated onto half-strength YPDA and incubated at 30°C. After 2-3 days, colony forming units (CFUs) of B55 were counted based on colony morphology. Identity

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[10] http://www.varianinc.com
[11] http://www.chlorofilms.org/index.php/crpVideo/display/videoid/46

was confirmed by 16S rDNA sequencing (Long *et al.*, 2010). The isolates were identified using the EzTaxon-e server[12] (Kim *et al.*, 2012) based on the 16S rRNA sequence data.

Plant growth promotion experiments in the glasshouse and field

For glasshouse experiments (Figure 3A), 10-day-old B55-inoculated or non-inoculated seedlings were planted into separate TEKU pots containing sand and lecaton. At 20 dpi, plants were transferred to 10 cm diameter round pots containing lecaton and sand. Pots were placed in a randomized design in the glasshouse (22° C, 65% humidity, 16 h light) in separate bottom containers to avoid cross-contamination. Plants were fertilized every other day with 50 mL distilled water amended with 0.6 g $Ca(NO_3)_2.4H_2O$ L^{-1} and 0.3 g Flory Basisdünger 1 L^{-1}. Survival of plants was monitored (24 dpi) and length of the longest leaf or stalk height was measured every other day. Total seed capsule number was determined at the end of the experiment (63 dpi). Colonization by B55 was measured 30 dpi; roots were collected and bacterial isolation was performed as described above. Bacterial identity was determined by 16S rDNA sequencing. Two independent experiments were carried out for the analysis of B55 effects on glasshouse-grown plants.

Field experiments were conducted at the at Brigham Young University's Lytle Ranch Preserve located in the Great Basin Desert, in SW Utah, USA. The release of transgenic plants was carried under APHIS notification 06-242-3r-3a and the seeds were imported under permit number 10-004-105m. Petri dishes containing 3-day-old B55-inoculated or non-inoculated WT and *35S-etr1* seedlings were shipped to the field station. Fourteen days after germination, the seedlings were transferred to pre-hydrated 50 mm peat pellets (Jiffy 703[13]) and seedlings were gradually adapted to the high light and low relative humidity of the habitat over a 2-week-period. Pre-adapted rosette-stage plants were transplanted into an irrigated field plot in size-matched quadruplets (consisting of one B55-inoculated and one non-inoculated WT and *35S-etr1* plant in a randomized design: Figures 5A-D), 31 dpi. Survival of the plants was assessed at 46 dpi, rosette diameters were measured 46, 62 and 73 dpi; stalk height measured at 62 and 73 dpi and the number of flowers was counted at 73 dpi. B55 colonization was quantified at 47 and 73 dpi for 5 randomly selected quadruplets of plants. Plants were carefully excavated and the loosely attached soil was removed before plants were wrapped in moistened paper towels and sent to the laboratory facility in Jena, Germany. Re-isolation of culturable bacteria was carried out as described, immediately after arrival (2 days after removal from the field). Other culturable, dominant resident bacterial isolates were counted based on colony morphology and the identity of representatives was determined by 16S rDNA sequencing. The experiment was conducted once for *35S-etr1* plants (2009 field season) and twice for WT (2009 and 2010 field seasons).

Data analysis

Data analysis was carried out with the StatView software package (SAS Institute) with a completely randomized analysis of variance. One-way and two-way ANOVAs followed by

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[12] http://eztaxon-e.ezbiocloud.net/
[13] http://www.jiffypot.com

Fisher's PLSD test or t-test were used to compare differences among treatments. Correlation analysis was performed with simple regression tests.

4.5   Results

Bacillus sp. B55 characteristics and *in vitro* PGP

Bacillus sp. B55 was isolated from the endosphere of an ET insensitive *35S-etr1 N. attenuata* plant grown in native Utah soil (Long *et al.*, 2010). Re-isolation experiments have shown that this Gram-positive bacterium is able to colonize the endosphere and rhizoplane of *N. attenuata* roots; with the rhizoplane typically harboring 10^3 more bacteria than the endosphere (data not shown).

Inoculation of WT and *35S-etr1* seeds with B55 revealed dramatic PGP effects on the *in vitro* growth of WT and especially *35S-etr1* seedlings. The ET insensitive transgenic line produces few root hairs and lateral roots (Long *et al.*, 2010) and tends to grow poorly on Petri dishes (Figure 1B). B55-inoculation enhanced WT and *35S-etr1* seedling growth significantly as clearly seen in seedling leaf surface area (62 and 105 % increase, respectively) and in the production of true leaves (three and six times more true leaves per seedling, respectively). Seedling chlorophyll *a* content increased by 45 and 20 % and chlorophyll *b* content by 80 and 24 % for WT and *35S-etr1*, respectively (Figure 1C). Although primary root length of WT and *35S-etr1* seedlings tended to decrease by 36 and 11 % after B55-inoculation, lateral root number increased significantly. On average, B55-inoculated WT and *35S-etr1* roots had 8 and 7 times more lateral roots, respectively (Figure 1D, left panels). Interestingly, B55 colonization of the endosphere of *35S-etr1* seedlings was more than 10 times higher than in WT, while rhizosphere colonization was similar (Figure 1D, right panels).

In order to understand the underlying mechanisms of the PGP effect, B55 was tested for known PGP traits. *In vitro* cultures of B55 produced 0.303 ± 0.026 µM α-ketobutyrate * (mg protein * h)$^{-1}$ ACCd (an enzyme that decreases ET production by the cleavage of the ET precursor, ACC) and 9.478 ± 2.522 µg * mL^{-1} IAA, an auxin analog. Furthermore, qualitative enzyme tests revealed that B55 is able to solubilize phosphate. However, seedling ET emissions and IAA contents were not significantly changed by B55 inoculation and WT rosette plant P contents were not altered by B55 inoculation (Figure 2).

PGP effects in the glasshouse

In the glasshouse, B55 inoculation increased the growth of both WT and *35S-etr1* plants (Figure 3). At 35 dpi, length of the longest rosette leaf of B55-inoculated WT and *35S-etr1* plants was almost 20% and more than 30% increased compared to non-inoculated plants, respectively (Figure 3B). Stalk heights of B55-inoculated WT and *35S-etr1* plants were 13 and 40% taller, respectively, compared to controls at 47 dpi (Figure 3b). Furthermore, rosette diameters of B55-inoculated WT plants correlated positively with B55 colonization (Figure 3C). The survival of B55-inoculated *35S-etr1* plants was increased by 20% compared to control *35S-etr1* plants; survival rate of WT plants, which was already close to 100%, was not affected (Figure 3D). B55-
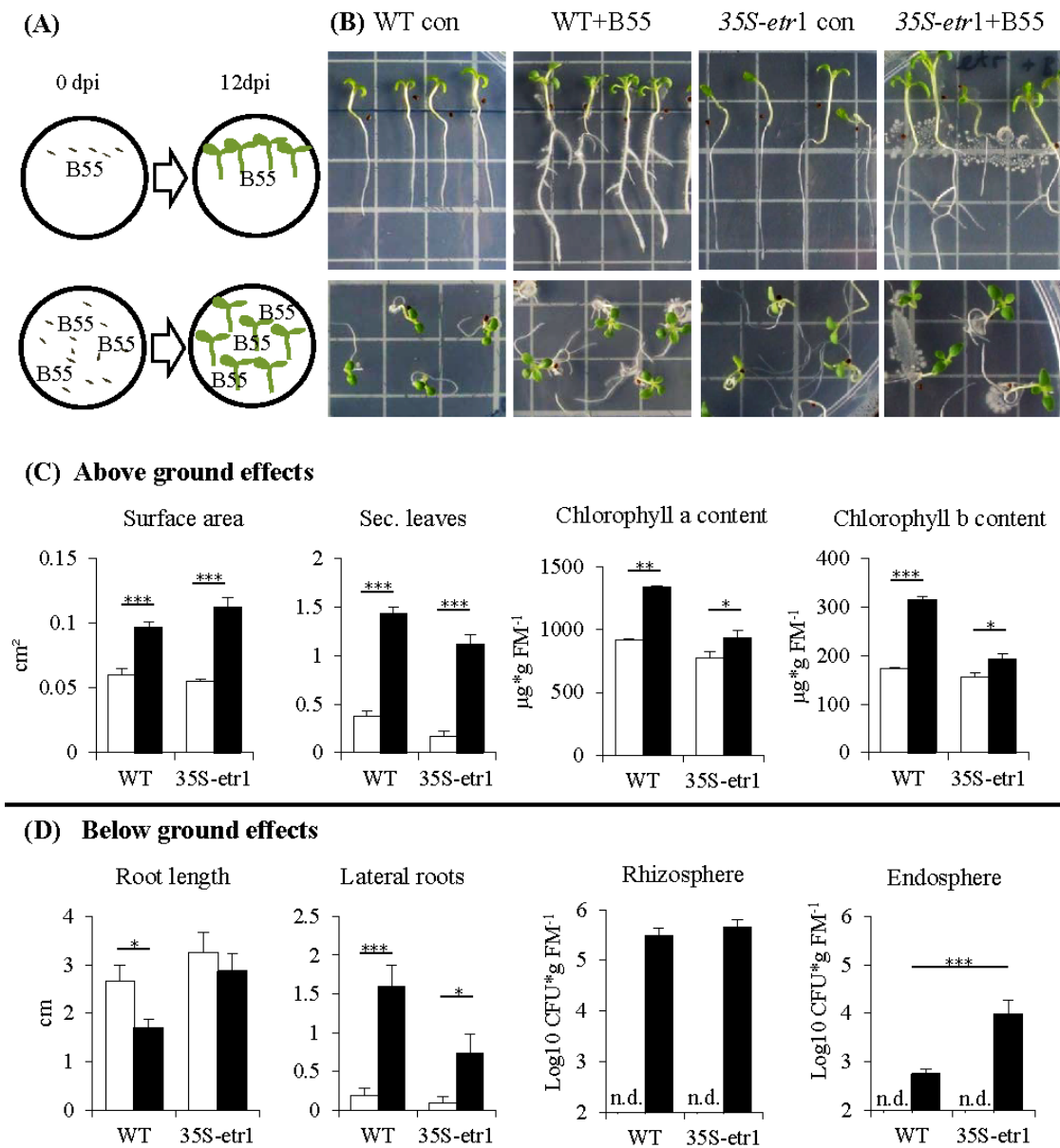
Figure 1 Effects of B55 inoculation on seedlings growth *in vitro*.
Experimental design (A). Effect of a B55 inoculation on WT and *35S-etr1* seedlings, grown vertically and horizontally (B). Mean (±SE) seedling surface area, number of true leaves, chlorophyll *a* and *b* content (C), primary root length, number of lateral roots and B55 colonization in the rhizo- and endospheres (D) of mock- or B55-inoculated WT and *35S-etr1* seedlings. White bars represent control treatments, black bars, B55 inoculated seeds. (PLSD test of an ANOVA between mock- and B55-inoculated plants: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). CFU: colony forming unit; FM: fresh mass; n.d: not detected. *n* = 4 replicate Petri dishes containing at least 20 seeds or 7 seeds (vertical placement), *n* = 5 replicate Petri dishes containing at least 20 seeds (for colonization).

inoculation also influenced the production of reproductive structures: B55-inoculated WT and *35S-etr1* plants yielded on average 2 and 1.5 more seed capsules, respectively, than control plants (Figure 3E). The seed production (number of seeds per capsule) was not affected by B55 (Figure 3F). B55-inoculated WT and *35S-etr1* glasshouse plants were similarly well colonized at 30 dpi (Figure 3G).

For these *in vitro* and glasshouse experiments, B55 treatment prior to germination resulted in strong PGP effects. To determine if B55 could similarly influence plant growth when plants were inoculated at a later stage of development, 20-day-old *N. attenuata* WT plants were inoculated by root-dipping. At 13 dpi, the length of the longest rosette leaf of B55-inoculated WT plants was significantly larger compared to control; even though the 14 % relative growth increase (Figure 4B) was less pronounced than the effects measured when seeds were inoculated prior to germination. Plants were colonized by ca. 1.8×10^2 and 10^6 log10 CFU*g FM^{-1} in the endosphere and rhizosphere, respectively.

PGP effects in the field

The consistency of PGP effects observed *in vitro* and in the glasshouse has rarely been tested under field conditions and the PGP effects of a native root associated bacterium had not been tested in its native host in the field. To conduct such a test, we examined the effect of B55 inoculation (of seeds) on the growth of WT plants, during two field seasons, and *35S-etr1* plants during one field season in their native habitat in SW Utah, USA. B55 inoculation strongly enhanced the survival and growth of the ET insensitive *35S-etr1* plants, whereas during the first field season (2009), effects on WT plants were barely detectable (Figure 5). At the end of the first field season experiment (73 dpi), *35S-etr1* rosette diameters and stalk heights were significantly increased by about 52 and 170%, respectively, compared to control *35S-etr1* plants (Figure 5E). During the first field season (2009), WT rosette growth did not benefit from B55 inoculation, however, stalks of B55-inoculated plants tended to grow faster than control plants (P = 0.06) (Figure 5E, right panel). During the second field season (2010), B55-inoculated WT plants exhibited significant increases in both rosette diameter and stalk height compared to controls (Figure 6). The effect of B55 inoculation on the survival of *35S-etr1* plants was dramatic, increasing plant survival by almost 20% (Figure 5F). The number of flowers was evaluated 62 dpi; and no significant difference was found between B55-inoculated and non-inoculated WT plants. Interestingly, the non-inoculated *35S-etr1* plants did not produce any flowers until the end of the experiment, while the B55-inoculated *35S-etr1* plants produced almost as many as the WT non-inoculated plants (Figure 5G). Re-isolation experiments revealed that with 4.7 and 4.9 log10 CFU*g FM^{-1}, B55-inoculated WT and *35S-etr1* plants were well colonized by B55 even after 47 days of growth in the field. Surprisingly, B55-like colonies were also identified from some roots of non-inoculated WT and *35S-etr1* plants (Figure 5H), suggesting that *Bacillus* sp. isolates were either resident in the field plot, or easily moved between inoculated and non-inoculated plants, perhaps via the watering channels of the field plot (see Figure 5C,D).
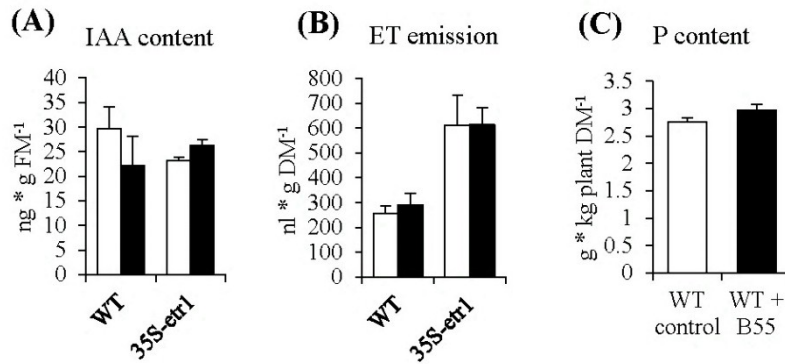
Figure 2 Effects of B55 inoculation on *N. attenuata* IAA content, ET emission and P content. Mean (±SE) IAA content (A) and ethylene emission (B) of *N. attenuata* WT and *35S-etr1* seedlings. White bars represent mock-inoculated seedlings; black bars represent seedlings inoculated with B55. Total P content of 38-day-old WT rosette plants (C). FM: fresh mass; DM: dry mass. *n* = 3 for IAA; 5 for ET; *n* = 6 for P.
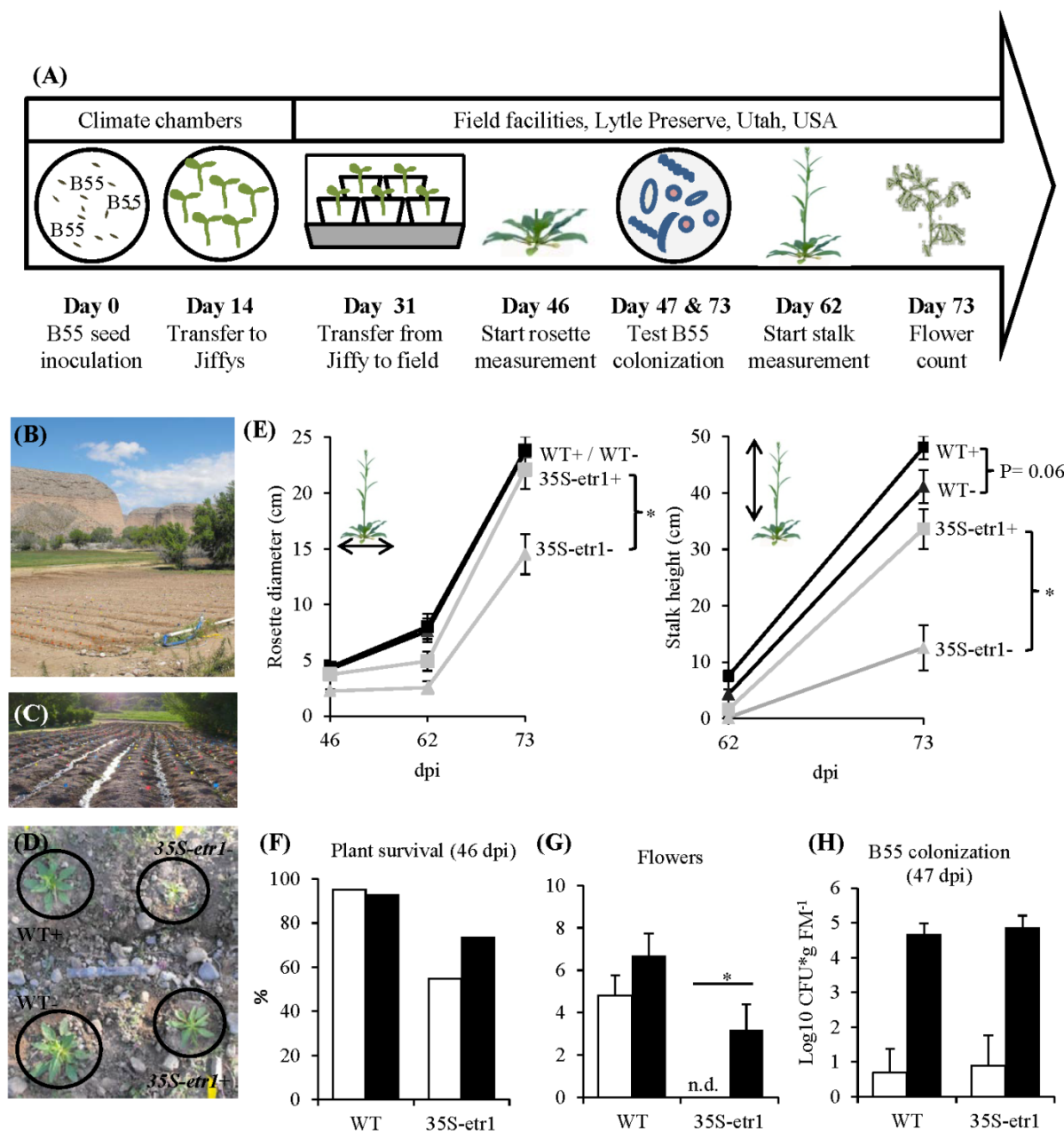
Figure 3 Effects of a B55 inoculation on glasshouse-grown plants.
Timeline of experiment (A). Mean (±SE) length of the longest rosette leaf and stalk height (B). Correlation between B55 colonization and WT rosette growth (C). Survivorship (D), seed capsule number (E), seed number per capsule (F) and B55 colonization (G) of B55-inoculated WT and *35S-etr1* plants. Except for (G), white bars represent control treatments, black bars, B55 inoculated plants. (PLSD test of an ANOVA between mock- and B55-inoculated plants: *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001). dpi: days post infection; CFU: colony forming unit; FM: fresh mass; n.d.: not detected. $n = 20$ for b and f; $n = 10$ for c; n > 80 for d; $n = 5$ for e.

Effects of B55 on the resident culturable bacterial community

We examined the influence of a B55 inoculation on the culturable, endophytic, naturally associated plant microbial taxa of plants after 73 days of growth in the field. B55 inoculation strongly affected the most abundant culturable bacterial communities associated with WT and *35S-etr1* roots. The analysis focused on bacterial strains found to colonize roots to the same degree as the introduced B55. B55 inoculated plants harbored twice as many bacteria of a greater diversity of bacterial taxa, compared to controls (Figure 7). In addition to B55, inoculated WT roots harbored *Pantoea* sp. Utah 2009-2 and *Pantoea* sp. Utah 2009-3 and *35S-etr1* roots harbored *Pantoea* sp. Utah 2009-2 and *Pseudomonas* sp. Utah 2009-4 (for accession numbers see Table 1). These bacterial genera were not detected in roots of control plants. No difference in colonization by B55 of the inoculated plants was observed, and B55 was not found amongst the dominant bacterial taxa of non-inoculated plants. The culturable bacterial communities of WT and *35S-etr1* control plants were dominated by one isolate: *Enterobacter* sp. Utah 2009-1. There was no difference in the extent of colonization by *Enterobacter* sp. Utah 2009-1 of the non-inoculated WT and the *35S-etr1* plants (Figure 7).

PGP effects of B55 on other transgenic *N. attenuata* lines

B55's dramatic PGP effects on the ET-insensitive genotype *35S-etr1* motivated us to compare PGP effects on other transgenic *N. attenuata* lines silenced in phytohormone signaling or defenses against herbivores or pathogens (Table 2). All lines inoculated with B55 (late stage inoculation) showed positive growth responses. Figure 8 shows the increase in growth of each line compared to non-inoculated plants. While the JA-deficient *N. attenuata* line, *ir-lox3,* was barely affected by B55 inoculation, *ir-mpk4* (impaired in herbivore-elicited phytohormone signaling) and *ir-gla1* (impaired in oxylipin synthesis) benefited the most from the interaction with B55. Interestingly, and in contrast to the results from the seed inoculation procedure, *35S-etr1* plants benefited less from the interaction compared to WTev, when inoculated at a later stage of development (20 days).
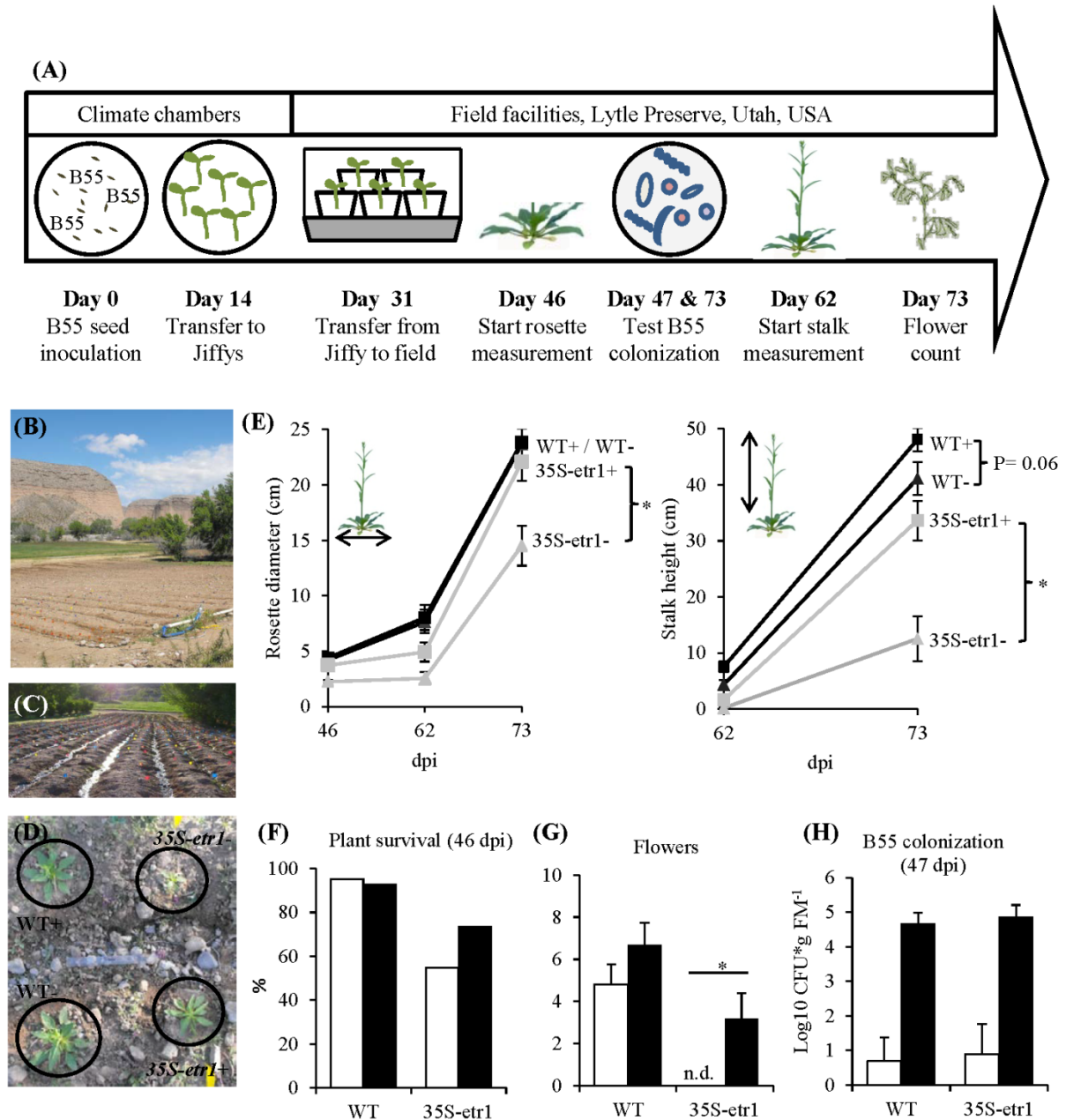
Figure 4 Effects of a "late-stage" B55 inoculation on WT plants.

