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The microbiome of medicinal plants and its potential for biocontrol and promotion of plant growth and quality

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Abstract

Past medicinal plant research primarily focused on bioactive phytochemicals. Currently, however, focus is shifting due to the growing recognition that a significant number of phytotherapeutic compounds are actually produced by associated microbes or through interaction with their host plant. Plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years. These medicinal plants provide a largely untapped and enormous bioresource of potential use in modern medicine, agriculture, and pharmaceuticals, yet their microbiome is mostly unknown. Therefore, the aim of this study was to analyse the microbiome of three different species of medicinal plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. and Thonn.). The plants cultivated on organically managed Egyptian desert farm were investigated and compared to surrounding field and desert soil. The soil microbiome of the desert ecosystem was comprised of a high abundance of Gram-positive, spore-forming bacteria (Firmicutes) which were ascertained to be of prime importance for pathogen suppression under arid conditions. A clearly plant-specific selection of the associated microorganisms was observed, whereas native desert antagonists with promising activity against a wide range of soil-borne phytopathogens were enriched in all investigated plant roots. The anthropogenically influenced ecosystem exhibited a higher microbial diversity and better ecosystem function for plant health in comparison to the natural desert soil. Conversely, several extremophilic bacterial groups decreased or completely disappeared from soil after agricultural use. The diazotrophic soil community was dominated by Alphaproteobacteria, and the nitrogen fixing microbiome was specific for each medicinal plant as well. While the rhizosphere inhabitants of the annual herbal Asteraceae were similar and dominated by potential root-nodulating rhizobia, the perennial *S. distichum* primarily formed associations with free-living nitrogen fixers. Rhizobia were ascertained in soil as well, but the free-living bacteria were most likely transmitted between plants. This high degree of plant-specificity identified plant species and their features as important drivers in functional diversity. The fungal microbiome was characterised by potential pathogens, and different desert habitats were screened for potential biological control agents adapted to the unique and arid conditions of desert farming to biologically control them. In a hierarchical evaluation including antifungal, antibacterial, and nematicidal activity, three promising drought and heat resistant biocontrol candidates were selected for field *ad planta* evaluation in comparison to three allochthonous Gram-negative strains already known for beneficial plant-microbe interactions in humid soils. The priming of chamomile seedlings with the autochthonous strains not only showed a stabilising effect on plant performance, but indigenous Bacillales strains were also able to elevate the plants' flavonoid production. These results demonstrate that a targeted bacterial treatment could influence the metabolic activity of the plant, and therefore represent one of many poorly understood links between the structure and metabolic profile of the plant-associated microbiome and the plant metabolome.

Zusammenfassung

Die Forschung an Arzneipflanzen konzentrierte sich bisher in erster Linie auf ihre bioaktiven Pflanzeninhaltsstoffe. Aufgrund der wachsenden Erkenntnisse, dass eine erhebliche Anzahl von phytotherapeutischen Verbindungen tatsächlich von assoziierten Mikroorganismen oder durch deren Interaktion mit ihrer Wirtspflanze produziert werden, hat sich der Fokus jedoch verlagert. Pflanzliche Arzneimittel sind in den meisten Teilen der Welt seit Tausenden von Jahren ein Teil der traditionellen Gesundheitsversorgung. Diese Arzneipflanzen bieten eine weitgehend unerschlossene und umfangreiche Bioressource mit möglicher Verwendung in der modernen Medizin, Landwirtschaft und Pharmaindustrie, ihr Mikrobiom ist aber größtenteils unbekannt. Das Ziel dieser Arbeit war es deshalb, die Mikrobiome von drei verschiedenen Arten von Arzneipflanzen (*Matricaria chamomilla* L., *Calendula officinalis* L. und *Solanum distichum* Schumach. und Thonn.) zu analysieren. Die Pflanzen, welche auf einer ökologisch bewirtschafteten ägyptischen Wüstenfarm angebaut wurden, wurden untersucht und mit dem umliegenden Acker- und Wüstenboden verglichen. Das Bodenmikrobiom des Wüstenökosystems beinhaltete eine große Fülle an Gram-positiven, sporenbildenden Bakterien (Firmicutes), für welche eine zentrale Bedeutung in der Unterdrückung von Pathogenen unter ariden Bedingungen festgestellt wurde. Es wurde eine deutlich pflanzenspezifische Auswahl der assoziierten Mikroorganismen beobachtet, wobei heimische Wüstenantagonisten mit einer vielversprechenden Aktivität gegen ein breites Spektrum von bodenbürtigen Phytopathogenen in allen untersuchten Pflanzenwurzeln angereichert wurden. Das anthropogen beeinflusste Ökosystem zeigte eine höhere mikrobielle Diversität und eine bessere Ökosystemfunktion für Pflanzengesundheit im Vergleich zum natürlichen Wüstenboden. Durch die landwirtschaftliche Nutzung sind jedoch mehrere extremophile bakterielle Gruppen zurückgegangen oder vollständig aus dem Boden verschwunden. Die diazotrophe Bodengemeinschaft war stark von Alphaproteobakterien dominiert; das stickstofffixierende Mikrobiom war ebenfalls für jede Arzneipflanze spezifisch. Während die Rhizosphärenbewohner der einjährigen krautigen Asteraceae ähnlich waren und von potenziell wurzelknöllchenbildenden Rhizobien dominiert waren, bildete der mehrjährige *S. distichum* vor allem Assoziationen mit freilebenden Stickstofffixierern. Die Rhizobien wurden auch im Boden nachgewiesen, aber die freilebendenden Bakterien werden möglicherweise zwischen Pflanzen übertragen. Dieses hohe Maß an Pflanzenspezifität identifizierte die Pflanzenspezies und ihre Eigenschaften als wichtigen Faktor der funktionellen Diversität. Das pilzliche Mikrobiom war von potenziellen Pathogenen gekennzeichnet. Um diese biologisch zu kontrollieren, wurden verschiedene Wüstenhabitate auf potenzielle biologische Kontrollstämme durchsucht, welche an die einzigartigen und ariden Bedingungen der Wüsten-Landwirtschaft angepasst sind. In einer hierarchischen Bewertungsskala, welche antimykotische, antibakterielle und nematizide Aktivität umfasste, wurden drei vielversprechende trockenheits- und hitzeresistente Biokontroll-Kandidaten für eine *ad planta* Beurteilung im Feld im Vergleich zu drei allochthonen Gram-negativen Stämmen, welche bereits für ihre nützlichen Pflanzen-Mikroben-Interaktionen in humiden Böden bekannt sind, ausgewählt. Die Behandlung von Kamillensetzlingen mit den autochthonen Stämmen zeigte nicht nur eine stabilisierende Wirkung auf die Pflanzenleistung, die heimischen Bacillales Stämme waren auch in der Lage, die Flavonoidproduktion der Pflanzen zu erhöhen. Diese Resultate beweisen, dass eine gezielte bakterielle Behandlung die metabolische Aktivität der Pflanze beeinflussen konnte und stellen damit eine von vielen kaum verstandenen Schnittstellen zwischen der Struktur und dem metabolischen Profil des pflanzenassoziierten Mikrobiomes und dem Metabolom der Pflanze dar.

Introductory Manuscript

Submitted as Review Article

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Submitted

The plant-associated microbiome: introduction into structure and function

All plant-associated microenvironments are colonised in high abundances by microorganisms, especially the nutrient-rich rhizosphere which hosts a plethora of microbes that are of central importance for plant nutrition, health and quality (Hiltner, 1904 in Hartmann et al., 2008; Berg, 2009; Mendes et al., 2011). Rhizospheres can host up to 10^{11} microbial cells per gram root with more than 30,000 different prokaryotic species (Berendsen et al., 2012). These rhizosphere microorganisms from a highly diverse reservoir of soil microbes are attracted by the rhizosphere's plant root secretions and rhizodeposits (Compant et al., 2010), and driven via the compositional variability of these exudates (Bais et al., 2006; Doornbos et al., 2012). Each plant species harbours a specific rhizosphere microbiome dependent of the present soil community (Smalla et al., 2001). Besides plant species, the composition and diversity of microbial rhizosphere communities is shaped by soil type and pedoclimate, plant health and developmental stage, climate and season, pesticide treatments, grazers and animals, and several other biotic and abiotic factors (Berg & Smalla, 2009). Some genera are ubiquitous and can be found distributed over the entire plant, such as the well-known plant-associated genera *Bacillus* and *Pseudomonas* (Berg et al., 2011). However, a high degree of specificity for each microenvironment was also observed via comparison of microbial colonisation patterns of different microhabitats (Berg et al., 2005b; Fürnkranz et al., 2012; Köberl et al., 2013). Certain plant-associated microorganisms including beneficials and pathogens are also passed down from previous generations via the seed (Hardoim et al., 2012; Hirsch & Mauchline, 2012), and likewise a transmission between plants through pollen grains was recently observed (Fürnkranz et al., 2012). Interestingly, the phylogenetically oldest land plants, mosses, transfer a highly diverse core microbiome of primarily potential beneficial bacteria from the sporophyte to the gametophyte and vice versa (Bragina et al., 2012).

Medicinal plants harbour a unique microbiome due to their unique and structurally divergent bioactive secondary metabolites that are most likely responsible for the high specificity of the associated microorganisms. In the past, medicinal plant research focused primarily on their ingredients, however recently the focus has shifted to include the structure and function of several medicinal plant microbiomes. Surprisingly, not only were the plants themselves able to produce substances with phytothereapeutic properties, but their associated microbes, especially endophytes, could as well (Table 1). The analyses of several Chinese medicinal plant microbiomes showed interesting results (*Ainsliaea henryi* Diels, *Dioscorea opposite*, *Potentilla discolor* Bge, *Stellera chamaejasme* L., *Ophiopogon japonicus* (Thunb) Ker-Gawl., *Juncus effusus* L. var. *decipiens* Buchen., *Rhizoma arisaematis* and others) (Li et al., 2008; Zhao et al., 2011), as each of them hosted a specific actinobacterial community and showed a remarkably high and diverse rhizospheric and endophytic colonisation with Actinobacteria featuring both antimicrobial and antitumour properties (Zhao et al., 2012). These Gram-positive and often spore-forming bacteria are promising biological control agents (BCAs), such as the genus *Streptomyces* that is a known and unique source of novel antibiotics (Goodfellow & Fiedler, 2010; Niraula et al., 2010; Nachtigall et al., 2011; Raaijmakers & Mazzola, 2012). Similarly, a high plant-specificity of associated microbial communities was also observed for pharmaceutical plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. and Thonn.) cultivated under desert farming conditions in Egypt (Köberl et al., 2013).

Medicinal plants: ingredients and applications

Plants contain numerous different biologically active compounds, and plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of

Table 1 Examples for bioactive phytometabolites where microorganisms are involved in their production.

Bioactive compound	**Therapeutic properties**	**Host plant**	**Producing microorganism**	**Reference**
Munumbicins	antibacterial, antimycotic, antiplasmodial	*Kennedia nigriscans*	*Streptomyces* sp.	Castillo et al, 2002
Kakadumycins	antibacterial, antiplasmodial	*Grevillea pteridifolia*	*Streptomyces* sp.	Castillo et al, 2003
Coronamycins	antimycotic, antiplasmodial	*Monstera* sp.	*Streptomyces* sp.	Ezra et al., 2004
Oocydin A	antimycotic (Oomycota)	*Rhyncholacis penicillata*	*Serratia marcescens*	Strobel et al., 1999a
Cryptocandin	antimycotic	*Tripterigeum wilfordii*	*Cryptosporiopsis quercina*	Strobel et al., 1999b
Colletotric acid	antibacterial, antimycotic	*Artemisia mongolica*	*Colletotrichum gloeosporioides*	Zou et al., 2000
Artemisinin	antiplasmodial	*Artemisia annua*	*Colletotrichum* sp.	Wang et al., 2001
Cochliodinol	antibacterial, antimycotic, anticancer	*Salvia officinalis*	*Chaetomium* sp.	Debbab et al., 2009
Botryorhodines	antimycotic, anticancer	*Bidens pilosa*	*Botryosphaeria rhodina*	Abdou et al., 2010
Pestacin and Isopestacin	antimycotic, antioxidant	*Terminalia morobensis*	*Pestalotiopsis microspora*	Strobel et al., 2002; Harper et al., 2003
Phomol	antiphlogistic, antibacterial, antimycotic, anticancer	*Erythrina crista-galli*	*Phomopsis* sp.	Weber et al., 2004
Podophyllotoxin	anticancer, antiphlogistic	*Podophyllum hexandrum*	*Alternaria* sp.	Yang et al., 2003
		Juniperus communis	*Aspergillus fumigatus*	Kusari et al., 2009a
Paclitaxel (Taxol)	anticancer	*Taxus brevifolia*	*Taxomyces andreanae*	Wani et al., 1971; Stierle et al., 1993
		Ginkgo biloba	*Alternaria* sp.	Kim et al., 1999
		Aloe vera	*Phoma* sp.	Immaculate et al., 2011
Camptothecin	anticancer, antiviral (HIV)	*Nothapodytes foetida*	*Entrophospora infrequens*	Puri et al., 2005; Amna et al., 2006
		Camptotheca acuminate	*Fusarium solani*	Kusari et al., 2009b
Maytansine	anticancer	*Putterlickia verrucosa*	*Actinosynnema pretiosum*	Wings et al., 2012
Rohitukine	antiphlogistic, anticancer, immuno-modulatory	*Dysoxylum binectariferum*	*Fusarium proliferatum*	Mohana Kumara et al., 2012
Subglutinols	immuno-modulatory	*Tripterigeum wilfordii*	*Fusarium subglutinans*	Lee et al., 1995

years. Traditional Chinese medicine (TCM), phytotherapeutic knowledge from the Mayans, the aboriginal medicine of Australia, and several other cultures comprise a huge spectrum of natural remedies that can be exploited as sources for new and effective therapeutic agents. Still widely practiced in the modern era, TCM supplies ethnopharmaceutical knowledge on over 5,000 plant species used for the treatment of numerous diseases and has already provided the basis for the discovery of many modern drugs, such as anticancer agents (Miller et al., 2012a, 2012b). In general, natural products play a highly considerable role in the drug discovery and development process, as about 26% of the new chemical entities introduced into the market worldwide from 1981 to 2010 were either natural products or those derived directly therefrom, reaching a high of 50% in 2010 (Newman & Cragg, 2012). Currently, research continues to show that a significant number of natural products are actually produced by microbes and/or microbial interactions with the host from whence they were isolated (Gunatilaka, 2006), and for several medicinal plants it is presumed that the plant-associated microbiome, especially the complex community of the endomicrobiome, is directly or indirectly involved in the production of bioactive phytochemicals. Presently, however, only a small subset of potential microbial strains could be definitively attributed to phytotherapeutic properties (Strobel et al., 2003, 2004; Chandra, 2012; Miller et al., 2012a, 2012b), and their relative contribution to the recognised valuable bioactivity of medicinal plants is not clear as of yet. In regards to the alarming incidence of antibiotic resistance in bacteria with medical relevancy, medicinal plants with antibacterial properties are of central importance as bioresources for novel active metabolites (Palombo & Semple, 2001). Likewise, there is an increasing need for more and better antimycotics to treat those with weakened immune systems who are more prone to developing fungal infections, such as from the AIDS epidemic, cancer therapy, or organ transplants (Strobel et al., 2003, 2004). For centuries, several phytotherapeutics have also been known for their antiphlogistic features, yet despite the progress within medical research, chronic inflammatory diseases such as asthma, arthritis,

and rheumatism remain one of the world's leading health problems (Li et al., 2003). Hypertension is another critical issue for human health and is a primary risk factor for stroke, heart disease, and renal failure. Many herbal remedies as well as foods, however, are known and effective folk medicines in the prevention and/or treatment of high blood pressure (Abdel-Aziz et al., 2011). Hence, nature must still harbour plenty of currently unknown active agents that may serve as leads and scaffolds for the development of desperately needed efficacious drugs for a multitude of diseases (Newman & Cragg, 2012). Today, globalisation has also had an impact on the use of medicinal plants and has proven beneficial in allowing greater access to these medicines for people all across the globe. For example, TCM plants are very popular in Europe, whereas the traditional German chamomile is primarily produced in Egypt. Three popular species of ethnobotanical plants (Fig. 1) with promising medicinal value and their bioactive properties are illustrated below.

***Matricaria chamomilla* L.:** The German chamomile *Matricaria chamomilla* L. (synonyms *Matricaria recutita* L. and *Chamomilla recutita* (L.) Rauschert) is well-known in folk medicine and one of the most favoured and most frequently used medicinal plants cultivated all over the world (Šalamon, 2004). It is believed to possess antiphlogistic, vulnerary, deodorant, bacteriostatic, antimicrobial, anticatarrhal, carminative, sedative, antiseptic, and spasmolytic properties (Murti et al., 2012). Sesquiterpenes (α-bisabolol, bisabolol-oxides A and B, farnesene), sesquiterpenelactones (chamazulene), acetylene-derivatives (spiroethers), as well as flavonoids (apigenin, quercetin, patuletin, luteolin) and their glucosides are some of the many compounds that are responsible for the medicinal properties (Ganzera et al., 2006; McKay & Blumberg, 2006).

***Calendula officinalis* L.:** The pot marigold *Calendula officinalis* L. is one of the best known versatile medicinal plants and is also a popular domestic remedy as well. The phytotherapeutic plant is rich in biologically active metabolites such as sesquiterpenes, alcohol, saponins, triterpenes, flavonoids, hydroxycoumarin, carotenoids (flavoxanthin,

auroxanthin, lutein, zeaxanthin), tannin, and essential oils (Roveroni-Favaretto et al., 2009). The entire plant, but especially the flowers and leaves, is antiphlogistic, antiseptic, spasmolytic, aperient, astringent, cholagogue, diaphoretic, emmenagogue, stimulative, vulnerary, and is attributed to a particularly positive impact on skin function (Ukiya et al., 2006; Butnariu & Coradini, 2012).

***Solanum distichum* Schumach. and Thonn.:** The semi-domesticated African nightshade *Solanum distichum* Schumach. and Thonn. (synonyms *Solanum indicum* ssp. *distichum* Schumach. and Thonn. and *Solanum anguivi* Lam.) is cultivated for culinary and medicinal purposes in many parts of Africa and the Arabian Peninsula (Bukenya-Ziraba, 2004). The edible fruits are rich in starch, calcium, vitamin A, ascorbic acid, and phosphate and contain steroid glycosides and glycol-alkaloids (Ripperger & Himmelreich, 1994; Zhu et al., 2000; Honbu et al., 2002; Abouzid et al., 2008). In traditional African folk medicine, these berries are claimed to guard against cardiovascular disorders (Abdel-Aziz et al., 2011), and their promising anti-hypertensive properties and low toxicity were validated in a rat model by Bahgat et al. (2008). Interestingly, *S. distichum* is resistant to many phytopathogens and is rarely infested by diseases and pests that affect many other *Solanum* species, such as against bacterial wilt caused by *Ralstonia solanacearum* (Bukenya-Ziraba, 2004).

Fig. 1 *Matricaria chamomilla* L. (A), *Calendula officinalis* L. (B), and *Solanum distichum* Schumach. and Thonn. (C).

Plant growth promotion and biological control by plant-associated microorganisms

Several rhizosphere microbes interact beneficially via different mechanisms with their host plant. They can have a direct plant growth promoting effect based on improved nutrient acquisition or hormonal stimulation, or indirectly affect the plant health by suppression of phytopathogens (Berg, 2009, Lugtenberg & Kamilova, 2009). Biofertilisers are microbes that supply the plant with nutrients, for example symbiotic root-nodulating rhizobia are the most prominent among the nitrogen-fixing microorganisms. Other microbial biofertilisers, such as mycorrhizal fungi and several rhizobacteria, are able to solubilise plant-available phosphate from either organic or inorganic bound phosphate (Lugtenberg et al., 2002). Microbes that hormonally promote plant growth are termed phytostimulators, and the phytohormone auxin, for instance, produced by fluorescent pseudomonads is one of the best understood examples. Some rhizobacteria, for example strains of the *Bacillus subtilis* group, stimulate plant growth using volatile organic compounds (VOCs) (Ryu et al., 2003; Kai et al., 2007). Indirectly, however, the plant growth can be promoted via biological control of phytopathogens. Pathogen growth can be inhibited by diffusible antibiotics or VOCs, toxins, biosurfactants, or extracellular cell wall-degrading enzymes, but microbial antagonism can also occur via degradation of pathogenicity factors like toxins, or simply by the competition for nutrients, minerals, or colonisation sites (Berg, 2009). Another possible way to reduce the activity of pathogenic microorganisms is the activation of the plant defence mechanisms, or the so called induced systemic resistance (ISR) triggered by certain non-pathogenic rhizobacteria. Flagella, lipopolysaccharides, siderophores, and several other bacterial components are thought to be involved in activating the non-pathogenic rhizobacteria-mediated ISR signalling pathway (van Loon et al., 1998; Lugtenberg & Kamilova, 2009).

Biological control of plant pathogens as well as plant growth promotion with microorganisms has been intensively studied over the past decades and is becoming a realistic

alternative to chemical pesticides and fertilisers in sustainable agriculture (Weller, 2007). Several microbial inoculants have already been successfully commercialised (Berg et al., 2009, 2013), but a specific biological control strategy for medicinal plants, which are increasingly affected by different soil-borne phytopathogens, has not been available until now. Traditional medicinal plants are often consumed raw, such as berries or other edible fruits, or in dried form as herbal brews or teas. Therefore, it is essential that any potentially harmful effect of the biocontrol agent on human health be avoided completely. In addition to the suppression of phytopathogens, antagonistic activity against potentially harmful human pathogens should also be considered in the biocontrol strategy (Berg et al., 2005a). The medicinal plant-associated microbiomes with their outstanding metabolic activities also provide a promising source for novel BCAs.

In conclusion, medicinal plants should be considered as meta-organisms that comprise both the plant themselves and their microbiome. As meta-organisms, they are a largely untapped and enormous bioresource for bioactive compounds and microorganisms of potential use in modern medicine, agriculture, and pharmaceutical industry. As such, more research is necessary to exploit this immense reservoir for mankind.

A case study: the microbiome of medicinal plants grown on a desert farm under organic management

In comparison to soils of humid areas, the soil microbiome of the Egyptian desert farm "Sekem" was comprised of a high abundance of Gram-positive, spore-forming bacteria primarily of the Firmicutes branch with 37% of the total bacterial soil community as revealed through a pyrosequencing-based amplicon sequencing approach (Köberl et al., 2011). However, a global soil community analysis including 32 libraries of 16S rRNA and 16S rRNA gene libraries from a variety of soils reported Firmicutes contribute a mean of only 2%

in the total bacterial soil community (Janssen, 2006). *Bacillus* and *Paenibacillus* play the key role in explaining this remarkably high abundance of Firmicutes in the investigated desert agro-ecosystem. These drought-resistant genera are of prime importance for pathogen suppression under arid conditions as nearly all isolated antagonists with activity against soil-borne phytopathogenic fungi could be affiliated to this taxonomic group. This is in direct contrast to humid soils, where primarily Gram-negative bacteria like *Pseudomonas* are responsible for the indigenous antagonistic potential (Berg et al., 2005b; Haas & Défago, 2005, Costa et al., 2006; Zachow et al., 2008). A significantly higher proportion of Firmicutes and antifungal isolates were observed in field soil from the Egyptian farm than in the surrounding desert soil uninfluenced by human activities. In general, the total bacterial soil microbiome of the anthropogenic ecosystem exhibited a higher diversity and better ecosystem function for plant health in comparison to the natural desert soil (Fig. 2). Due to the long-term agricultural use of the desert and the associated increasing occurrence of plant-pathogens, the indigenous antagonistic potential in soil was almost twice as high as in the uncultivated desert soil. However, the diversity of antagonistic bacteria was lower and highly dominated by isolates of the *Bacillus subtilis* group. The most efficient antagonists from the native desert soil belonged to *Streptomyces*, and *Bacillus* and *Paenibacillus* species were the most frequently isolated antagonists from all investigated arid habitats including both desert and agriculturally used soil, as well as from the rhizosphere and endorhiza of three different species of medicinal plants cultivated on the desert farm (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. and Thonn.). None of the plants are native to Egypt, and therefore were exposed to a previously unencountered microbiome. Interestingly, despite a clearly plant-specific selection of the associated bacterial microbiome, indigenous *Bacillus* and *Paenibacillus* strains of native desert soil with promising antagonistic properties against a wide range of soil-borne phytopathogens were enriched in all investigated plant roots. Conversely, several extremophilic bacterial groups, such as *Acidimicrobium*,

Rubellimicrobium, and *Deinococcus-Thermus* decreased or completely disappeared from soil after agricultural use (Köberl et al., 2011).

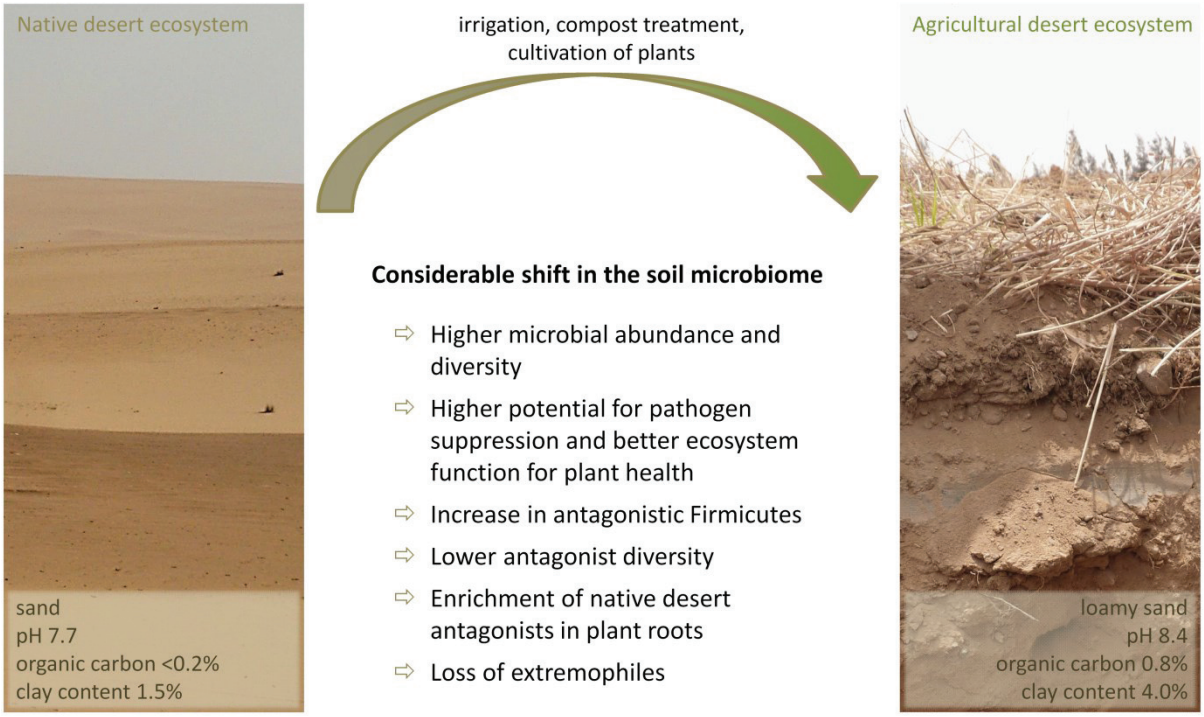

Fig. 2 Community shift in the soil microbiome after long-term agricultural use of the desert under organic management.

Nitrogen is an essential macronutrient for plants and one of the most yield-limiting factors in agricultural production systems throughout the world (Bhattacharjee et al., 2008; Orr et al., 2011). To gain insight into the indigenous community of diazotrophic plant growth promoting microorganisms that inhabit desert agro-ecosystems, community profiles of the *nifH* gene encoding the nitrogenase reductase subunit were assessed. A broad diversity and high abundance of diazotrophs were detected in all investigated habitats, thus underlining their importance in native and agricultural desert ecosystems. Due to watering and cultivation of desert soil, a considerable shift towards a higher abundance and diversity was also observed for the nitrogen-fixing community. Phylogenetic analyses distinguishing between the major *nifH* gene types (Zehr et al., 2003; Gaby & Buckley, 2012) revealed that all NifH

sequences from soil libraries were affiliated with the canonical *nifH* clusters I (conventional molybdenum nitrogenases) and III (molybdenum nitrogenases from anaerobes), while no sequences of alternative nitrogenases (cluster II) and *nifH* paralogs (clusters IV and V) were found. In general, the diazotrophic soil microbiota was highly dominated by NifH sequences related to Alphaproteobacteria. Each investigated medicinal plant cultivated on the desert farm harboured a specific root-associated diazotrophic microbiome. The rhizosphere inhabitants of *M. chamomilla* (Fig. 3) and *C. officinalis* were similar and both dominated by potential root-nodulating rhizobia acquired mainly from soil. Conversely, the rhizosphere of *S. distichum* was colonised in higher abundances by free-living nitrogen fixers most likely transmitted between plants as they were undetectable in soils. Although well known for taxonomic community structure (Berg & Smalla, 2009, Bulgarelli et al., 2012), this high degree of plant-specificity identified plants as important drivers for functional diversity as well (Köberl et al., unpublished). The total bacterial and fungal communities also revealed similar colonisation patterns between the medicinal plants *M. chamomilla* and *C. officinalis* compared to *S. distichum* (Köberl et al., 2013). This effect may have been intensified as a result of the close relationship between *M. chamomilla* and *C. officinalis* who both belong to the Asteraceae family and therefore produce more similar bioactive metabolites. Furthermore, both *M. chamomilla* and *C. officinalis* are annual herbal medicinal plants, while *S. distichum* is a perennial plant thus providing a longer timeframe to specifically select a stable associated microbiome.

In contrast to the highly specific bacterial communities associated with cultivated medicinal plants, fungal communities were less discriminative and characterised primarily by potential pathogens. Phytopathogenic species *Fusarium*, *Verticillium*, and several others were frequently identified, and, apart from *Rhizoctonia*, were the main soil-borne pathogens on the investigated desert farm that caused high yield losses on a wide host range of economically

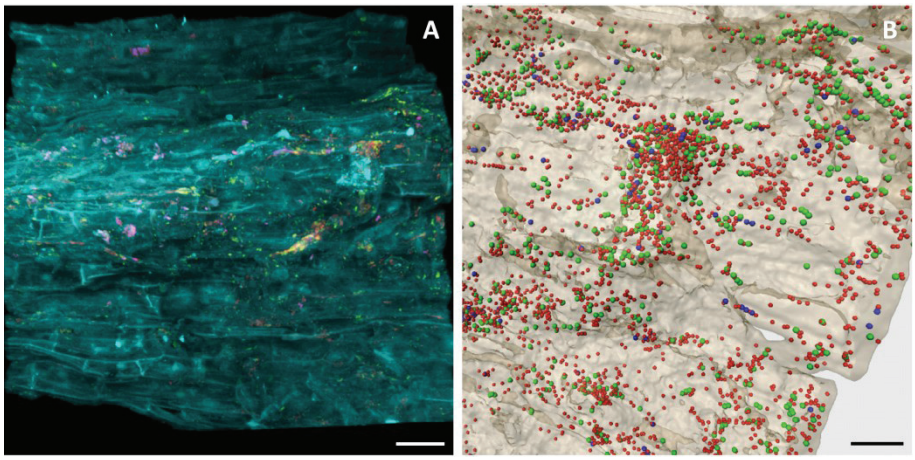

Fig. 3 *In situ* visualisation of the bacterial root colonisation of *Matricaria chamomilla*. Volume rendering (A) and three-dimensional reconstruction model (B) of confocal laser scanning microscopy stacks. (A) yellow = Alphaproteobacteria, pink = Betaproteobacteria, red = other eubacteria, cyan = root tissue, scale bar = 30 µm. (B) green = Alphaproteobacteria, blue = Betaproteobacteria, red = other eubacteria, beige = root tissue, scale bar = 15 µm.

important crops, including the medicinal plants. To biologically control these soil-borne diseases, different desert habitats were screened for potential BCAs adapted to the unique and arid conditions of desert farming. Due to this high content of potential plant pathogens in the fungal community, the selection of antagonists was focused on the indigenous bacterial microbiome. An *in vitro* screening of 1,212 bacterial isolates linked with the comprehensive ecological data resulted in an antagonist collection of 45 genotypically different antifungal strains. In a hierarchical evaluation including their antifungal properties against *Verticillium dahliae*, *Rhizoctonia solani* and *Fusarium culmorum* in addition to their antagonistic activity against the soil-borne plant pathogenic bacterium *Ralstonia solanacearum* and the nematode *Meloidogyne incognita*, three promising drought and heat resistant biocontrol candidates were selected: *Streptomyces subrutilus* Wb2n-11 isolated from desert soil in Sinai, *Bacillus subtilis* subsp. *subtilis* Co1-6 obtained from the rhizosphere of *C. officinalis*, and *Paenibacillus*

polymyxa Mc5Re-14 isolated from the endorhiza of *M. chamomilla*. Each belongs to risk group 1 and poses no risk for humans or the environment. These three potential BCAs have already shown promising *in vitro* plant growth promoting activities and stress tolerances; *B. subtilis* Co1-6 exhibited high drought and salt resistance, protease and glucanase activity, and the production of siderophores, *P. polymyxa* Mc5Re-14 had a lower tolerance to abiotic stresses in comparison to the *Bacillus* strain, but also tested positive for siderophores and glucanase activity, and the desert bacterium *S. subrutilus* Wb2n-11 showed hydrolytic degradation of chitin and glucan. All of them produced antibiotics against the nematode *M. incognita*, however their antibacterial activities were highly specific. While *B. subtilis* and *S. subrutilus* exhibited antagonistic suppression of the plant pathogen *R. solanacearum*, only the *Paenibacillus* isolate was active against the opportunistic human pathogen *Escherichia coli* (Köberl et al., 2013).

