Nicotiana attenuata microbiome characterization and plant-bacterial interactions from single isolates to consortia

Dissertation

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1 General introduction

1.1 Plant recruitment of bacterial communities

Bacteria are ubiquitous in nature and plants maintain close association with a multitude of microbes, which are collectively known as plant microbiota (van der Heijden & Schlaeppi, 2015). Plant-associated bacterial communities provide a variety of benefits to the host including nutrient procurement and protection from biotic and abiotic stress (Bulgarelli *et al.*, 2012b). Plant bacterial communities are sculpted by biotic factors such as genotypes, host species, developmental stages, and abiotic factors such as soil type, pH and environmental condition (Steenwerth *et al.*, 2008; Bulgarelli *et al.*, 2012b). Bacterial communities differ among plant species (Bulgarelli *et al.*, 2012a, 2015; Lundberg *et al.*, 2012; Peiffer *et al.*, 2013; Yeoh *et al.*, 2016), and, different parts of the plant, e.g. aboveground (phyllosphere) and belowground (root systems) parts harbor distinct bacterial communities (Bodenhausen *et al.*, 2013; Lebeis, 2014; Gilbert *et al.*, 2014). Root bacterial communities reside in a relatively stable environment, which is characterized by rather high amounts of nutrients due to root exudation to recruit bacterial communities (Badri *et al.*, 2009).

Four compartments exist along the soil-root continuum: bulk soil, rhizosphere, rhizoplane and endosphere (Fig 1) (Hacquard *et al.*, 2015).

Figure 1

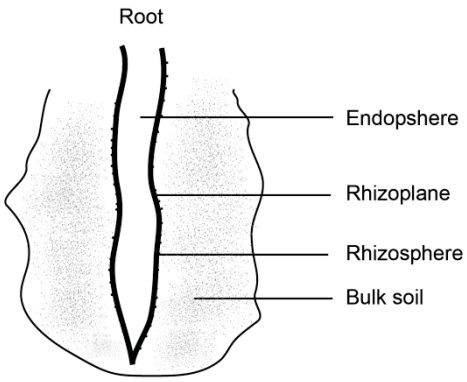

Figure 1: soil-root continuum

Undoubtedly, soil harbors higher bacterial diversity compared to other compartments. It has been estimated that one gram of soil contains 10^8 cells (Raynaud &

Nunan, 2014) and 10^4 bacterial species (Roesch *et al.*, 2007). However, exact numbers vary depending on the soil chemical properties and texture. The second most diverse compartment is the rhizosphere (soil directly in contact with roots) influenced by root exudation such as organic and inorganic ions sugars, vitamins, amino acids, purines (Dakora & Phillips, 2002; Hartmann *et al.*, 2008). Root exudation accounts for 10-16% of total pant nitrogen and ~11% of net photosynthetically fixed carbon, although these values depend on plant age and plant species (Jones *et al.*, 2009). The root compartment can be separated into two niches: rhizoplane and endosphere. The rhizoplane harbors bacterial communities, which tightly adhere to root surface, while bacteria inhabiting inside the roots are called endosphere (Hacquard *et al.*, 2015). Bacterial diversity is higher in bulk soil and gradually decreases towards the endosphere continuum (Bulgarelli *et al.*, 2012a; Lundberg *et al.*, 2012; Edwards *et al.*, 2015). The gradual shift of bacterial community from bulk soil to the endosphere is driven by root exudation, plant immunity and host adaptation. A two-step selection process drives the bacterial community shift from surrounding soil microbiome. First root exudation initiates the substrate driven community shift in rhizosphere followed by fine tuning of microbiome profile by host-genotype immunity for endosphere assembly (Bulgarelli *et al.*, 2012b). Despite recent advances in plant microbial profiling, factors driving the specific enrichment of some phyla are still largely unknown.

The term endophyte for bacteria residing inside the endosphere comes from the ancient Greek word ενδον (endon- within) and φυτόν (phyton -plant). Endophytic bacteria colonize the host tissue without causing any visible damage or elicit strong defense response. Endophytes are motile cells, which are freely residing inside the host tissue and not surrounded by any special membranes like endosymbionts. In 1887 Victor Gallipe postulated that bacterial endophytes are a subset of soil population (Galippe, 1887; Compant *et al.*, 2012) and recent studies based on next generation sequencing (NGS) are in complete agreement with Gallipe's postulation (Bulgarelli *et al.*, 2012b; Lundberg *et al.*, 2012; Edwards *et al.*, 2015). Bacteria or fungi isolated from surface-sterilized tissues are generally defined as endophytes, however, to prove the endophytic lifestyle of "putative endophytic bacteria" isolated from surface-disinfected tissues, unambiguous microscopic localization of bacteria inside the plant tissues is required either by tagging bacteria with reporter strains or *in situ* hybridization (Rosenblueth & Martínez-Romero, 2006; Reinhold-Hurek & Hurek, 2011).

1.2 Bacterial community analysis

Earlier plant-microbial community studies are based on the cultivation-based technique, which underestimates the diversity and only a small fraction of bacteria have been isolated and characterized, however, isolated microbes enable scientist to reveal the functional traits of microbes for plants (Turner *et al.*, 2013). The first generation of culture-independent techniques are based on molecular cloning of 16S rDNA and genetic finger print technique such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) widely used in plant microbial ecology studies. However, limitation of these techniques were co-migration of DNA fragments and it is not always possible to separate DNA fragments with low variation (Muyzer & Smalla, 1998). Recently, advances in high-throughput DNA sequencing technologies such as pyrosequencing (e.g. 454), reverse dye terminator (e.g., Illumina) and phospholinked fluorescent nucleotides (e.g. Pacbio) have allowed more in-depth studies of bacterial communities of plants, revealing a much larger diversity that includes many rare species (Lebeis, 2014). However, culture-independent approaches do not differentiate live from dead cells along with sequencing error and primer bias, which might lead to diversity overestimation. Complementation of both methods would allow us to better understand diversity and ecological context of plant bacterial communities. Up to date only very few studies have been performed combining both approaches. In particular, functional analyses studies based on synthetic communities are largely lacking.

1.3 Model plant *Nicotiana attenuata*

Nicotiana attenuata (TORR. EX WATSON- Solanaceae) is an annual wild tobacco plant, native to the Great Basin Desert, Utah. *N. attenuata's* germination is triggered from long-lived seed banks after sporadically occurring wildfires and the plants grow in a nitrogen rich soil (Baldwin & Morse, 1994; Preston & Baldwin, 1999). The synchronized germination causes them to grow in monoculture resulting in high intra-specific competition. As an annual plant with a short growing season. *N. attenuata* has to grow rapidly and produce a maximum number of seeds. The natural environment is further characterized by high light conditions and strong UVB exposure, though UVB-levels differ depending on time, with a maximum UVB irradiance of≈ 10 µmol m^{-2} s^{-1}. *N. attenuata* has been extensively studied for plant-herbivore interactions. After germination, from the emergence of radicle till flowering, *N. attenuata* is constantly bombarded by native herbivores from different feeding guilds (Baldwin, 2001; Kessler & Baldwin, 2002; Halitschke & Baldwin, 2003; Schwachtje & Baldwin, 2008;

Kallenbach *et al.*, 2012). *N. attenuata* employs both, direct and indirect defense responses to deter herbivores. Indirect defense means that they attract the predators of herbivores via emitting green leaf volatiles (Kessler & Baldwin, 2001; Allmann & Baldwin, 2010), whereas, in direct defense the plant itself produces toxic secondary metabolites such as nicotine, anti-digestive protease inhibitors and diterpene glycosides (DTGs) (Jassbi *et al.*, 2008; Heiling *et al.*, 2010). In *N. attenuata,* the JA defense response against the generalist herbivore *M. sexta* attack has been extensively studied, both under field and glass-house conditions (Diezel *et al.*, 2009). Herbivore attack leads to strong induction of JA (JA-burst), which in turn leads to the production of defense metabolites (Paschold et al., 2007; Wang & Wu, 2013). Being a native wild plant with short germination time along with availability of genetic transformation tools make *N. attenuata* into a good model system to investigate plant-bacterial interactions. For example, by using transgenic lines it has been shown that, *N. attenuata* root-associated microbial communities are influenced by the ability to produce (ir*ACO*) and perceive (35S *etr1*) the phytohormone ethylene (ET) (Long *et al.*, 2010).

1.4 Multifunctional role of phytohormone jasmonic acid (JA) in plants.

Jasmonates are a class of phytohormones including jasmonic acid (JA), 12-oxophytodienoic acid (12-OPDA), methyl jasmonate (MeJA) and JA-conjugated amino acids such as JA-leucine (JA-leu) and JA-isoleucine (JA-Ile) derived from the metabolism of fatty acids. Jasmonates regulate responses to biotic and abiotic factors. Especially JA plays a vital role in defense against herbivores and necrotrophic pathogens, and it also affects plant development (Gray, 2004; Wang & Irving, 2011), fruit ripening and root elongation (Hause *et al.*, 2000; Li *et al.*, 2004; Doornbos *et al.*, 2011b; Wang & Wu, 2013). Moreover, JA plays an important role in beneficial plant-microbe interactions, for example, it has been shown that, colonization of plant growth promoting fungi (PGPF) *Piriformospora indica* induces the upregulation of JA biosynthesis genes in barley roots, suggesting that plants might employ JA signaling molecules to regulate the colonization of fungi (Schäfer *et al.*, 2009). In addition, jasmonate signaling is involved in induced systematic resistance (ISR) when beneficial microbes colonizing the roots to prime the plants to enhance the defense against pathogens and herbivores (Pieterse *et al.*, 2014).

In *A. thaliana* it has been shown that activation of the JA defense pathway influences the rhizosphere bacterial community but not bulk soil (Carvalhais *et al.*, 2013).Whereas, in another study using the same model plant, Doornbos *et al.*, (2011a) demonstrated that rhizosphere bacterial communities are independent of the JA defense

pathway. Using a culture- dependent approach Kniskern *et al.*, (2007) reported that in JA deficient plants, leaf epiphytic bacterial diversity is higher than the endophyte diversity. Furthermore, numerous studies focused on plant-pathogen interactions, however, very little attention has been paid, if and how plant defense phytohormones shape their root and leaf associated bacterial communities and far less is known about the role of JA in leaf and root microbiota under field conditions. Given the natural variation in accumulation of JA levels among native populations of *N. attenuata* after herbivore attack (Kallenbach *et al.*, 2012) the species provides an ideal platform to investigate the role of JA in bacterial community composition. In *N. attenuata* JA signaling pathway, the enzyme allene oxide cyclase (AOC) - encoded by a single gene - converts 13S-OOH-18:3 to 12-oxo-phytodienoic acid (OPDA), followed by three cycles of β-oxidation and reduction of OPDA into JA (Bonaventure *et al.*, 2011); and silencing the expression of AOC leads to reduced JA signaling (Kallenbach *et al.*, 2012). In **manuscript I and II**, I used this previously characterized isogenic line impaired in JA biosynthesis (ir*AOC*) to investigate the role of JA in shaping leaf and root bacterial communities of field grown *N. attenuata*.

1.5 UV-B photoreceptor UV resistance locus 8 (UVR8) and response factor chalcone synthase (CHS).

Ultraviolet-B (UVB) radiation is a normal component of sunlight and its wavelength ranges from 280-315 nm and UVB exposure is harmful to any living organism, including microbial communities and causes direct DNA damage and inhibition of RNA transcription (Tilbrook *et al.*, 2013). Plants evolved a sophisticated way to perceive and respond to UVB exposure. In plants UVB light is perceived by UV resistance locus 8 (UVR8), which regulates the expression of genes for survival under sunlight. Under UVB exposure, plants accumulate UVR8 in the nucleus (Kaiserli & Jenkins, 2007). UVR8 is homodimer and upon UVB exposure it transforms into monomers and interacts with COP1, an E3 ubiquitin ligase, initiating UVR8 dependent induction of gene expression such as genes encoding for DNA photolyases, which repair damaged DNA(Tilbrook *et al.*, 2013), and genes encoding the first enzyme in flavonoid biosynthesis pathway, chalcone synthase (CHS). The first step of flavonoid pathway is catalyzed by CHS to synthesize naringenin chalcone, from which the diverse flavonoid end products are derived (Stafford, 1990), the resulting flavonoids are UV-protective compounds. In *A. thaliana*, UVR8 mutants are highly sensitive and unable to survive under constant exposure to UVB (Brown *et al.*, 2005). Plants exposed to UVB are

more resistant against herbivores and phytopathogens (Kuhlmann & Müller, 2011; Tilbrook *et al.*, 2013).

Different environmental conditions influence the shoot and root microbial communities of plants (Barnard *et al.*, 2013). In general, it has been postulated that the phyllosphere is exposed to variable environments characterized by fluctuating humidity, temperature and UV intensity (Lindow & Brandl, 2003). Peanut phyllosphere bacterial communities are influenced by UVB exposure and bacterial isolates retrieved from UVB exposed leaves are different from non-UVB exposed leaves (Jacobs & Sundin, 2001). In another study, Redford *et al.*, (2010) reported that UV- resistant bacterial phyla Deinococcus-Thermus and TM highly dominated the phyllosphere community of *Pinus ponderosa.* In *A. thaliana,* UVB exposure increases the resistance against the necrotrophic pathogen *Botrytis cinerea* via induction of UVR8 expression, which in turn regulates sinapate accumulation in leaves (Demkura & Ballaré, 2012). UVB induces the flavonoid biosynthesis pathway to produce phenolic sunscreen compounds. In legumes, flavonoid compounds are chemoattractants for *Rhizobia* to fix atmospheric nitrogen. Root secretion of flavonoids initiates the expression of nod factor genes in bacteria, which in turn leads to the formation of nodules on plant roots. Nodules are root organs, which are developed due to signal exchange between plants and *Rhizobia*. It has been shown that silencing the CHS gene in *Medicago truncatula* resulted in reduced levels of flavonoids and plants were unable to develop nodules (Wasson *et al.*, 2006). The role of UV exposure on phyllosphere bacterial community and the importance of flavonoid production on plant-*Rhizobia* associations have been extensively studied. However, the plant's response to UVB, and hence the role of its receptor UVR8 and the down-stream response CHS expression on root bacterial communities remain to be elucidated, in particular for plants whose native habitat is characterized by high UVB-radiation, such as the ecological model plant *N. attenuata*. In **manuscript IV**, I used a previously characterized isogenic line impaired in flavonoid biosynthesis (ir*CHAL*) resulted in reduced level of phenolic compound rutin and lack of floral volatiles such as benzylacetone and benzaldehyde emission (Kessler *et al.*, 2008) and a newly created ir*UVR8* isogenic line impaired in UVR8 expression generated by RNAi mediated transformation and a cross of both (ir*CHAL*xir*UVR8*) to investigate the role of UVB perception and response in shaping root bacterial communities.

1.6 Current agricultural practice and microbial phytopathogens.

The practice of growing the same crop year after year in the same field is defined as monoculture (Shipton, 1977) and is extensively used in modern industrialized agriculture due to economic benefits and low labor cost. However, the continuous practice of monoculture over the years can lead to an increase of plant diseases, due to accumulation of soil borne pathogens or adaptation of specialized pathogens to infect the specific plants (Cook & Weller, 2004). Plants are under attack by pathogenic microbes (fungal and bacterial) which are responsible for about 20% reduction in worldwide yield of food crops (Schumann & D'Arcy, 2006). In general, when a pathogen colonizes the plant host, results in disease symptoms like wilting, necrosis and chlorosis (Prell & Day, 2000).

In particular, diseases caused by soil borne fungal pathogens have a significant impact on global food production (Strange & Scott, 2005). Furthermore, fungal pathogens may cause catastrophic disease outbreak, because they form spores, which germinate under favorable conditions and are transported easily by water and wind. *Phytophthora infestans* is a destructive plant pathogen causing potato late blight and is best known for famous Irish potato famine (Goss *et al.*, 2014). Fungal pathogens such as *Fusarium spp, Alternaria spp, Gaeumannomyces graminis* and *Rhizoctonia solani* attack different host plants and cause catastrophic plant diseases which lead to complete destruction of fields (Deacon, 1991; Weller *et al.*, 2002; Adesina *et al.*, 2009). In soybean, *Fusarium virguliforme* induces sudden death syndrome (SDS) associated with wilting of stalk and rotting of root (Brar *et al.*, 2011), *Fusarium culmorum* and *Fusarium pseudograminearum* cause the Fusarium crown rot (FCR) disease, which have caused significant losses in wheat and cereals throughout the semiarid region of the world and especially in the Pacific Northwest of the United States (Cook, 1980; Burgess *et al.*, 2001; Hollaway *et al.*, 2013). The fungal pathogen *Alternaria alternata* induces black spot disease in pomegranate (Gat *et al.*, 2012), leaf blight in cotton (Bashan *et al.*, 1991) and leaf spot disease in cucumber (Vakalounakis & Malathrakis, 1988). In tobacco monoculture plantations - despite recent scientific advances in finding potential remedy to plant disease caused by fungal pathogens - various microbial pathogens have caused devastating diseases such as angular leaf spot (Wannamaker & Rufty, 1990), hairy root (Gelvin, 1990), hallow stalk & blackleg (Intyre *et al.*, 1977; Xia & Mo, 2007), verticillium wilt (Wright, 1968), powdery mildew (Cole 1964) and black shank (Gallup *et al.*, 2006). Overall, it still remains a challenge to protect monocultures from fungal outbreaks.

Tremendous repertoire of knowledge is available on crop plant-pathogen interactions, on the other hand, pathogen interaction with ecological model plants is scarce, mainly because of low incidence of disease occurrence in natural populations. For the past 15 years, we conducted field experiments at a field plot located at Lytle Ranch Preserve, Utah, USA using the coyote tobacco *N. attenuata* as a model plant. Continuous re-planting of *N. attenuata* in non-burned soil of the same field plot for the past 15 years (1998-2013) led to an increase in a number of dead plants due to a sudden wilt disease which is associated with black roots and collapse of the vascular system. For the past 3 years the infection rates aggravated despite the fact that *N. attenuata* is able to alter its phenotype in response to highly variable biotic stresses including herbivores and pathogens (Baldwin, 2001). Unknowingly, we had recapitulated the common agricultural dilemma of pathogen buildup by continuous cropping of this native plant under field conditions. In contrast to the defense response of *N. attenuata* to its native herbivore which has been intensively studied, however, the interaction of this plant to its native pathogen is poorly understood. In **manuscript III**, microbial causal agents of sudden wilt disease were isolated and tested under *in-vitro* conditions.

1.7 Plant growth promotion and biological control of plant pathogens.

A number of microbes associated with plant roots are able to promote plant growth (PGPB). Different direct mechanisms lead to growth promotion, such as helping plants to acquire nutrients e.g. biofertilizers (nitrogen, phosphorous), production of phytohormones (IAA, gibberellins) and stress controllers 1-amino cyclopropane-1- carboxylate (ACC) deaminase which lowers the plant ethylene levels (Lugtenberg & Kamilova, 2009). The associations of soil microbes to plants have been reported as early as 30 BC, in Virgil *Georgics* poem, it was mentioned that legume fields are more fertile which was partly due to the symbiotic relationship of legume plants to Rhizobia that are capable of fixing nitrogen from the air (Chew, 2002). Concurrently, Theophrastus (327-287BC) reported mixing of different soils as means of 'remedying defects and adding heart to the soil" (Tisdale *et al.*, 1985), with modern insight, this can be seen as enhancing the diversity of microbes resulting in positive effects on plants. In literature, there is growing evidence that, for sustainable agriculture, biofertilizers are the best alternative to chemical minerals to promote plant growth (Figueiredo *et al.*, 2011). It has been shown that, PGP effects of bacteria are species-dependent (Long *et al.*, 2008), however, far less is known, whether PGP effects are genotype

dependent. In **manuscript II**, I used the previously characterized isogenic line impaired in JA biosynthesis (ir*AOC*) to answer this question.

In contrast to direct plant growth promotion, the bacterial effect is considered as indirect if bacterial colonization prevents plant diseases caused by pathogens. This can be due to an antagonistic mechanism - growth inhibition of pathogens or siderophore production to outcompete pathogens for nutrients (Vessey, 2003; Lugtenberg & Kamilova, 2009; Figueiredo *et al.*, 2011). In agriculture different strategies such as fungicides, biocontrols and crop rotation are applied to minimize the disease incidence of single crop plantation (Curl, 1963; Whipps, 2001; Makovitzki *et al.*, 2007; Kobayashi & Crouch, 2009; Wang *et al.*, 2013). Crop rotation is known as cultivation of different crops in succession on the same field and its practice dates back to earliest dates of agriculture to suppress plant diseases (Curl, 1963). It is nowadays mainly replaced by the use of chemical treatments, such as fungicides which are used extensively in modern agriculture due to their efficacy to attack a wide range of phytopathogens and allows the continued re-planting of the same crop (Balba, 2007; Krämer *et al.*, 2012; Speck-Planche *et al.*, 2012). As a natural form of soil amendment, the addition of charcoal (commonly called biochar) to agricultural soil has been shown to improve various plant traits, including increased resistance against fungal pathogens (Lehmann *et al.*, 2011; Meller Harel *et al.*, 2012; Sohi, 2012).

The public concerns on environmental pollution and increasing resistance of the fungal plant pathogens due to the excessive use and misuse of synthetic chemicals have led to resurgence of research interest in bacterial and fungal biocontrols against fungal pathogens (Pal & Gardener, 2006; Bacon & Hinton, 2011). The term ‘biocontrol’ is the abbreviated synonym of ‘biological control agent (BCA)’ and the term refers to the use of bacteria or fungi to suppress the severity of diseases caused by pathogens in a single cropping system (Weller, 1988; Lugtenberg & Kamilova, 2009; Kobayashi & Crouch, 2009; Alabouvette *et al.*, 2009). Extensively studied mode of action of BCA with regard to their antifungal properties (Haas & Keel 2003; Haas & Défago 2005). In general, PGP bacteria or fungal endophytes can be used to mitigate plant diseases caused by fungal pathogens (Pliego *et al.*, 2010; Doornbos *et al.*, 2011b). Bacterial strains such as *Bacillus mojavensis* are used in maize plants to suppress disease caused by the fungal pathogen *F. verticilliodes* (Bacon & Hinton, 2011). Disease suppression activity by *Pseudomonas putida strain* 06909 against the pathogen *Phytophthora parasitica* is exerted by inhibiting fungal growth (Yang *et al.*, 1994). Fungal biocontrols such *Sporidesmium sclerotivorum* (del Rio *et al.*, 2002) and *Coniothyrium*

minitans (Jones *et al.*, 2004) are effective in mitigating diseases caused by *Sclerotinia* spp in soybean and lettuce respectively. But these studies have mostly focused on laboratory experiments and single species BCA; far less is known about how these biocontrols could be applied in field trials. In **manuscript III**, I used consortia of bacterial isolates along with other chemical treatments to prevent *N. attenuata* from sudden wilt disease under field and *in-vitro* conditions.

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1.8 Objective of the thesis

The ecological model plant *N. attenuata* has been extensively studied for native herbivore interactions, while a characterization of its microbiome and the functional role of the bacterial endophytes in an ecological context is lacking. I performed a systematic study of whether the phytohoromone JA and five different soil types sculpt the phyllosphere and root microbiota under natural conditions. The plant response to UVB exposure and the role of the UVB photoreceptor (UV resistance locus 8-UVR8) and response (chalcone synthase -CHS) on root colonization of UV-resistant bacteria *Deinococcus* were investigated under microcosm conditions. Furthermore, this thesis addresses a long festering agricultural problem: monoculture practice and fungal outbreak. Monoculture practice leads to pathogen buildup, which in turn leads to emergence of sudden wilt diseases. Native bacterial consortia were used as a biocontrol agent to prevent the plants from sudden wilt disease under field conditions. Microbiome characterization along with a characterization of the functional and ecological role of root-associated bacterial of *N. attenuata* were addressed in this thesis.

2 Manuscript overview and author contributions

Manuscript I

Analysis of plant-bacteria interactions in their native habitat: bacterial communities associated with wild tobacco are independent of endogenous jasmonic acid levels and developmental stages.

Rakesh Santhanam, Karin Groten, Dorothea G. Meldau, and Ian T. Baldwin

Published in *PLoS One* 2014, **9**(4):e94710

This manuscript investigates the role of the phytohormone jasmonic acid (JA) and developmental stages in shaping phyllosphere and root bacterial communities under native conditions. Previously characterized isogenic line impaired in JA biosynthesis (ir*AOC*) along with control empty vector (EV) plants were harvested from a field site at the plant's native habitat over five different developmental stages and subjected to culture dependent and independent 454 pyrosequencing method to characterize the bacterial community. Bacterial communities significantly differ between phyllosphere and root, whereas the capacity to produce JA and developmental stages do not shape the overall bacterial community of *N. attenuata* under field conditions.

R. Santhanam performed the experiments, analyzed the data and drafted the manuscript. I.T. Baldwin and K. Groten helped to draft the manuscript. R. Santhanam, K. Groten, D.G. Meldau and I.T.Baldwin conceived and designed the study.

Manuscript II

In wild tobacco *Nicotiana attenuata,* intraspecific variation among bacterial communities is mainly shaped by the local soil microbiota independently of jasmonic acid signal capacity.

Rakesh Santhanam, Ian T. Baldwin and Karin Groten.

Published in *Communicative & Integrative Biology* 2015, 8(2):e1017160.

In this short communication manuscript, we investigated, whether bacterial plant growth promoting effects (PGP) depend on endogenous JA production and we further corroborated the evidence of previously published data **(Manuscript I)** and demonstrate that the plant's capacity to produce JA seems not to be important for structuring root-associated bacterial communities. Pyrosequencing data were reanalyzed at qualitative level (presence-absence) and at order level to confirm that differences between leaf and root communities are not due to differences in abundance of certain bacterial communities, but mainly due to presence and absence of different bacterial communities in roots and leaves. Furthermore, we demonstrated that bacterial PGP effects are independent of endogenous JA production under glass-house condition.

R. Santhanam performed the experiments, analyzed the data and drafted the manuscript. I.T. Baldwin and K. Groten helped to draft the manuscript. R. Santhanam, K. Groten, and I.T.Baldwin conceived and designed the study.

Manuscript III

Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping

Rakesh Santhanam, Van Thi Luu, Arne Weinhold, Jay Goldberg, Youngjoo Oh and Ian T. Baldwin

Published in *PNAS* 2015, **112** : E5013–E5020.

In this study, we addressed the long festering agricultural problem of monoculture practise and fungal outbreak. The repeated planting of our model plant *N. attenuata* for past 15 years in the same field, led to emergence of sudden wilt disease caused by *Alternaria* and *Fusarium* disease complex, characterized by sudden collapse of vascular system associated with roots turning black. Under *in-vitro* conditions different chemical and biocontrol startegies were employed to find a potential remedy to prevent sudden wilt disease. Based on the *in-vitro* results we selected consortia of bacterial isolates (5-6) along with other 7 different treatments and tested them under field conditions. Bacterial consortia signicantly attenuated the death rate of sudden wilt disease in both 2013 and 2014 field seasons compared to other treatments. Furthermore, bacterial treatment does not alter the 32 ecological traits of *N. attenuata.*This study exemplifies the importance of an early window period for native bacterial recruitment of plants for long-lasting mutualistic realtionship.

R. Santhanam, V.T Luu, A.Weinhold contributed equally to this work. R. Santhanam, V.T Luu, A.Weinhold and I.T Baldwin drafted the manuscript and conceived and designed the study.

Manuscript IV

Native grown *Nicotiana attenuata* root microbiome is independent of soil types and plant responses to UVB increase *Deinococcus* root colonization

Rakesh Santhanam, Youngjoo Oh, Arne Weinhold, Van Thi Luu, Karin Groten and Ian T. Baldwin.

To be submitted to *New Phytologist*

Summary

Plants recruit microbial communities from the soil in which they germinate. Whether soil types sculpt the composition of root fungal and bacterial microbial communities under natural conditions, and whether UVB-exposure affects the root colonization of highly abundant species remains unknown. *Nicotiana attenuata* plants from 5 different natural populations were analyzed along with bulk soil by 454-pyrosequencing to characterize the root and soil microbiomes. Transgenic lines impaired in UVB perception (ir*UVR8*) and UVB responses (ir*CHAL*) were produced and used to test the colonization of the highly abundant (by pyrosequencing) taxon *Deinococcus* in microcosm experiments using a synthetic bacterial community and plants grown with visible light with/without UVB supplementation. Alpha and beta diversities of the bacterial and fungal communities differed significantly between soil and root but core root bacterial communities did not differ among locations. With UVB supplementation, wild type roots were colonized more by *Deinococcus*, while ir*UVR8* and ir*CHAL* were colonized less. *N. attenuata* plants recruits a core root microbiome irrespective of soil type under natural conditions, and *Deinococcus* root colonization is enhanced when plants are grown under UVB, and decreased when plants are unable to perceive and respond to UVB. A plant's response to UVB influences the composition of its microbiome.

R. Santhanam, performed the experiments, analyzed the data and drafted the manuscript. I.T. Baldwin and K. Groten helped to draft the manuscript. Y. Oh characterized the ir*UVR8* line R. Santhanam, V.T Luu, A.Weinhold, K. Groten and I.T Baldwin conceived and designed the study

Manuscript I

Analysis of plant-bacteria interactions in their native habitat: bacterial communities associated with wild tobacco are independent of endogenous jasmonic acid levels and developmental stages.

Rakesh Santhanam, Karin Groten, Dorothea G. Meldau, and Ian T. Baldwin

Published in *PLoS One* 2014, **9**(4):e94710

3 Manuscript I

OPEN ACCESS Freely available online

PLOS ONE

Analysis of Plant-Bacteria Interactions in Their Native Habitat: Bacterial Communities Associated with Wild Tobacco Are Independent of Endogenous Jasmonic Acid Levels and Developmental Stages

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Abstract

Jasmonic acid (JA) mediates defense responses against herbivores and necrotrophic pathogens but does it influence the recruitment of bacterial communities in the field? We conducted field and laboratory experiments with transformed *Nicotiana attenuata* plants deficient in jasmonate biosynthesis (ir*AOC*) and empty vector controls (EV) to answer this question. Using both culture-dependent and independent techniques, we characterized root and leaf-associated bacterial communities over five developmental stages, from rosette through flowering of plants grown in their natural habitat. Based on the pyrosequencing results, alpha and beta diversity did not differ among EV and ir*AOC* plants or over ontogeny, but some genera were more abundant in one of the genotypes. Furthermore, bacterial communities were significantly different among leaves and roots. Taxa isolated only from one or both plant genotypes and hence classified as 'specialists' and 'generalists' were used in laboratory tests to further evaluate the patterns observed from the field. The putative specialist taxa did not preferentially colonize the jasmonate-deficient genotype, or alter the plant's elicited phytohormone signaling. We conclude that in *N. attenuata*, JA signaling does not have a major effect on structuring the bacterial communities and infer that colonization of plant tissues is mainly shaped by the local soil community in which the plant grows.

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Introduction

Plants are inhabited by diverse bacterial communities which live in and on the plant's tissues. Bacteria which have been clearly shown to colonize tissues internally are termed "endophytes"; though in literature the term has also been extensively applied to bacteria or DNA extracted from surface-sterilized plant tissue [1,2]. In the introduction we will use the term for both definitions, but are aware that after surface-sterilization not all of the isolated bacteria or DNA might be strictly derived from internal tissues, as some bacteria may have survived the sterilization treatment, and that tissue-associated bacteria would be a more appropriate term for our own findings.

Endophytes or particular bacterial isolates can either have beneficial or detrimental effects on their hosts [1,2]; some are seed borne [3], but most bacteria are recruited from the surrounding soil during germination [4–6]. Thus, plants grown in different soils harbor highly diverse bacterial communities [7], and increasingly evidence for plant-soil feedbacks [8,9], likely mediated by root exudates, such as amino acids, sugars, fatty acids and organic acids, are being shown to affect soil bacterial communities. Many studies have revealed that plants only recruit a selection of the bacteria present in their immediate surroundings, and bacteria from the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria are found most frequently in roots [6,10–12]. However, further research is needed to determine how the soil microbiome and plant-microbe feedbacks influence the populations of bacterial communities.

Bacterial communities are highly diverse among the different tissues, and they are found in seeds, roots, leaves, stems, tubers, ovules and fruits [2,13]. For some plant species, roots harbor a greater number of bacterial taxa compared to stems and leaves and the communities differ in the different tissues [14,15]. The communities also differ among plant species [16] and genotypes [17,18] and change seasonally and developmentally [19,20]. Overall, it remains unknown if and how biotic or abiotic stresses and ontogeny affects the composition of the endophyte or tissue-associated bacterial community [21,22].

The phytohormones, salicyclic acid (SA), jasmonic acid (JA) and ethylene (ET) regulate responses to biotic and abiotic stresses [23–25] and play central roles in coordinating various aspects of developmental processes throughout the life cycle of plants, including flower morphogenesis, fruit formation or ripening, seed germination and root elongation [26,27], but they also play a major role in mediating defense responses against herbivores and pathogens [28,29]. Several plant-growth promoting (PGP) bacteria have been shown to enhance a plant's resistance against biotrophic and necrotrophic pathogens by increasing SA and JA levels,

respectively [30–33]. These studies have mostly focused on effects on the pathogen community; far less is known about how these phytohormones influence the bacterial communities. Only two studies have examined this question with *Arabidopsis* plants and with contradictory results [34,35].

In coyote tobacco (*Nicotiana attenuata*), it was previously shown that the root-associated bacterial community is influenced by ET perception and production [5]. However, the influence of JA and JA-inducible defenses on bacterial communities remains an open question. In this species, the herbivory-induced accumulation of ET, JA and JA-isoleucine (JA-Ile) is influenced by ontogeny; in particular the onset of flowering strongly reduces the inducibility of these three signaling molecules [36]. A systematic analysis of plant ontogeny and JA effects on the tissue-associated bacterial community composition is clearly needed for this native plant which has become a model for the study of ecological interactions.

In this study, *N. attenuata* (Solanaceae), a tobacco native to the Great Basin Desert that germinates after fires from long-lived seed banks to form monocultures in the nitrogen-rich soils of the post-fire habitat [43], was used as a model plant. Its defense reactions against attack from specialist (for example, *Manduca sexta*) and generalist herbivores have been extensively studied [44]. Natural or simulated attack by *M. sexta* larvae elicits strong JA signaling which in turn leads to the production of various defense responses [45]. This JA signaling can be silenced by knocking down the expression of a key enzymatic step in JA biosynthesis, allene oxide cyclase (AOC) which converts 13S-OOH-18:3 to 12-oxo-phytodienoic acid (OPDA), and is encoded by a single gene. OPDA is subsequently transformed into JA by reduction and three cycles of β-oxidation [46,47]. To date, the influence of JA signaling on the bacterial communities have not been thoroughly examined and plants impaired in JA biosynthesis provide an important tool to reveal the role of JA in shaping bacterial communities.

In this study we used plants differing in endogenous JA levels grown in their native habitat and tested the hypothesis that variation in JA signaling defense pathways, a trait that is known to be variable amongst different genotypes found within native *N. attenuata* populations [47] and to change over plant development [36] affects the root- and leaf-associated bacterial community composition and diversity. We planted two isogenic size-matched cohorts of *N. attenuata* plants with normal (EV) and impaired JA-biosynthesis (ir*AOC*) in their natural environment in Utah, USA, and harvested roots and leaves at 5 different developmental stages from rosette through flowering during the 2012 field season. Bacterial communities were retrieved by a combination of culture independent (pyrosequencing) and dependent approaches [37,38,40–42]. Statistical analyses and diversity indices were employed to assess the effects of JA signaling on the bacterial diversity in roots and leaves. In order to, further explore if the two genotypes specifically recruit particular bacterial isolates under *in-vitro* conditions. We investigated the effect of JA signaling on the bacterial colonization, but also if inoculation influenced the levels of JA, SA and ET after elicitation of EV plants by treating fresh puncture wounds with *M. sexta* oral secretion (OS_{MS}).

Materials and Methods

Plant material for field and glasshouse studies and sample collection in the field

For the field experiment, previously characterized, homozygous *Nicotiana attenuata* Torr. ex Watson empty vector plants (EV, A-03-9-1-1,[48]) and an isogenic transgenic line impaired in JA biosynthesis (ir*AOC*, A-07-457-1, [47]) were used. EV and ir*AOC* plants were germinated on Gamborg B5 as described in Krügel *et al.* [49], transferred to individual Jiffy pots and planted in size-matched pairs in a field plot located at Lytle Ranch Preserve, Great Basin Desert, Utah [50]. Plants were harvested at different developmental stages and rosette diameter and stalk length for each stage are shown in (Table S1 in File S1). At harvest plants were separated into roots and leaves and washed in tap water to remove the soil particles attached to the roots and transported to the laboratory on ice.

Isolation of culturable root- and leaf-associated bacteria

Two days after excavation from the field, bacterial isolation was carried out as described in [5]. Roots and leaves were surface-sterilized and a fraction stored at −80°C for pyrosequencing [51], while the remaining tissue was aseptically sectioned into smaller fragments and distributed onto three different isolation media: tap water-yeast extract agar (TWYE [52]), *Streptomycetes* isolation media (SIM [53]) and glucose-yeast extract agar (GYE [54]). Plates were incubated at 28°C for 4 d. After incubation, colonies were picked from plates, sub-cultured and stored in 50% glycerol solution at −80°C. The total number of bacterial isolates recovered from the respective media were 116 from EV roots (GYE-40, TWYE-42, SIM-34), 89 from ir*AOC* roots (GYE-32, TWYE-32, SIM-25), 107 from EV leaves (GYE-38, TWYE-41, SIM-28) and102 from ir*AOC* leaves (GYE-37, TWYE-35, SIM-30). The surface sterilization procedure efficacy was assessed by plating aliquots of the sterile distilled water used in the final rinse onto nutrient agar medium (Sigma, Steinheim, Germany) and incubated as described above. We did not observe any bacterial colonies on control plates.

Bacterial DNA extraction and 16S rRNA gene sequencing and identification

Genomic DNA was extracted from bacterial isolates and16S rRNA, PCR amplifications were performed according to Kim and Goodfellow [55] with minor modifications. Amplification of 16S rRNA gene was performed in a 20 µL final volume of Ready-mixTaq PCR reaction mix (SigmaAldrich) containing 2 µL of template DNA, 50 µM of primer 27F (5′-AGAGTTT-GATCCTGGCTCAG- 3′) and 1492R (5′- GGTTACCTTGT-TACGACTT- 3′, [56]. A negative control PCR mixture with sterile water was included in all PCR experiments. PCR products were purified using the QIAquick™ Gel Extraction Kit (QIA-GEN, Hilden, Germany) following the manufacturer's manual. Direct sequencing using the primer 783R (5′- CTACCAGGG-TAT C TAATCCTG -3′) was conducted with Big Dye Mix (Applied Biosystems, Foster City, CA, USA), and purification of the sequencing reactions was performed using the Nucleo-SEQ Kit (Macherey-Nagel, Düren, Germany). Analysis of all sequences was carried out in EzTaxon server (http://eztaxon-e.ezbiocloud.net/, [57])

Plant DNA extraction, sample pooling and bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), and 16S rRNA analysis

Total genomic DNA was extracted from all surface-sterilized root and leaf tissues using FastDNA™ Spin kit for soil (MP biomedicals). DNA of biological replicates at the respective developmental stages of the EV and ir*AOC* genotypes (n = 3–5) were pooled into one DNA sample (total number of pooled samples = 20): the concentration was determined by NanoDrop spectrophotometer, and diluted to a working concentration of 30 ng/µL before combining equal volumes. To evaluate the utility of 3 different primers, rosette-stage leaf and root samples were

used. Bacterial 16S rRNA genes variable regions from V4–V9 were amplified by the following 3 primers sets: 515F-806R: GTGCCAGCMGCCGCGGTAA - GGACTACVSGGGTATCTAAT [58]; 799F-1394R: ACCMGGATTAGATACCCKGACGGGCGGTGTGRTC, [59] and 939F-1394R: TTGACGGGGGCCCGCAC- ACGGGCGGTGTGRTC. The 799F-1394R primers were used in all subsequent analyses. Pyrosequencing bTEFAP was performed by Research & Testing Laboratories, Lubbock, TX, USA (RTL, www.researchandtesting.com).

Pyrosequencing 16S rRNA gene sequence analysis

The QIIME software package was used to analyse the high-quality reads using default parameters for each step [60]. Briefly, sequences were eliminated if the average quality scored <25, lengths were shorter than 200 bp, excess of 6 bases homopolymer runs, primer mismatch and ambiguous bases. USEARCH series of scripts were used to remove the chimer and noisy sequences followed by clustering of OTUs picking with 97% cut-offs [61]. Most abundant sequences were taken as representative sequence for each clusters and aligned to the Greengenes database [62] using PyNast algorithm with minimum percent identity at 80% [63]. FastTree was used to build the phylogenetic tree [64] and taxonomy was assigned using RDP classifier with a minimum support threshold at 80% [65]. OTUs with the same taxonomy at class and genera-level were pooled for description of community.

Statistical analysis

Primer E software v.6 [66] and QIIME software package were used for all statistical analyses. All samples were rarefied and OTUs present in ≤2 samples were not considered for further analysis. Alpha-diversity was determined by calculating the Shannon diversity index [67], Margalef's index [68], and Pielou's evenness index [69] based on OTUs 97% identity. The mean of 10 permutations of richness, evenness and diversity were used for an ANOVA analysis to compare 'genotype & tissues' (EVL, ir*AOC*L, EVR, ir*AOC*R). QIIME script OTU significance test (ANOVA) was applied to find out whether OTUs based on 97% identity are significantly associated with a specific sample type. The Uni-Frac distance metric was calculated as a measure of bacterial community similarity [70] for roots and leaves of each plant developmental stage. Its values range between 0 to1, and samples with a value of 1 have entirely different bacterial communities, while 0 indicates bacterial communities are identical among two samples. The Uni-Frac distance metric was also the basis for non-parametric analysis of similarities (ANOSIM) among samples [71] and non-parametric multidimensional scaling (MDS) to visualize the similarity of bacterial communities among genotype roots and leaves at the different developmental stages [72]. Alpha and beta diversity, Uni-Frac distance metric and Uni-Frac beta significance test were calculated based on 97% sequence identity. ANOVA followed by Fisher's PLSD test was used to compare means of CFU g^{-1} fresh mass (F-mass) of fresh roots or leaves, root length, plant biomass, leaf-surface area, and phytohormone levels (JA, SA, ET) using Infostat 2010 [73].

In vitro bacterial re-colonization assays

In order to examine whether JA influences the colonization pattern of bacteria isolated from the field, we selected ten bacterial strains exclusively isolated from either of the plant genotypes (EV or ir*AOC*, called 'putative specialist') and seven bacterial strains isolated from both genotypes, dubbed 'putative generalists', and *Bacillus sp* B55 from a previous study as positive control [74], for specific colonization assays. Roots of 7-day-old seedlings were dipped into 1 mL of bacterial suspensions for 1 min (OD_{600} = 1) and transferred to a Magenta^{TM} vessel box (W×L×H; 77 mm ×77 mm ×97 mm, Sigma, GA-7, Germany) filled with sand (0.7–1.2 mm grain size, Raiffeisen, Germany) and grown in a Vötsch chamber (22°C, 65% humidity, 16 h light). For single inoculations, seedlings of each genotype were individually inoculated with one of the selected 18 bacterial isolates or distilled water as control (Table S2 in File S1), while for the mixed inoculations, inoculation was conducted with a mixture of the 18 bacterial isolates (50 µL of each isolate at OD_{600} = 1). Two weeks after inoculation the length of the primary roots and the plant biomass were determined and the leaf surface area was analyzed using Adobe Photoshop C5 [74] and bacterial re-isolation was carried out as described above. Bacteria were identified by morphology and 16S rRNA sequencing.

Phytohormone levels after elicitation of specialist-inoculated glasshouse-grown plants

Phytohormone production (JA, SA, and ET) was determined in EV plants inoculated with two bacterial isolates retrieved only from EV (*Pseudomonas frederiksbergensis* A176, *Pseudomonas koreensis* A21) and ir*AOC* plants (*Kocuria palustris* B56, *Kocuria marina* D102), respectively. After surface-sterilization, EV seeds were incubated in bacterial solution and germinated as described above. Plants were grown according to [44] and at day 30, fully-expanded young rosette leaves (+2 nodal position, source leaf [36]) were mechanically wounded with a pattern wheel and the puncture wounds immediately treated with 20 µL of 1:5 diluted *M. sexta* oral secretion [75] or sterile distilled water. Leaves were harvested at 60 and 120 min after treatment. JA and SA levels were quantified with a UPLC-UV-ToF-MS according to [76]. For the determination of ET emissions, leaves were treated as above, and leaf discs (12 mm in diameter, 35.5±1 mg tissue) were punched from the mesophyll tissue on one side of the midvein, and placed in 4 mL transparent glass GC vials with screw lids and PTFE septa (Macherey-Nagel, Germany). Leaf discs were left to incubate for 5 h and ET was measured using a stop-flow set-up using a photoacoustic laser (ETD 300, Sensor Sense, and The Netherlands). Each vial was sampled for 5 min, which was sufficient to detect the entire peak of accumulated ET based on a stop-flow detached leaf method.