Timeline and experimental design (A). Mean (±SE) length of the longest leaf of mock- or B55-inoculated WT *N. attenuata* plants cultured in the glasshouse (B). WT plants were grown in a sand-filled Teku pots until day 20, before they were inoculated with B55 and transferred to 10cm round pots containing lecaton and sand. (PLSD test of an ANOVA between mock- and B55-inoculated plants: * $P < 0.05$). dpi: days post infection. $n = 15$.

Figure 5 Effects of a B55 inoculation on field-grown plants (field season 2009).
Timeline of experiment (A). Field plot (B). Plants were planted in rows separated by water channels (C), mock- and B55-inoculated WT and *35S-etr1* plants were planted in a quadruplets in a randomized design (D). Mean (±SE) rosette diameter and stalk height (E), survivorship (F), flower number (G) and B55 colonization (H). White bars represent control treatments, black bars, B55 inoculated plants. (PLSD test of an ANOVA between mock- and B55-inoculated plants: *$P < 0.05$) dpi: days post-inoculation; CFU: colony forming unit; FM: fresh mass; n.d.: not detected. $n = 40$ for WT-, WT+ and *35S-etr1+*; $n = 23$ for *35S-etr1-* at start of measurements (46 dpi); $n = 5$ for h.
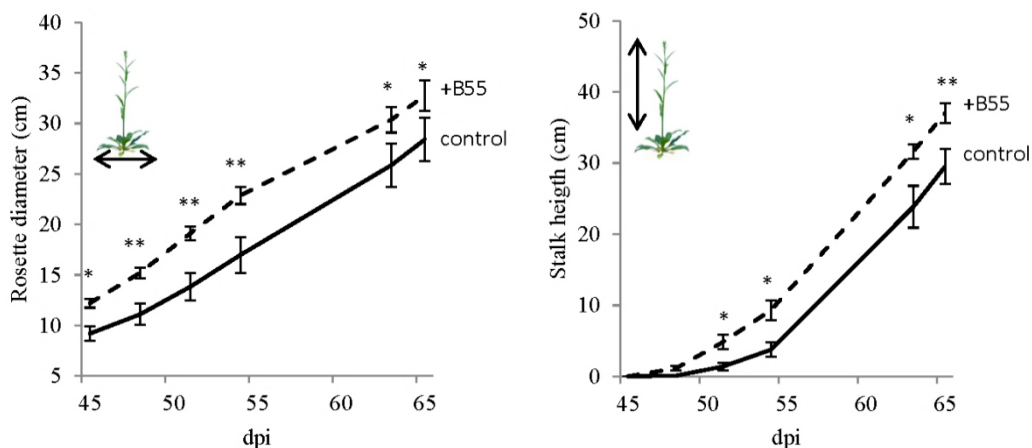
Figure 6 Effects of a B55-inoculation on field-grown WT plants (field season 2010).
Mean (±SE) rosette diameter and stalk height of B55-inoculated and mock-inoculated plants. Plants were grown in a paired design. Only pairs in which both plants survived until the end of the experiment were included in the analysis. * indicates a statistically significant difference between treatments as determined by a paired t-test (*$P < 0.05$; **$P < 0.01$) . dpi, days post infection. $n = 11$ pairs.
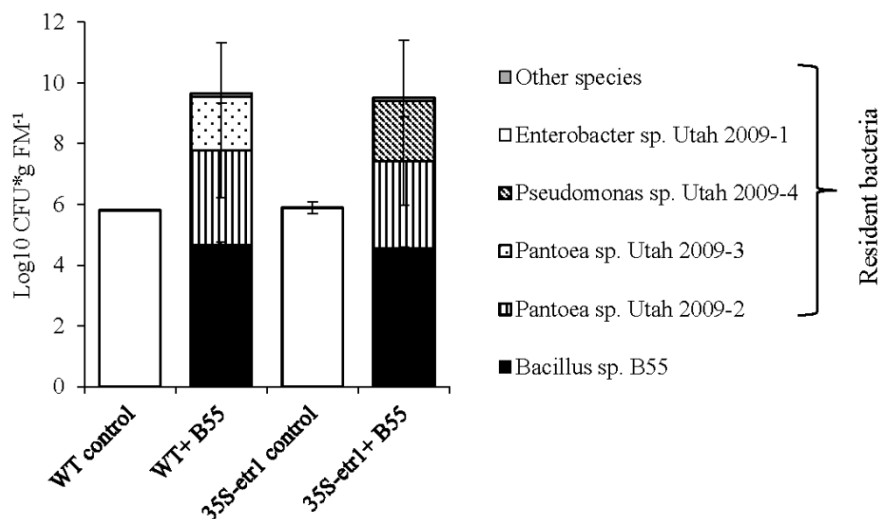

Figure 7 Effects of a B55 inoculation on the resident culturable bacterial community of field-grown plants.
Colony forming units of B55 and resident isolates at 73 dpi; see Table 1 for accession numbers of isolates. dpi: days post infection; CFU: colony forming units; FM: fresh mass. $n = 3$.
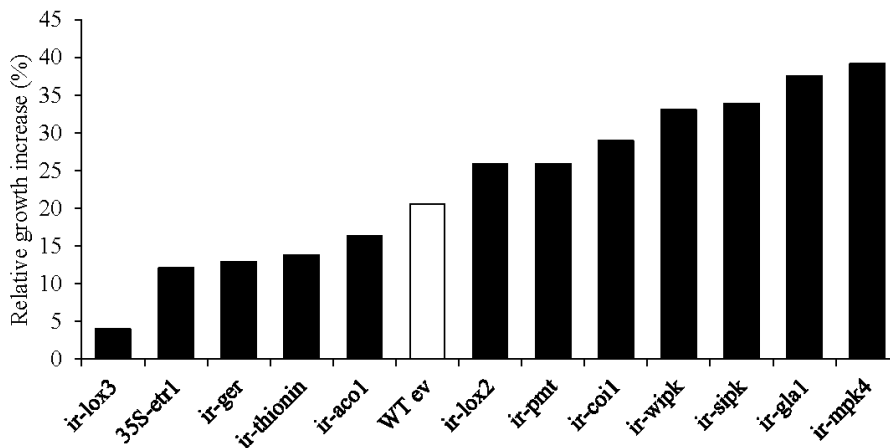
Figure 8 Growth responses of different transgenic *N. attenuata* lines to a B55 inoculation.
Relative B55-associated growth increase of different transgenic *N. attenuata* lines compared to non-inoculated plants. Twenty-day-old plants were inoculated with B55 or mock-inoculated (late stage-inoculation). Size of the longest leaf was measured on the day of inoculation and at the end of the experiment (12 or 14 dpi). Growth increase (%) of B55-inoculated plants was calculated relative to non-inoculated plants. See Table 2 for abbreviations of genes silenced by RNAi by expression of inverted repeat (*ir*) constructs in the transformed lines. dpi: days post infection. *n* = 10.

Table 1 Accession numbers of bacterial isolates used in this study.

Bacterial isolate	Genbank accession no.	Closest type strain neighbor	Reference
B55	JX101913	*Bacillus aryabhattai* B8W22	This study
Utah 2009-1	JQ172772	*Enterobacter cloacae* subsp. *dissolvens* LMG2683	This study
Utah 2009-2	JQ172773	*Pantoea gaviniae* A18/07	This study
Utah 2009-3	JQ172774	*Pantoea agglomerans* DSM 3493	This study
Utah 2009-4	JQ172775	*Pseudomonas lurida* DSM15835	This study

4.6 Discussion

Bacillus sp. isolates are natural root-associated bacteria of *N. attenuata* (Long *et al.*, 2010) and the particular isolate that we have named "B55", has dramatic PGP effects on WT and *35S-etr1* plants (a transgenic line impaired in ET perception), the line from which this bacteria was first isolated. Here, we show that this native PGPB not only improved *N. attenuata* WT's growth under *in vitro*, glasshouse and field conditions, but it also "rescued" many of the deleterious phenotypes of *35S-etr1* plants and dramatically increased the survival of this plant under field conditions, demonstrating PGP effect of a PGPB on its native host in nature.

Numerous studies have reported on bacterial mediated PGP effects *in vitro* and in the glasshouse, however, PGPB frequently fail under field conditions, probably due to their inability to colonize roots properly in a competitive environment (Compant *et al.*, 2010; Smyth *et al.*, 2011). Furthermore, bacterial formulations, previously reported to promote plant growth, often exhibited negative effects when applied in a natural environment. Particular physical and chemical properties of the soil are thought to interfere with the "targeted" plant species or the indigenous microbial community (reviewed in Aeron *et al.* 2011). If PGPB were selected from and hence adapted to the host plant's native habitat, the PGP effects might be made more consistent.

Our *in vitro* experiments demonstrated that many parameters associated with PGP (e.g. plant size, chlorophyll content, root branching), were increased in B55-inoculated seedlings. *35S-etr1* seedlings, which grow poorly compared to WT, benefited dramatically from the inoculation: seedling surface area doubled and the characteristic "unbranched" roots gave rise to seven times more lateral roots compared to control seedlings. These observations are consistent with those of López-Bucio *et al.* (2007), who found that a PGP *Bacillus megaterium* strain increased root hair and lateral root formation of *Arabidopsis thaliana* auxin (*aux1-7*) and ethylene (*eir1*) mutants, which tend to produce fewer root hairs and lateral roots compared to WT. Furthermore, chlorophyll *a* and *b* contents, previously reported to be lower in ET-insensitive plants (Grbic & Bleecker, 1995) were restored to WT control levels by B55 inoculation. B55's effects on *35S-etr1* seedling performance were associated with higher endosphere colonization: *35S-etr1* seedlings were found to be 10 times higher colonized than WT. Two types of explanations could account for the greater endosphere colonization despite similar rhizosphere colonization (Figure 1D): B55 was originally isolated from a *35S-etr1* plant and hence this genotype may have phenotypes that reflect its natural host. Native *N. attenuata* populations are known to be highly genetically diverse (Bahulikar *et al.*, 2004) and recent work has found that populations of *N. attenuata* harbor natural jasmonate-deficient ecotypes (Wu *et al.* 2008; I.T. Baldwin, unpublished results) and it would not be surprising if natural ET-deficient ecotypes, similar to those of the *35S-etr1* also occurred in these populations. Similar host specific associations have recently been reported by Weyens *et al.* (2011), demonstrating that the endophyte *Pseudomonas putida* W619 exerted positive effects only on the poplar accession it was originally extracted from. Second, *N. attenuata 35S-etr1* seedlings do not appear to restrict microbial entry and growth in their roots, pursuing an "open immigration policy" with regard to their endophytic microbial community (Long *et al.*, 2010). Similar results were found in an ET-insensitive *Medicago truncatula* line,

which was hypercolonized by rhizobia or *Klebsiella pneumoniae* 342, respectively (Penmetsa, 1997; Iniguez *et al.*, 2005). Interestingly, the rhizospheres of WT and *35S-etr1* plants were similarly colonized by B55. Hence the greater apparent benefit obtained by *35S-etr1* plants compared to WT may simply have resulted from the greater endosphere B55 colonization.

Glasshouse experiments with B55-inoculated and non-inoculated WT and *35S-etr1* plants were consistent with our *in vitro* findings. Inoculated *35S-etr1* plants grew similarly to non-inoculated WT plants and B55-inoculated plants yielded more seed capsules than their respective controls. Again, *35S-etr1* plants gained a greater growth benefit from B55 inoculation than WT plants did. Surprisingly in these glasshouse experiments, colonization of *35S-etr1* roots was similar to that of WT plants. We propose that higher colonization levels during seedling emergence and establishment (which are likely the most critical growth stages in nature) might be responsible for the observed durable PGP effects that lasted through all stages of development.

The 2009 field study was the most "successful" field planting of *N. attenuata 35S-etr1* plants to date. In the previous four field attempts usually more than 50% of the *35S-etr1* plants died before stem elongation and flowering, most probably due to high pathogen susceptibility and poor root development. Studies on the field performance of ET-sensing defective plants are rare, likely due to above-mentioned reasons. Bent *et al.* (2006) reported that field-grown ET-insensitive soybean were also highly susceptible to fungal pathogens. Geraats *et al.* (2007) analyzed the effect of bacterial antagonists on disease susceptibility of transgenic, ET-insensitive *N. tabacum* Tetr plants (which are also highly susceptible to fungal pathogens); here, bacterial antagonists were unable to alleviate infection, suggesting that one of the indirect PGP mechanisms, namely the suppression of soil-borne diseases and/or induction of systemic resistance (ISR) (Lugtenberg & Kamilova, 2009; Gamalero & Glick, 2011) are not likely involved.

Our results suggest that impaired ET perception augments the beneficial effects of B55 inoculation. Similar effects were observed by Lopez-Bucio *et al.* (2007) who concluded from their experiments that the PGP associated with a *B. megaterium* strain was independent of ET and auxin signaling. Recently, however, Camehl *et al.* (2010) reported that the beneficial interaction outcome between a PGP fungus (*Piriformospora indica*) and *A. thaliana* required functional ET perception and signaling; while WT plants benefited from the interaction, growth of *etr1*, *ein2* and *ein3/eil1* mutants was not promoted or even inhibited by the fungus. Similar results were found with *Glomus intraradices*'s parasitic interaction with *N. attenuata* plants (Riedel *et al.*, 2008) and is also likely the case with *P. indica's* PGP interaction with *N. attenuata* (Barazani *et al.*, 2005). The multiple functions of ET signaling and the specific interactions that occur among different partners (bacteria vs. fungi) are likely to account for these different results (Broekaert *et al.*, 2006; Hardoim *et al.*, 2008). B55's dramatic effects on *35S-etr1* plant growth prompted us to test its effect on other transgenic *N. attenuata* lines. A late-stage B55-inoculation enhanced growth of all lines tested (Figure 8) leading to the conclusion that the interaction of B55 with *N. attenuata* is independent of the (phytohormone) signaling pathways tested so far, including ET perception, and is likely a response of the particular accession that was used to produce all transformed lines. Interestingly, however, benefits gained by *35S-etr1* plants by a late-stage

Table 2 *Nicotiana attenuata* **lines used in Figure 8.**

All lines used were T_3 generation transformed plants harboring a single T-DNA insertion and silenced in the expression of the target gene, and fully characterized in the associated reference publication.

Genotype	Line code	Gene silenced and phenotype	Reference
ir-aco1	A-03-321-10	*ACC oxidase 1*, impaired in ET production	(von Dahl *et al.*, 2007)
ir-coi1	A-04-249-1	*coronatine insensitive 1,* impaired in JA perception	(Paschold *et al.*, 2007)
ir-ger	A-911-10	germin-like gene, impaired in responses to (a)biotic stresses	(Lou & Baldwin, 2006)
ir-gla1	A-09-849-2	*glycerolipase 1*, impaired in oxylipin biosynthesis	(Bonaventure *et al.*, 2011)
ir-lox2	A-04-52-2	*lipoxygenase 2*, impaired in traumatin and green leaf volatile production	(Allmann *et al.*, 2010)
ir-lox3	A-03-562-2	*lipoxygenase 3*, impaired in JA biosynthesis	(Allmann *et al.*, 2010)
ir-mpk4	A-07-119-4	*Mitogen activated protein kinase 4*, impaired in stomatal closure in response to (a)biotic stress; elevated chlorophyll contents	(Hettenhausen *et al.*, 2012)
ir-pmt	A-03-108-3-1	*putrescine N-methyl transferase,* impaired in nicotine production	(Steppuhn *et al.*, 2004)
ir-sipk	A-06-109-2	*Salicylic acid-induced protein kinase,* impaired in MAPK signaling and JA biosynthesis	(Meldau *et al.*, 2009)
ir-thionin	A-05-96-2	*pathogenicity-related gene 13*, impaired resistance to pathogens	(Rayapuram *et al.*, 2008)
ir-wipk	A-06-56-1	*wound-induced protein kinase,* impaired in MAPK signaling and JA biosynthesis	(Meldau *et al.*, 2009)
Ev	A-03-9-1-1	Empty vector (control)	(Zavala *et al.*, 2004)

inoculation (12 % growth increase) were lower than for WT (ca. 20%) and much reduced compared to that obtained by seed-inoculation (30%). The influence of B55 on *35S-etr1* seedlings' root structure seems to strengthen the young plant, an effect that lasts for the plant's life. This finding highlights the likely importance of a seedling's selection of PGPB into its rhizosphere at the onset of germination.

To analyze the mechanism behind B55's remarkable PGP effects, we tested B55 for known PGP traits. The ability of bacteria to produce IAA and ACCd is often considered a pre-requisite for PGP. For example, Long *et al*. (2008) found that ACCd production by endophytic bacteria was negatively correlated with plant ET production and positively with seedling root growth. Also IAA excretion into the rhizosphere is thought to promote root growth (Gamalero & Glick, 2011; Helman *et al.*, 2011). Even though B55 was found to produce substantially high concentrations of ACCd and IAA, WT and *35S-etr1* seedling IAA content and ET production were not affected by a B55-inoculation (Figure 2A,B). Furthermore, the high levels of ET production by *35S-etr1* seedlings were not changed, hence restoring ET sensing as possible PGP mechanism can be excluded (Figure 2B). Neither was the P content of WT plants changed by a B55 inoculation (Figure 2C). These findings point to other, unexplored PGP mechanisms.

B55 inoculation significantly changed the quantity and quality of the resident bacterial community of WT and *35S-etr1* plants. Even though we analyzed only the culturable bacterial community (which represents just a minute proportion of microbes interacting with plants), our results point towards the often underestimated importance of microbe-microbe interactions in facilitating PGP (reviewed by Berg & Zachow 2011). While B55-inoculated plants harbored several bacterial isolates, non-inoculated plants only housed *Enterobacter* sp. Utah 2009-1. Interestingly, an *E. cloaceae* sp. isolate has been described as having PGP effects on several plant species (reviewed in Jha *et al.* 2011); however, as for many plant-microbe interactions, host specificity and PGP occurs in a species-specific manner and hence determine the outcome of the interaction (Long *et al.*, 2008; Berg & Smalla, 2009). Further experiments will be carried out to unravel B55's antagonistic and synergistic actions on microbial communities and plant growth.

That B55 could alleviate some of the negative effects associated with the *35S-etr1* plant's inability to perceive ET, leads to the hypothesis that plants that harbor various mutations (such as phytohormone mutants) might be able to recruit particular microbes to help them compensate for their fitness deficiencies. As such, these facultative mutualistic associations with microbes could be viewed as part of the plant's extended phenotype, as proposed by Partida-Martinez and Heil (2011), a phenotype that deserves much more additional work.

4.7 Acknowledgements

4.8   References

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5 Manuscript III

Dimethyl disulfide, a bacterial volatile emitted by* Bacillus *sp. B55, promotes growth of* Nicotiana attenuata *and rescues the performance of an ethylene insensitive genotype

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5.1 Abstract

The volatile blend emitted by *Bacillus* sp. B55, a natural root-associated bacterium of *Nicotiana attenuata*, promotes the growth of wild-type (WT) and particularly, ethylene (ET)-insensitive *35S-etr1* plants. Dimethyl disulfide (DMDS), a sulfur (S)-containing compound volatile organic compound (VOC), is emitted by B55 and depleted from the headspace after a co-cultivation with seedlings in bi-partite Petri dishes. ^{35}S-labeling experiments demonstrated that ^{35}S is assimilated from the bacterial VOC bouquet and incorporated into the plants' proteins. Exposure of WT and *35S-etr1* seedlings to synthetic DMDS grown under different sulfate (SO_4^{-2}) supplies revealed genotype-dependent plant growth promotion (PGP) effects. For WT, only S-starved seedlings benefited from DMDS exposure; *35S-etr1* seedlings, which have an unregulated S metabolism (due to unregulated ET emissions and Yang cycle), benefited at all SO_4^{-2} supply rates. Exposure to B55 VOCs, and DMDS recovered many of the growth phenotypes of ET-insensitive plants, including the lack of root hairs, poor lateral root growth and low chlorophyll contents. We conclude that DMDS is a novel PGP agent, likely functioning by enhancing reduced S availability, particularly beneficial for *35S-etr1* plants due to their apparent impairments in S-metabolism. Because *N. attenuata*'s native soils are low in SO_4^{-2}, DMDS production by root-associated bacteria may be nutritionally essential for plants in nature.

5.2 Keywords

5.3   Introduction

Bacteria can affect plant performance through many different mechanisms of which improving access to nutrients, particularly to the limiting macronutrient nitrogen (N), is one direct means of plant growth promotion (PGP). The symbiosis between legumes and diazotrophic rhizobia is the best-studied example. Improved access to other nutrients such as phosphorus, iron or sulfate (SO_4^{-2}) have also been reported in mutualistic plant-microbe associations (Kloepper *et al.*, 1980; Rodriguez & Fraga, 1999; Banerjee & Yesmin, 2009). In addition, bacteria are known to alter a plant's hormone homeostasis through the production of phytohormones (e.g. auxins, cytokinins or gibberellins) but also by lowering ethylene (ET) levels, through the metabolism of the plant's ET precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), as a N source.

More recently, bacterial volatile organic compounds (VOCs) were shown to have PGP effects. The VOCs of *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937 were reported to increase the growth of *Arabidopsis thaliana* (Ryu *et al.,* 2003). The PGP effects were largely attributed to two volatiles, namely, 3-hydroxybutan-2-one (acetoin) and 2,3-butanediol, and experiments with the use of knockout mutants in 2,3-butanediol biosynthesis (BSIP1173 and BSIP1174) and the application of synthetic 2,3-butanediol strongly supported a role for 2,3-butanediol in PGP. Since this report, several other studies have examined bioactive volatile compounds responsible for PGP, as well as growth suppression (Farag *et al.*, 2006; Splivallo *et al.*, 2007; Vespermann *et al.*, 2007; Kai *et al.*, 2009; Kai & Piechulla, 2009; Gutiérrez-Luna *et al.*, 2010; Kai *et al.*, 2010; Blom, D. *et al.*, 2011; Blom, Dirk *et al.*, 2011; Velazquez-Becerra *et al.*, 2011; Weise *et al.*, 2012). For most of these, the underlying mechanisms and hormone signaling pathways involved remain to be discovered (Zhang *et al.*, 2007; Wenke *et al.*, 2012a).

The phytohormone ET plays multiple roles in plant development and defense (Pierik *et al.*, 2006; van Loon *et al.*, 2006): it regulates processes such as seedlings emergence, flower development and fruit abscission, as well as biotic and abiotic stress responses. (Davidson, 1949; Wilson, 1966; Rasmusse & Cooper, 1968; Pegg, 1976; Egley, 1980; Kahl *et al.*, 2000). Recently, several studies underscored ET's prominent role in mediating mutualistic plant-microbe interactions (Penmetsa, 1997; Iniguez *et al.*, 2005; Long *et al.*, 2010) and due to its gaseous nature, ET became the "candidate of choice" for the first studies on microbial VOC-mediated signaling processes in plants (Bailly & Weisskopf, 2012). Studying VOC-mediated effects in ET-signaling impaired *A. thaliana* plants, revealed that ET-insensitive *ein2* (*ethylene insensitive2*) mutants were non-responsive to VOC-mediated PGP by *B. amyloliquefaciens* IN937 and *Piriformospora indica* (Ryu *et al.*, 2003; Camehl *et al.*, 2010). Other ET-insensitive mutants, however, responded to the VOCs similarly as wild-type (WT) plants. Furthermore, ET emission by VOC-treated plants was found to be unaltered (Ryu *et al.*, 2003). On the other hand, recent transcriptome and proteome analyses of VOC-exposed *A. thaliana* plants revealed the regulation of genes and proteins involved in ET-signaling and biosynthesis (Kwon *et al.*, 2010). Although these studies suggest a role for ET in microbial VOCs-mediated interactions, unambiguous proof is still lacking.

Colloquially, ET is called the "senescence hormone" due to its role in flower and fruit development and leaf and fruit abscission. In the nineties, attempts to overcome or delay plant

senescence processes led to the creation of ET-insensitive plants, i.e. plants overexpressing a mutant *A. thaliana* ET receptor (*etr*1-1) (Chang *et al.*, 1993). Although these transgenic and mutant lines showed delayed senescence, impairing ET perception resulted in many negative growth effects which limited their agricultural utility (Bent *et al.*, 1992; Knoester *et al.*, 1998; Geraats *et al.*, 2002). Due to weak roots, which produce only few lateral branches and root hairs, lowered leaf chlorophyll contents and high disease susceptibility, ET-insensitive plants typically have difficulties in establishing growth, rendering them unable to survive in natural environments (Luschnig *et al.*, 1998; Clark *et al.*, 1999; Tholen *et al.*, 2007; Long *et al.*, 2010; Meldau *et al.*, 2012). To assess the effect of ET-insensitivity and the altered root morphology on plant-microbe associations, we employed a native plant system (coyote tobacco, *Nicotiana attenuata*) and identified natural root-associated bacteria which improve this plant's growth and survivorship. We isolated culturable microbes associated with *N. attenuata* WT and two transgenic lines impaired in ET signaling: an ET-insensitive (*35S-etr1*) and an ET-biosynthesis impaired line (*ir-aco1*) (Long *et al.*, 2010). We then investigated the intimate relationship between one isolate, *Bacillus* sp. B55, with its host in terms of PGP and observed dramatically increased survival and growth of *35S-etr1* plants grown in nature. Since B55 was isolated from *35S-etr1* plants grown in native soil and inoculating germinating seeds with a B55 greatly improved the growth and survivorship of this transgenic line in nature, we defined B55 as a part of the plant's "extended phenotype" (Meldau *et al.*, 2012). However, the mechanisms of B55's PGP remained elusive.