These three autochthonous Gram-positive strains were selected for *ad planta* evaluation in the field under desert farming conditions in comparison to three allochthonous Gram-negative strains already known for their beneficial plant-microbe interactions in humid soils: *Pseudomonas fluorescens* L13-6-12 isolated from the rhizosphere of potato (*Solanum tuberosum*), *Stenotrophomonas rhizophila* P69 from oilseed rape (*Brassica napus*) rhizosphere, and *Serratia plymuthia* 3Re4-18 from the endorhiza of potato (Lottmann & Berg, 2001; Wolf et al., 2002; Kai et al., 2007; Zachow et al., 2010; Alavi et al., 2013). The first results revealed that priming chamomile seedlings with the autochthonous strains not only showed a stabilising effect on plant performance, but *B. subtilis* Co1-6 and *P. polymyxa* Mc5Re-14 were also able to further elevate the plants' flavonoid production. Higher contents of the bioactive compounds apigenin-7-O-glucoside and apigenin, which belong to the major flavonoids of chamomile florets (Kato et al., 2008; Srivastava & Gupta, 2009), were measured in blossoms of plants treated with the two Bacillales strains compared to blossoms of other treatments and uninoculated control plants (Schmidt et al., 2012). These findings demonstrate

that a targeted bacterial treatment could influence the metabolic activity of the plant, and therefore represent one of the many poorly understood links between the structure and metabolic profile of the plant-associated microbiome and the plant metabolome.

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Desert farming benefits from microbial potential in arid soils and promotes diversity and plant health

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Desert Farming Benefits from Microbial Potential in Arid Soils and Promotes Diversity and Plant Health

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Abstract

Background: To convert deserts into arable, green landscapes is a global vision, and desert farming is a strong growing area of agriculture world-wide. However, its effect on diversity of soil microbial communities, which are responsible for important ecosystem services like plant health, is still not known.

Methodology/Principal Findings: We studied the impact of long-term agriculture on desert soil in one of the most prominent examples for organic desert farming in Sekem (Egypt). Using a polyphasic methodological approach to analyse microbial communities in soil as well as associated with cultivated plants, drastic effects caused by 30 years of agriculture were detected. Analysing bacterial fingerprints, we found statistically significant differences between agricultural and native desert soil of about 60%. A pyrosequencing-based analysis of the 16S rRNA gene regions showed higher diversity in agricultural than in desert soil (Shannon diversity indices: 11.21/7.90), and displayed structural differences. The proportion of Firmicutes in field soil was significantly higher (37%) than in the desert (11%). *Bacillus* and *Paenibacillus* play the key role: they represented 96% of the antagonists towards phytopathogens, and identical 16S rRNA sequences in the amplicon library and for isolates were detected. The proportion of antagonistic strains was doubled in field in comparison to desert soil (21.6%/12.4%); disease-suppressive bacteria were especially enriched in plant roots. On the opposite, several extremophilic bacterial groups, e.g., *Acidimicrobium, Rubellimicrobium* and *Deinococcus-Thermus,* disappeared from soil after agricultural use. The N-fixing *Herbaspirillum* group only occurred in desert soil. Soil bacterial communities were strongly driven by the a-biotic factors water supply and pH.

Conclusions/Significance: After long-term farming, a drastic shift in the bacterial communities in desert soil was observed. Bacterial communities in agricultural soil showed a higher diversity and a better ecosystem function for plant health but a loss of extremophilic bacteria. Interestingly, we detected that indigenous desert microorganisms promoted plant health in desert agro-ecosystems.

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Introduction

In contrast to desertification, which is recognised as a major threat to biodiversity, to convert deserts into arable, green landscapes is a global vision as well as competent answer to world hunger and climate change [1,2]. Desert farming, which generally relies on irrigation, is one way to this vision. Agriculture systems were already developed in arid landscapes by ancient cultures, yet nowadays, there is a dramatically increasing need for large-scale desert farming to feed the population. For example, in Egypt, desert farmland is expected to grow about 40% till 2017, but this needs about five billion m^3 of water a year [3]. These enormous amounts of water and the expected impact on the climate conditions are the major disadvantages of agriculture in the desert. While these problems are well-investigated, the effect on the bio-resource soil was yet not assessed. Moreover, there is still a gap of knowledge about the effect of management and land uses on the bacterial diversity of soils, which new molecular tools like metagenomics can help to close [4,5].

Deserts represent extreme environments for microorganisms [6]. Although the conditions varied strongly in the different regions of the world, all of them are characterised by a combination of extreme temperatures and desiccation, high soil salinity, low nutrient levels, high summer UV radiation levels, and physical instability caused by strong winds: all factors contribute to the visual appearance of a sterile environment. While early studies supported this "sterility" by very low levels of viable/cultivable microorganisms, applications of new methods in microbial ecology led to interesting new findings and showed a contrasting picture [6,7]. For example, in their global-scale study, Fierer & Jackson [8] found that the acidic soils of tropical forests harbour fewer bacterial taxa than the neutral pH soils of deserts. In McMurdo Dry Valleys, a hyperarid polar desert, microbial soil communities were relatively depauperate but harboured a broad range of previously unreported bacteria and fungi from polar regions [9]. In different sites in the Negev Desert, archaeal and bacterial diversity analysed by fingerprints using T-RFLP of the 16S rRNA genes was not constrained by precipitation, although the

taxonomic composition differed [10]. In soil of the Atacama Desert, a high diversity of microorganisms known for life in hypersaline environments was found by analysis of DGGE profiles [11]. Most of the desert microbial communities seem to be structured solely by a-biotic processes [6,7]. But, if adapted desert plants occurred, e.g. *Panicum* and *Stipagrostis* in Sinai or *Reaumuria negevensis* in Negev, they strongly shaped soil microbial diversity [12,13]. However, all these investigations showed a unique and extraordinary microbial diversity in desert soils. An understanding of diversity in such microbial communities can be used to assess potential effects of desert farming on soil ecosystem services like plant health [14]. Emerging problems with soil-borne pathogens limited the plant yield after several years often drastically. Due to their specific ecology soil-borne pathogens are difficult to suppress; disease-suppressive bacteria, which are able to antagonise and biologically control them, provide a promising and sustainable solution [15].

The objective of this study was to analyse the effect of desert farming on soil microbial diversity and on disease-suppressive bacteria. We studied microbial diversity in native Egyptian desert soil in comparison to the agricultural soil, which was used more than 30 years for organic agriculture in Sekem farms (www.sekem.com; Egypt). To study the role of plant-associated bacteria in the agricultural soil, we analysed microbial communities in the rhizosphere and endorhiza of cultivated medical plants. Sekem is not only one of the most prominent examples of organic farming in the desert; they were assigned for social entrepreneurship [16,17]. For this study, we used a broad set of methods including i) bacterial fingerprints using 16S rRNA PCR-SSCP (Single Strand Conformational Polymorphism) analysis to compare the communities at statistical level and identify the dominant bacterial taxa, ii) pyrosequencing-based 16S rRNA profiling to get a deeper insight into the soil communities, iii) a cultivation approach to assess the impact on disease-suppressive bacteria, and iv) a multivariate statistical analysis to identify environmental factors driving microbial communities. We demonstrate that long-term organic agriculture had a strong impact on microbial community structure and function, and identified highly specialised communities in all microenvironments.

Results

Molecular fingerprinting of microbial communities

To get a first overview about the structure of the bacterial communities, fingerprints were performed by SSCP analysis of 16S rRNA genes amplified from DNA obtained from desert and agricultural soil. In addition, we analysed bacterial communities from rhizosphere and endorhiza of the dominant plants German chamomile (*Matricaria chamomilla* L.), pot marigold (*Calendula officinalis* L.) and *Solanum distichum* Schumach. & Thonn. cultivated on farms. In comparison to the desert, in field soil an impressive diversity of bacteria was found (Fig. S1). According to cluster analyses, the composition of the bacterial community of agricultural soil differed significantly from the desert soil by approximately 60% of the bacterial strains (Fig. 1). In the bacterial community of desert soil two dominant bands could be detected, which were also abundant in all samples from the rhizosphere and endorhiza of all three investigated medical plants (Fig. S1). The two dominant bands were identified by partial 16S rRNA gene sequence analysis as *Ochrobactrum* sp. (closest database match *O. grignonense,* 99% similarity to NR_028901) and *Rhodococcus* sp.

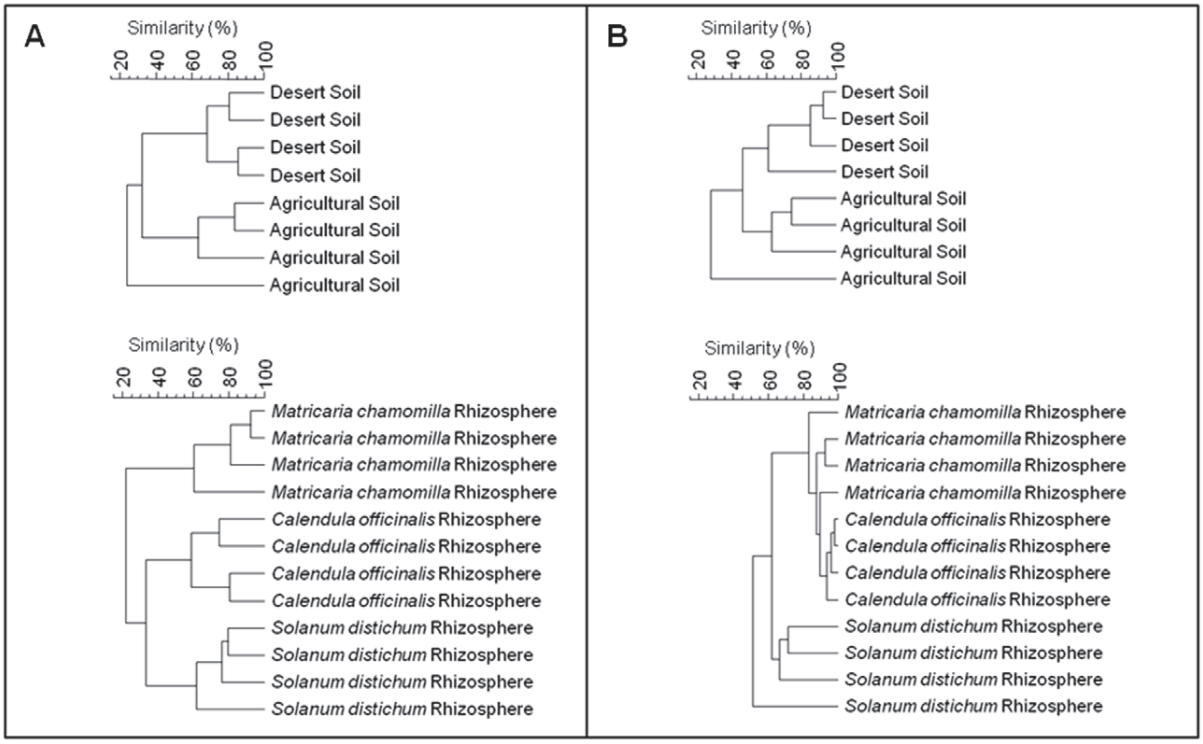

Figure 1. UPGMA dendrograms of total bacterial (A) and *Pseudomonas* (B) communities in soil and rhizosphere of the medical plants. The dendrograms were generated from the SSCP community profiles with GelCompar II. Following settings were used: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); similarity coefficient: curve based: Pearson correlation; position tolerances: optimisation: 4%, position tolerance: 1%.
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(closest database match *R. erythropolis*, 99% similarity to NR_037024). Further, *Bacillus* sp. was found nearly in all samples (closest database match *B. subtilis* subsp. *subtilis*, 99% similarity to NR_027552). For the rhizosphere as well as for the endorhiza of the medical plants a clear plant-specific effect of the bacterial communities was found (Fig. 1). They shared only 20% of the bacterial community, whereas the majority was determined by plant-specific bacteria.

Pyrosequencing-based 16S rRNA profiling of the bacterial community in soil

To deeply survey the diversity and the composition of the bacterial communities present in untreated desert soil and after 30 years of organic agriculture, a pyrosequencing-based analysis of partial 16S rRNA gene sequences (V4-V5 region) has been employed. In desert soil, we recovered 19,244 and in agricultural soil 33,384 quality sequences with a read length of $\geq$ 150 bp. Of all quality sequences 83.0% could be classified below the domain level; this proportion is in accordance with other pyrosequencing-based studies [18-20]. To determine rarefaction curves, operational taxonomic units (OTUs) were identified at sequence divergences of 3% (species level), 5% (genus level) and 20% (phylum level). The rarefaction analysis of the bacterial community in the desert soil in comparison to the agricultural soil is shown in Fig. 2. At a dissimilarity level of 20%, both curves show a clear saturation. Thus the surveying effort covered almost the full extent of taxonomic diversity at this level of genetic distance. Additionally, a comparison of rarefaction analyses with the number of OTUs estimated by the Chao1 richness estimator [20] revealed that in both soils over 90% of the estimated taxonomic richness was covered by the sequencing effort (Table 1). At the genus level (5% dissimilarity) the full extent of taxonomic diversity was not surveyed (42% and 31%). The computed Shannon indices of diversity (H') were much higher for the agricultural soil than for the desert soil, this indicates a higher bacterial diversity in soil due the agricultural use of the desert.

The 43,673 classifiable sequences obtained from both soil types together were affiliated with 18 different phyla. Proteobacteria (30.2%), Firmicutes (27.3%) and Actinobacteria (10.5%) were the dominant phyla (Fig. 3, Table S1). These dominant phyla were present in both soils. In detail, Firmicutes are highly enriched in agricultural soil (from 11.3% in desert soil to 36.6% in agricultural soil), Proteobacteria (46.0% in desert soil and 21.0% in agricultural soil) and Actinobacteria (20.7% in desert soil and 4.6% in agricultural soil) occurred in farmland in lower concentrations than in the surrounding desert. Further, in both soils Bacteroidetes (4.6% and 5.3%) and Gemmatimonadetes (1.4% and 1.9%) were present. Considering only phyla covering more than 1% of quality sequences, Acidobacteria (7.9%) and Planctomycetes (1.1%) were only found in the agricultural soil, and *Deinococcus-Thermus* (1.1%) was only detectable in the desert sand. These abundances of the phyla coincided with results from previously reported meta-analysis of bacterial community composition in soils and, despite the special soil type of the desert, the composition covers rather well with studies of completely different soils [18,20-22] with exception of Firmicutes. Most of the Firmicutes sequences were classified as belonging to the genus *Bacillus*; in the agricultural soil also *Paenibacillus* was found (5% of classified Firmicutes). In desert soil *Ochrobactrum* was the most abundant genus within the (Alpha-)Proteobacteria (79% of classified Proteobacteria) and *Rhodococcus* among the Actinobacteria (90% of classified Actinobacteria). The Acidobacteria in the agricultural soil are affiliated only with subdivision 6.

Quantitative analysis of bacterial abundances

A cultivation-independent approach (total 16S rRNA gene copy numbers using quantitative PCR) was combined with cultivation, which was also the basis for isolate screening, to assess bacterial abundances in the different microhabitats. Copy numbers in agricultural soil were 9.4 ± 0.5 $\log_{10}$ g^{-1} compared to 8.1 ± 1.1 $\log_{10}$ g^{-1} in desert soil (Fig. S2), and were not statistically significantly different. In contrast, abundances of culturable bacteria determined on R2A resulted in statistically significant higher abundances in agricultural soil (7.7 ± 0.4 $\log_{10}$ CFU g^{-1}) in comparison to desert soil (4.6 ± 0.6 $\log_{10}$ CFU g^{-1}). The rhizosphere of all three investigated medical plants was highly colonised by culturable bacteria: $\log_{10}$ CFU ranged from 7.8 ± 0.3 to 8.0 ± 0.2 g^{-1} fw. In contrast, in the endorhiza significantly lower CFUs were detected with $\log_{10}$ 2.0 ± 0.2 to 3.7 ± 0.8 g^{-1} fw.

Antagonistic potential of the bacterial community towards pathogenic fungi

A cultivation approach was used to analyse a functional aspect of the bacterial communities. To assess the indigenous anti-phytopathogenic potential, the antagonistic activity against three major soil-borne phytopathogenic fungi *Verticillium dahliae*, *Rhizoc-*

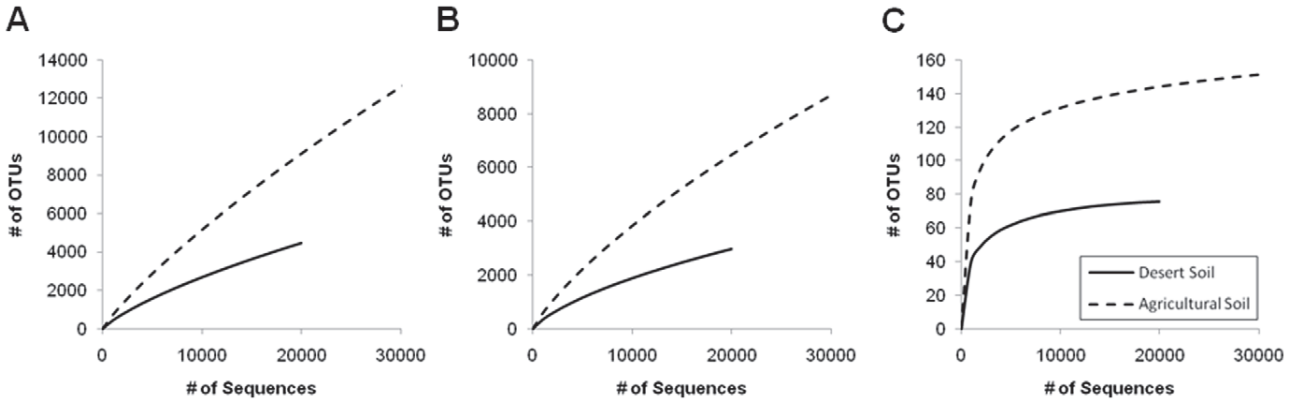

Figure 2. Rarefaction analyses of the two soil types. Rarefaction curves indicate that the diversity of bacterial phylotypes is higher in the agricultural soil compared to the surrounding desert soil. OTUs are shown at genetic distance levels of 3% (A), 5% (B) and 20% (C). Data were calculated by employing tools of the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu).
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Table 1. Species richness estimates obtained at 3%, 5% and 20% genetic dissimilarity from pyrosequencing of 16S rRNA from metagenomic DNA extracted from desert soil and agricultural soil.

	Shannon index[a] (H')			Rarefaction[b] (no. of OTUs)			Chao1[c] (no. of OTUs)			Coverage (%)		
	3%	5%	20%	3%	5%	20%	3%	5%	20%	3%	5%	20%
Desert Soil	7.90	7.04	3.02	4,465	2,967	76	13,278	7,012	77	33.6	42.3	98.8
Agricultural Soil	11.21	9.94	3.91	9,112	6,474	144	38,985	20,838	161	23.4	31.1	90.0

[a]a higher number indicates more diversity;
[b]the results from the rarefaction analyses are also depicted in Fig. 2;
[c]nonparametric richness estimator based on the distribution of singletons and doubletons.
doi:10.1371/journal.pone.0024452.t001

tonia solani and *Fusarium culmorum* was determined. From each microenvironment up to 200 isolates were randomly selected and assessed regarding their anti-phytopathogenic capacity *in vitro*. All isolated soil bacteria (199 isolates from desert soil and 155 isolates from agricultural soil) were screened by dual testing regarding their antagonistic activity towards *V. dahliae*, *R. solani* and *F. culmorum* (Table 2). In general, bacterial isolates obtained from the soil of the farm exhibited a higher *in vitro* antagonistic potential towards soil-borne phytopathogenic fungi in comparison to the bacteria isolated from the desert soil (agricultural soil 21.6±0.8%; desert soil 12.4±0.7%). From the agricultural soil, 17.4% (27 isolates) demonstrated *in vitro* broad-spectrum antagonism towards all three pathogens, from the desert soil 10.6% (21 isolates) were able to suppress the growth of all fungi tested. No enrichment of antagonists in the rhizosphere and endorhiza of the investigated medical plants was detected. In general, *M. chamomilla* and *S. distichum* showed a higher antagonistic potential than *C. officinalis*. Especially the endorhiza from *M. chamomilla* harboured a high proportion of antagonists. Whereas in the soil and in the rhizosphere could be found most antagonistic bacteria towards *F. culmorum*, in the endorhiza of the medical plants most antagonists showed antagonism towards *V. dahliae*.

To assess the diversity of bacterial antagonists, isolates with an activity towards at least two of the soil-borne pathogenic fungi (162 isolates) were characterised genotypically and identified by partial 16S rRNA gene sequencing (Fig. 4). Using restriction fragment length polymorphism, of the 16S rRNA (= amplified ribosomal RNA gene restriction analysis [ARDRA]), the antagonistic isolates could be clustered into six groups: (1) *Bacillus subtilis*, (2) *Bacillus cereus*, (3) *Bacillus endophyticus*, (4) *Paenibacillus/Brevibacillus*, (5) *Streptomyces*, and (6) *Lysobacter*. With the exception of the *Lysobacter* strain (only one isolate from the rhizosphere of *M. chamomilla*, closest database match *L. enzymogenes*), only Gram-positive antagonists were found. All antagonistic populations were dominated by Firmicutes; *Bacillus* and *Paenibacillus* could be isolated from all habitats. Interestingly, antagonistic isolates of the genus *Streptomyces* were found exclusively in desert soil.

To analyse the genotypic diversity within the taxonomic groups at population level, BOX PCR patterns of the whole bacterial genome were used. Especially within the large *Bacillus subtilis* cluster (123 isolates), a high genotypic diversity was found. At a cut off level of 80%, they could be divided into 37 genotypic groups. By partial 16S rRNA gene sequencing isolates were identified as *B. subtilis* subsp. *subtilis* and *spizizenii*, *B. vallismortis*, *B. mojavensis* and *B.*

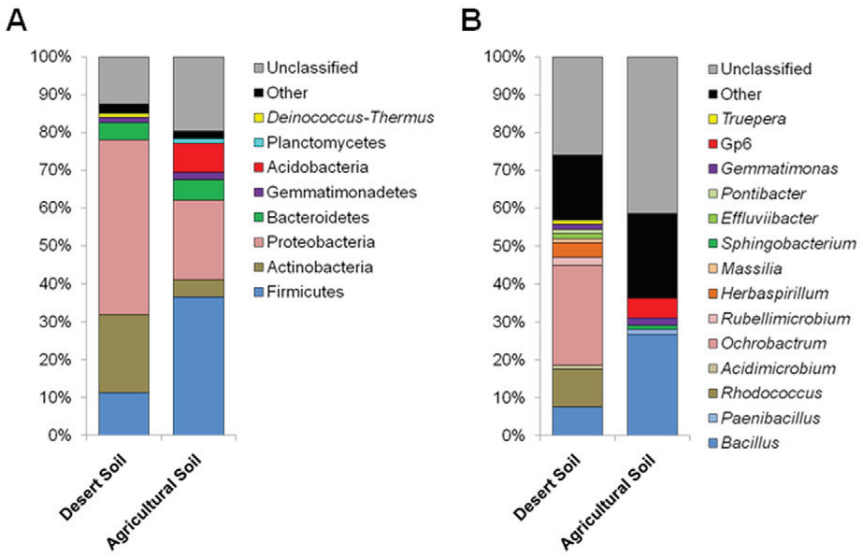

Figure 3. The bacterial communities in the two different soil types. Relative clone composition of major phyla (A) and genera (B) was determined by pyrosequencing of 16S rRNA from metagenomic DNA extracted from desert and agricultural soil. The identification of the closest strain based on 16S rRNA sequence similarity was achieved using the web server SnoWMAn 1.7 (http://snowman.genome.tugraz.at). Phylogenetic groups accounting for ≤1% of all quality sequences are summarised in the artificial group others.
doi:10.1371/journal.pone.0024452.g003

Table 2. Proportions of bacterial isolates antagonistic towards the soil-borne fungal pathogens *Verticillium dahliae*, *Rhizoctonia solani* and *Fusarium culmorum*.

		Proportion of antagonists (%)[a]		
Microhabitat	**Origin**	***V. dahliae***	***R. solani***	***F. culmorum***
Soil	Desert Soil	11.1±1.8	12.8±0.6	13.4±0.1
	Agricultural Soil	20.0±1.6	21.9±2.2	22.6±1.4
Rhizosphere	*Matricaria chamomilla*	12.5±2.9	8.3±0.7	13.0±1.8
	Calendula officinalis	9.0±0.5	7.1±0.1	10.1±3.1
	Solanum distichum	13.7±2.3	13.8±3.8	15.7±0.0
Endorhiza	*Matricaria chamomilla*	19.9±1.8	16.4±2.3	18.8±2.6
	Calendula officinalis	4.2±2.9	0.0±0.0	1.4±1.0
	Solanum distichum	13.5±5.1	10.4±5.8	12.5±5.8

[a]Data are averages of 1st and 2nd sampling ± confidences.
doi:10.1371/journal.pone.0024452.t002

atrophaeus. The *Paenibacillus/Brevibacillus* isolates could be divided into eight BOX clusters and *Bacillus endophyticus* into two. *Streptomyces* was subdivided in three genotypes, the closest database matches were *S. peucetius*, *S. scabiei* and *S. subrutilus*. Surprisingly, among the *Bacillus subtilis* group, isolates with identical BOX patterns could be detected in desert soil as well as in the agricultural soil, and also in rhizosphere and endorhiza of the medical plants (Fig. 5). Based on unique genotypic patterns and antagonistic potential, 45 promising biocontrol strains were selected of which 89% belonged to the Bacillales (Table S2).

Ecological factors driving the bacterial communities in soil

Indirect correspondence analysis (CA) based on the OTUs of soil species obtained by microbial fingerprinting showed the coherence and similarity of the different samples indicated by crowding points at a CA biplot (data not shown). Furthermore, the influence of the environmental factors on the bacterial soil communities was examined using the multivariate statistical analysis. A significant effect was proved for water supply (precipitation + irrigation) (regression coefficient: 0.3760), pH (0.3719) and, to a lower extent for organic carbon (0.1600) and soil quality (0.1011).

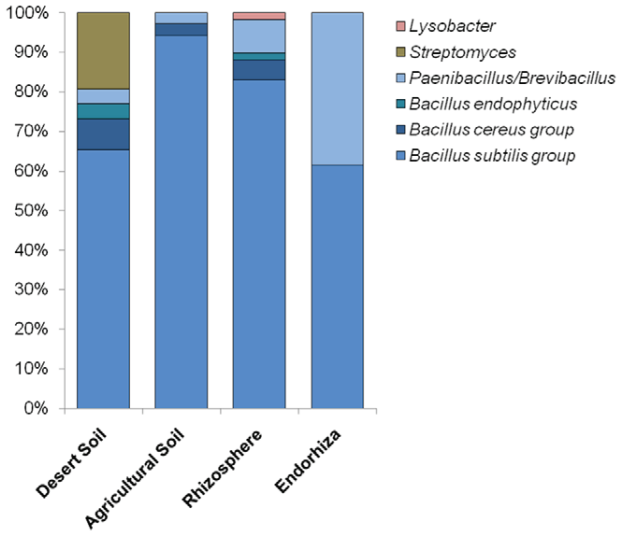

Figure 4. Diversity of bacterial antagonists with an activity towards pathogenic fungi. Isolates with activity against two pathogens were identified by partial 16S rRNA gene sequencing. Samples from rhizosphere and endorhiza include isolates from the medical plants *Matricaria chamomilla*, *Calendula officinalis* and *Solanum distichum*.
doi:10.1371/journal.pone.0024452.g004

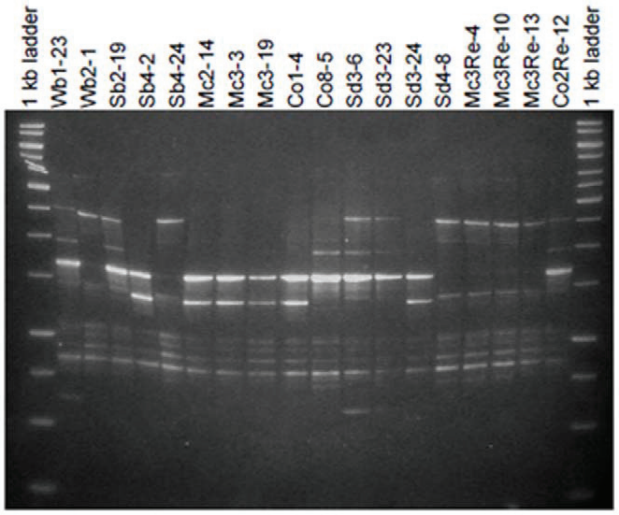

Figure 5. BOX PCR fingerprints of genetically very similar antagonists of the *Bacillus subtilis* group isolated from different microenvironments. The similarity in the dendrogram between them was more than 80%. Isolates were encoded by abbreviations: (1) soil type or plant species (Wb = desert soil; Sb = Sekem soil, Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*), (2) replicate (1–4), (3) microenvironment (Re = endorhiza, rhizosphere and soil have no further designation), and (4) consecutive number of the isolate per replicate.
doi:10.1371/journal.pone.0024452.g005

Discussion

Agriculture in deserts open new ways to solve diverse problems: produce enough food for poor regions e.g. in Africa, produce renewable crops for industrial applications and to capture and restore CO_2 in soil. However, agricultural use induces a drastic shift for the whole ecosystem, and risk assessments to evaluate the function are necessary. Here we analysed differences of microbial communities in undisturbed desert soil in comparison to desert soil, which was cultivated under organic (biodynamic) conditions for 30 years. Altogether, a strong impact of long-term agriculture on microbial community structure and function was identified, which will be discussed and assessed in detail.