Nucleotide sequence accession numbers

The sequencing data have been deposited in the European Nucleotide Archive- PRJEB4653, the isolates of the culture dependent approach are listed in Table S3.

Results

Primer pair 799F-1394R amplifies a minimum of chloroplast sequences with the highest diversity of bacterial sequences

Several primer combinations have been used to characterize the bacterial community by pyrosequencing. In order to find out which primer set had the highest specificity for bacterial sequences and retrieved the greatest diversity of taxa from our samples. We tested three different primer sets spanning the variable region of 16S rRNA from V4 to V9 (Figure S1A, [6]). For two primer sets, 515F-806R and 939F-1394R, between 94 and 97% of the reads were chloroplast sequences, and only 6 and 7 bacterial classes were amplified, respectively. With primer pair 799F-1394R less than 1% was chloroplast sequences (Figure 1) and reads associated with 9 bacterial classes were retrieved (Figure S1B). Shannon diversity

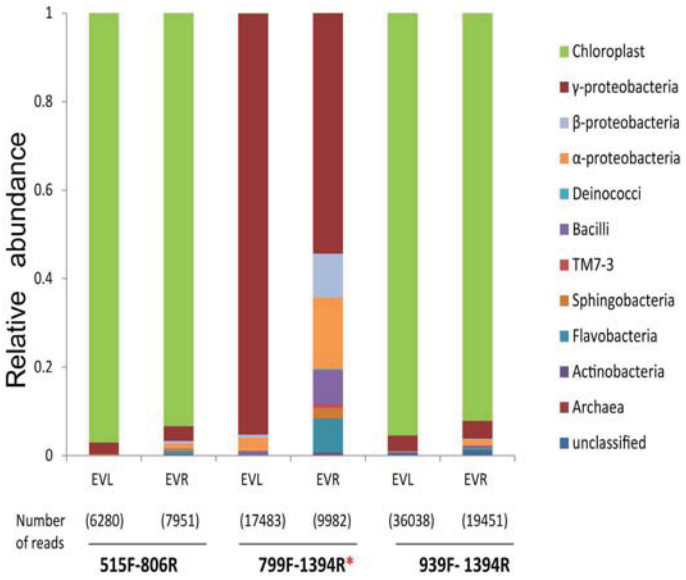

Figure 1. PCR primer pair 799F-1394R showed the lowest amplification of non-target chloroplast sequences comparing three different primer pairs. Relative abundance of bacterial classes recovered using three different primers (515F-806R, 799F-1394R & 939F- 1394R) from leaf and root samples of native field grown EV-genotype. Among these primers 799F-1394R amplified the lowest amount of reads matching with chloroplast sequences. Abbreviations: R, roots; L, leaves; *, PCR primer selected for further analysis.
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index and Margalef species richness were higher for the sequences retrieved by the 799F-1394R primer pair compared to those retrieved by the two others (Figure S1C,D). Based on these results, we selected 799F-1394R primers for further analyses.

Bacterial communities are independent of plant developmental stages

To investigate the influence of developmental stages on the plants' root- and shoot-associated bacterial communities, we analyzed the bacterial communities of EV and ir*AOC* roots and leaves grown in their native habitat in Utah, USA by pyrosequencing. The dataset comprised 6,500–19,000 reads for each sample (Figure S2). To compare and minimize heterogeneity among samples, all samples were rarefied to 6,374 reads per sample. The RDP Bayesian classifier assigned all the sequences to 14 different bacterial classes (Figure 2B,C). γ, β, α- proteobacteria along with Bacilli and Flavobacteria were identified in all samples independently of genotype, tissue and harvest time. Furthermore, bacterial classes such as δ, ε, proteobacteria along with Fusobacteria, Chlamydiae, candidate division TM7-3 and Acidobacteria appeared sporadically throughout the different developmental stages of roots of both genotypes.

The calculation of the weighted Uni-Frac beta-significance values, which identify pairs of samples that are significantly different from one other [70], indicated that root and leaf bacterial communities did not change significantly over development except for a few single time-points, which were only marginally different from one another (Table S4 in File S1). In order to visualize the similarity of bacterial communities among genotypes and tissues at the different developmental stages, a non-parametric multidimensional (MDS) ordination was constructed. The MDS plot shows that leaves are clustered closely together irrespective of developmental stages and genotypes, while roots showed an overall higher heterogeneity among samples, and samples of earlier developmental stages tend to group more closely together than from flowering plants independent of genotypes (Figure 3). Therefore we further evaluated if plant development like transition from rosette to flowering stages (young versus old) influences bacterial communities. We pooled the samples with stems not yet developed as young (rosette and elongated stage I) and elongated and flowering plants as old (I & II, elongated II & III and flowering I & II), because earlier studies showed that the JA outburst against insects dramatically shift from rosette to flowering transition [36]. However, neither alpha (Figure S3) nor beta diversity (ANOSIM, Table S5 in File S1) differed among these two developmental stages. We conclude that bacterial communities are largely independent of plant developmental stages irrespective of genotypes.

JA biosynthesis does not change the overall bacterial communities

In order to robustly evaluate whether endogenous JA levels influence bacterial communities we calculated the alpha and beta diversity indices of the leaf- and root-associated bacterial communities of EV and ir*AOC* genotypes. The Margalef's species richness, Pielou's evenness and Shannon diversity index were significantly different among 'genotypes & tissues' (Figure 4, Margalef's species richness ANOVA; $F_{3,16} = 17.26$, $p<0.001$, Pielou's evenness ANOVA; $F_{3,16} = 5.67$, $p = 0.007$, Shannon diversity ANOVA; $F_{3,16} = 9.27$, $p = 0.0009$); differences were due to discrepancies in bacterial communities among leaves and roots, but not genotypes. These findings are in accordance with the beta-

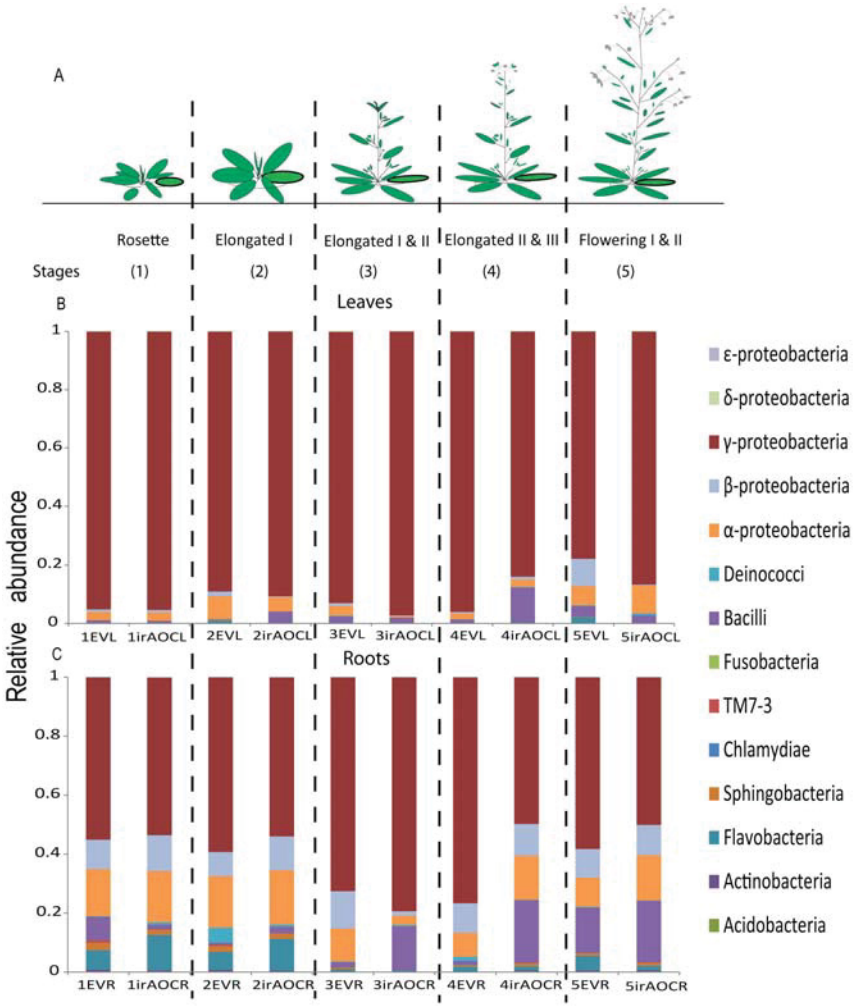

Figure 2. Native field grown EV and ir*AOC* leaf and root-associated bacterial communities (OTUs) are not influenced by developmental stages and jasmonic acid. In the culture-independent approach, at the class level relative abundance of operational taxonomic units (OTUs) of field-grown *N. attenuata* plants is independent of the developmental stages and the ability of the plant to produce jasmonates (JA). JA-producing empty vector (EV) plants and plants impaired in JA biosynthesis by silencing allene oxide cyclase (ir*AOC*) were grown in pairs at a field site in the plant's native habitat. Schematic representation of the plants' developmental stages at harvest **(A)**. Abundance of bacterial composition at the class level in EV and ir*AOC* leaves **(B)** and roots **(C)**. For abbreviations see Figure 1. All samples were rarefied to 6374 sequences.
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diversity analysis of similarities (ANOSIM, Table 1) based on the weighted Uni-Frac distance matrix. The same results described here at 97% sequence identity were obtained when we analysed the data at the class and genera level (data not shown). We conclude that overall bacterial communities are independent of JA biosynthesis.

Roots and leaves harbor distinct bacterial communities

To further elucidate the differences in bacterial communities among root and leaf samples we examined the abundance of 8 core OTUs at the class level which were present in roots and leaves, and covered ≥7–<90% of the total abundance across all samples. Abundance of five classes (Actinobacteria, Sphingobacteria, Flavobacteria, β, α- proteobacteria) were higher in roots compared to leaves, while γ-proteobacteria dominated the bacterial community of leaves. In contrast, candidate division TM7-3 and Acidobacteria bacterial classes were only retrieved from roots across all developmental stages irrespective of genotypes. At genera level, the abundance of 14 OTUs was significantly different among roots and leaves (Table S6 in File S1). In summary, both alpha and beta diversity (Figure 3&4 and

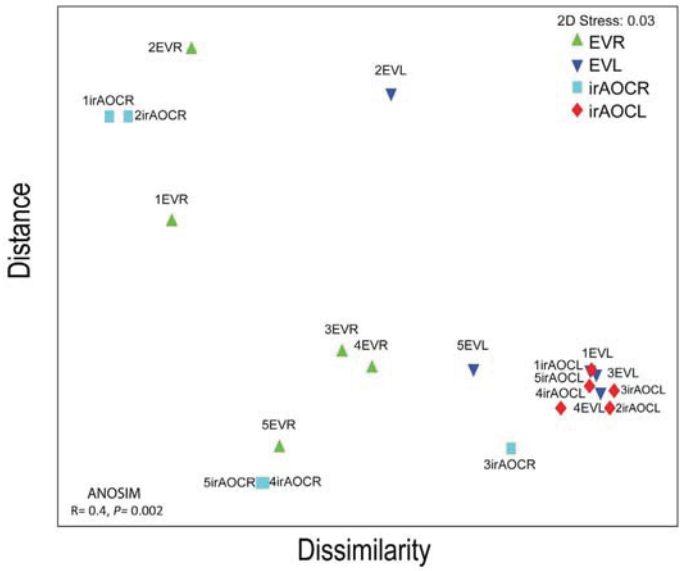

Figure 3. Based on the pyrosequencing results beta diversity of OTUs is influenced by tissues (leaves & roots) but not by genotypes (EV & ir*AOC*). In non-parametric multidimensional scaling (MDS) ordination, proximity of points reflects similarity. OTUs diversity among EV and ir*AOC* leaves and roots is highly similar, indicating bacterial communities are independent of JA and developmental stages but not tissues. Global ANOSIM among roots and leaves of both genotypes is significantly different ($p = 0.002$). MDS ordination and ANOSIM were determined by the weighted Uni-Frac distance metric based on OTUs rarefied to 6374 reads for each sample. Abbreviations: R, roots; L, leaves. Refer to Figure 2 for abbreviations and the experimental set-up in the field and harvest of plants.
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Table 1) variation analysis strongly suggest that root- and leaf associated bacterial communities are determined by the tissue type but not by the JA signaling capacity of the plants.

At the genera level 21 OTUs differ among plant genotypes

A more detailed analysis indicated that at the genera level, 21 OTUs differed significantly between the two genotypes; 9 OTUs were retrieved in higher abundance from EV roots and 12 from ir*AOC* roots ($n = 5$, $p<0.05$, Fisher's PLSD, Figure 5). In particular, OTUs corresponding to *Paenibacillus* and *Azospirillum* were much more abundant in ir*AOC* than EV roots. The differences among the genotypes were only found in roots, not leaves. Based on these results we conclude that roots harbor a greater diversity of bacterial communities compared to leaves, and the diversity of bacterial communities is largely independent of JA signaling, though the plants' capacity to produce JA may influence the occurrence and abundance of particular genera.

Putative EV and ir*AOC* specialist and generalist isolates did not show colonization specificity under *in-vitro* conditions

In addition to pyrosequencing, we employed a culture-dependent approach using the same plant material, because only cultured bacteria allow a functional analysis and further in-depth study of putative differences in colonization patterns. A total of 414 bacterial isolates were retrieved from surface-sterilized roots and leaves of both genotypes. Based on the 16S rRNA gene sequences, the isolates were assigned to 131 different species and 6 classes (116 and 89 isolates from EV, 107 and 102 from ir*AOC* roots and leaves, respectively, Table S3). A comparison of the genotypes revealed that 42 species (66 isolates) were only isolated from ir*AOC* plants, and we tentatively considered these as putative ir*AOC* specialists. Similarly, 51 species (121 isolates) were only recovered from EV plants (putative EV specialists), while 38 species (227 isolates) were found in both genotypes, and these we considered as generalists. Furthermore, members of the genera *Pseudomonas* colonized more frequently in EV plants (58 isolates) than ir*AOC* plants (22 isolates) over all developmental stages; whereas, the genera *Kocuria* was only retrieved from ir*AOC* plants (11 isolates, Table S3). Interestingly, eight genera isolated by the culture-dependent approach were also recovered in higher abundance from EV or ir*AOC* roots in the pyrosequencing approach (Figure 5).

To evaluate the reproducibility of the observed genotype-specific colonization patterns found in the field, we performed *in-vitro* re-colonization assays by inoculating seedlings either with single bacterial isolates or with a mixture of all bacterial isolates used in the single inoculations. We used the mixed inoculation procedure to recapitulate a more natural situation, and to evaluate if plants only recruit specific bacterial isolates from a mixture of cultures. Isolates were selected based on a) their difference in abundance in the two genotypes in the pyrosequencing and the culture-dependent approach and b) the beneficial effects of some species described in literature (Table S2 in File S1).

Overall, single and mixed inoculations with most bacterial isolates resulted in a poor colonization of leaves compared to roots of both genotypes (Figure 6B,C). Only 4 species were able to colonize both ir*AOC* and EV leaves. The colonization pattern of

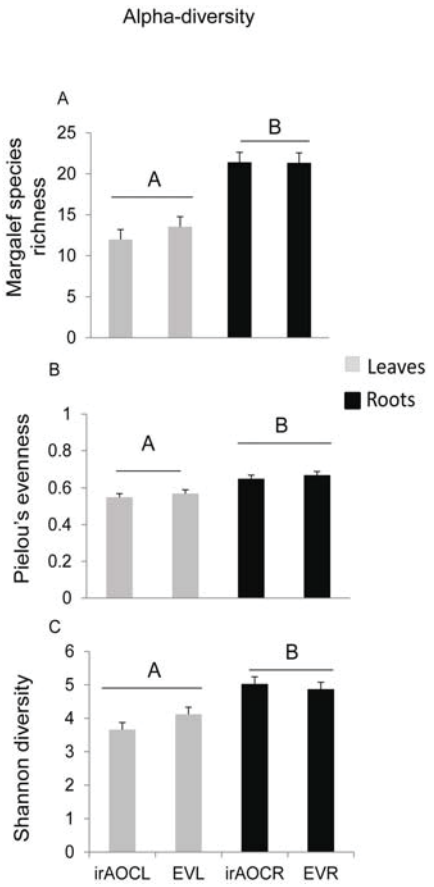

Figure 4. Alpha diversity of OTUs among leaves and roots of EV and ir*AOC* genotypes is significantly different. Based on the pyrosequencing results, alpha diversity indices such as Margalef's species richness (A), Pielou's evenness (B) and Shannon diversity (**C**) were significantly different among the 'genotypes & tissues' (EVL, ir*AOC*L, EVR, ir*AOC*R). Mean, ±SE, n = 5 subsampling (6374 sequences for each sample), different letters indicate significant differences, one-way ANOVA with Fisher's PLSD test; $P<0.05$.
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leaves and roots was independent of the putative specialist and generalist behavior observed in the field. In summary, under *in-vitro* conditions we could not confirm the apparent putative genotype-specific colonization behavior observed by some bacterial isolates in the field. The results are consistent with the hypothesis that bacterial colonization of plants is not primarily shaped by JA signaling, and depends upon the availability of individual bacterial isolates to infect plants in the soil at the plant's particular planting site.

Elicitation of JA, SA and ET did not differ among EV and ir*AOC* plants inoculated with the specialist taxa

We not only evaluated the effects of the plant's ability to elicit endogenous JA-signaling on its bacterial community but also if bacterial colonization alters the phytohormone levels in elicited plants. We inoculated EV seeds with two *Pseudomonas* species (*P. koreensis* A21, *P. frederiksbergensis* A176) isolated only from EV plants in the field and two *Kocuria* species found only in ir*AOC* plants (*K. palustris* B56, *K.marina* D102, Figure S4). These species were selected among other specialists because they were more abundant at different developmental stages, and were retrieved from both roots and leaves. We measured changes in SA, JA and ET production in leaves of rosette stage EV plants after elicitation by mechanical wounding followed by application of oral secretion of *M. sexta* (OS_{MS}). The production of all three phytohormones significantly increased after elicitation with OS_{MS} compared to wound and water elicitation only (ANOVA; JA, $F_{9,20} = 26.15$, $p<0.001$. SA, $F_{9,20} = 6.02$, $p<0.001$. ET, $F_{9,20} = 3.54$, $p<0.01$) (Figure S5). However, there was no significant difference in

Table 1. Pairwise ANOSIM analysis among EV and ir*AOC* genotype tissues of the culture-independent approach.

Pairwise Tests	Culture-independent	
Groups	**R-Statistic**	***P***
EVR and EVL	0.51	0.02
EVR and ir*AOC*L	0.83	0.008
ir*AOC*R and EVL	0.45	0.02
ir*AOC*R and ir*AOC*L	0.71	0.008
EVL and ir*AOC*L	0.06	0.73
EVR and ir*AOC*R	0.04	0.66
Global	0.45	0.002

Abbreviations: R, roots; L, leaves and for the experimental set up and approach see Figure 2.
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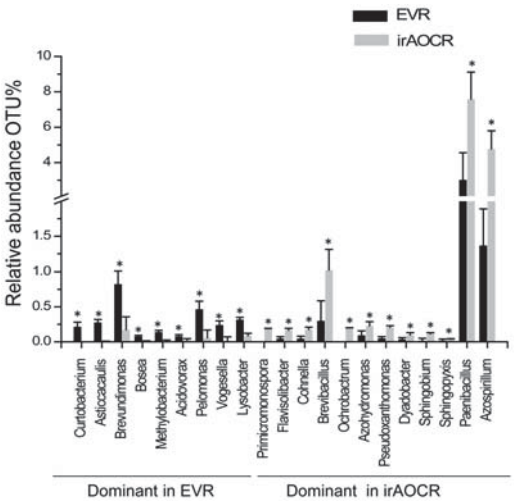

Figure 5. At the genera level, 21 OTUs differ significantly among the roots of the two genotypes (EVR and ir*AOC*R). OTU significance test was carried out with rarefied 6374 reads and OTUs which were significantly different were binned at the genera level. Mean, ±SE, n = 5, one-way ANOVA with Fisher's PLSD test; *$P<0.05$.
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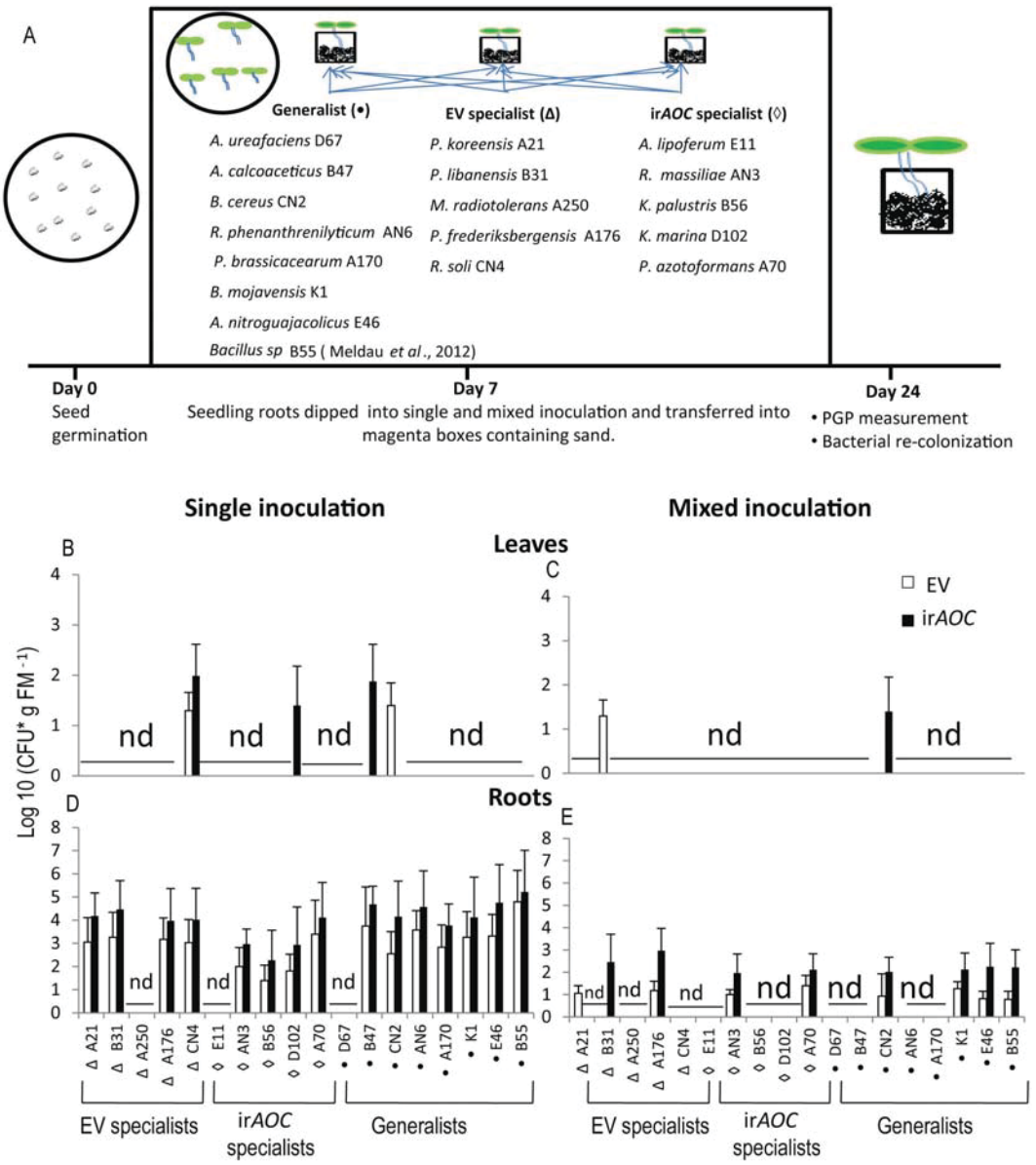

Figure 6. Cultured putative EV and ir*AOC* genotype specialist bacterial isolates did not colonize specifically to either genotype under *in-vitro* conditions. Bacterial colonization is independent of genotypes. Leaf (B,C) and root (D,E) colonization of EV and ir*AOC* plants grown under *in-vitro* conditions for 24 days after single or mixed inoculation of EV and ir*AOC* seedlings. Experimental set-up and list of species used (A): bacteria selected were either isolated only from EV or ir*AOC* genotypes (putative specialists) or generalists, isolated from both genotypes. Seven day old seedlings were inoculated by dipping their roots for 1 min into 1 mL of bacterial suspension of a constant OD = 1 at 600 nm of a single isolate or a mixture of all isolates (50 μL each of isolate). The identity of bacterial isolates was confirmed by morphology and 16S rRNA sequencing. Mean (±SE), CFU, colony-forming units; nd, not detected; FM, fresh mass; n = 6.
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phytohormone levels between the inoculation with the four bacterial isolates and water-treated controls (ANOVA; JA, $F_{4,12} = 0.328$, $p = 0.85$. SA, $F_{4,12} = 2.143$, $p = 0.15$. ET, $F_{4,12} = 0.31$, $p = 0.86$, Figure S5). Thus, inoculation of EV plants with field-observed putative specialist bacterial isolates did not influence the OS-elicitation of JA, SA and ET.

Discussion

In nature, plants are subjected to various biotic and abiotic stresses throughout their development which in turn may influence the composition of the bacterial communities [77,78]. In this study, we conducted a comprehensive pyrosequencing and culturing analysis of the temporal changes in bacterial communities of leaves and roots of plants grown in their native habitat. Additionally, we investigated the effects of JA signaling, and its associated defenses on the development of the plant's bacterial community because the literature on this topic is sparse and contradictory [34,35]. Our approach differs from previous studies which were either conducted under controlled glasshouse conditions [6,39] or used only culture-dependent techniques [34,35,79]. Our results not only support the results of earlier studies [6,35,39], but also provide new insights into root and shoot bacterial communities exposed to their natural environment.

Culture-independent approaches based on pyrosequencing are influenced by the primers and sequencing depth [58]. We tested three different primers, and sequences retrieved from two primer sets were largely chloroplast DNA, while 799F primer minimized the contamination of chloroplast DNA and excluded Cyanobacteria [12,59]. According to Ghyselinck *et al.* [18] this primer matches only with 78.5% of bacteria based on SILVA SSU ref 113 NR database, however, in our primer test 799F sequence reads resulted in higher alpha diversity and were assigned to more phyla (Figure S1) than the reads from the two other commonly used primers.

We tested the hypothesis that ontogeny alters community composition of roots and leaves, because inducible defense signaling mediated by JA is known to change dramatically at the rosette-flowering transition in *N. attenuata* [36]. Earlier studies indicated significant differences in bacterial populations over season in roots and leaves of soybean and rice [15,21,79,80]. For soybean plants it was shown that the density of bacteria decreased with age from vegetative growth to senescence [79], while diversity of *Pseudomonas* species decreased over development starting from young to senescent stages [79]. However, those studies only analyzed the culturable communities, while in the present study, based on deep sequencing of the 16S rRNA region, we found that bacterial communities are independent of plant developmental stages, and the weighted Uni-Frac beta significance test only showed a few pairs with marginally significant differences (Table S4 in File S1). Similarly, a comparison of alpha (Figure S3) and beta (Table S5 in File S1) diversity indices of the rosette-flowering transition indicated that bacterial communities are independent of developmental stages. Our findings are in agreement with the extensive study of Lundberg *et al.* [6] who investigated the influence of plant developmental stages of young versus old tissues on bacterial communities of *Arabidopsis*, which were grown in two different soil types in the glasshouse. This study also demonstrates that bacterial communities do not alter over developmental stages. We conclude that plant development does not have a major effect on community composition in native field grown *N. attenuata* plants.

In contrast to development, tissue type has a major effect on the bacterial community composition (Figure 3&4, Table 1), which was also observed for field grown soybean [79] and *Arabidopsis* [12] plants. In accordance with previous studies, the dominating bacterial class in leaves was γ- proteobacteria (Figure 2) [12]. At the genera level two OTUs, namely *Serratia* and *Enterobacter* heavily dominated the phyllosphere, while in many other studies *Pseudomonas* was the dominating genera [81,82]. Overall, roots recruited more diverse bacterial communities than the leaves (Figures 3, 4). Genera belonging to α- proteobacteria such as *Rhizobium* and *Azospirillum* (15–25% abundance) which are well-known to dominate the root communities of nitrogen fixing plants may have a positive effect on plant growth and health [21,83]. Based on these findings we assume that a core bacterial community is recruited from the soil, but roots and leaves provide different niches for bacterial growth. The roots' higher diversity may be due the secretion of root exudates and the direct contact of the roots with the soil microbiome [11,15], while bacterial communities of the leaves are additionally influenced by rain splashing of the soil, dust or wind [79].

The phytohormone JA is known to play a central role in plant defense against leaf-chewing herbivores, but it is also involved in induced systematic resistance (ISR) against pathogens [33]. However, influences of JA on bacterial communities at different plant developmental stages have so far received little attention [34,84]. Unlike, this study, Doornbos *et al.* [35] showed that the JA-response mutant *jar1* harbored significantly lower numbers of culturable bacteria compared to Co1-0 wild type, while a different study with the same *Arabidopsis* ecotype and a transgenic line impaired in the production of JA biosynthesis could not find any difference in the culturable leaf-associated bacterial communities [34]. A recent field study with 27 different maize genotypes also revealed small, but significant differences in diversity indices among genotypes [58]. Native *N. attenuata* plants are genetically diverse and individual plants of a population accumulate different amounts of JA after herbivore attack [47]. It would not be surprising if natural variation in JA accumulation also leads to the colonization with different bacterial communities that help a plant to compensate for JA-deficiencies, as we had previously demonstrated for ET-deficient plants [74,85]. However, our study did not support this expectation. Despite the differences in primary and secondary metabolites such as sugars, starch and nicotine between EV and ir*AOC* genotypes [86], results of our field study did not reveal any significant differences in alpha diversity indices (Figure 4), beta diversity variance or MDS plots (Figure 3, Table 1), though at the genera level some OTUs were significantly different between roots of EV and ir*AOC* plants (Figure 5). Unfortunately, up to now it is not possible to validate the pyrosequencing results experimentally. Therefore, we used selected isolates from the culture-dependent approach to test if they specifically colonize one of the genotypes. None of the isolates tested showed a preferred colonization of EV or ir*AOC* (Figure 6). In addition, EV and ir*AOC* specialist treatment of plants did not alter the OS-elicited accumulation of phytohormones (JA, SA, ET; Figure S5). These findings strongly suggest that neither the plants' capacities to produce JA, nor JA-elicited primary and secondary metabolites, play a major role in shaping root- and leaf-associated bacterial communities. However, it cannot be ruled out that JA has an effect on some genera which could not be retrieved by the culture-dependent approach. Furthermore, the *in-vitro* assay may not allow selective root colonization and recruitment of selected species.

This study demonstrates that the recruitment of root- and leaf-associated bacterial communities by *N. attenuata* in its native habitat

is independent of the developmental stages and JA signaling, but is mostly driven by the composition of the community that the plant first comes in contact with when it germinates from the seed bank and as plants grow, different tissues (roots and leaves) established distinct bacterial communities. The colonization of plants by bacterial communities appears to be opportunistic, and mainly depending on the local soil microbe population. However, under specific circumstances (e.g. biotic and abiotic stresses) these opportunistic interactions may become mutualistic and help plants to adapt to these stresses, as has been recently shown by Meldau *et al.* [74,85]. The mechanisms responsible for these opportunistic mutualisms need further investigation.

Supporting Information

Figure S1 Base position of primers on 16S rRNA and primer comparison with regard to diversity of bacterial classes covered. The variable base positions on the 16S rRNA gene of the three different primer pairs tested (A, 515F-806R, 799F-1394R & 939F- 1394R). The number of bacterial classes (B), Margalef species richness (C) and Shannon diversity (D) recovered by primer 799F- 1394R was higher than for the two other primer pairs tested. Results are based on a pooled EV leaf and root samples. Abbreviations: R, roots; L, leaves; *, primer pairs selected for further analysis.
(TIF)

Figure S2 Rarefaction curves based on pyrosequencing reads, describing the observed number of operational taxonomic units (OTUs) as a function of the sequencing reads per each root and leaf samples. The OTU richness is higher in roots than leaves. Partial 16S rRNA gene sequences were pooled into single OTUs at the cut off value of 97% similarity. For abbreviations see Figure S1, the vertical line indicates the number of reads subsampled from each sample (6374 reads) for normalization.
(TIF)

Figure S3 Root and leaf bacterial communities are independent of developmental stages. Alpha diversity indices were not significantly different among young and old developmental stages of EV and ir*AOC* genotypes. Samples without stem (Rosette and elongated stage I) were merged as young and plants with elongated stems and flowering (elongated I & II, elongated II & III and flowering I & II) pooled as old. Mean, ±SE, n = 2–3 different letters indicate significant differences, one-way ANOVA with Fisher's PLSD test; $P<0.05$.
(TIF)

Figure S4 EV and ir*AOC* genotype putative bacterial specialist isolates identified by the culture-dependent technique. Two *Pseudomonas* species (*P. frederiksbergensis*A176 (**Δ**), *P. koreensis*A21 (**Δ**)) were only isolated from EV plants, and two *Kocuria* species (*K. palustris*B56 (◇) and *K. marina* D102 (◇)) only from ir*AOC* field-grown plants in high numbers. These taxa were considered as putative specialists on their respective hosts. Mean (±SE), nd, not detected; n = 19. For the experimental set-up, harvest of plants and isolation of bacteria see Figure 2.
(TIF)

Figure S5 Inoculation with putative bacterial specialist isolates did not influence the different phytohormone elicitation. Phytohormone elicitation is independent of putative bacterial specialist inoculation. Elicitation of jasmonic acid (JA, B), salicylic acid (SA, C) and ethylene (ET, D) was not significantly different among plants inoculated with the specialist bacterial isolates from EV and ir*AOC* plants. Experimental design (A): Plants were seed-inoculated with different bacterial strains by incubating the seeds overnight in bacterial suspension (OD600 = 1). Rosette-stage EV leaves were wounded with a fabric pattern wheel followed by the application of oral secretion (OS) of *Manduca sexta* (wound + OS_{MS}, 20 µL) or water (Wound + water) to punctured wounds to faithfully mimic *M. sexta* larva attack. JA levels were measured 60 min and salicylic acid 120 min after treatment. Ethylene accumulated for 5 h after elicitation. Mean ±SE; FM, Fresh mass; n = 5.
(TIF)

File S1 Table S1,S2, S4, S5, S6: Table S1. Average rosette diameter and stalk length of native field grown *N. attenuata* at the time of harvest. Table S2. Cultured putative specialist and generalist bacterial isolates used in this study. Table S4. Uni-Frac beta diversity is not significantly different among developmental stages of EV and ir*AOC* genotypes tissues indicating that bacterial communities are independent of developmental stages. Table S5. Pairwise ANOSIM did not differ significantly among rosette (young) and elongated, flowering (old) developmental stages. Table S6. List of OTUs significantly different among EV and ir*AOC* leaves and roots at genera (or higher) level retrieved by pyrosequencing.
(PDF)

Table S3 Abundance of culturable bacterial species from surface-sterilized roots and leaves of EV and ir*AOC* genotypes over different plant developmental stages.
(XLSX)

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Author Contributions

Conceived and designed the experiments: RS KG DGM ITB. Performed the experiments: RS. Analyzed the data: RS. Contributed reagents/materials/analysis tools: RS. Wrote the paper: RS KG ITB.

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Figure S1

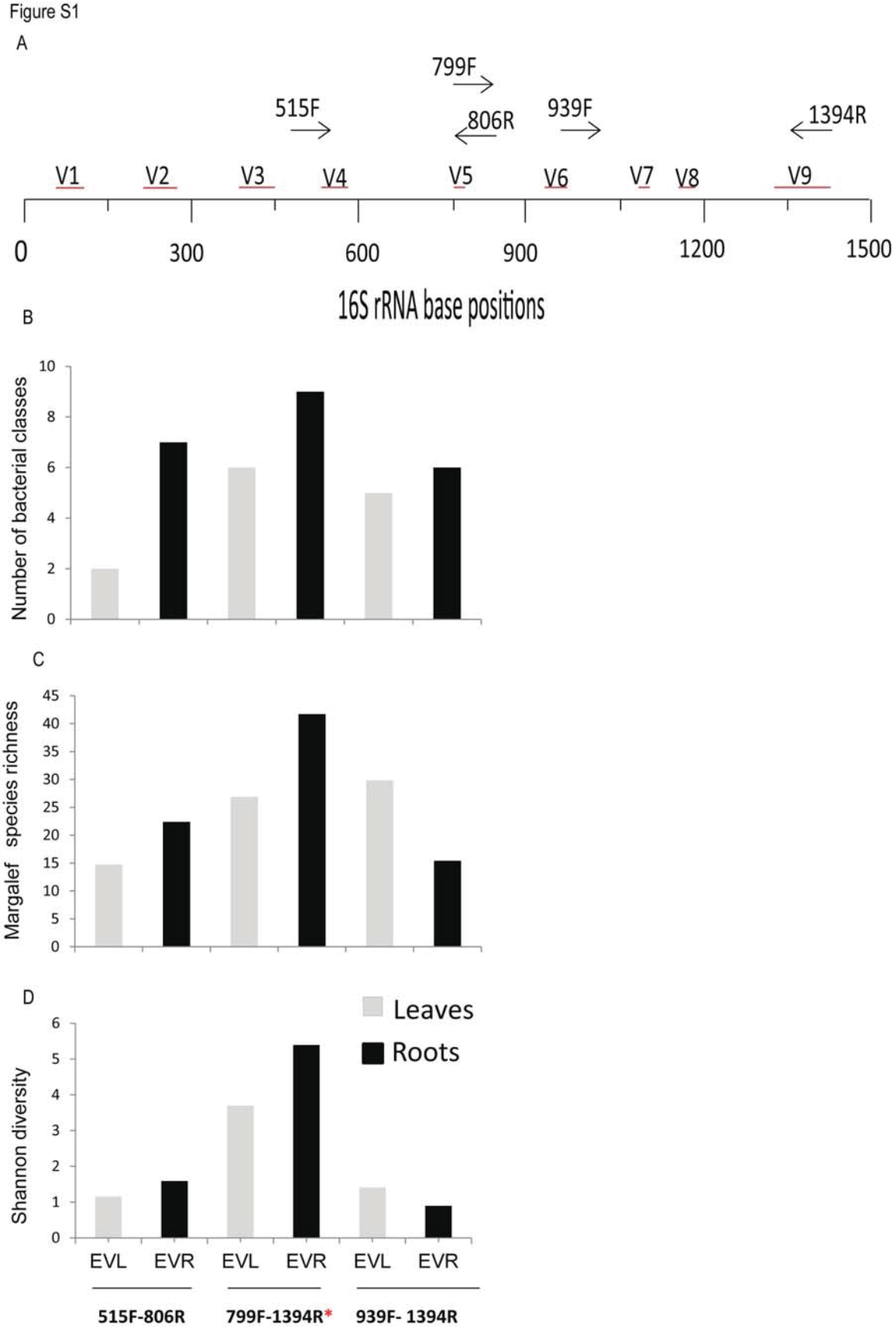
A
799F
515F
806R
939F
1394R
V1
V2
V3
V4
V5
V6
V7
V8
V9
0
300
600
900
1200
1500
16S rRNA base positions
B
Number of bacterial classes
C
Margalef species richness
D
Shannon diversity
Leaves
Roots
EVL
EVR
515F-806R
799F-1394R*
939F- 1394R

Figure S2

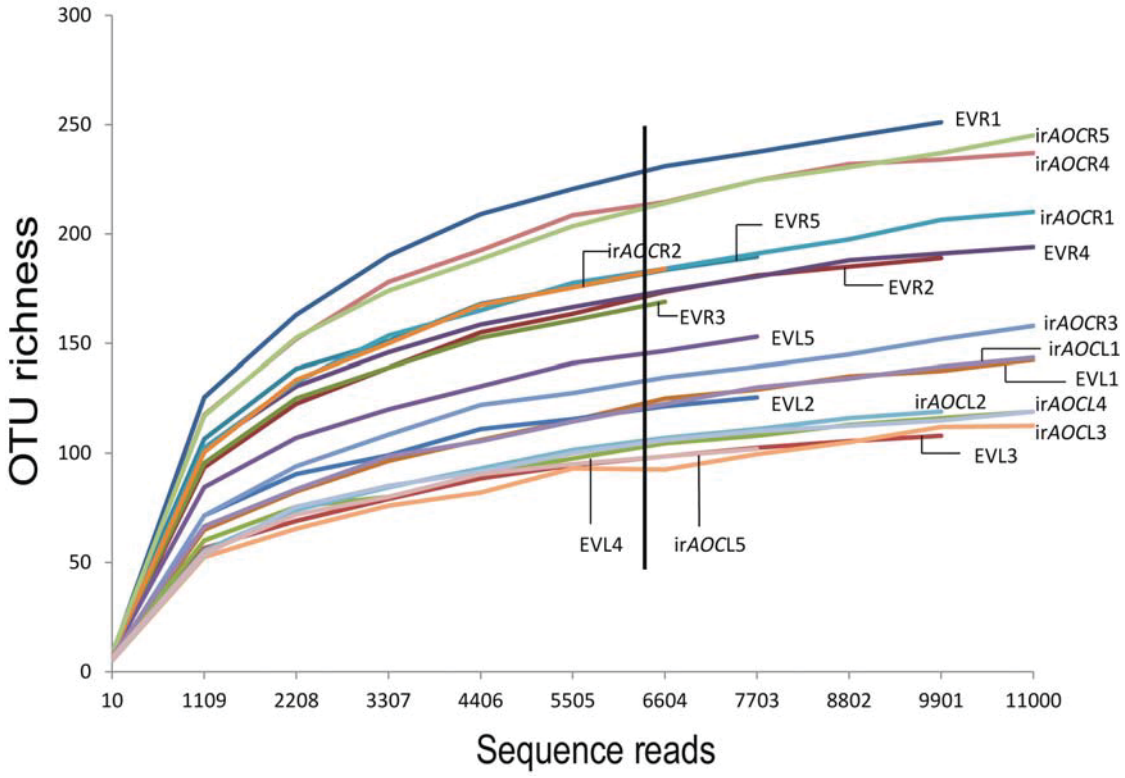
300
250
200
150
100
50
0
OTU richness
10
1109
2208
3307
4406
5505
6604
7703
8802
9901
11000
Sequence reads
EVR1
irAOCR5
irAOCR4
irAOCR1
EVR5
irAOCR2
EVR4
EVR2
EVR3
irAOCR3
EVL5
irAOCL1
EVL1
EVL2
irAOCL2
irAOCL4
irAOCL3
EVL3
EVL4
irAOCL5