Here we report one mechanism responsible for B55's remarkable PGP effects. We show that VOCs emitted by B55 promote seedling growth and identify an S-containing volatile organic compound, dimethyl disulfide (DMDS), which is released by B55 and confers the observed PGP effects. Interestingly, *35S-etr1* seedlings realize a greater benefit of exposure to the bacterium, its VOCs and DMDS than do WT plants. In addition, we demonstrate that *35S-etr1* plants suffer from an unbalanced S-metabolism and we propose that these plants benefit from their interaction with B55 by obtaining reduced S (in the form of bacterial DMDS) which helps to fulfill their enhanced S requirements which, in turn, likely results from an unregulated Yang cycle.

5.4 Results

B55 VOCs promote WT and *35S-etr1* seedlings growth

To evaluate if volatiles could account for the previously described plant growth promotion effect of B55 on WT and *35S-etr1* seedlings (Meldau *et al.*, 2012) we co-cultivated WT and *35S-etr1* seedlings with B55 for 12 d on bi-partite Petri dishes, where seedlings and bacteria only had contact through a shared headspace. After 12 d the surface area of seedlings exposed to the B55 VOCs was 5 and 8 times higher than that of WT and *35S-etr1* control plants, respectively (Figures 1A and B). B55 VOC-exposed WT and *35S-etr1* seedlings produced twice and almost 4 times as many true leaves (Figure 1C) and 5 and 26 times more lateral roots per cm primary root length (Figure 1D). Root length was 17 and 38 % greater when compared to WT and *35S-etr1* control seedlings, respectively (Figure 1E). In general, chlorophyll a and b contents were 14 and 18 % lower in *35S-etr1* seedlings compared to the respective WT treatment group. Exposure to B55 VOCs led to slight, non-significant, increases in chlorophyll a and b contents of

WT and *35S-etr1* seedlings (Supplemental Figure 1 online). These data demonstrate that VOCs emitted from B55 are sufficient to induce plant growth promotion in *N. attenuata* WT and *35S-etr1* plants.

Dimethyl disulfide is emitted by B55 and taken up by seedlings

We assumed that VOCs, which are absorbed by the plant, might be involved in PGP. We therefore analyzed the composition of the headspace volatile bouquet of 12 d old B55 co-cultivated with and without WT seedlings on a bi-partite Petri dish by SPME GC-MS. An abundant VOC produced by B55 (and not by media or seedlings) and which was depleted from the headspace when co-cultivated with seedlings, was the S- containing metabolite dimethyl disulfide (DMDS, CAS 624-92-0) (Figure 2). In order to evaluate whether bacterial DMDS was taken up by the seedlings or whether its production was suppressed by the seedlings' presence, we measured DMDS headspace concentrations after the co-cultivation of different numbers of WT seedlings with a standardized inoculation of B55. Increasing seedling number was correlated with decreases in DMDS headspace concentrations (Supplemental Figure 3A online). When 25 seedlings were grown together with B55 as little as 1 pg DMDS per Petri dish was found in the headspace; but up to 10 pg when only three seedlings were grown opposite of B55. Transcript abundance of the B55 *CYSTATHIONINE-ß-LYASE* gene (*CBL*), coding for an enzyme involved in bacterial DMDS production, was not altered by the presence of seedlings (Supplemental Figure 3B online). These results suggested that DMDS is adsorbed and potentially assimilated by the seedlings rather than its production suppressed by the presence of seedlings.

To test if DMDS was adsorbed and assimilated, the incorporation of volatile S into the seedlings' protein was evaluated. We performed an ^{35}S-labeling experiments using bi-partite Petri dishes, in which B55 and seedlings had only headspace contact and in which B55 was grown on minimal medium (M9) containing ^{35}S-labeled Na_2SO_4 as the sole S source (Figure 3A). ^{35}S of a ^{35}S-containing VOC (most probably DMDS) was found to be incorporated into WT seedlings' proteins when the seedlings growth medium was S-deprived (Figure 3B, supplemental Figure 4).

We found that a singly cultivated, 14 d old B55 colony accumulated in one day ca. one ng DMDS cm^{-2} colony. In general, DMDS production was found to be favored when B55 colonies were grown on a full medium such as YPD, high incubation temperatures, under light, and production increased with colony age (Supplemental Figure 5A-C online).

***35S-etr1* plants adsorb more ^{35}S than do WT plants**

Since *35S-etr1* seedlings benefited more from inoculation with B55 (Meldau *et al.*, 2012) and exposure to B55 VOCs (Figure 1) than do WT seedlings, we evaluated whether *35S-etr1* seedlings adsorbed more DMDS than do WT seedlings. Using a tri-partite Petri dish set up (Figure 4A), we found that S-starved seedlings (grown on MM2, that lack S) absorbed larger amounts of ^{35}S than did seedlings grown on SO_4^{-2} rich media (MM2, 2 mM SO_4^{-2}) (Figure 4B). But most interestingly, *35S-etr1* seedlings assimilated more ^{35}S than did WT seedlings, particularly for seedlings grown under high SO_4^{-2} supply; *35S-etr1* seedlings accumulated twice as much ^{35}S than did S-replete WT seedlings. These findings led to the hypotheses that i)

bacterial volatile S contributes to *N. attenuta*'s S nutrition when grown in a SO_4^{-2}-depleted environments and that ii) *35S-etr1* seedlings have higher S needs (Figure 4B).

35S-etr1 seedlings are impaired in their S metabolism

To evaluate the importance of S nutrition for *N. attenuata* WT and *35S-etr1* seedlings, we grew seedlings on MM2 containing different SO_4^{-2} concentrations (0, 50 or 1000 µM $MgSO_4x7H_2O$) and assessed growth parameters, as well as shoot S-containing metabolites (free Met and reduced GSHs). As expected, for 20 d old WT seedlings, all recorded growth parameters correlated positively with increasing medium SO_4^{-2} concentration (Figures 5A 1st column, 5B-C, white bars), except lateral root number, which decreased slightly with increasing SO_4^{-2} concentration (Figure 6A, white bars). *35S-etr1* seedlings however, did not show these correlations. Even under high SO_4^{-2} supply (1000 µM) they appeared abnormal in growth: compared to WT seedlings, leaves were enlarged, swollen and yellowish (Figure 5A 3rd column). This was also reflected in the large seedling surface area compared to WT (Figure 5B, white bars). Chlorophyll a content was only half that of WT seedlings, while lateral root number was consistently low (Figures 5C and 6A, white bars).

Sulfate concentration in the culture medium also affected S-containing metabolites. In WT seedlings, free Met and reduced GSH concentrations, were found to increase with media SO_4^{-2} concentrations (Figures 5C-D, white bars). The free Met level of *35S-etr1* seedlings grown at 50 or 1000 µM SO_4^{-2}, were 34 and 22 % lower compared to WT (Figure 5C). Also *35S-etr1* seedlings' reduced GSH levels were 67 and 58 % lower, compared to WT, grown under 50 and 1000µM SO_4^{-2} supply, respectively (Fig. 5D, white bars). Taken together our findings suggest that *35S-etr1* seedlings are impaired in their S metabolism.

DMDS promotes WT and *35S-etr1* seedling growth

To evaluate the contribution of DMDS to WT and *35S-etr1* seedlings' S nutrition, we applied the pure compound to the headspace of the seedlings. To find the effective dose, we spotted different amounts of DMDS (0, 0.5, 5, 50, 500 or 2000 µg) onto a cotton bud located in one compartment of a bi-partite Petri dish to WT seedlings sown on MM2, supplemented with different $MgSO_4x7H_2O$ concentrations [0, 50 or 1000 µM]) in the other compartment. After 17 d, we evaluated DMDS dose- and SO_4^{-2}- concentration dependent PGP effects (Supplemental Figure 6A online). The application of 2000 µg DMDS completely inhibited seed germination and seedling growth. For SO_4^{-2} starved seedlings (0 µM SO_4^{-2}), PGP was highest when 500 µg DMDS were applied. Seedlings grown on 50 or 1000 µM SO_4^{-2}, experienced no PGP in terms of seedling surface area, irrespective of how much DMDS was added. At a DMDS concentration of 500 µg, the growth of these seedlings was slightly inhibited (Supplemental Figure 6B online). All further DMDS application experiments were carried out with 500 µg (0.5 µL) DMDS Petri dish^{-1}. When seedlings were grown on MM2, supplemented with different concentrations of SO_4^{-2} and exposed to DMDS, PGP effects were strongest for SO_4^{-2}-starved seedlings (0 µM SO_4^{-2}, Figures 5 and 6, black bars), irrespective of the plant genotype.

For 20 d old WT seedlings grown on 0 µM SO_4^{-2} media, an addition of DMDS increased seedling surface area 3.5 times; chlorophyll a content by 400 % (Figures 5A 2nd column, 5B and

C, black bars) and the number of lateral roots by 65% (Figure 6A, black bars). DMDS application to WT seedlings grown under medium (50 µM) and high (1000 µM) SO_4^{-2} supply resulted in less pronounced PGP effects (Figures 5A 2nd column, 5B and C, black bars). However, when exposed to DMDS, the number of lateral roots was consistently increased, by 55 and 33 % for WT seedlings grown on 50 µM and 1000 µM SO_4^{-2}, respectively (Figure 6A, black bars).

In contrast to WT, *35S-etr1* seedlings realized dramatic PGP effects from the exposure to DMDS, irrespectively on which SO_4^{-2} concentration they were cultured (Figure 5A 4th column). Seedling surface area was increased for seedlings grown on 0 µM SO_4^{-2} by 3.3 times, but decreased by 22 and 30 % respectively, for seedlings grown on 50 and 1000 µM SO_4^{-2} supply (Figure 5B, black bars). Chlorophyll a content of seedlings increased by 328, 319 and 68 %, respectively for the 0, 50 and 1000 µM SO_4^{-2} concentration (Figure 5C and supplemental Figure 7A online, black bars). The number of lateral roots was 2, 2.7 and 2.8 times higher for *35S-etr1* seedlings grown on 0, 50 and 1000 µM SO_4^{-2} supply, respectively (Figure 6A, black bars). Strikingly, *35S-etr1* seedlings, which are normally root hair deficient, were found to produce root hairs when exposed to DMDS (Figure 6B).

The addition of DMDS increased free Met concentrations: SO_4^{-2} deprived (0 µM SO_4^{-2}) WT seedlings had 140 % higher Met levels (Figure 5C, black bars). WT seedlings grown under medium (50 µM) and high (1000 µM) SO_4^{-2} supply were hardly affected. In *35S-etr1* seedlings, free Met levels increased by circa 122, 48 and 22 % for 0, 50 and 1000 µM SO_4^{-2} concentration, respectively (Figure 5C, black bars).

WT's reduced GSH levels were increased by 900 and 85 % for 0 and 50 µM SO_4^{-2}, respectively. Seedlings grown under high SO_4^{-2} supply were not affected (Figure 5D, black bars). When *35S-etr1* plants were exposed to DMDS, reduced GSH concentration increased by circa 1000, 500 and 150 % for 0, 50 and 1000 µM SO_4^{-2}-grown seedlings, respectively (Figure 5D, black bars).

In summary, these data demonstrate that volatile DMDS positively effects plant growth and concentrations of S-containing metabolites when inorganic S supply is limited.

DMDS application affects transcript abundance of genes involved in SO_4^{-2} metabolism

Sulfate reduction is an energy-demanding process for plants (Oger *et al.*, 2004). Theoretically, the uptake of reduced S (e.g. in the form of DMDS) could save seedlings energy that could otherwise be invested in other plant physiological processes, such as growth or reproduction. Hence we were interested in whether the DMDS fumigation resulted in the down-regulation of genes involved in the S reduction pathway and analyzed the transcript abundance of the high affinity *SULFATE TRANSPORTER1* (Na*SULTR1*), *SULFITE REDUCTASE* (Na*SIR*) and *METHIONINE SYNTHASE 1* (Na*METSYN1*). Quantitative PCR revealed that genes involved in WT's SO_4^{-2} reduction were strongly regulated by the media SO_4^{-2} concentration (Figure 7, white bars). In WT seedlings, the SO_4^{-2} transporter, Na*SULTR1*, was highly induced under low SO_4^{-2} supply (Figure 7A, white bars). Also Na*SIR* and Na*METSYN1* transcript levels were induced when seedlings were S-deprived (0 µM SO_4^{-2}) (Figures 7B and C, white bars). While WT and *35S-etr1* seedlings, did not differ in Na*SULTR1* and Na*SIR* transcript levels (Figures 7A and B, white bars), the expression of Na*METSYN1* was significantly higher for *35S-etr1*

seedlings grown under medium (50 µM) and high (1000 µM) SO_4^{-2} supply than in similarly grown WT (P<0.0001) (Figure 7C, white bars).

Overall, the application of DMDS to WT and *35S-etr1* seedlings resulted in decreased transcript abundances of Na*SULTR1* and Na*SIR*. Transcript abundance of Na*SULTR1* was lowered by 36, 69 and 19 % for WT seedlings grown on 0, 50 and 1000 µM SO_4^{-2}, respectively. The abundance of Na*SIR* transcripts were reduced by 50 % for WT seedlings grown under medium (50 µM) and high (1000 µM) SO_4^{-2} supply. Also for *35S-etr1* seedlings, Na*SULTR1* transcripts decreased by 83, 91 and 32 % for 0, 50 and 1000 µM SO_4^{-2}, respectively. Compared to control treatments, Na*SIR* transcripts were decreased by 45 %, irrespective of the medium's SO_4^{-2} concentration (Figure 7B, black bars). WT and *35S-etr1* Na*METSYN1* transcript abundance was not affected by DMDS, with the exception of WT seedlings grown on 50 µM SO_4^{-2} in which Na*METSYN1* transcripts were increased by 36 %.

B55 VOC bouquet exposure mimics DMDS-induced effects

We tested whether the exposure of WT and *35S-etr1* seedlings to the complete B55 VOC bouquet, could mimic the growth responses observed for pure DMDS exposures when seedlings were grown under different SO_4^{-2} supply. Therefore, we grew WT and *35S-etr1* seedlings in one compartment of a bi-partite Petri dish on MM2, supplemented with different $MgSO_4 x7H_2O$ concentrations [0, 50 or 1000 µM]) and spotted 5 µL of a B55 suspension onto the other compartment containing 0.5xYPDA (Figure 8A). Growth parameters were evaluated after 15 d of growth.

In terms of surface area, only S-deprived WT and *35S-etr1* seedlings showed a positive reaction to B55 VOCs: WT and *35S-etr*1 seedling surface area was increased by 36 and 66 %, respectively (Figure 8B, black bars). For all other treatment combinations, no effects were observed. Seedling chlorophyll a content was not affected by B55 VOCs. For WT seedlings cultured on 1000 µM SO_4^{-2}, even negative effects were observed (Figure 8C, black bars). Interestingly, free Met levels of S-deprived WT and *35S-etr1* seedlings were not altered by exposure to B55 VOCs (Figure 8D, black bars). Free Met content, however, increased for WT and *35S-etr1* seedlings grown under medium (50 µM) and high (1000 µM) SO_4^{-2} supply, by 130 and 125 %, as well as, 100 and 180 %, respectively (Figure 8D, black bars). In the case of reduced GSH, opposite patterns were found. While WT and *35S-etr1* seedlings grown under medium (50 µM) and high (1000 µM) SO_4^{-2} supply did not show any changes, S-deprived seedlings did; WT and *35S-etr*1 reduced GSH contents increased by 160 and 130 %, respectively (Figure 8E, black bars).

Root architecture was influenced by B55 VOCs as well. WT seedlings grown under 0, 50 or 1000 µM SO_4^{-2} produced 3, 2.3 and 1.5 times more lateral roots compared to the respective control group (Figure 8F, black bars). The same was found for *35S-etr1* seedlings. Exposure to B55 VOCs resulted in 1.6, 3 and 3.5 times more lateral roots in seedlings cultured on 0, 50 or 1000 µM SO_4^{-2}, respectively (Figure 8F, black bars). These data demonstrate that the VOC bouquet emitted by B55 and pure DMDS applications induce similar S-dependent changes in plant growth and the concentrations of S-containing metabolites.

DMDS application affects ET production

Due to their ET-insensitivity, *35S-etr1* plants overproduce ET constitutively and in response to biotic elicitations and similar ET production responses are seen with WT plants rendered transiently ET-insensitive by treatments with the gas 1-methylcyclopropene (1-MCP) (von Dahl *et al.*, 2007). Ethylene production is tightly coupled to the Yang-cycle in which the S-containing amino acid Met is recycled (Miyazaki & Yang, 1987). The results of this study suggest that *35S-etr1* plants have an "unregulated" S metabolism. Hence we analyzed how the medium's availability of SO_4^{-2} and DMDS affect ET production.

As shown by von Dahl *et al.* (2007), the ET-insensitive *35S-etr1* plants show higher ET emissions compared to WT. While in WT seedlings ET emissions tended to increase with increasing SO_4^{-2} availability (simple regression analysis, P>0.05), the ET emission of *35S-etr1* seedlings was constitutively high, irrespective of the medium's SO_4^{-2} concentration (Supplemental Figure 8A online).

To evaluate whether an increased availability of reduced S, in the form of DMDS, facilitated ET production, we measured ET emission of WT and *35S-etr1* plants exposed to DMDS. Additionally we applied 1-MCP to plants to elicit transient ET-insensitivity. The 1-MCP treatment resulted in higher ET emissions of WT (260 % increase), as well as *35S-etr1,* plants (117 % increase); and interestingly, application of DMDS caused increased ET production only in ET insensitive plants: DMDS-treated *35S-etr1*, 1-MCP-treated WT and 1-MCP-treated *35S-etr1* plants had 57, 62 and 116 % increased ET emission compared to controls (Supplemental Figure 8B, online). The more DMDS was applied, the more ET was produced by ET insensitive *35S-etr1* plants (Supplemental Figure 8C online). Non-1-MCP-treated WT plants did not respond with an altered ET emission to the exposure to DMDS. ET-sensitivity of *35S-etr1* plants was not recovered by DMDS. These findings indicate that DMDS might increase S-availability which is in part used for ET production in ET-insensitive plants.

5.5   Discussion

Inexpensive high-throughput sequencing technologies are allowing for the complete characterization of the microbial communities in all types of eukaryotes and the differences that are being uncovered are fueling a plethora of hypotheses about the importance of microbes for the health and fitness of their hosts (*Nature* Vol. 488, Issue 7409, August 1st, 2012). Root associated microbes have long been thought to be important for the performance of plants, but few of these associations have been tested under real world conditions and little is known about their mechanism. Using a native plant system (coyote tobacco, *Nicotiana attenuata*) we are investigating the interaction of the naturally root-associated bacterium *Bacillus* sp. B55 which benefits this plant's growth and survivorship (Long *et al.*, 2010; Meldau *et al.*, 2012).

Here we demonstrate a volatile organic compound (VOC)-mediated plant growth promotion (PGP) effect which is based on a nutritional mechanism. We argue that the abundant sulfur (S)-containing VOC produced by *Bacillus* sp. B55, dimethyl disulfide (DMDS), contributes to *N. attenuata*'s S nutrition, by providing an inexpensive form of reduced S. Seedling vigor (in terms of size, chlorophyll content and root architecture) as well as S-

containing compounds (e.g. free Met and GSHs) were increased when seedlings were exposed to DMDS or the B55 VOC bouquet (Figures 1, 5 and 6). Additionally, we demonstrate that the ET-insensitive *35S-etr1 N. attenuata* plants suffer from an unbalanced S-metabolism and hence benefit from the interaction with B55 by obtaining reduced S in the form of bacterial DMDS (Figure 10). Furthermore, genes involved in the plant's S assimilation are down-regulated, perhaps reflecting an energy saving strategy, allowing energy that would be used for S reduction and assimilation to be otherwise invested in growth (Figure 7).

Bacterial VOCs are thought to play important roles in mutualistic or pathogenic plant-microbe communication and PGP (Bailly & Weisskopf, 2012; Wenke *et al.*, 2012b). However, only few bioactive VOCs, namely 2,3-butanediol, acetoin, DMDS, 2-pentylfuran and dimethylhexadecylamine, have been identified (Ryu *et al.*, 2003; Kai *et al.*, 2010; Zou *et al.*, 2010; Velazquez-Becerra *et al.*, 2011) and the signaling pathways as well as mechanisms involved in VOC-mediated PGP or growth depression remain largely unknown (Ryu *et al.*, 2003; Bailly & Weisskopf, 2012; Wenke *et al.*, 2012a).

Various groups of microorganisms and algae emit volatile S containing compounds (such as methanethiol, DMDS, dimethyl sulfide [DMS] or dimethyl trisulfide [DMTS]), but so do higher plants, like *Brassicaceae* sp., garlic or onion (Rumberger & Marschner, 2003; Doornbos *et al.*, 2010). The sulfurous, bioactive volatiles play diverse roles, affecting organisms across all kingdoms. S containing VOCs emitted from rotting *Brassicaceae* plant material or bacterial species like *Serratia plymuthica* IC1270 function as antimicrobials, suppressing fungi like *Verticilium dahliae*, *Fusarium spp.*, the tumor-producing bacterium *Agrobacterium tumefaciens* and phytopathogous nematodes (Kai *et al.*, 2009; Wang *et al.*, 2009; Huang *et al.*, 2010; Dandurishvili *et al.*, 2011). DMDS was first described to be produced by bacteria isolated from decomposing chicken (Freeman *et al.*, 1976). Surprisingly, DMDS along with other VOCs emitted by rhizobacteria *Pseudomonas fluorescens* B-4117 and *S. plymuthica* IC1270 was found to inhibit the cell-cell communication quorum-sensing (QS) network of various bacteria (Chernin *et al.*, 2011). Furthermore, DMDS functions as a plant defense compound against non-specialist herbivores feeding on *Allium porrum* (Dugravot *et al.*, 2003; Dugravot *et al.*, 2004) and as both oviposition repellent and attractant to different natural enemies of the cabbage root fly *Delia radicum* (Ferry *et al.*, 2007; Ferry *et al.*, 2009). Recently, Huang *et al.* (2012) described DMDS as an elicitor of Induced Systemic Resistance (ISR) in *Nicotiana benthamiana* against *Botrytis cinerea*. Additionally, S containing VOCs seem to enhance grape vine bud break (Kubota *et al.*, 2003; Vargas-Arispuro *et al.*, 2008). Whether DMDS mediates signaling processes involved in plant defense in *N. attenuata* is currently under investigation.

Here we provide evidence that DMDS plays a role in PGP by providing a form of reduced S for the plant. Our observations differ in part from those of Kai *et al.* (2010), which found that fumigations of *A. thaliana* seedlings with DMDS suppressed growth irrespective of the concentration applied. In our experimental set-up (MM2 medium supplemented with different amounts of SO_4^{-2}), we also found positive effects of 500 µg DMDS per Petri dish on the growth of *A. thaliana,* as we did with *N. attenuata* seedlings (Supplemental Figure 9 online). Very high DMDS concentrations (2000 µg / Petri dish), however, completely inhibited seedling growth (Supplemental Figure 6 online). These results are consistent with DMDS concentration- and

cultivation medium-dependent VOC-mediated PGP effects, indicating that the effect of DMDS depends on the environmental conditions.

In vitro pre-experiments have revealed that *N. attenuata* seedlings grow best under SO_4^{-2} supply higher than 50 µM (Supplemental Figure 10 online). Given that the SO_4^{-2} soil concentration is suboptimal for plant growth (ca. 22.5 µM SO_4^{-2}), DMDS production by root associated bacteria might play an important role in the plant's S nutrition in nature. Indeed, anthropogenic emissions of sulfur dioxide (SO_2) have practically ceased since the implementation of stringent controls over the use of high S fossil fuels (Smith *et al.*, 2011). Consequently, S input into soil has steadily decreased and SO_4^{-2} deficiency has become a limiting factor for plant growth (McGrath & Zhao, 1995). S is rated the 4th most important macronutrient (after nitrogen, phosphorus and potassium) and essential for plant and animal life. Being incorporated into compounds such as the essential amino acids cysteine (Cys) and methionine (Met), several co-enzymes, vitamins, thioredoxins and glutathiones, S availability determines crop quality and quantity (Zhao *et al.*, 1993; Zhao *et al.*, 1999). Sulfur deficiency directly affects a plant's primary metabolism, in large part through the suppression of the photosynthesis machinery via decreased chlorophyll contents and lowered synthesis of RUBISCO restricting carbon dioxide (CO_2) assimilation (Burke *et al.*, 1986; Gilbert *et al.*, 1997). This scenario is consistent with the growth effects we observed in *N. attenuata* S-deprived plants (Figures 5 and 6, white bars). Generally S is taken up by the roots from surrounding soil in the form of SO_4^{-2}. However, as several studies have shown, plants are also capable of using atmospheric S, SO_2 and hydrogen sulfide (H_2S), to meet their S needs. Recently, Chen *et al.* (2011) reported that H_2S application to *Spinacia oleracea* seedlings increased growth, chlorophyll content and other photosynthetic parameters. These, otherwise toxic volatiles, may become valuable S sources, particularly when S supply to the root is limited (DeKok *et al.*, 1997; Stuiver & De Kok, 2001; Durenkamp & De Kok, 2005). The PGP effects attributed to DMDS or B55 VOCs were greatest when *N. attenuata* seedlings were grown under SO_4^{-2}- limiting conditions (0 µM) (Figures 5 and 6). The application of DMDS compensated for SO_4^{-2} deficiency in the growth medium, consistent with the perspective of the aforementioned studies.