The composition of the bacterial communities in desert and agricultural soil differed strongly. In microbial fingerprints, both communities showed about 60% differences. Using a pyrosequencing-based approach of the 16S rRNA gene region, reasons for these differences could be identified. The relative abundance of Firmicutes in agricultural soil was significantly enhanced from 11 to 37%. Especially the proportion of 37% is remarkable because Janssen [22] reported them to contribute only a mean of 2% (range 0-8%) in the total bacterial soil community. *Bacillus* and *Paenibacillus* play the key role to explain this difference; they were dominant in the 16S rRNA gene amplicon library and represented 96% of the antagonists towards phytopathogens identified in the

culturable fraction. In addition, by microbial fingerprints we showed that this Gram-positive group was enriched in the rhizosphere as well as endorhiza of medical plants cultivated in Sekem. Interestingly, we found *Bacillus* isolates with the same BOX pattern in desert and field soil as well as in the rhizosphere and endorhiza of medical plants, which was confirmed also by our bacterial fingerprint analysis. Furthermore, identical 16S rRNA gene sequences were found for isolates as well as in the amplicon library. This is further evidence for enrichment of plant rhizosphere-specific bacteria from the soil. Moreover, it indicates that the antagonistic bacteria were enriched from desert soil and not from the compost treatment. Both, *Bacillus* and *Paenibacillus* are well-characterised plant-associated genera with antagonistic properties towards fungal plant pathogens [23]. While the proportion of Firmicutes was enhanced in field soil, several extremophilic bacterial groups, e.g. *Acidimicrobium*, *Rubellimicrobium* and *Deinococcus-Thermus* disappeared. Bacteria from all of these genera/phyla are either impossible or else extremely difficult to cultivate and only found in extreme environments by molecular analysis. For example, bacteria from the phylum *Deinococcus-Thermus* possess important adaptations such as resistance to environmental hazards, e.g., desiccation, ultraviolet radiation, high salinity, and high temperatures [24]. In general, the proportion of cultivable bacteria was lower in desert soil than in field soil, which was shown in the comparison between results obtained by cultivation and qPCR analysis. Based on our pyrosequencing data, bacterial communities in agricultural soil were characterised by a higher diversity than in desert soil (Shannon diversity indices: agricultural soil 11.21; desert 7.90). The high bacterial diversity found in the organically managed soil was shown for agriculture in the desert for the first time but was already reported for another organically managed system [14].

Additionally, for the function of the bacterial communities in desert and agricultural soil we found strong differences. The proportion of strains with antagonistic *in vitro* activity against soil-borne phytopathogens was statistically significantly enhanced in agricultural soil in comparison to desert soil. Other current studies showed also that organic farming methods can mitigate ecological damages caused by pests and pathogens by promoting natural enemies, analysed for example in the pathosystem potato – potato beetle [25] or grape – *Botrytis cinereia* [26]. Although the proportion of antagonistic strains was higher in agricultural soil, their diversity was much lower. All of the isolated antagonists belong to the *Bacillus/Paenibacillus* group. In contrast, in desert soil, diverse antagonistic *Streptomyces* species were identified, including *Streptomyces peucetius*, a species known to produce anthracycline antibiotics [27]. Another interesting fact is that members of the *Herbaspirillum* group, most of them known as N-fixing species, only occurred in desert soil. In most of the deserts, plants have a very short period to develop. This fact is well-known, when shortly after a rainfall millions of seedlings occur and colour the whole desert in green. Therefore, plants need plant growth promoting rhizobacteria, and *Herbaspirillum* strains belong to this group. Owing to compost treatment agricultural soils are saturated with nitrogen. These facts could explain that this important functional group had a lower abundance in field soil. Another genus with an interesting occurrence is *Orchrobactrum*. In desert soil *Ochrobactrum* was the most abundant genus within Proteobacteria and also in microbial fingerprints this genus was found in high abundance in soil but also in the rhizosphere/endorhiza of medical plants. Bacteria of this genus are known for its ambivalent interaction with eukaryotes, while they show plant growth promotion effects on plants, they can cause opportunistic infections in humans [28].

What are the reasons for the changes in structure and function of the bacterial community? The main factor, which explains the differences, is the continuous irrigation of farm land. This factor was identified by an indirect correspondence analysis. Precipitation in this arid region is general low (21-52 mm). The agriculture is completely dependent on irrigation water coming from the Nile or from local ground water. Irrigation systems were used to supply about 2,500-2,600 l m^{-3} per year. The aridity level was also one of the main factors that shaped the microbial community structure in patchy desert landscapes of Negev [29]. By the multivariate statistic, the pH of soil was identified as the second impact factor. This factor was often reported as main driver, e.g. in global studies of microbial communities in soil [8,18]. Another factor, which contributed to the shift in the bacterial community in a lower extend, is compost treatment. This was already shown for other examples in organic agriculture: due to the use of compost, studies have found that biodynamic farms have a significantly better soil quality than conventionally farmed soils but comparable to the soil quality achieved by other organic methods [14,17]. The compost treatment is responsible for nutrient and organic matter supply. On the other side, compost is known for an extremely high but also specific bacterial diversity. No evidence was found for an impact of these specifically adapted bacteria on soil communities. One factor, which could be not included in the statistical analyses, is plant-specific enrichment of bacterial communities. The extent of plant specificity was shown in a study of *Verticillium* host plants published by Smalla et al. [30], and later described for many other plant species [31]. In our study, we found a highly pronounced effect for each of the medical plants investigated. All three medical plants, which belong to the dominant herbs in Sekem and were included in the study, are known for their production of secondary metabolites. For example, German chamomile, for which we found the strongest effect, is used medicinally to treat sore stomach and irritable bowel syndrome. Chamomile plants produce the terpene bisabolol, and other active ingredients like farnesene, chamazulene, flavonoids and coumarin [32]. Some of them are known for their anti-microbial properties, and others, such as flavonoids often serve as signals in plant-microbe interactions [33].

In a final assessment, bacterial communities in agricultural soil showed a higher diversity and a better ecosystem function for plant health, which was measured as proportion of disease-suppressive bacteria. On the other side, there is a loss of extremophilic bacteria, which are typical inhabitants of desert soil. However, due to the fact that all farms are still surrounded by desert, we can conclude that also this specific diversity is maintained. The most interesting fact detected in our study was that indigenous desert microorganisms fulfil important functions in desert agro-ecosystems: *Bacillus* and *Paenibacillus* strains were enriched via plant roots from desert soil. This was shown at the population level using genotypic fingerprinting by BOX pattern, at community level by microbial fingerprints as well as in the metagenome.

Materials and Methods

The experimental design comprise samples from agricultural soil, rhizosphere and endorhiza samples from main medical crops cultivated in Sekem farms as well as samples from the surrounding desert soil from two different sampling times. All sampling sites are private property of the Sekem companies. The sampling was done in cooperation with Angela Hoffmann and Elshahat M. Ramadan (Sekem) with permission of Ibrahim Abouleish, the owner of Sekem, for a joint project. Therefore, no other permit was required. Samples from agricultural used soil were taken at the Sekem farm Adleya, located in the North-eastern desert region of

Egypt near Bilbeis (30°22′88"N; 31°39′41"E). The agriculture was completely dependent on irrigation water (2,607 l m^{-3} on average per year) coming from the Nile or from local ground water drillings; sprinkler and drip irrigation systems were used. The farmland soil was fertilised with compost that was produced on their own composting facility, where rice straw, water hyacinth, wood chips, organic waste, clay, chicken and cow manure was used as input materials. The compost was applied twice a year (May and September), during the preparation of the fields for the cropping season. The soil texture at the Sekem farm was classified by Luske & van der Kamp [17] as loamy sand (pH 8.4) with an organic carbon content of 0.8% and a clay content of 4%. Desert soil was collected from two sites in the surrounding desert uninfluenced by human activities (30°35'01"N; 32°25'49"E; 29°52′26″N, 31°13′1″E) and was classified as sand (pH 7.7) with an organic carbon content of <0.2% and a clay content of 1.5%. Desert soil was characterised by a low moisture level; plants were very scarce [17]. At each site, four composite samples of soil in a horizon of 10–30 cm depth were collected. Furthermore, from three different species of medical plants (German chamomile [*Matricaria chamomilla* L.], pot marigold [*Calendula officinalis* L.] and *Solanum distichum* Schumach. & Thonn.) planted on the Adleya farm (30°22′88"N; 31°39′41"E), roots with adhering soil were obtained. From each plant four independent composite samples consisted of 5–10 plants were taken. At the first sampling time (October 2009), *Matricaria chamomilla* and *Calendula officinalis* have been in the seedling stage, whereas the samples from the perennial *Solanum distichum* were taken from lignified plants. At the second sampling time (April 2010), all medical plants were in the flowering stage.

To isolate total community DNA from soil and from rhizosphere for all cultivation independent analyses, 5 g of soil/roots with adhering soil and 45 ml of 0.85% NaCl were mixed for 5 min on the vortex. For the isolation from the endorhiza, 5 g material of roots were surface-sterilised with 4% NaOCl for 5 min, then the roots were washed three times with sterile Aqua dest. After 10 ml sterile 0.85% NaCl were added the roots were homogenised using mortar and pestle. For isolation of total DNA from soil, rhizosphere and endorhiza 4 mL of the liquid parts were centrifuged at high speed (16,000×g, 4°C) for 20 min and resulting microbial pellets were stored at −70°C. In the desert soil, a lower concentration of DNA was expected. Therefore, for the isolation of total DNA the pellets of 10 ml supernatant were used. Total community DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, USA) according to the manufacturer's protocol and used for fingerprints and the deep-sequencing approach.

Fingerprinting of microbial communities by Single Strand Conformational Polymorphism Analysis (SSCP) was carried out as described by Schwieger & Tebbe [34]. Bacterial 16S rRNA gene sequences were amplified by PCR using the eubacterial primer pair Unibac-II-515f (5′-GTG CCA GCA GCC GC-3′) and Unibac-II-927r^{P} (5′-CCC GTC AAT TYM TTT GAG TT-3′) [35]. The PCR was performed by using a total volume of 60 µl containing 1 × Taq&Go (MP Biomedicals, Eschwege, Germany), 1.5 mM $MgCl_2$, 0.2 µM of each primer and 1 µl of template DNA (95°C, 5 min; 32 cycles of 95°C, 20 s; 54°C, 15 s; 72°C, 30 s; and elongation at 72°C, 10 min). For the analysis of the *Pseudomonas* community a nested PCR was performed. In a first PCR *Pseudomonas* were selectively amplified with primers F311Ps (5′-CTG GTC TGA GAG GAT GAT CAG T-3′) and 1459rPs^{P} (5′-AAT CAC TCC GTG GTA AAC GT-3′) [36] followed by a second PCR with the primer pair Unibac-II-515f/Unibac-II-927r^{P}. The reaction mixture for the first PCR (20 µl) was composed of 1 × Taq&Go, 2.25 mM $MgCl_2$, 0.5 mg/ml BSA, 1.5% DMSO, 0.2 µM of each primer and 1 µl of template DNA (94°C, 7 min; 30 cycles of 94°C, 45 s; 56°C, 2 min; 72°C, 2 min; and elongation at 72°C, 10 min). Samples served as templates for the second PCR. The obtained amplicons were separated using the TGGE Maxi system (Biometra, Göttingen, Germany) at 400 V and 26°C. Silver staining was used for the routine detection of DNA bands in SSCP gels [37]. Dominant bands were excised from SSCP gels as described by Schwieger and Tebbe [34]. Extracted DNA fragments were re-amplified by PCR and sequenced. For phylogenetic analysis and identification of related sequences, the obtained sequences were aligned with reference gene sequences from GenBank using BLAST algorithm.

Computer-assisted comparisons of SSCP generated community profiles were performed by using the software GelCompar II (Applied Maths, Kortrijk, Belgium). The cluster analysis was performed using following settings: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); similarity coefficient: curve based: Pearson correlation; position tolerances: optimisation: 4%, position tolerance: 1% [38]. Furthermore, correspondence analysis was used to answer the question whether a correlation exists (1) between the independently sampled microbial communities of the different sampling points and (2) between soil communities and environmental factors. The following environmental data were used: i) soil quality (sand, loam, semi-loam), ii) soil pH, iii) content of organic carbon and iv) water supply (sum of local precipitation per year [21, 52 mm] and irrigation). According to the distance of the bands, the SSCP gels were theoretically divided into operational taxonomic units (OTUs). The presence or absence of individual amplified product DNA bands in each group was scored. The obtained matrix was used to compare data statistically using the indirect correspondence analysis for unimodal data of the software package Canoco 4.5 [39].

To analyse the taxonomic composition of the soil bacterial community by a deep-sequencing approach, the hypervariable V4-V5 region of the 16S rRNA gene (*Escherichia coli* positions 515 to 927) was amplified in a nested PCR approach for pyrosequencing. In a first PCR the primer pair 27f/1492r [40] was used and in the second PCR V4-V5 region was amplified with the following primer set, containing the 454 pyrosequencing adaptors and sample specific tags (underlined): Unibac-II-515f_MID13 (5′-CGT ATC GCC TCC CTC GCG CCA TCA GCA TAG TAG TG GTG CCA GCA GCC GC-3′) respectively Unibac-II-515f_MID14 (5′-CGT ATC GCC TCC CTC GCG CCA TCA GCG AGA GAT AC GTG CCA GCA GCC GC-3′) and Unibac-II-927r_MID13-14 (5′-CTA TGC GCC TTG CCA GCC CGC TCA G CCC GTC AAT TYM TTT GAG TT-3′). The reaction mixture for the first PCR (20 µl) contained 1 × Taq&Go, 0.25 µM of each primer and 1 µl of template DNA (95°C, 5 min; 30 cycles of 95°C, 30 s; 57°C, 30 s; 72°C, 90 s; and elongation at 72°C, 5 min). The second PCR was performed by using 1 × Taq&Go, 1.5 mM $MgCl_2$, 0.4 µM of each primer and 2 µl of template DNA (95°C, 5 min; 32 cycles of 95°C, 20 s; 54°C, 15 s; 72°C, 30 s; and elongation at 72°C, 10 min). PCR products of four independent soil samples of the same habitat were pooled in equal volumes and purified by employing the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA). A total of 130 ng of amplified 16S rRNA gene product from each soil was required to construct the libraries for 454 pyrosequencing. For taxonomy-based analysis, the web server SnoWMAn 1.7 (http://snowman.genome.tugraz.at) [41] was employed. Sequences that were shorter than 150 bp in length or of low quality were removed from the pyrosequencing-derived data sets and following settings

were used: analysis type: BLAT pipeline; reference database: greengenes_24-Mar-2010; rarefaction method: MOTHUR; taxonomy: RDP; confidence threshold: 80%; include taxa covering more than: 1%. For rarefaction analysis and ascertainment of diversity indices, the data were normalised considering the same number of sequences to all samples using default settings in the open source software package QIIME (http://qiime.sourceforge.net), which allows analysis of high-throughput community sequencing data [42]. Rarefaction curves were calculated by using the tools aligner, complete linkage clustering and rarefaction of the ribosomal database project (RDP) pyrosequencing pipeline (http://pyro.cme.msu.edu) [43]. Shannon [44] and Chao1 [45] indices were calculated based on the complete linkage clustering data.

The same region of the 16S rRNA gene was amplified by quantitative PCR to determine the total bacterial abundances in desert and agricultural soil. Reactions were conducted in a total volume of 10 µl containing 1 × KAPA^{TM} SYBR® FAST qPCR MasterMix Universal (PEQLAB, Polling, Austria), 0.25 µM of each primer (Unibac-II-515f and Unibac-II-927r [35]) and 1 µl template DNA (95°C, 5 min; 35 cycles of 95°C, 20 s; 54°C, 15 s; 72°C, 30 s; and melt from 72 to 95°C). Rotor-Gene^{TM} 6000 real-time rotary analyser (Corbett Research, Sydney, Australia) was used for quantification of fluorescence. For absolute quantification the PCR amplified 16S rRNA gene fragment was cloned into a pGEM®-T Easy Vector (Promega, Mannheim, Germany). Serial dilutions of PCR fragments generated with the primers usp (5'-GTAAAACGACGGCCAGT-3') and rsp (5'-CAGGAAACAGCTATGACC-3'), which specifically bind to sides flanking the multi cloning side of the Vector, were used as standard for calculation of copy number. Concentrations determined by absolute quantification were calculated to copy number per g soil. Each replicate was analysed three times in two independent runs. Significances in the difference between desert and agricultural soil were calculated using the independent samples *t* test with PASW Statistics 18 (SPSS Inc., Chicago, USA).

Same cell suspensions as used for the isolation of total community DNA were used for isolation of bacteria from soil, rhizosphere and endorhiza: They were used for dilution and plating on R2A (Roth, Karlsruhe, Germany) in duplicates. Plates were incubated for 4 days at room temperature (RT) and colony forming units were counted to calculate the means of colonies (log_{10} CFU) based on fresh weight (fw). If possible, for each replicate 24 bacterial isolates were selected and subcultured on nutrient agar (NA). The isolates were purified and then stored at −70°C in nutrient broth (NB) (Sifin, Berlin, Germany) containing 15% glycerol. Isolates were encoded using a combination of letters and numbers indicating: (1) soil type or plant species (Wb = desert soil; Sb = Sekem soil, Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*), (2) replicate (1–4), (3) microenvironment (Re = endorhiza, rhizosphere and soil have no further designation), and (4) consecutive number of the isolate per replicate.

Altogether, 1,212 selected bacterial isolates were screened in dual-culture *in vitro* assays on Waksman agar (WA) [46] for their antagonistic potential towards *Verticillium dahliae* Kleb. V25, *Rhizoctonia solani* Kühn AG4, and *Fusarium culmorum* (Wm. G. Sm.) Sacc. E1. For *R. solani* and *F. culmorum* agar disks of 5 mm diameter with mycelia were directly cut out from PDA plates (Roth, Karlsruhe, Germany) and placed between the streaks of four bacterial isolates. *V. dahliae* was grown in liquid culture in Czapek Dox broth (Duchefa, Haarlem, Netherlands) at 20°C. 200 µl of the suspension containing hyphal fragments were plated onto the agar and after surface drying the isolates were placed on the same plate. Inhibition zones were measured after 4–7 days of incubation at RT. Each isolate was tested twice independently. From antagonistic isolates, DNA was prepared following the protocol of Berg et al. [46]. Amplified ribosomal RNA gene restriction analysis (ARDRA) using the restriction endonucleases *Hha*I (MP Biomedicals, Eschwege, Germany) and *Pst*I (New England Biolabs, Ipswich, UK) was used to group isolates at genus level. Isolates displaying similar ARDRA patterns were further analysed using BOX-PCR genomic fingerprinting. BOX-PCR fingerprints were performed using the BOX_A1R primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') as described by Rademaker and de Bruijn [47]. PCR conditions were used as specified by Berg et al. [28] and PCR products were separated by gel electrophoresis on 1.5% agarose gels. Antagonists with either individual ARDRA patterns or different BOX patterns (cut-off level 80%) were identified by partial 16S rRNA gene sequence analysis according to Berg et al. [46]. PCR product was sequenced with the Applied Biosystems 3130l Genetic Analyser sequencer, Data Collection v3.0, Sequencing Analysis v5.2 (Foster City, USA) at the sequencing core facility ZMF, Medical University of Graz, Austria. Obtained sequences were aligned with reference gene sequences from GenBank using BLAST algorithm. Sequences obtained were submitted to EMBL Nucleotide Sequence Database under accession numbers FR854236-FR854290.

Supporting Information

Figure S1 16S rRNA PCR-SSCP profiles of the bacterial communities in soil and endorhiza of the medical plants. Std.: 1 kb DNA ladder. The following bands were identified as: 1. *Ochrobactrum grignonense*, 99% similarity to NR_028901 and 2. *Rhodococcus erythropolis* 99% similarity to NR_037024.
(TIF)

Figure S2 Abundances of (A) total and (B) culturable bacteria in desert and agricultural soil. Data for total bacteria were ascertained by qPCR of the 16S rRNA genes and data for culturable bacteria by isolation on R2A. Averages of 16S rRNA gene copy numbers and viable counts per gram soil as log_{10} and confidences are shown.
(TIF)

Table S1 Relative composition of bacterial phyla, classes, orders, families and genera in desert and agricultural soil.
(DOC)

Table S2 Identification of selected bacterial antagonists isolated from different habitats.
(DOC)

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Author Contributions

Conceived and designed the experiments: GB HM EMR MK. Performed the experiments: MK. Analyzed the data: MK GB. Contributed reagents/materials/analysis tools: GB. Wrote the paper: MK GB.

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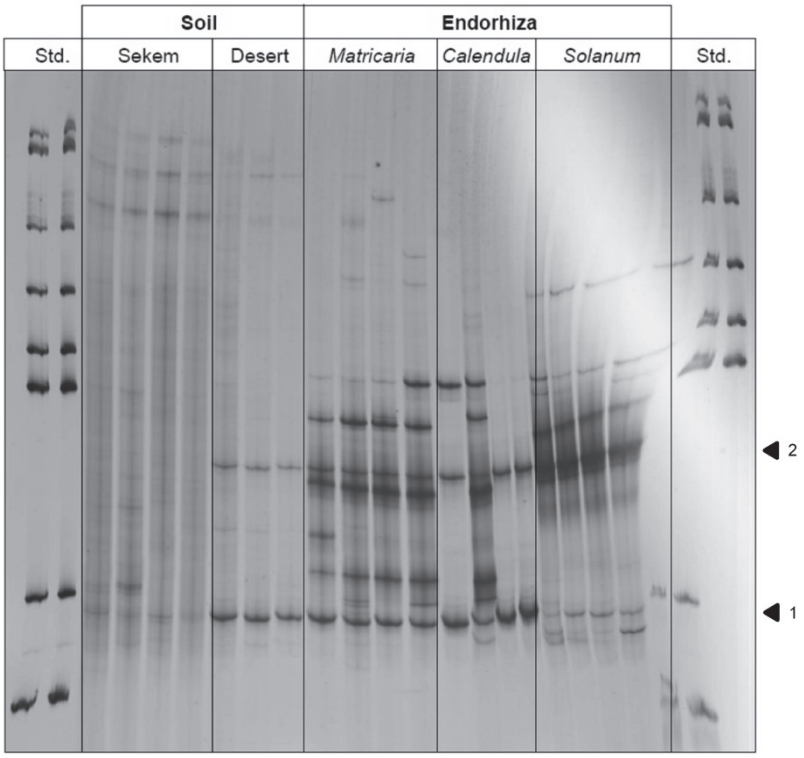
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Figure S1

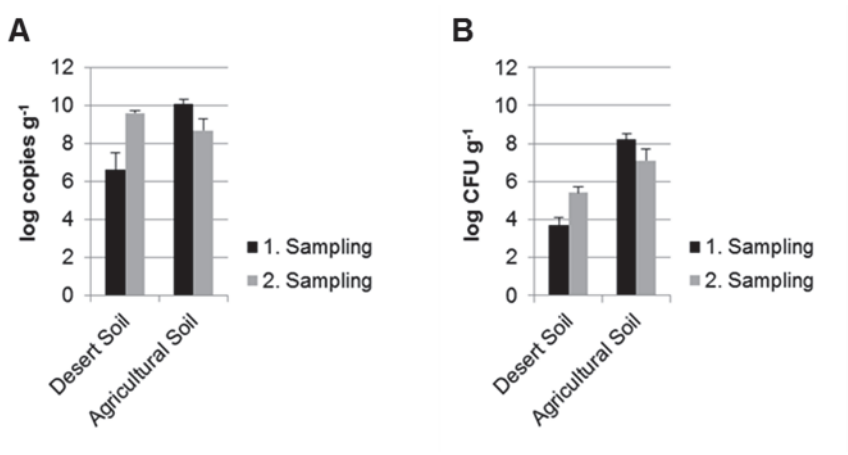
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Figure S2

Table S1. Relative composition of bacterial phyla, classes, orders, families and genera in desert and agricultural soil.

	Desert Soil[a]	Agricultural Soil[a]
Phylum Level		
Firmicutes	11.3%	36.6%
Actinobacteria	20.7%	4.6%
Proteobacteria	46.0%	21.0%
Bacteroidetes	4.6%	5.3%
Gemmatimonadetes	1.4%	1.9%
Acidobacteria		7.9%
Planctomycetes		1.1%
Deinococcus-Thermus	1.1%	
Other	2.5%	2.0%
Unclassified	12.4%	19.7%
Class Level		
Bacilli	10.9%	35.4%
Actinobacteria	20.7%	4.6%
Alphaproteobacteria	34.3%	6.7%
Betaproteobacteria	8.9%	6.7%
Gammaproteobacteria	1.6%	3.1%
Deltaproteobacteria	1.2%	4.2%
Sphingobacteria	3.3%	2.9%
Flavobacteria		2.0%
Bacteroidetes	1.0%	
Gemmatimonadetes	1.4%	1.9%
Acidobacteria		7.9%
Planctomycetacia		1.1%
Deinococci	1.1%	
Other	2.9%	3.0%
Unclassified	12.8%	20.6%
Order Level		
Bacillales	10.7%	35.2%
Actinomycetales	16.2%	2.3%
Rubrobacterales	2.0%	
Acidimicrobiales	1.1%	
Rhizobiales	29.5%	4.3%

Rhodospirillales	1.3%	1.1%
Rhodobacterales	2.5%	
Burkholderiales	8.4%	3.0%
Oceanospirillales		1.0%
Myxococcales		1.1%
Sphingobacteriales	3.3%	2.9%
Flavobacteriales		2.0%
Bacteroidales	1.0%	
Acidobacteriales		7.9%
Gemmatimonadales	1.4%	1.9%
Planctomycetales		1.1%
Deinococcales	1.1%	
Other	6.8%	7.6%
Unclassified	14.8%	28.7%
Family Level		
Bacillales	10.7%	35.2%
Actinomycetales	16.2%	2.3%
Rubrobacterales	2.0%	
Acidimicrobiales	1.1%	
Rhizobiales	29.5%	4.3%
Rhodospirillales	1.3%	1.1%
Rhodobacterales	2.5%	
Burkholderiales	8.4%	3.0%
Oceanospirillales		1.0%
Myxococcales		1.1%
Sphingobacteriales	3.3%	2.9%
Flavobacteriales		2.0%
Bacteroidales	1.0%	
Acidobacteriales		7.9%
Gemmatimonadales	1.4%	1.9%
Planctomycetales		1.1%
Deinococcales	1.1%	
Other	6.8%	7.6%
Unclassified	14.8%	28.7%
Genus Level		
Bacillus	7.6%	26.6%
Paenibacillus		1.4%
Rhodococcus	9.9%	

Acidimicrobium	1.1%	
Ochrobactrum	26.4%	
Rubellimicrobium	2.1%	
Herbaspirillum	3.8%	
Massilia	1.0%	
Sphingobacterium		1.2%
Effluviibacter	1.3%	
Pontibacter	1.0%	
Gemmatimonas	1.4%	1.9%
Gp6		5.3%
Truepera	1.1%	
Other	17.2%	22.3%
Unclassified	26.0%	41.4%

[a]determined by pyrosequencing of 16S rRNA from metagenomic DNA by using SnoWMAn 1.7.

Table S2. Identification of selected bacterial antagonists isolated from different habitats.

ARDRA group[a]	Isolate number	Microhabitat[b]	Closest database match[c]	Accession number	Similarity (%)
A	Wb2n-1	Desert Soil	*Bacillus vallismortis*	NR_024696	99%
A	Sb1-6	Agricultural Soil	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Sb3-5	Agricultural Soil	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Sb3-13	Agricultural Soil	*Bacillus atrophaeus*	NR_024689	99%
A	Sb3-21	Agricultural Soil	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	99%
A	Sb3-24	Agricultural Soil	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Sb4-14	Agricultural Soil	*Bacillus vallismortis*	NR_024696	99%
A	Sb4-23	Agricultural Soil	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Mc3-4	Mc Rhizosphere	*Bacillus mojavensis*	NR_024693	98%
A	Mc5-18	Mc Rhizosphere	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Mc5-19	Mc Rhizosphere	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Co1-6	Co Rhizosphere	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Co2-14	Co Rhizosphere	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	99%
A	Co7-19	Co Rhizosphere	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	100%
A	Sd1-14	Sd Rhizosphere	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	99%
A	Sd3-12	Sd Rhizosphere	*Bacillus subtilis* subsp. *subtilis*	NR_027552	100%
A	Sd3-21	Sd Rhizosphere	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	99%
A	Sd7-15	Sd Rhizosphere	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	100%
A	Mc1Re-3	Mc Endorhiza	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Mc2Re-2	Mc Endorhiza	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	99%
A	Mc2Re-9	Mc Endorhiza	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Mc2Re-18	Mc Endorhiza	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Mc2Re-21	Mc Endorhiza	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Mc3Re-13	Mc Endorhiza	*Bacillus subtilis* subsp. *subtilis*	NR_027552	98%
A	Mc5Re-2	Mc Endorhiza	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	100%
A	Mc5Re-15	Mc Endorhiza	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Sd2Re-10	Sd Endorhiza	*Bacillus mojavensis*	NR_024693	100%
A	Sd8Re-6	Sd Endorhiza	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	100%
A	Sd8Re-7	Sd Endorhiza	*Bacillus subtilis* subsp. *subtilis*	NR_027552	99%
A	Sd8Re-23	Sd Endorhiza	*Bacillus subtilis* subsp. *spizizenii*	NR_024931	100%
C	Wb1-13	Desert Soil	*Bacillus endophyticus*	NR_025122	99%
C	Mc4-18	Mc Rhizosphere	*Bacillus endophyticus*	NR_025122	99%

D	Wb2-3	Desert Soil	*Paenibacillus polymyxa*	NR_037006	99%
D	Sb3-1	Agricultural Soil	*Paenibacillus kribbensis*	NR_025169	99%
D	Mc2-9	Mc Rhizosphere	*Paenibacillus brasilensis*	NR_025106	99%
D	Mc5-5	Mc Rhizosphere	*Paenibacillus brasilensis*	NR_025106	99%
D	Mc6-4	Mc Rhizosphere	*Brevibacillus limnophilus*	NR_024822	99%
D	Mc2Re-16	Mc Endorhiza	*Paenibacillus brasilensis*	NR_025106	98%
D	Mc5Re-14	Mc Endorhiza	*Paenibacillus polymyxa*	NR_037006	99%
D	Sd5Re-24	Sd Endorhiza	*Paenibacillus brasilensis*	NR_025106	99%
E	Wb1n-4	Desert Soil	*Streptomyces scabiei*	NR_025865	98%
E	Wb2n-2	Desert Soil	*Streptomyces peucetius*	NR_024763	98%
E	Wb2n-11	Desert Soil	*Streptomyces subrutilus*	NR_026203	99%
E	Wb2n-23	Desert Soil	*Streptomyces peucetius*	NR_024763	98%
F	Mc1-3	Mc Rhizosphere	*Lysobacter enzymogenes*	NR_036925	99%

[a]the letters represent the different amplified rRNA gene restriction analysis patterns (A-F), group B (*Bacillus cereus* group) was completely excluded; [b]Mc…*Matricaria chamomilla*, Co…*Calendula officinalis*, Sd…*Solanum distichum*; [c]according to 16S rRNA gene sequencing.

Publication II

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Bacillus and *Streptomyces* were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt

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RESEARCH LETTER

Bacillus and *Streptomyces* were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt

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Abstract

Plant protection via disease-suppressive bacteria in desert farming requires specific biological control agents (BCAs) adapted to the unique arid conditions. We performed an ecological study of below-ground communities in desert farm soil and untreated desert soil, and based on these findings, selected antagonists were hierarchically evaluated. In contrast to the highly specific 16S rRNA fingerprints of bacterial communities in soil and cultivated medicinal plants, internal transcribed spacer profiles of fungal communities were less discriminative and mainly characterised by potential pathogens. Therefore, we focused on *in vitro* bacterial antagonists against pathogenic fungi. Based on the antifungal potential and genomic diversity, 45 unique strains were selected and characterised in detail. *Bacillus*/*Paenibacillus* were most frequently identified from agricultural soil, but antagonists from the surrounding desert soil mainly belonged to *Streptomyces*. All strains produced antibiotics against the nematode *Meloidogyne incognita*, and one-third showed additional activity against the bacterial pathogen *Ralstonia solanacearum*. Altogether, 13 broad-spectrum antagonists with antibacterial, antifungal and nematicidal activity were found. They belong to seven different bacterial species of the genera *Bacillus* and *Streptomyces*. These Gram-positive, spore-forming bacteria are promising drought-resistant BCAs and a potential source for antibiotics. Their rhizosphere competence was shown by fluorescence *in situ* hybridisation combined with laser scanning microscopy.