Figure S3

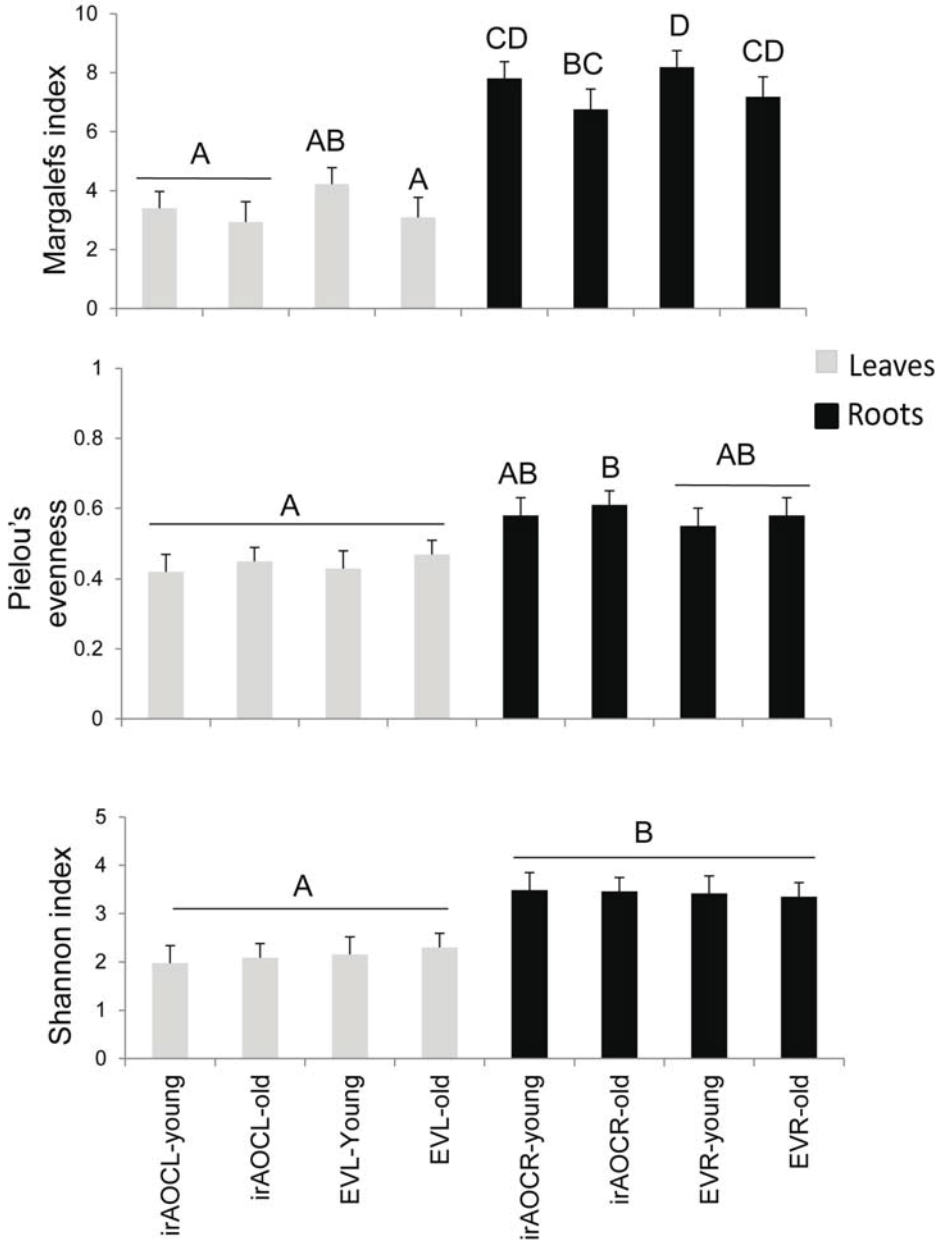
Margalefs index
10
8
6
4
2
0
A
AB
A
CD
BC
D
CD
Pielou's evenness
1
0.8
0.6
0.4
0.2
0
A
AB
B
AB
Shannon index
5
4
3
2
1
0
A
B
Leaves
Roots
irAOCL-young
irAOCL-old
EVL-Young
EVL-old
irAOCR-young
irAOCR-old
EVR-young
EVR-old

Figure S4

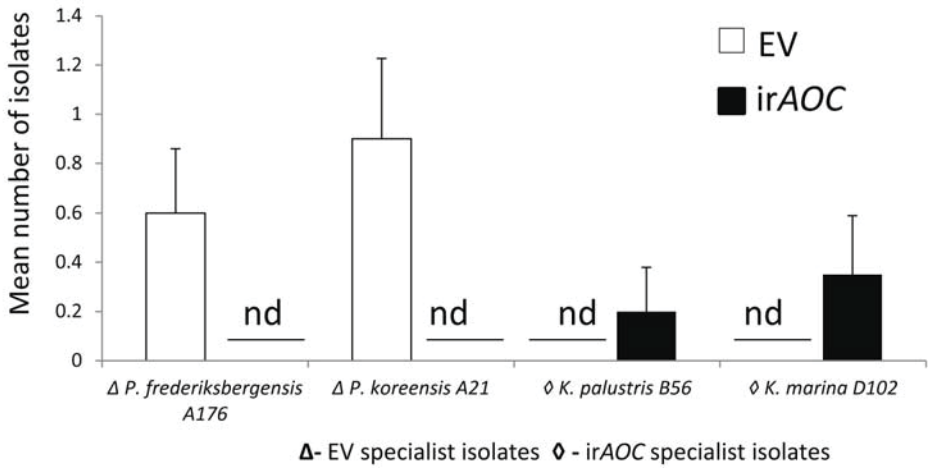
Mean number of isolates
1.4
1.2
1
0.8
0.6
0.4
0.2
0
EV
irAOC
nd
nd
nd
nd
Δ P. frederiksbergensis A176
Δ P. koreensis A21
◊ K. palustris B56
◊ K. marina D102
Δ- EV specialist isolates ◊ - irAOC specialist isolates

Figure S5

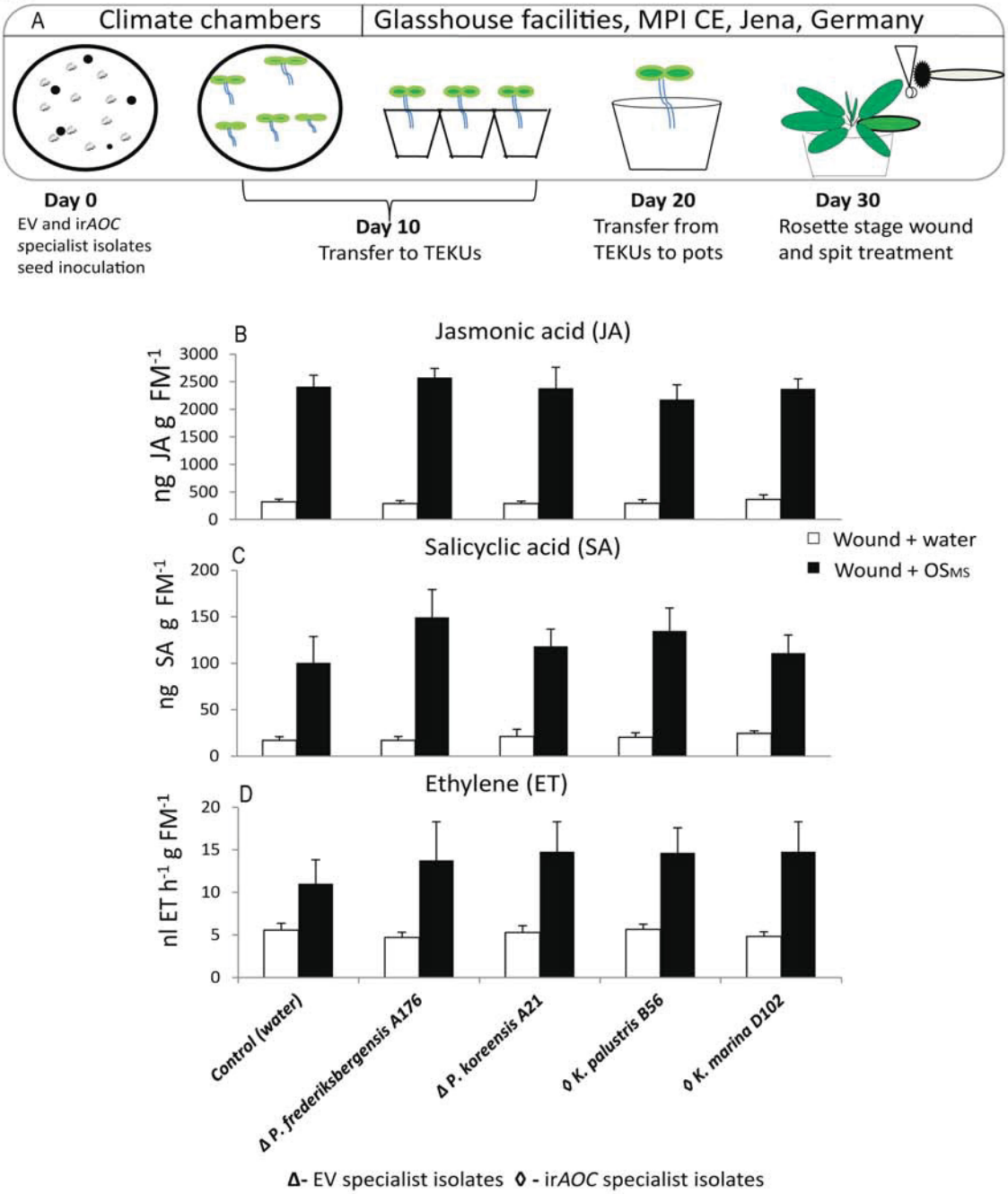
A
Climate chambers
Glasshouse facilities, MPI CE, Jena, Germany
Day 0
EV and irAOC specialist isolates seed inoculation
Day 10
Transfer to TEKUs
Day 20
Transfer from TEKUs to pots
Day 30
Rosette stage wound and spit treatment
B
Jasmonic acid (JA)
ng JA g FM-1
C
Salicyclic acid (SA)
ng SA g FM-1
D
Ethylene (ET)
nl ET h-1 g FM-1
Wound + water
Wound + OSMS
Control (water)
Δ P. frederiksbergensis A176
Δ P. koreensis A21
◊ K. palustris B56
◊ K. marina D102
Δ- EV specialist isolates ◊ - irAOC specialist isolates

Table S1. Average rosette diameter and stalk length of native field grown *N. attenuata* at the time of harvest.

	Rosette diameter (cm)		Stalk length (cm)	
Stages	EV	ir*AOC*	EV	ir*AOC*
Rosette (1)	9.3	9.5	0	0
Elongated I (2)	13	17	5	8
Elongated I &II (3)	15	18	14	25
Elongated II & III (4)	28	31	21	36
Flowering I & II (5)	38	40	40	54

N=3-5.

Table S2. Cultured putative specialist and generalist bacterial isolates used in this study.

Isolates	Nearest type strains	Characteristics	CFU (OD600=1)	References
A21	*Pseudomonas koreensis* Ps 9-14^T (AF468452)	Specialist for EV	28 X 10^{-7}	This study
B31	*Pseudomonas libanensis* CCUG 43190^T (AF057645)	Specialist for EV	40 X 10^{-7}	This study
A250	*Methylobacterium radiotolerans* CBMB20^T (AY683045)	Specialist for EV	24X 10^{-5}	This study
A176	*Pseudomonas frederiksbergensis* JAJ28^T (AJ249382)	Specialist for EV	12 X10^{-7}	This study
CN4	*Rhizobium soli* KCTC 12873^T (EF363715)	Specialist for EV	12 X 10^{-6}	This study
E11	*Azospirillum lipoferum* ATCC 29707^T (Z29619)	Specialist ir*AOC*	40 x10^{-5}	This study
AN3	*Rhizobium massiliae* KCTC 12121^T (AY341343)	Specialist ir*AOC*	40 X 10^{-4}	This study
B56	*Kocuria palustris* DSM 11925^T (Y16263)	Specialist ir*AOC*	11 X 10^{-6}	This study
D102	*Kocuria marina* KMM 3905^T (AY211385)	Specialist ir*AOC*	13 X 10^{-6}	This study
A70	*Pseudomonas azotoformans* CCUG 12536^T (D84009)	Specialist ir*AOC*	43 X 10^{-7}	This study
D67	*Arthrobacter ureafaciens* ATCC 7562^T (X80744)	Generalist for both genotypes	12 X 10^{-7}	This study
B47	*Acinetobacter calcoaceticus* ATCC 23055^T (AJ888983)	Generalist for both genotypes	70 X 10^{-7}	This study
CN2	*Bacillus cereus* ATCC 14579^T (AE016877)	Generalist for both genotypes	17 X 10^{-7}	This study
AN6	*Rhizobium phenanthrenilyticum* DSM 21882^T (FJ743436)	Generalist for both genotypes	80 X 10^{-4}	This study
A170	*Pseudomonas brassicacearum* DSM 13227^T (AF100321)	Generalist for both genotypes	90 X 10^{-7}	This study
K1	*Bacillus mojavensis* ATCC 51516^T (AB021191)	Generalist for both genotypes	30 X 10^{-7}	This study
E46	*Arthrobacter nitroguajacolicus* DSM 15232^T (AJ512504)	Generalist for both genotypes	16 X 10^{-7}	This study
B55	*Bacillus megaterium* ATCC 14581^T (D16273)	Generalist for both genotypes	20 X 10^{-5}	Meldau et al., 2012.

OD, optical density at 600nm; CFU, colony forming units.

Table S4. Uni-Frac beta diversity is not significantly different among developmental stages of EV and ir*AOC* genotypes tissues indicating that bacterial communities are independent of developmental stages.

Samples	1EVL	2EVL	3EVL	4EVL	5EVL	1irAOCL	2irAOCL	3irAOCL	4irAOCL	5irAOCL	1EVR	2EVR	3EVR	4EVR	5EVR	1irAOCR	2irAOCR	3irAOCR	4irAOCR	5irAOCR
1EVL	-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	*	*
2EVL		-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	*	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
3EVL			-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
4EVL				-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	*	*
5EVL					-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
1irAOCL						-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	*	*	n.s
2irAOCL							-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
3irAOCL								-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
4irAOCL									-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
5irAOCL										-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
1EVR											-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
2EVR												-	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
3EVR													-	n.s	n.s	n.s	n.s	n.s	n.s	n.s
4EVR														-	n.s	n.s	n.s	n.s	n.s	n.s
5EVR															-	n.s	n.s	n.s	n.s	n.s
1irAOCR																-	n.s	n.s	n.s	n.s
2irAOCR																	-	n.s	n.s	n.s
3irAOCR																		-	n.s	n.s
4irAOCR																			-	n.s
5irAOCR																				-

N= 6374 sequences for each samples, R, roots; L, leaves; * (0.01-0.05) marginally significant; n.s (> 0.1) not significant.

Table S5. Pairwise ANOSIM did not differ significatly among rosette (young) and elongated, flowering (old) deveoplmental stages.

Pairwise Tests		
Groups	R Statistic	Significance Level
EVR-young, EVR-old	0.917	0.1
EVR-young, irAOCR-young	0	1
EVR-young, irAOCR-old	1	0.1
EVR-young, EVL-young	0	0.667
EVR-young, EVL-old	1	0.1
EVR-young, irAOCL-young	1	0.333
EVR-young, irAOCL-old	1	0.1
EVR-old, irAOCR-young	1	0.1
EVR-old, irAOCR-old	0.704	0.1
EVR-old, EVL-young	0.5	0.1
EVR-old, EVL-old	0.852	0.1
EVR-old, irAOCL-young	1	0.1
EVR-old, irAOCL-old	0.963	0.1
irAOCR-young, irAOCR-old	1	0.1
irAOCR-young, EVL-young	0.5	0.333
irAOCR-young, EVL-old	1	0.1
irAOCR-young, irAOCL-young	1	0.333
irAOCR-young, irAOCL-old	1	0.1
irAOCR-old, EVL-young	0.417	0.1
irAOCR-old, EVL-old	0.926	0.1
irAOCR-old, irAOCL-young	0.833	0.1
irAOCR-old, irAOCL-old	0.852	0.1
EVL-young, EVL-old	0.083	0.4
EVL-young, irAOCL-young	0	1
EVL-young, irAOCL-old	0.083	0.4
EVL-old, irAOCL-young	-0.333	0.9
EVL-old, irAOCL-old	-0.148	0.9
irAOCL-young, irAOCL-old	-0.5	1
Global	0.619	0.001

Table S6. List of OTUs significantly different among EV and ir*AOC* leaves and roots at genera (or higher) level retrieved by pyrosequencing.

OTU numbers	Genus (or higher)	Leaves		Roots		ANOVA test	
		EVL	ir*AOCL*	EVR	ir*AOCR*	Communities (EVL*ir*AOCL*EVR* ir*AOCR*)	
		Average proportion %				$F_{3,16}$	*p*
Otu51	*Arthrobacter*	0^{A}	0^{A}	0.12^{B} (0.04)	0.15^{B} (0.06)	3.67	0.03
Otu92	*Phenylobacterium*	0^{A}	0^{A}	0.04^{B} (0.01)	0.04^{B} (0.01)	5.56	0.008
Otu123	*Nocardioides*	0^{A}	0^{A}	0.03^{B} (0.01)	0.04^{B} (0.01)	7.48	0.01
Otu27	*Rhizobium*	0^{A}	0^{A}	0.56^{B} (0.16)	0.39^{B} (0.2)	5.15	0.01
Otu60	*Cupriavidus*	0.01^{A} (0.009)	0.01^{A} (0.004)	0.09^{B} (0.01)	0.11^{B} (0.03)	7.24	0.0028
Otu63	*Enterobacter*	10.1^{C} (3)	7.2^{BC} (2.4)	2.65^{AB} (0.9)	0.9^{A} (0.3)	4.19	0.02
Otu28	*Methylophilaceae*	0.03^{A} (0.03)	0.03^{A} (0.03)	0.44^{B} (0.17)	0.71^{B} (0.2)	6.28	0.005
Otu285	*Pseudomonas*	0.03^{A} (0.01)	0.02^{A} (0.008)	0.15^{B} (0.05)	0.08^{AB} (0.01)	3.85	0.03
Otu56	*Agrobacterium*	0.05^{A} (0.03)	0.03^{A} (0.03)	0.14^{B} (0.04)	0.06^{AB} (0.02)	3.52	0.03
Otu0	*Serratia*	28^{B} (4)	14.23^{A} (4.3)	8.53^{A} (3)	6.86^{A} (2.1)	7.67	0.002
Otu13	*Mycoplana*	0.02^{A} (0.01)	0.02^{A} (0.02)	1.68^{B} (0.4)	1.8^{B} (0.8)	4.43	0.01
Otu24	*Achromobacter*	0.14^{A} (0.08)	0.01^{A} (0.01)	0.48^{AB} (0.16)	0.68^{B} (0.2)	3.64	0.03
Otu3	*Oxalobacteraceae*	1.69^{AB} (1.1)	0.34^{A} (0.1)	4.38^{BC} (0.9)	5.8^{C} (1.2)	6.58	0.005
Otu398	*Janthinobacterium*	0.01^{A} (0.004)	0.01^{A} (0.006)	0.26^{B} (0.05)	0.16^{B} (0.07)	7.08	0.003

R, roots; L, leaves; different letters indicates significant differences with Fisher's PLSD test; $P<0.05$.

Manuscript II

In wild tobacco *Nicotiana attenuata,* intraspecific variation among bacterial communities is mainly shaped by the local soil microbiota independently of jasmonic acid signal capacity.

Rakesh Santhanam, Ian T. Baldwin and Karin Groten.

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4 Manuscript II

SHORT COMMUNICATION

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In wild tobacco, *Nicotiana attenuata*, variation among bacterial communities of isogenic plants is mainly shaped by the local soil microbiota independently of the plants' capacity to produce jasmonic acid

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Keywords: *Nicotiana attenuata*, jasmonic acid, bacterial communities, leaf- and root-associated bacteria, plant growth promotion, opportunistic mutualism

The phytohormone jasmonic acid (JA) plays a central role in defense against necrotrophic pathogens and herbivores in *Nicotiana attenuata*. Recently Santhanam et al.[1] showed that JA does not have a major role in shaping the root- and shoot associated bacterial communities, though a few taxa differed among control (empty vector, EV) plants and plants impaired in their capacity to produce JA (ir*AOC*). In this addendum, we provide additional data showing that the composition of the plant bacterial communities is mainly shaped by tissue type. The qualitative data analysis revealed that at the order level, 5 bacterial OTUs formed a core community found in all tissues irrespective of genotypes, while 9 OTUs were different among roots and shoots. The heterogeneity among individual plants was high masking the potential genotype effect on bacterial communities. Using a culture-dependent approach, 3 of 18 bacterial taxa retrieved either only from one of the genotypes or from both had a growth promoting effect on EV and ir*AOC* seedlings. The data suggest that the local soil niche in which the roots grows is a major driver of the variability in root bacterial communities recruited by different individuals, and the plant growth-promoting effects of some taxa are independent of the genotype.

Plants harbor a diverse range of bacterial communities[2,3] which are influenced by many biotic and abiotic factors.[2] Several studies showed that tissue types such as leaves and roots influence the bacterial community composition, and harbor distinct communities.[1,4] It is often assumed that root bacterial communities are shaped by soil microbiota,[5-7] and leaf bacterial communities by air, sunlight irradiation, stomata and mineral content of the leaves.[8-10] However, 4 independent studies using *Arabidopsis* as a model system indicated that at the phylum level, core communities such as Actinobacteria, Bacteriodetes and Proteobacteria can be found in all roots independent of soil type and genotypes,[2,4,5-7] strongly indicating that bacteria do not randomly colonize roots, but certain phyla preferentially colonize plant roots.

In a previous study,[1] we analyzed leaf and root bacterial community of isogenic field grown plants impaired in JA-production (ir*AOC*) and control plants (empty vector, EV) by culture dependent and independent (pyrosequencing) approaches. Based on the quantitative data, we showed that leaf bacterial communities are different from those of roots.[1] Here, we demonstrate that based on qualitative data (presence and absence of OTUs at 97% similarity) leaf bacterial communities are clearly distinct from roots (**Fig. 1A**), and within each tissue type (root vs leaf) plants impaired in JA production and EV plants do not show a genotype-specific pattern. These data are consistent with our previous findings, and similar results were obtained by Bodenhausen et al,[4] who showed that leaf bacterial communities of *Arabidopsis thaliana* are different from those of roots and concluded that organ type (root vs leaf) type influences the composition of the bacterial communities.

Though root and leaf bacterial communities were clearly distinct,[1] a bacterial core community was present in all

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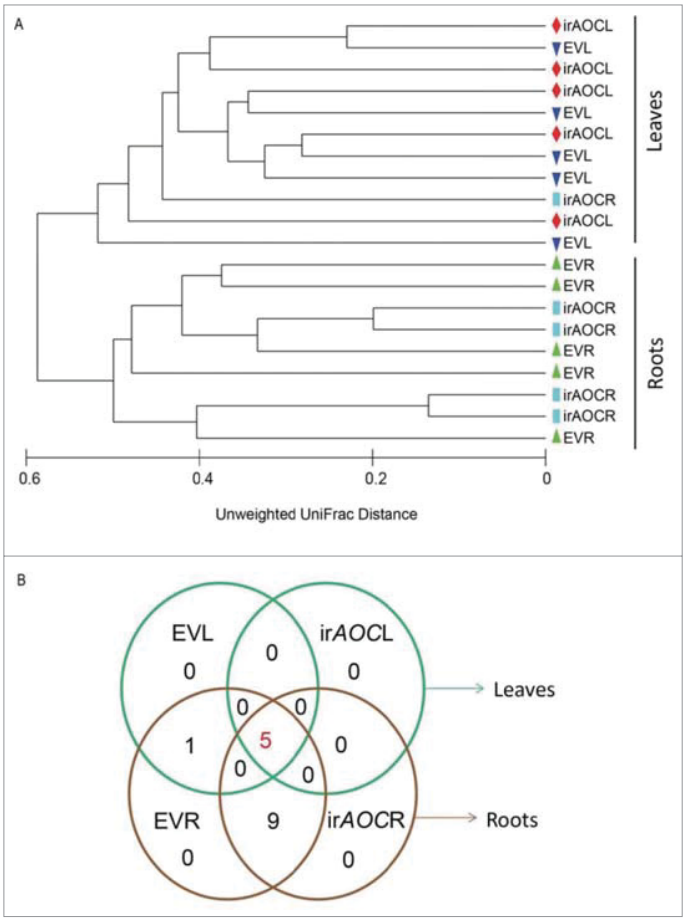

Figure 1. Bacterial communities are clearly different among roots and shoots (**A**), but not by the plant's capacity to produce jasmonic acid (JA). Venn diagram represents the core and root specific OTUs among EV and ir*AOC* genotype tissues (**B**). Here we assigned core communities as OTUs recovered from all tissue samples (n = 20) and root specific OTUs (n = 10) irrespective of genotypes. The group-average dendogram was constructed by unweighted UniFrac distance metric and the Venn diagram based on presence and absence data of OTUs at the order level in roots and shoots of EV and ir*AOC* plants. Only OTUs which were retrieved at least from all 5 replicates per tissue/genotype combination were considered, and not the total number of orders found. Abbreviations: R, roots; L, leaves

samples of field-grown *N. attenuata* plants irrespective of the genotype (**Fig. 1B**). At the order level, this core community consisted of 5 OTUs retrieved from all roots and shoots from 27 OTUs found in total. These 5 OTUs belonged to the bacterial phyla Bacteriodetes, Proteobacteria and Firmicutes. Additionally, 9 OTUs were present in all root samples, representing also 2 additional bacterial phyla, Actinobacteria and Deinococcus-thermus, indicating a root-specific enrichment. In accordance with our study, Actinobacteria were shown in a previous studies to be enriched in roots irrespective of soil types.[5,6] Deinococcus-thermus taxa are described as highly resistant to environmental hazards and can survive high doses of gamma and UV radiation.[11,12] Interestingly, *N. attenuata's* native habitat, the Great Basin Desert, Utah, USA[13] is characterized by high light intensities and high UV-B fluence rates.

In earlier studies, independent of the soil type, the bacterial community composition was found to be similar at the phylum level in different genotypes of the same and related species. Only few bacterial taxa were quantitatively different among plant genotypes[1,5-7] suggesting that genotypes have a minor role in structuring bacterial communities. Based on 16S rDNA gene pyrosequencing of 8 *Arabidopsis* ecotypes roots Lundberg et al[5] showed that of 778 OTUs, only 12 OTUs exhibited host genotype specific quantitative enrichment. In another study, using the same technique, Bulgarelli et al [6] found only one OTU was significantly different among 2 *Arabidopsis* ecotypes. In our study, based on ANOSIM β diversity, the overall bacterial diversity of leaves and roots of EV and ir*AOC* genotypes was not significantly different,[1] and we did not find a consistent clustering based on genotype (**Fig. 1A**) by qualitative data, and the bacterial community composition was highly heterogeneous among replicate plants based on qualitative and quantitative data. At the genera level, 21 OTUs significantly differed among EV and ir*AOC* roots,[1] and at the order level, 1 OTU was distinct among the genotypes (**Fig. 1B**). We hypothesize that sample-to-sample differences are due to differences in the local soil bacterial community in which the plant grows (**Fig. 2**), leading to the recruitment of soil-specific taxa. This hypothesis is in line with a large-scale study using 27 maize genotypes growing at 5 different locations in the US. Bacterial communities clearly clustered by soil, but not by genotype.[14]

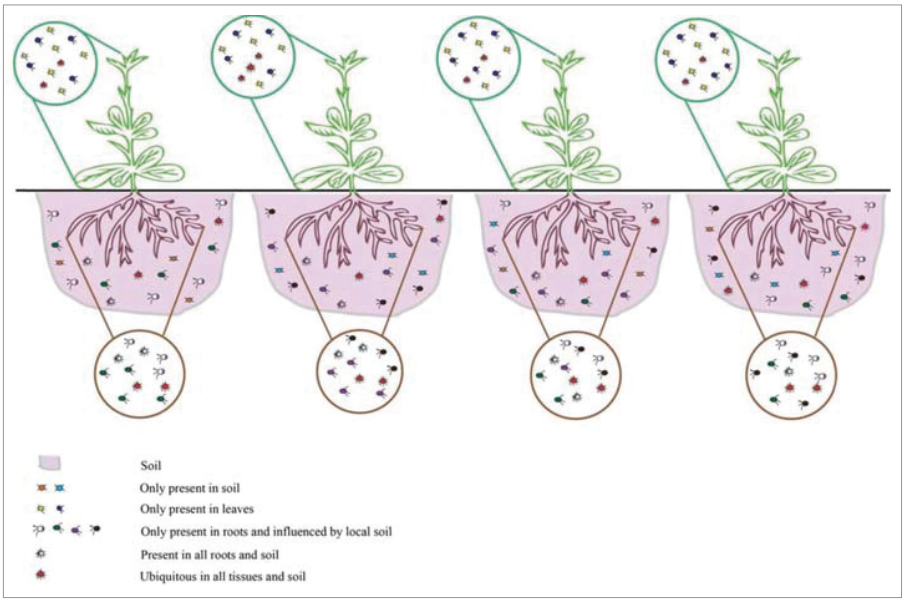

Figure 2. Summarizing scheme illustrating the intraspecific variation of root bacterial communities among individual plants of *N. attenuata*. Shoot bacterial endophytes are similar among individual plants and independent of the plant's capacity to produce JA, while root-associated bacterial communities are highly variable among different individuals irrespective of genotypes. We hypothesize that the intraspecific differences are due to local soil niches leading to the recruitment of plant-specific taxa that could be recruited for "opportunistic mutualisms."

Bacterial communities, which reside in plants can either have beneficial or detrimental effects on their hosts.[15,16] Detrimental effects are caused by necrotrophic and biotrophic pathogens [17,18] Beneficial effects can be direct or indirect, resulting in plant growth promotion. Direct plant growth promotion (PGP) can result from improved nutrient acquisition (e.g. nitrogen, phosphorous), the production of phytohormones (IAA, gibberellins) or the synthesis of stress modulators such as 1-amino cyclopropane-1- carboxylate (ACC) deaminase which lowers the plant's endogenous ethylene levels.[3,19] Indirect plant growth promotion can be due to the prevention or reduction of pathogen infection either by direct suppression, e.g., outcompeting pathogens for nutrients or by priming tissues for enhanced defense against pathogen or herbivore attack.[20,21] The enrichment of certain beneficial bacterial taxa can lead to opportunistic mutualisms between plants and microbes, as exemplified by a recent study of Meldau et al[22,23] showing that an ethylene insensitive *N. attenuata* genotype can recruit beneficial microbes to compensate its growth deficiency. PGP traits of certain bacterial isolates can be host dependent; e.g. Long et al[24] showed that bacterial strains isolated from *Solanum nigrum* roots were unable to promote growth of *N. attenuata*. However, in general, large numbers of bacterial isolates enhance growth of plants independent of their hosts.[3,19,20] The exact mechanism responsible for the recruitment and fine tuning of bacterial taxa from the local soil community remains to be elucidated. Some studies indicate that the lignin content and cell wall composition may play a role,[2,25] in addition to ethylene signaling.[26]

In our study, we isolated 414 bacterial strains from surface sterilized roots and leaves of both genotypes using a culture-dependent approach.[1] 18 strains were classified as putative specialists and generalists based on the isolation of a particular strain from either of the 2 plant genotypes (EV or ir*AOC*). PGP effects of these putative generalist and specialist bacterial strains were investigated (for experimental details see "In-vitro re-isolation" in Santhanam et al[1]). Three of the 18 strains (*B. cereusi* CN2, *P. azotoformans* A70 *A. nitroguajacolicus* E46, $n = 6$, $P < 0.05$, Fisher's PLSD) used in this assay significantly promoted plant growth with respect to plant biomass (ANOVA; $F_{19,90} = 6.81$, $P < 0.001$), leaf surface area (ANOVA; $F_{19,90} = 3.58$, $P < 0.001$) and primary root length (ANOVA; $F_{19,90} = 19.84$, $P < 0.001$, **Fig. 3**). Four isolates significantly reduced plant growth ($n = 6$, $P < 0.05$, Fisher's PLSD, **Fig. 3**), while 11 isolates had no significant effect (**Fig. 3**). The PGP effects did not depend on the plant's genotype from which they were

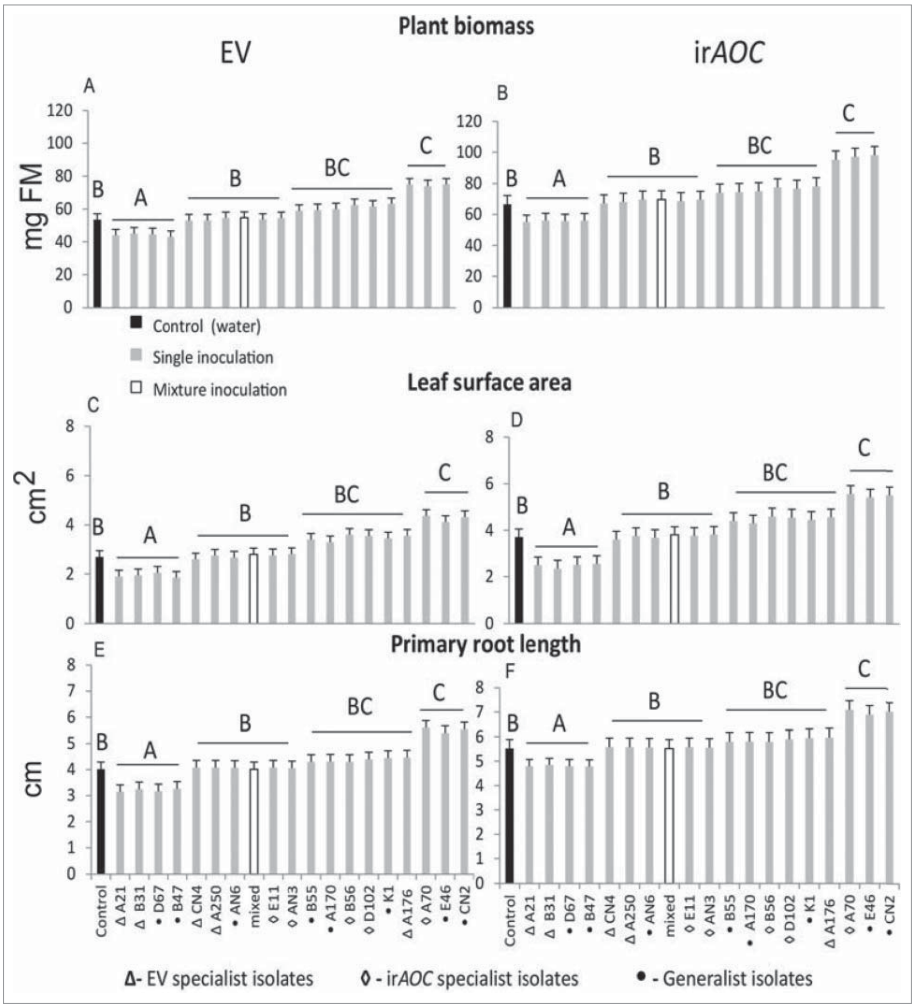

Figure 3. Putative genotype specialist and generalist bacterial taxa were isolated by culture-dependent approach and plant growth promoting (PGP) effects were performed under *in-vitro* conditions.[1] Plant biomass (**A and B**), leaf surface area (**C and D**) and primary root length (**E and F**) of EV and ir*AOC* plants were measured 24 d after 7-day old seedlings were inoculated. PGP effects of 3 bacterial taxa are independent of genotypes. The experimental setup is the same as described in Santhanam et al.[1] Mean (±SE, n = 6, different letters indicate significant differences among mock-and bacterial inoculations, one-way ANOVA with Fisher's PLSD test; $P < 0.05$).

isolated (2-way ANOVA plant biomass- bacterial type*genotype: p = 0 .98, leaf surface area- bacterial type*genotype: p = 0 .99, root length- bacterial type*genotype: p = 0 .97). Interestingly, inoculation with a mixture of all 18 bacterial isolates also did not promote growth, though - based on the single inoculations - plant growth promoting bacteria were present. We assume that under *in-vitro* conditions growth promotion by these bacterial isolates might be inhibited by competitive interactions among different isolates in mixed inoculations. In order to test whether colonization of bacterial isolates correlate with the PGP effects,

we performed a linear regression model with both genotypes; however, no correlation was observed between PGP effects and the colonization pattern (EV- plant biomass: $R^2 = 0$ .07, $p = 0$ .274, leaf surface area:$R^2 = 0.05$, $p = 0$ .337, root length:$R^2 = 0.05$,$p = 0$ .336; ir*AOC*- plant biomass: $R^2 = 0$ .074, $p = 0$ .258, leaf surface area:$R^2 = 0.04$, $p = 0$ .39, root length:$R^2 = 0.05$,$p = 0$ .319). This analysis corroborates that PGP effects were independent of quantitative root colonization and genotype and might be influenced by bacterial PGP traits such as production of IAA and ACC deaminase activity as exemplified by Long et al.[24]

Based on these results, we conclude that leaves and roots of field grown *N. attenuata* plants harbor distinct bacterial communities. Though all field-grown plants show a core community of the same bacterial orders, we assume the local soil niche determines the overall variability in the composition of the root-associated bacterial communities. Some of the recruited bacteria have a beneficial effect on plant growth independent of the genotype and may increase the plant's fitness depending on the environmental conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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5 Manuscript III

PNAS PLUS

Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping

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Plants maintain microbial associations whose functions remain largely unknown. For the past 15 y, we have planted the annual postfire tobacco *Nicotiana attenuata* into an experimental field plot in the plant's native habitat, and for the last 8 y the number of plants dying from a sudden wilt disease has increased, leading to crop failure. Inadvertently we had recapitulated the common agricultural dilemma of pathogen buildup associated with continuous cropping for this native plant. Plants suffered sudden tissue collapse and black roots, symptoms similar to a *Fusarium–Alternaria* disease complex, recently characterized in a nearby native population and developed into an in vitro pathosystem for *N. attenuata*. With this in vitro disease system, different protection strategies (fungicide and inoculations with native root-associated bacterial and fungal isolates), together with a biochar soil amendment, were tested further in the field. A field trial with more than 900 plants in two field plots revealed that inoculation with a mixture of native bacterial isolates significantly reduced disease incidence and mortality in the infected field plot without influencing growth, herbivore resistance, or 32 defense and signaling metabolites known to mediate resistance against native herbivores. Tests in a subsequent year revealed that a core consortium of five bacteria was essential for disease reduction. This consortium, but not individual members of the root-associated bacteria community which this plant normally recruits during germination from native seed banks, provides enduring resistance against fungal diseases, demonstrating that native plants develop opportunistic mutualisms with prokaryotes that solve context-dependent ecological problems.

Fusarium | microbiome function | plant disease resistance | *Nicotiana attenuata* | *Alternaria*

Eukaryotes maintain many complex relationships with the microbes they host, which can be so abundant and diverse that they frequently are considered a eukaryote's second genome. The complex relationships mediated by microbial associates are being revealed rapidly, thanks to the advances in sequencing, microbial culturing techniques, and the reconstitution of associated microbial communities in gnotobiotic systems (1, 2), even if some of these putative functional roles may need to be evaluated more critically (3).

When plants germinate from their seed banks, they typically acquire a selection of the diverse fungi and bacteria that exist in native soils, and a subset of this community becomes root-associated. The best characterized are the bacterial microbiomes of *Arabidopsis thaliana*. Approximately half of the bacterial community in the plant root is representative of the soil flora; the remainder is a conserved core consisting of a smaller number of bacterial lineages from three phyla: Actinobacteria, Proteobacteria, and Bacteroidetes (2, 4). Because these bacterial communities occur in nondiseased plants, they are thought to represent commensalistic or possibly mutualistic associations.

Root-associated microbes could benefit plants in many ways, and a recent review (5) highlighted the parallel functional roles of the microbiomes of the human gut and those of plant roots. The best-characterized beneficial functions for plants are (*i*) the plant growth-promoting rhizobacteria (PGPR), which promote growth by a variety of direct and indirect means that include increasing nutrient availability, interfering with ethylene (ET) signaling, and preventing diseases (6), and (*ii*) the bacteria that elicit induced systemic resistance (ISR) (7) by activating jasmonic acid (JA) and ET signaling (8). PGPR and ISR have been studied in a variety of cultivated and model plants, usually with model microbes (5), but little is known about their ecological context or whether they increase the growth and fitness of native plants. Whether PGPR and ISR functions occur among the well-characterized root-associated bacterial communities of *Arabidopsis*, either collectively or individually, also remains unknown.

The well-described agricultural phenomenon of disease-suppressive soils that harbor microbiomes that suppress particular soil-borne pathogens (9) illustrates the complexity of the dynamics involved. Native soils have a certain degree of pathogen-suppressive ability, frequently seen when a crop is grown continuously in a soil, suffers an outbreak of a disease, and subsequently becomes resistant to the disease (5). Perhaps the mechanisms involved are best understood in a root disease of wheat caused by *Gaeumannomyces graminis* var *Tritici* infections, known as "take-all" disease.

Significance

Plant roots associate with the diverse microbial community in soil and can establish mutualistic relationships with microbes. The genetic characterization of the plant microbiome (total microbiota of plants) has intensified, but we still lack experimental proof of the ecological function of the root microbiome. Without such an understanding, the use of microbial communities in sustainable agricultural practices will be poorly informed. Through continuous cropping of a seed-sterilized native plant, we inadvertently recapitulated a common agricultural dilemma: the accumulation of phytopathogens. Experimental inoculations of seeds with native bacterial consortium during germination significantly attenuated plant mortality, demonstrating that a plant's opportunistic mutualistic associations with soil microbes have the potential to increase the resilience of crops.

Author contributions: R.S., V.T.L., A.W., and I.T.B. designed research; R.S., V.T.L., A.W., J.G., Y.O., and I.T.B. performed research; I.T.B. contributed new reagents/analytic tools; R.S., V.T.L., A.W., J.G., Y.O., and I.T.B. analyzed data; and R.S., V.T.L., A.W., and I.T.B. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: Sequences for LK020799–LK021108 and LN556288–LN556387 have been deposited in the European Nucleotide Archive database and KR906683–KR906715 in The National Center for Biotechnology Information.

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After many years of continuous wheat cropping with several disease outbreaks, the disease suddenly wanes, apparently because of the build-up of antagonistic *Pseudomonas* spp. (9). Whether any of these interactions also occur in native plants remains unknown.

Nicotiana attenuata, a native annual tobacco of North America, germinates from long-lived seed banks to grow in the immediate postfire environment (10). When *N. attenuata* seeds germinate from their seed banks, they acquire a root-associated microbiome from their native soils which has been characterized by pyrosequencing and culture-dependent approaches (11–14). The composition of the root-associated microbiome is not influenced by a plant's ability to elicit JA signaling (14), but ET signaling, as mediated by the ability both to produce and to perceive ET, plays a decisive role in shaping the "immigration policy" for the root-associated microbiome (12). A certain *Bacillus* strain, B55, was isolated from the roots of an ET-insensitive *N. attenuata* plant (35S etr-1) and was able to rescue the impaired-growth and high-mortality phenotype of ET-insensitive plants under field conditions (15). Beneficial effects were attributed to B55's ability to reduce sulfur and produce dimethyl disulfide, which *N. attenuata* uses to alleviate sulfur deficiencies. This rescue provided one of the first demonstrations that the soil bacteria recruited by plants during germination can form opportunistic mutualistic relationships with their host based on the host plant's ecological context. Here we provide a second example that involves protection against a sudden wilt disease, which accumulated in a field plot after consecutive planting of *N. attenuata* seedlings.

Results and Discussion

Emergence of the Sudden Wilt Disease. For the past 15 y, we have planted the wild tobacco *N. attenuata* continuously in a field plot at Lytle Ranch Preserve, located in the plant's native environment of the Great Basin Desert, Utah. Seeds were germinated on sterilized medium, and young plants were first transferred to Jiffy peat pellets, to acclimate them to the environmental conditions, before they were planted in the field plot (Movie S1).

We observed the sporadic occurrence of a sudden wilt disease 8 y ago, which first affected elongated plants, causing them to wilt and die rapidly. In addition to the wilting symptoms, the normally white roots became black, and the two symptoms together (wilting plus black roots) were considered diagnostic of a plant being affected by the sudden wilt disease (Fig. S1). Plant mortality increased gradually over the years, and plants began to show symptoms at earlier developmental stages. By the end of the field season 2012, more than half (584 of 1,069) of the *N. attenuata* plants on the original (hereafter, "Old") plot, including different transgenic lines, showed these wilting symptoms and died; this value likely underestimates the actual death rate, because plants replaced during the early establishment stage (during the first 10 d after planting) were not included in this count. The sudden wilt disease seems to be specific for *N. attenuata*, because other plants or weeds growing on the plot were unaffected (Fig. S1). Interestingly, *Nicotiana obtusifolia*, which also is native to the Great Basin Desert, seemed to be less affected during the 2012 field season, because only 2 of 12 *N. obtusifolia* plants on the Old plot died. The emergence of the sudden wilt disease recapitulates a common agricultural dilemma that results from the accumulation of plant pathogens after continuous cropping and reuse of the same area for several years (16, 17). To avoid this problem, crop rotation is nearly as old as agriculture itself and entails the use of different crops in succession to interrupt the disease cycle of plant pathogens (18, 19). Because crop rotation was not an option for our research program, we compared the effectiveness of different disease-control methods, including biocontrols, fungicide treatment, and soil amendments, for *N. attenuata* planted in the Old plot.

***Alternaria* and *Fusarium* Fungal Phytopathogens Were Abundant in the Roots of Diseased Plants.** To identify and work with the microbial culprits of the sudden wilt disease, we isolated bacteria and fungi from the roots of diseased *N. attenuata* plants grown in the Old plot. A total of 36 fungal and 70 bacterial isolates were retrieved from the roots of diseased plants (Fig. 1 and Dataset S1). Based on the sudden wilt symptoms and the literature, we expected to find the bacterial plant pathogen *Ralstonia solanacearum*, because its ability to cause wilting symptoms in solanaceous plants is well known (20). Among 70 bacterial isolates, the only potential plant pathogens were *Pseudomonas tremae* and *Curtobacterium flaccumfaciens* (21), but both were recovered at low frequencies (≤2%). In contrast, isolates of plant pathogenic fungi of the *Fusarium* and *Alternaria* genera were abundant (Fig. 1). *Fusarium oxysporum* was the most abundant (25%), followed by *Fusarium solani* (22%) and different *Alternaria* species, which together represented ∼21% of the isolates.