Although major PGP effects could be attributed to bacterial VOCs *in vitro*, their role in nature remains elusive. To evaluate the contribution of B55's DMDS emission to a plants' S nutrition in nature, a bacterial mutant, unable to produce DMDS, would be required. Taking into account the challenge of transforming Gram-positive bacteria, as well as the fact that based on *in silico* analysis, the expression of three genes (*B. megaterium* QM B1551 *METHIONINE-γ-LYASE, CYSTATHIONINE-γ-LYASE, CYSTATHIONINE-β-LYASE*) involved in DMDS would be needed to be silenced, the utilization of transgenic plant lines, impaired in SO_4^{-2} reduction, might shed light on the relevance DMDS in nature. A molecular tool box for transforming *N. attenuata* has been developed over the last decade; but unfortunately, a transgenic line impaired in S reduction was not available to address our hypothesis, namely, that B55 VOCs / DMDS function as PGP agent by providing reduced S to the plant. However, the use of our ET-insensitive *35S-etr1* plant in the analysis of VOC-mediated PGP turned out to be serendipitous. While we were first only interested in unraveling the dramatic PGP effect conferred by B55 onto its host plant, *35S-etr1* (Meldau et al., 2012), we discovered that this line has impairments in S metabolism. We

found, that irrespective of the medium's SO_4^{-2} concentration, non-treated (control) *35S-etr1* plants exhibited an abnormal growth phenotype, which could be partially recovered by DMDS / B55 VOCs (Figure 5-9). A literature survey revealed that the *A. thaliana* gene *ETHYLENE INSENSITIVE LIKE*3 (At*EIL3*) shares the functional identity with a gene called *SULFUR LIMITATION1* (At*SLIM1*) (Maruyama-Nakashita et al., 2006). At*SLIM1* mutants were impaired in SO_4^{-2} uptake in low S media and had reduced growth. Our Na*ETR1* gene, however, did not show homology to any gene involved in S metabolism nor to the transcriptional regulator At*SLIM1*.

Due to the high ET emissions of *35S-etr1*, a phenotype coupled to ET-insensitivity (von Dahl *et al.*, 2007) (Supplemental figure 8A online), we assumed that *35S-etr1* plants might have an increased demand for S, in the form of Met, cycling in the Yang-Cycle. Compared to other S-containing compounds (i.e. reduced GSHs), free Met levels were comparatively high in non-treated (control) *35S-etr1* seedlings (Figure 5) and the transcripts of Na*METSYN1* accumulated to consistently higher levels, irrespective of the SO_4^{-2} concentrations the seedlings were cultivated on (Figure 8C). Also our labeling experiment (Figure 4) indicates that *35S-etr1* seedlings have a higher requirement for (reduced) S and assimilate more labeled S than do WT seedlings, consistent with our inference regarding the "unregulated" S metabolism and Yang-Cycle. We propose that ET-insensitive plants invest their S into the recycling of Met (in the Yang-Cycle) to supply the demands of their constitutively high ET emissions, which are maintained at the expense of plant growth. This is consistent with the results of Burstenbinder *et al.* (2007) and Katz *et al.* (2006), who found that Met availability restricts ET production. Hence we propose that the exposure of *35S-etr1* seedlings to DMDS (pure or as B55 VOC bouquet) compensates for the high S demand, rescuing seedling growth. ET insensitivity was, however, not affected by exposure to VOCs or DMDS (Supplemental Figure 8 online). Exposing ET-insensitive plants to DMDS amplified ET emissions, perhaps due to their increased reduced-S availability which consequently fueled the Yang cycle (Supplemental Figure 8B and C online).

Our experiments provide evidence consistent with the hypothesis that DMDS is assimilated by seedlings through the headspace (Figure 2 and Supplemental Figure 2 online). The labeled ^{35}S is incorporated into the seedling's protein after exposure to the bacterial VOC bouquet (Figures 3 and 4). However, other explanations exist; the radioactive signal might not necessarily have only originated from the assimilation of DMDS alone. Other S containing VOCs, not detected by methods we used (SPME coupled to GC-MS), might be produced by B55. For example, emission of methanethiol, a volatile precursor of DMDS, as well as other sulfurous VOCs, such as DMTS and DMS, are commonly produced VOCs of diverse microbes (Farag *et al.*, 2006; Kai *et al.*, 2010; Minerdi *et al.*, 2011). Furthermore, S-methyl pentanethioate (Supplemental Table 1 online), which is emitted in trace amounts, or the production of H_2S (not found in B55 VOCs, Supplemental Figure 11 online) might have contributed to the radioactive signal.

Although DMDS is an abundant VOC produced by B55, other non-sulfurous VOCs emitted by B55 (listed in Supplemental Table 1 online) have been reported to be produced by diverse bacterial taxa (Joffraud *et al.*, 2001; Chung *et al.*, 2002; Thiel *et al.*, 2010; Weise *et al.*, 2012) and might affect *N. attenuata* growth as well. The large amounts of CO_2 produced by co-

cultivated bacteria are known to positively affect seedling growth (Kai & Piechulla, 2009). With a CO_2 trapping experiment as described by Kai and Piechulla (2009) (see Supplemental Figure 12A), we found that the seedlings' affinity to CO_2 is much higher than that of the scavenging agent $Ba(OH)_2$ (Supplemental Figure 12B online). Thus it is not possible at this time to entirely exclude PGP effects resulting from DMDS from those resulting from CO_2 in the split plate assays. Additional experiments revealed, however, that even under reduced bacterial CO_2 exposure concentration, B55 VOCs still exerted positive effects on seedling growth; seedling surface area of seeds exposed to B55 VOCs and $Ba(OH)_2$ was as large as of those exposed only to the VOCs (Supplemental Figure 12C online).

In general, the PGP effects of seedlings grown on MM2 medium, co-cultivated with B55 were smaller than those observed after DMDS exposure (Figures 5, 6 and 8). This was particularly true for *35S-etr1* seedlings and might have two reasons. First, bacterial DMDS production might not be high enough to supply the S-requirements of growth on low SO_4^{-2} MM2 medium. Second, the many different VOCs emitted by B55 might act antagonistically and alter the growth response - a hypothesis which needs further investigation. Furthermore, we found that VOC effects in a Petri dish system are strongly influenced, not only by the bacterial culture medium, as reported by Blom, D. *et al.* (2011), but also by the seedlings' medium or a combination of both (compare Figure 1 with Figure 8).

Although the research on VOC-mediated PGP in plants is substantial and several VOCs have been identified, most work has been performed on a few model plants, as reviewed by Bailly and Weisskopf (2012) and Wenke *et al.* (2012b). How VOCs confer PGP effects on plants remains largely unknown. Our work has uncovered a new mechanism of PGP: a nutrient driven PGP effect of the S-containing VOC DMDS, likely functioning by enhancing S availability and reducing the energy requirements of S assimilation. Furthermore, we suggest that *35S-etr1* plants benefit most from the mutualistic association with B55 and DMDS emission due to their apparent impairments in S metabolism (Figure 9). Our work also demonstrates that by conducting research on naturally occurring and ecologically relevant interactions greatly facilities the study of these ubiquitous beneficial plant-microbe interactions. Our results show that the effects of bacterial compounds is highly context specific, depending on model system, plant genotype and bacterial strain, as one might expect of any co-evolved interaction. In addition, the work reveals the value of using transgenic lines of a native plant to uncover the interactions and their function.

The larger environmental context of this work is that anthropogenic emissions of sulfur dioxide (SO_2) have recently decreased with the implementation of pollution abatement measures. The cleaning of our air has decreased S inputs into soils, with the consequence that S deficiency has become a limiting factor for plant growth. While plants cannot exploit organically bound S, bacteria can. Uncovering the mechanisms of this native PGP plant-microbe interaction suggests the intriguing possibility of agronomic utility; by contributing to the host's S nutrition, B55 pre-inoculation of seeds could help reduce synthetic fertilizer inputs.

5.6 Materials and methods

Plant materials and bacterial strain

The 31 x selfed WT line of *Nicotiana attenuata* and the ET insensitive transgenic line *N. attenuata 35S-etr1* (A-03-328-8, fully described in von Dahl et al., 2007) and *Arabidopsis thaliana* Col-0 seeds were used in all experiments. Germination procedures have been described elsewhere (Ryu *et al.*, 2003; Long *et al.*, 2010). The plant growth promoting *Bacillus* sp. B55 strain was isolated from a *35S-etr1* plant grown in native Utah soil in 2008 (Long *et al.*, 2010). Unless noted otherwise, B55 was routinely grown on half-strength yeast peptone dextrose agar (YPDA; Sigma, Steinheim, Germany) at 30 °C. *N. attenuata* seedlings were grown, depending on the experimental set-up, on GB5 (with or without 1.2 % sucrose, Gamborg's B5 media, Duchefa, Haarlem, The Netherlands) or on modified minimal medium (MM2) supplemented with different amounts of SO_4^{-2} (0, 50 or 1000 µM $MgSO_4x7H_2O$). One liter MM2 contains 80 mg KNO_3, 65 mg KCl, 4.8 mg KH_2PO_4, 288 mg $Ca(NO_3)_2x4H_2O$, 8 mg NaFeEDTA, 0.75 mg KI, 6 mg $MnCl_2x4H_2O$, 2.65 mg $ZnSO_4x7H_2O$, 1.5 mg H_3BO_3 ; 0.13 mg $CuSO_4x5H_2O$, 0.0024 mg $Na_2MoO_4x2H_2O$ and 1.2 % sucrose, pH was adjusted to 6.8 (modifed after Becard & Fortin, 1988). To avoid magnesium deficiency in low SO_4^{-2} media and to exclude possible Cl^{2-} effects in high SO_4^{-2} media, magnesium concentration was balanced by the addition of 50 µM $MgCl_2x6H_2O$ to all MM2 media. Petri dishes were kept in a Percival growth chamber at 16/8 h day/night cycle, 155 µmol m^{-2} s^{-1} and 30/28 °C. Depending on the experimental design, *A. thaliana* seedlings were cultivated on half-strength Murashige and Skoog salt (MS, Duchefa, Haarlem, The Netherlands) agar medium or MM2 medium supplemented with different amounts of SO_4^{-2} (0, 50 or 1000 µM $MgSO_4x7H_2O$) in a York growth chamber (16/8 h day/night cycle, 190-220 µmol m^{-2} s^{-1}, 21 °C).

Seedling growth promotion by B55 volatile organic compounds

The effect of B55 volatile organic compounds (VOCs) was evaluated by spotting 5 µL of bacterial suspension (in sterile water, OD_{600}=1.0) onto one side of a bi-partite Petri dish containing 0.5xYPDA, while WT and *35S-etr1* seeds were transferred onto the other side containing GB5 medium, so that seedlings and bacteria shared only the common headspace. Seedling surface area was quantified after 12 d according to the video tutorial by Zach Jarou [http://www.chlorofilms.org/index.php/crpVideo/display/videoid/46]) using Adobe Photoshop CS5. Chlorophyll a and b contents were analyzed spectrophotometrically from an 80 % acetone extract using a TECAN plate reader (Tecan, Crailsheim, Germany). Number of true leaves and lateral root branches were determined by counting and primary root length was measured. Four replicates with at least 20 seedlings (horizontal placement) or 7 seedlings (vertical placement) per Petri Dish were carried out for each treatment. The experiment was repeated two times.

To test the effect of B55 VOCs on seedlings grown under different SO_4^{-2} supply, WT and *35S-etr1* seedlings were germinated in one side of a bi-partite Petri dish containing MM2 medium supplemented with different $MgSO_4x7H_2O$ concentrations [0, 50 or 1000 µM]). After 4 d, plates were opened under a safety hood and 5 µL of bacterial suspension (in sterile water, OD_{600}=1.0) or water were applied on the other side of the bi-partite dish, containing 0.5xYPDA.

Growth effects in terms of lateral root branches were assessed 11 d after sowing (vertical placement, n ≥ 4 Petri dishes with 4 seeds per dish). Seedling surface area, chlorophyll a and b content, free methionine (Met) and glutathion (GSH) levels were determined from 15 d old seedlings (horizontal placement, n≥ 4 Petri dishes with ca. 20 seeds per dish). The experiment was repeated twice for WT and once for *35S-etr1*.

Analysis of glutathiones (γ-glutamyl-cysteinyl-glycine)

The concentrations of reduced (GSH) and oxidized (GSSG) glutathion levels were determined according to the modified protocol by Rellan-Alvarez *et al.* (2006). Briefly, 50-70 mg fresh nitrogen (N_2)-ground shoot tissue were extracted with 200 μL ice-cooled extraction buffer (5 % [w/v] metaphosphoric acid, 0.1 % [v/v] formic acid, 1 % [w/v] Polyvinyl-polypyrrolidone (PVPP) and 10 ng/μL GSH internal standard [glycine-$^{13}C_2$,^{15}N, Sigma-Aldrich, Germany]) for 5 min using a Vortex. After centrifugation at 4°C for 20 min at 15 000 g, the pellet was re-extracted with 150 μL extraction buffer. Two hundred μL pooled extract was used for LC-MS analysis on a Varian 1200 triple quad spectrometer. Separation was performed on a Varian ProStar HPLC system (Econosil CN 5μm 250 x 4.6 mm, Alltech, Illinois, USA) with a mobile phase A: water, 0.05 % formic acid, 0.1 % acetonitrile and B: methanol. The elution profile was: 0-5 min, 5 % B; 5-12 min, 5-60 % B; 12-20 min, 60 % B. The flow rate of 1 mL min^{-1} was reduced to 250 μL min^{-1} by a LC Packings 1:4 fixed splitter. The triple quadrupole mass spectrometer was operated in multiple reaction monitoring (MRM) mode, using the following ion transitions: (*m/z* Q1 → Q3/collision energy): reduced GSH (*m/z* 306 → 143/19 V); (glycine-$^{13}C_2$,^{15}N)-GSH (*m/z* 309 → 146/19V); GSSG (*m/z* 611→ 305/25V).

Analysis of free methionines

Free Met content was assessed by extracting ca. 50 mg finely ground shoot sample in 80 % methanol for 5 min. The diluted extracts were directly analyzed by LC-MS/MS after a modified protocol by Jander *et al.* (2004). Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) and separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 μm, Agilent Technologies, Germany). Formic acid (0.05 %) in water and acetonitrile were employed as mobile phases "A" and "B", respectively. The elution profile was: 0-1 min, 3 % B; 1-2.7 min, 3-100 % B in A; 2.7-3 min 100 % B and 3.1-6 min 3 % B. The mobile phase flow rate was 1.1 mL min^{-1}. The column temperature was maintained at 25 °C. The liquid chromatography was coupled to an API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source operated in positive ionization mode. The instrument parameters were optimized by infusion experiments with pure standards (amino acid standard mix, Fluka, St. Louis, USA). The ionspray voltage was maintained at 5500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. MRM was used to monitor analyte parent ion → product ion: MRMs were chosen as following: Met (*m/z* 150→104), ^{13}C, ^{15}N-Met (*m/z* 156→109). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing. All samples were spiked

with ^{13}C, ^{15}N labeled amino acids (algal amino acids ^{13}C, ^{15}N, Isotec, Miamisburg, US) at a concentration of 10 ug of the mix per mL. The concentration of the individual labeled amino acids in the mix had been determined by classical HPLC-fluorescence detection analysis after pre-column derivatization with ortho-phthaldialdehyde-mercaptoethanol. Met was quantified by using ^{13}C, ^{15}N labeled Met as an internal standard.

Volatile collection and analysis

Volatile organic compounds of bi-partite Petri dishes containing medium (0.5xYPDA|GB5), B55, B55+WT seedlings (co-cultivation) or WT seedlings only were collected 14 d after inoculation by headspace sampling for 20 min with a SPME Divinyl-Carboxen-PDMS fiber (Sigma-Aldrich, Steinheim, Germany) and analyzed on a Varian CP-3800 GC coupled with a Varian Saturn 4000 ion trap MS in electron ionisation (EI; 70 eV) mode (Varian, Palo Alto, CA). The sample (SPME fiber) was injected into the GC and volatiles separated on a DB-5 column (30 m×0.25 mm I.D., 0.25 μm film thickness, Agilent, Boeblingen, Germany) with helium at a constant flow of 1 mL min^{-1} as the carrier gas. The injector temperature was at 230 °C; the oven temperature program was: 40 °C for 5 min, 185 °C at 5.0 °C min^{-1}, and a 30 °C min^{-1} ramp to 300 °C. EI spectra were recorded on Scan mode from *m/z* 40 to 300. DMDS was identified by library search and by comparison to synthetic DMDS (98 %, Sigma-Aldrich, Steinheim, Germany). Quantification of DMDS was performed in the linear range of detection based on calibration curves generated with increasing concentrations of commercial DMDS mixes in methanol. Other volatiles detected and identified by library search are summarized in Supplemental Table 2 online.

Characterization of dimethyl disulfide production by B55

The effect of age, colony size, culture medium, light, temperature and the presence of WT seedlings on the Petri dish headspace concentration of DMDS was evaluated. Therefore DMDS headspace concentration of B55 cultures of different age (ranging from 5 to 15 d), B55 cultures grown on different media (0.125x, 0.25x, 0.5xYPDA), under different temperature (26 and 30 °C) and light conditions (dark vs. 16 h light d^{-1}) were analyzed by GC-MS. The effect of seedlings on DMDS concentration in the headspace was analyzed by co-cultivating B55 with different amounts of seedlings (n= 3, 5, 10, 15 or 25 seeds) for 12 d on two-partite Petri dishes (GB5|0.5x YPDA).

Carbon dioxide experiments

In order to test whether elevated carbon dioxide (CO_2) levels arising from bacterial growth affect WT seedling growth, CO_2 was trapped with $Ba(OH)_2$ by the formation of $BaCO_3$ as described by Kai and Piechulla (2009). Trapping experiments were performed in tri-partite Petri dishes. Seedlings were grown as described above, except that the GB5 medium was amended with 1.2 % sucrose. The bacterial CO_2 production was indirectly assessed by the formation of $BaCO_3$. Five μL of B55 were applied onto 0.5xYPDA while WT seeds were co-cultivated or not. Seven mL of a fresh 0.1 M $Ba(OH)_2$ solution were filled in the third compartment and the plate was sealed two times with Parafilm. $BaCO_3$ formation was determined every day (d1-d6).

CO$_2$ production and effects were found to be immense (Supplemental Figure 12B online). To confine these effects in consecutive CO$_2$-trapping experiments, 5 µL of B55 suspension were applied on less nutritious medium (0.25xYPDA) and 6 d after sowing only. Leaf area was measured after 12 d and BaCO$_3$ dry mass was determined to assess the Ba(OH)$_2$ saturation level. Five replicates with at least 9 seedlings were performed.

Qualitative hydrogen sulfide test

H$_2$S production by B55 was tested qualitatively using lead acetate test strips (Sigma-Aldrich, Buchs, Switzerland). B55 cultures were set up according to the manual and color change from white to black, indicating the formation of lead sulfite, was checked after 36 h.

^{35}S-labeling experiments

Ten µL of B55 suspension (in sterile water; OD$_{600}$=1.0) were inoculated on 25 mL solid M9 medium supplemented containing 0.27 MBq Na$_2$^{35}SO$_4$ (Perkin-Elmer, Waltham, USA) (+^{35}S) as sole SO$_4^{-2}$ source in one compartment of a bi-partite Petri dish. In the other compartment WT seedlings were germinated on modified MM2 medium supplemented with 2 mM MgSO$_4$ x 7H$_2$O (+SO$_4$) or without MgSO$_4$x7H$_2$O (-SO$_4$) (Figure 3A). The plates were kept in a hood of the radioactive lab at RT at a 16/8 h day/night cycle and were illuminated with a self-build transportable lamp (stand: Trop Aquarienleuchte Typ E4/0-S 1x15 Watt; lamp: Osram L15W light color 954, Lumilux de Luxe [Osram, Munich, Germany]). Seventeen days after inoculation, seedlings were harvested in liquid N$_2$ for scintillation measurements and total protein extraction. Proteins were extracted with a buffer containing 100 mM Hepes, pH 7.5, 5 mM EGTA, 5 mM EDTA, 10 mM DTT, 10 mM Na$_3$VO$_4$, 10 mM NaF, 50 mM glycerol-2-phosphate, 1 mM PMSF, 10 % glycerol; separated with 10 % SDS-PAGE (Sodiumdodecylsulfate-Polyacrylamide Gelelectrophoresis), dried for 30 min on a gel dryer (583, BioRad, Bio-Rad Laboratories, München, Germany), exposed to positron imaging plate (FLA 3000 system, Fujifilm, Tokyo, Japan) and scanned after 7 d exposure. For scintillation counting 50-100 mg fresh mass of fine-ground frozen seedling tissue were incubated in 1 mL sodium hypochlorite solution for 1 h at 60 °C. After cooling to room temperature, 15 mL Hionic-Fluor (Perkin-Elmer, Waltham, USA) was added and scintillation counts were measured (Win Spectral 1414, Perkin-Elmer, Waltham, USA).

To determine whether *35S-etr1* seedlings adsorb higher concentrations of labeled S, the same experiment was carried out once more with tri-partite Petri dishes: one third containing WT seeds, the other *35S-etr1* seeds and the last third containing B55. After 20 d of co-cultivation, scintillation counts were measured. At least 6 Petri dishes were done for each treatment. This experiment was performed twice for WT and once for *35S-etr1*.

Application of synthetic dimethyl disulfide

Serial concentrations of DMDS in methanol (0.5, 5, 50, 500 and 2000 µg) were applied onto a cotton bud placed in an empty side of a bi-partite Petri dish while WT seeds were sown in the other side containing MM2 medium (supplemented with different MgSO$_4$x7H$_2$O concentrations [0, 50 or 1000 µM]). The volume of 0.5 µL (500 µg) DMDS / Petri Dish was

found to promote growth best for SO_4^{-2}-starved seedlings (Supplemental Figure 6 online) and further DMDS PGP experiments were carried out using this concentration.

Petri dishes containing WT or *35S-etr1* seeds sown on MM2 media on the one side and a cotton bud containing DMDS (or not) on the other side, were sealed three times with Parafilm and kept in a Percival growth chamber (16/8 h day/night cycle, 30/28 °C) for at least 17 d in vertical or horizontal position before PGP effects were evaluated in terms of seedling surface area, chlorophyll a and b content, lateral root number, free Met and GSH concentration. Five plates with at least 15 or 7 seeds were done for each treatment for horizontal and vertical placement, respectively. The experiment was repeated two times.

RNA extraction and quantitative real time polymerase chain reaction conditions

To analyze whether different SO_4^{-2} concentrations in the MM2 medium and DMDS application modulate the expression of genes involved in plant S metabolism, quantitative polymerase chain reaction (qPCR) was carried out. WT and *35S-etr1* seeds were sown on one side of a two-partite Petri dish containing MM2 medium (supplemented with different $MgSO_4x7H_2O$ concentrations [0, 50 or 1000 µM]) and 0.5 µL pure DMDS were applied or not onto a cotton bud positioned in the other side of a two-partite Petri dish. After 18 d of growth, seedlings were harvested, root and shoot separated and immediately frozen in liquid N_2. RNA was extracted from 200 mg fine-ground frozen material after the protocol of Kistner and Matamoros (2005).

To test whether the presence of WT seedlings affects B55's DMDS production, we analyzed the gene expression of the B55 *CYSTATHIONINE-B-LYASE* (Bm QM1551 *CBL*). CBL converts L-Met to methanethiol, the precursor of DMDS. Bacterial RNA was extracted from 10 d old colonies co-cultured on bi-partite Petri dishes with or without WT seedlings after the protocol of Majumdar *et al.* (1991). One loop of cells was harvested, suspended in 200 µL sterile water and mixed with ½ vol cold killing-buffer (20 mM Tris-HCl, pH 7,5; 5 mM $MgCl_2$; 20 mM sodiumazid) and centrifuged at 8500 rpm at 4 °C for 10 min. The supernatant was discarded and the pellet washed again with 1 mL killing buffer and centrifuged before the pellet was re-suspended in 500 µL lysis buffer (200 mM NaCl, 3 mM EDTA). The resulting suspension was transferred to a 2 mL Eppendorf tube containing 500 µL glass beads (0.25-0.5 mm, Karl Roth, Karlsruhe, Germany) and 500 µL phenol-chloroform-isoamylalcohol. The mixture was vortexed for 2 min at highest speed, cooled down on ice for 2 min and centrifuged at 4 °C for 5 min at 15000 rpm. The water phase was transferred to a new tube, re-extracted with 600 µL phenol-chloroform-isoamylalcohol. The supernatant was added to a new Eppendorf tube containing 600 µL chloroform-isoamylalcohol, vortexed for 5 min and centrifuged for 5 min at 13000 rpm, before the supernatant was re-extracted with 600 µL chloroform-isoamylalcohol. The resulting supernatant was mixed with 1/10 vol 3 M Sodium-acetate solution (pH 5.2) and 2.5 x vol ice-cold ethanol and inverted 10 times. The samples were kept for 2 h at -80 °C and then centrifuged at 4 °C and 15000 rpm for 25 min. The pellet was washed with 500 µL 70 % ice-cold ethanol (RNA grade) and centrifuged at 4 °C, at 15000 rpm for 20 min. The supernatant was removed and the pellet dried before it was suspended in DEPC water. The RNA was subjected to a DNase treatment using the RQ1 RNase-Free DNase kit (Promega, Mannheim, Germany). Copy DNA

(cDNA) synthesis using either Oligo-dT (plant) or random hexamers (bacteria) was carried out according to Mavrodi *et al.* (2012). qPCR was run on a Mx3005P qPCR system (Stratagene, Santa Clara, CA, USA). The gene-specific PCR products were detected with qPCR Core Kit for SYBR® Green I (Eurogentec, Seraing, Belgium). The *N. attenuata ELONGATION FACTOR1A* (Na*EF1a*) and *B. megaterium* QM B1551 *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (Bm QM1551 *GADPH)* genes served as internal standards for normalizing cDNA. Sequence accession numbers of *N. attenuata* genes tested (Na*SULTR1,* Na*SIR,* Na*METSYN1*) and primers used for qPCR are summarized in Supplemental Tables 2 and 3. qPCR conditions were set according to manufacturer's manual. The experiment was carried out once with $n \geq 4$.