Introduction

While desertification is recognised as a major threat to biodiversity, the conversion of desert soil into arable, green landscapes is a global vision (Clery, 2011; Marasco *et al.*, 2012). Desert farming, which generally relies on irrigation, is one way to potentially realise this goal. In Australia, Israel, California and Africa, desert farming areas are expanding. For example, desert farming in Egypt will have grown by 40% by 2017 (Reuters, 2007). However, emerging problems with soilborne pathogens, which can substantially limit crop yield, are often reported after several years of agricultural land use (Krikun *et al.*, 1982). These soilborne pathogens include various taxonomic groups, for example, fungi (*Fusarium culmorum*, *Rhizoctonia solani*, *Verticillium dahliae*), bacteria (*Ralstonia solanacearum*) and nematodes (*Meloidogyne incognita*) (Klosterman *et al.*, 2009; Messiha *et al.*, 2009; Neher, 2010). Because of its depleting effect on the ozone layer, the extensively used broad-spectrum soil fumigant methyl bromide was banned by the Montreal Protocol in 1987 and phased out in most countries by 2005. Now, there is an urgent demand for ecologically compatible and efficient strategies to suppress soilborne pathogens in both conventional and organic desert agriculture (Bashan & de-Bashan, 2010).

FEMS MICROBIOLOGY LETTERS

Biological control based on naturally occurring antagonists offers sustainable solutions for plant protection (Weller, 2007; Berg, 2009; Lugtenberg & Kamilova, 2009; Raaijmakers *et al.*, 2009). However, beneficial plant–microorganism interactions are highly specific, and only a few broad-spectrum antagonists have been reported (Zachow *et al.*, 2008; Hartmann *et al.*, 2009). Gram-negative bacteria, especially those from genus *Pseudomonas*, were identified as the dominant members of the indigenous antagonistic communities under humid conditions (Berg *et al.*, 2005; Haas & Défago, 2005; Costa *et al.*, 2006; Zachow *et al.*, 2008) and as a major group of disease-suppressive bacteria through pyrosequencing (Mendes *et al.*, 2011). Although there are problems with the formulation and shelf life of *Pseudomonas*, strains have still been developed as commercial BCAs (Weller, 2007; Berg, 2009). Gram-positive bacteria have also been widely used as BCAs and plant growth-promoting rhizobacteria (PGPRs), even though their ability to colonise the rhizosphere has been controversial (Hong *et al.*, 2009; Fan *et al.*, 2011). Their ability to form durable, heat-resistant endospores allows for easy formulation (Emmert & Handelsman, 1999; Adesemoye *et al.*, 2009), but their use as BCAs in desert agroecosystems is not been established so far.

Desert soils are characterised by arid conditions, which include a combination of extreme temperatures and desiccation, high soil salinity, low nutrient levels, high UV radiation levels and physical instability caused by strong winds (Cary *et al.*, 2010). In one of the most prominent examples of organic desert farming in Sekem (Egypt), we found a strong correlation between long-term organic agriculture and bacterial community composition in soils. Bacterial communities in agricultural soil showed a higher diversity and a better ecosystem function for plant health compared to the surrounding natural desert soil (Köberl *et al.*, 2011). A comprehensive analysis explained these structural differences: the proportion of Firmicutes represented by antagonistic *Bacillus* and *Paenibacillus* in field soil was significantly higher (37%) than in the desert soil (11%). In contrast, Actinobacteria occurred in farmland in lower concentrations (5%) than in the desert (21%), and antagonistic isolates of *Streptomyces* were only isolated from native desert soil (Köberl *et al.*, 2011). A high presence of Actinobacteria in soil of the North American Sonoran Desert was also found by 454-pyrotag analyses (Andrew *et al.*, 2012) as well as in soil of the hyperarid Atacama Desert in north-west Chile (Neilson *et al.*, 2012). From the latter, several so far unknown *Streptomyces* spp. were recently described (Santhanam *et al.*, 2012a,b, 2013). In addition, a study examining soil bacterial communities in the Negev Desert in the south of Israel even revealed a higher abundance of Actinobacteria in barren soils compared to soils under shrub canopies (Bachar *et al.*, 2012). However, the indigenous desert microbiome should contain BCAs that are adapted to the specific biotic and abiotic conditions of desert habitats as well as strains that produce novel bioactive compounds, because the genus *Streptomyces* is known as a unique source of novel antibiotics (Goodfellow & Fiedler, 2010; Niraula *et al.*, 2010; Nachtigall *et al.*, 2011). The potential for both has been until now poorly understood and used.

The objective of this study was to analyse microbial communities from agricultural desert habitats (e.g. from the rhizospheres and endorhiza) in comparison with the surrounding desert soil for their biocontrol potential and to specifically select and characterise broad-spectrum antagonists against soilborne pathogens regarding this potential.

Materials and methods

Experimental design and sampling

Microbial diversity in organic desert farming was studied at Sekem farms (www.sekem.com) in Egypt (30°22′88″N, 31°39′41″E) in comparison with surrounding desert soil (30°35′01″N, 32°25′49″E; 35°59′0″N, 41°2′0″E). The sampling strategy is described in detail in Köberl *et al.* (2011). Briefly, at each site, four composite samples of soil in a horizon of 0–30 cm depth were collected. Furthermore, roots with adhering soil were obtained from three different species of medicinal plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. and Thonn.) planted on a Sekem farm. From each plant species, four independent composite samples consisting of 5–10 plants were taken. Samplings were performed in October 2009 and in April 2010. Physico-chemical data of the soil are provided in Luske & van der Kamp (2009).

Microbial fingerprints from single-stranded conformational polymorphism analysis of the ITS and 16S rRNA region (PCR-SSCP)

Total community DNA was isolated from bulk soil, rhizosphere and endorhiza of the medicinal plants according to Köberl *et al.* (2011). Fingerprinting of microbial communities by SSCP was performed as described by Schwieger & Tebbe (1998). Amplification of the fungal internal transcribed spacer (ITS) fragment was performed by a nested PCR approach with primer pairs ITS1/ITS4 and ITS1/ITS2^{P} (White *et al.*, 1990). Nested PCR was performed as described by Zachow *et al.* (2008). SSCP analysis of bacterial 16S rRNA gene sequences is specified in Köberl *et al.* (2011). Sequences of excised and re-amplified

bands were submitted to EMBL Nucleotide Sequence Database under accession numbers FR854281-FR854290, FR871639-FR871646 and HE655458-HE655480.

SSCP profiles of the microbial communities generated with universal fungal and bacterial primers were further applied for multivariate analysis. According to the distance of the bands, the SSCP gels were theoretically divided into operational taxonomic units (OTUs). The presence or absence of individual amplified product DNA bands in each group was scored. OTUs served as response variables for principal component analysis (PCA) using Canoco 4.5 for Windows (Lepš & Smilauer, 2003). Matrices based on Pearson correlation were subjected to significance tests of pairwise similarities by applying permutation analyses ($P < 0.05$) using the permtest package of R statistics version 2.13.1 (The R Foundation for Statistical Computing, Vienna, Austria) with 10^5 random permutations of sample elements (Kropf *et al.*, 2004; R Development Core Team, 2011).

Screening for *in vitro* activity against soilborne bacteria and nematodes

Forty-five promising strains with antagonistic activity against pathogenic fungi (Köberl *et al.*, 2011) were tested for antibacterial activity against *Ralstonia solanacearum* 1609 and B3B. The activity of all isolates against both *R. solanacearum* strains was identical; therefore, the data in Table 2 are presented in singular form. For the screening, yeast peptone glucose (YPG) medium was used, and Tetrazolium Violet (Sigma-Aldrich, Saint Louis, USA) was added to the medium prior to pouring as a redox indicator of bacterial growth (Adesina *et al.*, 2007; Tsukatani *et al.*, 2008).

For testing the activity of the selected antagonists towards the phytopathogenic nematode *Meloidogyne incognita* (Kofoid and White) Chitwood, culture supernatants from the bacteria were prepared. For this, the bacterial isolates were grown at 28 °C for 24 h on R2A agar (Merck, Darmstadt, Germany). A preculture was grown over night from a single colony in 5 mL of tryptic soy broth (TSB) (Merck) with 50 mg L^{-1} rifampicin at 28 °C with shaking at 150 r.p.m. 200 μL of the preculture were added to 100 mL sterile TSB and incubated for 24 h at 28 °C with shaking. The bacteria were then removed from the culture by centrifugation at 7500 ***g*** for 20 min, followed by sterile filtration of the supernatants through membranes with 0.22 μm pore size. The sterile culture supernatants were kept at 4 °C until application. To study the effect of extracellular bacterial products on the mortality of *M. incognita* juveniles (J2), 500 μL of a juvenile suspension containing approximately 100 freshly hatched J2 was mixed with 1 mL of each bacterial filtrate in a Petri dish with 500 μL of an antibiotic solution containing 300 mg L^{-1} streptomycin and 300 mg L^{-1} penicillin to suppress microbial growth. Each treatment was replicated 4 times. Controls consisted of TSB, water and a culture supernatant of the nonantagonistic strain *Escherichia coli* JM109, respectively. All dishes were kept at 25 ± 2 °C in the dark. Numbers of motile and nonmotile nematodes were counted after 6, 12, 24 and 48 h using a binocular microscope. To distinguish between nonmotile and dead J2, the nematodes were transferred to water at the end of the exposure time. Juveniles that did not recover and become motile again were considered dead. The rate of mortality was determined using linear regression of the percentages of dead J2 after 0, 6, 12 and 24 h.

Fluorescence *in situ* hybridisation (FISH) and confocal laser scanning microscopy (CLSM)

Samples were fixed in 4% paraformaldehyde and stained by in-tube FISH according to the protocol of Cardinale *et al.* (2008). An equimolar mixture of Cy3-labelled EUB338, EUB338II and EUB338III probes (Amann *et al.*, 1990; Daims *et al.*, 1999) was used for the detection of all bacteria and a Cy5-labelled HGC236 probe (Erhart *et al.*, 1997) for the detection of Actinobacteria. As a negative control, nonsense FISH probes labelled with both fluorochromes (NONEUB; Wallner *et al.*, 1993) were applied. Confocal images were obtained using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems GmbH, Mannheim, Germany).

Results

Molecular fingerprinting of microbial belowground communities

All investigated SSCP fingerprints of the ITS and 16S rRNA gene fragments from both the rhizosphere and endorhiza of the medicinal plants and bulk soil showed a high diversity. According to the statistical cluster analysis, there is a clear plant-specific effect on both communities in the rhizosphere (Fig. 1, Table 1). Furthermore, microenvironment-specific SSCP patterns of the microbial communities were detected, and statistically significant differences between the rhizosphere and the endorhiza of the medicinal plants were calculated (Fig. 1, Table 1). Additionally, plant-associated microenvironments were compared with the surrounding soil. The composition of the bacterial and fungal communities in soil differed significantly from the plant-associated communities (*P* values: fungal communities 0.0241; bacterial communities 0.0266) and between agricultural and desert soil (*P* values: fungal communities 0.0291; bacterial communities 0.0289).

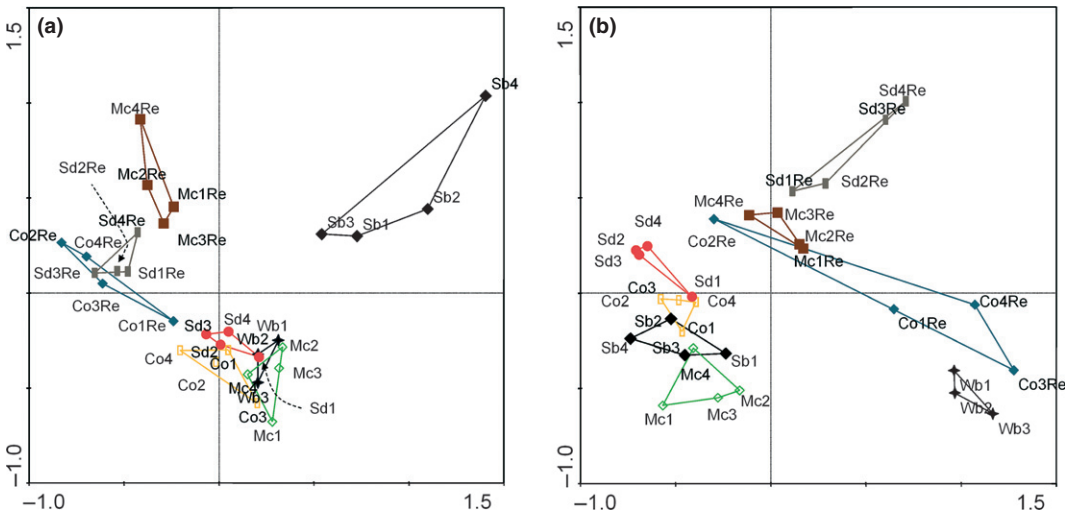

Fig. 1. PCA of OTUs identified by SSCP fingerprinting for fungal (a) and bacterial (b) communities. Samples were encoded using a combination of letters and numbers indicating (1) soil type or plant species (Wb = desert soil, Sb = Sekem soil, Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*), (2) replicate (1–4) and (3) microenvironment (Re = endorhiza, rhizosphere and soil have no further designation).

Table 1. Statistical analysis of microbial fingerprints obtained by PCR-SSCP.

	Fungal communities	Bacterial communities
Microenvironment	*P* values for pairwise comparisons between medicinal plants*	
Rhizosphere†		
Mc-Co	0.0276	0.0281
Co-Sd	0.0284	0.0286
Mc-Sd	0.0296	0.0286
Endorhiza†		
Mc-Co	0.0297	0.0556
Co-Sd	0.0719	0.0283
Mc-Sd	0.0282	0.0293
Medicinal plant	*P* values for comparisons between rhizosphere and endorhiza*	
Matricaria chamomilla	0.0290	0.0287
Calendula officinalis	0.0288	0.0287
Solanum distichum	0.0287	0.0281

*Analysed by permutation test ($P < 0.05$) using R statistics.
†Mc, *Matricaria chamomilla*; Co, *Calendula officinalis*; Sd, *Solanum distichum*.

The fingerprints of the fungal community represented a high diversity in all microenvironments and were similar for the first and second samplings (Fig. 2). In general, potential plant pathogens were frequently found within the fungal communities. In fingerprints from both samplings, *Alternaria* (closest database match *Alternaria tenuissima*, 100% similarity to JN620417) and *Fusarium* (closest database matches *Fusarium chlamydosporum*, 100% similarity to HQ671187 and *Fusarium solani*, 99% similarity to FJ865435) were most commonly found. *Alternaria* was also found in desert soil from Sinai (first sampling) as well as from Saqqara (second sampling). In addition, *Cladosporium* (teleomorph *Davidiella*) was identified in fingerprints from both samplings. In rhizosphere and soil samples from the first sampling, *Epicoccum* (closest database match *Epicoccum nigrum*, 100% similarity to JN578611) was assigned to a dominant band. In soil from the Sinai desert, the black fungus *Aureobasidium* (closest database match *Aureobasidium proteae*, 99% similarity to JN712490) was additionally identified. Similarly, *Verticillium dahliae* (closest database match *V. dahliae* var. *longisporum*, 100% similarity to AB585937) was identified as a dominant band found in almost all plant samples from the second sampling time, which apart from *Fusarium* spp. was one of the main soilborne phytopathogens on the Sekem farms. In samples from the second sampling, the obligate root-infecting pathogen *Olpidium* (closest database match *Olpidium brassicae*, 99% similarity to AB625456), belonging to the fungal phylum Chytridiomycota, and *Sarocladium* (closest database match *Sarocladium strictum*, 100% similarity to JN942832; previously recognised in *Acremonium*) were found. Although several other ITS fragments were not identified, due to this high content of potential phytopathogens in the fungal communities, the selection of antagonists was focused on the bacterial communities.

Detailed characterisation of selected antagonistic strains

A screening of 1212 bacterial isolates resulted in 162 antifungal antagonists against the main fungal soilborne

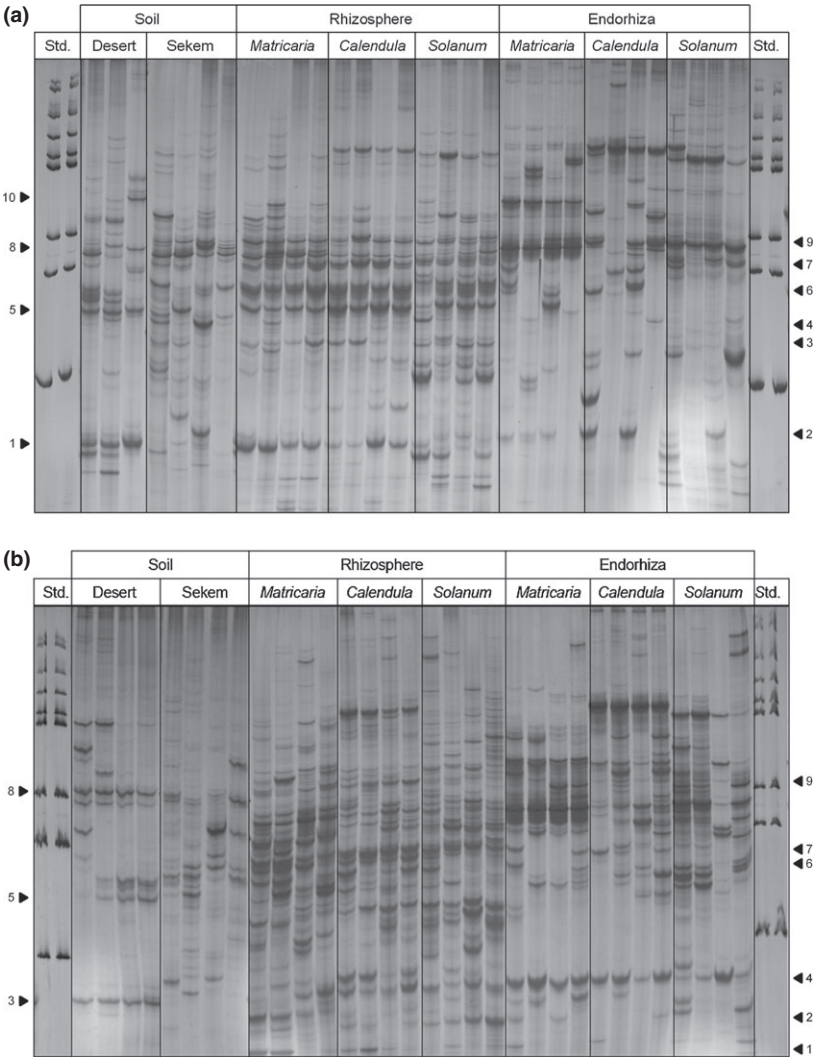

Fig. 2. ITS PCR-SSCP profiles of the fungal communities in soil, rhizosphere and endorhiza of the medicinal plants from first (a) and second (b) sampling time. Std.: 1 kb DNA ladder. (a) From fingerprints of the first sampling (October 2009), the following bands were identified as: 1. *Epicoccum nigrum*, 100% similarity to JN578611; 2. *Pichia jadinii*, 99% similarity to FJ865435; 3. *Gibellulopsis nigrescens*, 100% similarity to JN187998; 4. *Emericella nidulans*, 99% similarity to JN676111; 5. *Alternaria tenuissima*, 100% similarity to JN620417; 6. *Davidiella tassiana*, 99% similarity to JN986782; 7. *Fusarium chlamydosporum*, 100% similarity to HQ671187; 8. *Exserohilum rostratum*, 99% similarity to JN179081; 9. *Fusarium solani*, 99% similarity to FJ865435; 10. *Aureobasidium proteae*, 99% similarity to JN712490. (b) From the second sampling (April 2010), the following bands were identified: 1. *Cryptococcus carnescens*, 99% similarity to GU237051; 2. *Olpidium brassicae*, 99% similarity to AB625456; 3. *Preussia minimoides*, 96% similarity to AY510422; 4. *Verticillium dahliae* var. *longisporum*, 100% similarity to AB585937; 5. *Alternaria tenuissima*, 100% similarity to JN620417; 6. *Fusarium chlamydosporum*, 99% similarity to EU556725; 7. *Cladosporium cladosporioides*, 100% similarity to JN986781; 8. *Ulocladium oudemansii*, 100% similarity to FJ266488; 9. *Sarocladium strictum*, 100% similarity to JN942832.

pathogens (*V. dahliae*, *R. solani* and *F. culmorum*) (Köberl *et al.*, 2011). These fungi were identified in Sekem soil by cultivation and, with the exception of *R. solani*, in the molecular fingerprinting analyses. Altogether, 45 genotypically unique antifungal strains were selected to assess their antibacterial activity against *R. solanacearum* (Table 2). Of these isolates, 33.3% were able to inhibit the growth of the soilborne bacterial pathogen *in vitro*, including most isolates of *Streptomyces* (3 of 4 isolates) and some strains of the *Bacillus subtilis* group (12 of 30 isolates).

Plant-parasitic nematodes often positively interact with soilborne fungal pathogens. Therefore, the selected bacterial isolates were additionally evaluated *in vitro* for their effects against juveniles of the root-knot nematode *M. incognita*. All bacteria accumulated inhibitory substances in the culture medium to some degree, while the medium itself and water had no effect. The percentage of dead J2 continuously increased during the incubation period of 48 h reaching over 70% for 11 strains with a maximum of 89% for strain Mc5Re-2, while only 28% of J2 were dead in the *E. coli* control (Table 2). On average, the increase in mortality was highest within the first 12 h of exposure and declined thereafter. The ten most efficient strains caused between 47% and 63% mortality in the first 24 h, with the highest rates observed for strains Sb4-23, Mc5Re-2, Mc1Re-3 and Sb3-24 (Fig. 3). The seven most efficient antagonists were all isolates of *Bacillus subtilis* obtained from either agricultural soil or from the endorhiza of *M. chamomilla*.

In situ visualisation of Actinobacteria in the rhizosphere

FISH-CLSM analysis confirmed generally high bacterial abundances and occurrence of Actinobacteria in below-ground habitats under arid conditions. Using an

Table 2. List of selected bacterial antagonists isolated from different microenvironments with their antagonistic properties.

			Antagonistic activity towards‡					
							Meloidogyne incognita¶	
ARDRA group*	Isolate number	Closest database match† (accession number), similarity (%)	*Verticillium dahliae*§	*Rhizoctonia solani*§	*Fusarium culmorum*§	*Ralstonia solanacearum*	Dead J2 after 48 h (%)‖	Mortality rate (% J2 per day)**
A	Wb2n-1	*Bacillus vallismortis* (NR_024696), 99%	+	++	+	+	73 ± 6	49 ± 4
A	Sb1-6	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	–	+	+	54 ± 4	32 ± 2
A	Sb3-5	*Bacillus subtilis* subsp. *subtilis* (NR_027552, 99%	+	++	+	+	46 ± 3	25 ± 3
A	Sb3-13	*Bacillus atrophaeus* (NR_024689), 99%	+	++	+	+	33 ± 3	17 ± 1
A	Sb3-21	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	+	–	68 ± 7	52 ± 4
A	Sb3-24	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	++	+	–	78 ± 7	57 ± 4
A	Sb4-14	*Bacillus vallismortis* (NR_024696), 99%	+	+	+	–	45 ± 5	23 ± 1
A	Sb4-23	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	+	+	84 ± 5	63 ± 3
A	Mc3-4	*Bacillus mojavensis* (NR_024693), 98%	+	++	++	+	67 ± 8	30 ± 2
A	Mc5-18	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	++	++	++	–	29 ± 2	14 ± 2
A	Mc5-19	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	–	+	35 ± 4	17 ± 2
A	Co1-6	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	++	++	++	+	70 ± 7	37 ± 3
A	Co2-14	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	+	++	–	72 ± 12	40 ± 5
A	Co7-19	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	++	+	+	–	48 ± 5	26 ± 1
A	Sd1-14	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	++	–	56 ± 5	35 ± 3
A	Sd3-12	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 100%	+	+	++	–	29 ± 2	17 ± 1
A	Sd3-21	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	+	–	57 ± 4	35 ± 5
A	Sd7-15	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	+	++	+	–	43 ± 4	26 ± 2
A	Mc1Re-3	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	++	++	–	80 ± 4	56 ± 7
A	Mc2Re-2	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	+	+	83 ± 4	54 ± 4
A	Mc2Re-9	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	++	–	61 ± 3	38 ± 2
A	Mc2Re-18	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	++	–	82 ± 2	50 ± 6
A	Mc2Re-21	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	–	+	++	–	66 ± 5	46 ± 3
A	Mc3Re-13	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 98%	+	+	+	+	61 ± 3	43 ± 3
A	Mc5Re-2	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	+	+	+	–	89 ± 3	59 ± 3

Table 2. Continued

			Antagonistic activity towards‡					
							Meloidogyne incognita¶	
ARDRA group*	Isolate number	Closest database match† (accession number), similarity (%)	*Verticillium dahliae*§	*Rhizoctonia solani*§	*Fusarium culmorum*§	*Ralstonia solanacearum*	Dead J2 after 48 h (%)‖	Mortality rate (% J2 per day)**
A	Mc5Re-15	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	+	+	33 ± 2	22 ± 1
A	Sd2Re-10	*Bacillus mojavensis* (NR_024693), 100%	++	++	++	–	52 ± 7	24 ± 2
A	Sd8Re-6	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	+	+	+	+	22 ± 2	13 ± 2
A	Sd8Re-7	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	++	++	++	–	24 ± 2	12 ± 1
A	Sd8Re-23	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	++	+	+	–	26 ± 2	14 ± 1
C	Wb1-13	*Bacillus endophyticus* (NR_025122), 99%	–	+	+	–	21 ± 2	14 ± 2
C	Mc4-18	*Bacillus endophyticus* (NR_025122), 99%	–	+	+	–	56 ± 5	21 ± 2
D	Wb2-3	*Paenibacillus polymyxa* (NR_037006), 99%	–	+	+	–	49 ± 4	34 ± 4
D	Sb3-1	*Paenibacillus kribbensis* (NR_025169), 99%	+++	++	+	–	44 ± 6	23 ± 1
D	Mc2-9	*Paenibacillus brasilensis* (NR_025106), 99%	++	++	+	–	64 ± 6	24 ± 1
D	Mc5-5	*Paenibacillus brasilensis* (NR_025106), 99%	++	–	++	–	58 ± 5	26 ± 1
D	Mc6-4	*Brevibacillus limnophilus* (NR_024822), 99%	+++	–	++	–	77 ± 4	39 ± 2
D	Mc2Re-16	*Paenibacillus brasilensis* (NR_025106), 98%	++	+	–	–	57 ± 9	31 ± 4
D	Mc5Re-14	*Paenibacillus polymyxa* (NR_037006), 99%	++	+	++	–	52 ± 3	38 ± 1
D	Sd5Re-24	*Paenibacillus brasilensis* (NR_025106), 99%	++	+	++	–	20 ± 2	11 ± 2
E	Wb1n-4	*Streptomyces scabiei* (NR_025865), 98%	+	++	+	+	70 ± 2	47 ± 4
E	Wb2n-2	*Streptomyces peucetius* (NR_024763), 98%	++	++	+	+	66 ± 3	40 ± 1
E	Wb2n-11	*Streptomyces subrutilus* (NR_026203), 99%	+++	+++	+	+	76 ± 7	48 ± 6
E	Wb2n-23	*Streptomyces peucetius* (NR_024763), 98%	++	+++	+	–	26 ± 3	15 ± 1
F	Mc1-3	*Lysobacter enzymogenes* (NR_036925), 99%	+	++	++	–	63 ± 6	23 ± 2

*The letters represent the different amplified rRNA gene restriction analysis patterns (A-F); group B (*Bacillus cereus* group) was completely excluded (Köberl *et al.*, 2011).

†According to 16S rRNA gene sequencing.

‡Dual culture assay: +…0–5 mm, ++…5–10 mm, +++…> 10 mm radius of zone of inhibition, –…no suppression.

§Results of a previous study performed by Köberl *et al.* (2011).

¶Control with *Escherichia coli* showed 28% dead J2 after 48 h, and a mortality rate of 21%, at controls with media and water both values were 0%.

‖± Standard deviation.

**Determined by linear regression of the percentages of dead J2 after 0, 6, 12 and 24 h, ± error of slope.

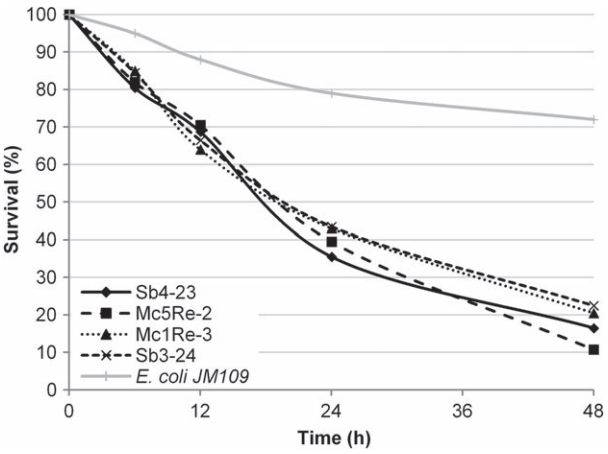

Fig. 3. *In vitro* effects of extracellular bacterial products on the mortality of *Meloidogyne incognita* juveniles. Depicted are the impacts of the four most efficient isolates in comparison with the control with *Escherichia coli* JM109.

Actinobacteria-specific probe, some of these bacterial colonies could be identified in the rhizosphere of *Matricaria chamomilla* as well when grown under organic desert farming conditions (Fig. 4).

Discussion

One of the major challenges of the 21st century will be to develop an environmentally sound and sustainable crop production. Desert agriculture opens up new possibilities to address diverse problems: to produce enough food for poor regions, to produce renewable crops for industrial applications, and to capture and restore CO_2 in soil. The accumulation of soilborne pathogens is another important ecological problem, which can cause dramatic yield losses. To solve this problem, we analysed associated microbial communities, which were found specific for each plant species and microhabitat. ITS profiles of fungal communities were less discriminative than bacterial fingerprints and characterised mainly by potential pathogens. Therefore, we selected bacterial antagonists against these and the well-known pathogens.

The dominance of Gram-positive bacteria in the group of antagonists in plant-associated and soil communities under arid conditions is in contrast to other studies performed under humid, temperate climate conditions. Here, mainly members of the genus *Pseudomonas* were found as antagonists (Berg *et al.*, 2006; Costa *et al.*, 2006; Weller, 2007), as it is well-studied for its beneficial plant–microorganism interaction (Haas & Défago, 2005; Lugtenberg & Kamilova, 2009). To verify our result, *Pseudomonas*-selective medium was used to monitor *Pseudomonas* isolates (King *et al.*, 1954), but only a few colonies were detected (data not shown). This differing ecology between arid and humid environments can be explained by the extreme abiotic conditions, such as the combination of extreme temperatures and desiccation, high soil salinity, low nutrient levels and high UV radiation levels in deserts. Recently, in a farm located in the northwestern desert region of Egypt, Marasco *et al.* (2012) reported a predominant role of *Bacillus* within the plant growth-promoting microbiome associated with the drought-sensitive pepper plant, which supported this conclusion. In addition, in the rhizosphere of Antarctic vascular plants, another extreme environment, Firmicutes were also identified as the most abundant phylum using a deep-sequencing approach (Teixeira *et al.*, 2010). However, in the microbiome of the sugar beet rhizosphere, Firmicutes represent 20% of the bacterial phyla with Proteobacteria as the dominant member (39%) (Mendes *et al.*, 2011). *Bacillus*, *Paenibacillus* and *Streptomyces* are spore-forming bacteria, and spore production aids in survival under suboptimal conditions

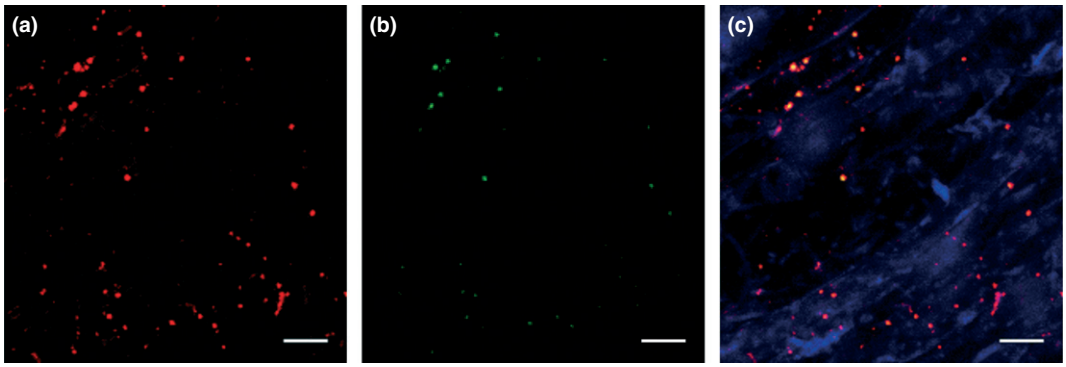

Fig. 4. *In situ* visualisation of Actinobacteria in the rhizosphere of *Matricaria chamomilla*. Fluorescent *in situ* hybridisation (FISH) showed a high colonisation of chamomile roots with bacteria in general (a), of which some colonies could be identified as Actinobacteria (b). The overlay (c) of the fluorochrome signals (a and b) with the autofluorescence of the root (blue) shows examples for Actinobacteria (yellow) amidst other eubacteria (red). Scale bar = 5 μm.