Wilt diseases in solanaceous plants can be caused by various pathogens, such as *Fusarium* wilt (*F. oxysporum*) or bacterial wilt (*R. solanacearum*) (22, 23). Because *Fusarium* spp. and *Alternaria* spp. were isolated in abundance from diseased roots, we considered them to be the potential causal agents of the sudden wilt disease. The repeated planting of *N. attenuata* violated the natural disease-avoidance strategy of the plant's normally ephemeral, fire-chasing populations and likely led to an accumulation of pathogens. Moreover because our experimental procedures use sterile medium for germination and a preadaption period in Jiffy peat pellets, the roots' contact with the bacterial community in the native soil in the field occurs weeks after germination, and one of the strong inferences of this study is that the recruitment of beneficial microbes occurs soon after germination. Hence, these plants may lack the opportunity to recruit microbes from the surrounding soil at an early stage of their development and therefore lack the appropriate microbial community required for pathogen resistance. Whether plants acquire bacteria during the early stage of growth in Jiffy pellets is not known, but if they do, then these bacterial recruits are unable to protect the plants against the wilt disease. Furthermore, previous work (14) demonstrated that isogenic field-grown *N. attenuata* plants harbor highly divergent bacterial root communities that likely reflect spatial differences in soil microbial communities; from this variability we infer that microbes acquired during growth in the Jiffy pellets do little to shape the plant bacterial community that is retained throughout growth in the field (14). An additional vulnerability factor that likely contributed to the accumulation of specialized pathogens (24) is that our plantation populations are de facto genetic monocultures, in stark contrast with the high genetic diversity of native populations, which likely is a result of the long-lived seed banks and the differential recruitment of different cohorts into populations after fires (25, 26).

In Vitro Tests of Fungicide, Bacterial, and Fungal Treatments Reduced *N. attenuata* Seedling Mortality. A native fungal outbreak was used to develop an in vitro pathosystem for *N. attenuata* with native isolates (13). In this study, we used this pathosystem to test different strategies of minimizing the occurrence of the sudden wilt disease in the field.

For the in vitro tests, we used two fungal isolates: *Fusarium oxysporum* U3, isolated from the roots of diseased *N. attenuata* plants from the Old plot, and *Alternaria* sp. U10 from the established pathosystem described in ref. 13. With these fungi we examined biocontrol strategies and fungicide application that could provide resistance. Biocontrols are beneficial microbes that protect plants from microbial pathogens (27). For the biocontrol treatments, we used four native fungal isolates, *Chaetomium* sp. C16, C39, and C72 and *Oidodendron* sp. Oi3, which were isolated from diseased plants but were reported to be potential biocontrol agents (28, 29), and six native bacterial isolates (*Arthrobacter nitroguajacolicus* E46, *Bacillus cereus* CN2, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176), which had been

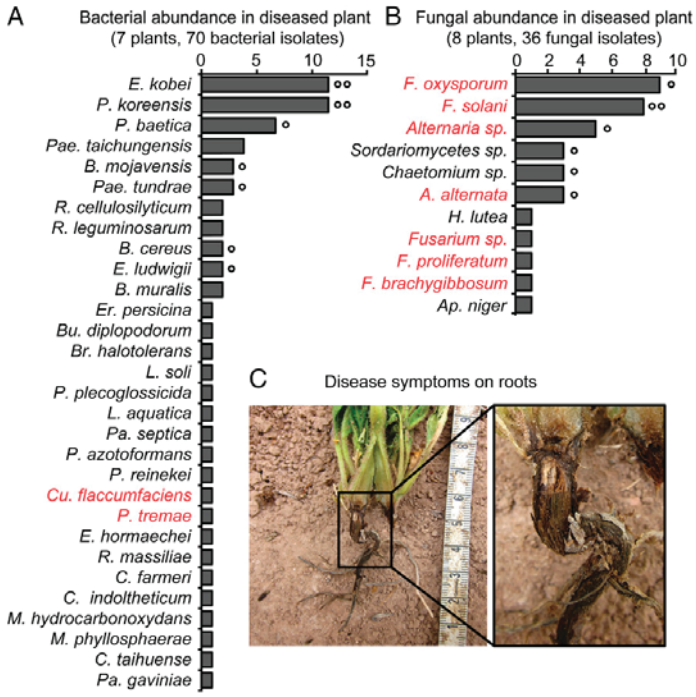

Fig. 1. Abundance of bacteria and fungi isolated from the roots of diseased *N. attenuata* plants. Abundance of culturable bacteria and fungi isolated from native field-grown plants exhibiting the sudden wilt disease symptoms. Potential plant pathogens are in red font. (*A*) Only two potential bacterial pathogens (*C. flaccumfaciens* and *P. tremae*) were found in the 70 members of the bacterial community retrieved from the roots of seven diseased plants. (*B*) In contrast, potential fungal pathogens (*Alternaria* and *Fusarium*) were abundant among the 36 culturable isolates of the fungal community from the roots of eight diseased plants. Isolates, which were found in two or more or four or more plants are indicated by (°) and (°°), respectively. Bacterial genus acronyms: B, *Bacillus*; Br, *Brevibacterium*; Bu, *Budvicia*; C, *Chryseobacterium*; Ci, *Citrobacter*; Cu, *Curtobacterium*; E, *Enterobacter*; Er, *Erwinia*; L, *Leifsonia*; M, *Microbacterium*; P, *Pseudomonas*; Pa, *Pantoea*; Pae, *Paenibacillus*; R, *Rhizobium*. Fungal genus acronyms: A, *Alternaria*; Ap, *Aspergillus*; F, *Fusarium*; H, *Hypocrea*. (*C*) Symptoms of the sudden wilt disease in field-grown *N. attenuata* plants included black coloration of the roots. For details see Fig. S1.

isolated from the roots of healthy *N. attenuata* plants from the same field location (12, 14). The selection of these bacterial isolates was based on in vitro plant growth-promoting effects on *N. attenuata* (15, 30); the isolates had been reported as biocontrol agents in the literature (28, 29, 31).

The treatment of the seeds with fungicide significantly reduced seedling mortality when seedlings were challenged with *Fusarium* sp. U3 and *Alternaria* sp. U10 [U3 $t_{(1,8)} = 2.52$, $P < 0.03$; U10 $t_{(1,8)} = 8.23$, $P < 0.0001$, *t*-test] (Fig. S2). The treatment of the seeds with bacteria was most effective when all six strains were mixed, which significantly reduced mortality from both fungal pathogens [U3 $F_{7,32} = 6.6$, $P < 0.0001$; U10 $F_{7,32} = 9.1$, $P < 0.0001$, ANOVA, least significant difference (LSD)] (Fig. S2). Fungal isolates showed inconsistent effects, and some appeared to have negative effects on plant growth. Two fungal isolates, *Chaetomium* sp. C72 and *Oidodendron* sp. Oi3, were selected for field experiments because they reduced seedling mortality in seedlings inoculated with *Fusarium* sp. U3 ($F_{4,13} = 11.961$, C72, $P < 0.0001$; Oi3, $P < 0.05$, ANOVA, LSD) (Fig. S2) without negatively affecting subsequent seedling growth.

In summary, we selected the mixed bacterial inoculation, two fungal isolates (C72 and Oi3), and the fungicide for large-scale tests in the diseased Old plot. The use of biocontrol strains recently has become a popular alternative to conventional chemical treatments. However, biocontrol bacterial strains that can protect plants from phytopathogens under in vitro conditions frequently are less successful under glasshouse conditions and even might be detrimental under field conditions; this context dependence makes the screening of potential biocontrol candidates challenging (32). The use of bacterial or fungal isolates native to the host plant may increase the success rate in screening experiments, because these microbes are likely to be better adapted to their host and its associated environmental conditions than are generalist strains retrieved from culture collections (33). In agriculture, the use of such locally adapted isolates has been shown to decrease the incidence of *Fusarium* wilt disease in peanut plant (34).

Inoculation with Native Bacterial Isolates Significantly Attenuates Disease Incidence in the Field Without Slowing Plant Growth. For the field experiments in 2013, we included soil amendment as a third disease-control strategy and combined these strategies to produce seven different treatment groups: control, bacteria, fungi, fungicide 1×, fungicide 5×, charcoal, and charcoal plus fungicide (combined treatment) (Fig. 2). Because the germination of *N. attenuata* seeds is elicited by smoke, which initiates growth in burned soil, we simulated this soil condition by adding shredded charcoal as a soil amendment at the time of planting (Fig. S3). The application of pyrolyzed plant material (biochar) is a common farming practice that has been shown to have several beneficial effects on plants, increasing crop yields and mitigating

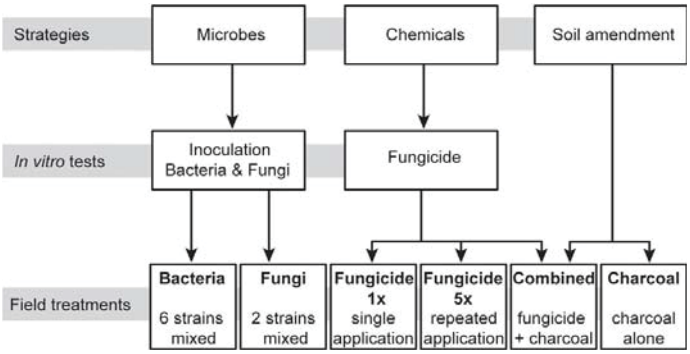

Fig. 2. Workflow of the three main strategies and the treatments used for 2013 field experiment. Of the three main strategies pursued to curb the spread of the disease in the field, the inoculation with microbes (bacteria or fungi) and fungicide treatment were first evaluated under in vitro conditions in the laboratory (Fig. S2). The mixed inoculation with six bacterial isolates, two fungal isolates, and the treatment with a commercially available fungicide in vitro reduced the mortality of *N. attenuata* seedlings infected with native isolates of fungal pathogens (*Fusarium* sp. and *Alternaria* sp.), and these treatments were selected for the field experiments. The combination of the strategies resulted in seven different treatments (including control treatment) that were deployed for the 2013 field experiment. All treatments were applied to *N. attenuata* seedlings before or during their planting into the field. The repeated fungicide treatment (fungicide 5×) was reapplied four times at 1-wk intervals after planting.

disease symptoms (35, 36). For a slow release of the fungicide, we combined the charcoal and fungicide treatments and presoaked the charcoal with the fungicide solution (combined treatment).

A total of 735 *N. attenuata* plants from the seven treatment groups were planted in the Old plot. As a control experiment, 261 plants were randomly assigned to the seven treatment groups and planted into the New plot; the two plots are located about 900 m apart, and the New plot had been used only during the previous two growing seasons without any signs of the sudden wilt disease (Fig. S3). The plants from the seven treatment groups were planted in a block in a randomized design (Fig. S4). In the Old plot, the first dead plants were observed quite early. Because these plants were still small, with a rosette diameter of about 5 cm, the black coloration of the roots was not always visible. In such cases, the cause of death could not be assigned to the sudden wilt disease and was categorized as "only wilting symptoms" (Fig. S4). Most of the plants with only wilting symptoms were observed in three treatment groups (fungi, charcoal, and combined) and contributed to the overall high mortality of these groups (Fig. S4). Three days later (15 d post planting, dpp), the majority of the newly dying plants showed the characteristic black roots (Fig. S1), as did the great majority of plants that subsequently died (Fig. S4).

The treatments fungicide 1×, fungicide 5×, and charcoal showed no significant mortality reduction compared with the control treatment, and the fungi and combined treatments, even at 15 dpp, showed elevated mortality rates compared with the controls (Fig. 3). Only the plants inoculated with the mixed bacteria showed a consistently attenuated death rate with a statistically significant reduction compared with the control plants at 15 and 18 dpp p ($P < 0.05$, G test) (Fig. 3). Over the 22-d observation period, the increase in plant mortality showed two peaks at 15 and 30 dpp (Fig. S4). At the end of the observation period, 219 of 735 plants (26.7%) on the Old plot had died, and 20.2% showed all the symptoms of the sudden wilt disease (Fig. 3 and Fig. S4). As in the previous season, *N. obtusifolia* plants showed lower mortality than *N. attenuata* and seemed to be substantially more resistant to the sudden wilt disease (Fig. S4). In contrast, none of the plants from the seven treatment groups on the New plot died or showed symptoms of the sudden wilt disease, even though all treatments and planting procedures were performed identically on the Old and New plots.

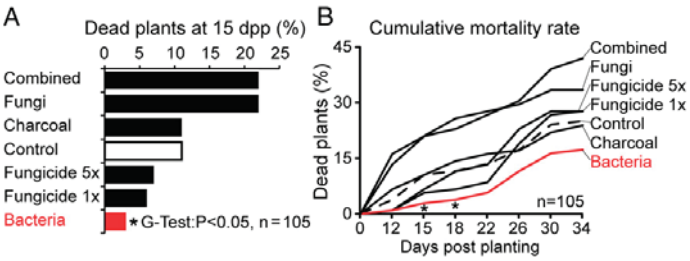

Fig. 3. Efficiency of the different treatments in reducing the mortality of field-grown *N. attenuata* plants (2013 field season). Plants in the different treatment groups (fungi, charcoal, fungicide 5×, fungicide 1×, bacteria, and combined charcoal + fungicide) were planted together with control plants in a randomized design on the Old (diseased) field plot (see Materials and Methods). (*A*) Plant mortality at 15 dpp was significantly reduced in the bacterially treated group compared with the control plants (G test: $P < 0.05$, $n = 105$ plants per group). (*B*) The increase in plant mortality was observed every 3 or 4 d for a 22-d observation period. The plants receiving the bacterial treatment had the lowest overall mortality rate. For details of the spatial distribution of plants and the rate of change in mortality see Fig. S4.

In addition to mortality, we quantified the growth (rosette diameter and stalk height) of all plants from both field plots. The combined and fungi-treated plants had the highest mortality rates and the strongest reductions in growth on both field plots (Fig. 4) (Old plot, rosette diameter, $F_{6,515} = 10.13$; $P < 0.0001$, fungal and combined $P < 0.05$, ANOVA, LSD; stalk height, $F_{6,515} = 23.66$, $P < 0.0001$ fungal and combined $P < 0.05$, ANOVA, LSD at 34 dpp. New plot, rosette diameter, $F_{6,254} = 4.09$, $P = 0.0006$, fungal and combined $P < 0.05$, ANOVA, LSD; stalk height, $F_{6,254} = 14.36$, $P < 0.0001$, fungal and combined $P < 0.05$, ANOVA, LSD). The remaining treatments (charcoal, fungicide 1×, and fungicide 5×) did not reduce mortality and these plants were distinctly smaller on the Old plot (Fig. 4). The mixed bacteria treatment did not reduce plant growth on either field plot and was the only treatment that consistently reduced plant mortality. We conclude that although the bacteria mixture provided a biocontrol effect against the pathogen, it did not significantly increase plant growth (Old plot, rosette diameter, $F_{6,515} = 10.13$. $P < 0.0001$, bacteria $P = 0.9$, ANOVA, LSD, stalk height; $F_{6,515} = 23.66$, $P < 0.0001$, bacteria $P = 0.9$, ANOVA, LSD; for New plot data, see Table S1).

Bacteria Inoculation Did Not Influence Other Plant Performance Traits. Because our group has studied plant–herbivore interactions with plants germinated under sterile conditions, we were interested in understanding if the bacterial inoculation would alter the expression of traits known to be involved in *N. attenuata's* defense responses to attack from its native herbivore communities. We quantified the constitutive and herbivore-induced levels of phytohormones, secondary metabolites, and volatiles as well as plant biomass, reproductive output, and herbivore damage from the native herbivore community in bacterially inoculated plants. None of the 32 parameters analyzed indicated differences between the bacteria-treated and control plants (Table S1), demonstrating that the bacterial inoculations specifically influenced pathogen resistance but not traits essential for herbivore resistance.

Consortium of Bacteria Provide the Protection. A combination of multiple biocontrol strains can provide improved disease control over the use of single organisms (31). Therefore, under in vitro conditions we examined the effect of bacterial consortia, each lacking a particular strain that had proved effective during the 2013 field season (Fig. S5). Because of regulatory reasons, one strain (CN2), which was classified as a potential S2 strain in Germany, had to be excluded from further experiments (*SI Materials and Methods*), reducing the mix to five isolates. Consortia lacking the isolates K1, E46, or A176 (mix minus K1, mix minus E46, and mix minus A176) were significantly less effective in reducing mortality in seedlings inoculated with *Alternaria* sp. U10 than the mix of all five strains ($F_{6,48} = 34.9$, $P < 0.0001$, ANOVA, LSD), indicating that these strains are essential for the protective effect. Deleting the other two strains (mix minus B55 and mix minus A70) did not change seedling mortality, indicating that these bacteria alone could not protect plants effectively from the sudden wilt disease (Fig. S5). Based on these results, the consortia were split into subgroups including either two (B55 + A70) or three bacteria (K1 + A176 + E46), and these subgroups were evaluated in another field trial in 2014. Consistent with the results from the in vitro experiments, the inoculation with three bacteria (K1 + A176 + E46) or the mixture of all five bacteria (K1 + A176 + E46 + B55 + A70) reduced mortality rates in the field by 36 and 52%, respectively (Fig. 5). The inoculation with only two strains (B55 and A70) had no effect. This result indicates that the protection is not explained purely by a founder effect in which rapid root colonization blocks a niche from being colonized by other microbes, including

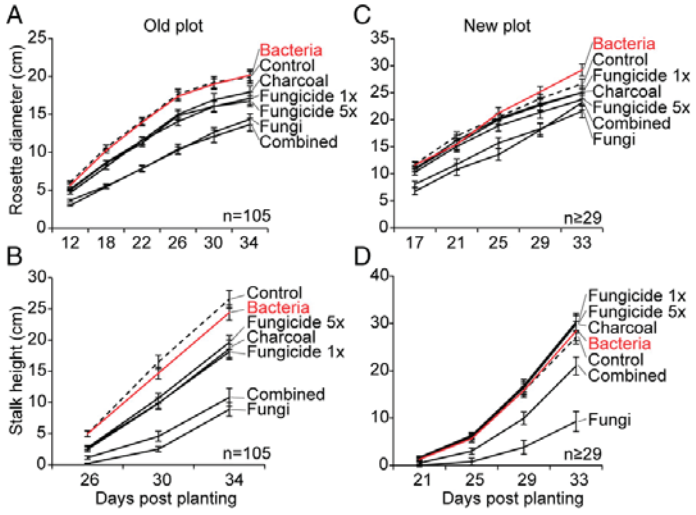

Fig. 4. Growth parameters of plants in the different treatment groups in two field plots. *N. attenuata* plants from the different treatment groups (bacteria, charcoal, fungicide 1×, fungicide 5×, fungi, and combined treatment with charcoal + fungicide) were planted together with control plants in 2013 into two field plots (Old and New), and their growth parameters (rosette diameter and stalk height) were quantified. (*A* and *B*) Mean rosette diameter and stalk height of the different treatment groups compared with control plants (dotted line) grown in the Old (diseased) plot (± SEM; $n = 105$ plants per group). (*C* and *D*) Mean rosette diameter and stalk height of plants from the different treatment groups compared with control plants (dotted line) grown in the New plot (± SEM; $n \geq 29$ plants per group). A comprehensive characterization of 32 traits known to be important for insect resistance and general ecological performance, including hormone levels and defense parameters (Table S1), was conducted on plants grown in the New plot to evaluate the effect of bacterial inoculation on traits not directly related to fungal pathogen resistance.

pathogens. The strongest mortality reduction in the field was achieved when these two strains were included in the bacterial mixture (Fig. 5), indicating that they do contribute important synergistic effects to the other strains of the consortium. Because of the lower replicate number (about half as many plants as in 2013), the 2014 results were not statistically significant ($P > 0.05$, $n = 45$, G test). Mixtures of commercial biocontrol strains sometimes combine multiple mechanisms of action to enhance the consistency of disease control (37). These synergistic mechanisms include the many different forms of antibiosis, biofilm formation, and founder effects as well as mechanisms that function indirectly through the host by eliciting systemic resistance (e.g., ISR) (7, 38).

Persistence of Biocontrol Bacteria in Late-Stage Plants. For effective suppression of pathogens under competitive natural conditions, biocontrol strains need to be excellent colonizers and persist as root endophytes (39, 40). In the development of commercial biocontrol agents, the focus has long been on *Pseudomonas* and *Bacillus* taxa because of their efficient root-colonizing capacity and their direct pathogen antagonistic characteristics associated with the production of lytic enzymes and antibiotics (41). From our mixed inoculations that included native *Pseudomonas* and *Bacillus* taxa, four of six strains were reisolated from the surface-sterilized roots of 2013 field-grown flowering-stage plants harvested 34 dpp. While the *Bacillus* isolates (K1 and CN2) dominated the bacterial root isolates, *Pseudomonas* strains (A176 and A70) were recovered at lower frequencies (Fig. S6). Furthermore, an additional test of the robustness of the bacterial association was performed with a second inbred ecotype of *N. attenuata*, originally collected from Arizona, which was preinoculated with the five isolates at the seed germination stage and planted in the New plot along with the Utah ecotype. All five isolates could be reisolated at the end of the growing season (Fig. S7). Because the Arizona ecotype had been planted only on the New plot, and no plants were lost to the sudden wilt disease, we performed in vitro assays to evaluate the disease-suppressive effect of the bacterial consortium for this ecotype. The consortium of five bacterial isolates also reduced the mortality rate of a Arizona ecotype [$t_{(1,20)} = 17.682$, $P < 0.001$, t-test] (Fig. S7). This result indicates that the consortium of isolates provides protection to a second *N. attenuata* ecotype. The reproducibility and persistence of the members of the mixed bacterial consortium in two *N. attenuata* ecotypes planted over two field seasons demonstrates that these native bacterial taxa establish stable associations with *N. attenuata* roots at germination which persist throughout growth under field conditions.

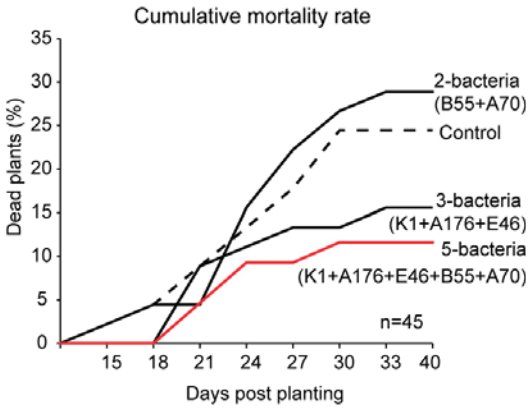

Fig. 5. Reproducibility of the disease-suppression effect of bacterial consortia in the 2014 field season. Based on the results of in vitro tests (Fig. S5) we parsed the bacterial consortia into two groups of two (B55 + A70) or three (K1 + A176+ E46) bacteria and compared these groups with the mixture of the five isolates (K1 + A176 + E46 + B55 + A70) in protecting inoculated seedlings from the sudden wilt when planted into the Old plot. Preinoculated plants were planted together with control plants in a randomized design on the Old (diseased) plot (2014 field season; see *Materials and Methods*). Inoculation with three bacteria (K1 + A176 + E46) or five bacteria (K1 + A176 + E46 + B55 + A70) reduced plant mortality by 36% and 52%, respectively, compared with control plants at 40 dpp ($n = 45$ plants per group). The inoculation with two bacterial strains (B55 + A70) had no significant effect in reducing the rate of death compared with noninoculated control plants.

Opportunistic Mutualisms and the Opportunities They Afford Agriculture. Soil arguably harbors the world's most diverse microbial communities, and when seeds germinate from their seed banks in native soils, they have the opportunity to recruit particular microbial taxa from these marketplaces of potential microbial partners (42). Microbial interactions are commonly categorized as being pathogenic, commensalistic, or mutualistic, as if these traits were fixed features of host and microbial taxa, but most are likely to be context dependent, shifting along the functional spectrum depending on environmental conditions or during the life cycle of the microbe or the plant (43). Root microbiomes are notoriously diverse (2, 4), and some of the diversity may arise from particular microbes being of benefit only to particular hosts under particular conditions or stresses, such as drought or pathogen infestation (44). As shown in this study and others (31), beneficial microbial communities can be acquired from the soil at an early stage during germination and establish beneficial associations that last throughout the entire life cycle of the plants. If plants lack such an early colonization, as in our previous planting procedure, they are exposed suddenly to the field microbiota during planting. Allowing the plant to interact with bacteria either on agar plates or during the Jiffy stage may fill empty niches of the root environment and allow plants to cope better with soil-derived pathogens.

To understand the mechanisms by which a consortium of microbes is recruited soon after germination and maintained in a context-specific manner will require a better understanding of the chemical signals that plants release as they germinate and the opportunities that differences in root morphology and growth afford microbes for colonization. Although organic acids [e.g., malic acid (45)] and certain secondary metabolites [e.g., benzoxazinoids (46)] have been found to mediate the recruitment of particular microbes under in vitro conditions, untargeted metabolomics and genomic approaches are sorely needed to evaluate the processes that are involved when plants are grown under real-world conditions. Crops, likewise, could benefit from location-specific consortia, depending on the region and type of soil in which they are grown.

These opportunistic mutualisms that plants develop with their root-associated microbes have great potential to increase the resilience of crop yields to the ever-changing landscape of abiotic and biotic stresses in agriculture, as many others have argued (31, 33, 47, 48). This work demonstrates that native plants use this strategy, and considerably more attention needs to be focused on the issue for crop plants. Have crop plants lost such abilities, and do they differ from their wild ancestors regarding their root-associated microbiota (49)? Certainly we should reconsider agricultural practices, such as the use of nonspecific antimicrobial seed treatments, that could thwart this important recruitment process. Moreover each plant species likely benefits from recruiting a specialized consortium of bacteria, which needs to be evaluated separately for each plant system. Likewise, evidence of phytoprotective roles of microbes from in vitro experiments should be evaluated under agricultural conditions, because certain microbes (e.g., those used in our fungal treatment) could prove to be

detrimental under field conditions. Progress is being made in rapidly querying, in a high-throughput manner, the ability of the diverse soil microbial communities from around the globe to synthesize antimicrobial secondary metabolites (50). We infer from the research reported here that native plants have been querying the soil microbial community throughout evolutionary history to help them solve context-specific challenges, and we need to empower our crop plants to do the same.

Materials and Methods

Plant Material and Culture Conditions. Wild-type *N. attenuata* Torr. Ex S. Watson seeds of the "Utah" ecotype were collected originally from a population at the DI (Desert Inn, 37.3267N, 113.9647W) ranch in Utah in 1989. For all in vitro and field experiments, wild-type seeds of the 31st inbred generation were surface sterilized and germinated on Gamborg's B5 plates (Duchefa) as previously described (51). Seeds of the "Arizona" ecotype were used in the 22nd inbred generation.

Isolation of Bacteria and Fungi from Field-Grown Plants. Field-grown *N. attenuata* plants at rosette and elongated stages that displayed symptoms of the sudden wilt disease were used for the isolation of potential plant pathogenic bacteria as described in ref. 12. Isolation of potential pathogenic fungi was carried out as described in ref. 13. Identification of bacterial and fungal isolates was performed as previously described (13, 14). The reisolation of the preinoculated bacteria was performed likewise using surface-sterilized roots of healthy plants to enrich endophytic bacteria. For detailed information, see *SI Materials and Methods*.

Plant Treatments in the Field. Field experiments were conducted at a field station at the Lytle Ranch Preserve in Utah. For the 2013 field season, seeds were inoculated with the mixed bacterial solution (*Arthrobacter nitroguajacolicus* E46, *Bacillus cereus* CN2, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176) or two native fungal isolates (*Chaetomium* sp. C72 and *Oidodendron* sp. Oi3). For the fungicide treatment, Jiffy pots (Jiffy 703, jiffygroup.com) were soaked with 15 mL of 1% fungicide solution (Landor; Syngenta) one night before planting. For the charcoal treatment, ~100 g of charcoal was added to the soil surrounding each plant before planting. For the combined treatment the charcoal was presoaked with 25 mL of 5% fungicide solution (Landor; Syngenta). Size-matched plants of each treatment group were planted in a randomized design (735 on the Old plot and 261 on the New plot). For the repeated fungicide (fungicide 5×) treatment, plants were watered weekly with 50 mL of 1% fungicide solution. For the 2014 field season, bacterial consortia consisting of two (B55 + A70), three (K1 + A176 + E46), or five (K1 + A176 + E46 + B55 + A70) bacteria were used for seed inoculation, and 180 plants from the different treatments were planted into the Old plot. See *SI Materials and Methods* for additional experimental details; Table S1 lists the 32 ecological traits used to characterize bacterially inoculated plants planted into the New plot.

Nucleotide Sequence Accession Numbers. The sequencing data for LK020799–LK021108 and LN556288–LN556387 have been deposited in the European Nucleotide Archive and KR906683–KR906715 in The National Center for Biotechnology Information. Also see Dataset S1.

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Supporting Information

Santhanam et al. 10.1073/pnas.1505765112

SI Materials and Methods

Isolation of Bacterial and Fungal Pathogens from Diseased *N. attenuata* Plants. Elongated and rosette field-grown *N. attenuata* plants displaying symptoms of the sudden wilt disease were shipped to the laboratory facility in Germany within 2 d of excavation. The isolation of potential plant pathogenic bacteria was performed as described in ref. 12 using nutrient agar (Sigma) and Casamino acid-peptone-glucose agar adapted for the isolation of *R. solanacearum* (52) supplemented with antifungal agents, cyclohexamide and nystatin, both at 25 μg/mL. Isolation of potential pathogenic fungi was carried out as described in ref. 13 on potato dextrose agar (PDA; Fluka) and water agar (53) supplemented with the antibacterial agents streptomycin and penicillin, both at 20 μg/mL. Bacterial and fungal genomic DNA extraction, amplification of 16S rDNA, and internal transcribed spacer and direct sequencing and identification were performed as described in ref. 14 for bacterial isolates and ref. 13 for fungal isolates.

In Vitro Seedling Mortality Assays. Two plant-protection strategies, (microbial and chemical), were evaluated with an in vitro assay for the protection they afford to *N. attenuata* seedlings against two different fungal pathogens: *Alternaria* sp. U10, which was isolated from a native population of diseased *N. attenuata* plants in Utah (13), and *Fusarium oxysporum* U3, which was isolated from the roots of diseased plants from the Old plot in 2012.

Potential fungal and bacterial biocontrol isolates were native root-associated isolates from field-grown *N. attenuata* plants (14). Six native bacterial isolates, *Arthrobacter nitroguajacolicus* E46, *Bacillus cereus* CN2, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176, were chosen because they had promoted plant growth in a previous experiment (30) or had been reported in the literature to be potential biocontrol strains. The four native fungal isolates, *Chaetomium sp*. C16, C39, and C72 and *Oidodendron* sp. Oi3, were isolated from diseased plants of the Old field plot; these isolates were not reported in the literature as being pathogenic but had potential biocontrol effects (28, 29). We tested these strains with seedlings challenged with *Fusarium* and *Alternaria* fungal pathogens in vitro. *Bacillus cereus* CN2 was used only in the 2013 experiments because its classification as an S2 strain by the German authorities prohibited its further use.

Potential biocontrol bacterial and fungal isolates were maintained on nutrient agar and PDA medium, respectively. Surface-sterilized *N. attenuata* seeds were incubated for 12 h with mixed bacterial cultures by combining equal concentrations of all six bacterial isolates (E46, CN2, B55, K1, A70, and A176) from fresh individual bacterial cultures grown overnight to a working concentration of 10^{-4} cfu/mL in sterile H_2O. For the partitioned consortia experiments, five different mixes were made by excluding single individual isolates from the consortia: e.g., mix minus K1 treatment consisted of four different bacteria but not K1 (A70 + A176 + B55 + E46). In this fashion, the following different bacterial consortia were made: mix minus A70, mix minus A176, mix minus B55, and mix minus E46. Arizona ecotype seeds also were treated with the consortium of five bacterial isolates. For each potential biocontrol fungal strain, mycelium was harvested from two 14-d-old fungal plates and dissolved in 1 mL of sterile H_2O to incubate sterile *N. attenuata* seeds for 5 min. For the fungicide treatment, surface-sterilized seeds were treated for 5 min with 1 mL of undiluted fungicide solution (Landor; Syngenta) containing fludioxonil (25 g/L), difenoconazol (20 g/L), and tebuconazol (5 g/L). Bacteria-, fungi-, and fungicide-treated seeds were germinated on Gamborg's B5 plates (GB5; Duchefa). After 7 d, germinated seedlings were inoculated with fungal pathogens by placing 1-cm-diameter plugs from 14-d-old PDA in the center of seedling plates (see Fig. S2*A* for the experimental set-up) and were incubated in a growth chamber (22 °C, 65% humidity, 16 h light). Dead seedlings were counted 26 d postinoculation from 10 replicate plates, and the mean percentage of seedling mortality was calculated.

Plant Treatments in the Field. For the field experiments, wild-type seeds were germinated on Gamborg's B5 plates (Duchefa) and preinoculated with the mixed bacterial solution or with fungal mycelium, as described above. About 16 d after germination, seedlings were transferred into 50-mm Jiffy peat pots (Jiffy 703, jiffygroup.com) prehydrated with tap water and were placed under shaded conditions for more than 2 wk to adapt the seedlings to the strong sun and relative low humidity of the Great Basin Desert. Well-grown and adapted seedlings were transplanted into the Old plot (54). A detailed description of the field-planting procedure is provided in the supplemental movie (Movie S1).

For the 2013 field season, shortly before planting, Jiffy pots were watered with 10 mL of mixed bacterial or fungal solution. The mixed bacterial cultures were generated by scraping the separately cultivated strains (E46, CN2, B55, K1, A70, and A176) from well-grown nutrient agar plates and diluting them in tap water. The bacterial strains were mixtures of equal volumes pooled from the appropriate strains; all solutions were cloudy, indicating a visually estimated very high OD. The mixture of two potential biocontrol fungal isolates (*Chaetomium* sp. C72 and *Oidodendron* sp. Oi3) was prepared in a similar manner by scrapping fungal mycelium from 10 fully grown PDA plates, equally pooled, and diluted in water. For the fungicide treatment, Jiffy pellets were soaked with 15 mL of 1% fungicide solution (Landor; Syngenta) one night before planting. For the charcoal treatment, Royal Oak All-Natural Hardwood Lump Charcoal (Walmart) was chipped into small pieces, and ∼100 g were added to the soil surrounding each plant before planting. For the combined treatment (charcoal and fungicide), 100 g of chipped charcoal were presoaked with 25 mL of 5% fungicide solution (Landor; Syngenta) before planting (Fig. S3 *A* and *B*). The treatments were designed to be easily applicable (for a future scale-up) and directly performed during germination (bacteria and fungi), during the Jiffy stage (bacteria, fungi, and fungicide 1×), or during planting (charcoal and combined) (Fig. S3); only the repeated fungicide (fungicide 5×) treatment was performed after planting also. Size-matched plants were planted in a randomized design into the field plot on the Lytle Ranch Preserve, Utah. The experiment was conducted on two separate field plots; of the 819 plants planted in the Old (diseased) field plot, 735 plants were randomly assigned to the seven different treatment groups, as were 261 plants in a New plot (control). The two field plots are ∼900 m apart and are separated by a river (Fig. S3*E*). During planting, each plant was fertilized with 5 g of Osmocote Smart-Release Plant Food (19-6-12 N-P-K) (Scotts-Sierra Horticulture) suitable for slow nutrient release over 4 mo. The plants in the repeated fungicide (fungicide 5×) treatment were watered every seventh day with 50 mL of 1% fungicide solution poured into a hole located 8–10 cm from the plant. Growth parameters and plant mortality were recorded every 3 or 4 d for a period of 22 d.

For the 2014 field experiment, three bacterial consortia consisting of two (B55 + A70), three (K1 + A176 + E46), or five (K1 + A176 + E46 + B55 + A70) bacteria were tested. Only the seeds were treated with the bacterial mixtures, and in total 180 plants (n = 45 for each group) were planted into the Old field plot (diseased) as described above. The mortality of all plants was recorded every 3 d for 22 d. To evaluate further the reproducibility of the bacterial association with roots, in the 2014 field season, *N. attenuata* inbred genotypes originally collected from Arizona and Utah were treated with a mixture of five bacterial isolates and were planted at the New field plot as mentioned above.

Phenotypic Characterization of Bacterially Treated Plants in the Field. A selection of 32 morphological and chemical traits (Table S1) known to be important in mediating *N. attenuata's* ecological performance were measured in 7–21 pairs of control plants and plants treated with the mixture of six native root-associated bacteria that had been planted in the New field plot. Replicate values given in Table S1 reflect the number of pairs of plants used in a given analysis.

Leaf chlorophyll content was measured in the largest nonsenescent leaf using a Minolta SPAD-502 (Konica Minolta Sensing, Inc.). Plant growth and reproduction parameters and herbivore damage were assayed once, as previously described (55), before the plants were destructively harvested for biomass measures.

Measurements of foliar and floral volatiles were conducted using polydimethylsiloxane (PDMS) tubes to absorb volatiles from headspace samples as previously described (56). Herbivore-induced plant volatiles were elicited by wounding leaves followed by the immediate application of *Manduca sexta* oral secretions (W+OS) (25), and ventilated polyethylene terephthalate (PET) containers were immediately placed around the wounded leaves and around similar mature, nonsenescent control leaves. PDMS tubes were placed in the PET containers for 1 h immediately after treatment and then were removed and stored in tightly closed amber 1.5-mL GC vials (56); a new PDMS tube was placed in the PET container and left there until 48 h after treatment, at which time it also was removed and stored. To measure floral volatiles, a PDMS tube was placed inside a ventilated PET container enclosing a single, newly opened flower as previously described (56) and was exposed to the floral headspace for 12 h, from 20:00–8:00 the following day; then the PDMS tube was removed and stored as described (8), and a new PDMS tube was placed in the headspace, incubated from 8:00 until 20:00, and stored. PDMS samples were kept in sealed vials until thermal desorption (TD)-GC-quadrupole MS (QMS) analysis was performed as described (56). TD-GC-QMS analysis was performed on a TD-20 thermal desorption unit (Shimadzu, www.shimadzu.com) connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu). An individual PDMS tube was placed in an 89-mm glass TD tube (Supelco, www.sigmaaldrich.com), desorbed, and analyzed as described (56). Compounds were identified by comparison of spectra and Kovats retention indices against libraries (Wiley, National Institute of Standards and Technology, eu.wiley.com/WileyCDA/WileyTitle/productCd-1118615964.html), and, where possible, by comparison with pure standards.

The phytohormones JA and JA-Ile and secondary metabolites nicotine, chlorogenic acid, caffeoyl putrescine, dicaffeoyl spermidine, rutin, and hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) were induced by W+OS treatment (57–59). The first stem leaf was collected as a control sample before the W+OS treatment, and the second and third stem leaves were collected 1 or 48 h after the W+OS treatment for phytohormone or secondary metabolite analysis, respectively. Approximately 100 mg of frozen leaf samples were homogenized in liquid nitrogen and extracted, and the phytohormone and secondary metabolite concentrations were quantified as described in ref. 60. A Varian 1200 LC-ESI-MS/MS system (Varian) was used for phytohormones analysis, and HPLC (Agilent-HPLC 1100 series) coupled to a photodiode array and evaporative light scattering detector (HPLC-PDA-ELSD) was used for the secondary metabolite analysis.

Variables measured in microbially treated and untreated plants were compared using individual t tests. Wilcoxon pairwise tests were used when a variable did not meet the assumptions of parametric tests. All statistics were performed in R-Studio (R Core Team, 2012). P values were not corrected for multiple tests.

Bacterial Reisolation from Field-Grown Control and Preinoculated Plants. Root-associated bacteria were reisolated from surface-sterilized roots as described above using control and bacterially preinoculated plants harvested from both field plots in the 2013 field season (seven roots for each treatment from the Old field plot and three roots for each treatment from the New field plot). In the 2014 field season, the roots of five plants were analyzed when the plants had reached the early flowering stage. Bacterial plates were incubated at 28 °C for 4 d, and colonies were picked, subcultured, purified, and identified by 16S rDNA sequencing as described above.

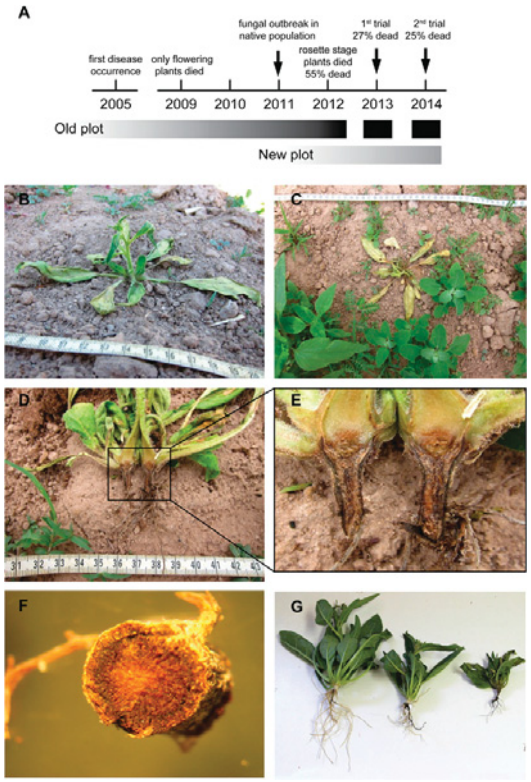


Fig. S1. Symptoms of the sudden wilt disease. (*A*) Disease symptoms first occurred only sporadically in our field plot in elongated and flowering plants and later were also observable in rosette-stage plants. Regular field experiments on the Old plot were ended in 2012 because of the unacceptably high plant mortality. Total plant mortality was recorded during the last three field seasons. (*B*) Sudden wilt disease symptoms characterized by dry or flaccid leaves developed within 1 or 2 d in *N. attenuata* plants. (*C*) The wilting was specific to *N. attenuata*; surrounding plants were not affected, and the surrounding soil usually was still moist. (*D–F*) The signature characteristic of a diseased plant was the development of black roots; the discoloration was visible on the outside as well as in longitudinal sections. The occurrence of wilting together with the black roots was diagnostic of a plant being affected by the sudden wilt disease. (*G*) Plants with differently pronounced disease levels were observed during the 2014 field season and illustrate the course of the disease. Wilting first was observed only in elongated plants (here with mild symptoms and mainly white roots) but during the last three field seasons appeared in younger plants also (here with marked symptoms and completely black roots).

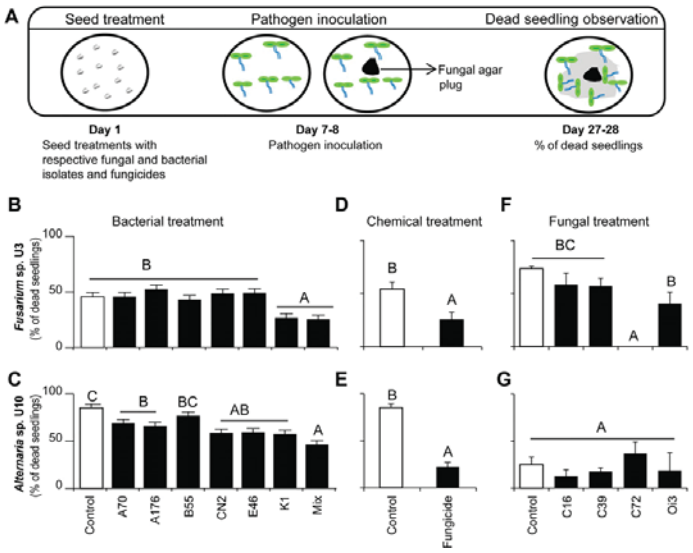


Fig. S2. Inoculation with a mixture of native bacteria, a fungicide, and two native fungal isolates reduced seedling mortality under in vitro conditions. (*A*) Schematic of the in vitro experimental setup. Seedling mortality was observed in separate infection assays using two fungal pathogens that previously had been isolated from diseased *N. attenuata* plants in a native population and characterized: *Fusarium* sp. U3 and *Alternaria* sp. U10 (3). (*B* and *C*). Evaluation of six native bacterial isolates for potential biocontrol abilities. The seeds were inoculated with individual cultures of *P. azotoformans* A70, *P. frederiksbergensis* A176, *B. megaterium* B55, *B. cereus* CN2, *A. nitroguajacolicus* E46, or *B. mojavensis* K1 or with a mixture of all strains (*SI Materials and Methods*). The mixed inoculation of all six strains had the strongest effects against *Fusarium* sp. U3 and *Alternaria* sp. U10 and was selected as the treatment for the field experiments. (*D* and *E*) The fungicide seed treatment (Landor; Syngenta) significantly reduced seedling mortality of *N. attenuata* seedlings infected with fungal pathogens. (*F* and *G*) Native fungal isolates were tested for possible biocontrol abilities. The seeds were inoculated with *Chaetomium* sp. (C16, C39, and C72) or *Oidodendron* sp. (Oi3) before being infected with fungal pathogens. The C72 and Oi3 treatments were chosen for field experiments because they reduced the mortality of seedlings inoculated with *Fusarium* sp. U3. Bars represent mean seedling mortality (± SEM, $n = 10$ plates); the different letters above the bars indicate significant differences in a one-way ANOVA with Fisher's protected least significant difference (PLSD) test; $P < 0.05$.