Ethylene measurements and glasshouse dimethyl disulfide treatments

Sterilized WT and *35S-etr1* seeds were sown in sterile three-necked flasks containing 25 mL MM2 medium, supplemented with different $MgSO_4x7H_2O$ concentrations [0, 50 or 1000 µM]), tightly sealed, and cultivated in a Percival growth chamber (16/8 h day/night cycle, 155 µmol m^{-2} s^{-1}, 30/28 °C). After 17 d, flasks containing seedlings were subjected to ET measurements using a photoacoustic spectrometer (INVIVO, Sankt Augustin, Germany), as described by von Dahl *et al.* (2007); flasks containing only medium served as blanks. The experiment was carried out twice, with n=4 flasks containing ca. 20 seeds.

To test the effect of ET insensitivity on DMDS-related ET emission, 41 d old WT and *35S-etr1* plants (routinely grown in 10 cm-round pots containing sand and lecaton, for growth conditions see Meldau et al, 2012) were wrapped in a transparent plastic bag and exposed overnight to 100 µL 0.1 % 1- MCP (1-Methylcyclopropene, an ET-receptor inhibitor) or the solvent 0.75 % KOH+NaOH only (von Dahl *et al.*, 2007). The next day plants were treated with 10 µL DMDS or not. One day later, three leaves were wounded and the whole rosette was cut, transferred to a three-necked-flask, in which the ET accumulated for 5 h before it was measured. The experiment was carried out once with 5 replicates per treatment combination.

To observe DMDS-dose dependent effects, 32 d old non-inoculated WT and *35S-etr1* plants were wrapped in transparent plastic bags and fumigated for 24 h with 0.5, 2.5, 5.0 or 10.0 µL DMDS. The next day, three leaves were wounded and the whole rosette was harvested and ET accumulated for 5 h before emissions from DMDS treated or control WT and *35S-etr1* plants were measured. The experiment was carried out once with 5 replicates per treatment combination.

Statistical analysis

Data analysis was performed with the StatView software package (SAS Institute) with a completely randomized analysis of variance. One-way and multiple way ANOVAs followed by Fisher's PLSD test or t-test were used to compare differences among treatments. Correlation analysis was performed with simple regression tests.

5.7 Acknowledgements

5.8 Figures
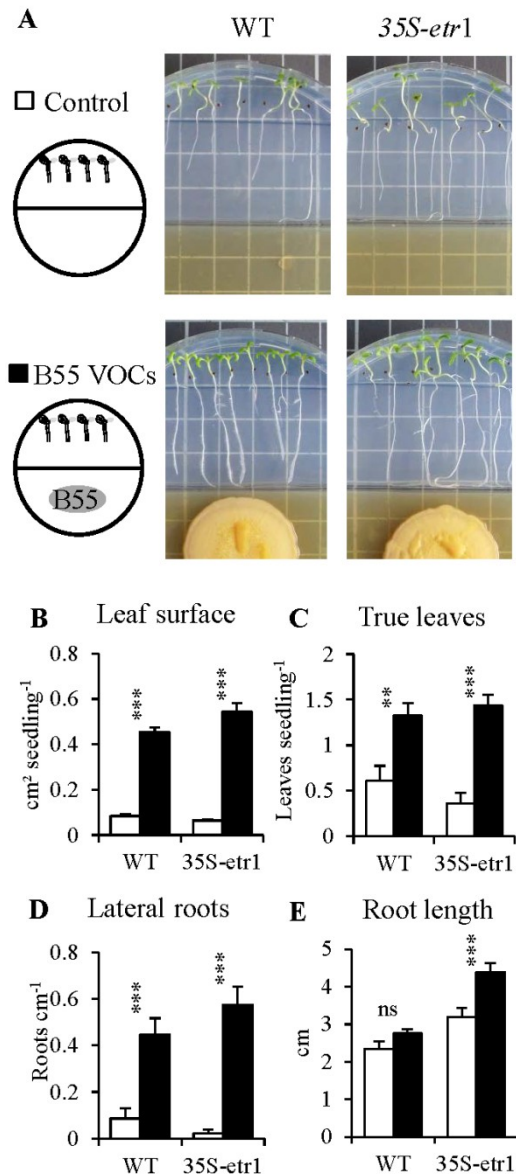
Figure 1. Effect of B55 Volatile Organic Compounds on Seedling Growth

Experimental design and effect of B55 VOCs. WT and *35S-etr1* seedlings were co-cultivated with or without B55 for 12 d in bi-partite Petri dishes containing GB5 medium (seedlings) and 0.5xYPDA (B55) (A).

    (B) Mean (±SE) seedling surface area.
    (C) Mean (±SE) number of true leaves.
    (D) Mean (±SE) number of lateral roots.
    (E) Mean (±SE) primary root length.

For (B) to (E) a Student's t-test between mock- and B55 VOCs treated seeds was performed with ** P< 0.01; *** P< 0.001. VOCs= volatile organic compounds; ns= not significant. n= 4 Petri dishes with 20 (B and C) or 7 seeds (D and E).
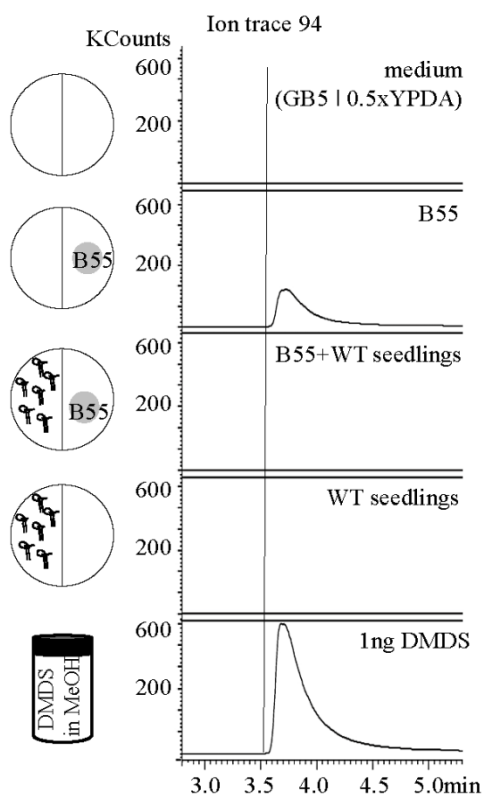
Figure 2. Depletion of Headspace DMDS after Co-cultivation of B55 with Seedlings
GC-MS spectra of the selected molecule ion 94 of cultivation medium, B55 alone, co-cultivation (B55+WT seedlings), WT seedlings alone and a DMDS standard. DMDS = dimethyl disulfide.
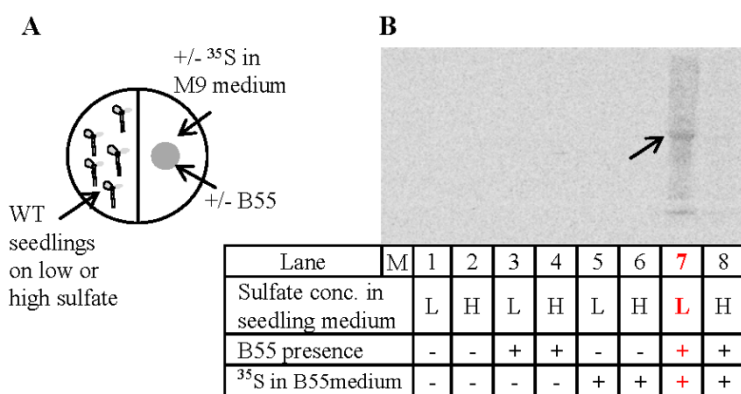

Figure 3. Incorporation of ^{35}S-labeled Volatile Sulfur into *N. attenuata* Seedling's Protein
(A) Set up of the experiment.
(B) Radioactivity screen and table summarizing treatment combinations. The arrow depicts the incorporation of ^{35}S into WT seedling protein.
L= low SO_4^{-2} concentration; H= high SO_4^{-2} concentration; M= marker.
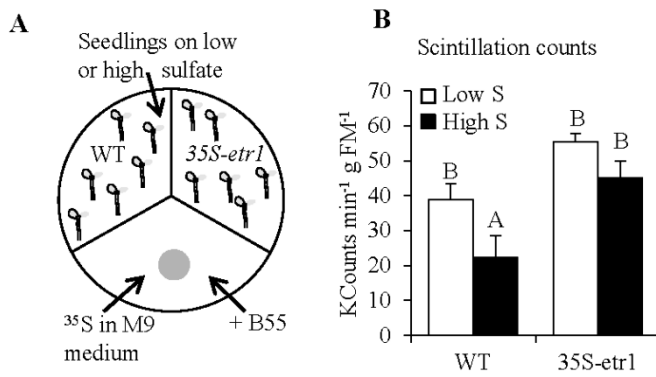
Figure 4. Uptake of ^{35}S-labeled Volatile Sulfur by *N. attenuata* Seedlings

(A) Set up of the experiment.
(B) Scintillation counts (±SE) of WT and *35S-etr1* total seedling tissue after 20 d of co-cultivation with B55. A PLSD test of an ANOVA between B55 VOCs treated WT and *35S-etr1* seedlings grown on low or high sulfate concentration was carried out. Different letters depict statistically differences at P< 0.05. S= sulfate; FM= fresh mass. n≥ 6 Petri dishes with 15 seeds.
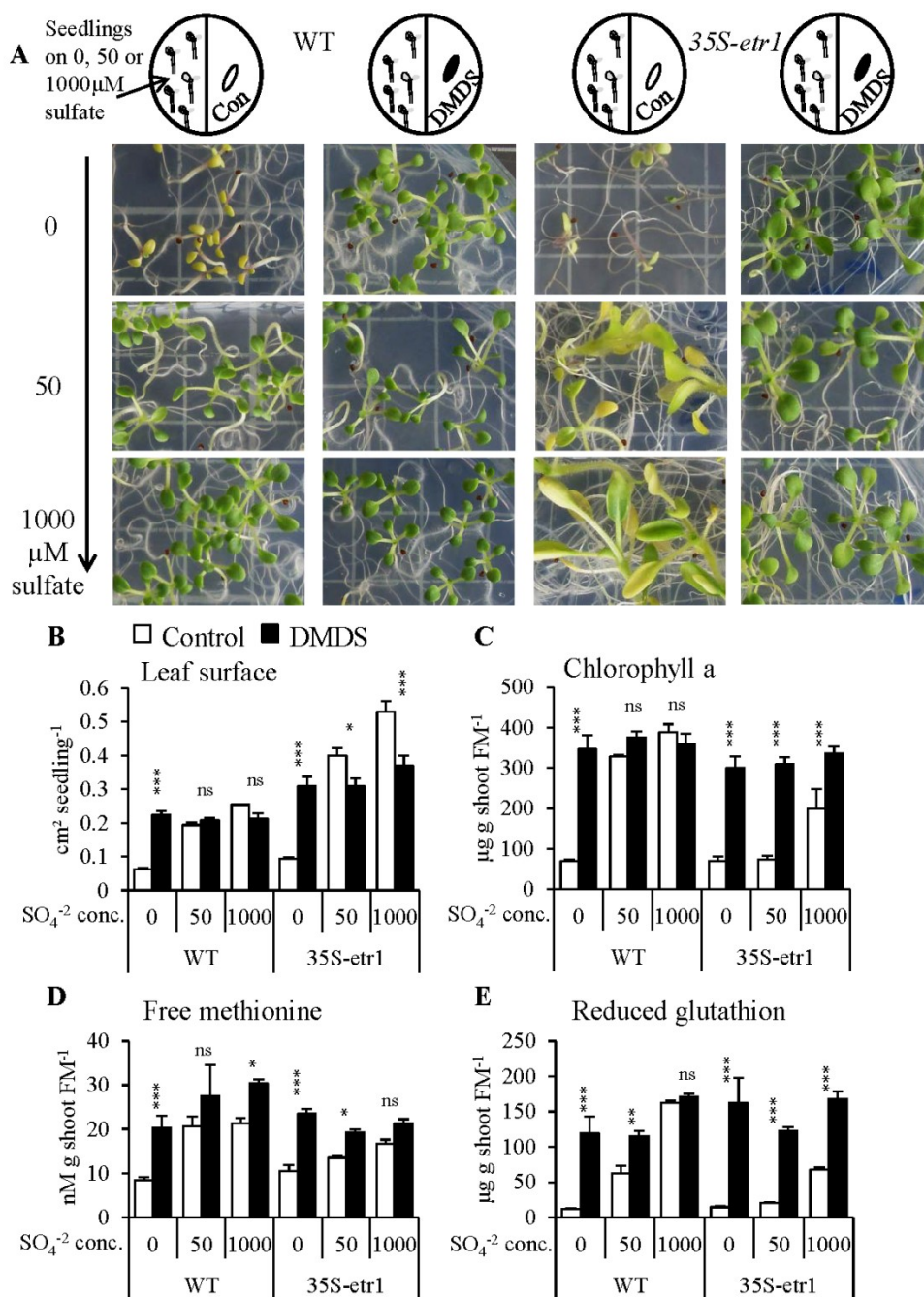
Figure 5. Effect of DMDS on Seedling Shoot Growth and S-containing Compounds

Experimental design and effect of DMDS on 20 d old WT and *35S-etr1* seedlings. Seedlings were grown in bi-partite Petri dishes containing MM2 medium supplemented with different sulfate concentrations (0, 50, 1000 µM MgSO$_4$*7H$_2$O) (A).

(B) Mean (±SE) shoot surface area of mock- or DMDS treated WT and *35S-etr1* seedlings.
(C) Mean (±SE) chlorophyll a content of mock- or DMDS treated WT and *35S-etr1* seedlings.
(D) Mean (±SE) free methionine content of mock- or DMDS treated WT and *35S-etr1* seedlings.

(E) Mean (±SE) reduced glutathion content of mock- or DMDS treated WT and *35S-etr1* seedlings.

For (B) to (E) a PLSD test of an ANOVA between mock- and DMDS treated seedlings was performed with * $P< 0.05$; **$P< 0.01$;*** $P< 0.001$. DMDS= dimethyl disulfide; FM= fresh mass; ns= not significant. n= 4 Petri dishes with 20 seeds.
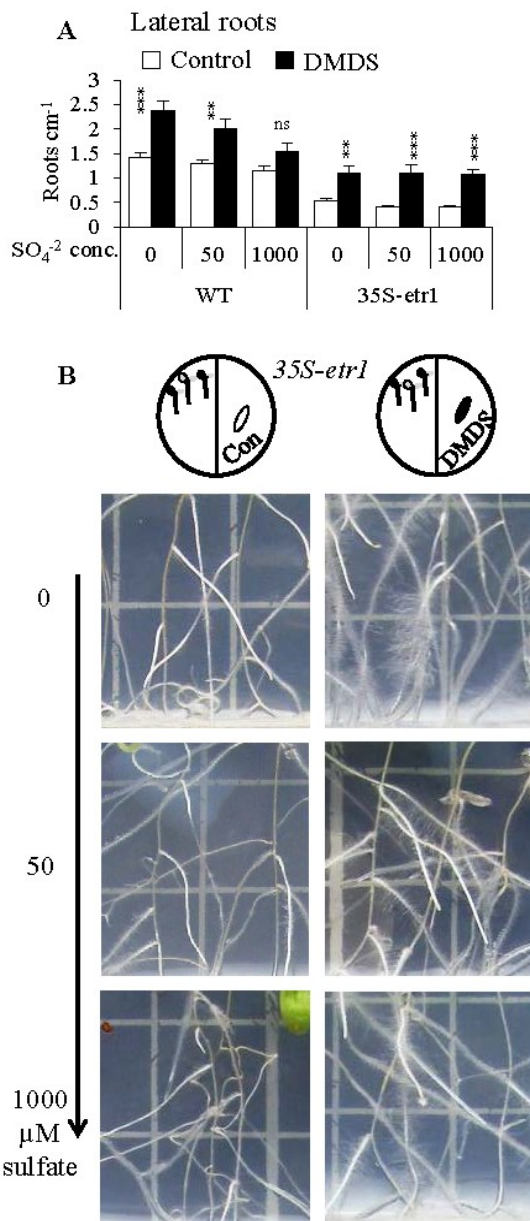
Figure 6. Effect of DMDS on Seedling Root Architecture

WT and *35S-etr1* seedlings were grown for 17 d in bi-partite Petri dishes containing MM2 medium (vertical placement) supplemented with different sulfate concentrations (0, 50, 1000 μM $MgSO_4 * 7H_2O$) and treated with or without DMDS.

- (A) Mean (±SE) lateral root number. A PLSD test of an ANOVA between mock- and DMDS-treated seedlings was performed with ** P< 0.01; *** P< 0.001. DMDS= dimethyl disulfide; ns= not significant. n= 4 Petri dishes ≥ 4 seeds.
- (B) Effect of DMDS on *35S-etr1* root hair formation.
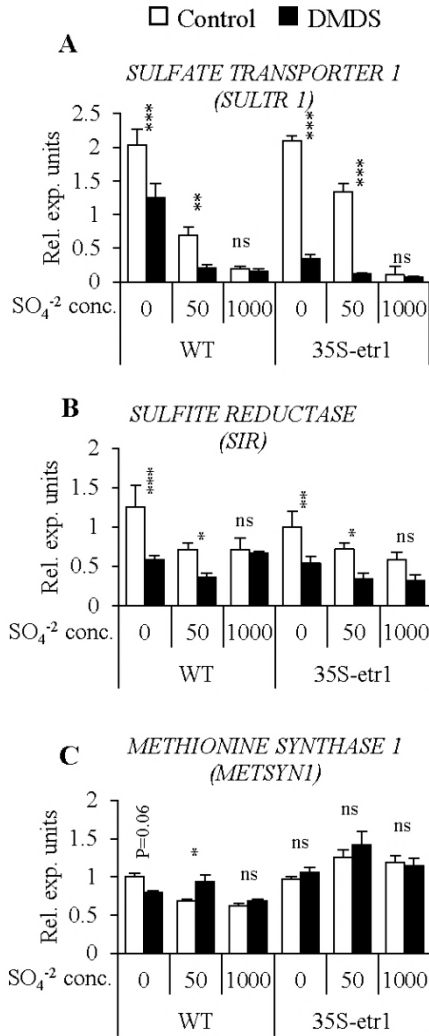
Figure 7. Effect of DMDS on the Expression of *N. attenuata* Genes Involved in S Metabolism

WT and *35S-etr1* seedlings were grown for 18 d in bi-partite Petri dishes containing MM2 medium supplemented with different sulfate concentrations (0, 50, 1000 μM $MgSO_4 \cdot 7H_2O$) and treated with or without DMDS.

    (A) Mean (±SE) relative transcript abundance of *SULFATE TRANSPORTER1 NaSULTR1.*

    (B) Mean (±SE) relative transcript abundance of *SULFITE REDUCTASE NaSIR*

    (C) Mean (±SE) relative transcript abundance of *METHIONINE SYNTHASE1 NaMETSYN1.*

For (A) to (C) a PLSD test of an ANOVA between mock- and DMDS- treated seedlings was performed with * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. DMDS= dimethyl disulfide; ns= not significant. $n \geq 4$, except for A, $n \geq 3$.
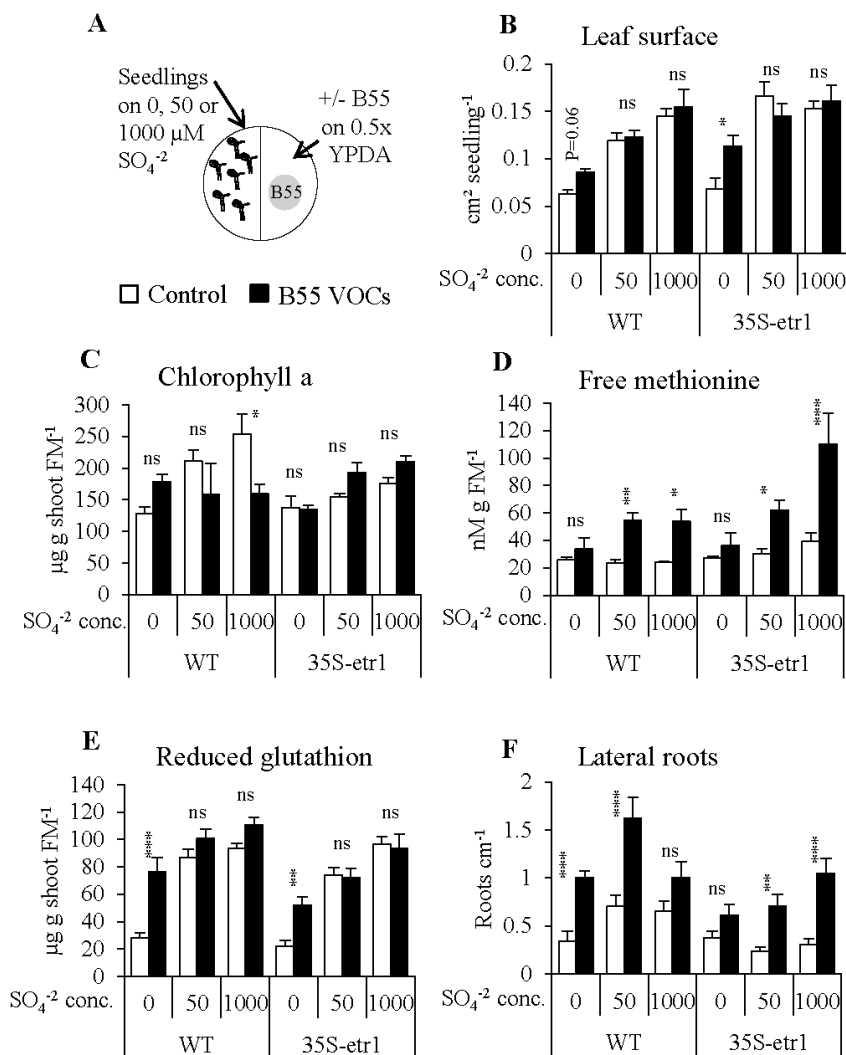
Figure 8. Effect of B55 VOCs on Seedlings Grown under Different Sulfate Supply
WT and *35S-etr1* seedlings were co-cultivated for 15 d (B-E) or 11 d (F) with or without B55 in bi-partite Petri dishes. Seedlings were grown on MM2 medium supplemented with different sulfate concentrations (0, 50, 1000 μM $MgSO_4*7H_2O$); B55 was grown on 0.5x YPDA.

- (A) Experimental design.
- (B) Mean (±SE) shoot surface area of mock- or VOC-treated WT and *35S-etr1* seedlings.
- (C) Mean (±SE) chlorophyll a content of mock- or VOC-treated WT and *35S-etr1* seedlings.
- (D) Mean (±SE) free methionine content of mock- or VOC-treated WT and *35S-etr1* seedlings.
- (E) Mean (±SE) reduced glutathione content of mock- or VOC-treated WT and *35S-etr1* seedlings.
- (F) Mean (±SE) lateral root number of vertically grown, mock- or VOC-treated WT and *35S-etr1* seedlings.

For (B) to (F) a PLSD test of an ANOVA between mock- and VOC-treated seedlings was performed with * $P < 0.05$; **$P < 0.01$; *** $P < 0.001$. VOC= volatile organic compound; FM= fresh mass; ns= not significant. $n \geq 3$ Petri dishes with 20 (B-E) or 4 seeds (F).
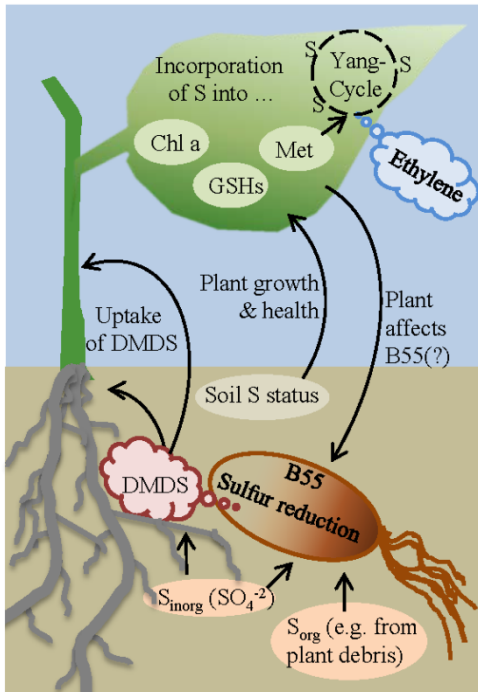
Figure 9. Proposed Model Summarizing the Volatile Organic Compound-mediated Mutualistic Interaction between *Bacillus* sp. B55 and its Native Host *Nicotiana attenuata* Sulfur (S) availability can limit plant growth in many habitats. The *N. attenuata* root-colonizing bacterium B55 reduces (organic [org]) S and emits dimethyl disulfide (DMDS), which can be taken up by the plant. The already reduced S can be channeled into S-containing compounds (e.g. glutathiones [GSHs] or the amino acid methionine [Met]) and S-related metabolic pathways, leading to biosynthesis of chlorophyll (Chl a) or ethylene. By saving the plant's energy for reducing inorganic (inorg) S, plant growth is promoted by B55. In turn, the plant might affect B55's growth and DMDS production positively.