(Nicholson, 2002). However, it is still unclear whether these Gram-positive bacteria were alive and active in soil. Once considered their habitat, the soil may simply just serve as a reservoir (Hong *et al.*, 2009). While rhizosphere colonisation was recently shown by the BCA *Bacillus amyloliquefaciens* FZB42 (Fan *et al.*, 2011), we also found Actinobacteria colonisation as well.

Bacillus/*Paenibacillus* and *Streptomyces* species are well-known for their biocontrol potential (Schisler *et al.*, 2004; Berg, 2009). Several strains of *Bacillus subtilis* are already in use as biological pesticides (Fan *et al.*, 2011), and the antagonistic potential of *Paenibacillus polymyxa* towards a wide range of mycotoxin-producing fungi such as *F. culmorum* is well documented (Tupinambá *et al.*, 2008). Furthermore, a broad disease-suppressive activity has been detected for strains of *Lysobacter* (Postma *et al.*, 2011), the only Gram-negative genus selected. Despite this fact, we know that the biocontrol effect and mode of action are strongly strain-specific (Berg *et al.*, 2006; Berg, 2009). In our study, we detected plant species and microhabitat-specific bacterial antagonists, but also strain specificity was confirmed. Altogether, 13 broad-spectrum antagonists with antibacterial, antifungal and nematicidal activity were found which belong to seven different bacterial species of the genera *Bacillus* (*B. atrophaeus*, *B. mojavensis*, *B. subtilis* subsp. div., *B. vallismortis*) and *Streptomyces* (*S. peucetius*, *S. scabiei*, *S. subrutilus*). On their basis, biocontrol products specifically for arid conditions can be developed.

In this study, we linked ecological data with the selection strategy for antagonists. Within the fungal community, mainly potential phytopathogens were identified. Therefore, we focused on the selection of bacterial antagonists. In the cultivation-independent and dependent approach, strains of *Bacillus*/*Paenibacillus* were found as the key players in bacterial communities in arid agricultural systems. Conversely, members of the genus *Streptomyces* were important in the natural desert ecosystem. This was also confirmed by a comparative deep-sequencing approach of desert and field soil (Köberl *et al.*, 2011). Gram-positive, spore-forming bacteria of the genera *Bacillus*, *Paenibacillus* and *Streptomyces* were selected using our hierarchical procedure; all of them belong to risk group 1 (no risk for humans and the environment) and are promising drought-resistant and heat-resistant biocontrol candidates. Furthermore, they showed a remarkable antibiotic activity.

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Manuscript I

Submitted as Journal Article

The high diversity and abundance of diazotrophs underline their importance for native and agricultural desert ecosystems

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The high diversity and abundance of diazotrophs underline their importance for native and agricultural desert ecosystems

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Submitted

Abstract

Diazotrophs provide the only biological source of fixed atmospheric nitrogen in the biosphere; they are the key for plant-available nitrogen especially in natural or organically managed agricultural ecosystems. Less is known about their diversity and function in arid ecosystems. By a methodological approach combining *nifH*-specific qPCR, fingerprints, amplicon pyrosequencing and FISH-CLSM, nitrogen-fixing underground communities in Egypt were compared. Although the diazotrophic microbiota in the desert ecosystem were characterised by a generally high abundance and diversity – the highest reported until now compared with other ecosystems, statistically significant differences were found between both soil types (native and agriculturally used), between the different microhabitats (bulk soil, rhizosphere, endorhiza), and between the three investigated medicinal plants. We observed a considerable community shift from desert to agriculturally used soil demonstrating a higher abundance as well as a higher diversity in the agro-ecosystem. In comparison to the rhizosphere, the endorhiza was characterised by lower abundances and a subset of species. Comparing root-associated communities of plants grown in desert farming, the most remarkable differences were found. While the microbiomes of *Matricaria chamomilla* and *Calendula officinalis* were similar and dominated by potential root-nodulating rhizobia mainly acquired from soil, the perennial *Solanum distichum* formed primarily associations with free-living nitrogen fixers most likely transmitted between plants as they are undetectable in soils. The results underline the importance of diazotrophs in desert ecosystems, identified plants as important drivers for functional diversity and can explain the underlying mechanism of the phenomenon of rapidly flowering deserts as well.

Keywords: desert farming/medicinal plants/nitrogen-fixing communities/organic agriculture

Introduction

Nitrogen is one of the most yield-limiting factors in agricultural production systems throughout the world and an essential macronutrient for plants (Orr *et al.*, 2011). Nitrogen-fixing microorganisms provide the only natural source of fixed atmospheric nitrogen in the biosphere (Gaby and Buckley, 2012). The capability for nitrogen fixation is widely dispersed among prokaryotic taxa including very divergent, distantly related bacteria and archaea (Zehr and Turner, 2001; Zehr *et al.*, 2003). Biological nitrogen fixation by diazotrophic microorganisms with the input of recycled organic wastes, such as manure or compost, is considered as sustainable alternative to chemical nitrogen fertilisers as well as possibility to reduce rates of fertiliser application (Martínez *et al.*, 2003; Yang *et al.*, 2008). The dispersal of inorganic nitrate into surface and groundwater, which often leads to eutrophication and severe environmental and health problems, can be largely avoided by biological inoculants (Orr *et al.*, 2011).

The *nifH* gene that encodes the nitrogenase reductase subunit is highly conserved over all nitrogenase types and has become the marker gene for studies of phylogeny, diversity, and abundance of diazotrophic microorganisms (Zehr and Turner, 2001). Phylogenetic analyses of *nifH* genes have revealed five primary clusters of gene homologous (Raymond *et al.*, 2004; Gaby and Buckley, 2012). Although a wide range of environments have already been investigated for *nifH* gene diversity (Orr *et al.*, 2011; López-Lozano *et al.*, 2012), the global census of diazotrophic diversity remains far from complete (Gaby and Buckley, 2011, 2012). Recently developed next generation sequencing techniques in combination with network analyses will allow deeper insight into the function of microbial communities involving nitrogen fixation. This has already been recently shown in marine habitats (Farnelid *et al.*, 2013), but not in terrestrial ecosystems.

Also in arid terrestrial ecosystems, plants play an important role for primary production as well as for microbial life (Marasco *et al.*, 2012). Plant specificity of associated bacteria as well as co-evolution of plant-microbe interactions is well-studied (rev. in Berg and Smalla, 2009; Burgarelli *et al.*, 2012; Oldroyd, 2013). Root exudates and especially bioactive metabolites are one of the most important drivers of this selecting effect from the soil microbiome (Bais *et al.*, 2006). Because we found a high specificity in the structural microbial diversity associated with medicinal plants (Köberl *et al.*, 2013), we hypothesised that this high specificity is visible in functional diversity as well. All three selected plants German chamomile (*Matricaria chamomilla*), pot marigold (*Calendula officinalis)* and African nightshade (*Solanum distichum)* are well-known for their anti-microbial effects and bioactive ingredients (McKay and Blumberg, 2006; Ukiya *et al.*, 2006; Bahgat *et al.*, 2008). Although the highly specific association between *Rhizobium* cultivars and legumes is well-investigated (Oldroyd, 2013), less is known about specific free-living nitrogen-fixing bacteria.

The objective of this research was to study the nitrogen-fixing communities in the endorhiza, rhizosphere of medicinal plants and bulk soil of long-term organically managed agricultural soil from the Sekem farms in Egypt in comparison to unexploited native desert soil. Therefore, we used a methodological approach combining *nifH*-specific qPCR, fingerprints, amplicon pyrosequencing and FISH-CLSM. Our previous research showed a higher antagonistic potential against soil-borne pathogens, a higher overall bacterial diversity, and better ecosystem function for plant health in soil used for desert agriculture (Köberl *et al.*, 2011, 2013). Based on these results, we hypothesised that the agricultural use of desert soil, especially crop rotation with leguminous cover crops, also enhances the diversity of the nitrogen-fixing underground community. However, we expected also a high abundance of diazotrophs in desert soil, which could explain the rapid plant development and phenomenon of flowering deserts.

Materials and Methods

Experimental design and sampling

Nitrogen-fixing communities were studied at the organically managed Sekem farm Adleya (www.sekem.com) located in the North-eastern desert region of Egypt near Bilbeis (30°22’88"N, 31°39’41"E). Physico-chemical data of the soil is provided in Luske and van der Kamp (2009). The diazotrophic community of bulk agricultural soil was investigated in comparison to the community of native desert soil from Sinai (30°35'01"N, 32°25'49"E). Profiles of the *nifH* gene in communities associated with the rhizosphere and endorhiza of three different species of medicinal plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. and Thonn.) were studied and compared. The sampling strategy and total community DNA isolation is described in detail in Köberl *et al.* (2011).

Quantification of microbial nifH genes by qPCR

To determine *nifH* gene abundances, quantitative PCRs were performed according to Hai *et al.* (2009) with some modifications. Reactions were conducted in a total volume of 10 µl containing 1× KAPA SYBR FAST qPCR MasterMix Universal (PEQLAB, Polling, Austria), 0.6 mg ml^{-1} BSA, 0.125 µM of primers nifH-F and nifH-R (Rösch *et al.*, 2002) and 0.8 µl template DNA dilutions (95 °C, 10 min; 39 cycles of 95 °C, 45 s; 55 °C, 45 s; 72 °C, 45 s; and melt from 72 to 95 °C). Rotor-Gene 6000 real-time rotary analyser (Corbett Research, Sydney, Australia) was used for quantification of fluorescence. For absolute quantification, the PCR amplified *nifH* gene fragment from *Erwinia carotovora* DSM549 was ligated into the pGEM-T Easy Vector (Promega, Mannheim, Germany) and transformed into *E. coli* DH5α. Serial dilutions of PCR fragments generated with the vector-specific primers usp and rsp (Köberl *et al.*, 2011) were used as standard for calculation of *nifH* gene copy

numbers. Concentrations determined by absolute quantification were calculated to copy number per g soil or fresh weight (fw) of plant. Each replicate sample was analysed in duplicates in three independent runs. Statistical analysis was performed with PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) using the independent samples *t*-test for differences between desert and agricultural soil, and the Games-Howell post hoc test for plant samples.

Fingerprints from single-stranded conformational polymorphism analysis of the nifH gene (PCR-SSCP)

Fingerprinting of microbial communities using SSCP was conducted as described by Schwieger and Tebbe (1998) specifically adapted for *nifH* gene fragment separation by Bragina *et al.* (2011). Comparisons of SSCP-generated *nifH* profiles were performed using GelCompar II 5.1 (Applied Maths, Kortrijk, Belgium) and statistically assessed (Kropf *et al.*, 2004; R Development Core Team, 2011).

nifH gene profiling using 454 pyrosequencing

The nitrogenase gene *nifH* was amplified according to a nested PCR protocol with primers designed by Zani *et al.* (2000). The first PCR was performed again with the primer pair nifH4/nifH3 as described above for SSCP analysis. Amplicons served as templates for the second PCR (30 µL) with the primer pair nifH1 and nifH2 designed by Zehr and McReynolds (1989) that contained the 454 pyrosequencing adaptors, linkers, and sample-specific tags (Supplementary Table S1). Accordingly, 3 µL template dilutions were added to 1× Taq&Go, 1.5 mM $MgCl_2$ and 0.2 µM of each primer (95 °C, 5 min; 30 cycles of 95 °C, 1 min; 65.5 °C, 1 min; 72 °C, 30 sec; and elongation at 72 °C, 5 min). Obtained PCR products were purified by employing the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). For rhizosphere samples, PCR products of two independent PCR reactions were pooled. For soil

samples, respective PCR products from four independent replicate samples were pooled from each habitat in equal volumes. Pyrosequencing read libraries were generated by Eurofins MWG (Ebersberg, Germany) using the Roche 454 GS-FLX+ Titanium sequencing platform.

Primer sequences were cropped, reads with low quality (minimum average base quality score 20) and a read length shorter than 200 bp were removed and remaining sequences were translated into their amino acid sequence using the tool FrameBot of RDP's FunGene Pipeline (http://fungene.cme.msu.edu/FunGenePipeline). All subsequent analyses were done on amino acid sequence datasets that were normalised to the same number of sequences within a habitat (5 217 sequences per soil sample and 553 sequences per rhizosphere sample) using the in-house developed Perl script Subsetify 1.4 (10 times random re-samplings followed by subset formation) (Bragina *et al.*, 2013). Amino acid sequences were aligned and clipped at the same alignment reference position (~ 108 amino acids) by using ClustalX 2.1 (Larkin *et al.*, 2007). OTUs were classified and rarefaction curves were constructed based on the distance matrices of amino acid sequences at 0%, 4% and 8% dissimilarity (Farnelid *et al.*, 2011) using mcClust and rarefaction of RDP's FunGene Pipeline. Diversity indices were ascertained based on the clustering data (Shannon, 1997; Chao and Bunge, 2002). Representative sequences at 8% dissimilarity were selected for the following taxonomic and phylogenetic analysis (Farnelid *et al.*, 2011) where clusters with less than 1% of relative abundance were not designated. Nearest relatives were retrieved using the search tool tblastn of the NCBI database.

A neighbour-joining tree with 100 bootstrap replications was created with the tools seqboot, protdist, neighbor, consense and fitch of PHYLIP 3.69 (Felsenstein, 1989). The phylogenetic tree was visualised and edited in MEGA4 (Tamura *et al.*, 2007). A heat map showing the number of sequences for each OTU was added. As an out-group root, a partial sequence of the light-independent photochlorophyllide reductase subunit L (BchL) from

Chlorobaculum tepidum (accession number AAG12203) was selected. Chlorophyllide reductases share a small, but significant degree of similarity with NifH (Zehr and Turner, 2001).

A profile clustering network analysis was performed in order to highlight single OTUs (8% dissimilarity) with considerable differences between the rhizospheres of the medicinal plants. The network analysis was carried out with OTUs exhibiting a mean read change between plants of more than 1% of the normalised data set. If the ratio of mean OTU read numbers exceeded two, the OTUs were regarded as altered and assigned to the respective profile. Visualisation of the network was carried out using Cytoscape 2.8.2 (Smoot *et al.*, 2011). Significant differences between medicinal plants were calculated with Metastats (White *et al.*, 2009). P values were computed using a combination of the nonparametric *t*-test, exact Fisher's test and the false discovery rate with 1 000 permutations.

Fluorescence in situ hybridisation (FISH) and confocal laser scanning microscopy (CLSM)

Samples were fixed in 4% paraformaldehyde and stained using in-tube FISH according to the protocol of Cardinale *et al.* (2008). For the detection of Alphaproteobacteria, the Cy5-labelled ALF968 probe (Loy *et al.*, 2007) was used. Betaproteobacteria were detected with the ATO488-labelled BET42a probe applied together with an unlabelled competitor probe to avoid unspecific hybridizations (Manz *et al.*, 1992). An equimolar mixture of Cy3-labelled EUB338, EUB338II and EUB338III probes (EUB338MIX; Amann *et al.*, 1990; Daims *et al.*, 1999) was used for the detection of all bacteria. As a negative control, non-sense FISH probes labelled with all three fluorochromes (NONEUB; Wallner *et al.*, 1993) were applied. Confocal images were obtained using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems GmbH, Mannheim, Germany). Three-dimensional models were generated with Imaris 7.0 (Bitplane, Zurich, Switzerland).

Results

Abundances of nifH genes in different underground communities

Analysis of *nifH* gene copy numbers resulted in a statistically significant higher abundance in the agriculturally used soil (6.0 ± 0.3 $\log_{10}$ g^{-1}) in comparison to the native desert soil (4.4 ± 0.7 $\log_{10}$ g^{-1}) (Figure 1). Among the medicinal plant-associated microenvironments, rhizospheres showed significantly higher *nifH* gene copy numbers than endorhiza samples. Calculated abundances in the rhizospheres ranged from 7.9 ± 0.1 to 8.3 ± 0.1 $\log_{10}$ g^{-1} fw and were not significantly different between medicinal plants. Conversely, the endorhiza of the perennial *S. distichum* was more significantly colonised by nitrogen-fixing microorganisms (5.9 ± 0.1 $\log_{10}$ g^{-1} fw) than the endorhizas of the annual Asteraceae *M. chamomilla* (4.7 ± 0.3 $\log_{10}$ g^{-1} fw) and *C. officinalis* (4.9 ± 0.2 $\log_{10}$ g^{-1} fw).

Molecular fingerprinting of diazotrophic underground communities

SSCP fingerprints of *nifH* genes in microbial communities revealed a first insight into the diversity (Supplementary Figure S1). According to the statistical comparison analysis (Supplementary Figure S2), the diazotrophic community composition of field soil differed significantly from the less diverse desert soil ($p = 0.0286$) by approximately 70%. Rhizospheres exhibited significantly different profiles to bulk agricultural soil ($p = 0.0005$) as well as to the inner tissue of the root ($p < 0.0001$). Also, statistically significant differences in nitrogen-fixing rhizosphere communities were detected between the three different medicinal plants (p values between 0.0285 and 0.0290). Approximately half of the *nifH* gene community was shared by all three plants; *M. chamomilla* and *C. officinalis* were more similar to each other, but still nearly 40% of their communities were determined by plant-specific nitrogen fixers. Dominant bands were identified as *Rhizobium* sp., *Bradyrhizobium* sp. and *Burkholderia* sp. Furthermore,

Paenibacillus spp. were assigned to dominant endorhiza bands. Throughout all plant-associated microenvironments, several cyanobacteria from the genera *Anabaena* and *Nostoc* were found. Additionally, the *nifH* gene sequence of the methanogenic archaeon *Methanocella* was identified.

Pyrosequencing-based nifH profiling

To gain deeper insight into the diazotrophic community composition and diversity in soils and medicinal plant rhizospheres, a pyrosequencing-based analysis of the *nifH* gene was employed. Normalised NifH sequence libraries were rarefied at three cut-off levels (0%, 4% and 8% amino acid dissimilarity) (Supplementary Figure S3). Sequences from both soil types (10 434 reads) were classified into 361 OTUs with a dissimilarity cut-off of 8% (desert soil 118 OTUs; agricultural soil 290 OTUs) and rhizosphere sequences (6 636 reads) were clustered into 400 OTUs (86 to 124 OTUs per sample). At a genetic dissimilarity level of 8%, the coverage of Chao1 estimated richness reached 61.1% and 56.9% for desert and agricultural soil, respectively (Supplementary Table S2). The calculated Shannon diversity indices (H') were much lower for the desert soil than for the agricultural soil indicating higher diazotrophic diversity due the agricultural use of the desert. The coverage of the rhizosphere samples was between 78.9 and 42.0%, and Shannon indices barely showed differences; their values ranged from 3.26 to 3.92 at 8% dissimilarity.

Among quality amino acid sequences, 70.6% of soil reads and 68.1% of rhizosphere reads could be taxonomically assigned to at least class level (Figure 2). The desert soil in particular exhibited an overwhelming dominance of NifH sequences related to Alphaproteobacteria (82.4%). Field soil revealed a high proportion of unclassified sequences, yet still containing 41.0% Alphaproteobacteria. In regards to phyla with greater than 1% of quality reads: 1.9% of field soil sequences could be affiliated to Deltaproteobacteria, 10.8% to

Bacilli, 1.2% to Clostridia, 2.8% to Spirochaetes, and 1.1% to Cyanobacteria. Rhizosphere samples also revealed a high abundance of Alphaproteobacteria (58.0-15.4%). Additionally, Betaproteobacteria were found in all samples (55.7-1.3%). Higher proportions of Alphaproteobacteria were found in the rhizospheres of the Asteraceae (*M. chamomilla* 55.2-39.8% and *C. officinalis* 58.0-42.1%), in comparison to Betaproteobacteria that were more dominant in the rhizosphere of *S. distichum* (55.7-27.1%). Gammaproteobacteria were additionally found in the rhizosphere of *S. distichum* (19.7-2.4%) and of *M. chamomilla* (≤1.8%), yet Deltaproteobacteria were only identified in the *S. distichum* rhizosphere (≤2.2%). Among Firmicutes, Bacilli were found associated with *M. chamomilla* (≤17.2%), and Clostridia with *M. chamomilla* (≤1.6%) and *C. officinalis* (≤1.3%). Cyanobacteria were found in the rhizospheres of *M. chamomilla* (≤2.7%) and *C. officinalis* (≤1.8%).

Representative sequences of the dominant alphaproteobacterial NifH clusters in native desert soil showed 99-100% of similarity to the NifH sequences of the genus *Rhizobium* (Supplementary Table S3). Further, *Bradyrhizobium* and *Mesorhizobium* were also found. The diversity within Alphaproteobacteria was higher in the agriculturally used soil with 40.9% identified as *Rhizobium*, 12.9% as *Agrobacterium*, 38.2% as *Methylocystis*, 5.1% as *Bradyrhizobium* and 2.9% as *Methylocella*. Classifiable Bacilli in unplanted field soil were identified as *Paenibacillus*, and all cyanobacterial reads revealed the genus *Anabaena* as the closest hit. Deltaproteobacteria, Clostridia, and Spirochaetes could not be identified at the genus level (<95% of sequence similarity). In all three investigated medicinal plant rhizospheres, alphaproteobacterial sequences of the genera *Rhizobium*, *Ensifer* and *Bradyrhizobium* were found, and *Agrobacterium* was identified in the rhizospheres of the Asteraceae. *Methylocystis* was found associated with roots of *C. officinalis* and *S. distichum*, and *Azospirillum* with *M. chamomilla* and *S. distichum*. Clusters of Betaproteobacteria found in all rhizospheres were identified as *Ideonella* and *Derxia*, and *Zoogloea* was found

associated with both Asteraceae. In the rhizospheres of *C. officinalis* and *S. distichum*, nitrogen-fixing *Burkholderia* and *Azoarcus* were additionally identified. *Dechloromonas* was found in rhizospheres of *M. chamomilla* and *S. distichum*, and *Azospira* and *Azonexus* were only associated with the *S. distichum* root. Classifiable Gammaproteobacteria in the rhizospheres of *M. chamomilla* and *S. distichum* were affiliated with the genus *Azomonas*. All deltaproteobacterial reads found associated with *S. distichum* were classified in the genus *Geoalkalibacter*, and all classifiable Bacilli reads in the rhizosphere of *M. chamomilla* were identified as *Paenibacillus*.

In a phylogenetic tree distinguishing between the major *nifH* gene types, all NifH sequences from the soil sample libraries were affiliated with the canonical *nifH* clusters I and III (Figure 3). Conventional molybdenum nitrogenases (cluster I) were dominated by Alphaproteobacteria (closest related to *Rhizobium*, *Methylocella*, *Bradyrhizobium*, *Methylocystis*, *Mesorhizobium*, *Skermanella* and *Agrobacterium*), but also contained sequences from Cyanobacteria (*Anabaena*), Actinobacteridae (*Frankia*), and Bacilli (*Paenibacillus*). Reads affiliated with the molybdenum nitrogenases from anaerobes (cluster III) were most closely related to Spirochaetes (*Treponema*), Clostridia (*Clostridium*, *Acetobacterium* and *Desulfosporosinus*), and Deltaproteobacteria (*Desulfovibrio*). No sequences of alternative nitrogenases (cluster II) and *nifH* paralogs (clusters IV and V) were found in the soil libraries.

A profile clustering network analysis was applied to gain better insight into the differences between the diazotrophic communities of the three medicinal plant rhizospheres (Figure 4). OTUs equally distributed among all three rhizospheres were neglected in this network. The rhizosphere profiles revealed more similar diazotrophic communities between *M. chamomilla* and *C. officinalis*, which were dominated by potential root nodule bacteria. NifH sequences of OTUs identified as genera *Ensifer*, *Rhizobium* and *Bradyrhizobium* were

found in significantly higher abundances in the rhizospheres of Asteraceae. Conversely, the rhizosphere of *S. distichum* was colonised in greater numbers by nitrogen-fixing Beta-, Gamma-, and Deltaproteobacteria. Significantly higher read counts were obtained for OTUs identified as *Ideonella*, *Dechloromonas*, *Azoarcus*, *Azospira* and *Azonexus*. For each medicinal plant, several specific OTUs were found within the nitrogen-fixing community.

In situ visualisation of bacterial rhizosphere colonisation

FISH-CLSM analyses revealed high rhizospheric colonisation. A high abundance of Alphaproteobacteria in the rhizosphere of medicinal plants grown under arid desert farming conditions was confirmed as shown for *M. chamomilla* with Alpha- and Betaproteobacteria-specific probes (Figure 5). Among the root associated bacteria, they were clearly dominant over Betaproteobacteria and were found as both single cells and in large colonies in close interaction with other undefined eubacteria.

Discussion

An immense diversity and high abundance of diazotrophic communities was detected in all investigated arid habitats, thus strongly supporting their important role in native and agricultural desert ecosystems. However, although the diazotrophic microbiota were characterised by a generally high diversity and abundance, statistically significant differences were found between both native and agriculturally used soil, between the different microhabitats endorhiza, rhizosphere and bulk soil, and between the medicinal crops grown in desert farms.

All microenvironments were inhabited primarily by proteobacterial nitrogen fixers, and the desert soil was noticeably dominated by non-symbiotic Rhizobiales. Similar results were reported by López-Lozano *et al.* (2012) for soil in the Chihuahuan Desert in Mexico

investigated using *nifH* gene clone libraries. However, using deep sequencing we found a much higher diversity: NifH sequences from soils could be classified into 361 OTUs and rhizosphere reads into 400 OTUs at a genetic dissimilarity level of 8%. Moreover, pyrosequencing techniques for *nifH* gene sequencing allow for the analysis of more sequences than those analysed for the global study published two years before (Gaby and Buckley, 2011). The high NifH diversity was mainly focussing on bacteria; the only suggestion for archaeal nitrogen fixation in these unique desert habitats was found with *Methanocella* in the endorhiza, especially from *C. officinalis*. The *nifH* genes are widely found in many methanogens, but not all of them are capable of nitrogen fixation. A complete *nif* operon that enables the organism to undergo nitrogen fixation was recently detected in the genome of *Methanocella conradii* HZ254 (found as closest database match in this study) (Lü and Lu, 2012). However, the genome of the closely related species *Methanocella paludicola* SANAE only contains genes similar to *nifH* that are neither part of an operon with other *nif* genes nor associated with the nitrogen fixation function (Sakai *et al.*, 2011). Through comparisons with the metagenomically reconstructed genome sequence of the Rice Cluster I archaeon RC-I_{MRE50} for which a full component of the genes for nitrogenase was found (Erkel *et al.*, 2006), Sakai *et al.* (2011) already discussed an inter-species physiological difference between the nitrogen fixation capabilities among the members of the order Methanocellales.

After long term organic desert agriculture, a considerable shift in the diazotrophic soil community towards both higher abundance and diversity was observed. The agricultural use of desert soil, especially by crop rotation with leguminous cover crops like alfalfa or clover, enhances the diversity and activity of the nitrogen-fixing underground community as well. Nitrogen-fixing microorganisms in deserts play an indispensable role for both plant development and growth, yet desert soils are characterised by harsh environmental conditions, e.g. extreme temperatures and desiccation, high soil salinity, low nutrient levels, high UV

radiation levels and physical instability caused by strong winds (Cary *et al.*, 2010), that can be transformed into a more manageable environment by watering and cultivating plants. With the same primer pair, we calculated much higher abundances of *nifH* genes in the rhizospheres of the investigated medicinal plants in Egypt than Hai *et al.* (2009) in the rhizosphere of *Sorghum bicolor* grown in Burkina Faso. Only a fraction of the medicinal plant-associated nitrogen fixers were able to invade the root and successfully colonise the inner tissue. Abundances calculated for endorhizas totalled 58-71% of the appropriate rhizosphere, and the diversity of nitrogen-fixing microorganisms in the inner part of the root was noticeably lower. Although we expected a higher abundance of nitrogen fixers in desert soil, all habitats influenced by desert agriculture contained a higher amount of potential nitrogen fixers. However, native desert soil also showed an impressive diversity and relatively high abundance in contrast to other ecosystems, thus explaining the rare phenomenon of rapidly flowering desert ecosystems after rainfall. In contrast to previous studies describing desert microbial communities to be structured solely by abiotic processes (Cary *et al.*, 2010), in addition we identified plants as important drivers for the functional diversity in arid soil ecosystems.

Most impressively, the high specificity for each plant species is at the functional level. While specific structures of microbial communities were previously described (rev. in Berg and Smalla, 2009), this was confirmed for the structure of *Arabidopsis* cultivars using amplicon libraries (Bulgarelli *et al.*, 2012). In this study, we found evidence for the specificity of the diazotrophic communities associated with the three plant species. In general, diazotrophic communities found in the medicinal plant-associated microenvironments (rhizosphere and endorhiza) were more similar between the two Asteraceae in comparison to the *Solanum*, and the *nifH* gene profiles revealed a higher abundance of Alphaproteobacteria associated with the roots of the Asteraceae. Conversely, the nightshade showed a higher

proportion of Betaproteobacteria. Previous investigations of the total bacterial as well as fungal communities already revealed such similar colonisation patterns between *M. chamomilla* and *C. officinalis* compared to *S. distichum* (Köberl *et al.*, 2013). Both Asteraceae are well-known annual medicinal plants and produce several flavonoids, which can explain their similar microbiomes. Although the blood pressure lowering effects of the African *S. distichum* were experimentally shown, its bioactive ingredients are not all identified (Bahgat *et al.*, 2008). Nitrogen-fixing alphaproteobacterial genera were also massively represented in both soils where plants primarily acquire their associated microbial community (Berg and Smalla, 2009). The question remains, however, as to where the Beta- and Gammaproteobacteria originated as they were identified neither in the desert nor in agriculturally conditioned bulk soil. Transmission through seeds or pollen would be possible, as recent studies on pumpkin flowers uncovered that the surface of pollen grains is densely colonised by Beta- and Gammaproteobacteria (Fürnkranz *et al.*, 2012). Plant seeds are well known carriers of seed-borne pathogens, but also of beneficial microorganisms (Hardoim *et al.*, 2012). Altogether, we could support our hypothesis that high specificity is also visible in the functional diversity of plant-associated bacteria.

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Supplementary information is available.

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Titles and legends to Figures

Figure 1 Abundances of *nifH* genes in bulk soils, rhizosphere, and endorhiza of medical plants detected by qPCR. Wb = desert soil, Sb = agricultural soil; Mc = *M. chamomilla*, Co = *C. officinalis*, Sd = *S. distichum*; Re = endorhiza, rhizosphere has no further designation. Averages of *nifH* gene copy numbers per gram soil or fresh weight as $\log_{10}$ and confidences are shown. Significant differences between samples ($p \leq 0.05$) are indicated by different letters.