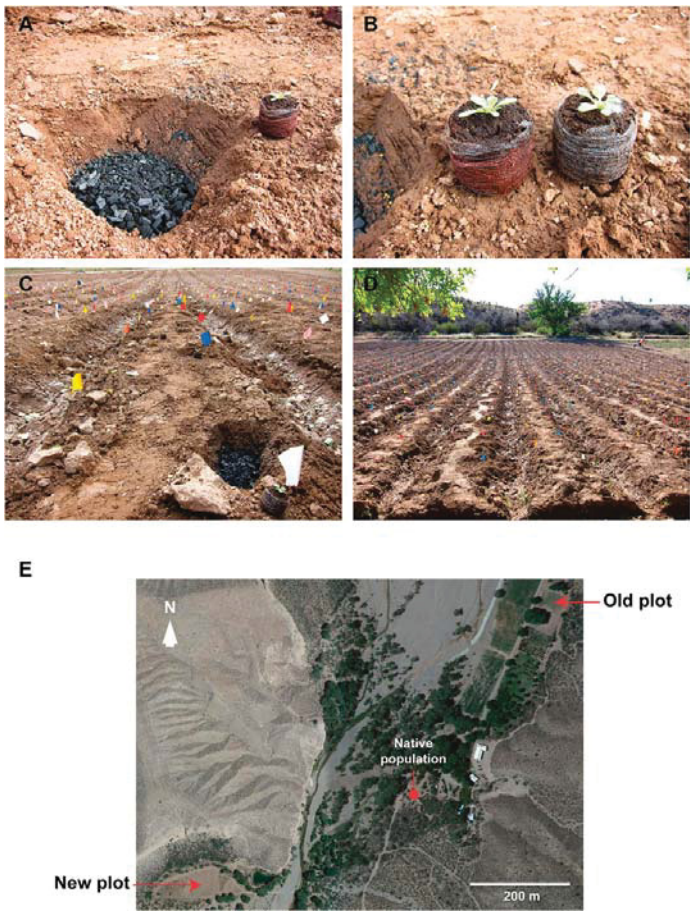


Fig. S3. Planting of the treatment groups during the 2013 field season. (*A*) Before planting, charcoal was added to the charcoal and combined treatment groups. (*B*) Jiffy pots were treated before planting; the applied fungicide solution can be seen by the red color. (*C* and *D*) Treatment groups were planted in a randomized design on the lower section of the Old field plot (N 37.1463 W 114.0198), and, as a control experiment, also on the New plot (N 37.1412 W 114.0275). (*E*) Google Maps view of Old and New field plots ~900 m apart.

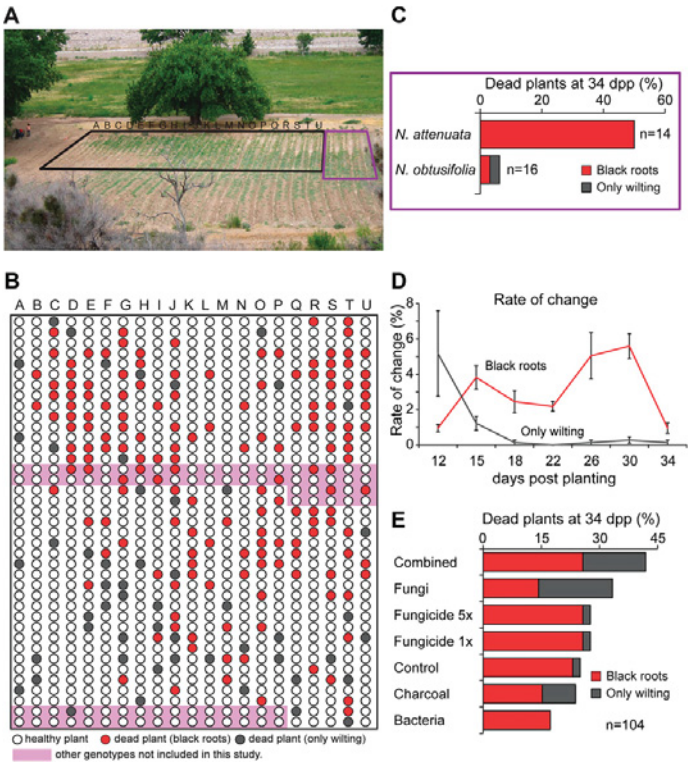


Fig. S4. Overview of the Old field plot and the rate of change in plant mortality during the 2013 field season. (*A*) The Old field plot during the 2013 season. The rectangle indicates the lower area of the plot where plants had been planted. This portion of the plot was selected because the greatest number of diseased plants was found in this area during the 2012 growing season. (*B*) Schematic illustrating the spatial distribution of plants (from the rectangle shown in *A*), distinguishing plants with the sudden wilt symptoms (wilting and black roots) and plants with only wilting symptoms. The occurrence of the sudden wilt disease was not distributed equally throughout the plot. (*C*) Mortality rate of *N. obtusifolia* planted together with *N. attenuata* in an adjacent block of a separate experiment. (*D*) The development of plant mortality is shown as the rate of change (percentage dead plants at one observation minus dead plants at previous observation). The error bars reflect the differences among the treatments. Plants with only wilting symptoms were observed mainly at the early time points. (*E*) Percentage of dead plants at 34 dpp showing black roots or only wilting symptoms. Most plants with only wilting symptoms were found in the fungi, charcoal, and combined (charcoal + fungicide) treatments.

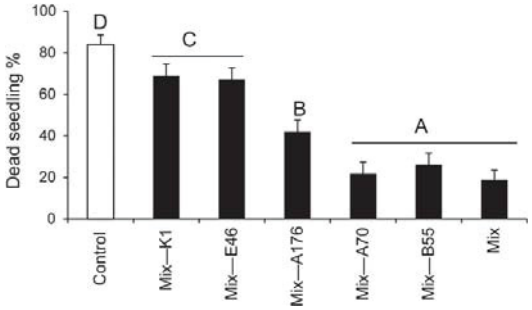


Fig. S5. In comparison with the effects of seedling inoculation with a mixed bacterial consortium consisting of five taxa, the individual absences of three bacterial isolates (K1, E46, and A176) significantly increased seedling mortality under in vitro conditions. Different mixed bacterial consortia were evaluated for potential biocontrol abilities against *Alternaria* sp. U10. Mixed bacterial consortia lacking one bacterial isolate (e.g., mix minus K1, mix minus E46, and mix minus A176) significantly increased seedling morality. Based on these results, the three-bacteria mixed consortium (K1 + A176 + E46) and two-bacteria mixed consortium (B55 + A70), along with the mixture of all five bacteria, were selected as treatments for the 2014 field experiments. Bars represent mean seedling mortality (± SE; n = 7–10); the different letters above the bars indicate significant differences in a one-way ANOVA with Fisher's PLSD test; $P < 0.05$. For the experimental set-up see Fig. S2*A*.

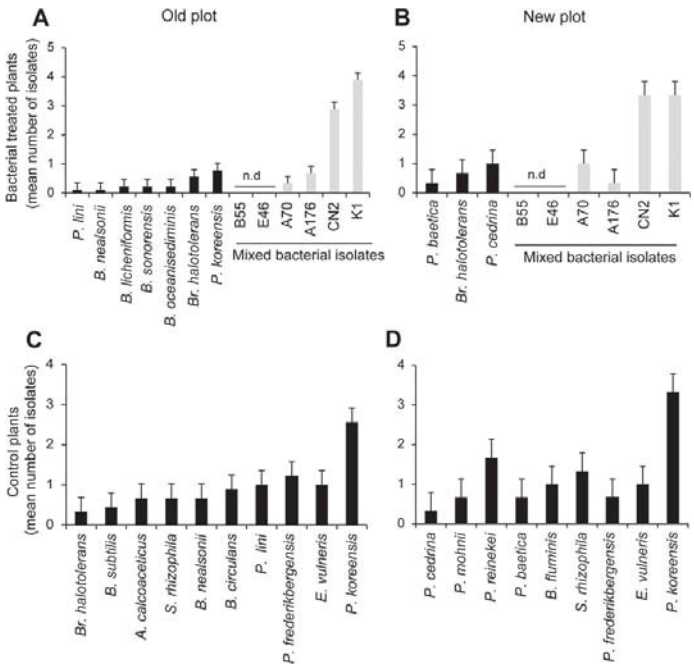


Fig. S6. Reisolation of the bacteria from healthy field-grown plants at the flowering stage demonstrated the persistence of the inoculated bacterial taxa. In 2013, healthy plants from the control and bacterial treatment groups were harvested at the early flowering stage from both field plots, and the culturable bacterial consortium were isolated. (*A* and *B*) Four of the six native bacterial taxa used in the bacterial mix (seed and Jiffy treatment) persisted throughout growth under field conditions and were reisolated from the roots of plants from both field plots. The inoculated roots showed strong colonization by *B. cereus* CN2 and *B. mojavensis* K1. The bars represent the mean number of isolates (± SEM; Old field plot: n = 7 roots, 70 isolates; New field plot: n = 3 roots, 30 isolates). (*C* and *D*) The control plants showed natural colonization by *P. frederiksbergensis*, which was also used in the bacterial mixture as A176. Bacterial genus acronyms: A, *Acinetobacter*; B, *Bacillus*; Br, *Brevibacterium*; *E*, *Escherichia*; P, *Pseudomonas*; S, *Stenotrophomonas*.

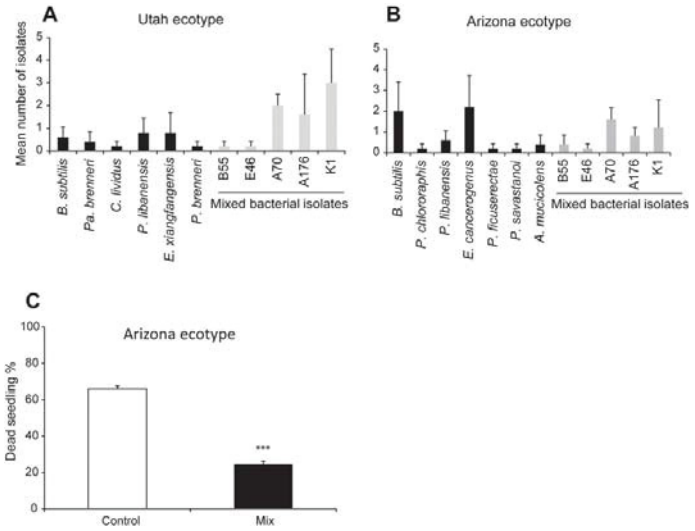
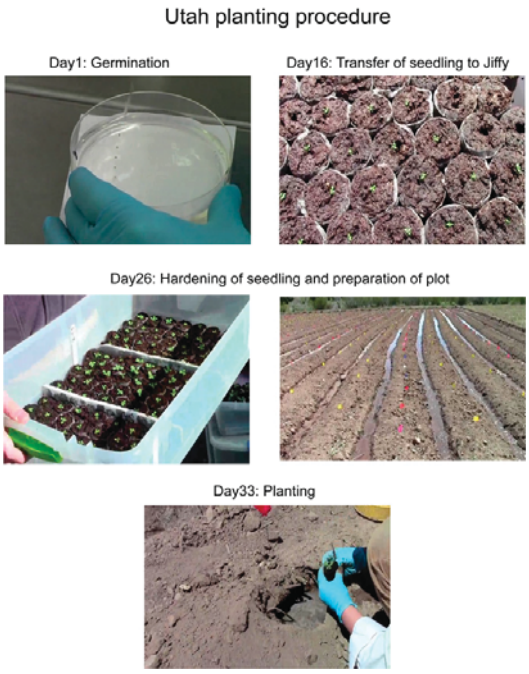


Fig. S7. Reproducibility of the reisolation of the mixed bacterial consortium from Utah and Arizona genotypes in the 2014 field season and the protection effect of the consortium for the Arizona ecotype. Bacterially treated Utah and Arizona genotypes were harvested at the early flowering stage from the New field plot, and the culturable bacterial consortium were isolated. (*A* and *B*) All five native bacterial taxa used in the bacterial inoculation treatment (seed treatment only) persisted throughout growth under field conditions of both ecotypes. The persistence of the inoculated bacteria taxa within both ecotypes demonstrates the consistency of the bacterial association with *N. attenuata*. The bars represent the mean number of isolates (± SEM; $n = 5$ roots, 50 isolates). Bacterial genus acronyms: A, *Achromobacter*; B, *Bacillus*; C, *Ciceribacter*; E, *Enterobacter*; P, *Pseudomonas*; Pa, *Pantoea*. (*C*) Under in vitro conditions, the consortium of five mixed bacterial isolates significantly reduced the mortality of the *N. attenuata* Arizona ecotype inoculated with *Alternaria* sp. U10 (± SEM; $n = 11$, $P < 0.001$, *t*-test).

Table S1. Traits important for *N. attenuata*'s ecological performance and insect resistance were compared between bacterially treated and control plants grown in the New field plot during the 2013 field season

Variable	Test	*n*	Test statistic	*P* value
Plant-growth parameters				
Root length	*t* test	21	−0.162	0.872
Shoot length	*t* test	21	0.222	0.826
Root/shoot ratio	*t* test	21	−0.454	0.653
Rosette diameter	*t* test	21	0.318	0.753
Stem length	*t* test	21	−0.313	0.756
Number of branches	*t* test	21	−0.267	0.791
Number of buds	*t* test	21	−0.976	0.335
Number of flowers	Wilcoxon	21	204.500	0.696
Number of seed capsules	Wilcoxon	21	201.000	0.522
Chlorophyll	*t* test	12	−1.258	0.222
Herbivore damage				
Grasshopper damage	Wilcoxon	21	187.500	0.342
Mirid damage	Wilcoxon	21	186.000	0.338
Noctuid damage	Wilcoxon	21	174.000	0.238
Flea beetle damage	Wilcoxon	21	230.000	0.727
Tree cricket damage	Wilcoxon	21	231.000	0.697
Flower volatiles				
Benzyl acetone flower volatile	Wilcoxon	16	106.000	0.415
Leaf volatiles 1 h after W+OS				
3(Z)-hexen-1-ol	Wilcoxon	16	30.000	0.491
2(E)-hexen-1-ol	Wilcoxon	16	30.000	0.491
Putative alpha-pinene	Wilcoxon	16	22.500	0.886
3(Z)-hexenyl acetate	Wilcoxon	16	23.000	0.950
3(Z)-hexenyl isobutanoate	Wilcoxon	16	32.000	0.345
3(Z)-hexenyl butanoate	Wilcoxon	16	24.500	1.000
3(Z)-hexenyl 2-methylbutanoate	*t* test	16	−0.907	0.383
3(Z)-hexenyl valerate	*t* test	16	−0.879	0.400
Putative sesquiterpene oxide	Wilcoxon	16	25.000	0.950
Leaf volatiles 48 h after W+OS				
1-Hexanol	*t* test	16	0.884	0.396
3(Z) hexen-1-ol	Wilcoxon	16	45	0.19
Putative alpha-pinene	Wilcoxon	16	29.5	0.81
3(Z)-hexenyl isobutanoate	Wilcoxon	16	36	0.721
3(Z)-hexenyl butanoate	*t* test	16	0.336	0.742
Putative alpha-terpineol	*t* test	16	1.442	0.174
3(Z)-hexenyl 2-methylbutanoate	Wilcoxon	16	40	0.442
3(Z)-hexenyl valerate	Wilcoxon	16	38	0.574
Alpha-duprezianene	Wilcoxon	16	44	0.227
Putative sesquiterpene oxide	*t* test	16	2.185	0.048
Phytohormones at W+OS (0 h)				
JA	*t* test	7	−0.266	0.7945
JA-Ile	*t* test	7	−0.171	0.867
ABA	*t* test	7	−1.49	0.162
Phytohormones 1 h after W+OS				
JA	*t* test	7	0.068	0.9473
JA-Ile	*t* test	7	2.042	0.0659
ABA	*t* test	7	0.011	0.991
Secondary metabolites at W+OS (0 h)				
Nicotine	*t* test	7	0.442	0.6661
Caffeoylputrescine	*t* test	7	0.426	0.6792
Chlorogenic acid	*t* test	7	0.132	0.8973
Dicaffeoyl spermidine	*t* test	7	0.124	0.9032
Rutin	*t* test	7	−0.325	0.7506
HGL-DTGs	*t* test	7	−0.325	0.7507
Secondary metabolites 48 h after W+OS				
Nicotine	*t* test	7	0.247	0.8094
Caffeoylputrescine	*t* test	7	0.545	0.5977
Chlorogenic acid	*t* test	7	1.513	0.1585
Dicaffeoyl spermidine	*t* test	7	1.521	0.1566
Rutin	*t* test	7	−0.759	0.4624
HGL-DTGs	*t* test	7	−0.759	0.4624



Movie S1. Utah field-planting procedures.

Movie S1

Dataset S1. Fungal and bacterial isolates retrieved from diseased roots

Dataset S1

6 Manuscript IV

Native grown *Nicotiana attenuata* root microbiome is independent of soil types and plant responses to UVB increase *Deinococcus* root colonization

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Chapter VI

6.1 Summary

- Plants recruit microbial communities from the soil in which they germinate. Whether soil types sculpt the composition of root fungal and bacterial microbial communities under natural conditions, and whether UVB-exposure affects the root colonization of highly abundant species remains unknown.
- *Nicotiana attenuata* plants from 5 different natural populations were analyzed along with bulk soil by 454-pyrosequencing to characterize the root and soil microbiomes. Transgenic lines impaired in UVB perception (ir*UVR8*) and UVB responses (ir*CHAL*) were produced and used to test the colonization of the highly abundant (by pyrosequencing) taxon *Deinococcus* in microcosm experiments using a synthetic bacterial community and plants grown with visible light with/without UVB supplementation.
- Alpha and beta diversities of the bacterial and fungal communities differed significantly between soil and root but core root bacterial communities did not differ among locations. With UVB supplementation, wild type roots were colonized more by *Deinococcus*, while *irUVR8* and *irCHAL* were colonized less.
- *N. attenuata* plants recruits a core root microbiome irrespective of soil type under natural conditions, and *Deinococcus* root colonization is enhanced when plants are grown under UVB, and decreased when plants are unable to perceive and respond to UVB. A plant's response to UVB influences the composition of its microbiome.

Keywords: *Deinococcus*, UVB-radiation, root microbiome, *Nicotiana attenuata, chalcone synthase, pyrosequencing, UVR8, fungal and bacterial root communities.*

6.2 Introduction

Soil is a highly diverse ecosystem which harbors diverse communities of microbes (Doornbos *et al.*, 2011b; Bulgarelli *et al.*, 2012b). Plants, anchored in soil with their roots, which are colonized by diverse bacterial and fungal communities (Hassan & Mathesius, 2012; Hacquard *et al.*, 2016). The interaction among plants and microbes is well-characterized for some partners, in particular for plant-pathogens, plant-growth promoting bacteria and for bacterial and fungal species which act as biocontrol agents (Compant *et al.*, 2005; Lugtenberg & Kamilova, 2009; Santhanam *et al.*, 2015b). However, only recently, due to the rapid development of next generation sequencing (NGS) platforms, it is possible to characterize the plants' microbiome in their entirety (Lebeis, 2014). Previous studies that characterized the microbial communities from biotic and abiotic samples relied heavily on cultivation-based techniques, which are highly biased, underestimate the microbial diversity, and only recently has it been possible to cultivate 60% of root bacterial communities identified by pyrosequencing (Bai *et al.*, 2015). NGS enables researchers to identify and quantify the entire microbial population including rare and uncultivable taxa. The technique is mainly based on the 16S rDNA consisting of the variable regions V1-V9 to characterize the bacterial communities, and on the18S rDNA or ITS region for fungal communities. DNA-based NGS community analysis, do not differentiate living from dead cells and hence overestimate diversity with results influenced by primer selection (Ghyselinck *et al.*, 2013; Lebeis, 2014).

Several NGS based studies using different plant species demonstrated that different plant parts such as leaves and roots harbor different microbial communities (Bodenhausen *et al.*, 2013; Santhanam *et al.*, 2014). Similarly, roots and soils harbor distinct microbial communities with root communities being lower in diversity than that of the soil (Bulgarelli *et al.*, 2012a). When the same plant species was grown in different soils, the plants harbored a core set of OTUs belonging to a limited number of phyla, indicating selective recruitment of bacterial communities by the roots from a pool of microbes in the soil (Bulgarelli *et al.*, 2012a; Lundberg *et al.*, 2012). A field study with 27 modern inbred lines of maize grown at different locations in the US also found a similar rhizosphere microbiome across all samples largely independent of soil types (Peiffer *et al.*, 2013). Interestingly, a comparison of the root microbiota of *Arabidopsis* and its relatives arrived at a different conclusion – the composition of the bacterial communities depended more on environment than on host and microbe-microbe interaction (Schlaeppi *et al.*, 2013). Although the conclusions of these studies are not

fully consistent, they all indicate that plants harbor only a subset of the soil microbiome. However, it remains largely unknown which plant and environmental factors favor colonization of particular microbial taxa under natural conditions.

Among the few plant factors that have been investigated in detail regarding their effect on the composition of a plant's endophyte community, one focus has been on phytohormones (Doornbos *et al.*, 2011a; Pieterse *et al.*, 2014). For example, the levels of salicylic acid influenced the root microbiome of *A. thaliana* under glasshouse condition (Lebeis *et al.*, 2015), *N. attenuata's* ability to perceive and produce ethylene has been reported to be a key determinant of composition of the root-associated bacterial communities (Long *et al.*, 2010), while the plant's ability to produce jasmonic acid (JA) did not significantly alter its root bacterial colonization (Santhanam *et al.*, 2014). Solar UVB radiation is part of the natural light and its intensities vary depending on the region and the time of the day. The effect of UVB on plant responses was intensively studied (Frohnmeyer & Staiger, 2003). Previous studies showed that UVB radiation has various effects on plants including damage to DNA, alterations in transpiration and photosynthesis, changes in growth, development and morphology, and rearrangement of secondary metabolites (Davidson & Robson, 1986; Caldwell *et al.*, 1994, 2007; Mazza *et al.*, 1999; Ruhland *et al.*, 2005; Nawkar *et al.*, 2013; Jenkins, 2014; Kataria *et al.*, 2014).

Several studies have emphasized the importance of UVB radiation effects on pathogens, herbivores and soil (Avery *et al.*, 2004; Rinnan *et al.*, 2005; Caldwell *et al.*, 2007; Piccini *et al.*, 2009; Niu *et al.*, 2014). UVB-exposed plants tend to be more resistant to attack from herbivores (Tilbrook *et al.*, 2013), and in *A. thaliana* UVB exposure confers cross-resistance to the fungal necrotrophic pathogen *Botrytis cinerea* through increases in sinapate accumulation, a phenolic whose biosynthesis is regulated by UVR8 (Demkura & Ballaré, 2012). UVB-radiation was found to change the community composition of the culturable bacterial community of field-grown peanut leaves (Jacobs & Sundin, 2001). Although UVB induced changes in the microbiome in aboveground tissue of plants were shown, our understanding of UVB induced changes in the plant's response on the root microbiome remains still largely unknown.

It is well known that a plant's chemical composition, as well as its root exudates are influenced by UVB exposure (Caldwell *et al.*, 2007; Hectors *et al.*, 2014; Kaling *et al.*, 2015) and that UVB exposure results in the dramatic increase in phenolic sunscreens (Mazza *et al.*,

1999, 2000; Izaguirre *et al.*, 2007). In plants, UVB is perceived by UV RESISTANCE LOCUS 8 (UVR8), a photoreceptor specific for UVB radiation (Rizzini *et al.*, 2011), which induces the expression of chalcone synthase (CHS); the key regulator of flavonoid biosynthesis. *A. thaliana* uvr8 mutants are highly susceptible to the damage caused by high UVB radiation and have reduced levels (CHS). Among other roles, flavonoids are important UV-screens that provide protection from high UVB radiation (Brown *et al.*, 2005). Additionally, flavonoids act as a chemoattractants for *Rhizobia,* nitrogen-fixing bacteria that forms root nodules on legume plants (Hassan & Mathesius, 2012). Silencing the expression of chalcone synthase resulted in less nodulation and low *Rhizobium* colonization (Wasson *et al.*, 2006; Abdel-Lateif *et al.*, 2013). While a number of studies have investigated the role of CHS and flavonoids on recruiting beneficial *Rhizobium* species, it remains unknown if and how recruitment is related to the perception of UVB by the UVR8 receptor and its downstream responses that include the elicitation of CHS.

Coyote tobacco (*Nicotiana attenuata)* germinates from long-lived seed banks in the typically nitrogen rich soils of post-fire environment (Lynds & Baldwin, 1998; Preston & Baldwin, 1999; Baldwin, 2001). The natural environment of *N. attenuata* plants, the Great Basin Desert, Utah, USA is further characterized by high light conditions and strong UVB irradiance. The importance of UVB for *N. attenuata's* herbivory defense has been demonstrated - UVB radiation along with 17-hydroxygeranyllinalool diterpene glycosides provide resistance against mirid attack under field conditions (Dinh *et al.*, 2013). During and after germination, *N. attenuata* seedlings recruit microbes from the surrounding soil. Both culture-dependent and -independent approaches have been employed to study the microbiomes of *N. attenuata* plants grown in a field plot (Long *et al.*, 2010; Santhanam *et al.*, 2014; Groten *et al.*, 2015).

The aim of this study was to systematically investigate whether the soil shapes the root bacterial and fungal communities of native-grown *N. attenuata* populations using 454 pyrosequencing. For a robust comparison, we used natural populations which have not been altered for breeding purposes and grown at different native sites, and an inbred wild type line grown at the field plot in the same area. Furthermore, in a microcosm experiment using a synthetic bacterial community consisting of four native strains isolated from the field grown plants, we tested the hypothesis that the plant's response to UVB radiation, its perception (UVR8) and response (CHAL) influence the root colonization of the UV-resistant bacteria *Deinococcus* which are highly abundant in *N. attenuata* roots under natural conditions. This

experiment was based on a previously characterized RNAi silenced line impaired in the expression of CHS (Kessler *et al.*, 2008), and a newly generated isogenic line silenced in the expression of UVR8, and a cross of both.

6.3 Materials and Methods:

Sample collection

N. attenuata plants were collected in 2013 from five different locations in the Great Basin Desert Utah, USA: Burn (N 37.3332 W 113.9388), Pahcoon Spring (N 37.2381 W 113.8284), Wash (N 37.1396 W 114.0278), Gate (N 37.1428 W 114.0224) and Lytle field plot (N 37.1463 W 114.0198) (Fig.1a). To investigate genotypic variation and robustly validate the core root microbiome of *N. attenuata,* WT-31st inbred plants from Lytle plot were included to compare its root microbiome to native populations. Furthermore, the data from this study were compared with previous study with plants harvested from Lytle field plot (Santhanam *et al.*, 2014). For plants of the Lytle field plot, wild type (WT) *Nicotiana attenuata* Torr. Ex S. Watson seeds of the 31st inbred generation were surface sterilized and germinated on Gamborg's B5 plates (Duchefa) as previously described (Krügel *et al.*, 2002) and transferred to individual Jiffy pots and planted in a field plot located at Lytle Ranch Preserve, Utah, USA as described in (Diezel *et al.*, 2009). For the other four locations, plants were naturally grown and not privately owned or protected in any way, so no special permits were required to collect the samples. We harvested 9 plants from Gate (G), Wash (W) and Lytle (L) locations and 3 plants from Burn (B) and Pahcoon Spring (P). Differences in the numbers of plants harvested among sites was due to limited availability of plants in the native populations. After harvesting, samples were washed in running tap water and dried and transported to the laboratory, MPI-CE, Jena, Germany. Bulk soils were collected, adjacent to the sampled plants. Total P, Ca and K were determined by ICP-MS (ICP-Atomic Emission Spectrometer "Optima 3300 DV" (PerkinElmer) after microwave-based digestion, and total C and N were determined by combustion (Vario EL II"-Elementar Analysensysteme GmbH, Hanau). Chemical properties of soil samples were measured at the Max-Planck Institute for Biogeochemistry as described (Quesada *et al.*, 2010) (http://www.biogeosciences.net/7/1515/2010/bg-7-1515-2010.html).

DNA extraction, sample pooling and PCR amplification

Total DNA was extracted from 3 root and soil samples from each location using the FastDNA™ Spin kit for soil (MP biomedicals). For the G, W and L locations, 9 roots were pooled to create 3 samples, thus in total, 30 samples (15 from each compartment) were used. The concentration of DNA was determined by a Nano Drop ND 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and diluted to a working concentration of 30ng/µL. For 454 pyrosequencing, samples were sent to MR.DNA (http://mrdnalab.com/), Texas,

USA. Variable regions from V5–V9 of the bacterial 16S rDNA gene were amplified by the following primer sets: 799F-1394R: ACCMGGATTAGATACCCKG-ACGGGCGGTGTGRTC, (Chelius & Triplett, 2001). Based on our previous research (Santhanam *et al.*, 2014), we selected primer set 799F-1394R, because it excludes amplification of chloroplast and Cyanobacteria. For fungal community analysis primers ITS1F (CTTGGTCATTTAGAGGAAGTAA, Gardes & Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC, White *et al.*, 1990) were used.

Analysis of pyrosequencing data and statistical analysis.

The QIIME software package was used to analyze the reads using default parameters for each step (Caporaso *et al.*, 2010). QIIME, Primer E software v.6 package (Clarke & Gorley, 2006) and R version 3.1.1 were used for all statistical analysis. For details, see SI.

Silencing of *NaUVR8*, *NaCHAL* and their combination

To generate ir*UVR8* plants, we cloned a 319 bp fragment of *NaUVR8*gene as an inverted repeat construct into a pRESC8 transformation vector containing a hygromycin (*hptII*) resistance gene as selection maker (Fig. S6a). *N attenuata* plants were transformed using the LBA4404 strain of *Agrobacterium tumefaciens* using the transformation method described in Krügel *et al.*, (2002). Three independently transformed lines were screened according to Gase *et al.* (2011). All three lines met the requirements of homozygosity and showed silencing efficiencies of greater than 95% (Figure S6c).One of best silenced line, 260-10-1 was grown in the field at the Lytle Ranch Preserve in Utah for a detailed functional analysis (see SI). The cross between ir*UVR8* (260-10-1) and ir*CHAL* (283-1, previously described in Kessler *et al.*, 2008) homozygous plants was created by hand-pollination using ir*UVR8* and ir*CHAL* plants as a pollen donor and pollen acceptor, respectively in the glasshouse. The hemizygote ir*UVR8* x ir*CHAL* plants showed reduction of ≈ 95% in *NaUVR8* and of ≈75% in *NaCHAL* expression compared to those of EV plants (data not shown, personal communication by Y. OH). For details, see SI.

Field release

For field releases, we germinated empty vector (EV - WT plants transformed with an empty vector construct as control) and ir*UVR8* on Gamborg's B5 medium as described above. The transgenic seeds were imported and plants released under US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) permit and notification numbers 12-320-103m and 13-350-101r, respectively.

Growth measurements and photosynthetic capacity

Plant growth parameters were measured every 5 days (N=15) and net photosynthetic rates and stomatal conductance were measured using a LI-6400XT portable photosynthesis analysis system (Li-COR Bioscience, Lincoln, NE, USA) with 2000 μmol $m^{-2}s^{-1}$ of photosynthetically active radiation, 400 μmolCO_2 m^{-2} s^{-1} of reference CO_2 concentration. 1st stalk leaf was used for all measurement, and values were generated from 4 size-matched undamaged, EV and ir*UVR8* plants growing on the field plot of the Lytle Ranch Preserve.

Isolation of *Deinococcus*

The 31st inbred generation of *N. attenuata* WT seeds were surface-sterilized and germinated as described above. Ten days after germination, seedlings were transferred to Teku pots containing native soil collected from five different locations from the Great Basin Desert, Utah, USA (see sample collection and Fig. 1). Native soils were diluted with sterile autoclaved sand (1:1). Plants were grown under glasshouse condition for three weeks, maintained at a day/night cycle of 16h (26–28°C)/ 8h (22–24°C), supplemented by 600 W or 400 W high-pressure sodium lamps (Philips Sun-T Agro), and 45-55% relative humidity. After three weeks, roots were harvested and gently washed with sterile water to remove soil, and roots were crushed for 3 mins followed by inoculations of three different media, Glucose Yeast Extract (GYE), R2A and Tryptone Yeast Extract (TGY). Plates were incubated at 28°C for one week, and pink-colored colonies were picked from plates, sub-cultured and stored in 50% glycerol solution at −80°C. Bacterial isolates were identified based on 16S rDNA gene sequencing as described in (Santhanam *et al.*, 2014). Three bacterial strains showed 100% (603bp) similarity with the highly abundant core OTU12140 and strain D61 was used for further experiments.

Microcosm experiment

Microcosm experiments with four different bacterial isolates representing four different phyla *Arthrobacter nitroguajacolicus*- E46 (Actinobacteria), *Pseudomonas frederiksbergensis*- A176 (Proteobacteria), *Bacillus mojavensis*- K1 (Firmicutes) isolated from field-grown roots from an earlier study (Santhanam *et al.*, 2014) and *Deinococcus citri*- D61 (Deinococcus-Thermus). Seeds of *N. attenuata* WT (31st inbred generation) and of transgenic lines impaired in UVB perception (ir*UVR8*, A-12-260-10-1) and flavonoid biosynthesis (ir*CHAL* , A-06-283-1-1) (Kessler *et al.*, 2008) and a cross of both (ir*CHAL*xir*UVR8*) were surface sterilized and germinated as described above. Ten days after germination, seedlings were transferred to a Magenta™ vessel box (W×L×H; 77 mm ×77

mm ×97 mm, Sigma, GA-7, Germany) filled with sand (0.7–1.2 mm grain size, Raiffeisen, Germany) and 50 mL of Ferty B1 fertilizer (Planta Düngemittel, Regenstauf, Germany, http://www.plantafert.de/) and inoculated with a mixture of the 4 bacterial isolates by combining equal concentration of individual isolates (E46+A176+ K1+ D61) from overnight-grown single cultures to obtain a working concentration of 10^{-6} CFU/mL. Seedlings were grown in a Vötsch growth chamber (22°C, 65% humidity, 16 h light / 8h dark). Half of the plants were exposed to UVB (≈ 1.5 umol m^{-2} s^{-1}, 290-315 nm) for 4h per day from 10 AM to 2 PM. These time-points were selected based on observations of UVB intensity in *N. attenuat*a' native habitat in May to June 2014 (Fig S6d, the UVB intensity of a representative day is shown, May 21th 2014). 36 days after inoculation, roots were harvested and stored at -80°C for quantification of bacterial communities by qPCR.

Primer specificity and quantification of bacteria

Bacterial species specific primers designed using Primique software (Fredslund & Lange, 2007); for details see SI. For quantification of root bacterial colonization in the microcosm experiment, the same primers and procedures used for qPCR described in SI were used (Fig. S7). All samples were run in triplicates and the average Ct values are reported. For the generation of standard curves, PCR products from four primers were diluted (10- fold serial dilutions 1 to 10^{-8} ng μL^{-1}) after measuring the PCR products using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, USA). For qPCR, the following cycling parameters were used: 95°C for 30s, followed by 35 cycles of 63°C for 60 s, 72°C for 60s and 95°C for 30s. Subsequently, a melting curve analysis was conducted by increasing the temperature from 60°C to 95°C within 20 min. The PCR efficiency of all four reactions was from 95-105%. Based on the standard curves, absolute copy numbers of specific 16S rDNA templates were calculated as described by Lee *et al.*(2006, 2008). 16S rDNA gene copy numbers were normalized by number of operons from nearest neighbor strains (https://rrndb.umms.med.umich.edu/) (Stoddard *et al.*, 2015) and log10 transformed. qPCR products were purified and sequenced as described above.

Nucleotide sequence accession numbers

The sequencing data have been deposited in the European Nucleotide Archive-PRJEB13826, the isolates of the culture-dependent approach are listed in Table S2. *NaUVR8* is available at DDBJ/EMBL/GenBank (gene ID KX094971).

6.4 Results

Native root microbiomes are dominated by Actinobacteria, Deinococcus-Thermus and TM7 bacteria phyla and Ascomycota fungal phyla irrespective of locations.

To investigate the variability of the *N. attenuata* root bacterial communities across native populations of plants, we sampled native grown *N. attenuata* roots and bulk soil from five different locations at the Great Basin Desert, Utah, USA: Burn (B), Gate (G), Lytle (L-field plot), Pahcoon (P) and Wash (W) (Fig. 1a). Based on the chemical properties of the soils, Burn (B) and Pahcoon (P) soils are distinct from those from the Gate (G), Lytle (L) and Wash (W) populations (Fig. S1a), which clustered together in the analysis. The bacterial and fungal communities were determined based on barcoded 454 pyrosequencing of 16S rDNA and ITS respectively, using the QIIME platform.

For downstream analysis of bacterial communities, singletons and Archaea were removed, and the number of sequences per sample rarified to 1310 reads, which resulted in a total of 5122 OTUs from 22 taxa at the phyla level based on 97% similarity (Fig. 1b). Among these phyla, seven (Actinobacteria, Bacteroidetes, Chloroflexi, Proteobacteria, Firmicutes, Deinococcus-Thermus and TM7) were shared between roots and soils and strongly dominated the root and soil communities representing more than 98% of relative abundance, while 15 phyla representing less than 2 % of the total relative abundance were only recovered from soils but not from roots.

The dominant phyla in the roots were Actinobacteria (36%), Deinococcus-Thermus (23%) and candidate division TM7 (19%) and these represent 78% of the total relative abundance (Fig. 1b). These dominant phyla were subjected to Generalized Least Squares (GLS) to find out whether they are significantly enriched in roots compared to soils or if they differ among the locations. Phyla significantly differed between compartments - soils vs. roots (Fig S2, Actinobacteria- L ratio=9.4, p=0.002; Deinococcus-Thermus- L ratio=9.4, p=0.002; TM7- L ratio=7.81, p=0.005), but were independent of the locations of the populations (Actinobacteria- L ratio=4.8, p=0.2; Deinococcus-Thermus- L ratio=5.6, p=0.2; TM7- L ratio=4.5, p=0.3). In contrast, the phylum Chloroflexi (60%, Fig. 1A) was significantly more abundant in soils than in roots (Fig. S2, Chloroflexi- L ratio=51.3, p=<0.0001) and also differed significantly amongst locations (Chloroflexi- L ratio=27.9, p=<0.0001, pairwise Tukey, Burn:Lytle, t-ratio= -5.8, p=0.0017; Burn: Pahcoon, t-ratio= -3.1, p=0.02; Burn:Wash, t-ratio= -4.1, p=0.003; Gate:Lytle, t-ratio=-4.3, p=0.001;

Gate:Wash, t-ratio=-3.5, p=0.01; Lytle: Pahcoon, t-ratio=4.4, p=0.01; Wash: Pahcoon, t-ratio=-3.5, p=0.01).

In order to robustly evaluate whether locations and soil types shape *N. attenuata's* root bacterial communities, we calculated the alpha diversity parameters, Shannon diversity (Shannon & Weaver, 1964) and Margalef's species richness (Magurran, 1991) as a measure of diversity and richness within the samples. These were calculated based on the OTUs at 97% sequence similarity. For bacteria, both indices are significantly higher in soils compared to roots (Fig. 2 a,b: ANOVA, Margalef- $F_{9,20} = 27.1$, p=<0.0001, Shannon- $F_{9,20} = 13.9$, p=<0.0001). The higher bacterial diversity in soils was mirrored by a higher number of OTUs in soils (2843 OTUs) than in roots (1283 OTUs); and only (498 OTUs) were shared amongst root and soil samples of 5122 OTUs in total. The beta diversity among the samples was determined based on the Bray-Curtis-dissimilarity matrix for the analysis of similarities (ANOSIM) and non-metric multidimensional scaling ordination (NMDS). ANOSIM revealed that, root bacterial communities are significantly different from soils (R=0.87, p=0.001) and in NMDS plots, root and soil bacterial communities clustered separately, independent of locations (Fig. 2c).

Fungal reads were rarified to 5500 reads and resulted in a total of 1546 OTUs based on the 97% similarity criteria. The OTUs corresponded to five phyla present in all root and soil samples: Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota with a clear dominance of Ascomycota with more than 85% of the total relative abundance in roots except for one site, and 45 to 70% in soil (Fig S3). There was no significant difference among the enrichment of Ascomycota in the two compartments (soil vs. root, L ratio=2.01, p=0.15), but the abundance of Ascomycota significantly differed among locations (L ratio=31.23, p=<0.0001, pairwise Tukey, Burn:Lytle, t-ratio= -3.9, p=0.007, Gate:Lytle, t-ratio=-3.1, p=0.03).

In contrast to the results obtained for bacteria, alpha diversity indices of fungal communities, Shannon and Margalef species richness, differed significantly amongst the samples from the different populations (Fig. S4; ANOVA, Shannon:$F_{9,20} = 4.7$, p = 0.001, Margalef: $F_{9,20} = 5.4$, p = 0.0008) without showing a clear influence from the compartment (roots vs. soils). Similarly, beta diversity ANOSIM among fungal communities of soils and roots from the different locations differed significantly (R=0.7, p=0.001), and in NMDS plot based on the Bray-Curtis dissimilarity matrix, root samples clustered predominantly in the

upper part of the plot away from the soil samples which clustered in the lower part. However, consistent clustering of replicates based on sampling location was not observed, mainly due to the high variance amongst the samples (Fig. S4).

In summary, native grown *N. attenuata* roots specifically recruit a subset of the total bacterial communities, which were dominated by the phyla Actinobacteria, Deinococcus-Thermus and TM7 from soils, while fungal communities did not show a specific enrichment in roots. Ascomycota dominated the taxa recovered from both roots and soils.

Core root bacterial and fungal communities of native grown *N. attenuata* roots

To further investigate which taxa constitute the core root bacterial communities and if at a lower taxonomic level different taxa colonize the roots depending on the locations, we examined the root bacterial communities at 97% similarity OTUs. 49 OTUs of a total of 1781 OTUs were shared amongst all root samples (Fig. 3); this core community represented 45 to 78 % of the total relative abundance and belonged to only six phyla (Deinococcus-Thermus, Actinobacteria, TM7, Chloroflexi, Proteobacteria and Bacteroidetes), including two phyla (Proteobacteria, Bacteroidetes) which constituted less than 9% of the total relative abundance. Among the 49 core OTUs, 23 OTUs differed significantly amongst the soil and root compartments (Table S1). Most of the significant OTUs belong to the phyla Deinococcus-Thermus and Actinobacteria while other taxa tended to be underrepresented (Table S1). Unique bacterial OTUs at each location varied from 6.4 % (PR), 6.9% (WR), 7.5% (BR), 9.4% (GR) and 18.5% (LR) of total relative abundance. In summary, the high abundance of core OTUs across the samples and a very low relative abundance of unique OTUs among the samples, indicate that sampling locations had a very minor role in shaping *N. attenuata's* root bacterial communities.

The unique fungal communities harbored by roots ranged from 1 (GR) to 25% (LR) of total relative abundance (Fig. S5). In contrast, 20 OTUs belonging to the phylum Ascomycota were found in all roots irrespective of locations. These 20 core root OTUs represent nearly 45 to 75% of the total relative abundance at three sampling locations (WR, PR, LR, which were not particularly similar with regard to their soil chemistries), while their relative abundance in all soil samples and two of the root samples (BR, GR) was less than 20%. From these results we infer that the plants enrich their core fungal taxa in the roots, but the recruitment of fungi from soil is less selective than it is for bacteria, and as a

consequence, sampling location had a larger influence for the root fungal communities than it did for the bacterial communities.

Deinococcus-Thermus taxa are enriched in *N. attenuata* roots and the isolation and culturing of *Deinococcus citri*

We compared the highly abundant phyla found in this study with the most abundant phyla found in roots of *Arabidopsis*, barley and sugarcane which are particularly well-studied (Bodenhausen *et al.*, 2013; Schlaeppi *et al.*, 2013; Bulgarelli *et al.*, 2015; Yeoh *et al.*, 2016) to evaluate if *N. attenuata* plants growing in nature harbor species specific phyla. We specifically compared data sets from previous studies that were based on primers that amplify the V5-V9 hypervariable region of the 16S rDNA gene, as we had amplified in the present study, and selected data sets that minimized primer bias due to the targeting of different regions.