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5.10 Supplemental figures and tables
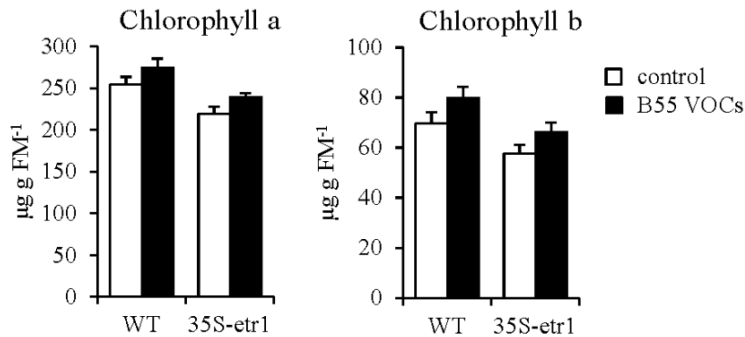
Supplemental Figure 1. Effect of B55 Volatile Organic Compounds on Seedling Chlorophyll Content
Mean (±SE) chlorophyll a and b content of mock- or B55 VOC-treated WT and *35S-etr1* seedlings grown on GB5 medium. VOC= volatile organic compound; FM = fresh mass. n= 4 Petri dishes with 20 seeds.
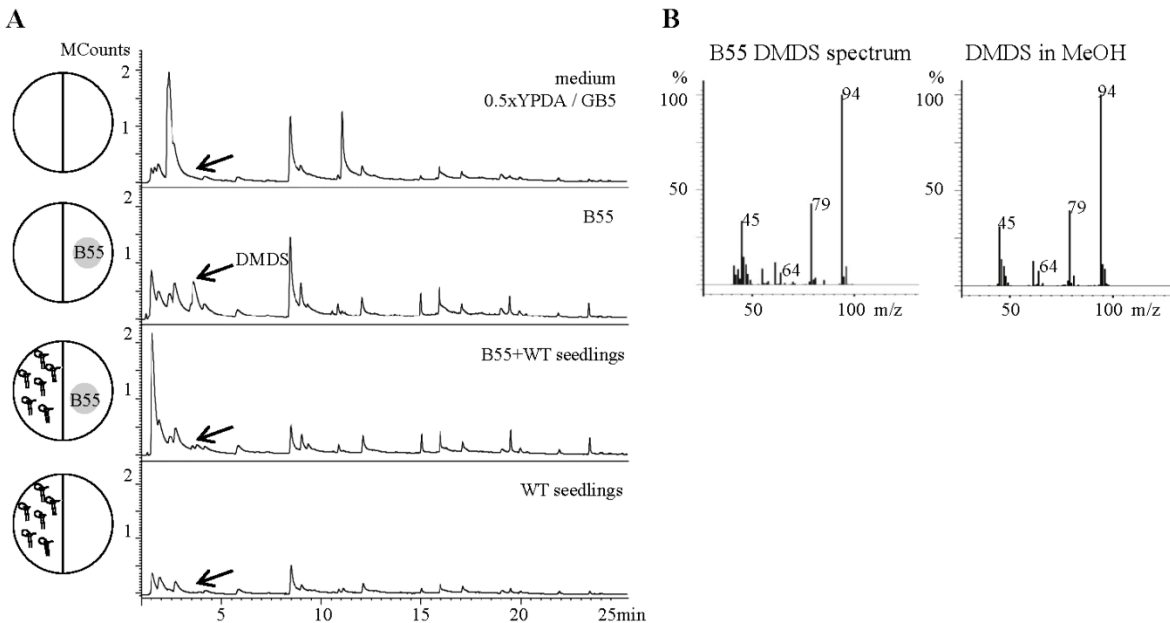
Supplemental Figure 2. Chromatographic Profile of B55 Volatile Organic Compounds
(A) Total ion current (TIC) GC-MS spectra of cultivation medium, B55 alone, co-cultivation (B55+WT seedlings), WT seedlings alone. The arrow depicts dimethyl disulfide (DMDS). The compound was identified as DMDS by comparison to an authentic standard.
(B) DMDS mass spectra obtained from B55 VOC bouquet (left panel) and DMDS standard (right panel).

A DMDS headspace conc.

B *B55 CYSTATHIONINE-ß-LYASE*

$R^2 = 0.8082$
$P<0.0001$
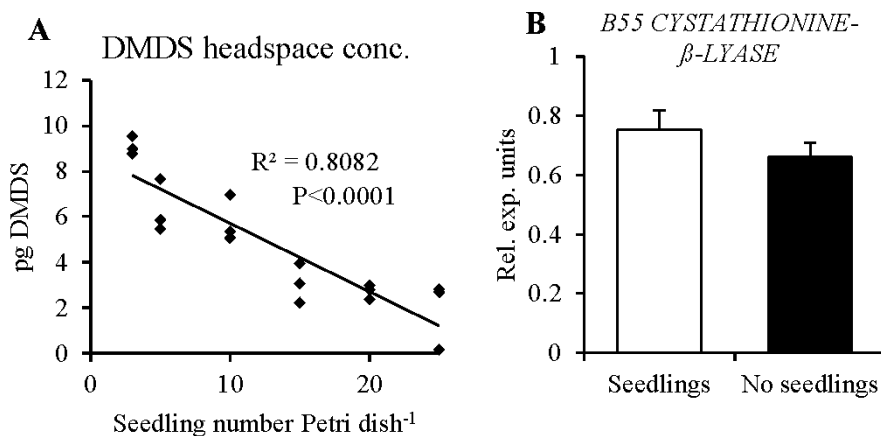
Supplemental Figure 3. Dimethyl Disulfide Emission by B55 as Affected by Seedlings

(A) Regression line showing the correlation between seedling number Petri dish[-1] and the headspace concentration of DMDS detected after 12 d of co-cultivation of B55. DMDS= dimethyl disulfide. A simple regression analysis was performed. n= 3 Petri dishes with 20 seeds.

(B) Transcriptional abundance (±SE) of the B55 *CYSTATHIONINE-ß-LYASE* (*CBL*) gene as affected by seedlings' presence. CBL performs the enzymatic conversion of L-methionine to methanethiol, before the latter is spontaneously converted to DMDS. n≥ 4.
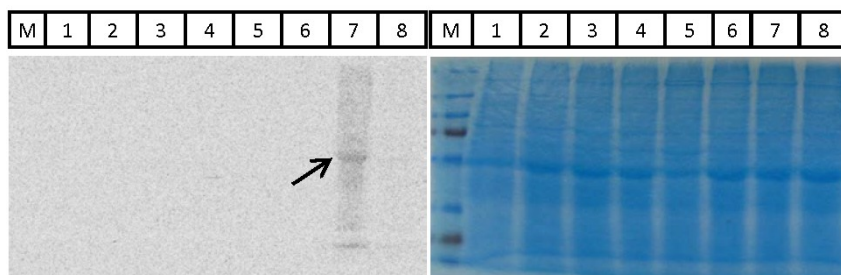
Supplemental Figure 4. Radioactive Screen and Protein Loading Gel
The arrow depicts the incorporation of 35S into WT seedling protein (Lane 7).

A Effect of medium

B Effect of age

C Effect of light and temperature

$y = 0.0101e^{4.9188x}$

$P=0.0003$
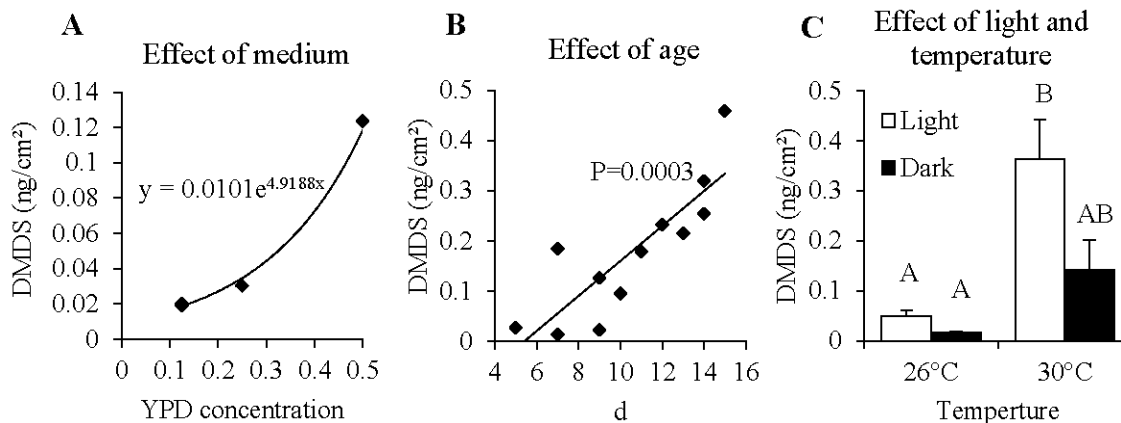
Supplemental Figure 5. Characteristics of DMDS Production

(A) Effect of medium (16 d) on B55 DMDS production.

(B) Effect of colony age (5-16 d) on B55 DMDS production. Bacteria were cultivated on 0.5xYPDA. A simple regression analysis was performed.

(C) Effect of light and inoculation temperature (10 d) on B55 DMDS production (±SE). Bacteria were cultivated on 0.5xYPDA. A PLSD test of an ANOVA between light and cultivation temperature was performed. Different letters depict statistically significant differences at $P< 0.05$. n= 3.

For (A) to (C) DMDS= dimethyl disulfide.

A

0   50   1000 µM sulfate

DMDS
conc.
2000 µg

500 µg

50 µg

5 µg

0.5 µg

Control
(MeOH)

B Shoot surface area   ☐ 0 µM   ▨ 50 µM   ■ 1000 µM sulfate
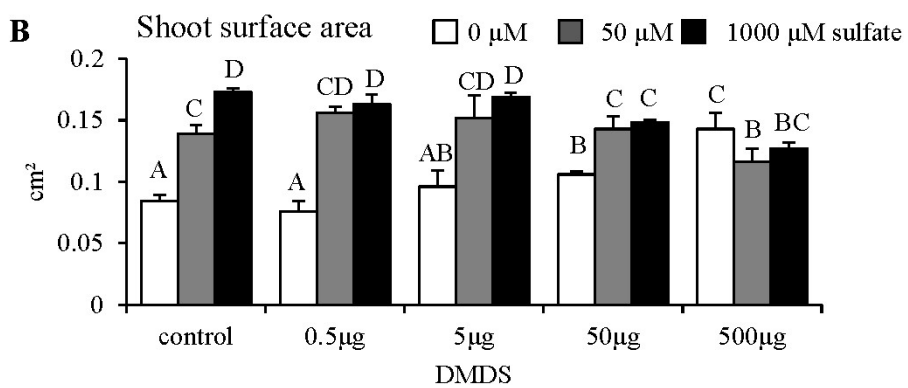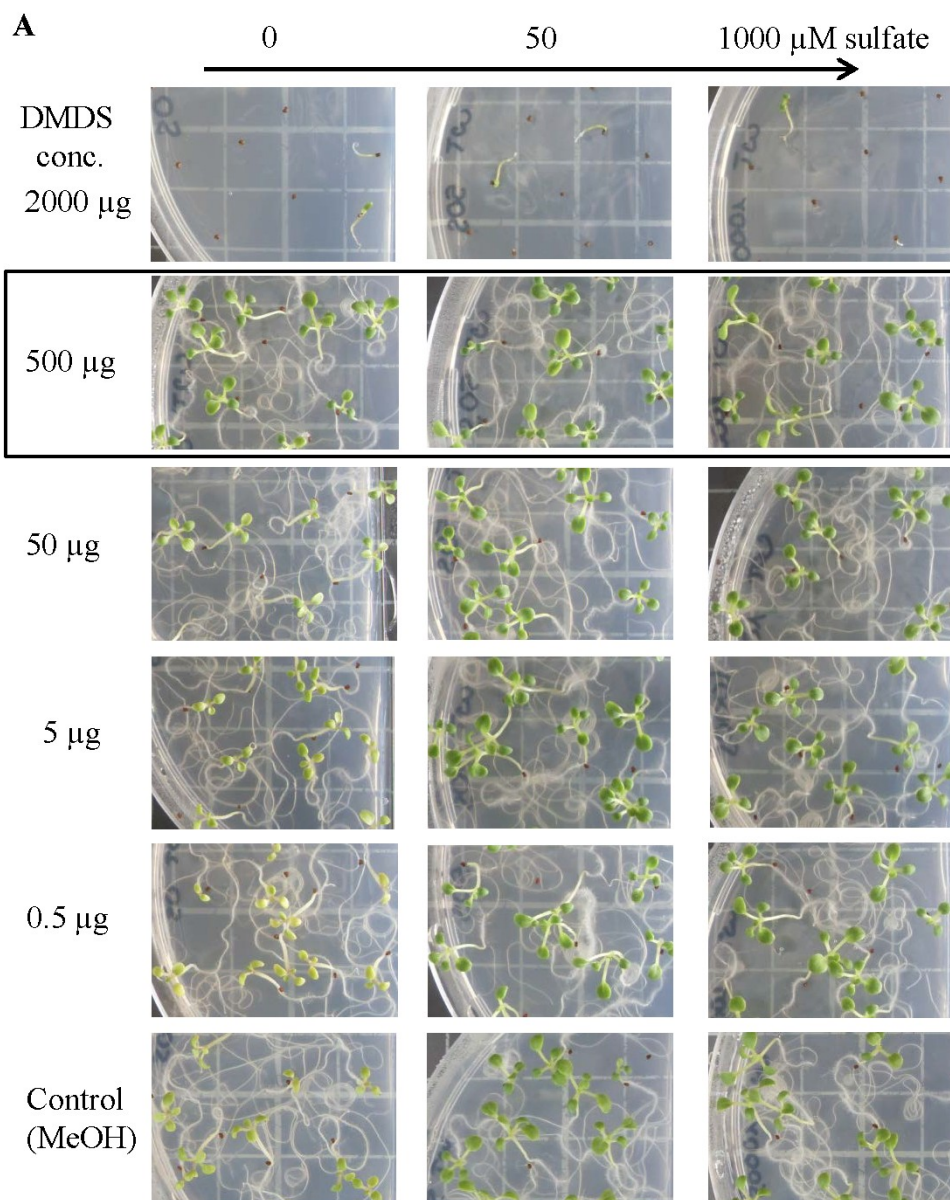
Supplemental Figure 6. Serial Application of DMDS

WT seedlings were grown for 17 d on MM2 medium supplemented with different sulfate concentrations and exposed to 0, 0.5, 5, 50, 500 μg DMDS diluted in MeOH.

- (A) Effect of different DMDS concentrations on WT seedling growth.
- (B) Mean (±SE) seedling surface area. A PLSD test of an ANOVA between different DMDS concentrations and medium sulfate supply was carried out. Different letters depict statistically significant differences at P< 0.05. n= 4 Petri dishes with 20 seeds.
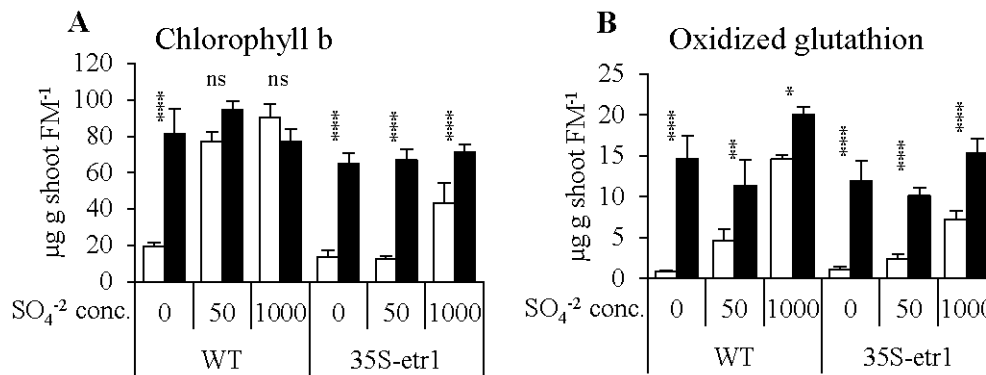
Supplemental Figure 7. Effect of DMDS on Chlorophyll b and Oxidized Glutathion Content

WT and *35S-etr*1 seedlings were grown for 20 d in bi-partite Petri dishes containing MM2 medium supplemented with different sulfate concentrations (0, 50, 1000 μM $MgSO_4 * 7H_2O$) and treated with or without DMDS.

- (A) Mean (±SE) chlorophyll b content of mock- or DMDS-treated WT and *35S-etr1* seedlings. n= 4 Petri dishes with 20 seeds.
- (B) Mean (±SE) oxidized glutathion content of mock- or DMDS treated WT and *35S-etr1* seedlings. n= 4 Petri dishes with 20 seeds.

For (A) and (B) a PLSD test of an ANOVA between mock- and DMDS treated seedlings was carried out with * P< 0.05; ** P<0.01; *** P< 0.001. DMDS= dimethyl disulfide; FM= fresh mass; ns= not significant.
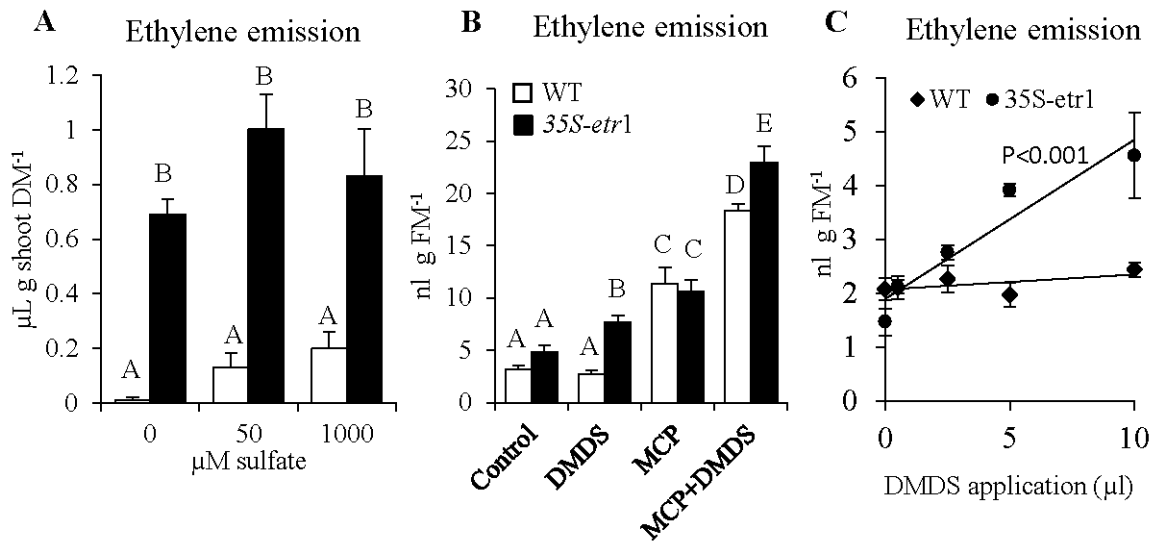
Supplemental Figure 8. Effect of Genotype and DMDS on Ethylene Production

    (A) Mean (±SE) ET emission of 17 d old WT and *35S-etr1* seedlings grown on MM2 media containing different sulfate concentrations. DM= dry mass. n= 4 flasks with 20 seeds.

    (B) Mean (±SE) ET emission of 41 d old glasshouse grown WT and *35S-etr1* plants fumigated with / without DMDS, 1-MCP or a combination of DMDS and 1-MCP. DMDS= dimethyl disulfide; MCP = 1-Methylcyclopropene; FM= fresh mass. n= 5.

    (C) Regression line showing correlation between DMDS application and ET emission of 32 d old glasshouse grown WT and *35S-etr1* plants treated with different amounts of DMDS. DMDS= dimethyl disulfide; FM= fresh mass. n= 5.

For (A) and (B) a PLSD test of an ANOVA between plant line and treatment combinations was performed. Different letters depict statistically significant differences at $P < 0.05$.

A

B
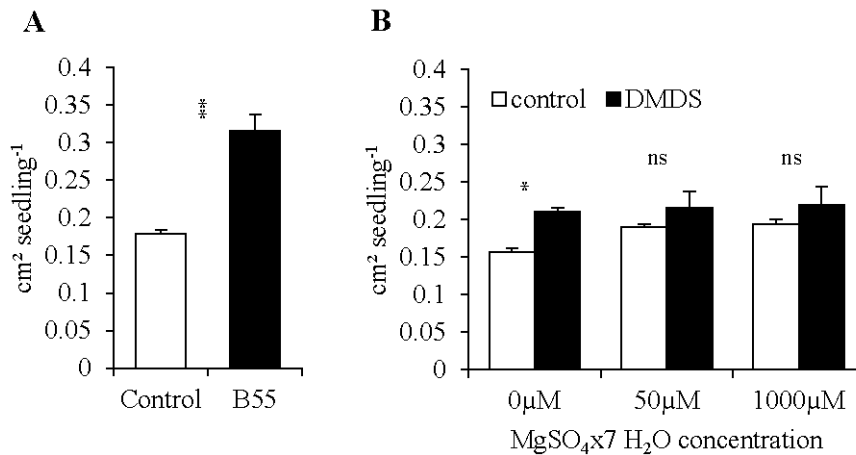
Supplemental Figure 9. Effect of B55 VOCs and DMDS on *Arabidopsis thaliana* Col-0 Seedlings

(A) Mean (±SE) seedling surface area of 14 d old *A. thaliana* Col-0 seedlings grown on 0.5x MS and exposed to B55 VOCs (bi-partite Petri dish). Student's t-test between mock- and B55 VOCs treated seeds was performed with **P< 0.01.

(B) Mean (±SE) seedling surface area of 17 d old *A. thaliana* Col-0 seedlings grown on MM2 supplemented with different sulfate concentrations and exposed to 0.5 μL pure DMDS Petri dish^{-1} (bi-partite Petri dish). A PLSD test of an ANOVA between mock- or DMDS-treated seeds with *P< 0.05 was performed.

For (A) and (B) VOCs = volatile organic compounds; ns = not significant. n= 3 Petri dishes with 15 seeds.
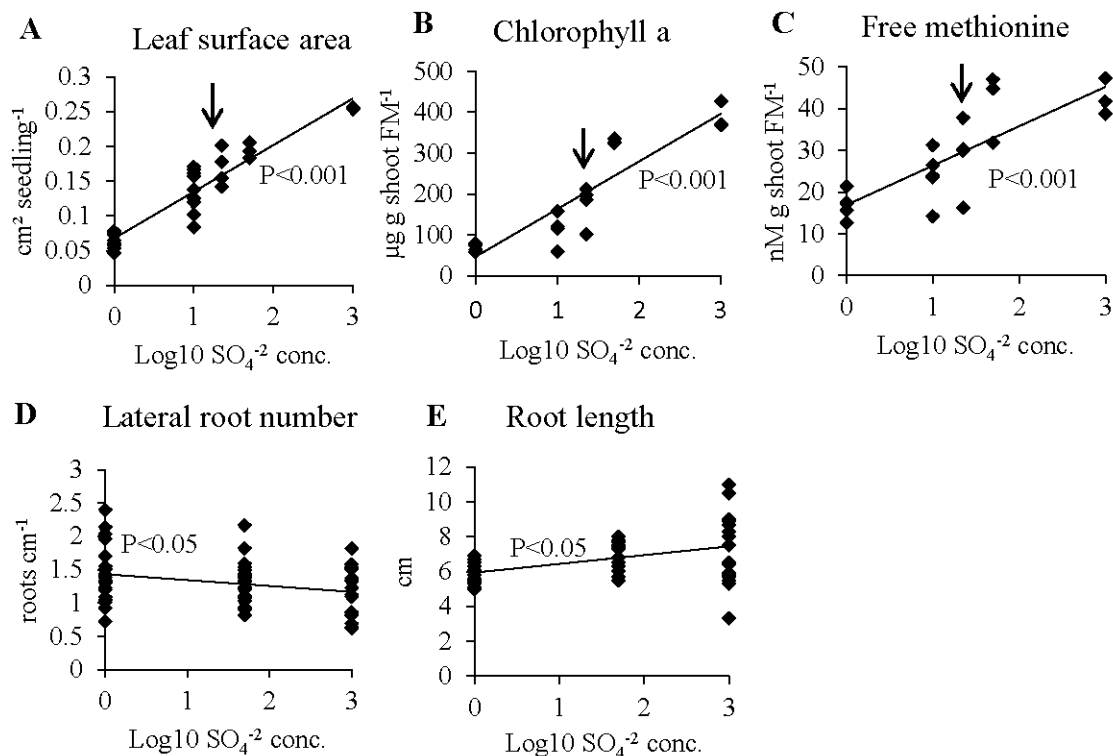
Supplemental Figure 10. Sulfate Effects on *In Vitro* Plant Growth

WT seedlings were grown for 20 d on MM2 medium supplemented with different sulfate concentrations. The arrow depicts native soil sulfate concentration (= ca. 22.5 µM). Regression lines showing the correlation between sulfate concentration in growth medium and

    (A) shoot surface area. n≥ 4 Petri dishes with 20 seeds.
    (B) chlorophyll a content. n≥ 4 Petri dishes with 20 seeds.
    (C) free methionine levels. n≥ 4 Petri dishes with 20 seeds.
    (D) lateral root number. n≥18 roots.
    (E) root length. n≥ 18 roots.