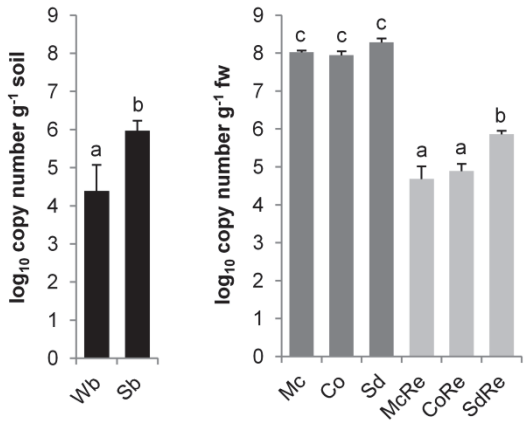
Figure 2 Taxonomic classification of NifH sequences in bulk soils and associated with the rhizosphere of medical plants obtained by 454 amplicon sequencing. Wb = desert soil, Sb = agricultural soil, Mc = *M. chamomilla*, Co = *C. officinalis*, Sd = *S. distichum*. NifH amino acid sequences were classified at class (a) and genus (b) level. From each medical plant, four independent replicate samples were investigated separately. PCR products of soil replicates were pooled to one composite sample per soil type. Clusters containing less than 1% of quality sequences were not designated. Multi-coloured charts at the legend are shown for each soil or rhizosphere type correspondingly.

Figure 3 Phylogenetic composition of diazotrophic communities in desert (Wb, squares) and agricultural soil (Sb, circles). Each library comprised 5 217 high quality NifH sequences clustered at 8% dissimilarity. Neighbour-joining phylogenetic tree was constructed with both one representative sequence per each OTU and the closest database match (accession numbers in brackets). A partial sequence of the light-independent photochlorophyllide reductase subunit L (BchL) from *Chlorobaculum tepidum* (AAG12203) was used as an out-group. Reliability of the tree topology was evaluated by bootstrap analysis with 100 re-samplings (bootstrap values > 50% are indicated as black circles). Sequences were affiliated to the

canonical *nifH* clusters I and III. Numbers of sequences in each cluster are indicated in a heat map. OTUs containing less than 1% of the normalised data set were not phylogenetically designated. The scale bar indicates 0.2 amino acid substitutions per site.

Figure 4 Profile clustering network analysis of NifH sequence libraries of rhizosphere samples from *M. chamomilla*, *C. officinalis* and *S. distichum* at a dissimilarity level of 8%. The abundance values for OTUs with a mean read change between plants of more than 1% of the normalised data set were used. If the ratio of mean OTU read numbers exceeded 2, the OTUs were regarded as altered and assigned to the respective profile. Node sizes of OTUs correspond to the relative abundance of the total data set; nodes matching to abundances of 0.5% and 10% were added as reference points. Distributions between plants are displayed by widths of connection lines. Significances ($p \leq 0.05$) are indicated by coloured node borders: red node borders indicate significances between connected and all not linked profiles, green is used for significances between *M.* and *Calendula*, orange for significances between *Calendula* and *Solanum*, and blue for significances between *Matricaria* and *Solanum*; nodes with black borders showing no significant differences. Black node labels indicate a similarity to the taxonomic node label (closest database match) of ≥ 95%, whereas gray node labels have a similarity < 95%.

Figure 5 *In situ* visualisation of the bacterial colonisation in the rhizosphere of *M. chamomilla*. Volume rendering (a), corresponding XY, XZ, YZ projections (b), and a three-dimensional reconstruction model (c) of confocal laser scanning microscopy stacks. (a-b) yellow = Alphaproteobacteria, pink = Betaproteobacteria, red = other eubacteria, cyan = root tissue (c) green = Alphaproteobacteria, blue = Betaproteobacteria, red = other eubacteria, beige = root tissue. Scale bar = 30 μm.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b
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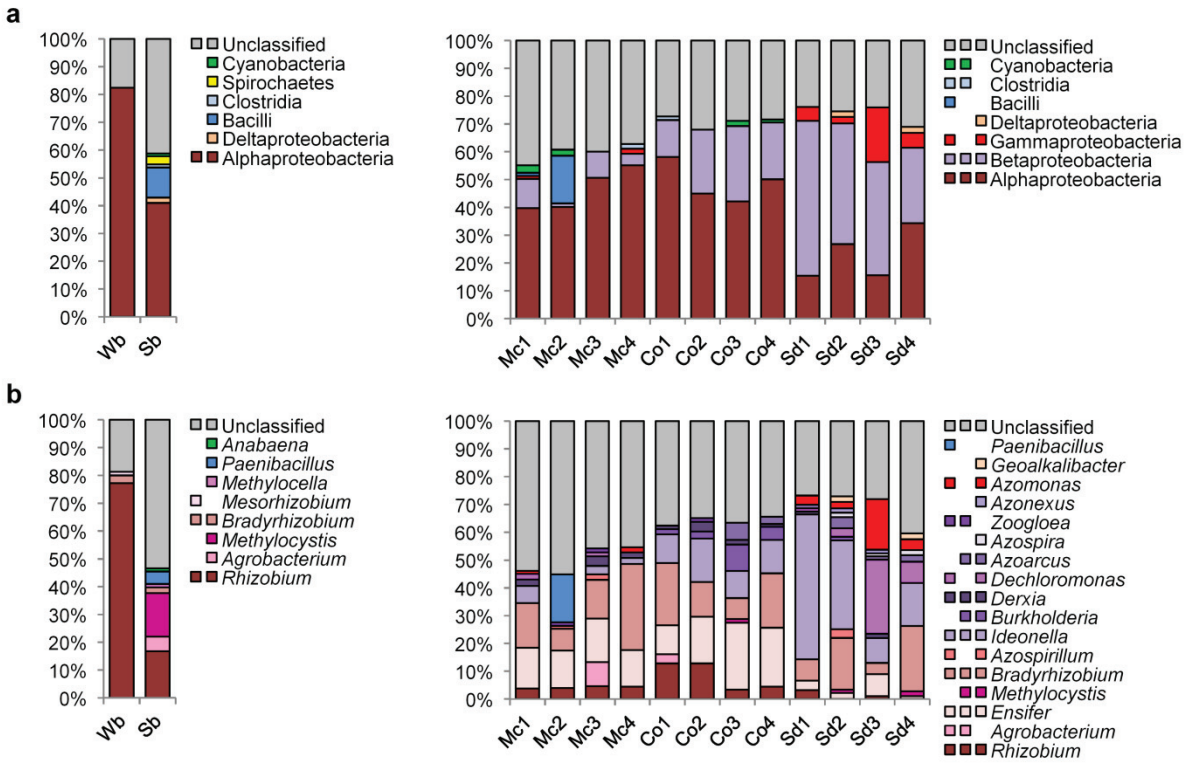
b
Mc
Co
Sd
McRe
CoRe
SdRe

Figure 1


a
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90%
80%
70%
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0%
Wb
Sb
Unclassified
Cyanobacteria
Spirochaetes
Clostridia
Bacilli
Deltaproteobacteria
Alphaproteobacteria
Mc1
Mc2
Mc3
Mc4
Co1
Co2
Co3
Co4
Sd1
Sd2
Sd3
Sd4
Unclassified
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Clostridia
Bacilli
Deltaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
b
Unclassified
Anabaena
Paenibacillus
Methylocella
Mesorhizobium
Bradyrhizobium
Methylocystis
Agrobacterium
Rhizobium
Unclassified
Paenibacillus
Geoalkalibacter
Azomonas
Azonexus
Zoogloea
Azospira
Azoarcus
Dechloromonas
Derxia
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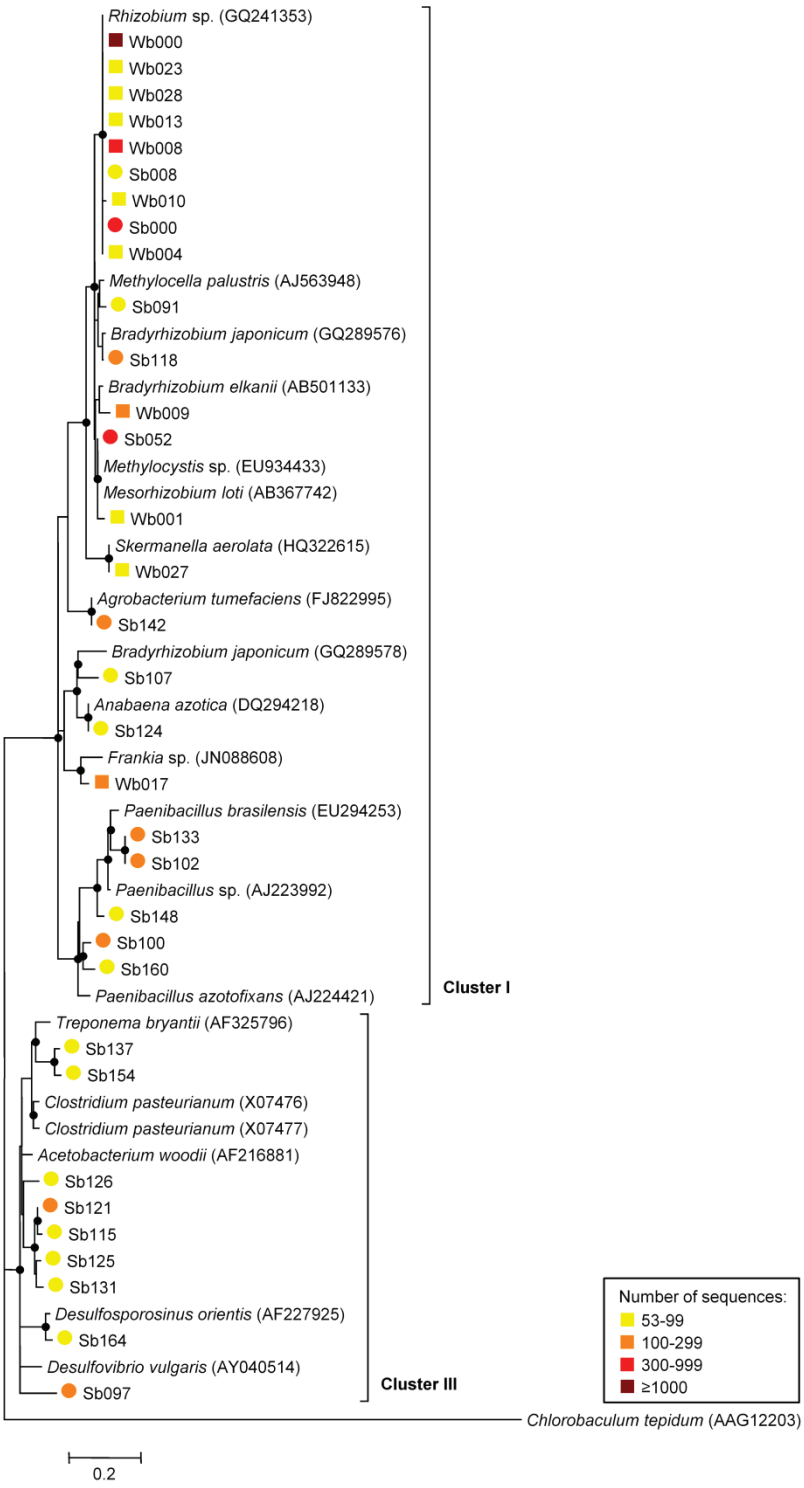
Ideonella
Azospirillum
Bradyrhizobium
Methylocystis
Ensifer
Agrobacterium
Rhizobium

Figure 2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Wb028
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Wb010
Sb000
Wb004
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Sb091
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Sb118
Bradyrhizobium elkanii (AB501133)
Wb009
Sb052
Methylocystis sp. (EU934433)
Mesorhizobium loti (AB367742)
Wb001
Skermanella aerolata (HQ322615)
Wb027
Agrobacterium tumefaciens (FJ822995)
Sb142
Bradyrhizobium japonicum (GQ289578)
Sb107
Anabaena azotica (DQ294218)
Sb124
Frankia sp. (JN088608)
Wb017
Paenibacillus brasilensis (EU294253)
Sb133
Sb102
Paenibacillus sp. (AJ223992)
Sb148
Sb100
Sb160
Paenibacillus azotofixans (AJ224421)
Cluster I
Treponema bryantii (AF325796)
Sb137
Sb154
Clostridium pasteurianum (X07476)
Clostridium pasteurianum (X07477)
Acetobacterium woodii (AF216881)
Sb126
Sb121
Sb115
Sb125
Sb131
Desulfosporosinus orientis (AF227925)
Sb164
Desulfovibrio vulgaris (AY040514)
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Number of sequences:
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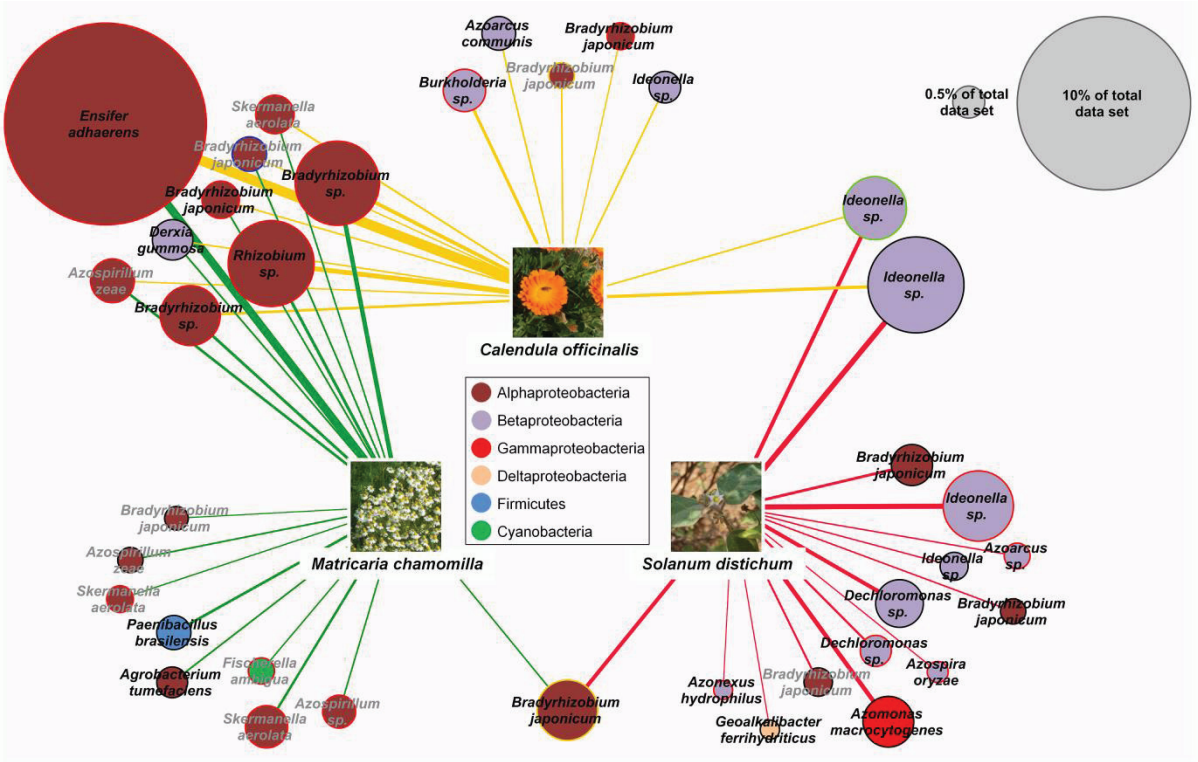
100-299
300-999
≥1000
Chlorobaculum tepidum (AAG12203)
0.2

Figure 3


Azoarcus communis
Bradyrhizobium japonicum
Burkholderia sp.
Bradyrhizobium japonicum
Ideonella sp.
0.5% of total data set
10% of total data set
Ensifer adhaerens
Skermanella aerolata
Bradyrhizobium japonicum
Bradyrhizobium sp.
Bradyrhizobium japonicum
Derxia gummosa
Rhizobium sp.
Azospirillum zeae
Bradyrhizobium sp.
Ideonella sp.
Ideonella sp.
Calendula officinalis
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Deltaproteobacteria
Firmicutes
Cyanobacteria
Bradyrhizobium japonicum
Azospirillum zeae
Skermanella aerolata
Paenibacillus brasilensis
Agrobacterium tumefaciens
Fischerella ambigua
Skermanella aerolata
Azospirillum sp.
Matricaria chamomilla
Bradyrhizobium japonicum
Solanum distichum
Bradyrhizobium japonicum
Ideonella sp.
Azoarcus sp.
Ideonella sp.
Dechloromonas sp.
Bradyrhizobium japonicum
Dechloromonas sp.
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Azonexus hydrophilus
Bradyrhizobium japonicum
Geoalkalibacter ferrihydriticus
Azomonas macrocytogenes

Figure 4

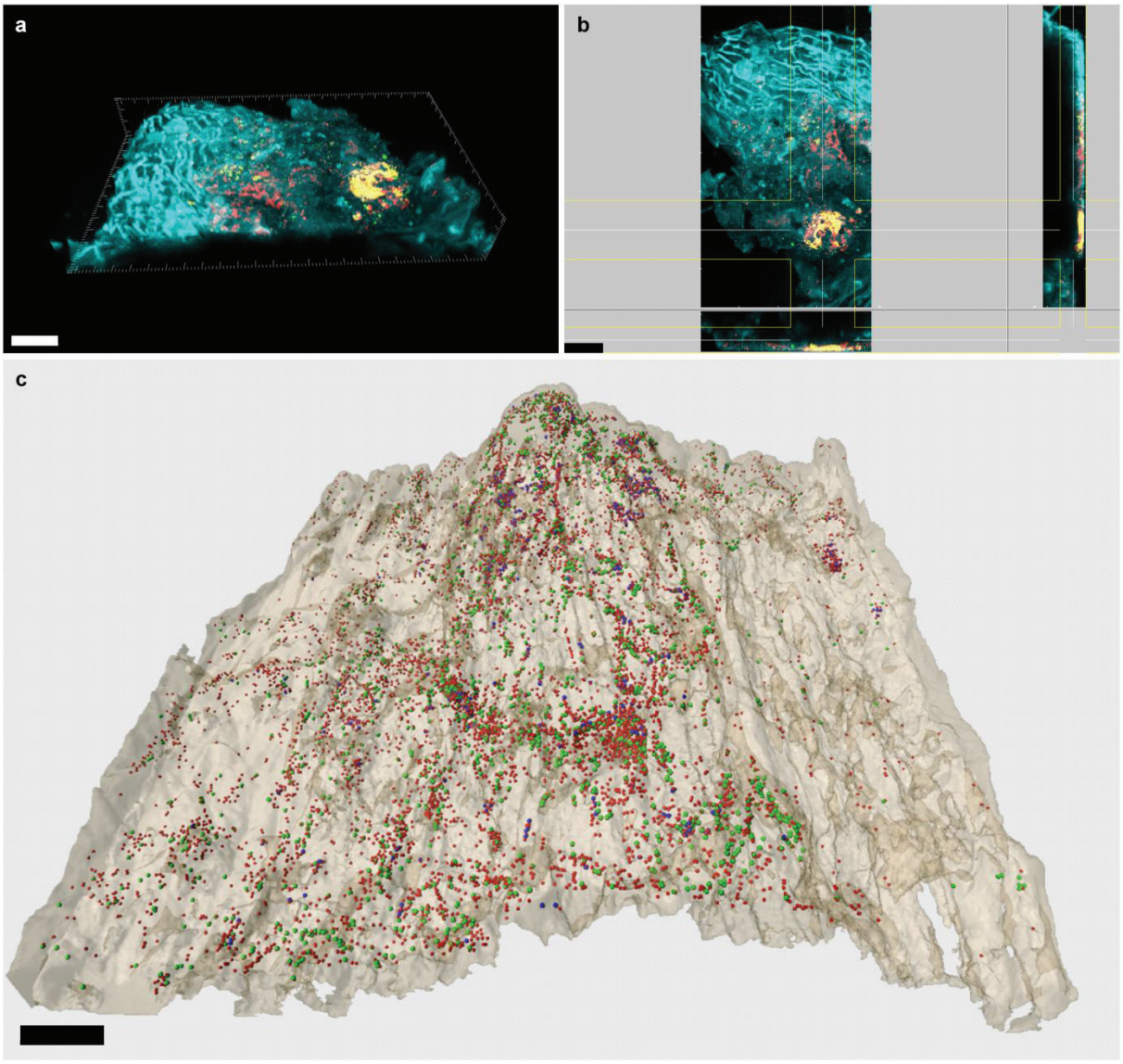

Figure 5

Supplementary Information

Table S1 Custom primers including 454 pyrosequencing adaptors (bold), linkers (italic) and sample specific tags (underlined).

Name	Primer sequence
nifH1_MID21	**CGTATCGCCTCCCTCGCGCCA***TCAG*CGTAGACTAGTGYGAYCCNAARGCNGA
nifH1_MID22	**CGTATCGCCTCCCTCGCGCCA***TCAG*TACGAGTATGTGYGAYCCNAARGCNGA
nifH1_MID23	**CGTATCGCCTCCCTCGCGCCA***TCAG*TACTCTCGTGTGYGAYCCNAARGCNGA
nifH1_MID24	**CGTATCGCCTCCCTCGCGCCA***TCAG*TAGAGACGAGTGYGAYCCNAARGCNGA
nifH1_MID25	**CGTATCGCCTCCCTCGCGCCA***TCAG*TCGTCGCTCGTGYGAYCCNAARGCNGA
nifH1_MID26	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACATACGCGTTGYGAYCCNAARGCNGA
nifH1_MID27	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACGCGAGTATTGYGAYCCNAARGCNGA
nifH1_MID28	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACTACTATGTTGYGAYCCNAARGCNGA
nifH1_MID29	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACTGTACAGTTGYGAYCCNAARGCNGA
nifH1_MID30	**CGTATCGCCTCCCTCGCGCCA***TCAG*AGACTATACTTGYGAYCCNAARGCNGA
nifH1_MID31	**CGTATCGCCTCCCTCGCGCCA***TCAG*AGCGTCGTCTTGYGAYCCNAARGCNGA
nifH1_MID32	**CGTATCGCCTCCCTCGCGCCA***TCAG*AGTACGCTATTGYGAYCCNAARGCNGA
nifH2_454	**CTATGCGCCTTGCCAGCCCGC***TCAG*ADNGCCATCATYTCNCC

Table S2 Richness estimates and diversity indices obtained at 0%, 4% and 8% amino acid dissimilarity for NifH sequence libraries of bulk soil and rhizosphere samples.

Sample[a]	Quality reads[b]	Clusters[c] (OTUs)			Chao1[d] (OTUs)			Coverage (%)			Shannon[e] (H')		
		0%	4%	8%	0%	4%	8%	0%	4%	8%	0%	4%	8%
Wb	5 217	2 487	543	118	18 564	1 050	193	13.4	51.7	61.1	6.20	4.77	1.87
Sb	5 217	3 228	881	290	22 195	1 680	509	14.5	52.4	56.9	7.17	5.41	3.92
Mc1	553	435	230	120	3 990	613	186	10.9	37.5	64.5	5.84	4.77	3.92
Mc2	553	390	206	96	3 479	410	137	11.2	50.2	70.1	5.51	4.48	3.58
Mc3	553	434	226	103	3 834	558	155	11.3	40.5	66.6	5.82	4.68	3.70
Mc4	553	450	263	124	5 292	677	210	8.5	38.8	59.1	5.87	4.92	3.83
Co1	553	415	217	101	2 642	495	146	15.7	43.8	69.2	5.73	4.61	3.67
Co2	553	452	245	111	6 057	633	202	7.5	38.7	55.1	5.88	4.78	3.69
Co3	553	414	210	91	2 209	448	115	18.7	46.9	78.9	5.71	4.49	3.45
Co4	553	422	219	97	3 942	831	166	10.7	26.3	58.4	5.72	4.60	3.62
Sd1	553	425	207	86	4 417	421	168	9.6	49.2	51.2	5.73	4.44	3.26
Sd2	553	446	240	118	3 506	806	281	12.7	29.8	42.0	5.88	4.80	3.89
Sd3	553	431	221	97	4 481	609	162	9.6	36.3	59.8	5.66	4.23	3.30
Sd4	553	476	227	105	5 159	586	190	9.2	38.8	55.3	6.03	4.74	3.74

[a]Wb = desert soil, Sb = agricultural soil, Mc = *M. chamomilla*, Co = *C. officinalis*, Sd = *S. distichum*. Numbers indicate the independent replicate sample.

[b]quality reads were normalised to the same number of sequences.

[c]rarefaction curves are depicted in the Supplementary Figure S3.

[d]nonparametric richness estimator based on the distribution of singletons and doubletons.

[e]a higher number indicates more diversity.

Table S3 Relative composition of bacterial classes identified in the NifH sequence libraries of soils and medicinal plant rhizospheres at genus level.

	Desert Soil[a]	Agricultural Soil[a]	Closest database matches (GenBank accession number)	Similarity (%)
Alphaproteobacteria				
Rhizobium	93.7%	40.9%	*Rhizobium* sp. (GQ241353)	99-100%
Agrobacterium		12.9%	*A. tumefaciens* (FJ822995)	100%
Methylocystis		38.2%	*Methylocystis* sp. (EU934433)	100%
Bradyrhizobium	3.4%	5.1%	*B. elkanii* (AB501133)	95%
			B. japonicum (GQ289576)	99%
Mesorhizobium	1.6%		*M. loti* (AB367742)	98%
Methylocella		2.9%	*M. palustris* (AJ563948)	98%
Bacilli				
Paenibacillus		41.5%	*P. brasilensis* (EU294253)	96%
			Paenibacillus sp. (AJ223992)	95%
Cyanobacteria				
Anabaena		100%	*A. azotica* (DQ294218)	100%

	Matricaria chamomilla[a]	*Calendula officinalis*[a]	*Solanum distichum*[a]	Closest database matches (GenBank accession number)	Similarity (%)
Alphaproteobacteria					
Rhizobium	8.2-9.9%	8.2-28.5%	≤21.2%	*Rhizobium* sp. (GQ241353)	99-100%
Agrobacterium	≤17.1%	≤5.6%		*A. tumefaciens* (FJ822995)	96%
Ensifer	23.9-36.8%	18.1-57.1%	3.2-51.2%	*E. adhaerens* (JX081993)	97-100%
Methylocystis		≤3.0%	≤5.3%	*Methylocystis* sp. (AF378718)	99%
Bradyrhizobium	19.4-56.1%	18.0-39.0%	25.6-69.6%	*B. japonicum* (GQ289574)	95-97%
				Bradyrhizobium sp. (JX079649)	95-98%
				B. japonicum (GQ289566)	95%
Azospirillum	≤3.9%		≤11.5%	*A. amazonense* (GU256445)	100%
Betaproteobacteria					
Ideonella	≤58.6%	36.0-77.0%	22.2-93.5%	*Ideonella* sp. (AY231580)	97-99%
Burkholderia		11.0-34.7%	≤2.9%	*Burkholderia* sp. (JN247661)	98%
Derxia	≤47.8%	5.3-15.7%	≤3.6%	*D. gummosa* (AB089484)	100%
Dechloromonas	≤19.0%		1.9-65.3%	*Dechloromonas* sp. (AJ563286)	100%
				Dechloromonas sp. (JX154811)	100%
				Dechloromonas sp. (JX154844)	98%
Azoarcus		≤22.7%	2.6-9.2%	*A. communis* (U97116)	99%
				Azoarcus sp. (EF158389)	100%
Azospira			≤6.7%	*A. oryzae* (U97115)	98%
Zoogloea	≤100%	≤5.5%		*Z. oryzae* (AB201045)	98%
Azonexus			≤3.3%	*A. hydrophilus* (EF626686)	99%
Gammaproteobacteria					
Azomonas	100%		64.3-100%	*A. macrocytogenes* (AY644349)	99%
Deltaproteobacteria					
Geoalkalibacter			100%	*G. ferrihydriticus* (DQ660332)	98%
Bacilli					
Paenibacillus	≤100%			*P. brasilensis* (EU294253)	98%
				Paenibacillus sp. (AJ223992)	95%

[a]clusters with less than 1% of quality sequences were not designated.

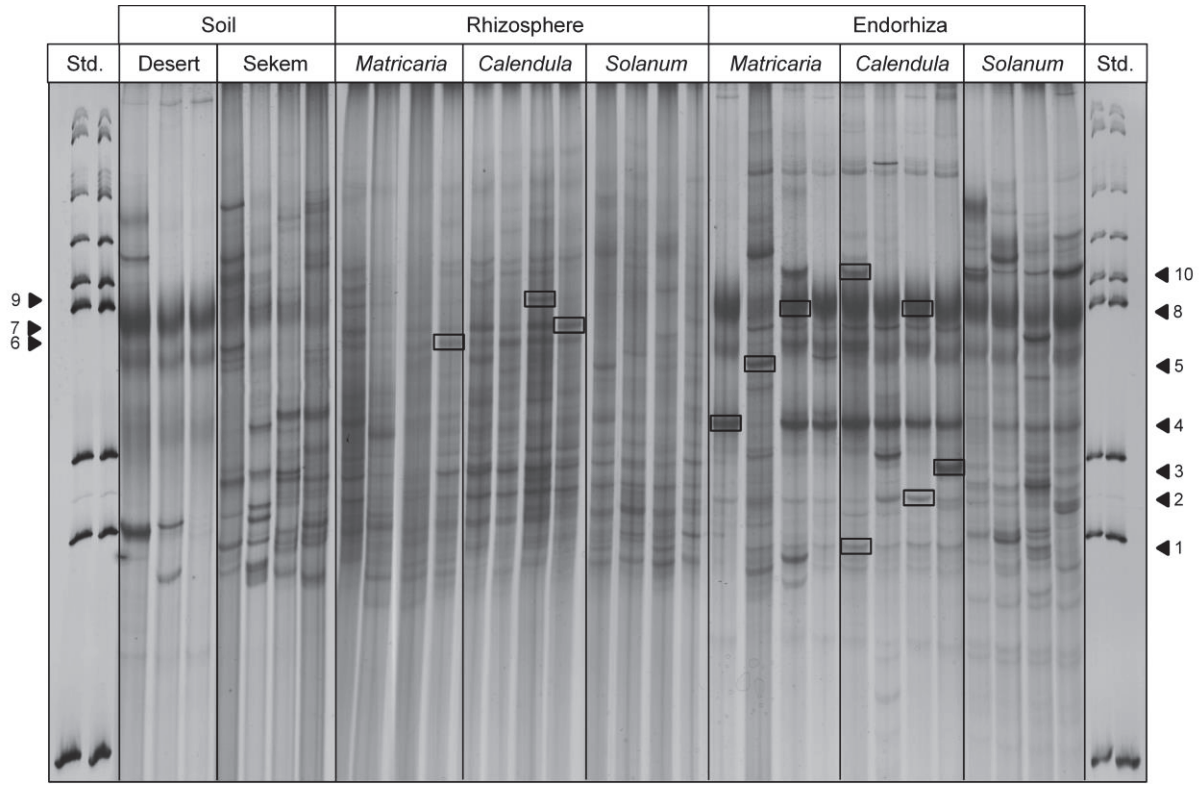

Figure S1 *nifH* PCR-SSCP profiles from desert and agricultural soil as well as the rhizosphere and endorhiza of the medical plants *M. chamomilla*, *C. officinalis* and *S. distichum*. Std.: 1 kb DNA ladder. Closest database matches of the following bands were identified: 1. *Bradyrhizobium japonicum*, 90% similarity to GenBank accession number GQ289576; 2. *Paenibacillus terrae*, 95% similarity to CP003107; 3. *Methanocella conradii*, 96% similarity to CP003243; 4. *Paenibacillus polymyxa*, 96% similarity to HM146187; 5. *Nostoc* sp., 93% similarity to BA000019; 6. *Nostoc punctiforme*, 90% similarity to CP001037; 7. *Anabaena* sp., 92% similarity to HQ836215; 8. *Rhizobium* sp., 95% similarity to FN666268; 9. *Anabaena* sp., 95% similarity to L04499; 10. *Burkholderia xenovorans*, 89% similarity to EF158805.