Interestingly, the comparison of the core bacterial communities reported from the roots of *Arabidopsis*, barley, sugarcane and *N. attenuata* revealed that taxa from the bacterial phyla Deinococcus-Thermus and TM7 appear to be specific to *N. attenuata* (Fig. 4a). The most abundant OTU from the phylum Deinococcus was OTU12140 which was highly abundant in all roots and shared 100% similarity with the *Deinococcus citri* NCCP-154^{T} type strain (Fig. 4b). Phylum TM7 is a candidate division and is not culturable, while Deinococcus-Thermus is the second most abundant phylum in *N. attenuata* roots and is culturable. Interestingly, *Deinococcus citri*, as well as other Deinococcus-Thermus species - are well known for their high resistance to UV and gamma radiation and abilities to survive in harsh desert environments (Cox *et al.*, 2010). These conditions characterize *N. attenuata*'s native habitat, the Great Basin Desert in Utah, USA, with a maximum UVB irradiance of≈ 10 μmol m^{-2} s^{-1} (Fig S6d). It is well known that a plant's chemical composition, as well as its root exudates are influenced by UVB exposure (Caldwell *et al.*, 2007; Hectors *et al.*, 2014; Kaling *et al.*, 2015) and that UVB exposure results in the dramatic increase in phenolic sunscreens (Mazza *et al.*, 1999, 2000; Izaguirre *et al.*, 2007) and that perception of UVB fluence is mediated by the UVR8 receptor (reviewed in Jenkins, 2014). Therefore, we hypothesized that specific enrichment of Deinococcus-Thermus taxa in *N. attenuata* roots may reflect the plant's responses to UVB exposure, and that colonization may be mediated by UVB perception by *NaUVR8* and its downstream response, the expression of NaCHAL, which regulates the production of phenolic compounds which are known to protect plants from UV-damage (Li *et al.*, 1993; Agati & Tattini, 2010). To test this hypothesis, (1) we

isolated a native *Deinococcus citri* strain by growing *N. attenuata* on the same native soils from which the root samples used in the pyrosequencing analysis were derived. Colonies which showed the characteristic purple color were picked; sequencing analysis revealed that of 33 candidates, 6 isolates belonged to *Deinococcus* and two to *Deinococcus citri* (Fig. 4c, Table S2). Isolate *Deinococcus citri* D61 was selected for further experiments. (2) We generated stably transformed lines of the 31st inbred generation of *N. attenuata* that were silenced in the expression of *NaUVR8* and (3) crossed these with the previously characterized lines, ir*CHAL* that are silenced in an important response to UVB fluence, namely the production of the phenolic sunscreen compounds (Kessler *et al.*, 2008).

Generation and characterization of *NaUVR8*-silenced plants

As UVB is a crucial environmental factor for *N. attenuata* under natural conditions, we analyzed the transcript levels of *NaUVR8* in 17 different tissues of native *N. attenuata* plants collected close to Pahcoon, and thus exposed to the same environmental conditions as the samples taken at one of the locations further investigated in the present study (Fig 1). Interestingly, *NaUVR8* is not only expressed in above-ground tissues such as flower buds, leaves, and stalk, which are known to perceive and respond to light, but also in the transition part (root-to-shoot junction) and in below-ground tissues (primary and lateral roots, Fig 5a). These results suggest that *NaUVR8* has yet unknown functions in below-ground tissues and might interact with bacterial microbial communities in the soils. For an in-depth functional analysis we generated *NaUVR8*-silenced transgenic plants transformed with an inverted-repeat (ir) RNAi construct of *NaUVR8* (ir*UVR8* plants, Fig S6a). The characterization of one of the best *NaUVR8*-silenced lines (ir*UVR8-260-10-1)* in the field significantly lower net photosynthetic rates and also lower stomatal conductance than EV control plants (Fig. 5b). Photosynthetic rates correlated with growth parameters such as rosette diameter, and stalk diameter being also slightly reduced in ir*UVR8* plants compared to EV (Fig. 5c,d). Our data is consistent with previous studies of *UVR8*-knockout plants which showed growth defects (Favory *et al.*, 2009; Wargent *et al.*, 2009) and lower photosynthetic capacity (Davey *et al.*, 2012; Kataria *et al.*, 2014) compared with wild-type plants grown under high UVB fluences.

UVB irradiance increases *Deinococcus citri*-D61 root colonization of WT host plants, but decreases colonization of plants impaired in the perception of and response to UVB

To evaluate thoroughly the influence of UVB perception and downstream response on *Deinococcus* root colonization, we established a microcosm experiment including WT, ir*UVR8*, ir*CHAL* impaired in flavonoid biosynthesis (Kessler *et al.*, 2008) and a cross of both

ir*UVR8*xir*CHAL* plants to investigate not only the importance of UVB perception, but also to elucidate if an active UVB response or protection system is relevant for *Deinococcus* colonization. In order to mimic natural conditions, plants were grown in microcosms under glasshouse (no-UVB) light conditions which were experimentally supplemented with UVB. Seeds were inoculated with a synthetic bacterial community consisting of four bacterial isolates representing the four different phyla which are highly abundant in natural communities - *Deinococcus citri*- D61 (Deinococcus-Thermus), *Arthrobacter nitroguajacolicus*- E46 (Actinobacteria), *Pseudomonas frederiksbergensis*- A176 (Proteobacteria), *Bacillus mojavensis*- K1 (Firmicutes). The latter three species were selected based on their plant growth promoting effects (Santhanam *et al.*, 2014, 2015b). Species-specific primers were designed (Fig. S7) to evaluate the abundance of bacterial isolates by quantitative PCR (qPCR).

Isolate A176 (Proteobacteria) dominated the root community of *N. attenuata* followed by D61 (Deinococcus-Thermus), E46 (Actinobacteria) and K1 (Firmicutes) (Figs. 6 & S8). In WT plants, UVB supplementation significantly increased the colonization of *D. citri* compared to non-UVB supplemented plants (Fig 6, ANOVA, $F_{7,32}=6.3$, $p<0.0001$), while root colonization of the other bacterial isolates was not significantly altered, though A176 and K1colonization tended also to increase but not that of E46 (Fig. S8, ANOVA, A176-$F_{7,32}=1.9$, $p=0.1$, E46- $F_{7,32}=1.2$, $p=0.3$, K1- $F_{7,32}=1.8$, $p=0.1$).

In contrast to the response in WT plants, UVB supplementation decreased root colonization of *D. citri* D61 in the all three transgenic lines, ir*UVR8*, ir*CHAL* and ir*CHAL*xir*UVR8* (Fig. 6). From these results we infer that a plant's physiological responses to UVB exposure, which normally enhance root colonization, are altered when the perception and downstream signaling induced by UVB light are silenced, so that *D. citri* 61 colonization is impaired. *D. citri* D61 root colonization differ significantly amongst the genotypes ($F_{3,32}=6.7$, $p=0.001$), treatment (UVB vs non-UVB, $F_{1,32}=4.5$, $p=0.04$) and the interaction of both factors (genotypes*treatment, $F_{3,32}=7.2$, $p=0.0007$). Thus, *NaUVR8* and *NaCHAL* probably play additional roles in root bacterial colonization, independent of their mediation of a plant's UVB responses.

In summary, these results indicate that *N. attenuata*'s responses to UVB radiation, that include both perception of and responses to UVB fluence, are important factors affecting the recruitment of root bacterial communities

6.5 Discussion

Plants maintain extensive close relationships with soil microbial communities, but how the host plant and its responses to environmental factors determine root colonizing by fungal and bacterial communities remains largely unknown. In this study, we describe the root microbiota of native *N. attenuata* plants grown at different locations in their native habitat, and analyzed the importance of UVB and the plants' capacity to perceive and respond to UVB for root colonization with selected native bacterial strains. The majority of work on microbiomes of plants were performed in the glasshouse with native soil (Bulgarelli *et al.*, 2012a; Lundberg *et al.*, 2012; van der Heijden & Schlaeppi, 2015), and a few studies were done in the field with crop plants (Peiffer *et al.*, 2013; Edwards *et al.*, 2015). Both approaches are different from native populations exposed to the full spectrum of environmental factors in the field and not bred for needs of humans, thus loosing many important plant traits. In particular the production of secondary metabolites might be altered due to breeding (Degenhardt *et al.*, 2009; Meyer *et al.*, 2015). Glasshouse conditions are known to alter the chemical composition of the host, and pot-bound plants grow differently from the field (Kaur *et al.*, 2012). Our results are consistent with the idea that each plant species harbors a characteristic core root bacterial community, while locations and soil types have only a minor effect. In contrast the recruitment of root fungal communities is less specific. Using synthetic microbial communities based on isolates recovered from the same native soils used for the community characterization, we demonstrate that the relative abundance of the UV-resistant species *Deinococcus citri* D61 increased when the host plant was exposed to UVB. The relative colonization rate decreased in plants impaired in UVB perception and in the production of phenolic sunscreens, suggesting that physiological and metabolic changes induced by environmental factors (here, UVB radiation) affect the root bacterial community composition at the species level.

N. attenuata root bacterial communities from five different locations are all dominated by Actinobacteria, Deinococcus-Thermus and TM7, resulting in 78% relative abundance of these three phyla in the roots. Previous studies also found Actinobacteria to be highly abundant in roots of *A. thaliana*, barley, rice, sugarcane (Bulgarelli *et al.*, 2012a; Lundberg *et al.*, 2012; Bodenhausen *et al.*, 2013; Peiffer *et al.*, 2013; Edwards *et al.*, 2015; Yeoh *et al.*, 2016), but the other dominant phyla in the roots of these species were Proteobacteria, Firmicutes and Bacteroidetes (Schlaeppi *et al.*, 2013; Edwards *et al.*, 2015). In *N. atten*uata roots, these three phyla represented less than 15 % of the total relative

abundance, indicating that plants preferentially recruit specific phyla, but different species recruit different communities (Schlaeppi *et al.*, 2013). The dominating phyla were significantly enriched in roots compared to soils, irrespective of the locations where plants were grown (Figs. 1 & S2). Alpha and beta diversity indicies of soil and root communities were significantly different for both bacterial and fungal, communities (Fig. 2a,b & Fig. S4a,b) as well as in NMDS plots, were soil and root samples clustered separately (Fig. 2c), even though the chemical properties of soils were different (Fig S1). Despite of genotypic variation among native populations (Kallenbach *et al.*, 2012), the core bacterial communities of *N. attenuata* constituted 45-78% of relative abundance irrespective of locations (Fig. 3) and consist of phyla Actinobacteria, Deinococcus-Thermus and TM7, Proteobacteria, Bacteroidetes, Chloroflexi. In an independent study in the same area, these phyla except of Chloroflexi, dominated the *N. attenuata* root bacterial communities of field grown 31st inbred WT and an isogenic line impaired in the production of phytohormone JA (ir*AOC*) (Santhanam *et al.*, 2014, 2015a). Taken these results together, we conclude that *N. attenuata* recruits a core bacterial community irrespective of locations and genotypes. Similar findings were reported for *Arabidopsis* grown on two different soil types in the glass house and for a field study with maize across the Eastern part of the US, which also showed that overall bacterial communities amongst genotypes did not differ and genotypes had a limited effect on shaping the root bacterial communities (Lundberg *et al.*, 2012; Peiffer *et al.*, 2013; Schlaeppi *et al.*, 2013).

In contrast to bacterial root colonization, fungal colonization of roots was less specific. Soil and root communities did not clustered by locations (Fig. S4a,b) and differences in alpha diversity occurred amongst individual locations, but there was no clear distinction among locations and root samples (Fig. S4 c), indicating that plants recruit fungi less specifically, but also independently of the soil types. In contrast to these results, soil types had a strong effect on fungal communities of *Populus deltoides* in the glasshouse (Bonito *et al.*, 2014) and field (Shakya *et al.*, 2013). In *N. attenuata*, the phylum Ascomycota dominated the fungal communities with more than 85% of total relative abundance in all roots (Fig. S3). Similar results were obtained with *P. deltoides* and also in a previous study with *N. attenuata* plants grown on a field plot in the same area in Utah, USA (Groten *et al.*, 2015) in which Ascomycota represented 90% of the relative abundance of the root fungal community. The high affinity of the phylum Ascomycota to different plant species on different soils may reflect their beneficial effects on plants. Among the 20 core OTUs in *N. attenuata* roots

which all belonged to the phylum Ascomycota, a Blast search revealed highest similarity of two OTUs to *Epicoccum* and *Preussia*; both of these genera are described in literature as producing antifungal compounds (Fávaro *et al.*, 2012; Mapperson *et al.*, 2014). *Trichoderma,* well-known for its plant growth promoting and biocontrol effects was also present in some roots (Larkin & Fravel, 2001). Based on these results we suggest that *N. attenuata* has a limited capacity to discriminate fungal root colonization, but fungal endophytes may help to protect the plants from pathogen infection.

Until now, the biotic and abiotic factors playing a role in the recruitment of Rhizobia with have been extensively studied (Hassan & Mathesius, 2012). Furthermore, the role of ethylene in *N. attenuata* bacterial recruitment has been investigated (Long *et al.*, 2010), but mostly it remains elusive, which mechanisms enable plants to specifically recruit other bacterial species under natural conditions. Here we tested the hypothesis that plant's physiological response to the abiotic factor UVB-radiation leads to a specific enrichment of the phylum Deinococcus-Thermus, and specifically to an enrichment of the highly abundant OTU 12140 matching with type strain *Deinococcus citri* NCCP-154^{T}. A high abundance of the phylum Deinococcus-Thermus and TM7 was also found for the phyllosphere of *Pinus ponderosa* (Redford *et al.*, 2010), while in most root bacterial community studies its abundance is low (Bodenhausen *et al.*, 2013; Schlaeppi *et al.*, 2013; Bulgarelli *et al.*, 2015; Yeoh *et al.*, 2016). The phylum is known to include UV-and gamma radiation resistant species (Makarova *et al.*, 2001), and *P. ponderosa* as well as *N. attenuata* are exposed to high light intensities in their native habitat. All these studies, addressed the influence of UV on microbial communities and changes in communities might be due to survival of the UV resistant bacteria. However, the plant's response to UV, and its effects on bacterial communities, remains largely unknown. Moreover, based on NGS results a putative functional role can only be deduced from correlation and could not be tested. Therefore, we additionally used a culture-dependent approach and isolated *Deinococcus citri* which shows 100% sequence similarity with the highly abundant OTU 12140, and used this strain to elucidate the role of UVB, its perception and down-stream response in *Deinococcus* colonization in plant roots.

In plants, upon UVB exposure, UVB photoreceptor UVR8 induces the flavonoid biosynthesis enzyme (CHAL) to protect plants from DNA and protein damage (Kliebenstein *et al.*, 2002; Rizzini *et al.*, 2011). The best tool to dissect the role of a gene is the use of plants specifically impaired in its expression, and to thoroughly characterize these plants in

comparison to plants with a fully functional gene. Therefore, we generated RNAi silenced *N. attenuata* lines impaired in UVB perception (ir*UVR8*) and characterized them in their native habitat. The plants showed growth defects such as smaller rosette and stalk diameter, and lower photosynthetic capacity compared with EV-control plants. The same general effects, but more severe, were observed in previous studies with *UVR8*-knockout plants: impaired growth (Favory *et al.*, 2009; Wargent *et al.*, 2009), lower photosynthetic capacity (Davey *et al.*, 2012; Kataria *et al.*, 2014) and lower phenolic compound levels (Favory *et al.*, 2009; Demkura & Ballaré, 2012) compared with wild-type plants grown under high UVB fluences. .The line characterized in the field was used together with a transgenic plant impaired in flavonoid production (ir*CHAL*) and a cross of both (ir*UVR8*xir*CHAL*) inoculated with a synthetic bacterial community consisting of bacteria from 4 different phyla performed in a growth chamber

To complement the NGS study with a functional analysis to reveal the ecological importance of highly abundant taxa, we conducted microcosm experiment with the two transgenic plants impaired in UVB perception (ir*UVR8*). Under UVB supplementation, *D. citri* D61 was significantly increased in colonization of WT plants (Fig. 6), but not for the three other bacterial species which were co-inoculated with *D. citri*. Based on this result we suggest that UVB-induced metabolic changes specifically enhance the *Deinococcus* root colonization. It has been shown that UVB induces the biosynthesis of flavonoids (Markstädter *et al.*, 2001; Quattrocchio *et al.*, 2006; Liu *et al.*, 2012; Eichholz *et al.*, 2012; Hectors *et al.*, 2014) and the secretion of flavonoid compounds by roots serves as chemoattractant for the initiation of nodulation followed by bacterial *Rhizobium* colonization in legumes (Hassan & Mathesius, 2012). Further experiments are required to understand the mechanism in detail, but it is tempting to speculate that the UVB induced flavonoid production leads to increased *Deinococcus* colonization. This hypothesis is supported by the three transgenic plants ir*UVR8*, ir*CHAL* and ir*UVR8*xir*CHAL* which showed lower colonization rates after additional UVB supplementation compared to non-UVB supplemented plants (Fig. 6b). Furthermore, ir*UVR8* plants have lower net photosynthetic rates and may provide less sugar/nutrients for the bacteria (Fig. 5b). However, the absolute colonization rates are highest in plants impaired in UVB perception and response under non-UVB conditions (Fig. 6b). Therefore, an additional signal seems to regulate *Deinococcus* colonization. The plant's normal response to UVB might be important for restricting root colonization by *Deinococcus.* This further suggests that plants have mechanisms to regulate

and restrict bacterial colonization. The detailed mechanism behind the *Deinococcus* colonization of *N. attenuata* under UVB-irradiation will be investigated in future studies.

In summary, the present study demonstrates that only a minor part of the root bacterial communities was sculpted by the environment, while more than 80% are specifically enriched in *N. attenuata* roots independent of soils and locations. In contrast, root fungal communities were less specifically acquired. A functional analysis mimicking natural conditions in a microcosm experiment revealed that under UVB supplementation *Deinococcus* colonization increased in WT but not in the plants impaired in UVB perception (ir*UVR8*) and response (ir*CHAL*). These data suggest that UVB-irradiation is an important factor not only plants' adapted to, but also their bacterial partners. However, despite current progress, more work is still required to fully understand the mechanism and metabolic processes responsible for increased *Deinococcus* colonization in nature with high PAR and high UVB irradiance.

6.6 Main figures

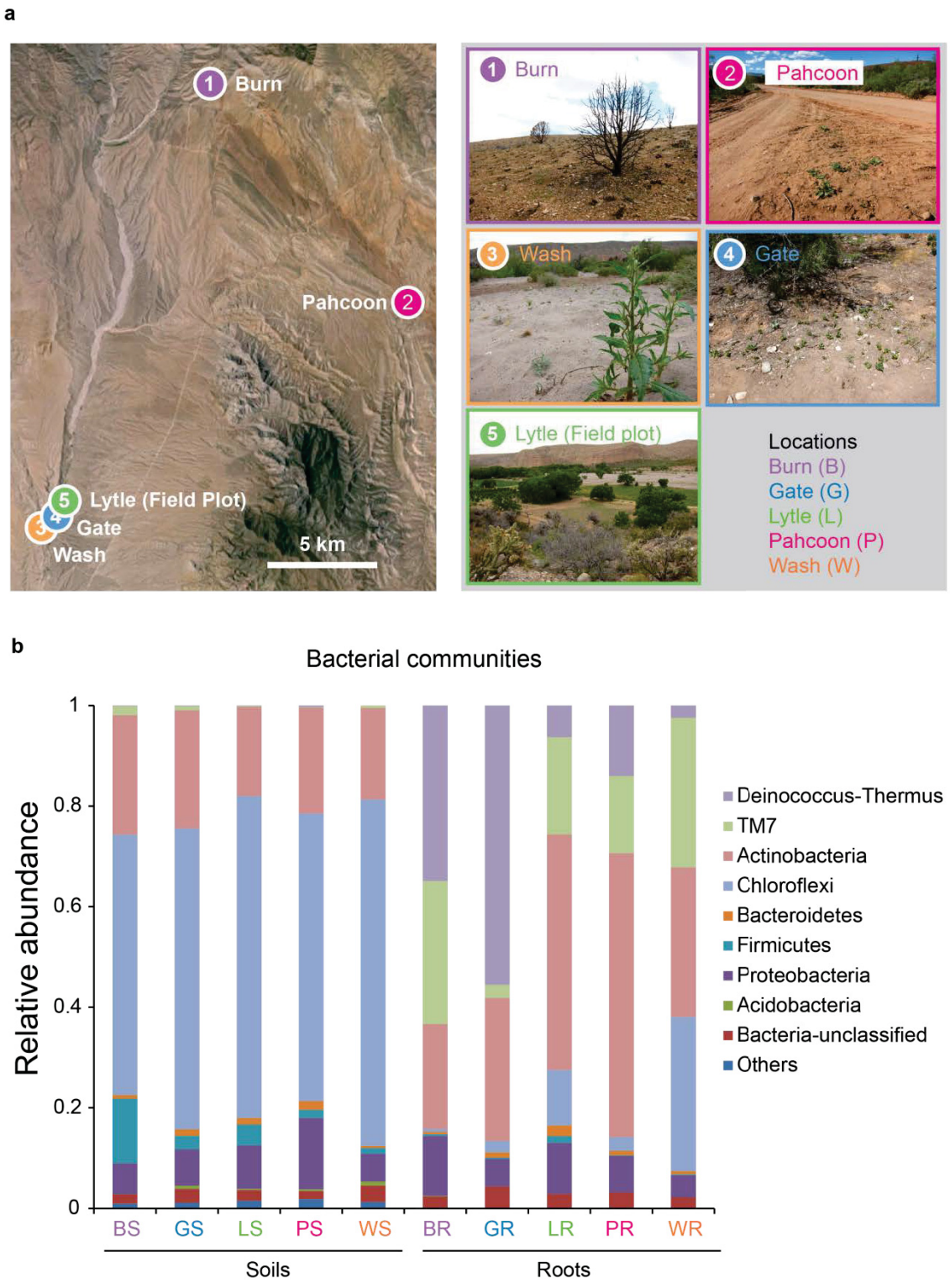


Figure 1: Native grown *N. attenuata* plant roots recruit distinct bacterial communities compared to soils

(A) Location and pictures of the five different *N. attenuata* native populations used in this study in the Great Basin Desert Utah, USA. *N. attenuata* root (R) and soil (S) samples were collected from these locations. (B) Relative abundance of root and soil bacterial communities at the phylum level. DNA was extracted from roots and soils; the V5-V8 variable region of 16S rDNA amplified, sequenced by 454 pyrosequencing and analyzed using the QIIME platform. Each bar shows the average relative bacterial abundance of three biological replicates. Only the most abundant bacterial phyla are shown; the remaining phyla represent <1% of relative abundance. They include Armatimonadetes, Chlamydiae, Chlorobi, Cyanobacteria, FBP, Fibrobacteres, Gemmatimonadetes, Nitrospirae, OP3, Planctomycetes, SBR1093, Tenericutes, Verrucomicrobia and Acidobacteria. Abbreviations- Burn (B), Gate (G), Lytle-field plot (L), Pahcoon (P) and Wash (W).

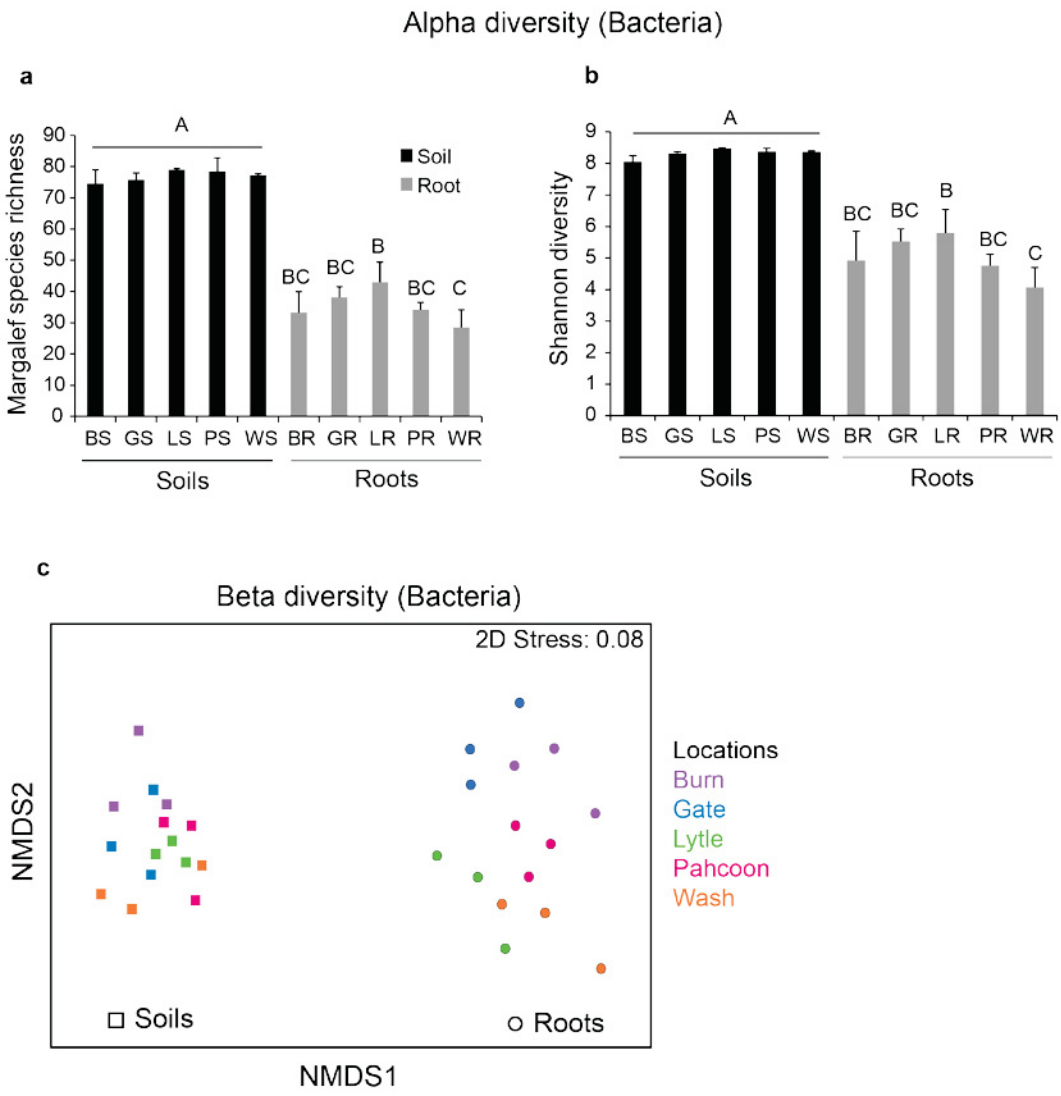


Figure 2: Native grown *N. attenuata* roots recruit their bacterial communities independently of soil type

The alpha diversity indices Margalef species richness (A) and Shannon (B) are significantly higher in soil compared to roots irrespective of locations (mean, ±SE, N = 3, different letters indicate significant differences, one-way ANOVA with Fisher's PLSD test; $p<0.05$). (C) Non-parametric multidimensional scaling (NMDS) ordination based on the Bray-Curtis dissimilarity matrix shows distinct clusters of root and soil bacterial communities. Each dot corresponds to a different sample, the color represents the location, and the shape indicates soil (□) and root (○). Native grown *N. attenuata* plants recruit similar bacterial communities across the different locations and soil types. Abbreviations: S-soil, R-root; B-Burn, G-Gate, L Lytle-field plot, P-Pahcoon, W-Wash.

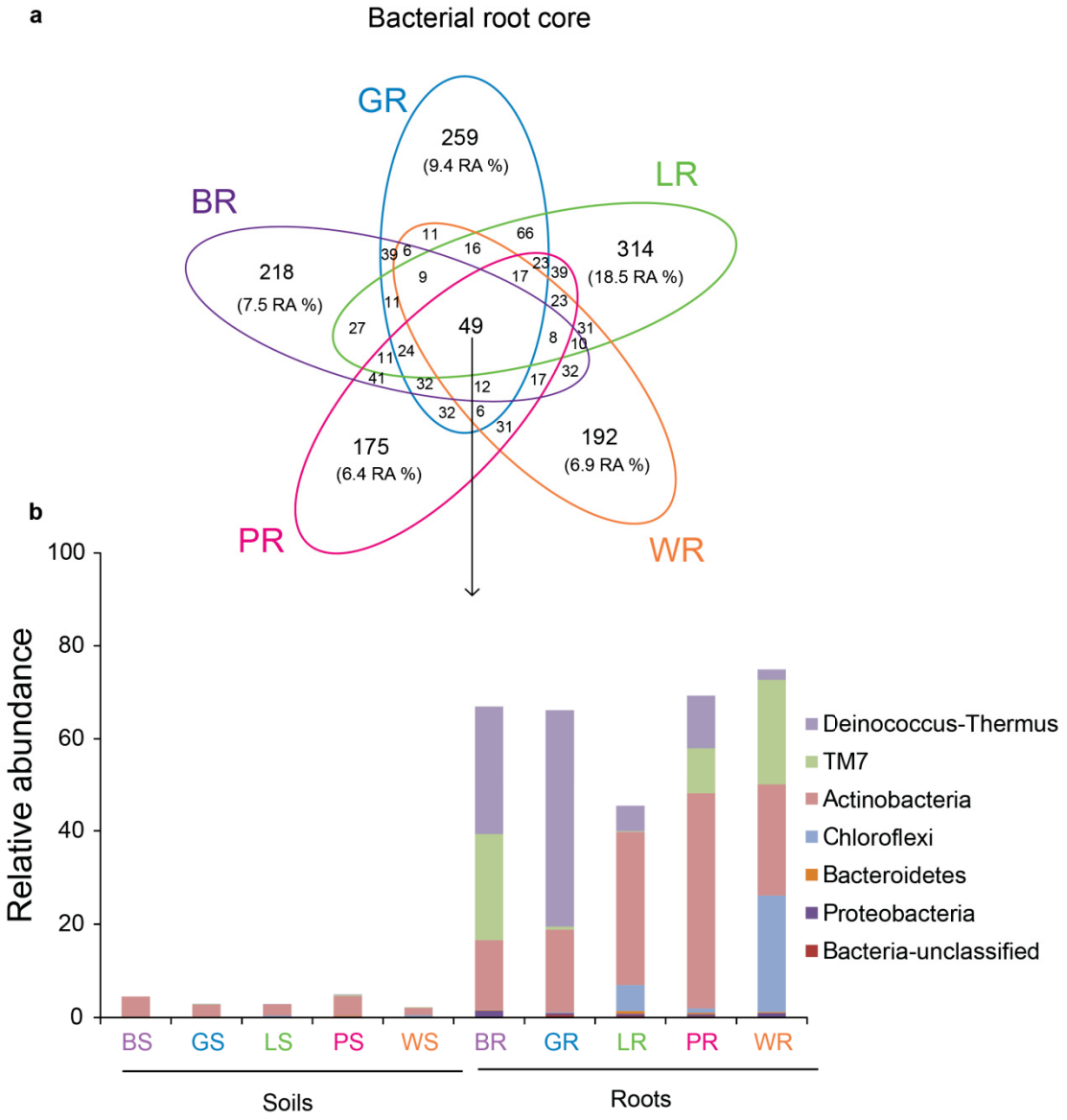


Figure 3: The core root microbiota consists of 49- OTUs and is largely independent of locations and soil type

(A) *N. attenuata*, harbors 49 core root OTUs of a total of 2847 OTUs. Location and soil type had very little effect on shaping the root bacterial communities. Unique OTUs (97% similarity) constitute only 6 to 9.5 % (except Lytle: LR-18%) of the total relative abundance of root bacterial communities. (B) The core root bacterial community constitutes nearly 45-70% of total relative abundance. The core OTUs belong mainly to the dominant root phyla Actinobacteria, Deinococcus-Thermus and TM7.

a Six most abundant root enriched bacterial phyla among *Nicotiana attenuata*, *Arabidopsis thaliana*, *Hordeum vulgare* and *Saccharum officinarum*.

Nicotiana attenuata	*Arabidopsis thaliana*	*Hordeum vulgare* (barley)	*Saccharum officinarum* (sugar cane)
Actinobacteria	Actinobacteria	Actinobacteria	Actinobacteria
Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes
Proteobacteria	Proteobacteria	Proteobacteria	Proteobacteria
Chloroflexi	Chloroflexi	Chloroflexi	Chloroflexi
TM7	Firmicutes	Firmicutes	Firmicutes
Deinococcus-Thermus	AD3	Nitrospirae	Spirochaetes

Bacterial phyla (rank): Core communities, Species specific, Intersection.

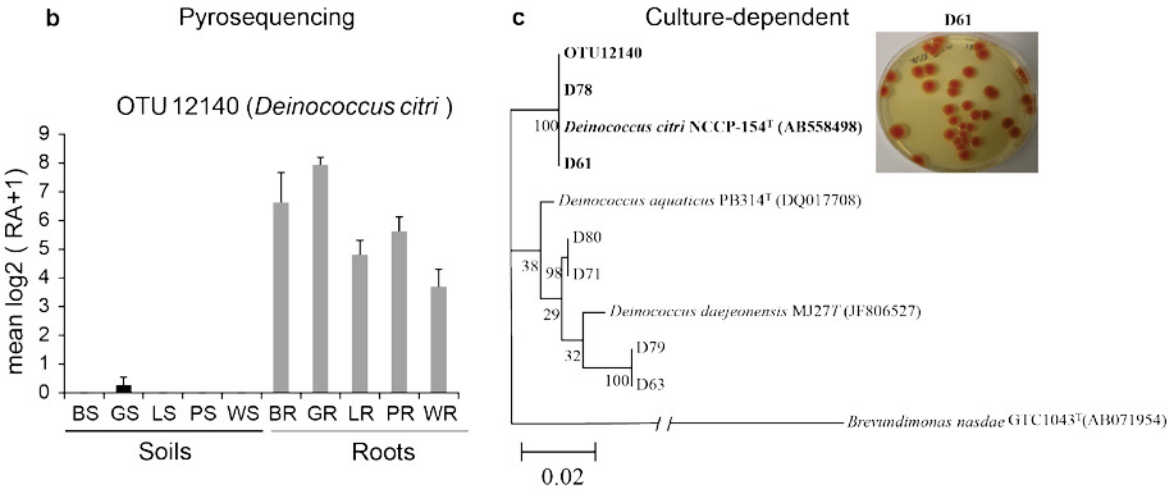


Figure 4: Phylum Deinococcus-Thermus - whose members are known to be UV-resistant - is highly abundant in *N. attenuata* roots compared to other plant species whose root microbiomes have been thoroughly analyzed; *Deinococcus citri* was isolated from seedlings grown in native soil.

(A) Comparison of the most abundant bacterial phyla found in plant roots of different species based on the V5-V8 hypervariable region of 16S rDNA gene. In *N. attenuata* roots, members of the bacterial phyla Deinococcus- Thermus and Tm7 are highly abundant, while Firmicutes members are depleted compared to other plant species. *N. attenuata*- this study, *Arabidopsis thaliana*- Bodenhausen *et al.,* 2013, Schlaeppi *et al.,* 2014. *Hordeum vulgare* (barley)- Bulgarelli *et al.,* 2015, *Saccharum officinarum* (sugar cane)- Yeoh *et al.,* 2015. (B) Within the phylum Deinococcus- Thermus, OTU 12140 is highly abundant and shares 100%

similarity with the type strain *Deinococcus citri* NCCP-154^{T} (Eztaxon database); N=3. For abbreviations see Fig.1. (C) Neighbor-joining tree based on V5-V8 hypervariable region of 16S rDNA gene depicts similarities among *Deinococcus* isolates from seedlings grown on soil samples retrieved from the five locations analyzed in this study. In total 6 *Deinococcus* strains were isolated and D61 and D78 isolates share 100% similarity with OTU 12140. Numbers at the nodes are percentage of bootstrap values based on 1,000 resampled datasets. Bar: 0.02 substitutions per nucleotide position.

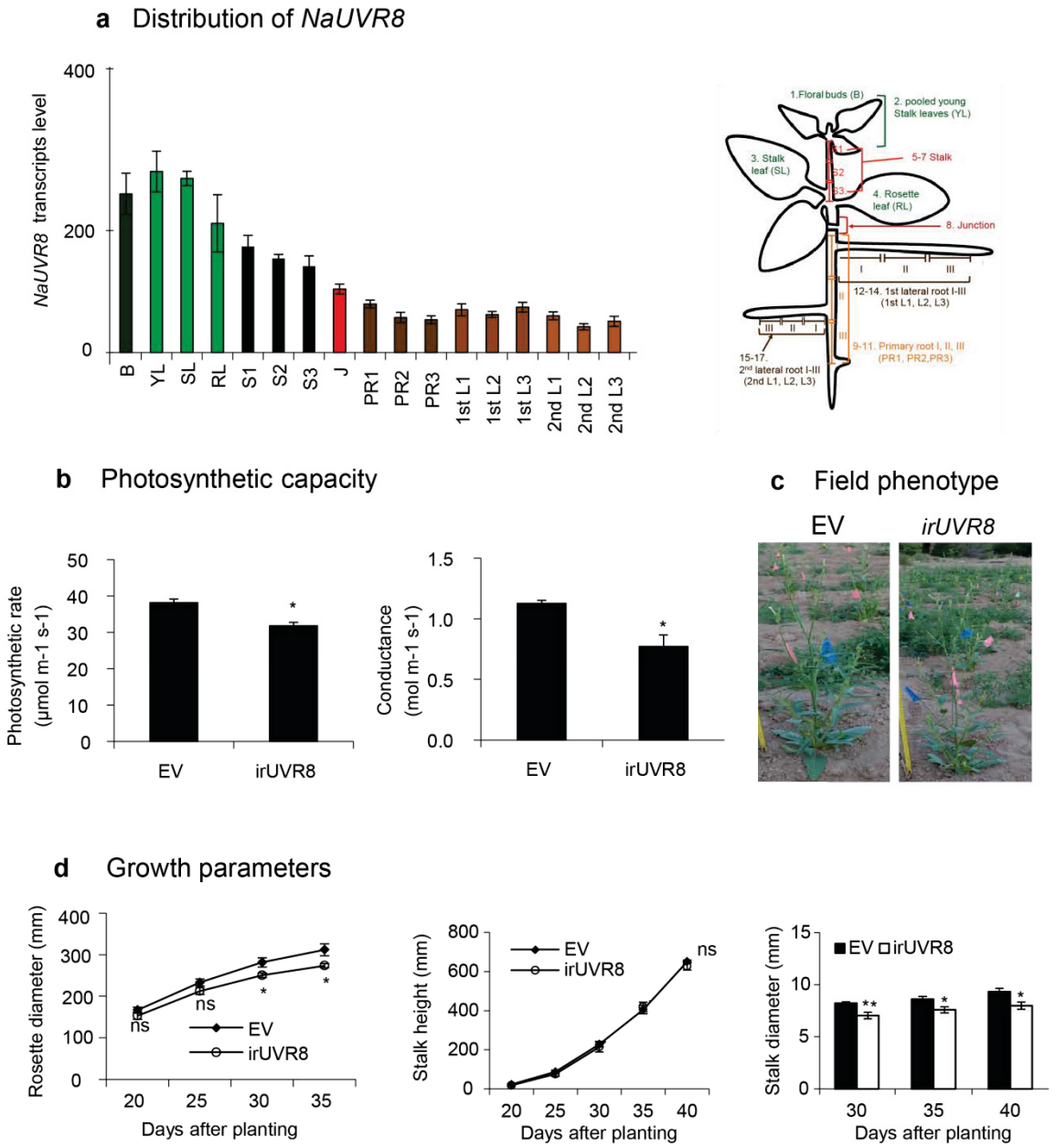


Figure 5: *NaUVR8* transcript distributions in different tissues of native *N. attenuata* plants, and phenotype of *irUVR8* plants grown under field conditions

(A) 20 different tissues of native *N. attenuata* plants were collected in their native habitat, the Great Basin Desert, Utah, USA, and transcript abundance of *NaUVR8* in these tissues was determined by quantitative real time PCR (qPCR). Bars indicate *EF1a*-normalized relative transcript abundances (N = 7±SE). (B) EV and *irUVR8* plants were grown in the field (the Great Basin Desert, Utah, USA), and growth and development monitored for 40 days (May to June 2014, N = 15, mean±SE). *irUVR8* plants showed a slight decrease in photosynthetic

capacity compared to EV plants. Mean ±SE (N = 4) net photosynthetic rates and conductances in EV and *irUVR8* plants with the following parameters: photosynthetically active radiation = 2000 $\mu molm^{-2}s^{-1}$, $CO_{2\ ref}$ = 400 $\mu mol_{CO2}\ m^{-2}\ s^{-1}$. (C, D). *irUVR8* plants displayed smaller rosette and stalk diameters, but stalk heights were not significantly different from those of EV plants. Asterisks (B and D) indicate significant differences between EV and ir*UVR8* plants determined by Student's t-test at each measurement time (*P≤0.05, **P≤ 0.01).

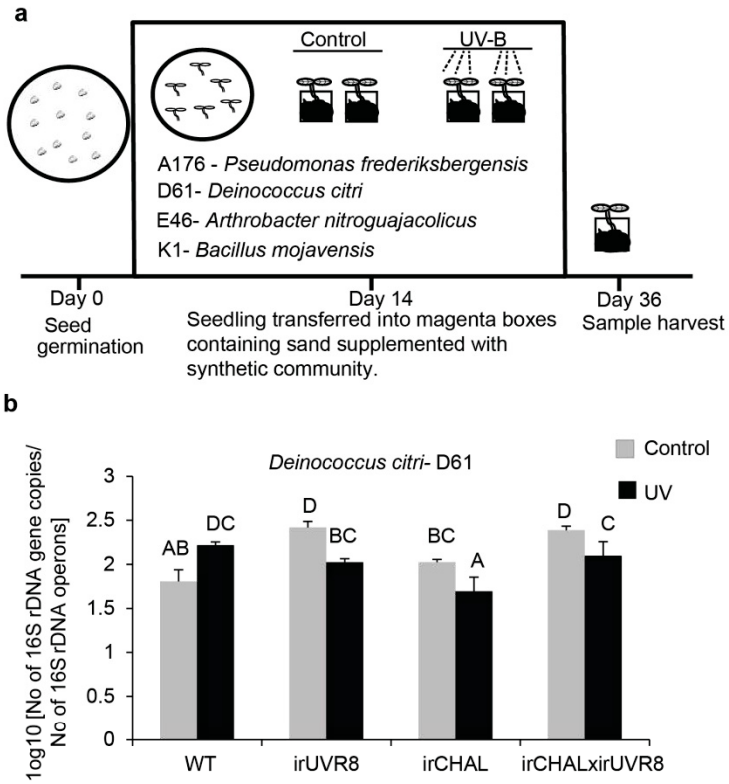


Figure 6: UVB supplementation significantly increases the root colonization with *Deinococcus citri*- D61 in WT plants but decreases in plants impaired in UVB perception and response

(A) Schematic representation of the experimental set-up in the growth chamber and list of bacterial species used for seedling inoculations. (B) Determination of relative bacterial abundance in the roots by qPCR with species-specific primers of the four bacterial species used in the experiment. With UVB supplementation, *Deinococcus citri*- D61 colonization significantly increased in wild type (WT), whereas, colonization of *D. citri*- D61 decreased in plants silenced in the expression of the UV-receptor UVR8 (ir*UVR8*) and in the expression of a core biosynthesis enzyme of UVB inducible flavonoids (ir*CHAL*) and a cross of both (ir*CHALxirUVR8)*. 16S rDNA gene copy numbers are normalized by number of operons from nearest neighbor *Deinococcus gobiensis* (rrnDB). Mean, ±SE, N = 5, different letters indicate significant differences, one-way ANOVA with Fisher's PLSD test; $p<0.05$.

6.7 Supplemental figures

A Chemical properties of the soils from the five different locations.

Sites	%C	%Cinorg	%Corg	%N	C/N	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	P (mg/kg)
Burn Soil	2.64	1.78	0.86	0.05	52.8	74010	18978	7988	645
PahcoonSoil	1.26	0.84	0.42	0.030	42	36917	9322	3111	273
Wash Soil	2.05	1.1	0.95	0.050	41	51202	20130	9158	656
Gate Soil	1.86	1.41	0.45	0.060	31	45930	22553	13696	758
Lytle Soil	2.45	1.28	1.17	0.090	27.22	49249	21255	14959	751

B Dendrogram based on Pearson correlation of chemical properties of the soils.