For (A) to (E) a simple regression analysis was performed. FM= fresh mass.
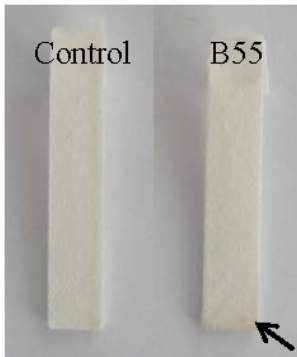
Supplemental Figure 11. Hydrogen Sulfide (H_2S) Production by B55

H_2S production was tested qualitatively by lead acetate strips. The arrow depicts a slight color change from white to gray, indicating low H_2S production.
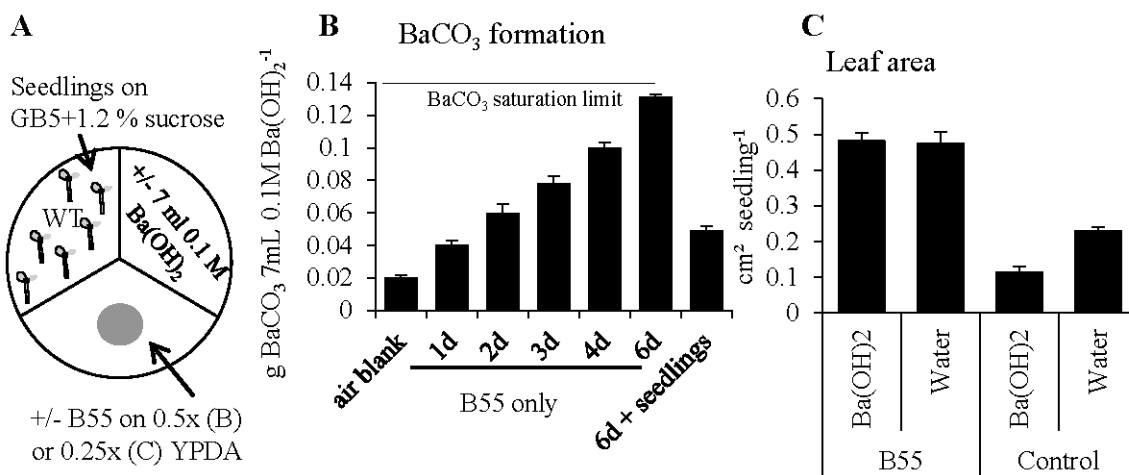
Supplemental Figure 12. Effect of Carbon Dioxide on Seedling Growth

   (A) Experimental set up.

   (B) Mean (±SE) $BaCO_3$ formation of B55 grown on 0.5xYPDA (alone, 1-6 d) or with WT seedlings (6 d). $BaCO_3$ formation of 6 d old B55 co-cultivated with seedlings was much lower compared to 6 d old B55 colonies cultivated alone, infering that seedlings have a higher affinity to CO_2 than $Ba(OH)_2$. n= 3 Petri dishes (with or without 15 seeds).

   (C) Mean (±SE) surface area of 12 d old WT seedlings co-cultivated with or without B55 in the presence of $Ba(OH)_2$ or water. Bacteria were cultivated on 0.25xYPDA. n≥ 5 Petri dishes with 15 seeds.

Supplemental Table 1 Volatile compounds exclusively emitted by B55 (but not by the medium itself) as identified by library search.

RT (min)	Identity
3.53	2-pentanol
3.79	2-butanone
10.37	S-methyl pentanethioate
10.61	unknown
11.23	2-heptanone, 5- or 6- methyl
13.76	benzyl alcohol

Supplemental Table 2 Gene accession numbers

Organism	Gene	Genbank accession nr.
Bacillus sp. B55	16S rRNA, partial	JX101913
N. attenuata	*SULFATE TRANSPORTER1, SULTR1*	Pending
N. attenuata	*SULFITE REDUCTASE, SIR*	Pending
N. attenuata	*METHIONINE SYNTHASE1, METSYN1*	Pending

Supplemental Table 3 Primers used in this study

Gene	Primer 1 (5'-3')	Primer 2 (5'-3')
Na*EF1A*	CCACACTTCCCACATTGCTG	CGCATGTCCCTCACAGCAAA
Na*SULTR1*	GTCTCACTCTTGTTAGGTACTC	GCAGCGTGAGATAGGAAGTC
Na*SIR*	TGCCTGAGTGGGAATTCAAGA	CCTGAGTGCCTTCTTCATCG
Na*METSYN1*	TGTAGGTTAGTCTGCCCTCTG	GCTGGTATTGTAATAAGGAAATCC
Bm QM B1551 GADPH	GATCGCCTTCAGCAGCTTC	GCTATGCGTGTTCCACTCC
Bm QM B1551 CBL	TCTACAGCAGCCGATTGACC	CCACAACTAGACCGCCAATG

6 General discussion

Plant productivity is threatened by multiple factors, including biotic and abiotic stresses (e.g. pests and diseases, as well as nutrient depletion or drought). For example, the loss of agricultural productivity of crops due to pathogen infection is estimated at 13% (Lal, 1998; Pimentel *et al.*, 2000; Pimentel *et al.*, 2005). Also native plant populations suffer from pathogen attack and malnutrition (Gilbert, 2002) and hence they must have developed measures to confine fitness losses. One environmentally friendly possibility to restrict losses in agriculture is the utilization of free-living microbes, so-called plant growth-promoting bacteria (PGPB), as bio-inoculants (i.e. bio-protectants, bio-stimulants or bio-fertilizers) (Kloepper *et al.*, 1989; Kaymak, 2010). While mutualistic microbes have been studied intensely in an agricultural context, their role in the pest control and nutritional status of native plants has - with the exception of few model plant species (i.e. *Arabidopsis thaliana* or *Medicago truncatula*) - been insufficiently investigated (van Wees *et al.*, 2000; Zinniel *et al.*, 2002; Dong *et al.*, 2003; Van der Ent *et al.*, 2009; Schwachtje *et al.*, 2011; Jorquera *et al.*, 2012; Pineda *et al.*, 2012).

This dissertation aimed at unraveling the role of mutualistic bacterial associations of a native plant, coyote tobacco (*Nicotiana attenuata*), inhabiting the Great Basin Dessert in the USA. Because it germinates after fires to form monocultures where there is little competing vegetation, this annual pioneer plant is subjected to various kinds of biotic stresses, e.g. high intraspecific competition, herbivory and pathogen pressure (Baldwin *et al.*, 1994; Bahulikar *et al.*, 2004), making it a good model to study the relevance of associated mutualistic microbes for the survival of wild plants in the rough-and-tumble of their natural environments.

In my dissertation I assessed the relevance of plant ethylene (ET) signaling in the recruitment of mutualistic bacteria to the plant's root endosphere, and found that the culturable bacterial community of *N. attenuata* is determined by soil type rather than ET signaling (**Manuscript I**). Furthermore, I assessed the importance of a beneficial native bacterium, *Bacillus* sp. B55, in the growth and fitness of wild-type (WT) plants and an ET-insensitive line (*35S-etr1*) in nature (**Manuscript II**). Finally, I report on mechanisms responsible for B55's remarkable plant growth-promoting (PGP) effects. I can show that volatile organic compounds (VOCs) emitted by B55 promote seedling growth, and identify a sulfur (S)-containing VOC, dimethyl disulfide (DMDS), which is released by B55 and confers the observed PGP effects (**Manuscript III**).

6.1 Factors shaping *N. attenuata*'s culturable microbial community

Numerous studies have addressed the effects of soil type and plant genetic make-up (species, genotype or cultivar) on the assembly of indigenous root-associated bacterial communities (Badri & Vivanco, 2009; Aira *et al.*, 2010; Weinert *et al.*, 2010; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012); still, it remains unclear which factor (genotype or soil) dominates the assembly process (Berg & Smalla, 2009). While plant species effects are substantial, effects of transgenic modifications in crops, which, for example, facilitate the release of certain substances into the rhizosphere (e.g. phytases, which allow for better phosphate uptake) or the

accumulation of molecules in the root (e.g. zeaxanthin, a plant pigment, which is target for enhanced production in crops to improve human visual capacity) are marginal and comparable to cultivar-specific differences (George *et al.*, 2009; Weinert *et al.*, 2009; Chun *et al.*, 2011). On the other hand, the salicylic acid and JA signaling pathways have been reported to shape the indigenous leaf and rhizosphere microbial community of *A. thaliana* plants (Kniskern *et al.*, 2007; Hein *et al.*, 2008). Another study, using the model plant *A. thaliana* and a transgenic line overexpressing defensive glucosinolates in roots, reported effects on the microbial rhizosphere composition (Bressan *et al.*, 2009). From these reports, it appears that manipulating evolutionary and ecologically relevant traits in plant defense affects the structure of plant microbial communities, while the expression of "novel", evolutionarily irrelevant compounds does not affect this assembly.

In our study (**Manuscript I**), we assessed the root microbial communities of three different *N. attenuata* genotypes grown in four different native soil types. Specifically, we were interested in the role of ET signaling in the recruitment of endophytic bacteria, since endophytes are thought to establish a more stable interplay with their host (Hardoim *et al.*, 2008). The gaseous plant hormone ET has been shown to play an important role in plant-pathogen interactions (van Loon *et al.*, 2006), but effects of ET signaling on a plant's indigenous microbial community remained so far unknown.

Signaling effects are best studied using transgenic lines, which vary only in the expression of the gene of interest (although pleiotropic effects can occur) (Bergelson *et al.*, 1996). Hence, an untransformed wild type (WT) control, one transgenic line impaired in ET biosynthesis (*ir-aco1*) and one impaired in ET perception (*35S-etr1*) were used. Our study revealed that the soil type the plants were cultured in, generally determined the microbial assembly in the root endosphere. Although we also observed genotype-dependent changes in diversity, namely that ET signaling-impaired plants harbored a less diverse microbial community than WT, these were marginal. Hence, we concluded that soil factors are more important in shaping *N. attenuata's* root endosphere bacterial community than ET signaling. Furthermore, great variability in total colonization and species composition across the samples supports the hypothesis that stochastic processes prevail in community assembly, as discussed by Hardoim *et al.* (2008).

Recent advances in high-throughput sequencing technologies revolutionized research in plant-microbe interactions, and new technologies like pyrosequencing might be applied to obtain a more complete view of the ET-associated microbial community assembly, including non-culturable bacteria and fungi. The approach we followed (extracting culturable bacteria and assaying them on one culture medium) selects for fast-growing, easy-to-culture and very abundant bacterial isolates (i.e. *Bacillus* and *Pseudomonas* sp.), and draws an incomplete picture. Hence, conclusions about the magnitude of ET effects, we reasoned, should be treated with caution. Nevertheless, a recent study by Doornbos *et al.* (2010), using ET-insensitive tobacco plants (Tetr18) and a culture-independent approach, came to a similar finding: the plant's ET-insensitivity had only marginal effects on the rhizosphere microbial community composition. They attributed the minor changes in community structure to an increased susceptibility of Tetr18 plants to opportunistic microbes than to impairments in ET-signaling (Doornbos *et al.*, 2010). Furthermore, we found, as other studies before, that the overexpression of a mutated version of

the *A. thaliana* ET receptor (AtETR1) resulted in pleiotropic effects, giving rise to a severe root phenotype (lack of root hairs, few lateral braches, lack of gravitropism) (Luschnig *et al.*, 1998; Clark *et al.*, 1999), which might in turn affect the microbial communities to a greater extent than ET signaling itself. Innovative techniques, like inducible and transient manipulation of gene expression, might help to separate ET signaling from the pleiotropic effects of the mutant receptor (Koo *et al.*, 2009).

Although ET-signaling-mediated changes in bacterial species diversity were marginal (**Manuscript I**), they might nevertheless impact plant health, as it is often the case for the occurrence of a single pathogenic microbe or the inoculation of a PGP strain. Several studies working with *single* microbial isolates reported on the importance of ET in modulating mutualistic plant-microbe interactions; and the hormone was shown to determine the balance between mutualism and pathogenicity (Dong *et al.*, 2003; Camehl *et al.*, 2010). Performing *in vitro* inoculation assays using selected bacterial isolates, we confirmed plant genotype-specific colonization by *Pseudomonas thivervalensis,* which exclusively entered the ET-insensitive line *35S-etr1* (**Manuscript I**). We infer that changes the root biochemistry, e.g. in the metabolome or root exudates of *35S-etr1* plants might promote this specific interaction. Similar effects have been shown before by Oger *et al.* (2004). They found that the production of opines in transgenic *Lotus corniculatus* roots led to 10^4-fold enrichment of opine-utilizing bacterial isolates (e.g. *Agrobacterium* sp.) in the rhizosphere. Ongoing research on *N. attenuata* root exudates suggests that ET signaling indeed affects root exudate composition and hence might facilitate these specific plant genotype-microbe interactions (M. Bonilla, unpublished results).

From our findings and the related literature, it becomes clear that the role of ET in mutualistic plant-microbe is not well understood. In-depth analyses using state-of-the-art technologies, like culture-independent analyses of plant microbial communities as well as innovative plant genetic manipulation strategies, should be employed to disentangle ET-mediated effects at the microbial community level as well as specific (single strain) plant-microbe interactions.

6.2   Plant growth promotion under natural conditions

Endophytic bacteria are defined as bacteria living inside plants without causing visible damage. Their interactions with their host plants can, however, range from beneficial to detrimental (Ryan *et al.*, 2008). With the implementation of environmentally friendly agricultural measures (e.g. the introduction of integrated pest management), beneficial (or mutualistic) bacteria attracted notice as potential alternatives to pesticides and fertilizers. Here, endophytic bacteria represent promising candidates as "bio"-protectants and "bio"-fertilizers, since they compete with pathogens for the same niche (Berg & Hallmann, 2006).

After the isolation of *N. attenuata* endophytic bacteria (**Manuscript I**), we were interested in their PGP potential and hence screened the bacteria for two factors commonly associated with PGP: the production of an auxin homolog (IAA) and 1-aminocyclopropane-1-carboxylate deaminase (ACCd) activity (Khalid *et al.*, 2004; Long *et al.*, 2008). We found, however, that only one-half of all IAA- and / or ACCd-producing bacteria had PGP effects *in vitro*. This finding is in accordance with Smyth *et al.* (2011), who tested 15 bacterial strains for

the expression of PGP traits and their effects on wheat growth. The authors found no correlation between the presence of PGP traits, and the PGP performance of these strains under *in vitro* and glasshouse conditions. Interestingly, one isolate (*P. fluorescens* MKB37) that exhibited only neutral effects in the *in vitro* studies finally displayed the strongest PGP effects in the glasshouse (Smyth *et al.*, 2011). While the presence of a bacterial trait increases the chances of PGP, it does not necessarily confer the expected effects, as was shown for the bacterial production of IAA (Barazani & Friedman, 1999; Long *et al.*, 2008) and ACCd activity (Schwachtje *et al.*, 2012). Hence, results of *in vitro* PGP screens should be challenged critically, and, clearly, other mechanisms and intensive plant-microbe communication are involved in plant growth enhancement.

From the isolated bacteria (**Manuscript I**), the influence of one isolate, namely *Bacillus* sp. B55, on WT and *35S-etr1* growth was analyzed in detail (**Manuscript II**). Specifically, we were interested in the real-world stability of the dramatic PGP effects we observed for WT and especially *35S-etr1* seedlings when inoculated with B55 under *in vitro* conditions. While *35S-etr1* plants grow happily in the glasshouse and produce a roughly similar biomass and seed number as WT, this impaired plant line is unable to survive in nature (**Manuscript II**). Inoculation with B55 significantly enhanced *in vitro* seedling growth of both WT and *35S-etr1* plants. But, while B55 displayed only neutral effects on WT growth in nature, it rescued the performance of the hampered ET-insensitive *35S-etr1* plants (field season 2009). We repeated the field study in the next year (field season 2010), and surprisingly B55 was found to enhance WT plant growth as well. Our findings indicate that B55's PGP effects in nature can vary substantially and might depend on multiple (a)biotic factors (e.g. soil type, climate) as proposed by Smyth *et al.* (2011). Similar observations have been made for several, commercially available bio-inoculants, which are agriculturally exploited; their efficiency was found to be subjected to strong variation (Lucy *et al.*, 2004; Kaymak, 2010; Aeron *et al.*, 2011). Plant growth promotion might become obvious only when a plant is subjected to stress (e.g. nutrient deficiencies); on the other hand, the outcome can switch completely under prosperous environmental conditions. For example, B55 exhibited PGP only when the plants were grown under low nutrient supply (D. Meldau, unpublished results). This is in concurrence with Partida-Martinez and Heil (2011), who argue that every biotic interaction is conditional and can harbor a continuum of outcomes. Not only abiotic factors (e.g. climate, nutrient supply), but also complex interactions like microbial facilitation, suppression or co-operation influence this conditional outcome and should not be neglected (Li & Alexander, 1986). We found, for example, that inoculation of seeds with B55 affected quantity and diversity of the resident bacteria interacting with field-grown WT and *35S-etr1* plants (**Manuscript II**). Experiments aimed at the re-construction of natural microbial communities might yield information on the complex interplay in the plant's rhizo- and endosphere.

6.3   Microbial VOCs for enhanced plant growth

The effects of bacterial VOCs on plant growth are enticing and have been previously demonstrated in several studies; the underlying mechanisms and signaling pathways are poorly understood (Bailly & Weisskopf, 2012; Wenke *et al.*, 2012). One VOC emitted in substantial

amounts by B55 (and multiple other microbes) is the S-containing VOC DMDS (Farag *et al.*, 2006; Kai *et al.*, 2009; Minerdi *et al.*, 2011). While DMDS and other sulfurous VOCs have been demonstrated to act as diverse signaling molecules in many biotic interactions, DMDS has also been shown to affect *A. thaliana* seedling growth negatively (Kai *et al.*, 2010). Our study (**Manuscript III**), however, reveals DMDS as PGP agent that supports seedling growth, especially when seedlings' access to sulfate is limited. The exposure of WT and *35S-etr1* seedlings to DMDS or the B55 VOC bouquet resulted not only in enhanced seedling growth, but also in an increased accumulation of S-containing metabolites (i.e. free methionine or the antioxidant glutathione [GSH]). Furthermore, transcript studies indicate a down-regulation of the plant's sulfate reduction pathway. Our results demonstrate that the effects of VOCs are highly context-specific, depending on plant genotype and microbial identity, as one might expect of any co-evolved interaction. Indeed, multiple factors have been demonstrated to shape the interaction outcome: plant developmental stage, growth medium and exposure time, microbial strain identity, inoculum size, and culturing conditions (Blom *et al.*, 2011).

Although bacterial VOCs exert substantial effects in closed systems, their role in nature remains elusive. Research on VOC-mediated plant growth modulation in complex soil systems is a tricky endeavor, due to experimental limitations imposed by tractable spatial arrangements and the gaseous nature of VOCs. So far, studies have been mainly restricted to *in vitro* systems (using split-plate set ups in which plants and microbes communicate only through the shared headspace) and hence the relevance of bacterial VOCs for PGP effects in nature is questionable. Nevertheless, there is common consensus that microbial VOCs shape plant growth in nature, as reviewed by Bailly and Weisskopf (2012). Bailly and Weisskopf (2012) propose two explanations for this phenomenon: first, plants are predisposed for the use of VOC signals (e.g. in plant-plant and plant-herbivore communication); second, the spatially close interaction between microbes and roots in the soil environment supports the accumulation of, and hence favors communication via, VOCs.

The use of bacterial mutants unable to produce specific VOCs of interest presents the method of choice to shed light on the importance of VOCs in plant growth modulation (Ryu *et al.*, 2003). But not all microbes can be easily silenced in genes of interest, as it is the case for many Gram-positive bacteria, including B55, mainly due to cell wall constraints (Rattanachaikunsopon & Phumkhachorn, 2009). Additionally, in the case of bacterial DMDS production, three genes involved in the biosynthesis of methanethiol (the precursor of DMDS) would be needed to be silenced in *Bacillus* sp. B55, (**Manuscript III**). Furthermore, bacterial gene silencing might sometimes be lethal or associated with pleiotropic effects. Hence, the utilization of transgenic *plant* lines, silenced in the trait of interest, might shed light on the relevance of bacterial VOCs in nature. Indeed, a molecular tool box for transforming *N. attenuata* has been developed, representing an alternative to bacterial gene silencing. Unfortunately, a transgenic line impaired in S reduction was not available to address our hypothesis, namely, that B55 VOCs / DMDS function as PGP agent by providing reduced S to the plant. However, the use of our ET-insensitive *35S-etr1* plant in the analysis of VOC-mediated PGP turned out to be serendipitous (**Manuscript III**). Although we found no direct connection between ET insensitivity and DMDS in terms of signaling, we hypothesize from our experiments that *35S-*

etr1 plants benefit most from the mutualistic association with B55 (**Manuscript II**) and DMDS emission (**Manuscript III**) due to their apparent impairment in S metabolism: *35S-etr1* seedlings having lower levels of chlorophyll a and b and GSH, while ET emissions are high. Since the S-containing amino acid Methionine (Met) is required for ET biosynthesis, we propose that ET-insensitive plants invest their S into the cycling of Met (in the Yang-Cycle) to supply the demands of their constitutively high ET emissions, which are maintained at the expense of plant growth. Ongoing research (sulfate uptake experiments and gene expression studies) aims at elucidating *35S-etr1*'s impairments in the S reduction pathway in more depth. To further dissect the role of B55's DMDS emission in *N. attenuata*'s S nutrition, supplementation assays using plants transiently interrupted in sulfate reduction pathway, e.g. by using Virus Induced Gene Silencing (VIGS), need to be carried out. Based on our hypothesis that the plant can use B55's DMDS as a reduced S source, B55-inoculated VIGS-silenced plants should perform better than non-inoculated individuals.

Elemental S is not only vital for a plant's "primary" metabolism, but is a constituent of the defense metabolites of many plants. For example, a study by Hoeller *et al.* (2010) reports on "sulfur-induced resistance" (SIR) of tobacco plants against the tobacco mosaic virus (TMV). The authors attribute SIR to increases in GSH levels (a ubiquitous S-containing antioxidant) of plants grown under high sulfate supply. Furthermore, S-containing defense metabolites of the order Brassicales contribute up to 30 % of the plant's total S content; glucosinolates representing the most famous example (Rausch & Wachter, 2005). It was shown that sulfate availability determines the plant's glucosinolate concentration (Falk et al., 2007); and just recently, Kruse et al. (2012) reported on the positive correlation between sulfate availability, glucosinolate concentration, and increased resistance of *A. thaliana* plants to the fungal pathogen *Alternaria brassicicola*. Hence it would be intriguing to test whether B55 inoculation (or the application of its VOCs) can confer similar effects on the defense of *N. attenuata* and/or *A. thaliana*.

Plants cannot exploit organically bound S, which is estimated to account for 95% of a soil's total S (Scherer, 2001), while microbes can. Furthermore, because atmospheric S inputs into soils have decreased due to the implementation of pollution abatement measures, S deficiency has become a problem in many soils (McGrath & Zhao, 1995; Smith *et al.*, 2011). Given that plants can use volatile S, the inoculation of S-containing VOC emitting microbes into the field might help the plant to meet their S requirements and to reduce fertilizer inputs. To assess the role of B55's volatile S-compounds in *N. attenuata*'s S-nutrition under more complex conditions, PGP experiments using B55-inoculated and non-inoculated *N. attenuata* cultivated in native soils which are either depleted or replete in sulfate and supplemented with an organic S source will be carried out.

Cleary, it is time to move research on VOC-mediated plant growth modulation to the field. However, this will require the development of innovative technologies, which allow for the analyses of rhizosphere VOCs in a complex soil system and differentiation of their origin (plant, introduced microbe or indigenous microbes).

6.4 Can plants selectively recruit "helper bacteria"?

As for every living organism, a plant's growth phenotype is not only determined by genetic factors, but also by the environment. Orchids, for example, strongly depend on specific fungal partners that provide energy (in the form of carbon) for germination (Kruse *et al.*, 2012). In another study, the fungus *Paraphaeosphaeria quadriseptata* was reported to confer thermotolerance to cacti, probably by interfering with the heat-shock protein signaling of the host (Turbyville *et al.*, 2006). These two examples represent extreme cases of dependence, in which the free-living microbe ensures growth and survival of its plant host.

It is further hypothesized that impaired plants selectively recruit "helper bacteria" to balance their deficiencies. Indeed, recently, several studies have shown that biotic stresses can result in the enrichment of beneficial microbes in the plant's rhizosphere (Berendsen *et al.*, 2012). For example, Mavrodi *et al.* (2012) found that different wheat irrigation practices, which are accompanied by the outbreak of certain plant diseases, can promote the build-up of indigenous populations of antibiotic-producing pseudomonads, which in turn have the capacity to suppress these diseases. Another study on strawberry reported on an increased population size of the HCN-producing biocontrol agent, *Pseudomonas* sp. LBUM300, and a stimulation in its hydrogen cyanide (HCN) production after a *Verticillium dahliae* infection (DeCoste *et al.*, 2010).

In our studies, B55, a bacterial isolate originally extracted from a *35S-etr1* plant, benefitted growth and survival of seed-inoculated *35S-etr1* plants more than WT. These ET-insensitive plants also seem to strongly depend on B55 as bacterial associates to facilitate growth and propagation in nature (**Manuscript II**). We hence propose that *35S-etr1* plants selectively recruit B55, and that B55-mediated PGP occurs in a genotype-specific manner. Indeed, in a related study, Long *et al.* (2008) could show that PGP bacteria favored only the growth of the host plant they were originally isolated from (*Solanum nigrum*) and not a closely related species (*N. attenuata*). Perhaps there are similar intraspecific differences in the PGP effects of individual bacterial isolates.

We further hypothesized that plants deficient in ET signaling would select for a subgroup of PGPB which produce IAA and ACCd to ameliorate ET signaling-related stresses (Hardoim *et al.*, 2008). Our results however, did not support this hypothesis (**Manuscript I**). But, in the course of our studies, we discovered that we were actually looking for the wrong bacterial trait. While we first only asked to which extent ET insensitivity has effects on the bacterial community assembly, we later discovered that *35S-etr1* plants are impaired in S metabolism, and that they hence preferentially associate with B55, which produces reduced S in the form of the volatile organic compound DMDS (**Manuscript III**). Indeed, the endosphere of *35S-etr1* seedlings produced ten times more colonies of B55 than WT (**Manuscript II**), supporting the hypotheses of the selective recruitment of, and dependence on, "helper microbes"; two phenomenona which will require more attention in future.