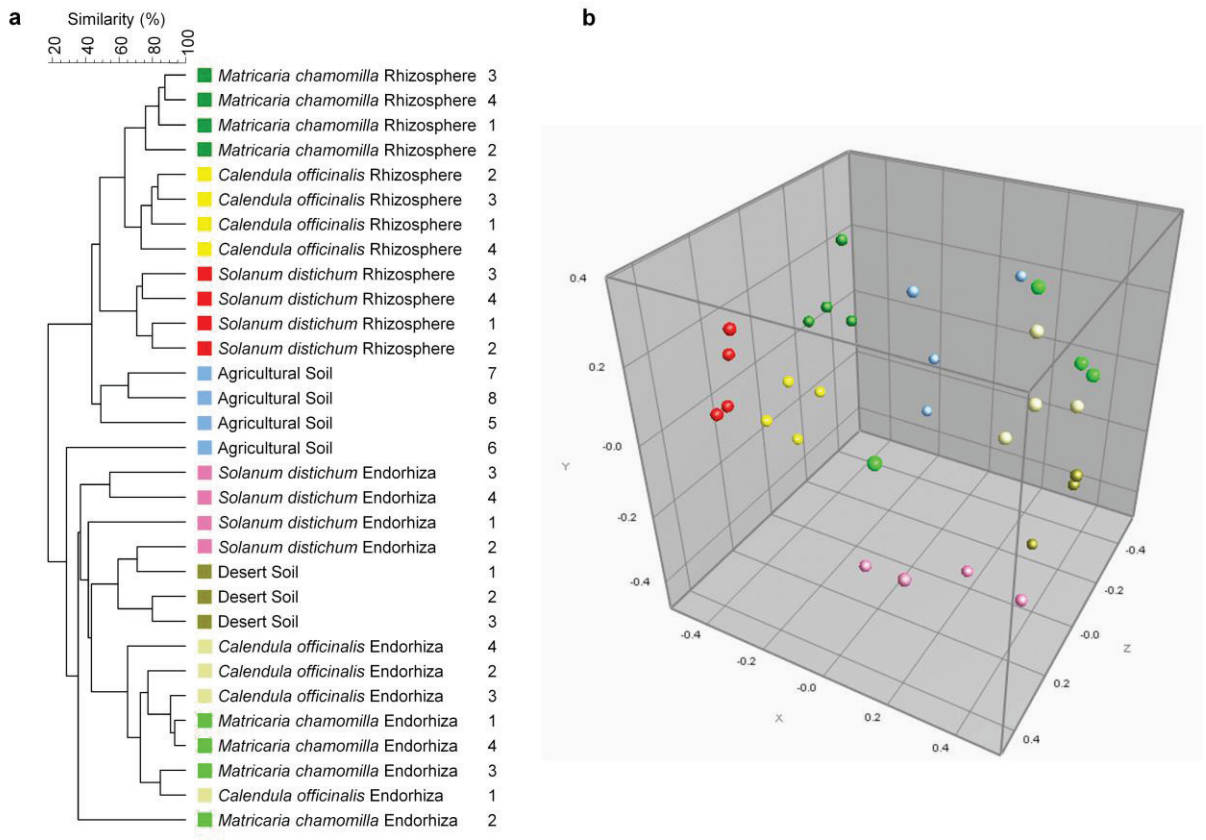

Figure S2 Comparison analysis of PCR-SSCP profiles of the *nifH* gene patterns in bulk soil, rhizosphere and endorhiza of the medical plants. (a) Unweighted pair group method with arithmetic mean (UPGMA) tree. Numbers indicate independent replicate samples. The dendrogram was generated with GelCompar II using Pearson correlation and an optimisation of 0%. (b) Multidimensional scaling (MDS) ordination plot based on Pearson similarity matrix. Colours correspond to squares in the tree.

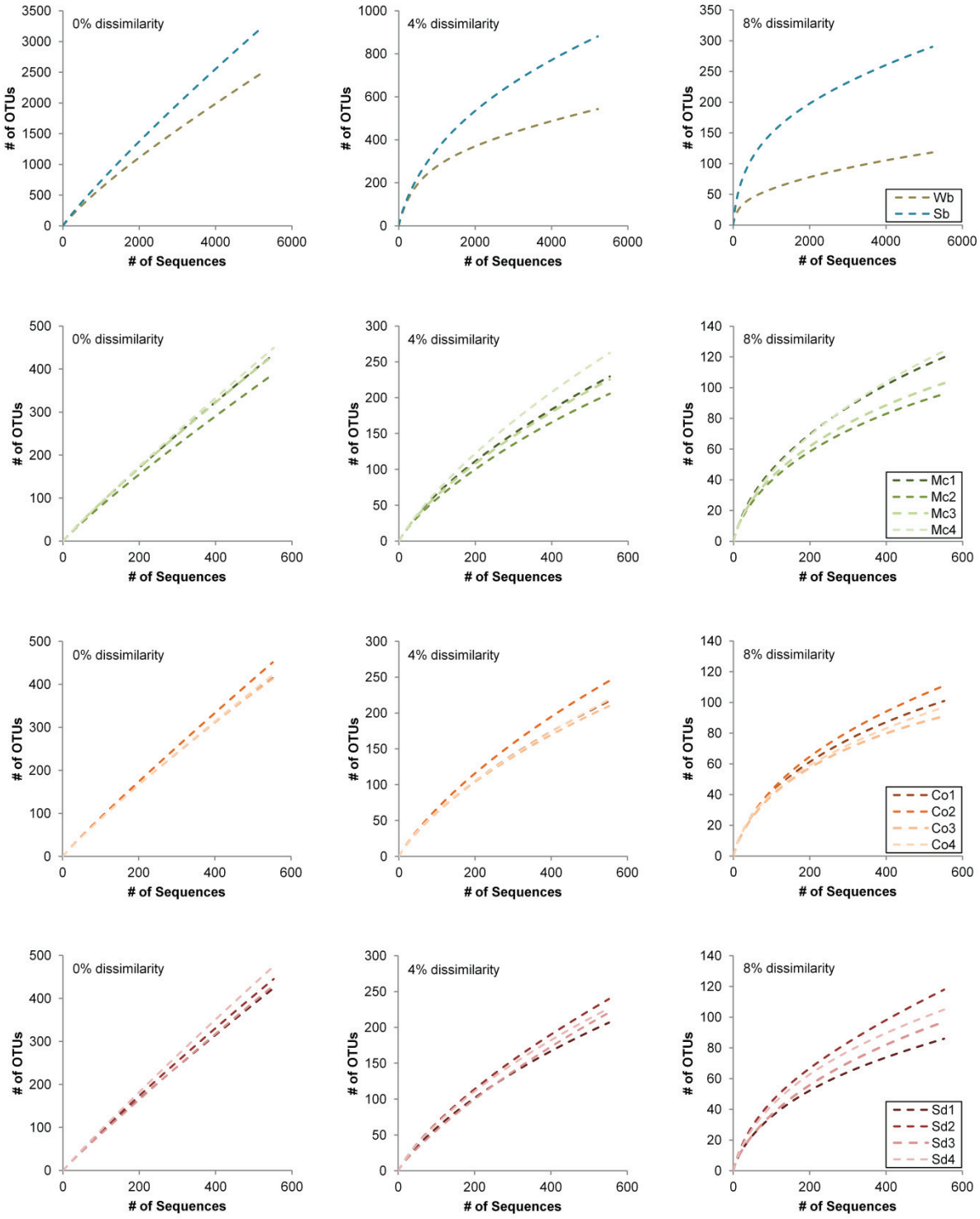

Figure S3 Rarefaction analyses for NifH sequence libraries of bulk and rhizosphere soil samples. Wb = desert soil, Sb = agricultural soil, Mc = *M. chamomilla*, Co = *C. officinalis*, Sd = *S. distichum*. For the medical plants, curves are presented from four indipendent replicate samples per species. OTUs are shown at amino acid distance levels of 0%, 4% and 8%.

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Using ecological knowledge and molecular tools to develop effective and safe biocontrol strategies

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Using Ecological Knowledge and Molecular Tools to Develop Effective and Safe Biocontrol Strategies

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1. Introduction

Today's farming systems undermine the well-being of communities in many ways: farming has destroyed huge regions of natural habitats, which also implies a loss of species and their ecosystem services (Sachs et al., 2010). Plant protection measures also causes problems for human health (Horrigan et al., 2002), and agriculture is responsible for about 30% of greenhouse-gas-emission (IPCC, 2007). Furthermore, emerging, re-emerging and endemic plant pathogens continue to challenge our ability to safeguard plant growth and health worldwide (Miller et al., 2009). Therefore, one of the major challenges for the 21st century will be an environmentally sound and sustainable crop production.

Microbial inoculants containing microorganisms with beneficial plant-microbe interactions have a great potential to contribute to this objective (Berg, 2009; Bhattacharjee et al., 2008). Over the past 150 years, research has demonstrated repeatedly that bacteria and fungi have an intimate interaction with their host plants and are able to promote plant growth as well as to suppress plant pathogens (Compant et al., 2005; Lugtenberg & Kamilova, 2009; Weller et al., 2002; Weller, 2007; Whipps, 2001). All plant-associated microenvironments, especially the rhizosphere, are colonized in high abundances by antagonistic microbes (Berg et al., 2005a). Between 1 and 35% of the microbial inhabitants showed antagonistic capacity to inhibit the growth of pathogens *in vitro* (Berg et al., 2002, 2006). The proportion of isolates, which express plant growth promoting traits is much higher in general, and was found up to 2/3 of the cultivable population (Cattelan et al., 1999; Fürnkranz et al., 2009; Lottmann et al., 1999). Diverse microbial inoculants, which were selected from this promising indigenous potential, are already on the market. In recent years, the popularity of microbial inoculants has increased substantially, as extensive and systematic research has enhanced their effectiveness and consistency (Berg, 2009).

New molecular and microscopic techniques are one reason for progress in biocontrol research. These techniques have enhanced our understanding about the plant and especially

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the rhizosphere as a microbial ecosystem and resulted into more effective screening strategies for bioactive microbes. In this chapter we will discuss these points first in general and in a second part with three representative examples.

2. Molecular and microscopic tools in biocontrol research

Molecular and microscopic tools can be used to study the ecology of single plant growth promoting rhizobacteria (PGPR) or biological control agent (BCA) strains or to analyse the structure and function of the target microbial community. In a first step we will analyse the use of methods for single strains (Table 1). Here, molecular fingerprints using repetitive elements in the genome (Rademaker & de Bruijn, 1997) can be used at several levels of biocontrol research. While the functions of many of these repetitive sequence elements are still unknown, they have proven to be useful as the basis of several powerful tools for use in microbial ecology. The repetitive, sequence-based PCR or rep-PCR DNA fingerprint technique uses primers targeting several of these repetitive elements and PCR to generate unique DNA profiles or 'fingerprints' of individual microbial strains (Ishii & Sadowsky, 2009). In screening strategies, these fingerprints can be applied to differentiate strains at population level and to select only unique isolates (Berg et al., 2006; Faltin et al., 2004). In a later stage, these highly reproducible fingerprints can be used for identity check and quality control. Genome sequencing also offers a tool to study PGPRs in great detail. Strains of *Pseudomonas fluorescens*, one of the dominant and cosmopolitan plant-associated species (Weller, 2007), were the first sequenced strains (Paulsen et al., 2005). Genomic information allowed the analysis of the mode of action, detailed investigations of interactions as well as optimisation of fermentation and formulation processes (rev. in Gross & Loper, 2009). De Bruijn et al. (2007) used genome mining to discover unknown gene clusters and traits that are highly relevant in the life style of *P. fluorescens* SBW25. Proteomic and transcriptomic studies are interesting to study the function of BCAs. For example, Garbeva et al. (2011) studied transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors (*Bacillus, Brevundimonas* and *Pedobacter*), which demonstrated that Pf0-1 shows a species-specific response to bacterial competitors. In another transcriptomic study published by Hassan et al. (2010), a whole genome oligonucleotide microarray was developed for *P. fluorescens* Pf-5 and used to assess the consequences of a gacA mutation: GacA significantly influenced transcript levels of 10% of the 6147 annotated genes in the Pf-5 genome including genes involved in the production of hydrogen cyanide, pyoluteorin and the extracellular protease. Transcriptomic studies can also lead to new insights into plant responses on BCAs: *Pseudomonas*-primed barley genes indicated that, as is the case in dicots, jasmonic acid plays a role in host responses (Petti et al., 2010). A new tool is metabolomics, which allow the analysis of metabolites *in situ*. This is not only a technique to answer questions about the activity *ad planta*, it is also important for registration procedures, which are still a high hurdle on the way to the market. Frimmersdorf et al. (2010) used a metabolomic approach to show how *Pseudomonas aeruginosa* adapts to various environments. In addition, analysis of the mobilome of strains can result in interesting findings for biocontrol research as shown for *P. fluorescens* Pf-5 by Mavrodi et al. (2009), in which mobile genetic elements contain determinants that contribute to Pf-5's ability to adapt to changing environmental conditions and/or colonize new ecological niches. Studying the colonisation of plants has been greatly facilitated by the application of fluorescent proteins which are used as vital markers and reporter genes (rev. in Bloemberg, 2007). These new insights have changed our understanding about

colonisation; many of the strains analysed showed an endophytic life style (Chin-A-Woeng et al., 1997; Zachow et al., 2010), and the "root shield", which was hypothesized in former times, was rarely found in contrast to single cells and micro-colonies. Raman-FISH combines stable-isotope Raman spectroscopy and fluorescence *in situ* hybridization for the single cell analysis of identity and function (Huang et al., 2007a). This potential has been demonstrated through the discriminant functional analysis of Raman spectral profiles (RSP) obtained from the soil and plant-associated bacterium *P. fluorescens* SBW25; results suggests that SBW25 growth in the phytosphere is generally neither carbon-catabolite-repressed nor carbon-limited (Huang et al., 2007b).

Molecular tools were also used to analyse target habitats of biocontrol studies (Table 1). Cultivation-based methods to analyse plant-associated bacteria only address the culturable fraction, which are thought to represent only a small proportion (0.1 to 10%) of the total bacteria present in soil and in the rhizosphere (Amann et al., 1995). The analysis of nucleic acids directly extracted from plant microenvironments opened the chance to study a much broader spectrum of microbes (Table 1). Most frequently ribosomal RNA gene fragments are amplified from total community DNA and subsequently analysed by fingerprinting techniques: Terminal restriction fragment length polymorphism (T-RFLP), single-strand conformation polymorphism (SSCP), denaturing/temperature gradient gel electrophoresis (D/TGGE) using universal/specific primers (Schwieger & Tebbe, 1998; Smalla et al., 2007). Application of these fingerprinting techniques resulted in important findings such as plant-specific microbial communities (Smalla et al., 2001), the impact of cultivars on microbial communities (Milling et al., 2004) or the structure of endophytic communities (Rasche et al., 2006). Fingerprinting techniques are often used to analyse the structure of plant-associated communities and can also be used to study functional aspects. For example, Briones et al. (2002) found cultivar-specific differences for ammonia-oxidizing bacteria (AOB) in rice rhizospheres by a multiphasic approach including DGGE of the amoA gene, analysis of libraries of cloned amoA, fluorescently tagged oligonucleotide probes targeting 16S rRNA of

Objective/Level	Isolates: BCAs and pathogens	Microbial communities
Molecular fingerprints	Rep-PCR (BOX)	T-RFLP, SSCP, D/TGGE using universal/specific primers
Genomic information	Genome sequencing	Metagenome
Functions Functional diversity	Transcriptomics (RNA-based) Proteomics (Protein-based)	Metatranscriptome Metaproteome
Bioactive compounds	Metabolome	Metabolome
Adaptation/evolution	Mobilome	Metamobilome
Visualisation/activity	GFP/DsRed labelled strains, CLSM Raman spectroscopy and fluorescence *in situ* hybridization (FISH)	FISH-CLSM

Table 1. Molecular and microscopic tools in biocontrol research.

AOBs as well as metabolism rates obtained by the 15N dilution technique. Other techniques have a great impact on our functional understanding; this was shown for example for transcriptome profiling (Mark et al., 2005; Yuan et al., 2008), microarrays (Sanguin et al., 2006; Weinert et al., 2011) *in vivo* expression technology and differential fluorescence induction promoter traps as tools for exploring niche-specific gene expression (Rediers et al., 2005), new methods for the *in situ* analysis of antifungal gene expression using flow cytometry combined with green fluorescent protein (GFP)-based reporter fusions (de Werra et al., 2008), barcode pyrosequencing (Gomes et al., 2010), and ultra deep sequencing (Velicer et al., 2006). Stable isotope probing (SIP) used to determine bacterial communities assimilating each carbon source in the rhizosphere of four plant species resulted in plant species specific patterns (Haichar et al., 2008). Metagenomic approaches have been established to analyse the plant-soil interface (Erkel et al., 2006; rev. in Leveau, 2007).

3. Using ecological knowledge to screen and evaluate biocontrol agents

The advanced techniques discussed above should be integrated into strategies to screen and evaluate biocontrol agents (Fig. 1). Of primary importance is the life cycle of the pathogen. This can result in new targets for biocontrol; one example is the impact of zoospores on pathogenic oomycetes, which are primary targets for suppression (de Bruijn et al., 2007; Raaijmakers et al., 2010). Furthermore, it is also important to understand the target microenvironment of plants. Plant specificity is one critical point but also knowledge about the structure and function of the microbial communities. There are strategies to select BCAs from the indigenous antagonistic potential as well as to use ubiquitous, cosmopolitan BCAs (Zachow et al., 2010). If a BCA is selected, an evaluation strategy is needed to assess their potential for commercialization.

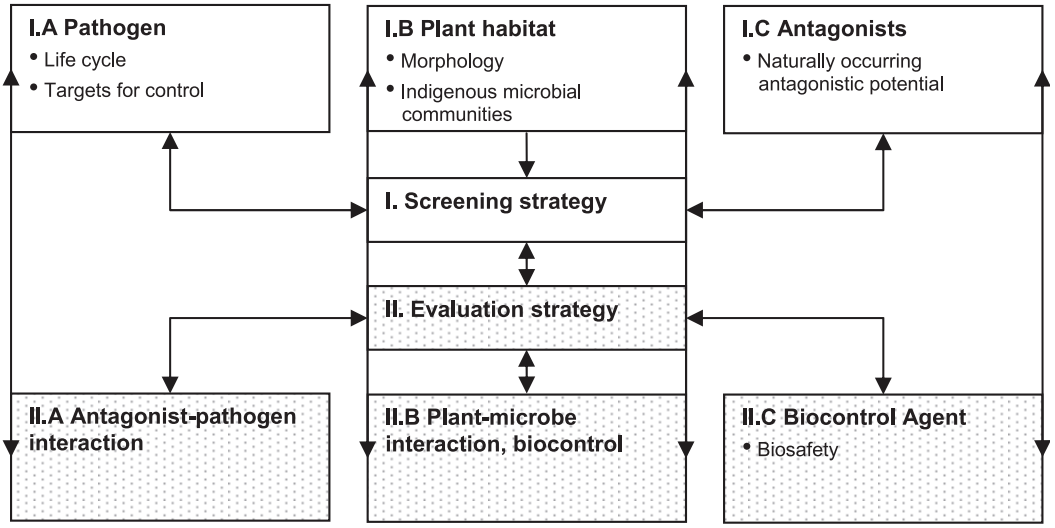

Fig. 1. Integration of ecological knowledge into screening and evaluation strategies.

Knowledge about the effect of BCAs under greenhouse and field conditions presents the basis for this evaluation. However, often inconsistent effects make the decision difficult. Detailed analyses of plant-microbe and pathogen-microbe interactions under different environmental conditions can help to optimize the biocontrol effect under practical conditions. Another aspect, which should be integrated in an early phase of evaluation, is

biosafety. Many BCAs fail here due to problems with human or environmental health. Due to the fact that the whole program to investigate toxicology is time-consuming and expensive, alternative test systems should be used, e.g. the *Caenorhabditis elegans* assay (Zachow et al., 2009) or Duckweed (*Lemna minor*) as a model plant system for the study of human microbial pathogenesis (Zhang et al., 2010).

4. Examples for screening and evaluation strategies

4.1 Strategy to control soil-borne pathogens on medical plants under organic conditions in Egypt

On the SEKEM farms in Egypt desert land was converted into arable land, and biodynamic agriculture is operated for over 30 years now (www.sekem.com). Today SEKEM is carrying out organic agriculture on more than 4100 hectares and has the largest market for organic products outside Europe and North America. They produce organic foods, spices, tea, cotton textiles and natural remedies. However, the cultivation especially of medical plants is more and more affected by soil-borne phytopathogens, which lead to significant yield losses. The objective of our study was to develop a specific biocontrol strategy for desert farming.

An important factor was to find out, whether and how the highly specialized natural microbial communities of the desert soil are affected by agriculture and watering. To examine the impact of organic agriculture on bacterial diversity and community compositions in desert soil, soil from a SEKEM farm in comparison to the surrounding desert soil were assessed by a pyrosequencing-based analysis of partial 16S rRNA gene sequences. When appropriate primers are chosen, in a pyrosequencing analysis with short reads the microbial diversity is represented almost as reliably as with near-full-length sequences (Will et al., 2010). Fragments encompassing the V4-V5 region of the 16S rRNA gene provide estimates comparable to those obtained with the nearly complete fragment (Youssef et al., 2009). In desert soil 19244 and in agricultural soil 33384 quality sequences with a read length of ≥ 150 bp were recovered. Using different data bases, 83.0% of all quality sequences could be classified below the domain level, in the range of the percentage of classified 16S rRNA gene sequences of other pyrosequencing-based studies (Lauber et al., 2009; Lazarevic et al., 2009; Will et al., 2010). The computed Shannon indices of diversity (H') (Shannon, 1997) were much higher for agricultural soil than for desert soil (H' at a dissimilarity level of 20%: SEKEM soil 4.29; desert soil 3.54); this indicates a higher bacterial diversity in soil due the agricultural use of the desert. A comparison of rarefaction analyses with the number of operational taxonomic units (OTUs) estimated by the Chao1 richness estimator (Chao & Bunge, 2002; Will et al., 2010) revealed that at this genetic distance the surveying effort in both soils covered almost the full extent (over 97% in both soils) of taxonomic diversity. This was also shown by a clear saturation of both curves in the rarefaction analysis (data not shown). The 43673 classifiable sequences obtained from both soil types together were affiliated with 18 different phyla. Dominant groups were especially Proteobacteria (30.2%), Firmicutes (27.3%) and Actinobacteria (10.5%). These dominant phyla were present in both soils. In detail, Firmicutes were highly enriched in agricultural soil (from 11.3% in desert soil to 36.6% in SEKEM soil), Proteobacteria (46.0% in desert soil and 21.0% in SEKEM soil) and Actinobacteria (20.7% in desert soil and 4.6% in SEKEM soil) occurred in SEKEM in lower abundances than in the surrounding desert. In addition, in both soils Bacteroidetes (4.6% and 5.3%) and Gemmatimonadetes (1.4% and 1.9%) were

present. Whereas Acidobacteria (7.9%) and Planctomycetes (1.1%) were only present in the agricultural soil, *Deinococcus-Thermus* (1.1%) was only detectable in the desert sand. These abundances of the phyla are coextensive with results from previously reported meta-analysis of bacterial community composition in soils and, despite the specific soil type of the desert, the composition covers rather well with studies of completely different soils (Hansel et al., 2008; Janssen, 2006; Lauber et al, 2009; Will et al., 2010). However, greatly different from all reported studies was the high abundance of Firmicutes. Janssen (2006) reported them to contribute only a mean of 2% (range 0 – 8%) in the total bacterial soil community. Most of the Firmicutes sequences were classified as belonging to the genus *Bacillus*; in the agricultural soil also the phylogenetically related genus *Paenibacillus* was found (5% of classified Firmicutes). In desert soil, *Ochrobactrum* was the most abundant genus within the (Alpha-)Proteobacteria (79% of classified Proteobacteria) and *Rhodococcus* among the Actinobacteria (90% of classified Actinobacteria). The Acidobacteria in the agricultural soil are affiliated only with subdivision 6.

Additionally to the pyrosequencing analysis, the composition of the bacterial as well as fungal community in the two different soil types was investigated by SSCP analysis of rRNA gene fragments (Bassam et al., 1991; Schwieger & Tebbe, 1998). Furthermore, the composition of the microbial community in rhizosphere and endorhiza of three different species of medical plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. & Thonn.) grown under organic conditions on SEKEM farms were examined. According to the cluster analysis prepared on the basis of SSCP community fingerprints, the agricultural soil in bacterial as well as in fungal community composition strongly differed from the desert soil. As shown in the pyrosequencing analysis, in comparison to the desert in soil of the SEKEM farm an impressive diversity of bacteria, expressed as various bands in the gel, was found (data not shown). In the bacterial community of the desert soil, two dominant bacterial bands could be detected, which were also visible in all samples from the endorhiza of all three investigated medical plants. This shows that bacteria are taken up by the plants from the soil, and that soil is the main reservoir for biological control agents. The two dominant bands were identified by partial 16S rRNA gene sequence analysis as *Ochrobactrum* sp. (closest database match *O. grignonense*) and *Rhodococcus* sp. (closest database match *R. erythropolis*). Further, nearly in all samples *Bacillus* sp. was found (closest database match *B. subtilis*). By SSCP analysis and also by the pyrosequencing approach, *Ochrobactrum* and *Rhodococcus* could be detected as dominant bacteria. However, both genera include opportunistic human pathogens (*O. anthropi*, *R. equi*). Several studies provided evidence that similar or even identical functions are responsible for beneficial interactions with plants and virulence in humans (Berg et al., 2011). For *Ochrobactrum* was already detected the production of plant growth hormones and siderophores and also an antifungal activity towards several phytopathogens was described (Chakraborty et al., 2009). *Ochrobactrum* was found in diverse environmental niches, like rhizosphere, soil, sediments and activated sludge (Berg et al., 2005b). *Rhodococcus* could also be found in a broad range of environments, including soil, water and eukaryotic cells. This genus includes also a phytopathogenic species causing leafy gall formation on a wide range of host plants, *R. fascians* (Goethals et al., 2001). The fungal community fingerprints included a quite high diversity in all microenvironments. As an example, SSCP profiles of fungal communities in rhizosphere and endorhiza are shown in Figure 2. A dominant band, which was found nearly in all samples, was identified as

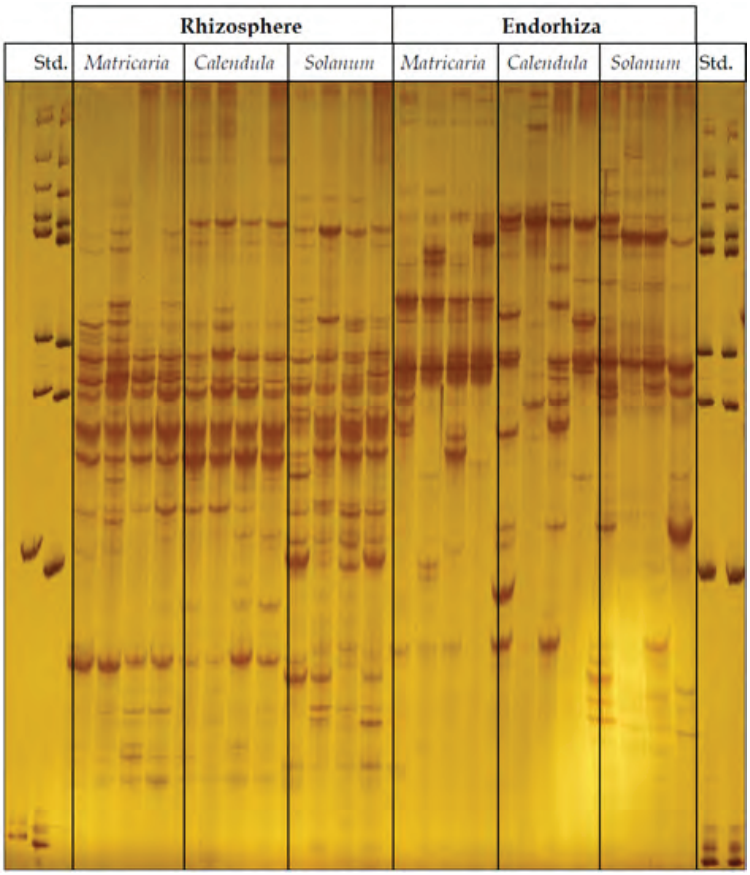

Fig. 2. SSCP profiles of the fungal communities in rhizosphere and endorhiza of the medical plants. Four independent replicates per plant and microenvironment were loaded onto the gel. Std.: 1 kb DNA ladder.

Verticillium dahliae, which is one of the mainly occurring soil-borne phytopathogens on the SEKEM farms. In general, mainly potential plant pathogens were found within the fungal communities. The obligate root-infecting pathogen *Olpidium,* belonging to the fungal phylum Chytridiomycota, was found especially in the rhizosphere and endorhiza of *Matricaria chamomilla*. *Alternaria* and *Acremonium* were found primarily in the rhizosphere samples. According to the generated dendrograms, a clear plant specificity of the bacterial and fungal communities in the rhizosphere as well as in the endorhiza was found (Fig. 3). Furthermore, microenvironment-specific SSCP patterns of the bacterial and the fungal communities were detected (data not shown). There were significant differences between the rhizosphere and the endorhiza of the medical plants. In general, samples from the rhizosphere generated more bands than samples from the endorhiza of the medical plants, which indicate that a sub-set of rhizobacteria was able to invade the root.