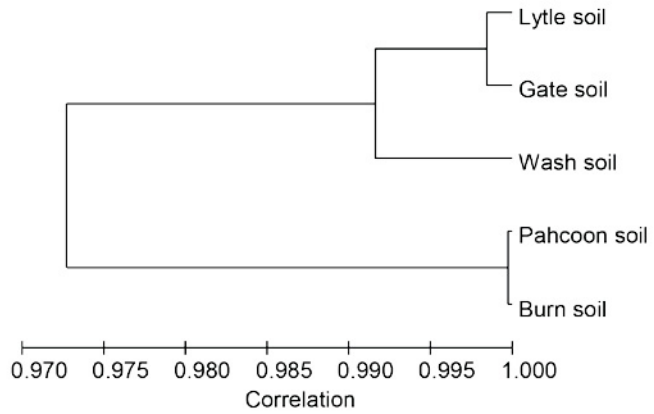


Figure S1: Chemical properties of the soils retrieved from five different locations at the Great Basin Desert, Utah, USA.

(A) Chemical properties of soils C: carbon, Cinorg: inorganic carbon, Corg: organic carbon, N: nitrogen, Ca: calcium, K: potassium, Mg: magnesium, P: phosphorus. (B) Dendrogram based on Pearson correlation shows that Phacoon and Burn soil properties differ from the three other locations.

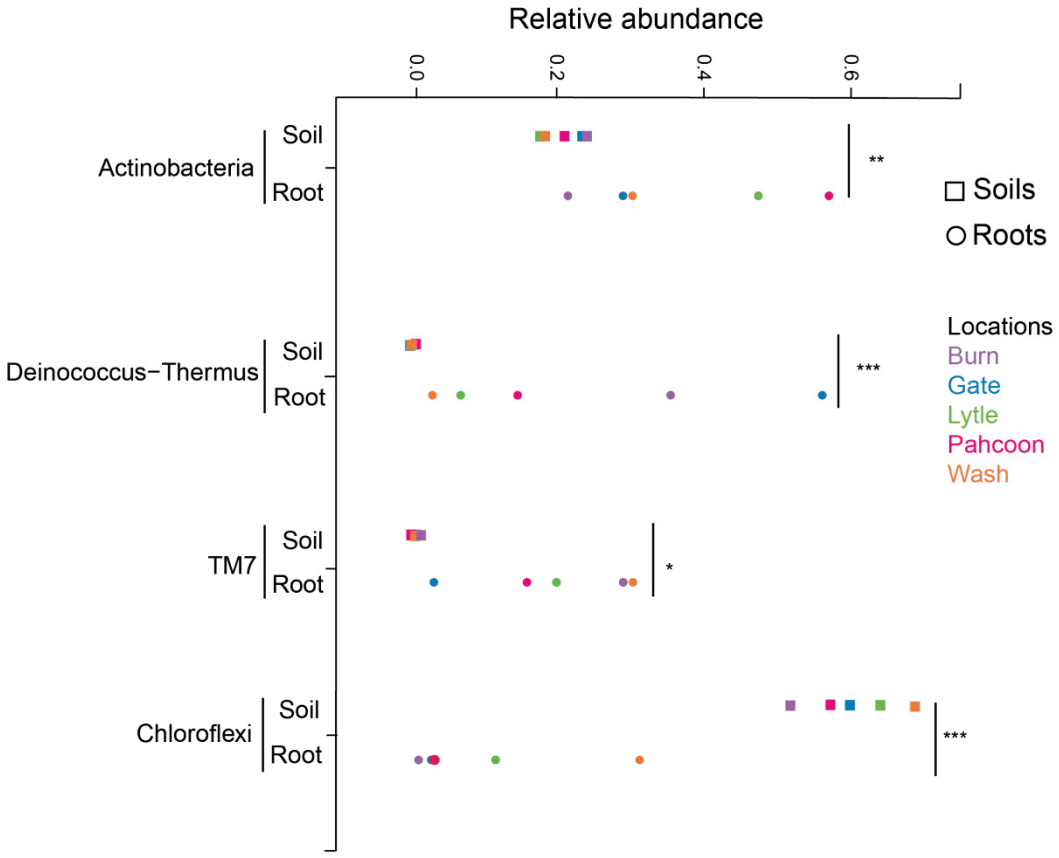
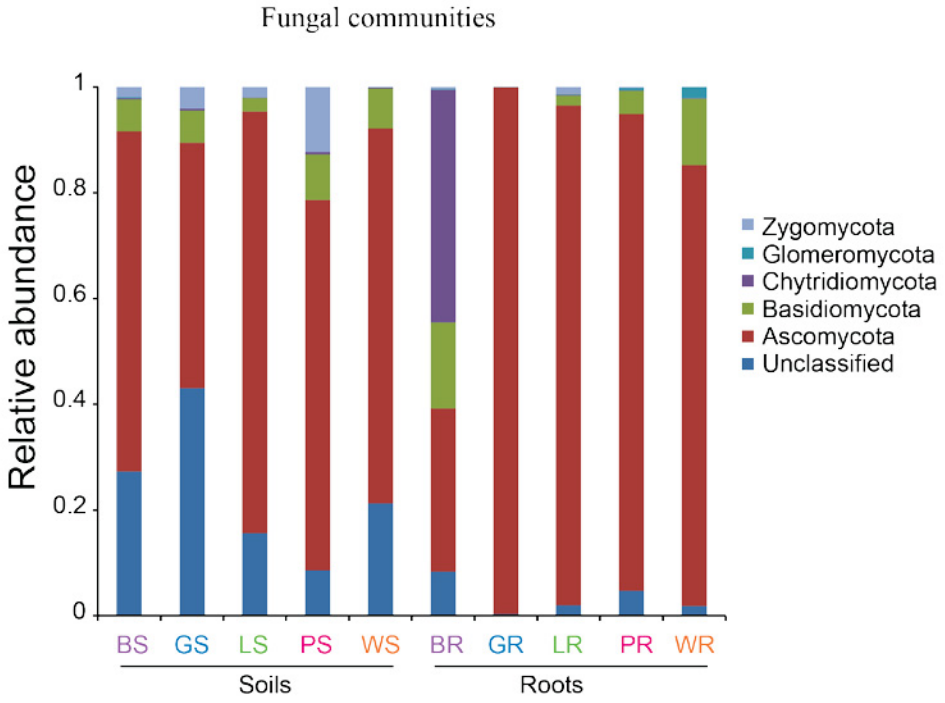


Figure S2: *N. attenuata* roots specifically recruit bacteria of the phyla Actinobacteria, Deinococcus-Thermus and TM7 from native soils

Relative abundance of four phyla enriched in roots or soils. Three bacterial phyla - Actinobacteria, Deinococcus-Thermus and TM7 - are significantly enriched in roots compared to soil (GLS, compartment: soil vs roots, Tukey HSD,* $p<0.05$, ** $p<0.01$, ***$p<0.001$), while the phylum Chloroflexi is highly abundant in soil. Each colored point represents the average of three replicates taken from soils (□) or roots (○) at the five different locations.



Figures S3: Fungal communities of native grown *N. attenuata* plant roots

The same samples as described in Fig. 1 were used for fungal community analysis and ITS1 - 4 regions were sequenced by 454 pyrosequencing. The relative abundance of fungal phyla of native grown *N. attenuata* roots (R) and corresponding soils (S) are depicted. The phylum Ascomycota dominated the root and soil fungal communities. The fungal phyla present in soils and roots are not clearly distinct, and most phyla are present in both compartments.

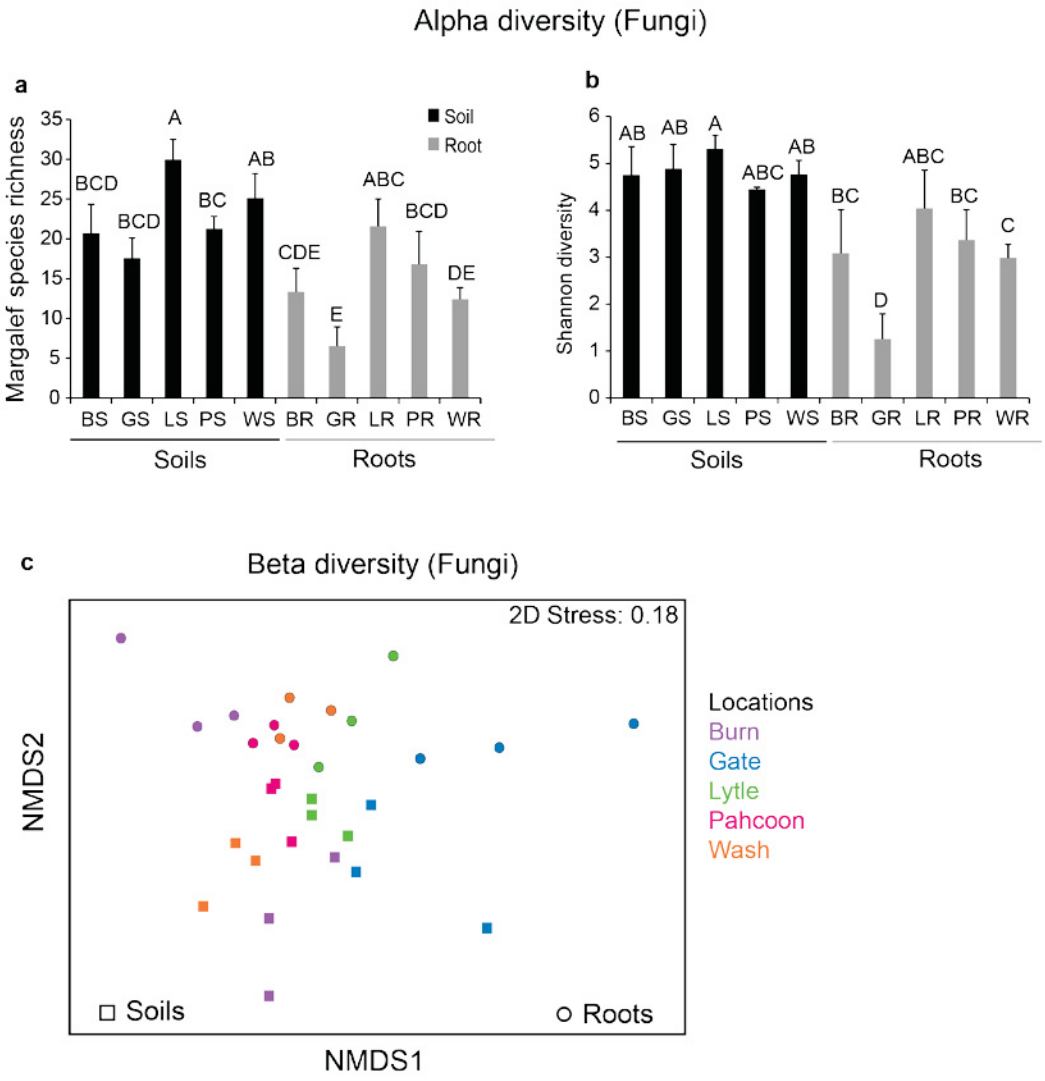
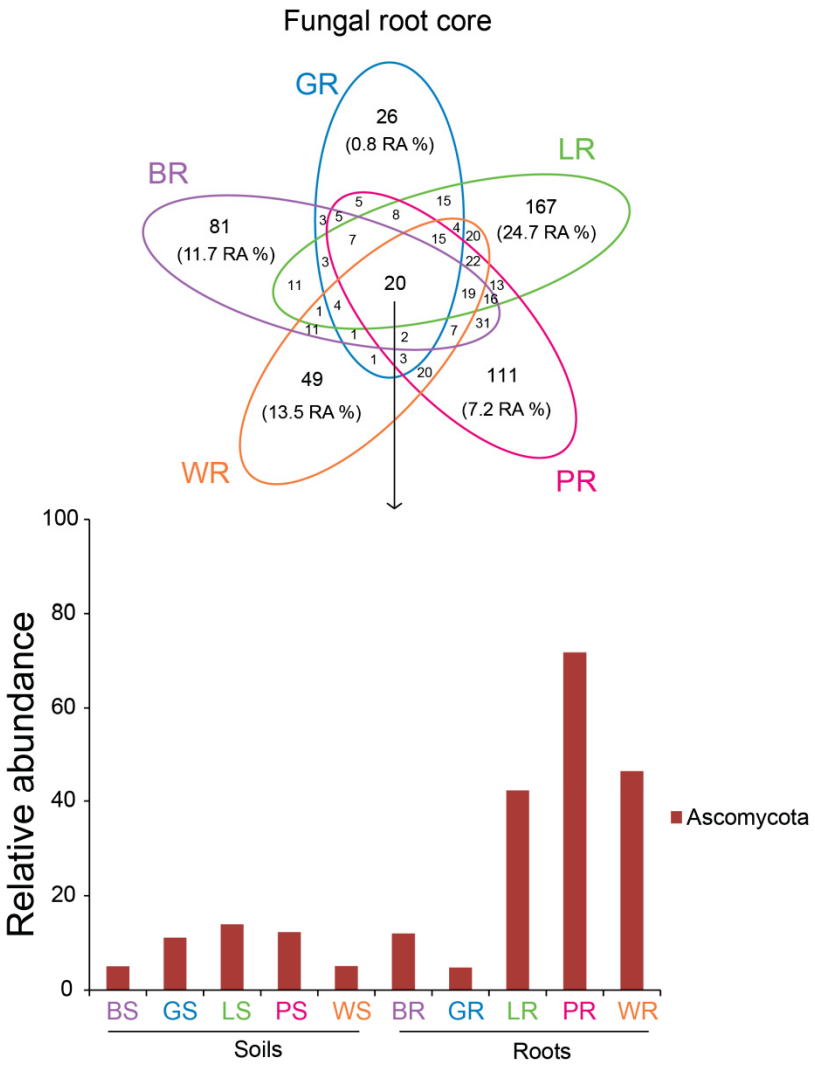


Figure S4: Alpha and beta diversity of root and soil fungal communities.

Alpha diversity indices, (A) Margalef species richness and (B) Shannon are not significantly different among soils and roots except for Gate location (GS & GR), (Mean, ±SE, N = 3, different letters indicate significant differences, one-way ANOVA with Fisher's PLSD test; $p<0.05$). For abbreviations see Figure 3. (C) In NMDS ordinations based on the Bray-Curtis dissimilarity matrix, root fungal communities partially clustered separately from soils. Each point corresponds to a different sample, the color represents the location, and the shape indicates soils (□) or roots (○), respectively.



Figures S5: The fungal core community of *N. attenuata* roots comprises 20 OTUs belonging to the phylum Ascomycota; their abundance does not show a distinctive pattern between soils and roots.

Core OTUs result from the intersection of the shared root fungal OTUs identified at the five natural populations of *N. attenuata*. Locations and soil types had a very minor effect on composition of the fungal root communities and unique OTUs for each location range from 1 to 25%. Relative abundance of these OTUs is depicted for each location and for roots and soils and ranges from about 10 to 70%.

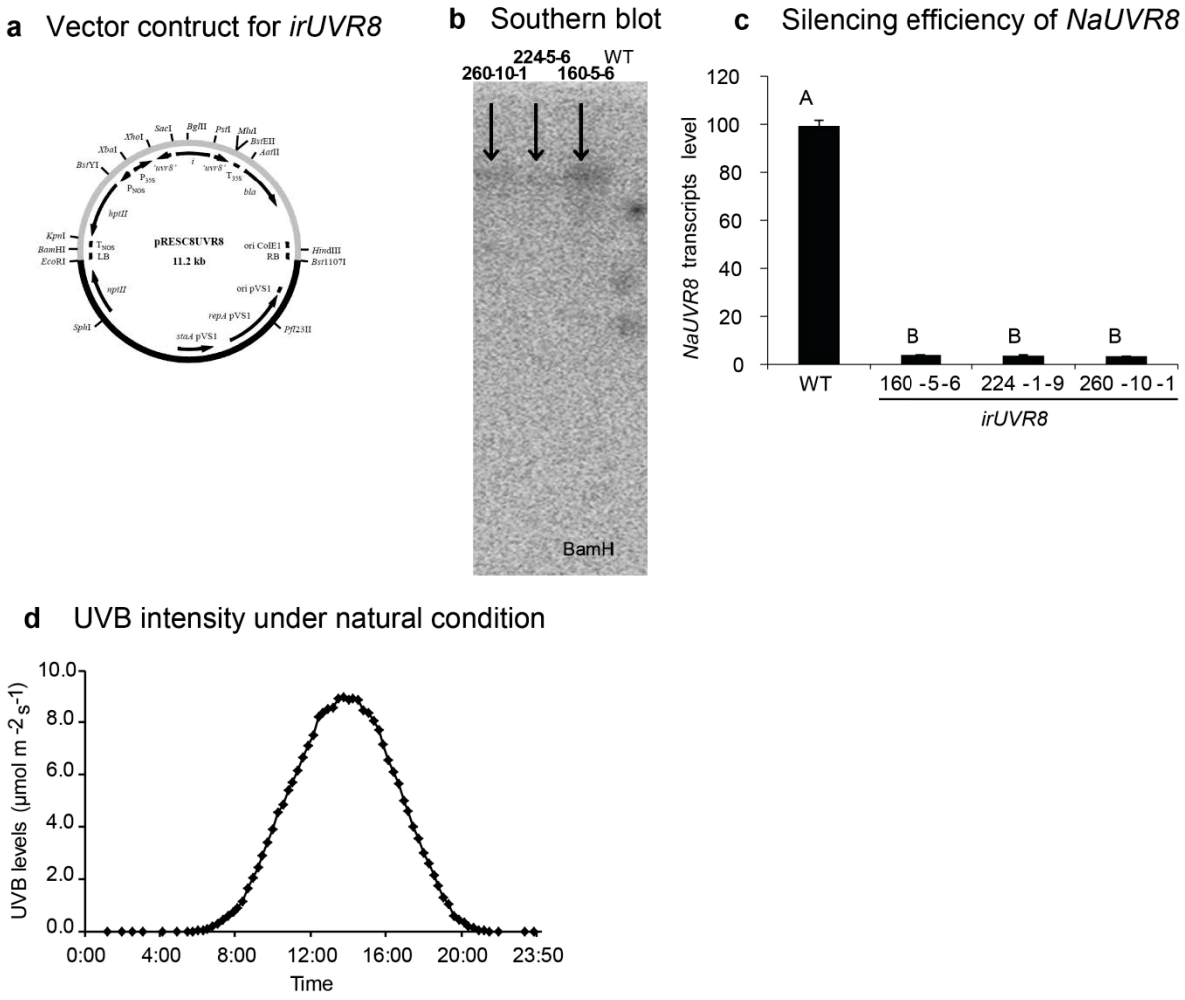
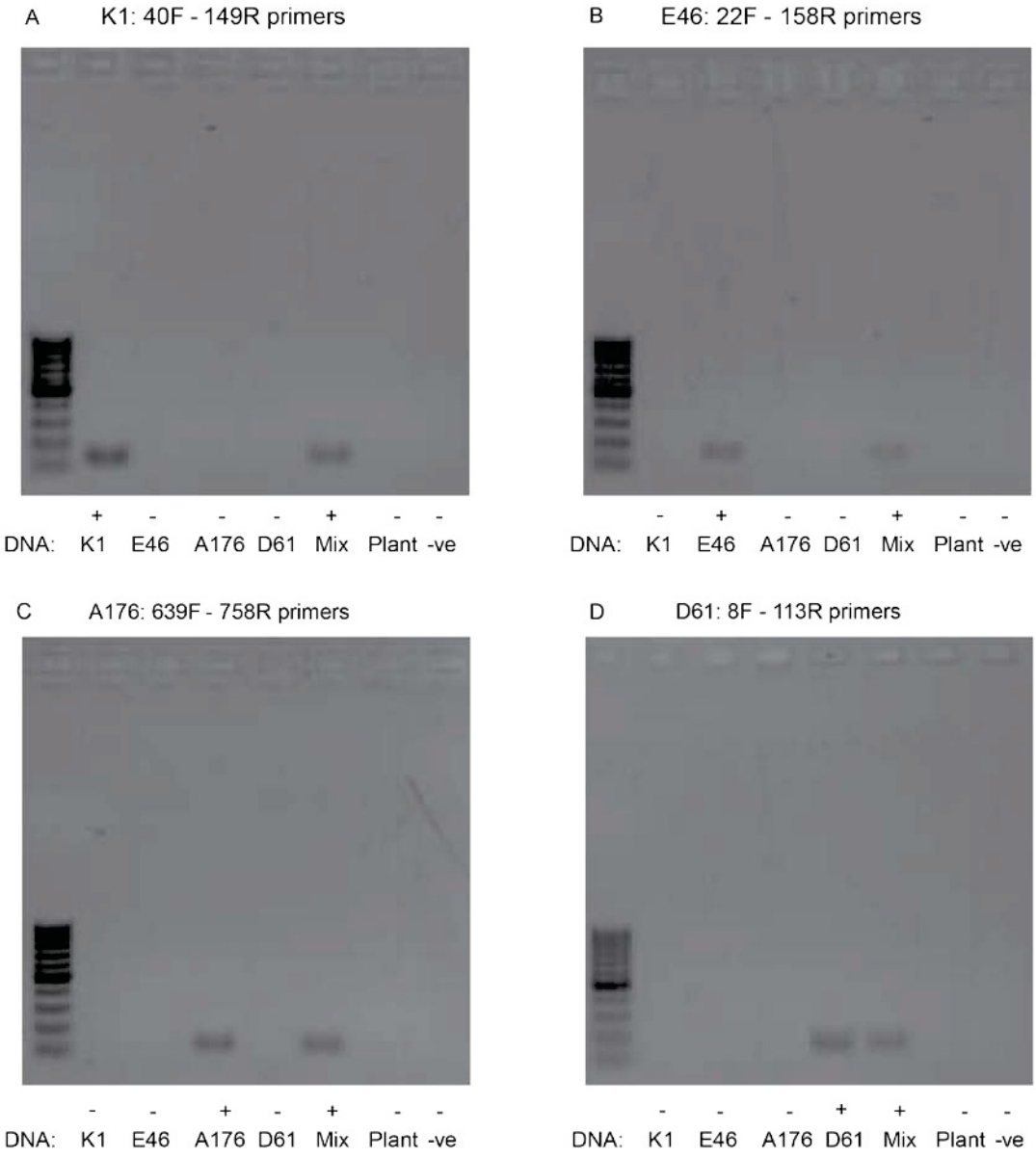


Figure S6. ir*UVR8* plants were transformed with an inverted repeat (ir) *NaUVR8* construct and UVB intensity under natural conditions.

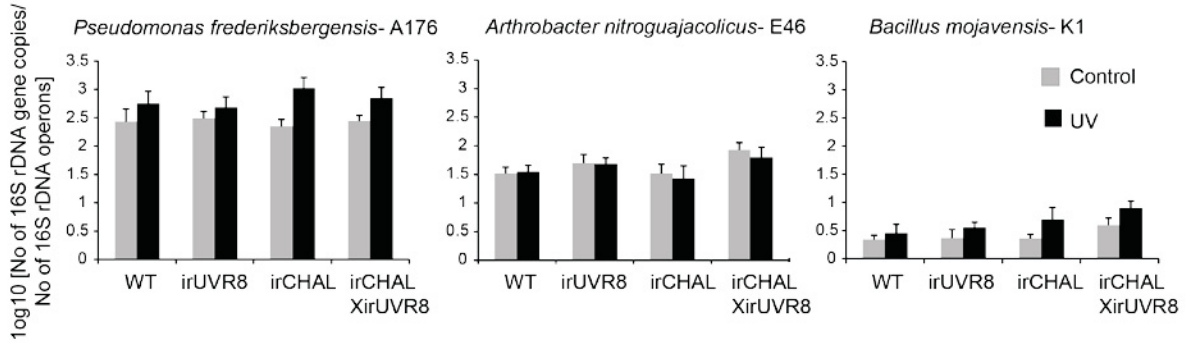
(A) The *pRESC8UVR8* vector used for *Agrobacterium tumefaciens*-mediated transformation of *N. attenuata* plants. (B) Southern blot to determine the number of T-DNA insertions in the genomes of three independent stably transgenic *irUVR8* lines. DNA gel blot of genomic DNA digested with BamHI enzyme and hybridized with a probe coding for the hygromycin resistance gene located between right and left T-DNA borders of the transformation vector *pSESC8UVR8*. The arrows indicate single copy insertion and *irUVR8-260-10-1* was selected for further experiments. (C) Transcript abundances of *NaUVR8* were determined by qPCR in the leaves of EV and three independent ir*UVR8* plants (260-10-1, 224-5-6, and 160-5-1). (D) UVB intensity under natural conditions. Bars indicate *EF1a*-normalized relative transcript abundances ± SE (N=4). Different letters indicate significant differences among the genotypes determined by ANOVA ($P \leq 0.05$).



Figures S7: Bacterial species specific primers.

Agarose gels stained with ethidium bromide showing a single PCR product band only in the sample containing the corresponding bacterial species and in the bacterial mix, but not in water (-ve) and plant controls. Species specific primers were designed based on 16S rDNA gene of the four bacterial strains used in this study. (A) *Bacillus mojavensis*- K1, 40F- 5'CAAGTCGAGCGGACAGATG 3' and 149R 5'ACAAGCATCCGGTATTAGCC 3'. (B) *Arthrobacter nitroguajacolicus*- E46, 22F- 5'CGGCGTGCTTAACACATG 3' and 158R- 5'CGTCAGACAGTCATATCCG 3', (C) *Pseudomonas frederiksbergensis*- A176, 639F 5'CGAGCTAGAGTATGGTAGAGG 3' and 758R 5'TCGCACCTCAGTGTCAGTATC 3',

(D) *Deinococcus citri*- D61, 8F-5'AGTCGGACGATTGGCTTC 3' and 113R 5' ACATCACGTATTAGCGTCTC 3'. DNA was extracted from the four bacterial species and plants. For the mix sample, equimolar concentrations of each bacterial species were mixed. PCR was performed with 35 cycles and the PCR product separated on a 1.5% agarose gel.



Figures S8: Root bacterial colonization by strains A176, E46 and K1 is not influenced by UVB in WT, and also not in the plants impaired in UVB perception and responses

Results were obtained from the same experimental set-up as shown in Fig. 6. Bacterial abundances of strain A176, E46 and K1 were determined by qPCR with species-specific primers (Fig. S7). Colonization of bacterial isolates A176, E46 and K1 are independent of the genetic background of plants. 16S rDNA gene copy numbers are normalized by number of operons from nearest neighbor *Pseudomonas chlororaphis, Arthrobacter aurescens, Bacillus subtilis* (rrnDB) for respective strains. Mean, ±SE, N = 5, different letters indicate significant differences, one-way ANOVA with Fisher's PLSD test; $p<0.05$.

Table S1: Of 49 core bacterial OTUs, 23 OTUs from *N. attenuata* significantly differed among soil and root samples (separate excel sheet).

Table S2: Bacterial isolates retrieved from roots of *N. attenuata.*(separate excel sheet)

6.8 *New Phytologist* Supporting Information

Article title: Native grown *Nicotiana attenuata* root microbiome is independent of soil types and plant responses to UVB increase *Deinococcus* root colonization

Authors: Rakesh Santhanam, Youngjoo Oh, Arne Weinhold, Van Thi Luu, Karin Groten and Ian T. Baldwin

Article acceptance date: Click here to enter a date.

The following Supporting Information is available for this article:

Methods S1 Analysis of pyrosequencing data:

The QIIME software package was used to analyze the reads using default parameters for each step (Caporaso *et al.*, 2010b). Sequences, were removed if the average quality scored <25, lengths were shorter than 200 bp, excess of 6 bases homopolymer runs, primer mismatch and ambiguous bases. Most abundant sequences were taken as representative sequence for each clusters and aligned to the Greengenes database (McDonald *et al.*, 2012), using PyNast algorithm with minimum percent identity at 80% (Caporaso *et al.*, 2010a). After alignment, USEARCH series of scripts were used to remove the chimer and noisy sequences followed by clustering of OTUs picking with 97% cut-offs (Edgar *et al.*, 2011). Taxonomy was assigned using RDP classifier with a minimum support threshold at 80% (Wang *et al.*, 2007). OTUs with the same taxonomy at phyla level were pooled for description of community. Fungal reads were processed with default settings, and chimer were removed as mentioned above, followed by OTU picking as representative sequence for each cluster and alignment to the ITS database from UNITE (http://unite.ut.ee) using the PyNast algorithm with a minimum percent identity at 80% (Caporaso *et al.*, 2010a)

Method S2: Statistical analysis.

QIIME, Primer E software v.6 package (Clarke & Gorley, 2006) and R version 3.1.1 were

used for all statistical analysis of both bacterial and fungal communities. Library "g plots" were used to construct Venn diagram based on the 97% OTUs similarity. To find out the statistical significance of core OTUs, we conducted statistical comparisons with log2 transformed (log2(RA+1)) per mill values, as described (Schlaeppi *et al.*, 2013), with p values adjusted using the Bonferroni correction for multiple testing. Generalized linear squares (GLS) from library "nmle" was used to assess the effect of 'soil' and 'root' on community composition among locations based on the phylum rank. R version 3.1.1 were used for ANOVA followed by Fisher's PLSD and Student t-test for pairwise comparisons.

Method S3 Identification of *N. attenuata UVR8* and plant transformation

Comparing publicly available sequences at NCBI of UVR8 from *Arabidopsis* and other closely related Solanaceae species.e.g. *Solanum lycopersicum*, and *N. tabacum* we identified homologs in *N. attenuata* using the *N. attenuata* 454 transcriptome data base (Gase & Baldwin, 2012) To generate *irUVR8* plants, we cloned a 319 bp fragment of *NaUVR8*gene as an inverted repeat construct into pRESC8 transformation vector containing a hygromycin (*hptII*) resistance gene as selection maker (Fig. S6a). *N attenuata* plants were transformed using the LBA4404 strain of *Agrobacterium tumefaciens* using transformation method described in (Krügel *et al.*, 2002). Homozygous transgenic lines were selected by screening of T2 generation seeds that showed hygromycin resistance, and T-DNA insertions were confirmed by Southern blot hybridization, using genomic DNA from selected lines and ^{32}P-labeled PCR fragment of the *hptII* gene as hybridization probe (Fig. S6b). Quantitative real time PCR was used to select the best silenced transgenic lines: *irUVR8*-160-5-6, 224-1-9, and 260-10-1, which showed more than 95% reduction of NaUVR8 transcript compared to EV plants. We tested the 260-10-1 line in the field because several studies have shown that higher background irradiance (PAR) levels ameliorated the UVB induced damages (Adamse & Britz, 1992; Caldwell *et al.*, 1994; Flint & Caldwell, 1996; Kakani *et al.*, 2003); and it is

known that *Arabidopsis* plants grown in the laboratory chamber with UVB supplementation (≈100-200 µmol m^{-2} s^{-1}, and 1-2 µmol m^{-2} s^{-1}) show a severe phenotype. Although ir*UVR8* plants were grown under an extremely high UVB environment (max. ≈ 12 µmol m^{-2} s^{-1}), the growth defects of *irUVR8* plants was not as severe as in *Arabidopsis*. Based on these findings we assume that the differences in the phenotyes between *Arabidopsis* and *N. attenuata* are due to the extremely high PAR levels combined with high UVB irradiance for *N. attenuata*. This assumption is supported by severe UVB induced damage phenotype - strong necrosis, curly leaves, and low reproductivity- of *irUVR8* plants grown in a climate chamber which has much lower UVB irradiance as well as substantially lower PAR levels (≈ 400 µmol m^{-2} s^{-1}PAR and ≈ 1.3 µmol m^{-2} s^{-1} UVB) compared to the field station (data not shown).

Ir-construct sequence of *NaUVR8*

GGAGCAAATATGGTCAACTAGGACATGGTGATTTTGAGGATCATCTTTTCCCTCATAAGGTT
CAAGCTTTGCATGGCAGTTTTACTTCTCAGATATCAGGTGGTTGGAGGCATACCATGGCGCT
TACTGCTGATGGAGAACTTTATGGTTGGGGTTGGAACAAGTTTGGACAAGTTGGTGTTGGTG
ACAATGTTGATCATTGTTCACCTGTACAAGTGAAATTTCCACATGATCAGAAAGTAATTCTG
ATTTCATGCGGATGGAGGCACACACTTGCTGTTACAGAAAGACAGAATGTCTTTTCCTGGGG
AAGAGGTAC

Method S4: Quantitative real time PCR

Total RNA was extracted from approximately 100 mg of frozen leaf tissues with Trizol method, followed by DNase-I treatment (Fermentas) according to the manufacturer's instructions. Remaining DNase was removed by phenol extraction and precipitated with addition of 3 M sodium acetate (pH 5.2) and pure ethanol. The cDNA was prepared from 1 µg of total RNA using Revert Aid^{TM} H Minus reverse transcriptase (Fermentas) and oligodT primer (Fermentas). Quantitative real-time PCR was conducted with synthesized cDNA using the core reagent kit for SYBR Green I (Eurogentec) and gene-specific primer pairs (Supplemental Tables S1) using Mx3005P PCR cycler (Stratagene). Relative gene expression was calculated from calibration curves obtained by analysis of dilution series of cDNA

samples, and the values were normalized by the expression of housekeeping gene *NaEFα1* (*N. attenuata* elongation factor alpha 1). All reactions were performed using the following qPCR conditions: initial denaturation step of 95°C for 30s, followed by 40 cycles each of 95°C for 30 s, 58°C for 30s and 72°C for 1min, followed by melting curve analysis of PCR products.

Primer sequence of *NaUVR* and *EFa1* for SYBR-qPCR

NaUVR8_For: 5'-AGGGGAGAGGATGGACAACT

NaUVR8_Rev: 5'-TGGAGTTTCCATGACCCAAT

Na_EF1a_For: 5'-CCACACTTCCCACATTGCTGTCA

Na_EF1a_Rev: 5'- CGCATGTCCCTCACAGCAAA

Method S5 Primer specificity of bacterial isolates

Bacterial species specific primers were designed using Primique software (Fredslund & Lange, 2007). Specificity of primers was tested against bacterial isolates and plant DNA by PCR. The rimer pair was considered to be specific if only a single PCR product was visible in an agarose gel in samples containing DNA of the respective species. PCR amplification was performed in a 20 µL final volume of ReadymixTaq PCR reaction mix (Sigma Aldrich) containing 1 µL of template DNA (1ng/ µL) and 300 nM for each primer under the following PCR conditions: 95˚C for 1 min, followed by 35 cycles of denaturation at 95˚C for 60 s, annealing at 63˚C for 60 s and primer extension at 72˚C for 60 s, followed by a final extension at 72˚C for 10 min. The quality of the PCR reaction was examined by running an aliquot of the PCR product in 1.2% (w/v) agarose containing ethidium bromide

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Author Contribution:

R. Santhanam, performed the experiments, analyzed the data and drafted the manuscript. I.T. Baldwin and K. Groten helped to draft the manuscript. Y. Oh characterized the *irUVR8* line. R. Santhanam, V.T Luu, A.Weinhold, K. Groten and I.T Baldwin conceived and designed the study.

7 General Discussion

All eukaryotic organisms maintain complex interactions with their microbial partners, for example the potential of gut microbiota influencing host nutritional and health status is well documented (Cummings & Macfarlane, 1997; Flint *et al.*, 2012). Similar to the animal gut, for plants the root is the primary organ for water uptake and nutrients, which is surrounded by a complex microbial community (Hacquard *et al.*, 2015). A growing body of evidence indicates that the composition of root bacterial communities, the so-called root microbiome, is a "secondary genome" (Rout & Southworth, 2013), and can have significant impact on plant health and development. These impacts can have serious influences on plant yield quantity and quality, as well as developmental changes and biotic and abiotic stress tolerance (Mendes *et al.*, 2011; Panke-Buisse *et al.*, 2015). Burgeoning human populations, dramatically increased the food demand, which in turn increased the urgency and importance of how the microbiome can be exploited to increase crop yield and to reduce crop loss caused by phytopathogens (Lareen *et al.*, 2016). Furthermore, a novel approach is needed to tackle abiotic stress such as drought, flood and increased salinity caused by global warming and climates change.

This study aims to reveal factors shaping the *N. attenuata* phyllosphere and root microbiota under natural conditions, by both culture-dependent and -independent 454 pyrosequencing method. Furthermore, this study addresses the agricultural dilemma associated with monoculture practice and fungal outbreak and its potential solutions. In **manuscript I**, I used a previously characterized isogenic line impaired in the production of the JA to investigate the role of JA and different developmental stages in shaping the leaf and root bacterial communities. I demonstrate that JA and developmental stages of plants do not influence the bacterial community composition, however, leaf and root community composition are significantly different. In **manuscript II**, I investigated, whether bacterial PGP effects are dependent on endogenous JA production and revealed that bacterial PGP effects are independent of the plant's capacity to produce JA. In **manuscript III,** monoculture practice of our model plant led to emergence of sudden wilt disease and by using native root associated bacterial consortia (5-6 isolates) as a biocontrol agent, sudden wilt disease was reduced in two consecutive field seasons (2013 and 2014). Finally, in **manuscript IV,** I investigated**,** whether soil from five different locations at their native habitat, the Great basin Desert, sculpt the root bacterial and fungal microbial community; and

I found that, soil types have limited influence on sculpting *N. attenuata's* root microbiota. Based on the pyrosequencing results, UV-resistant bacteria Deinococcus-Thermus were highly abundant in *N. attenuata* roots, irrespective of location. Furthermore, the role of the plant's response to UVB exposure and its perception (UVR8) and induction of flavonoid biosynthesis (CHS) in the recruitment of the UV resistant bacterium *Deinococcus* was investigated in a microcosm experiment, and I demonstrate that, plant response to UVB exposure significantly increased the *Deinococcus* colonization.

7.1 Influence of plant host factors on *N. attenuata* leaf and root bacterial communities.

Plants distinguish pathogenic from non-pathogenic bacteria via the plant defense systems, for example the plant cell surface contains pattern recognition receptors (PRRs), it distinguishes potential pathogens by conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS), flagellae and other cell wall components (Zipfel, 2008) and non-pathogenic bacteria are recognized by microbe-associated molecular patterns (MAMPs) (Bittel & Robatzek, 2007). Recognition of any PAMP, leads to activation of a defense signaling cascade to enhance plant immunity against pathogens (Van Wees *et al.*, 2008). Plant defense against phytopathogens is regulated by a complex network of phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). Appropriate defense response is important for plant fitness, because activation of defense may have detrimental effects on plant growth (growth-defense dilemma; Herms & Mattson, 1992). In general, plant pathogens are divided into biotrophic and necrotrophic pathogens based on their lifestyle. Biotrophs feed on the living host, necrotrophs kill the living host and feed on them (Glazebrook, 2005). In plants, the phytohoromone SA plays a vital role against biotrophic pathogen, whereas, JA against necrotrophic pathogens (Glazebrook, 2005).

Previous studies, using *A. thaliana* as a model plant, investigated the role of JA in leaf epiphytic and endophytic bacterial community by low resolution cultural dependent method (Kniskern *et al.*, 2007) and by analyzing the rhizosphere bacterial communities using pyrosequencing and DGGE method (Doornbos *et al.*, 2011a; Carvalhais *et al.*, 2013). Both approaches came to contrasting conclusions. Carvalhais *et al* (2013) used 16S rDNA gene amplicon pyrosequencing to determine the role of JA signaling pathway in rhizosphere community by applying MeJA, which activates the JA signaling pathway and concluded that MeJA treatment significantly altered the rhizosphere community composition. Whereas, Doornbos *et al* (2011) by using DGGE fingerprinting, demonstrated that bacterial community

composition was not different in *jar1* mutant deficient in JA signaling compared to control Col-0 plant; and foliar application of MeJA did not significantly altered the community composition neither. Given these contrasting findings, our ecological model plant *N. attenuata*, which is genetically diverse among individual plants of a population, accumulates different levels of JA after herbivore attack (Kallenbach *et al.*, 2012). Based on this it is tempting to speculate that, natural JA variant genotypes bacterial communities might differ and could be influenced by abundance of necrotrophic community or recruit bacterial communities to compensate their deficiency as exemplified by Meldau *et al* (2013), who showed ethylene insensitive plants specifically recruit *Bacillus* sp B55 to enhance plant growth by sulphur production.

Contrary to our expectation, in **manuscript I** leaf and root bacterial communities are not sculpted by JA. Alpha (within samples) and beta (between samples) diversity indices, were not significantly different among ir*AOC* vs EV leaves or ir*AOC* vs EV roots, indicating that JA did not shape the leaf and root bacterial community. Our findings are in contrast with earlier results from Kniskern *et al* (2007), who showed that, leaf bacterial richness was higher in *fad3/7/8* mutant deficient in JA accumulation compared to controls. The findings of Kniskern *et al* (2007) are based on culture dependent approach, which underestimates the diversity richness and is prone to false interpretation. However, in recent deep pyrosequencing study Lebeis *et al* (2015) showed that root bacterial communities are independent of JA by using mutants such as *DEPS, JEN, NJ* which are all deficient in JA response. Whereas SA influences the root bacterial communities and the dominant phylum Actinobacteria is replaced by Proteobacteria in the endosphere of mutants which lack response to SA. Interestingly, no changes were observed in either Actinobacteria or Proteobacteria abundance among control and JA deficient isogenic lines in this study and Lebeis *et al* (2015). It is surprising that in *N. attenuata* despite the difference in primary and secondary metabolites such as, nicotine, starch and sugar between EV and ir*AOC* genotype (Machado *et al.*, 2013) overall bacterial community composition is not influenced by JA. To further validate the results of the culture independent method, we employed microcosm experiment with bacterial isolates retrieved only from EV or ir*AOC* genotypes named as putative "specialist" and from both the genotypes as putative "generalist". Under microcosm experiment, both putative specialist and generalist colonized both genotype, EV and ir*AOC*. These results further corroborate the conclusion of culture independent method, that *N. attenuata* bacterial community is not sculpted by the plant's capacity to produce JA.

7.2 Plant developmental stages and bacterial communities

In **manuscript I,** I demonstrate that overall bacterial communities of five different plant developmental stages did not differ among tissues or genotypes, even though, in *N. attenuata* inducible defense signal mediated by JA dramatically changes from rosette to flowering stages (Diezel *et al.*, 2011). Results of this study corroborated the findings of Lundberg *et al*. (2012) using a pyrosequencing approach. The authors demonstrated that plant developmental stages of 8 different genotypes of *Arabidopsis* grown under glasshouse condition, harvested at different time points such as appearance of inflorescence meristem and fruiting stage did not have major effects on overall community composition. Whereas, study based on culture- dependent methods, demonstrated that density of bacteria decreased with age from vegetative growth to senescence in soya bean plant (Kuklinsky-Sobral *et al.*, 2004). Another study based on DDGE finger printing method, showed that plant growth influences the bacterial community composition of potato roots (Andreote *et al.*, 2008; Van Overbeek & Van Elsas, 2008). In general, composition of root exudates is known to change throughout the developmental stages (Doornbos *et al.*, 2011b; Bulgarelli *et al.*, 2012b), however, in the present study plant developmental stages do not have a major role in shaping leaves and root communities of field grown *N. attenuata.*

7.3 Tissue type influences bacterial community composition

In **manuscript I**, in contrast to JA and developmental stages, tissue type had a significant impact on bacterial communities. Alpha and beta diversity of leaves and roots were significantly different, indicating that bacterial community composition is shaped by tissue type. Similar findings were reported in potato and *Arabidopsis* (Berg *et al.*, 2005; Bodenhausen *et al.*, 2013). In this study, roots were dominated by Actinobacteria, Sphingobacteria, Flavobacteria, β, α- Proteobacteria, whereas leaves were dominated by γ-Proteobacteria. In leaves, among γ-Proteobacteria namely, *Enterobacter* and *Serrati* were highly abundant, unlike lettuce – its phyllosphere community was dominated by *Pseudomonas* (Rastogi *et al.*, 2012), while in roots, nitrogen fixing bacteria such as *Rhizobium* and *Azospirillum*, belonging to α-Proteobacteria dominated the communities. These bacteria have potential beneficial growth effects on plants. Furthermore, alpha diversity (species richness) is higher in roots compared to leaves. This might be due to close association of roots with soil, which harbors even more diverse microbial communities. The differences among tissue types might be due to total bacterial population of leaf and root tissues. The phyllosphere bacterial abundance is ~10^6 cells/g (Rastogi *et al.*, 2012) and by

contrast roots harbor 10^8 cells/g dry weight tissue (Hardoim *et al.*, 2008). It has been shown that total community size is directly proportional to diversity (Kniskern *et al.*, 2007). Another reason for the differences in diversity might be that root exudates acts as a chemoattractant to recruit bacterial communities from soil, whereas the leaf surface is rather nutrient poor and bacteria are exposed to variable environments such as UV radiation, humidity and fluctuating temperature (Lindow & Brandl, 2003).