To support these hypotheses, and to gain deeper insights into the role of bacterial DMDS in the host's S nutrition, the frequency of DMDS-producing bacterial strains associating with WT under different inorganic sulfate supplies could be explored in the field. Furthermore, it has been shown that genetic variability within *N. attenuata* populations is high (Bahulikar *et al.* 2004), and

this may lead to the emergence of genotypes which are unable to respond properly to environmental signals (e.g. herbivory). Kallenbach *et al.* (2012), for example, reported that two out of one hundred plants in a natural *N. attenuata* population have reduced JA signaling capacities. Because jasmonate-mediated defense is costly, these genotypes might experience a fitness advantage when herbivore pressure is low. It hence can be supposed that genetic diversity has evolved also in other phytohormone pathways, e.g. in ET-signaling. Thus, to test our hypothesis that ET-overproducing plants preferentially associate with sulfurous VOC-producing bacteria to meet their S needs, native *N. attenuata* populations should be screened for naturally occurring individuals with increased ET emissions and, their microbial associates should be tested for DMDS production.

6.5 The take home message: Plant-microbe interactions should be studied in an ecologically relevant context

Analyzing both, the root endosphere microbial community and the specific interaction between *N. attenuata* and *Bacillus* sp. B55, my dissertation aimed at moving from a holistic to a reductionist approach. Although a plant's fitness is finally defined by multiple factors, analyzing specific interactions can be rewarding, as I could show for the intimate interaction between *N. attenuata* and B55. My findings allow for the conclusion that stressed plants can recruit "little helpers" to balance for their fitness deficiencies. Furthermore, it becomes clear that benefits of mutualistic plant-microbe interactions may only become apparent under adverse conditions, like nutrient limitation or impairments in plant physiology.

My work demonstrates that conducting research on naturally occurring and ecologically relevant interactions strongly facilitates the study of mutualistic plant-microbe interactions. To understand how plant-microbe interactions work in nature, we should investigate them in their evolutionary and ecological context, rather than subjecting plants to "alien" environments (as it is the case for 99 % of all crops (Pimentel *et al.*, 2005)) and PGPB they would never have associated with over the course of evolution. Intensive research on mechanisms in microbe-mediated PGP is needed to turn PGPB into a promising tool for use in sustainable agriculture.

6.6 References

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7 Summary

Plant-bacteria interactions are vital for plant growth and fitness. Endosphere-inhabiting mutualistic bacteria are thought to establish stable and intimate relationships with their host plant, often providing services including plant growth-promotion (PGP) by improving nutrient availability or disease protection. How plants select for their mutualistic, endophytic companions, however, remains mostly elusive. Furthermore, the multiple mechanisms by which mutualistic bacteria might enhance plant productivity have rarely been explored. In this thesis, I present work which aims at shedding light on these intriguing questions.

In my dissertation I assessed the relevance of plant ethylene (ET) signaling in the recruitment of culturable mutualistic bacteria to the root endosphere of the wild tobacco species *Nicotiana attenuata*. Wild-type (WT) and transgenic lines either deficient in ET biosynthesis (*ir-aco1*) or ET perception (*35S-etr1*) grown in native soils were employed for the analysis (**Manuscript I**). This survey revealed that the culturable bacterial community of *N. attenuata*'s roots is mainly composed of *Pseudomonas* and *Bacillus* sp. Furthermore, bacterial diversity was strongly determined by the soil type and, to a lesser extent, by plant ET signaling: the ET signaling-impaired genotypes fostered a less diverse bacterial endophyte community. In terms of PGP, about 50% of the isolated strains conferred positive effects on seedling growth. Usually, the ability of the bacteria to interfere with the plant's hormone homeostasis (i.e. by the production of auxins [indole-3-acetic acid, IAA] and 1-aminocyclopropane-1-carboxylate deaminase [ACCd]), is thought to be responsible for PGP effects, but, interestingly, the mere presence of these traits did not necessarily result in seedling growth promotion, implying that multiple mechanisms as well as fine-tuned plant-microbe communication are involved in PGP.

In a consecutive study, the PGP effects conferred by one bacterial isolate, *Bacillus* sp. B55 (extracted from an ET-insensitive *35S-etr1* plant) were analyzed in detail (**Manuscript II**). Inoculation with B55 increased growth and fitness of WT and the impaired *35S-etr1* plants *in vitro*, in the glasshouse and in the field. Most interestingly, *35S-etr1* plants, which develop a poor rooting system and are highly susceptible to diseases and hence barely survive in nature, realized greater benefits from B55 inoculation than WT. Notably, *35S-etr1* roots were much more strongly colonized by B55 than WT roots, leading to the hypothesis that ET-impaired plants recruit, and promote the growth of, beneficial companions. Despite analyzing a broad range of PGP characteristics for which B55 tested positive, the mechanisms behind B55's dramatic PGP remained elusive.

Searching for novel bacterial traits mediating the observed PGP effects, I could show that volatile organic compounds (VOCs) emitted by B55 promote seedling growth (**Manuscript III**). A sulfur (S)-containing VOC, dimethyl disulfide (DMDS), which was released by B55 and conferred the observed PGP effects, was identified. Again, *35S-etr1* seedlings realized a greater benefit of the exposure to B55's VOCs and DMDS than WT plants. In addition, I found that *35S-etr1* seedlings suffer from "impaired" S-metabolism and hence propose that these plants benefit

greatly from their interaction with B55 by obtaining reduced S (in the form of bacterial DMDS) which might help to fulfill their S requirements.

Analyzing both, the root endosphere microbial community and the specific interaction between *N. attenuata* and *Bacillus* sp. B55, my dissertation aimed at drawing a bow from a holistic to a reductionist approach. Although a plant's fitness is finally defined by multiple factors, analyzing specific interactions can be rewarding, as I could show for the intimate interaction between *N. attenuata* and B55. My findings allow for the conclusion that stressed plants can recruit "little helpers" to balance for their fitness deficiencies – an observation which we should pay more attention in future. Furthermore, it becomes clear that benefits of mutualistic plant-microbe interactions may only become apparent under adverse conditions, like nutrient limitation or impairments in plant physiology.

In conclusion, my thesis demonstrates that conducting research on naturally occurring and ecologically relevant interactions greatly facilities the study of beneficial plant-microbe interactions. In addition, my studies reveal the value of using transgenic lines to uncover these interactions and their functions.

8 Zusammenfassung

Pflanzen-Bakterien Interaktionen sind bedeutend für das Wachstum und die Fitness der Pflanzen. Mutualistische Bakterien, welche die Endosphäre der Pflanzen besiedeln können, bilden stabile und enge Beziehungen zu ihrem Wirt aus und fördern zudem oft das Gedeihen, indem sie zum Beispiel. die Nährstoffverfügbarkeit erhöhen, oder die Pflanze vor Krankheiten schützen. Welche Faktoren bei der Anwerbung nützlicher Endosphären-Besiedler durch die Pflanze zum Tragen kommen, ist kaum erforscht. Auch ist wenig über die zahlreichen Wirkungsmechanismen der Pflanzenwachstumsförderung durch Bakterien bekannt. Diese Doktorarbeit widmet sich der Untersuchung dieser unzureichend erforschten Themen.

In meiner Dissertation untersuchte ich die Bedeutung des Pflanzenhormon's Ethen (ET) auf die Selektion kultivierbarer, mutualistischer Bakterien in die Wurzelendosphäre des wilden Tabaks, *Nicotiana attenuata*. Genetisch unveränderte Wildtyp (WT)- Pflanzen, sowie transgene Linien, entweder defekt in der ET-Biosynthese (*ir-aco1*) oder der ET-Wahrnehmung (*35S-etr1*) wurden in natürlichen Böden aus Utah gezogen und für die Analyse genutzt (**Manuskript I**). Spezies der Gattungen *Pseudomonas* and *Bacillus* dominierten die kultivierbare, endophytische Bakteriengemeinschaft. Zudem wurde die Zusammensetzung hauptsächlich durch den Bodentyp, zu einem geringen Maß auch durch Signaltransduktionsprozesse des Pflanzenhormons ET bestimmt: *ir-aco1* und *35S-etr1* Pflanzen verfügten über eine weniger artenreiche Bakteriengemeinschaft. In Reinkultur inokuliert, beeinflusste ungefähr die Hälfte aller Bakterienisolate das Keimlingswachstum positiv. Jedoch führte das bloße Vorhandensein von wachstumsfördernden Merkmalen bei Bakterien (z.B. die Produktion von Hormonen [hier Auxin, Indol-3-essigsäure, IAA] oder der 1-Aminocyclopropan-1-carbonsäure Deaminase [ACCd]) nicht in jedem Fall zu einer Wachstumsförderung. Umgekehrt wurde die Hälfte aller wachstumsfördernden Bakterienisolate negativ auf die Produktion von IAA und ACCd getestet. Dies führt zu dem Schluss, dass diverse, unbekannte Mechanismen, sowie intensive Kommunikation zwischen Wirt und Bakterium bei der bakteriell-bedingten Pflanzenwachstumsförderung eine Rolle spielen.

In darauffolgenden Studien, untersuchte ich die Effekte eines ausgewählten Isolates (*Bacillus* sp. B55) auf das Wachstum von WT und *35S-etr1* Pflanzen im Detail (**Manuskript II**). Die Inokulation von B55 führte zu einer beträchtlichen Wachstums- und Fitnesssteigerung im Labor und im Feld. Dabei profitierte der ET-unempfindliche Genotyp *35S-etr1*, aus welchem B55 ursprünglich isoliert wurde, stärker von der Interaktion als WT. Zudem wurden *35S-etr1* Pflanzen deutlich stärker kolonisiert als WT, was auf eine Selektion und Anreicherung von wachstumsfördernden Bakterien durch diesen beeinträchtigten Genotypen schließen lässt. Obwohl B55 auf etliche, bekannte wachstumsfördernde Charakteristika positiv getestet wurde, blieb der eigentliche Mechanismus unbekannt.

Auf der Suche nach „neuen" Mechanismen der Wachstumsförderung, wurde deutlich, dass die von B55 abgesonderten, volatilen organischen Substanzen (VOCs) einen positiven Einfluss auf das Keimlingswachstum haben (**Manuskript III**). Dabei wurde eine stark flüchtige,

schwefelhaltiger Substanz, Dimethyl Disulfide (DMDS), welche von B55 emittiert und in Reinstoffapplikationen die zuvor beobachteten Wachstumseffekte erzielte, identifiziert. Während für WT nur mangelhaft mit Sulfat versorgte Keimlinge von DMDS profitierten, führte die DMDS-Gabe bei *35S-etr1* Keimlingen, unabhängig von der Verfügbarkeit an Sulfat, zu Wachstumssteigerungen. Zudem stellte ich im Zuge der Untersuchungen fest, dass ET-unempfindliche Pflanzen „Störungen" im Schwefelhaushalt aufweisen. Sie profitieren vermutlich deshalb im besonderen Maße von der Interaktion mit B55, indem sie den volatilen, schon reduzierten Schwefel in Form von DMDS nutzen, um ihren Schwefelbedarf zu decken.

Mit der Erforschung der *N. attenuata*-assoziierten, mutualistischen Bakteriengesellschaften bis hin zur detaillierten Analyse eines wachstumsfördernden Wirkmechanismus', spannt meine Dissertation einen Bogen vom holistischen zum reduktionistischen Forschungsansatz. Auch wenn letztlich die Fitness der Pflanze von multiplen Faktoren bestimmt wird, lohnt es sich einen Blick ins Detail (hier auf das Bakterienisolat B55) zu wagen. Meine Ergebnisse lassen den Schluss zu, dass im Wachstum gestörte Pflanzen vermehrt helfende Mikroben anwerben können, um ihr Überleben zu sichern – eine Beobachtung, welcher in Zukunft mehr Beachtung geschenkt werden sollte. Zudem wird deutlich, dass die Vorteile mutualistischer Pflanzen-Mikroben-Interaktionen oft nur unter bestimmten Bedingungen, wie z. Bsp. bei Nährstofflimitierung oder Störungen in der Pflanzenphysiologie, zum Tragen kommen.

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Zou C, Li Z, Yu D. 2010. *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran. *Journal of Microbiology* **48**(4): 460-466.

10 Erklärungen

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Dorothea Meldau
Jena, den 12.12.2012

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller Universität Jena ist mein erstes Promotionsverfahren überhaupt.

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Dorothea Meldau
Jena, den 12.12.2012

11 Curriculum vitae

Dorothea Meldau, Dipl. Biol.

Maiden name: Sonntag

Date of Birth: 8th May 1984

Place of Birth: Heiligenstadt, Thuringia

Nationality: German

Family status: Married, one child

Education

Since Apr 2008	Doctoral researcher at the Friedrich Schiller University, Jena, DE
Oct 2002 - Mar 2008	Studies at the Georg-August-University Göttingen, DE; Degree: Diploma, Grade: 1.4
Sep 1994 - Jun 2002	Käthe-Kollwitz Gymnasium, Lengenfeld / Stein, DE; Degree: Abitur, Grade: 1.1

Research Experience

Since Apr 2008	Dissertation, Max Planck Institute for Chemical Ecology (Prof. I.T. Baldwin), Department of Molecular Ecology, Jena, DE
Jun 2007 - Mar 2008	Diploma thesis at the Devision of Molecular Phytopathology & Mycotoxin Research (Prof. P. Karlovsky), Department of Crop Sciences, Georg-August University Göttingen, DE and at CAS in Botany (Prof. N. Mathivanan), University of Madras, Chennai, IN
Oct 2005 - Apr 2006	Short term project at the Palaeoenvironmental Research Unit, (Prof. M. O'Connell), National University of Ireland, Galway, IRE
Dec 2006 - Jun 2007	Student work force at the Department of X-ray Crystallography (Dr. M. Wahl), Max Planck Institute for Biophysical Chemistry, Göttingen, DE

Teaching Experience

2008 - 2012	Supervision of Bachelor students and interns
Oct 2006 - Dec 2006	Tutor at the Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Faculty of Biology, Georg-August-University Göttingen, DE
Oct 2003 - Sep 2004	Tutor at the Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Faculty of Biology, Georg-August-University Göttingen, DE

Experience Abroad

Jun 2007 - Dec 2007	Diploma research project at CAS in Botany, University of Madras, Chennai, IN
Aug 2005 - May 2006	Self-organized research stay at the National University of Ireland, Galway, IRE

Grants and Awards

Nov 2012	Poster Prize; 3rd International Conference on Microbial Communication (MiCom), Jena, DE
May 2012	Runners up Poster Prize; 28th New Phytologist Symposium "Functions and ecology of the plant microbiome", Rhodes, GR
Feb 2012	Travel grant to attend the 28th New Phytologist Symposium "Functions and ecology of the plant microbiome", Rhodes, GR
Apr 2008 - Mar 2012	Fellowship of the Jena School of Microbial Communication (JSMC), DE
Jun 2007 - Dec 2007	DAAD short-term internship for conducting Diploma-related practical work

Language Skills

German	native language
English	fluent

11.1 List of Publications

Scientific publications

Long H. Hoang, <u>Dorothea G. Sonntag</u>, Dominik D. Schmidt, Ian T. Baldwin. 2010. The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception. *New Phytologist* 185(2): 554-567.

<u>Dorothea G. Meldau,</u> Long H. Hoang, Ian T. Baldwin. 2012. A native plant growth promoting bacterium, *Bacillus* sp. B55, rescues growth performance of an ethylene insensitive plant genotype in nature. *Frontiers in Plant Science* **3**: DOI: 10.3389/fpls.2012.00112.

Oral Presentations

<u>Dorothea Meldau</u>, Long H. Hoang, Stefan Meldau, Hendrik Wünsche and Ian T. Baldwin. A native plant growth promoting bacterium, *Bacillus megaterium* B55, rescues growth performance of an ethylene insensitive plant genotype in nature. Invited institute seminar at the Julius Kühn Institute in Braunschweig, DE, Feb 2012

<u>Dorothea Meldau</u>, Long H. Hoang, Stefan Meldau and Ian T. Baldwin. Bacterial body odors make plants happy. MiCom 2011 - 2nd International Student Conference on Microbial Communication, Jena School for Microbial Communication, Jena, DE, Sep 2011

<u>Dorothea Sonntag</u>, Long H. Hoang, Ian T. Baldwin.The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception. JSMC/ILRS Symposium, Jena School for Microbial Communication/International Leibniz Research School, Jena, DE, Oct 2009

<u>Dorothea Sonntag</u>, Long H. Hoang, Ian T. Baldwin. Modifications in ethylene homeostasis of *Nicotiana attenuata* grown in native soils influence diversity of endophytic bacteria in roots. JSMC Symposium 2008, Jena School of Microbial Communication, Jena, DE, Dec 2008

Poster Presentations

<u>Dorothea Meldau</u>, Stefan Meldau, Long H. Hoang, Stefanie Underberg, Hendrik Wünsche and Ian T. Baldwin. The smelly road-how *Bacillus* sp. B55 promotes plant growth. 3rd International Student Conference on Microbial Communication, Jena School for Microbial Communication, Jena, DE, Nov 2012

Arne Weinhold, <u>Dorothea Meldau</u>, Rakesh Santhanam, Stefan Schuck, Thi Van Luu, Karin Groten and Ian T. Baldwin. Microbial Interactions with *Nicotiana attenuata*. SAB Meeting 2012, MPI for Chemical Ecology, Jena, DE, Oct 2012

<u>Dorothea Meldau</u>, Long H. Hoang, Stefan Meldau, Hendrik Wünsche and Ian T. Baldwin. The smelly road - how *Bacillus* sp. B55 promotes plant growth. 28th New Phytologist Symposium "Functions and ecology of the plant microbiome", New Phytologist Trust, Rhodes, GR, May 2012

<u>Dorothea Meldau</u>, Long H. Hoang, Stefan Meldau and Ian T. Baldwin. Bacterial body odors make plants happy. ICE Symposium, MPI for Chemical Ecology, Jena, DE, Sep 2011

Arne Weinhold, Ian T. Baldwin, Karin Groten, Stefan Meldau, <u>Dorothea Sonntag</u>, Antje Wissgott. Plant Microbe Interactions - From Koch's postulates to Microbial Communities. SAB Meeting 2010, MPI for Chemical Ecology, Jena, DE, Oct 2010

Long H. Hoang, <u>Dorothea Sonntag,</u> Stefan Meldau and Ian T. Baldwin. Volatiles of bacterial endophytes: sulfur nutrition and plant growth promotion. ICE Symposium, MPI for Chemical Ecology, Jena, DE, Jun 2009

<u>Dorothea Sonntag</u>, Mario Kallenbach, Karin Groten and Ian T. Baldwin. Exploring the molecular basis of the interaction between *Nicotiana attenuata* and *Glomus intraradices*. JSMC Symposium 2008, Jena School of Microbial Communication, Jena, DE, Dec 2008

<u>Dorothea Sonntag</u>, Long H. Hoang and Ian T. Baldwin. Effect of plant genotype and soiltype on native bacterial endophyte community in *Nicotiana attenuata*. Plant Interactions with the Environment, Universität Neuchâtel, Neuchâtel, CH, Sep 2008

Stefan Meldau, Meredith Schuman, Hendrik Wünsche, <u>Dorothea Sonntag</u>, Baldwin I.T. How sedentary plants behave in a mobile word. 3rd Interdisciplinary PhD Net Meeting 2008, The Art of Science and the Science of Art, Max-Planck-Gesellschaft, München, DE, Aug 2008

Acknowledgements

12 Acknowledgements

Thanks to …

- My supervisor Prof. **Ian T. Baldwin** who gave me the opportunity to pursue a PhD project in his lab and who supported me throughout all the years. You taught me how to raise hypotheses, to develop and push a project, to evaluate scientific progress, to critically question results, to "see the bigger picture" and finally to write a paper. I really learned a lot!
- My Thesis Committee, consisting of **Prof. Erika Kothe** and **Dr. Karin Groten** for the yearly meetings and co-supervision.
- **Karin** for supervising my first PhD project, although it never ripened to a publication. Thanks for your advice and help with regard to scientific and personal issues.
- My colleague and friend **Long**. When I started my PhD I felt pretty lost in a lab dominated by plant-herbivore research. You introduced me to the lab/institute, showed me where to find things, whom to ask if I needed help and taught me many microbiological methods. I liked our teamwork, the rigorous discussions and the fruitful cooperation which resulted in three joint publications.
- **Dr. Jianqiang Wu**, my office mate and friend. I miss you a lot (although Matthias Erb is a worthy successor)! Thanks for your competent advice, for answering my many "little" questions, for the regular "in between chats", for organizing social activities (International Dinner), for answering the phone when my parents were calling and the delicious Chinese food!
- **Matthias Erb** who moved to "my" office more than 6 months ago. I hope you did not regret you decision… Thanks for your friendship, your permanently "open ear", instant practical help and advice, and patience when I was talking with my students. Big thanks for critically reading my manuscript and thesis!
- All the past and current office mates: **Merjin, Long**, the BYU intern **Austin**, **Jianqiang, Variluska, Pilar, Abbie** and **Matthias**.
- **Merry, Mario** and **Alex** for their competent and repetitive instructions to GC-MS and LC-MS and their patience.
- Our "technical stuff": **Klaus, Thomas, Matthias S., Antje, Susi, Wibke** and **Eva** for help with molecular biology issues, ordering supplies, running sequencing reactions, submitting sequences to NCBI, cloning and transformation and providing seeds.
- The Microbial folks: **Arne, Stefan S., Lynn, Karin, Van and Rakesh** for sharing experience and information.
- All the people who were planting, watering and measuring numerous plants, counting endless herbivores and digging up roots under the scorching Utah sun: **Celia, Danny, Ian, Silke, Maria, Arne, Matthias E.**

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- The bygone **Wu-Group** consisting of **Christian, Hendrik, Dahai, Maria, Stefan M**. and the Big Master **Dr. Wu**! Being - scientifically speaking - initially a "lone wolf", you integrated me into your group! Also our regular social activities enriched my life a lot!
- **Wibke** and **Susi**: thanks for your friendship, the many lunches and coffees together as well as cheerful out-of-lab activities.
- **Merry**, who did proof-reading of all kind of English drafts, including my thesis introduction and discussion. Thanks for your friendship and yummy cakes!
- My friend **Hendrik**. As far as I remember you were the first person I was talking to when I came to lab… Thanks for your friendship, the cheerful and sometimes serious chats (about the financial crisis etc.) and the scientific cooperation!
- **Stefan M.**'s group (**Martin, Christoph** and **Ivan**) for their participation in the regular "out-of-lab" evening meetings.
- My many HIWIs: **Andreas Große, Stefan Gischkat, Christine Fischer, Tim Sehrt**, **Amy**, **Anke Rauch, Christine Lembke** and **Julia Heise** for watering and measuring endless plants, for help with bacterial re-isolation and colony counting, for RNA extraction, PCR as well as sowing, harvesting and grinding of numerous seedlings.
- The interns: **Prabin Bajgain, Sudeep Ghimire, Frederike Wenig** and **Sascia Hilck** for their commitment, enthusiasm for my projects and technical assistance.
- My Bachelor and Master students: **Christine, Stefanie**, **Sascia** and **Pilar** for their dedicated and valuable work, inspiring discussions and fresh ideas.
- Our secretary **Evelyn** for managing all organizational issues.
- All current and past **Eco-Group** and **Eco-Lab** members for providing a pleasant working atmosphere – I don't list names here! The chance that I miss one is too high!
- **Michael Reichelt** for measuring hundreds of amino acid samples, **Christine Mißbach, Jürgen Rybak**, and **Ewald Große-Wilde** for the introduction into CLSM and help with microscopy, **Holger Merker** for supplying microbial strains and chemicals ("This makes at least equal contribution"), **Marco Kai** for inspiring discussions about microbial VOCs and critical comments on my manuscript, **Bettina Raguschke** for the supply of chemicals.
- The **Gardeners** and **Tamara** for planting and taking care of many, many plants.
- The **library team, IT department**, **administration** and **workshop people** for their help and support in accessing papers and introducing me to Endnote, for troubleshooting PC "problems", taking care of administrative and ordering issue and building / modifying experimental set-ups.
- The **Jena School for Microbial Communication (JSMC)** for four, and the **MPG** for one year of generous financial support. Special thanks to **Carsten Thoms** for managing the JSMC, organizing symposia, activities, workshops etc.
- The **JSMC fellows** (especially the **first cohort**) with which I shared many cheerful social activities and workshops.

- The **Jenaer Graduierten Akademie (JGA)** who gave me the opportunity to broaden my non-scientific knowledge, to acquire soft and teaching skills, and to meet interesting people by the participation in several JGA courses.
- My friend, flat mate, collaborator and husband **Stefan** for his endless support, rigorous scientific discussions and advice, his love and affection. For cheering me up when I'm down and stressed, for motivating me, for organizing and running experiments during pregnancy and maternity leave, for taking care of our son Gabriel and, and, and…
- My son **Gabriel** and my soon to be baby. You made me work concentrated and efficiently, and to be productive while having a 7 ½ / 5 week only! You own the talent to cheer me up, distract and freshen me when my thoughts are stuck in scientific spheres.
- Besonderer Dank gilt auch meiner **Familie**. Hier möchte ich vorallem meine **Eltern** erwähnen. Ihr habt mich in all meinen Vorhaben unterstützt. Unzählige Male habt Ihr die zwei Stunden Zug- / Autofahrt auf Euch genommen, um auf Gabriel aufzupassen, damit ich meine Promotion vorantreiben konnte. Tausend Dank auch für Eure Gastfreundschaft, die Ihr meinen in- und ausländischen Kollegen und Freunden entgegengebracht habt.
- Lieben Dank auch meinen **Geschwistern, Marga, Lisi** und **Jo** (mit **Freddy**) für ihre Unterstützung.