7.4 Plant growth promoting effects are independent of genotypes.

Numerous researches focused on JA response against necrotrophic pathogens, however, the influence of JA on plant beneficial bacterial isolates is largely unknown. In **manuscript II**, we investigated whether PGP effects depend on the ability of plants to produce endogenous JA. We used ir*AOC* and EV putative "specialist" and "generalist" bacterial isolates from **manuscript I**. Out of 18 putative specialist and generalist isolates of EV and ir*AOC* plants, only three isolates, the EV specialist *Pseudomonas azotoformans* A70, the ir*AOC* specialist *Arthrobacter nitroguajacolicus* E46 and the generalist *Bacillus ceres* CN2, significantly promoted plant growth compared to controls. *P. azotoformans* A70 is well known to fix nitrogen (Anzai *et al.*, 2000), and the observed plant growth promotion could be due to improved nitrogen nutrition. *B. ceres* CN2 and *A. nitroguajacolicus* E46 might enhance plant growth by phosphate solubilization or sulphur oxidation. In contrast to the observations of others (El Zemrany *et al.*, 2006) *Azospirillum lipoferum*-E11 did not promote plant growth in the present study. Loss of activity of *A. lipoferum*-E11 might be due to species specific effects. It has been shown by Long *et al.*, (2008) that bacterial isolates from *Solanum nigrum* are unable to promote growth of *N. attenuata* and vice-versa. In this study, surprisingly a mixed inoculation did not enhance growth under *in-vitro* conditions, and growth was similar to that of control plants. These contrary results might be explained by competition for resources among different species or their ability to colonize the plant under competitive conditions. Thus, the effects of a single inoculation with specific bacterial isolates do not necessarily reflect the influence of individual bacterial isolates within a complex mixed community. Understanding bacterial interactions, such as competition for resources and their localization in particular niches in a mixed community are important for understanding successful colonization in nature.

7.5 *N. attenuata* root microbiome

In **manuscript I and II**, we demonstrated that *N. attenuata*'s root microbiome is independent of JA and developmental stages. Based on these, we further hypothesized that

soil might sculpt *N. attenuata*'s root microbiome. In **manuscript IV** we addressed the hypothesis whether root microbiota of *N. attenuata* are different among soil types, which differ in chemical properties and locations. A growing body of evidence highlights that plant root microbial communities are sculpted by various environmental factors (Bulgarelli *et al.*, 2012b; van der Heijden & Schlaeppi, 2015). Among them, soil types may influence the root microbiota of plants. Soil types differ in structure, pH, minerals, texture and availability of nutrients. The physio-chemical properties of soil creates the ecological niches, which determine the availability of microbial communities for plants to select (Berg & Smalla, 2009; Philippot *et al.*, 2013; Bulgarelli *et al.*, 2015). For instance, soil pH and nutrient availability such as phosphate, carbon and nitrogen affect the pathogenic crop pathogen, fungi and beneficial microbes (Duffy *et al.*, 1997; Lacey & Wilson, 2001; Toljander *et al.*, 2008; Dumbrell *et al.*, 2009)

In **manuscript IV**, we harvested native grown *N. attenuata* along with bulk soil from five different locations at their native habitat the Great Basin Desert, Utah. All samples were subjected to culture-independent pyrosequencing approach to analyze the bacterial and fungal communities. In general, for both fungal and bacterial community alpha diversity was higher among soil samples compared to roots. These results were consistent with the findings of other pyrosequencing studies of *A. thaliana*, maize, sugarcane, barley, rice, grapes, which were carried out under glasshouse and field conditions (Bulgarelli *et al.*, 2012a, 2015; Lundberg *et al.*, 2012; Bodenhausen *et al.*, 2013; Peiffer *et al.*, 2013; Shakya *et al.*, 2013; Gilbert *et al.*, 2014; Bonito *et al.*, 2014; Edwards *et al.*, 2015; Yeoh *et al.*, 2016) demonstrating that soil harbors huge microbial diversity. Furthermore, overall root bacterial community is significantly different from soil and roots and soil samples clustered separately in NMDS plot, independent of locations, even though chemical properties of five soil types differed among each other, though differences were not strong. Findings of this study are in accordance with other pyrosequencing studies. It was shown that under glasshouse conditions there is a big overlap among the root bacterial communities in *A. thaliana*, though soils types are chemically different and from two different continents (Bulgarelli *et al.*, 2012a and Lundberg *et al.*, 2012). These results clearly demonstrate that plants specifically recruit particular bacteria from soil, irrespective of soil types; and plant selection of bacteria from a pool of bacteria in the soil may depends on other environmental factors. Though, *N. attenuata* native populations are genetically diverse (Bahulikar *et al.*, 2004; Kallenbach *et al.*, 2012) the core bacterial communities of *N. attenuata* from native populations and 31st inbred line from

Lytle plot were similar, irrespective of genotypes. Core bacterial communities consist of Actinobacteria, Deinococcus-Thermus and TM7, Proteobacteria, Bacteroidetes, Chloroflexi. The same bacterial communities dominated also the roots of field grown *N. attenuata* EV and ir*AOC* plants harvested in 2012 and described in **manuscript I.** Based on these results, we conclude that *N. attenuata* recruits core bacterial community irrespective of genotypic variabilities in natural populations, and bacterial communities are consistent over time and independent of location. Also for a crop plant, maize, the roots of 27 genetically diverse inbred lines grown at five field sites harbored similar microbial communities (Peiffer *et al.*, 2013) and only 5% variation was observed amongst the genotypes. In *Arabidopsis*, core communities of 12 genotypes were similar, when grown under glass house conditions (Lundberg *et al.*, 2012; Schlaeppi *et al.*, 2013). Taken together, the present study clearly demonstrates that plant genotypes have limited effect on shaping root bacterial communities.

In all recent deep sequencing studies the phylum Actinobacteria dominated the root microbiota of different plant species such as *A. thaliana, A. lyrata, A. halleri* and *Cardamine hirsute*, maize, sugarcane, barley, rice and 12 different *A. thaliana* genotypes (Bulgarelli *et al.*, 2012a, 2015; Lundberg *et al.*, 2012; Peiffer *et al.*, 2013; Schlaeppi *et al.*, 2013; Edwards *et al.*, 2015; Yeoh *et al.*, 2016). In **manuscript I** and **IV** the phylum Actinobacteria also dominated the root microbiota of *N. attenuata*, irrespective of genotypes, soil types and locations indicating a specific recruitment of Actinobacteria by plants from soil. Among Actinobacteria, bacterial members belonging to the genus *Streptomyces* are highly abundant and are well known for the production of secondary metabolites (bio-active compounds) (Firáková *et al.*, 2007), shown to inhibit the growth of phytopathogens (Mendes *et al.*, 2011; Cha *et al.*, 2016). *Streptomyces* can also to promote plant growth (Gopalakrishnan *et al.*, 2013; Lin & Xu, 2013; Viaene *et al.*, 2016). Bulgarelli *et al.* (2012a) showed that, specific enrichment of Actinobacteria in *Arabidopsis* roots compared to untreated wooden splinters was due to cues from metabolically active root host cells. However, the metabolic cues and molecular mechanism behind this specific recruitment needs further research. Other bacterial phyla such as Firmicutes, Proteobacteria, Bacteroidets, Chloroflexi quantitatively differ among all plant species.

For fungi, the phylum Ascomycota dominates the root microbiota, irrespective of plant species (Shakya *et al.*, 2013; Bonito *et al.*, 2014; Groten *et al.*, 2015). The high affinity of the phylum Ascomycota to different plant species might also be due to beneficial effects on plants. Fungal species belonging to the Ascomycota are well known for the production of

antifungal compounds and plant growth promoting effects (Larkin & Fravel, 2001; Newsham, 2011; Mapperson *et al.*, 2014)

Many phytopathogenic fungi and bacterial species co-evolved with plants and show species-specific pathogenesis (Raaijmakers *et al.*, 2009). In addition, it has been shown that different plant species recruit different bacterial communities, even though they were grown in the same field. For example, bacterial rhizosphere communities of oilseed rape, potato and strawberry harbored species-specific rhizosphere bacterial communities and the differences in community composition among the species became more pronounced in the second year when the plants were grown on the same field again (Smalla *et al.,* 2001). The molecular basis of this host specificity is not fully explored. To find out if *N. attenuta* harbors specific bacterial communities, in **manuscript IV,** we compared the results of this study with other pyrosequencing studies of barley, sugarcane and *A. thaliana* (Bodenhausen *et al.*, 2013; Schlaeppi *et al.*, 2013; Yeoh *et al.*, 2016). These studies were selected because the primers used all cover the V5-V9 regions of 16S rDNA to avoid an inappropiate comparison. NGS studies are primer biased and one have to be cautious in interpreting and comparing the results when different primer pairs, in particular when they are targeting different regions, are used (Ghyselinck *et al.*, 2013). Interstingly, the phyla Deinococcus-Thermus and TM7 are specific to *N. attenuata,* while other bacterial phyla such as Actinobaceria, Chloroflexi, Bacteroidetes and Proteobacteria are shared among all plant species. The phylum Deinococcus-Thermus is highly abundant and well known for UV and gamma resistant traits and can survive in harsh desert conditions (Battista, 1997; Cox *et al.*, 2010). These conditions are also found in the native habitat of *N. attenuata*, which is characterized by high light intensities and high UVB fluence rates and arid desert condition. Based on this, we tested the hypothesis that Deinococcus-Thermus colonization increases when exposed UVB radiation and colonization is regulated by UVB perception receptor UVR8 and response enzyme chalcone-synthase (CHAL).

In **manuscript IV**, results obtained by the culture-independent approach were complemented with culture-dependent experiments to reveal the functional role of highly abundant taxa at community level. We isolated *Deinococcus citri* from seedlings grown on native soil from the Great Basin Desert Utah. One of the isolates shared 100% similarity with *Deinococcus citri* NCCP-154^{T} type strain and with the highly abundant OTU-12140 found in the culture-independent approach. To analyze the role of molecular cues in *Deinococcus* colonization, we used a previously characterized ir*CHAL* line impaired in the expression of

CHAL (Kessler *et al.*, 2008) and a newly generated RNAi mediated isogenic line impaired in UVB perception (ir*UVR8*) and a cross of both (ir*CHAL*xir*UVR8*), and evaluated root bacterial colonization in a microcosm experiment with a synthetic bacterial community consisting of three bacterial isolates representing three different phyla from **manuscript I** and *Deinococcus citri* D61 from **manuscript IV.** Under UVB supplementation, *D. citri* D61 colonization significantly increased in wild type (WT) plants, which able to respond normally to UVB, but not in the isogenic lines impaired in UVB perception (ir*UVR8*) and response (ir*CHAL*). The effect was only observed for *D. citri* and not for the three other bacterial species tested. These results indicate that UVB might alters the plant's metabolism in favor of *D. citri* colonization, and UVR8 and CHAL play a role in root this process. UVB exposure increases the production of flavonoids in plants (Brown *et al.*, 2005). Flavonoids can be exuded by plant roots and may specifically attract *D. citri*, similar to the attraction of Rhizobia by legumes. For legumes it has been shown that flavonoids are a chemoattractant for the colonization of *Rhizobia* leading to the formation of root nodules (Hassan & Mathesius, 2012). Furthermore, the decreased colonization rates in the transgenic lines impaired in UVB perception and response could be due to a reduced transfer of carbohydrates to the roots, as these plants are also impaired in photosynthetic rates. However, the detailed mechanism and metabolic cues responsible for *Deinococcus* colonization of *N. attenuata* have to be addressed in future experiments.

7.6 Agricultural dilemma and poly-microbial solutions.

In **manuscript III,** we addressed the long festering problem of monoculture practice and fungal outbreak and the ability of plants to acquire native beneficial microbes to solve ecological-context dependent problem. Plant disease caused by soil-borne phytopathogens causes dramatic crop yield loses all over the world (Weller *et al.*, 2002; Raaijmakers *et al.*, 2009). The global population is estimated to reach 10 billion by 2050 and intensive monoculture cropping is a way forward to feed the ever growing population. However, practice of intensive monoculture leads to pathogen buildup followed by disease outbreak and leading to crop failure (Schippers *et al.*, 1987; Oyarzun *et al.*, 1993).

The ecological model plant *N. attenuata* has been planted on a field plot in its native habitat for the past 15 years. The continuous replanting within the same area in an agricultural like style led to the appearance of a sudden wilt disease, which seemed to be highly specific for *N. attenuata*, as other plants such as *N. obtusifolia* or weeds growing on

the same plot seemed not to be affected. Sudden wilt disease is characterized by sudden collapse of vascular system associated with roots turning black. The emergence of the sudden wilt disease 8 years ago recapitulates a common agricultural dilemma that resulted from the accumulation of plant pathogens from continuous cropping and re-using the same area for several years.

In general, plants recruit beneficial bacterial communities from soil (Gaiero *et al.*, 2013) In a recent study, using rice has a model plant it was demonstrated that bacterial community structures were largely established 1 day after germination and steady-state of bacterial composition was reached at day 13 (Edwards *et al.*, 2015). We speculate that a native plant population of *N. attenuata* might acquire beneficial microbial communities from the soil at an early stage during germination and establishes mutualistic associations throughout the plant life cycle (Compant *et al.*, 2010; Doornbos *et al.*, 2011b). However in our field experimental set-up *N. attenuata* seeds are germinated on sterile media and transferred into the field soil. The first contact of plant roots with soil bacteria happens when the plants are planted in the field plot (two to three weeks after germination).This is in contrast to the native way of growth, and these seedlings lack the opportunity to acquire mutualistic microbial associations from the surrounding soil at an early stage of development. The lack of early exposure to beneficial soil bacteria and the artificial re-planting at the same field site deprived *N. attenuata* from its native behavior of an annual fire-chasing plant.

Many different pathogens can cause wilting symptoms in Solanaceous plants, and Fusarium wilt, Granville wilt or verticillium wilt are common plant diseases which show similar vascular wilt symptoms in tomato, potato and tobacco plants (Bonde, 1939; Larkin & Fravel, 2001; Tjamos *et al.*, 2004; Rodríguez-Molina *et al.*, 2007; Chen *et al.*, 2013). Granville wilt is a devastating disease of *Nicotiana tabacum* and caused by the bacterial plant pathogen *Ralstonia solanacearum* (Katawczik & Mila, 2012). Despite considerable efforts during the isolation of potential bacterial pathogens, we could not identify or culture a single *Ralstonia* isolate from diseased plant roots, even though we used CPG-medium which is suitable for the specific isolation of *R. solanacearum* (Kelman 1954). Furthermore, other tobacco associated bacterial pathogens such as *Pseudomonas syringae* pv. *tabaci* (Wannamaker & Rufty, 1990), *Agrobacterium rhizogenes* (Gelvin, 1990), *Erwinia carotovora* subsp. *carotovora* (Intyre *et al.*, 1977; Xia & Mo, 2007) or *Rhodococcus fascians* were not among the isolates retrieved from the diseased roots. However, the number of potential plant pathogens among all isolates was very low and only *Curtobacterium*

flaccumfaciens and *Pseudomonas tremae* have been reported for being able to infect certain host plants (Gardan *et al.*, 1999; González *et al.*, 2005), whereas based on literature they do not cause diseases in tobacco plants (Bull *et al.*, 2010, 2012). *Alternaria* and *Fusarium* fungi dominated the fungal community of roots from sudden wilt disease infected *N. attenuata* plants and might be the potential causal agents.

In agriculture, different strategies, such as crop rotation with non-host crops, soil amendments, soil solarisation or fumigation, treatment with chemical fungicides, inoculation with biocontrol strains are applied to control plant diseases (Curl, 1963; Krupinsky *et al.*, 2002; Tjamos *et al.*, 2004; Makovitzki *et al.*, 2007; Wang *et al.*, 2013). In this study, different strategies such as native bacterial PGP from **manuscript I & II** and fungi, along with chemical fungicide were employed under *in-vitro* conditions. A major advantage of culturable isolates is that they can be used for a functional analysis e.g., PGP and biocontrol efficiency. In this study we used bacterial consortia to mimic natural heterogeneity. Based on the *in-vitro* study bacterial consortia, the fungal isolates *Chaetomium sp*. (C72) and *Oidodendron sp* (Oi3) and chemical fungicide (Landor) significantly reduced the death mortality rate and were selected for field trail. In the 2013 field trail, 3 major strategies (7 treatments) were tested: fungicide treatment, biocontrol strains and soil amendment and a combination of two treatments (fungicide+charcoal). In nature, *N. attenuata* germinates in charred debris of a post-fire environment, triggered by smoke-derived cues (Baldwin & Morse, 1994). To mimic these conditions in the field, we added charcoal during the planting process as soil amendment and alternative treatment. Among 7 treatments, only bacterial consortia protected the plants from sudden wilt disease. Interestingly, charcoal, fungicide, a combination of fungicide+ charcoal and fungal biocontrol had higher mortality rate than controls. The discrepancies in performance of fungal isolates might be due to a lack of compatibility among co-inoculated fungal isolates, because in contrast to *in-vitro* conditions (single fungal isolates) we used a mixture (C72+Oi3) of fungal isolates in the field trial. To our surprise, fungicide treatment was ineffective and had a negative effect on plant growth under field conditions. This might be due to a loss of activity due to high temperatures in the field. Alternatively the fungicide treatment may prevent the native AMF or other beneficial fungi to interact with plants. The selection of fungicides for agricultural practice should take these findings into consideration.

Many biocontrol strains might be very effective under *in vitro* conditions, but less successful under glasshouse conditions and might even be unfeasible under field conditions

(Deacon, 1991), which makes the screening for potential biocontrol candidates still a big challenge (Lugtenberg & Kamilova, 2009). To test the robustness and reproducibility of the 2013 field trail, we repeated the experiment with bacterial consortia in 2014. Bacterial consortia reduced the plant mortality in the field in both years. Furthermore, we tested, whether bacterial consortia influenced the ecological traits of *N. attenuata* and whether the protection effect depends on genotype. Therefore, we used a second inbred ecotype of *N. attenuata*, originally collected from Arizona. The bacterial consortia did not influence the 32 ecological traits of *N. attenuata* investigated, and the protective effect was independent of genotypes.

We used bacterial isolates as potential biocontrol strains, which were native to *N. attenuata*, since they might be better adapted to their host or the environmental conditions than organisms isolated from other plant species (Cook, 1993; Köberl *et al.*, 2013). Biocontrol strains have to be excellent root colonizers for an effective suppression of plant pathogens under competitive natural conditions (Lugtenberg *et al.*, 2001; Doornbos *et al.*, 2011b). In 2013 field trial, from our consortia inoculations, 4 out of 6 strains were re-isolated from roots of flowering-stage plants. From the 2014 field season, all native bacterial taxa from the consortia were retrieved. Differences in re-isolation among these two field seasons might be due to culture-dependent bias. However, re-isolation of these consortia from both field seasons demonstrate that the native bacterial taxa used here, are efficient root colonizers and persist till early flowering stage. Synergism among these bacterial consortia might be responsible for protection effect. It has been suggested, that the combination of multiple biocontrol strains may provide improved disease control compared to the use of a single organism (Raupach & Kloepper, 1998; Sessitsch *et al.*, 2004; Compant *et al.*, 2010; Chen *et al.*, 2013). Beside multiple synergistic effects, the mixed inoculation might induce systematic resistance (ISR) or better colonization of ecological niches (Lugtenberg & Kamilova, 2009; Figueiredo *et al.*, 2011; Doornbos *et al.*, 2011b; Glick, 2012). In nature, *N. attenuata* recruits beneficial microbial community from the soil when it germinates from the seed bank and this recruitment mostly appears to be opportunistic, driven by biotic and abiotic stresses. Sometimes this opportunistic interaction leads to opportunistic mutualism between plants and microbes where both partners benefit. This has been demonstrated in a recent study: ethylene insensitive *N. attenuata* genotype recruits beneficial microbes to compensate for its growth deficit (Meldau *et al.*, 2012, 2013). The mechanism behind the biocontrol efficiency is currently under investigation.

7.7 Conclusion and future perspectives

The present dissertation work underpins the importance of studying plant- microbe interaction in an ecologically relevant context. Simply knowing the microbial population, without understanding the community context, precludes organism based insights into community function and dynamics. NGS descriptive analyses were complemented with functional based analysis to reveal the ecological context of microbial communities and demonstrated that, *N. attenuata* overall bacterial community compositions are not shaped by JA or soils and PGP effects of bacterial isolates are independent of JA. *N. attenuata* specifically recruits certain microbial taxa irrespective of soils and locations. Recent reports based on NGS studies along with findings of this study clearly demonstrate that plants preferentially recruit species of the phylum Actinobacteria, which reside inside the root without causing any negative symptoms to plants. Findings of these studies could be used to develop Actinobacteria-based biological solutions for agricultural problems such as pathogen outbreak or to improve yields. Furthermore, I show that the plant's response to UVB irradiation increased the colonization of *Deinococcus*. These findings help to elucidate the environmental factors changing the plant's metabolism, to modulate the root microbiome for the benefit of plants. The detailed mechanism how abiotic factors lead to physiological and metabolic changes in the plants which drive changes in the root microbiome will be addressed in future studies.

Realizing the principles behind the root-microbiome interactions may help us to generate stable beneficial communities in agricultural soils. And the question is whether it is stable and maintained throughout the plant ontogeny. Previous reports demonstrated that, plants selectively recruit and maintain stable beneficial microbial communities to suppress the phytopathogens through production of secondary metabolites (Weller *et al.*, 2002; Doornbos *et al.*, 2011b). In this thesis, I addressed the agricultural dilemma and monoculture practice led to pathogen buildup and emergence of the sudden wilt disease caused by *Alternaria* and *Fusarium* disease complex. Moreover, my results support the importance of early colonization of bacterial consortia at the time germination to establish the long lasting beneficial relationship to prevent the plant from sudden wilt disease. Farming methods, which support the recruitment and maintenance of these beneficial microbial communities in the roots, could provide a way for sustainable agricultural practice. The agricultural implications of this work are far-reaching and considerable more attention needs to put on current agricultural practice using non-specific antimicrobial compounds for seed treatment, which

thwart the important recruitment process. This study shows that plant develop opportunistic mutualism with their microbial partners to solve context-dependent ecological problems. However, more mechanistic investigations are needed to reveal the synergism behind the bacterial consortia protection effect and plant metabolic cues responsible for recruitment of bacterial consortia.

Despite recent advance in NGS studies, our knowledge is still limited to the molecular and genetic basis of plant-beneficial interaction in an ecological and evolutionary context. Future NGS studies have to include multipartite interactions e.g. plant-bacteria-fungi and should be complemented by culture-dependent approach with microcosm synthetic microbial communities to reveal functional role of microbial communities in ecological context. Instead of focusing on taxonomic relatedness, more attention needs to be given to functional groups of microbes in microbial community analysis. Moreover, microbial ecological studies have to move from a reductionist approach (single organism) to a holistic approach (consortia), which reflects natural conditions to take microbe-microbe interactions for sustainable agricultural practice into account.

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8 Summary

Plants rely on soil microbes for beneficial interactions i.e., growth promotion, nutrient availability and disease suppression. Biotic and abiotic factors shaping the root microbiome are still largely unknown. Furthermore, why and how plants acquire soil microbes during germination and their ecological and functional roles remain elusive. Most of the high-throughput sequencing studies are limited to model or crop plants and lack the complementation of a culture-dependent approach to reveal functional traits of dominant and rare microbial communities. Until now, poly-microbial disease complex and poly-microbial biocontrol application received little attention in the scientific community. In this dissertation, my work aims to address these intriguing questions.

During my research work, I investigated the influence of the phytohormone jasmonic acid (JA) and plant developmental stages on leaf and root bacterial communities in wild tobacco *N. attenuata*. Roots and leaves of field grown plants of a previously characterized, isogenic line impaired in JA biosynthesis (ir*AOC*) and an empty vector (EV) control line were harvested over five different developmental stages from rosette to flowering stages. A 454- pyrosequencing and culture dependent method were employed to characterize the bacterial communities. Neither JA nor plant developmental stages shaped the bacterial communities; however, tissue type had a major effect on community composition. Roots harbored higher bacterial diversity compared to leaves, irrespective of genotypes. The culture-independent results were further validated by a culture-dependent approach and demonstrated that JA does not influence the bacterial community composition of *N. attenuata*. Furthermore, bacterial plant growth promoting (PGP) effects are independent of the plant's ability to produce endogenous JA.

Moreover, I hypothesized abiotic factor such as soil types and the plant's response to UVB exposure shape the *N. attenuata* root microbiome. To test this hypothesis, we harvested native grown *N. attenuata* from different locations at the Great Basin Desert, Utah. Deep-sequencing analysis revealed that root bacterial communities were independent of soil types. Moreover, root bacterial communities from five different locations clustered separately compared to soil types in a non-metric multi-dimensional scaling plot (NMDS), although chemical properties of soils are different. Diversity of soil samples is higher compared to roots and plants selectively recruit the microbial communities from soil, irrespective of location and genotypes. However, fungal recruitment is less specific by plants. Interestingly,

in *N. attenuata*, the phylum Deinococcus-Thermus is unique compared to other plant species and more abundant in roots than in soils, which indicates selective enrichment of *Deinococcus* members in *N. attenuata* roots. Based on the UV resistant trait of Deinococcus-Thermus and the native habitat of *N. attenuata* characterized by high UVB fluence rates.We hypothesized that, plants response to UVB exposure and its UVB perception receptor UV RESISTANCE LOCUS 8 (UVR8) and response -flavonoid biosynthesis enzyme chalcone synthase (CHAL) – influences the colonization of Deinococcus- Thermus. We generated an isogenic line impaired in *UVR8* expression (ir*UVR8*) and used it along with a previously characterized UVB response line (ir*CHAL*). In a microcosm experiment with a synthetic bacterial community, *Deinococcus* highly colonized wild type roots under UVB exposure, but not the isogenic lines impaired in UVB perception (ir*UVR8*) and response (ir*CHAL*).

In a consecutive study, I addressed the current agricultural dilemma and long festering problem of monoculture practice and fungal outbreaks. The continuous usage of a field plot in *N. attenuata*'s native habitat using an agricultural set-up for the past 15 years led to the emergence of sudden wilt disease caused by fungal pathogen *Fusarium* and *Alternaria* disease complex. Sudden wilt disease is characterized by sudden collapse of the vascular system associated with roots turning black. Three different strategies such as microbial biocontrol, chemical fungicide and soil amendment were tested under field conditions. Among 7 different treatments, only bacterial consortia protected the plant from sudden wilt disease in 2013 field trial. In the consecutive year field trial 2014, bacterial consortia attenuated the sudden wilt disease, demonstrating the robustness of the protection effect. Bacterial protection effect is independent of genotypes and does not influence ecological traits of *N. attenuata.* Furthermore, bacterial consortia can be re-isolated from previously bacterial inoculated healthy plants indicating that these native consortia are excellent root colonizers.

In conclusion, this work demonstrates that *N. attenuata*'s microbiome is not significantly sculpted by JA, plant developmental stages and soil types, whereas, tissue types and UVB supplementation influence it. In addition, this thesis addresses the importance of poly-microbial solutions to enhance the plants' tolerance against poly-microbial disease complexes in an agricultural context; and it delineates the functional and ecological role of procurement of microbial communities by plants during germination.

9 Zusammenfassung

Pflanzen interagieren mit Bodenmikroorganismen, die eine positive Wirkung auf sie haben, wie z.B. Wachstumsförderung, Verbesserung der Nährstoffverfügbarkeit und Erhöhung der Toleranz gegen Krankheiten. Biotische und abiotische Faktoren, die das Mikrobiom der Wurzel beeinflussen, sind jedoch noch weitgehend unbekannt. Darüber hinaus ist nach wie vor nicht geklärt, warum und wie Pflanzen während der Keimung Bodenmikroorganismen erwerben, und welche ökologischen und funktionellen Rollen sie spielen. Die meisten Hochdurchsatz-Sequenzierungs-Studien beschränken sich auf Modell- oder Kulturpflanzen und es fehlt an der Ergänzung der Untersuchungen durch kultivierungsabhängige Verfahren, um die funktionalen Merkmale von dominanten und seltenen mikrobiellen Gemeinschaften aufzuklären. Bisher wurden polymikrobiellen Krankheitskomplexen und polymikrobieller Biokontrolle in der wissenschaftlichen Gemeinschaft wenig Beachtung geschenkt. Ziel der Dissertation war, diese interessanten Fragen zu beantworten.

In meiner Arbeit, untersuchte ich den Einfluss des Phytohormons Jasmonsäure (JA) und unterschiedlicher Pflanzenentwicklungsstadien in wildem Tabak (*N. attenuata*) auf Blatt- und Wurzelbakteriengemeinschaften. Wurzeln und Blätter wurden über fünf verschiedene Entwicklungsstadien vom Rosetten- bis zum Blütenstadium von im Feld gewachsenen Pflanzen -einer bereits charakterisierten isogenen Linie, die in der JA-Biosynthese (ir*AOC*) beeinträchtigt ist, und einer Linie, die nur mit dem Vektor (EV) transformiert wurde - geerntet. 454- Pyrosequenzierung und kultivierungsabhängige Verfahren wurden verwendet, um die bakterielle Gemeinschaft zu charakterisieren. Weder JA noch Pflanzenentwicklungsstadien beeinflussten die Bakteriengemeinschaft; der Gewebetyp hatte jedoch eine große Wirkung auf die Zusammensetzung der bakteriellen Gemeinschaft. Wurzeln beherbergten höhere bakterielle Vielfalt im Vergleich zu Blättern, und zwar unabhängig vom Genotyp. Die kultivierungs-unabhängigen Ergebnisse wurden durch einen kultivierungsabhängigen Ansatz bestätigt und die Untersuchungen zeigen, dass JA nicht die Zusammensetzung der Bakteriengemeinschaft von *N. attenuata* beeinflusst. Weiterhin sind bakterielle Effekte, die das Pflanzenwachstum fördern, unabhängig von der Fähigkeit der Pflanze, JA zu erzeugen.

Darüber hinaus, habe ich vermutet, dass Faktoren wie Bodentypen und die Antwort der Pflanzen auf UVB-Bestrahlung das Wurzelmikrobiom von *N. attenuata* prägen. Um diese

Hypothese zu testen, haben wir Wurzeln und Boden von natürlichen *N. attenuata* Populationen von verschiedenen Orten in der Great Basin Wüste, Utah, gesammelt. Die tiefe Sequenzanalyse ergab, dass Wurzelbakteriengemeinschaften weitgehend unabhängig von Bodentypen bzw. Standorten sind. Außerdem gruppierten in einer nichtmetrischen multidimensionalen Analyse (NMDS) die Bakteriengemeinschaften der Wurzeln getrennt von den verschiedenen Standorten /Bodentypen, obwohl die chemischen Eigenschaften der Böden unterschiedlich sind. Die bakterielle Vielfalt des Bodens ist höher im Vergleich zu Wurzeln. Das bedeutet, dass Pflanzen selektiv die mikrobiellen Gemeinschaften aus dem Boden rekrutieren, und zwar unabhängig vom Standort. Im Gegensatz dazu ist für Pilzgemeinschaften die Rekrutierung weniger selektiv. Interessanterweise wurde nur in Wurzeln von *N. attenuata* das Phylum Deinococcus-Thermus im Vergleich mit anderen Pflanzenarten nachgewiesen, und die hohe Abundanz in Wurzeln im Vergleich zum Boden deutet auf eine selektive Anreicherung der Gattung *Deinococcus* in *N. attenuata*-Wurzeln hin. Aufgrund der bekannten hohen Resistenz von *Deinococcus* gegenüber UV-Licht und dem natürlichen Lebensraum von *N. attenuata*, der durch hohe UVB-Strahlungsraten gekennzeichnet ist, wurde die Hypothese aufgestellt, dass die pflanzliche Reaktion auf UVB-Strahlung, der Rezeptor für die Wahrnehmung von UVB UV-Resistenz LOCUS 8 (UVR8) und die Signalantwort, das Enzym Chalkonsynthase für die Flavonoidbiosynthese (CHAL), molekulare Signale darstellen, um *Deinococcus-Thermus* zu rekrutieren. Wir kreiierten eine isogene Linie, die in der Expression von *UVR8* (ir*UVR8*) beeinträchtigt ist, und verwendeten sie zusammen mit einer zuvor charakterisierten Linie, die in der Flavonoid biosynthese beeinträchtigt ist (ir*CHAL*). In einem Mikrokosmosexperiment mit einer synthetischen Bakteriengemeinschaft, erhöhte sich die Kolonisation mit *Deinococcus* von Wildttyp-Wurzeln unter zusätzlicher UVB-Bestrahlung, aber nicht in den isogenen Linien, die in der UVB-Wahrnehmung (ir*UVR8*) und -Antwort (ir*CHAL*) beeinträchtigt sind.

In einer weiteren Studie beschäftigte ich mit einem aktuellen landwirtschaftlichen Dilemma und dem bereits seit langem bekannten Problem der Monokulturpraxis und Krankheitsausbrüchen. Die kontinuierliche Nutzung eines Versuchsfelds im natürlichen Lebensraum von *N. attenuata* für die letzten 15 Jahre ist vergleichbar mit einem landwirtschaftlichen Anbau und führte zur Entstehung einer plötzlichen Welkekrankheit, die durch den Fusarium und Alternaria Krankheitskomplex verursacht wird. Plötzliche Welkekrankheit ist durch einen akuten Zusammenbruch des Gefäßsystems gekennzeichnet, und die Wurzeln färben sich in diesem Zusammenhang schwarz. Drei verschiedene

Bekämpfungsstrategien, nämlich mikrobielle Biokontrolle, chemische Fungizidbehandlung und Bodenverbesserung, wurden unter Feldbedingungen getestet. Von 7 verschiedenen Behandlungskombination schützten in einem Feldversuch im Jahr 2013 nur bakterielle Konsortien die Pflanzen vor plötzlicher Welkekrankheit. Im Feldversuch im folgenden Jahr, 2014, reduzierten Bakterienkonsortien wiederum die plötzliche Welkekrankheit, was die Robustheit der Schutzwirkung dieses Ansatzes belegt. Die bakterielle Schutzwirkung ist unabhängig von Genotypen und hat keinen Einfluss auf ökologische Merkmale von *N. attenuata*. Darüber hinaus können bakterielle Konsortien aus zuvor mit Bakterien geimpften gesunden, im Feld gewachsenen Pflanzen reisoliert werden, was darauf hinweist, dass diese einheimischen Konsortien ausgezeichnete Wurzelkolonisatoren sind.

Zusammenfassend zeigt diese Arbeit, dass das Mikrobiom von *N. attenuata* nicht maßgeblich von JA, Pflanzenentwicklungsstadien und Standort geformt wird, während Gewebetypen und zusätzliche UV-B-Bestrahlung die mikrobiellen Wurzelgemeinschaften verändern. Darüber hinaus befasst sich diese Arbeit mit der Bedeutung von polymikrobiellen Lösungen zur Erhöhung der Toleranz der Pflanzen gegenüber polymikrobiellen Krankheitskomplexen im landwirtschaftlichem Kontext, und sie umreißt die funktionale und ökologische Rolle der Rekrutierung von mikrobiellen Gemeinschaften durch die Pflanze während der Keimung.

10 Bibliography

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11 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.

_____

Rakesh, Santhanam.

Jena, 06, 2016

12 Curriculum vitae

Rakesh Santhanam

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Mobile: 017684316960.

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Nationality: Indian.

Sex: Male.

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ACADEMIC QUALIFICATIONS

April 2012 – present **Max-Planck Institute for Chemical Ecology,** Jena, Germany.

PhD studies in the Department of Molecular Ecology under supervision of Prof. Ian T. Baldwin and Dr. Karin Groten.

Title "Nicotiana attenuata microbiome characterization and plant-bacterial interactions from single isolates to consortia."

Short description: Microbiota profiling of the model plant *Nicotiana atteunata* via next generation sequencing. Revealing biomechanism of plant protection against sudden wilt disease by pretreatment with a consortium of native bacteria.

2008-2009 **Newcastle University,** Newcastle upon Tyne, United Kingdom.

M.Sc in Industrial and Commercial Biotechnology, School of Biology.

Grade: Merit.

Research project Title: Selective isolation and characterization of novel soil streptomycetes.

Short description: Large numbers of putatively novel neutrophilic streptomycetes were isolated from a hay meadow soil, UK. Among 126, *Streptomyces* isolates- five isolates found to be novel.

2004-2008 **Anna University**, Chennai, Tamil Nadu, India.

B.Tech in Biotechnology, Engineering.

Grade: First class.

EMPLOYMENT

April 2012-present **MPI for Chemical Ecology,** Jena, Germany PhD fellowship.

May 2010-Jan 2012 **Demuris Ltd.** Newcastle University, United Kingdom.

Position- Junior Research Scientist.

Cultivation of diverse actinomycetes from marine and terrestrial habitats, followed by antibiotic bioassay screening against clinical pathogens and reporter strains, detection of novel bioactive compounds using chromatography techniques and mass spectrometry.

Supervision of M.Sc student project and preparation of monthly laboratory research reports for management meetings.

Sept 2009 - May 2010 **Microbial Laboratory.** School of Biology, Newcastle University.

Position-Guest Scientist.

Initial studies focussed on my M.Sc project along with the isolation of actinomycetes from marine sponges, Mariana Trench and Atacama Desert soils by culture dependent and culture independent techniques. This research resulted in four publications.

Supervision of undergraduate project students and running the laboratory in the absence of the research technician.

RESEARCH SKILLS

Microbes: Isolation and functional characterization of bacterial and fungal isolates from biotic and abiotic samples.

Bioassays: Characterizing functional traits of bacterial isolates e.g. bioactive natural products, enzyme production, biofilm formation, siderophore production and adaptation of bioassays to the systems used.

Bioinformatics: DNA/Protein sequence analysis, phylogenetics, pyrosequencing and microbiota community analysis.

Chemistry: Chromatographic techniques; HPLC, TLC and Mass spectrometry (MS/MS).

Microscopy: SEM, Fluorescent, confocal and light sheet microscopy.

Molecular Biology: Fluorescence *in situ* hybridization (FISH), qPCR, Gene cloning, DGGE, Finger printing and primer design.

Protein Biochemistry: Protein purification, SDS-PAGE , Western blotting and Southern blotting.

ICT: Statistics, R, bioinformatics tools; Clustal X, Bioedit, MEGA, PHYLIP, TREECON, QIIME. MS Office; MS Word, Excel, PowerPoint. Operating system: Windows and Ubuntu.

Curriculum vitae

AWARDS

- 1st poster prize-IMPRS symposium 2016.
- Awarded PhD scholarship from Max-Planck Society.
- Selected among 12 students from Europe for the EU-funded microbial workshop "Facilis 2015", Milan. All costs were covered for the selected scientists.
- EPSO Young Plant Scientist Award: among the top 8 applicants short-listed by the jury.

EXTRACURRICULAR ACTIVITIES.

- Cooking, swimming, spinning and cricket.

LANGUAGES.

- English, Tamil and Telugu.

MEDIA REPORTS.

- Max-Planck newsletter: http://www.mpg.de/9373626/bacteria-tabacco-root-disease.
- EPSO newsletter: http://www.epsoweb.org/node/18.

PUBLICATIONS

- **Santhanam, R.**, Oh, Y, Weinhold, A., Thi Luu, V. Groten K, Baldwin, I. T. (2016). Native grown *N. attenuata* root microbiome is independent of soil type and UVB increases root colonization of *Deinococcus*. *New Phytologist* (to be submitted).
- **Santhanam, R.**, Thi Luu, V., Weinhold, A., Goldberg, J., Oh, Y, Baldwin, I. T. (2015). Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping. *Proceedings of the National Academy of Sciences of the United States of America*. Doi 10.1073/pnas.1505765112.
- **Santhanam R**, Baldwin IT and Groten K (2015). "In wild tobacco, *Nicotiana attenuata*, variation among bacterial communities of isogenic plants is mainly shaped by the local soil microbiota independently of the plants' capacity to produce jasmonic acid" *Communicative & Integrative Biology*, e1017160.
- **Santhanam R**, Groten K, Meldau DG, and Baldwin IT (2014) "Analysis of plant-bacteria interactions in their native habitat: bacterial communities associated with wild tobacco are independent of endogenous jasmonic acid levels and developmental stages" *PLoS ONE*, 9:e94710.

- Aizenberg-Gershtein, Y., Izhaki, I., **Santhanam, R**., Kumar, P., Baldwin, I. T., Halpern, M.(2015). Pyridine-type alkaloid composition affects bacterial community composition of floral nectar. *Scientific Reports*, 5: 11536.
- Groten, K., Nawaz, A., Nguyen, N. T. H., **Santhanam, R**., Baldwin, I. T. (2015). Silencing a key gene of the common symbiosis pathway in Nicotiana attenuata specifically impairs arbuscular mycorrhizal infection without influencing the root-associated microbiome or plant growth. *Plant, Cell and Environment*, 38(11), 2398-2416.
- **Santhanam R**, Huang Y, Rong X, and Goodfellow M (2013) *Streptomyces erringtonii* sp. nov. & *Streptomyces kaempferi* sp. nov., isolated from a hay meadow soil *Antonie Van Leeuwenhoek* 103(1):79-87
- **Santhanam R**, Huang Y, Rong X, Andrews B.A, Asenjo A.J and Goodfellow M (2013) *Streptomyces bullii* sp.nov., isolated from a hyper-arid Atacama Desert soil. *Antonie Van Leeuwenhoek* 103(2):367-73.
- Rateb ME, Hallyburton I, Houssen WE, Bull AT, Goodfellow M, Santhanam R, Jasparsa M and Ebel R (2013). Induction of diverse secondary metabolites in Aspergillus fumigatus by microbial co-culture. RSC Advances (3):14444-14450.
- **Santhanam R**, Okoro C.K, Huang Y, Rong X, Bull A.T, Andrews B.A, Asenjo A.J , Weon H.Y and Goodfellow M (2012) *Streptomyces deserti* sp.nov., isolated from hyper-arid desert soil. *Antonie Van Leeuwenhoek* 101(3):575-81.
- **Santhanam R**, Okoro C.K, Bull A.T, Huang Y, Rong X, and Goodfellow M (2012) *Streptomyces atacamensis* sp.nov., isolated from hyper-arid desert soil. *International Journal of Systematic and Evolutionary Microbiology* 62:2680-2684.

Selected Oral presentation

- **Santhanam R.** (2015). Native bacterial consortia protect their host plant *Nicotiana attenuata* from fungal sudden wilt disease via synergistic mechanism. Talk presented at ICE SYMPOSIUM, MPI FOR CHEMICAL ECOLOGY, Jena, DE.
- **Santhanam R.** (2015). Native root-associated bacteria protect their host plant from a fungal sudden-wilt disease via Induced Systematic Resistance and allelopathy. Talk presented at 14TH IMPRS SYMPOSIUM, MPI FOR CHEMICAL ECOLOGY, Dornburg, DE.

- **Santhanam R.** (2014). Bacterial community interactions with *Nicotiana attenuata*. Talk presented at FACILIS- course organized by US-EU task force on environmental microbiology at UNIVERSITY OF MILAN, Milan, Italy.
- **Santhanam R.** (2014). Bacterial community interactions with *Nicotiana attenuata*. Talk presented at LIMNOLOGISCHES KOLLOQUIUM, FRIEDRICH-SCHILLER-UNIVERSITÄT JENA, INSTITUTE FÜR ÖKOLOGIE, AQUATIC GEOMICROBIOLOGY/LIMNOLOGY, Jena, DE.
- **Santhanam R.** (2014). Bacterial endophytic community interactions with phytohormone JA and developmental stages. Talk presented at 13TH IMPRS SYMPOSIUM, MPI FOR CHEMICAL ECOLOGY, Dornburg, DE.

Selected Poster presentation:

- **Santhanam R., Luu V.T., Weinhold A., Groten K., Baldwin I.T.** (2016). Native root-associated bacteria protect their host plant from a fungal sudden-wilt disease via synergistic mechanism Poster presented at VAMM ANNUAL CONFERENCE, Jena, DE.
- **Santhanam R., Luu V.T., Weinhold A., Groten K., Baldwin I.T.** (2016). Native root-associated bacteria protect their host plant from a fungal sudden-wilt disease via synergistic mechanism. Poster presented at15TH IMPRS SYMPOSIUM, MPI FOR CHEMICAL ECOLOGY, Dornburg, DE.
- **Santhanam R., Luu V.T., Weinhold A., Baldwin I.T.** (2014). Opportunistic root associated bacteria protect *N. attenuata* from a wilt disease which arose from continuous cropping. Poster presented at ICE SYMPOSIUM, MPI FOR CHEMICAL ECOLOGY, Jena, DE.
- **Ferrieri A., Gulati J., Gaquerel E., Machado R.A.R., Erb M., Santhanam R., Weinhold A., Wang M., Wilde J., Groten K., Oh Y., Fragoso V., Kim S., Xu S., Baldwin I.T.** (2014). Roots: where it all starts. Poster presented at SAB MEETING 2014, MPI FOR CHEMICAL ECOLOGY, Jena, DE.
- **Santhanam R, Groten K., Baldwin I.T.** (2013). Endophytic bacterial communities at species and class level are complex and differ between field grown *Nicotiana attenuata* plants, independent of their developmental stages and JA. Poster presented at 12TH IMPRS SYMPOSIUM, MPI FOR CHEMICAL ECOLOGY, Jena, DE.

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