The major problems in the cultivation of plants on SEKEM farms are caused by the soil-borne pathogenic fungi *Verticillium dahliae* Kleb., *Rhizoctonia solani* J.G. Kühn and *Fusarium culmorum* (Wm.G. Sm.) Sacc. as well as by the soil-borne pathogenic bacterium *Ralstonia solanacearum*. Although grown in organic agriculture, which aims to minimize the impact on the environment by practices such as crop rotation, using pathogen resistant cultivars, and the use of organic manure (compost) instead of synthetic fertilizers (Schmid et al., 2011), they have an increasing importance. One reason is an intensive growing of a limited number

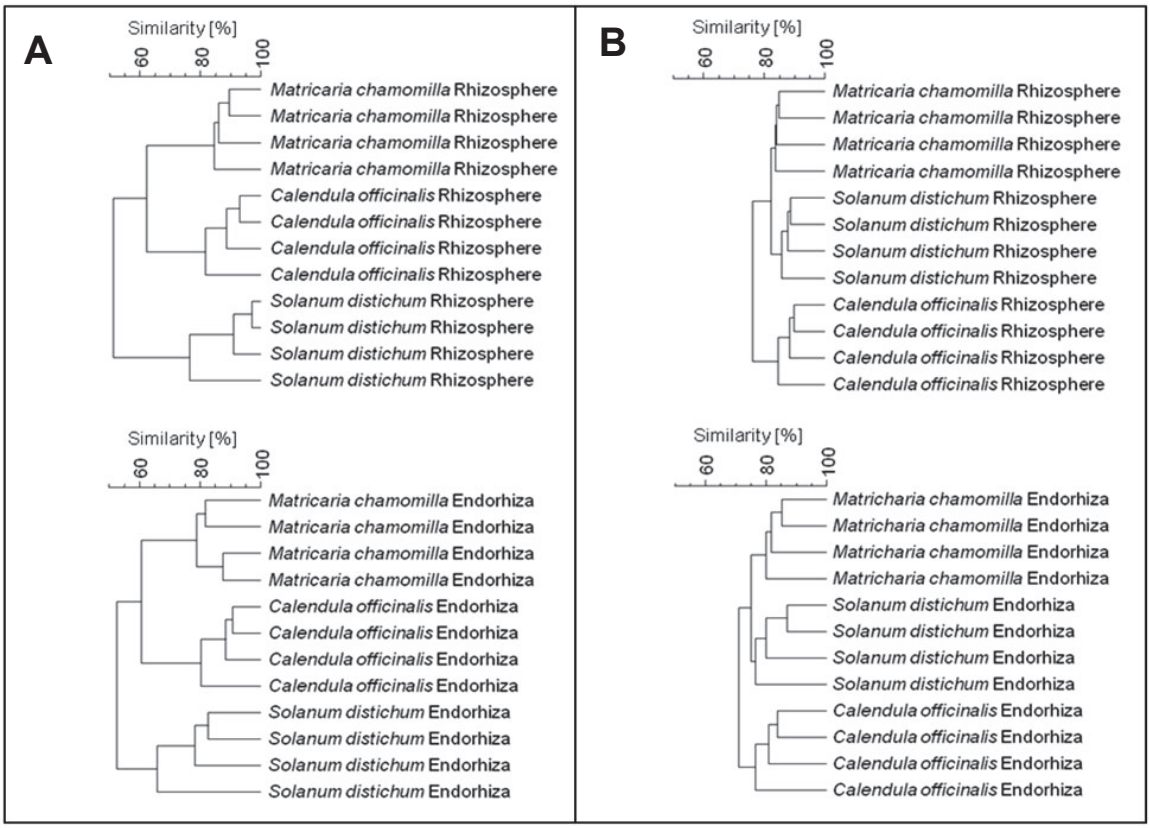

Fig. 3. UPGMA dendrograms of bacterial (A) and fungal (B) communities in rhizosphere and endorhiza of the medical plants. The dendrograms were generated from the SSCP community profiles with GelCompar II. The following settings were used: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); similarity coefficient: band based: dice; position tolerances: optimization: 4%, position tolerance: 1%.

of crops in short rotations. Here, biocontrol agents should solve these problems and help to suppress soil-borne pathogens on a natural way. Although BCAs are already on the market, our biocontrol product will be optimized for desert farming – regarding soil, weather, pathogen species, etc. For this reason, autochthonous bacteria were isolated from rhizosphere and endorhiza of medical plants as well as from bulk soil collected in SEKEM farms, and were evaluated for their potential for biocontrol. In a first step, the dual-culture assay was used to find out the antagonistic potential towards the pathogenic fungi (Berg et al., 2002, 2005a). A total of 1589 bacterial isolates were screened for their ability to inhibit *in vitro* the growth of *Verticillium dahliae*, *Rhizoctonia solani* and *Fusarium culmorum*. Bacterial isolates obtained from the soil of the SEKEM farm exhibited a higher *in vitro* antagonistic potential towards soil-borne phytopathogenic fungi in comparison to the bacteria isolated from the desert soil (SEKEM 21.6 ± 0.8%; desert 12.4 ± 0.7%). From the agricultural soil 17.4% (27 isolates) demonstrated antagonism towards all three pathogens, from the desert soil 10.6% (21 isolates) were able to suppress the growth of all fungi tested. Already the desert soil harbours a high proportion of antagonists, which were augmented by organic agriculture in SEKEM soil. The soil from the farm seems to be supplied with antagonists in such an optimal way, that there was no detectable enrichment of antagonists in the rhizosphere and endorhiza of the investigated medical plants. In general, *Matricaria*

chamomilla and *Solanum distichum* showed a better antagonistic potential than *Calendula officinalis*. Especially the endorhiza from *Matricaria chamomilla* harbours a high proportion of antagonists. Whereas in the soil and in the rhizosphere could be found most antagonistic bacteria towards *Fusarium culmorum*, in the endorhiza of the medical plants most antagonists were found towards *Verticillium dahliae*.

In a next step, the antagonistic mechanisms of all isolates, which showed an activity towards at least two of the investigated pathogenic fungi (162 isolates), were investigated *in vitro* with a special focus on fungal cell wall degrading enzymes (β-1,3-glucanase, chitinase and protease) (Chernin et al., 1995; Grube et al., 2009) and siderophore-production (Schwyn & Neilands, 1987). Production of chitinase could be detected for 8.0% of the antagonists; *Lysobacter enzymogenes* followed by all isolates of *Streptomyces* showed a high chitinolytic activity. Glucanase activity was shown for nearly all isolated antagonists (93.8%); only the isolates of the *Bacillus cereus* group were not able to degrade β-1,3-glucan. Casein degradation by protease could be shown at 80.9% (*Bacillus* sp. and *Lysobacter* sp.). The production of siderophores was shown for all antagonists except the isolates of *Paenibacillus* sp. (93.2%).

To avoid investigations with genetically similar strains, amplified rRNA gene restriction analysis (ARDRA) of the 16S rRNA gene with the restriction endonuclease *Hha*I (Zachow et al., 2008) and BOX polymerase chain reaction fingerprints (Berg et al., 2002; Rademaker & de Bruijn, 1997) of the antagonistic isolates were performed. A representative selection of promising biological control agents was identified by partial 16S rRNA gene sequencing. The use of ARDRA of the 16S rRNA gene with the restriction enzyme *Hha*I led to the separation of isolates clustered into five groups (data not shown); within groups the similarity of the band patterns was 100% identical: *Bacillus subtilis* group, *Bacillus cereus* group, *Paenibacillus*, *Streptomyces* and *Lysobacter*. Except *Lysobacter* (only one isolate from the rhizosphere of *Matricaria chamomilla*) only gram-positive antagonists were found. All microenvironments were dominated by antagonists from the Firmicutes branch. *Bacillus* and *Paenibacillus* could be isolated from all habitats. Antagonistic isolates of the genus *Streptomyces* were found exclusively in desert soil. Especially within the large ARDRA cluster of the *Bacillus subtilis* group containing 123 isolates, analysis of the BOX PCR fingerprints showed a high genotypic diversity. At a cutoff level of 80%, they could be divided into 39 genotypic groups. The genus *Paenibacillus* could be divided into 11 BOX clusters, *Streptomyces* was subdivided in three genotypes. According to the ARDRA and BOX dendrograms, 46 preferably genotypically different strains were selected to test them on their antibacterial activity towards *Ralstonia solanacearum* (Adesina et al., 2007) and *Escherichia coli*. The cluster of the *Bacillus cereus* group was completely excluded for further investigations, because of some human pathogenic strains belonging to this taxonomic group. Most isolates of the genus *Paenibacillus* (identified as *P. brasilensis* and *P. polymyxa*) were able to inhibit *in vitro* the growth of *E. coli* (7 of 11 isolates), but these strains showed no antagonistic activity towards *R. solanacearum*. The growth of *R. solanacearum* was inhibited by 32.6% of the selected antagonists: most isolates of *Streptomyces* (3 of 4 isolates) and some strains of the *Bacillus subtilis* group (12 of 30 isolates).

Organic amendments like manure, compost and cover crops positively affected the disease suppressiveness of SEKEM soil. During decomposition of organic matter in soil, the ecosystem is subjected to oligotrophication. The ratio of oligotrophic to copiotrophic organisms changes during microbial succession, and this has been associated with general disease suppression (van Bruggen & Semenov, 2000; Garbeva et al., 2004). Our cultivation-

independent approaches showed an extraordinary high Firmicutes level in SEKEM soils. By cultivation and characterization, the antagonistic role of *Bacillus* and *Paenibacillus* (both Firmicutes) was identified. Both are well-known and potent in biocontrol (Berg, 2009; Schisler et al., 2004; Tupinambá et al., 2008). These gram-positive bacteria have a natural formulation advantage due to their ability to form durable, heat-resistant endospores (Emmert & Handelsman, 1999). *Lysobacter* was the only gram-negative genus identified (Park et al., 2008). This is in contrast to the majority of other studies, where members of the *Pseudomonas* genus play a major role (Haas & Défago, 2005; Weller et al. 2007). Due to the fact that the proportion of antagonistic strains in soil and root is already high, biocontrol strategies could aim to enhance the diversity of the antagonistic community by application of *Lysobacter, Pseudomonas* or *Serratia* strains. However, in our study we selected promising candidates, which will be tested *ad planta* in comparison to these often used antagonists.

4.2 Strategy to control Fusarium wilt in bananas in Uganda

The banana family Musaceae includes monocotyledonous plants of the genera *Ensete*, *Musa* and *Musella*. Most important is the genus *Musa* comprising 50 to 100 species and cultivars including those with edible fruits like dessert or cooking banana, species with inedible fruits like ornamental bananas or those used for fibres production (Li et al., 2010). In many countries in Africa, Latin America, Asia or the Caribbean, banana production is an important source of income. Banana is the fourth important staple food after rice, wheat and milk in Uganda, the country with the highest per capita consumption per year of cooking banana and the second largest producer after India in the world. Farmers have to deal with several problems as plant pests and diseases, climate change or soil depletion. Diseases caused by fungi, bacteria and viruses are the most limiting factors of high quality production. Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*), is the most severe disease in banana plants, which leads to high yield losses (Ploetz, 2006). An infestation with the phytopathogen compromises the water and nutrient transport that can cause, in the worst case, the death of the plant. *Foc* belongs to the *F. oxysporum* species complex, which is distributed in a broad range of soils and causes serious symptoms on numerous host plants. Despite its ubiquitous occurrence, a morphological identification is difficult and is based primarily on the structure and abundance of asexual reproductive structures and on cultural characterizations (Fourie et al., 2011). The species is divided into more than 150 *formae specialis* and further subdivided in races, depending on the affected plant cultivars. *F. oxysporum* persists in soil as immobile chlamydospore until germinating by utilizing nutrients released from plant roots. The life cycle of the fungus commences with a penetration of the spore germ tube or the mycelium of the plants root tip. Further, wounds facilitate the endophyte an entrance of the potential host. When the mycelium entered the xylem vessel, it travels upwards through the plant. In later stages, microconidia are produced, which are distributed in the vessel system and germinate when their movement is stopped. This decreases water and nutrient transport, resulting in severe wilt and eventually death of the plant. Early symptoms of an infestation are reddish brown colouration of the xylem, a yellowing of old leaves and a beginning of wilt. In advanced stages, pseudostem coating leaves collapse and die. The pseudostem sometimes splits. Internally, xylem vessels of the roots and the rhizome turn reddish-brown as the fungus grows through the tissue (Aboul-Soud et al., 2004; Daly & Walduck, 2006). Different studies with bananas and banana plants *in vitro* and *in vivo* have shown that plants harbour fungal and bacterial organisms with antagonistic potential towards plant pathogens (Cao et al.,

2005; de Costa et al., 1997; Lian et al., 2008). However, an efficient strategy to control fungal pathogens especially *Foc* is still missing. In our study, we used molecular techniques to study banana-associated microbial communities in detail and focus on endophytes, which have a great potential for biocontrol of vascular diseases.

For screening of antagonists the rhizosphere, the endosphere and bulk soil of Ugandan banana plants were analysed. The term endosphere refers to the pseudostem of the plant, which is not lignified. Bananas grown in four different fields (variants) in Central Uganda characterized by different manure systems and/or agro-forest systems were sampled. In the first step, bacterial and fungal abundances in the microhabitats were examined. Surprisingly, the highest bacterial abundances with $\log_{10}$ 9.4 ± 0.1 g^{-1} fw were calculated for the endosphere followed by the rhizosphere with $\log_{10}$ 8.4 ± 0.3 g^{-1} fw and soil with $\log_{10}$ 7.7 ± 0.3 g^{-1} fw from R2A medium. Similar values for all microhabitats ranging from $\log_{10}$ 6.2 ± 0.2 g^{-1} fw for rhizosphere followed by soil and endosphere with almost same abundances of $\log_{10}$ 5.5 ± 0.3 g^{-1} fw and $\log_{10}$ 5.4 ± 0.3 g^{-1} fw were estimated for fungal isolates on synthetic nutrient-poor agar (SNA). A total of 1152 bacterial isolates from different media as R2A, MacConkey (for enrichment of Enterobacteriaceae) and King's B medium (for enrichment of *Pseudomonas*) and 586 fungi from SNA medium were randomly selected and screened *in vitro* for their antagonistic potential towards the pathogens. The target pathogen was also isolated from bananas in Uganda. Interestingly, different fungal species were identified: *F. oxysporum* f.sp. *cubense, Fusarium chlamydosporum,* and *Colletotrichum musae.* The latter are known as "low" pathogens; however, strains of all three species were integrated in the screening strategy. The antagonistic activity of bacteria or fungi towards the pathogen evaluated by the method of Berg et al. (2006) ranged from 3 – 6%. Altogether 37 highly active bacterial and 36 fungal strains were further characterized. ARDRA genotyping was able to distinguish bacteria on genus level into *Pseudomonas*, *Bacillus*, *Burkholderia* and *Serratia.* With repetitive BOX PCR a further characterization on population level was performed. Members of the genus *Burkholderia* were more diverse than those of *Serratia* (Fig. 4).

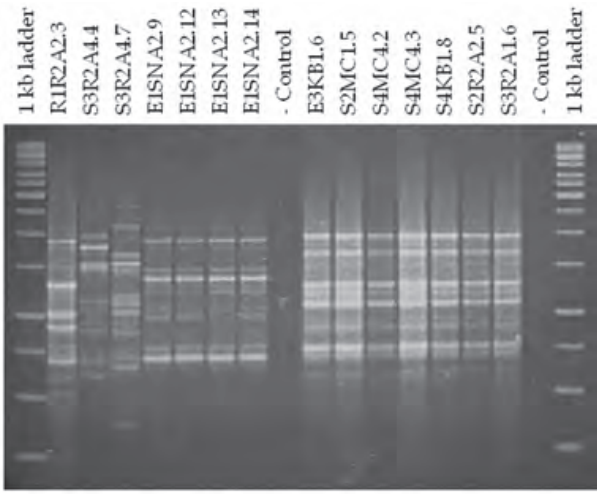

Fig. 4. BOX analysis on species level of bacterial antagonists. First seven isolates were identified as *Burkholderia* species and the other seven as *Serratia marcescens*. For identification of isolates the following abbreviations were used: a) habitat with R for rhizosphere, S for soil and E for endosphere, b) number of variant from 1 to 4, c) medium isolated from MC for MacConkey agar, KB for King's B agar, R2A for R2A agar and SNA for synthetic nutrient-poor agar d) number of replicate from 1 to 4 and e) number of isolate from 1 to 14.

Additionally, the best antagonists were screened for their ability to produce lytic enzymes like glucanase or protease, which are known for their positive influence in combating fungal pathogens by enzymatic degradation of the cell wall (Kamensky et al., 2003). Further, the production of siderophores, short-chained quorum sensing molecules and the auxin indole-3-acetic-acid (IAA) was investigated, which are involved in plant growth promoting processes. The results indicated that 100% of the tested isolates produced an active protease, while only a single isolate, which was identified as *Bacillus indicus*, was able to degrade glucan. Nearly all strains (94.6%) produced siderophores but only 21.6% isolates, belonging to the genera *Pseudomonas* and *Burkholderia*, released quorum sensing molecules. Seven isolates were positively tested for production of IAA, all of them identified as *Serratia marcescens*. To characterize fungal isolates, morphological groups were identified. Sequencing analysis of the ITS region indicated, that the majority of isolates belong to the genera *Penicillium*, *Paecilomyces*, *Fusarium* and *Mortierella*. All of them include known biocontrol strains, some actually tested in *Musa* spp. like non-pathogenic *F. oxysporum* strains (Kidane, 2008).

Cultivation independent analyses include the fingerprint method SSCP, quantitative PCR (qPCR), metagenome analysis and confocal laser scanning microscopy (CLSM) in combination with fluorescence *in situ* hybridization (FISH). Using SSCP fingerprints, a high specificity was shown for each microenvironment of banana, particularly for the endosphere. The patterns obtained from the bacterial community using universal primers were highly diverse, especially for rhizosphere and soil. This is a typical picture for environmental samples, especially for soil. A detection of bacterial species ranges up to 100 most dominant ones. This problem can be solved by using of more specific primers, e.g. for *Pseudomonas* or Enterobacteriaceae. Using both in analyses, specific patterns for each habitat appeared. Surprisingly, comparing all fields with different treatments or environmental influence, bacterial, enteric and fungal community didn't show distinct patterns. This could be explained by a high specificity of banana-associated bacteria independent from the site. The *Pseudomonas* community was more sensitive, but each site showed an individual pattern. In our study, we found that Enterobacteriaceae were extraordinarily present in and around cultivated banana plants. Therefore, further investigations on the microhabitat-specific communities were performed using a metagenomic approach. The sequences (1944 – 23800) obtained after pyrosequencing were aligned with databases and identified on genus level. In Figure 5 taxa including more than 1% of the totally analysed community were presented. Each habitat harboured a specific arrangement of genera. In the two rhizosphere variants, more than 40% of the identified genera are members of the *Enterobacter* community, followed by *Serratia*, *Pantoea* and *Klebsiella* with almost 40% and some other genera making up less than 20%. The bacterial composition in the endosphere differed from the rhizosphere samples with a lower number of *Enterobacter* and higher presence of the genus *Raoultella*. The highest species richness was shown for the soil sample, with the dominant genus *Pantoea* with known plant growth promoting species (Bonaterra et al., 2005; Braun-Kiewnick et al., 2000). *Serratia*, *Klebsiella* and *Enterobacter* represented together more than 40% of the analysed species. The analysis illustrates that depending on the investigated microhabitat, different species dominated. For the majority of the listed genera, species with growth promoting abilities are described. In different parts of the plant, diverse species play a key role, like *Enterobacter* in rhizosphere or *Pantoea* in soil and endosphere. To complement pyrosequencing data, a further assessment of *Pseudomonas* and Enterobacteriaceae was performed with quantitative PCR. Similar results were measured for both communities; the highest copy numbers g^{-1} fresh material of enterics and pseudomonads were detected in endosphere with $\log_{10}$ 8.4 ± 0.5 for *Pseudomonas* and $\log_{10}$ 7.9 ± 0.2 for Enterobacteriaceae

followed by rhizosphere with $\log_{10}$ 7.2 ± 0.6 and $\log_{10}$ 6.8 ± 0.4 and last with $\log_{10}$ 6.3 ± 0.8 for enterics in soil. In the *Pseudomonas*-specific analysis, no data for soil were received due to values under the detection limit. With confocal laser scanning analysis (CLSM) detection of different bacterial classes as Alpha-, Beta- and Gammaproteobacteria was performed to illustration our data. Due to the fact, that the number of enterics was extraordinary high in prior analyses, the focus in microscopy was also set on *Enterobacteriaceae*. The microscopic analysis confirmed the previous results, with the detection of a high number of *Enterobacteriaceae* in the endosphere and also lower detection in rhizosphere.

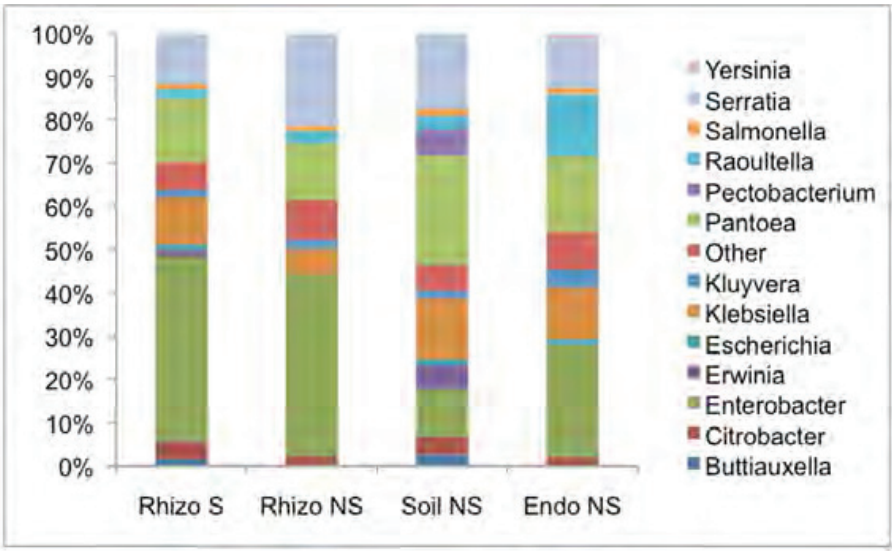

Fig. 5. Genera of the Enterobacteriaceae community associated with banana plants. Two rhizosphere samples under influence of agro-forest (shaded = S) and not (non-shaded = NS) and one sample from soil and endosphere in comparison. DNA was amplified with enterics-specific primers and analysed by pyrosequencing and identification with the web server SnoWMAn 1.7. The pipeline used was BLAT, NCBI database was selected and included taxa covering more than 1%. Phylogenetic groups accounting for ≤ 1 % of all quality sequences are summarized in the artificial group others.

This multiphasic approach showed that the pseudostem of banana – the endosphere – is a unique microenvironment in plants. It is characterized by extremely high microbial abundances, a high diversity and specificity, but a low proportion of antagonistic strains. Enterics play a key role in the bacterial community; they are dominant and represent a cluster of antagonists. However, they also contain human and plant pathogenic species. The endosphere should be the target habitat for biocontrol strategies: the number of strains with a beneficial plant impact should be enhanced here. We have isolated promising strains of *Pseudomonas*, *Bacillus*, *Burkholderia* and *Serratia,* which are interesting candidates for *ad planta* experiments. However, it is necessary to pay attention to the enteric community in bananas, especially to the pathogens.

4.3 Strategy to control a multi-species disease in the Styrian oilseed pumpkin

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a pumpkin variety that bears its name according to its origin of cultivation that is the Austrian district Styria. The specialty of this cultivar is the absence of a wooden seed shell that facilitates the production of pumpkin seed oil. Beside the culinary aspect of this dark green oil it is

famous as a very healthy nutritional supplement containing high levels of polyunsaturated fatty acids, antioxidants, vitamins A, B1, B2, B6, C, D, E and counteracts diseases of bladder and prostate.

In recent years, dramatic yield losses of Styrian oil pumpkin were reported in Styria due to black rot of pumpkins caused by *Didymella bryoniae* (Auersw.) Rehm, anamorph *Phoma cucurbitacearum* (Fr.) (Huss et al., 2007). The ascomycete has a broad host range within the Cucurbitaceae and causes symptoms on vegetative plant parts known as gummy stem blight (Keinath et al., 1995). It spreads from temperate to tropical regions of the world (Sitterly & Keinath, 1996). Fruits, leaves and flower scars are invaded by the pathogen and it can also be seed-borne (Lee et al., 1984; Ling et al., 2010; de Neergaard, 1989; Sitterly & Keinath, 1996). By cultivation-independent SSCP fingerprinting of the fungal ribosomal internal transcribed spacer (ITS) region in combination with DNA sequencing and BLAST analysis (Altschul et al., 1997), it was detected as well in roots of oil pumpkin (data not shown). This underlines the potential establishment of the pathogen even in soils (Bruton, 1998). The analysis of the phenotypic and genotypic variability of the pathogen across different oil pumpkin fields in Styria resulted in a remarkable high morphological versatility in contrast to a low genetic diversity (Zitzenbacher, pers. communication). Styrian oil pumpkins are also affected by bacterial pathogens *Pectobacterium carotovorum* subsp. *carotovorum* and subsp. *atrosepticum*, *Pseudomonas* spp. and *Xanthomonas cucurbitae* causing soft rot of pumpkins and leaf diseases (Huss, 2011). The transport of these bacterial phytopathogens by the fungus was observed in vitro (Zitzenbacher, pers. communication) suggesting synergistic interactions between them in the course of co-infections.

In order to manage microbial diseases of Styrian oil pumpkin based on autochthonous bacterial and fungal antagonists, initial studies to discover the microbial diversity associated with this host plant were conducted. Roots, female flowers and fruit pulp from three different oil pumpkin cultivars ("Gleisdorfer Ölkürbis", "Gleisdorfer Diamant" and "GL Maximal") at a field site in Styria were collected. Root samples were taken at three time points (before flowering, time of flowering, fruits well developed). Bacterial genera *Pseudomonas* and *Bacillus* that are known for their plant beneficial interactions (Haas & Défago, 2005) were analysed by SSCP analysis. Data revealed a greater impact of the microhabitat on community structure for *Pseudomonas*, whereas the plant stage had a stronger impact for *Bacillus* populations. Female flowers as possible gates for bacterial and fungal infections were analysed in more detail. For *Bacillus* and *Pseudomonas* and ascomycete communities, no effect of the plant cultivar on population structure was observed. However, in the flower, the communities are well-structured. FISH-CLSM studies revealed a dense bacterial colonisation of pollen grains that act as propagation vehicles between pistils especially for Alphaproteobacteria (Fig. 6) and shaped in this way the bacterial community structure of the oil pumpkin anthosphere.

To obtain oil pumpkin-associated microorganisms for testing their antagonistic properties against *D. bryoniae* and bacterial pathogens, bacterial and fungal strains were isolated from oil pumpkin cultivars and microhabitats as described above. Endophytes were cultivated from roots and fruit pulp. In addition, seed borne microbial strains were obtained from aforementioned varieties by the isolation from roots, stems and leaves from plants that seeds were surface sterilized and grown under gnotobiotic conditions. Finally 2320 isolates (1748 bacteria and 572 fungi) were subjected to dual culture assays against *D. bryoniae* A-220-2b to test their antagonistic potential against this pathogen. Of tested bacteria, 7.3% inhibited growth, whereas 12.4% of observed fungi showed either growth inhibition or overgrowth of *D. bryoniae* (Fig. 7).

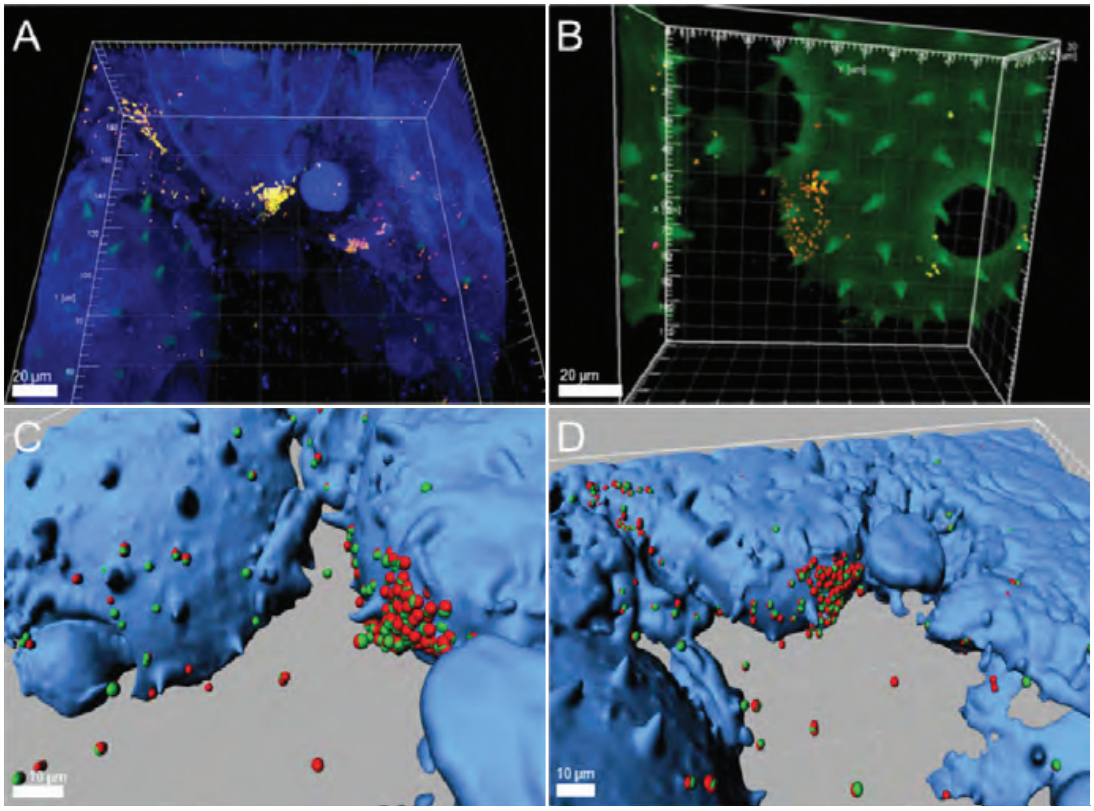

Fig. 6. FISH-stained bacteria colonising pollen grains located on pistils of oil pumpkin (GL Opal) visualized by CLSM. A) Alphaproteobacteria (in yellow) and not taxonomically classified bacteria (in red) labelled with ALF968-Cy5 and EUB338MIX-Cy3. B) Alphaproteobacteria labelled with ALF968-Cy5 (yellow), Firmicutes labelled with LGC354MIX-FITC (pink) and taxonomically undefined bacteria (in red) labelled with EUB338Mix-Cy3. C,D) 3D rendered image (Imaris software) of overall bacterial communities (in red) labelled with EUB338MIX-Cy3 and Alphaproteobacteria (red and green) labelled with ALF968-Cy5.

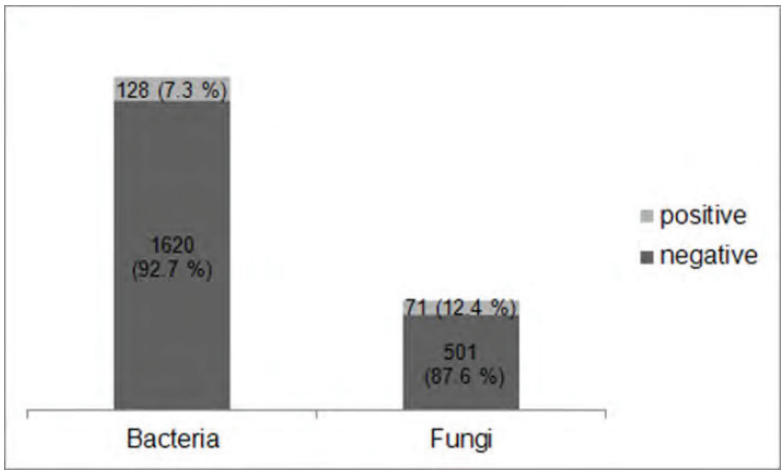

Fig. 7. Amount of oil pumpkin-associated bacterial and fungal isolates positively or negatively tested for *in vitro* antagonism against *D. bryoniae* A-220-2b.

Potential antagonists (128 bacteria and 71 fungi) were subsequently screened *in vitro* for effects on growth inhibition of *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 and *Xanthomonas cucurbitae* 6h4 to find broad-spectrum antagonists. Altogether, 32% of fungal as well as 49% of bacterial *D. bryoniae* antagonists were positively tested against at least one, 34% of tested prokaryotes against at least two and 6% of investigated bacterial strains against all three bacterial phytopathogens, whereas no fungal *D. bryoniae* antagonist was effective against more than one bacterial pathogen (Fig. 8).

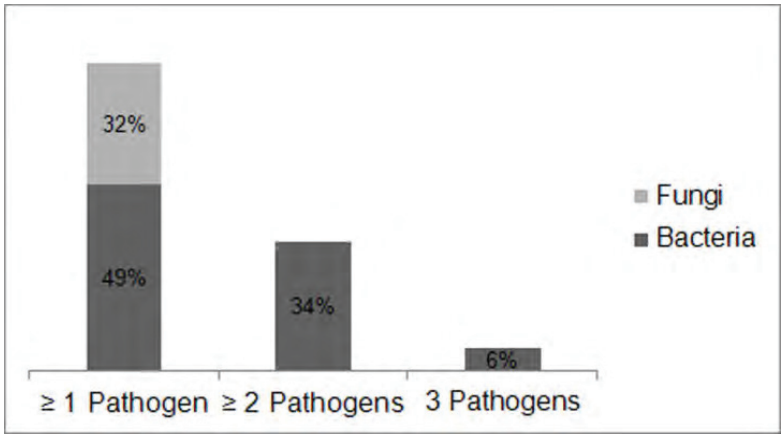

Fig. 8. Percentage of fungal and bacterial *D. bryoniae* antagonists positively tested against at least one, two or all three bacterial pathogens *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 *and Xanthomonas cucurbitae* 6h4.

Broad-spectrum antagonists that have the potential to suppress *D. bryoniae* as well as at least two bacterial phytopathogens of oil pumpkin were characterized genotypically by ARDRA. This resulted in a grouping of 43 bacterial isolates into four different genera: *Pseudomonas*, *Paenibacillus*, *Serratia* and *Lysobacter*. As a relative high number of isolates belong to *Paenibacillus* and *Lysobacter* they were further analysed by BOX PCR (Rademaker & de Bruijn, 1997) to get insight into the intra-genera diversities. Within the group of *Paenibacillus* a negligible variability between BOX patterns was observed in contrast to strains of *Lysobacter* that were divided into five groups. Finally five potential broad-spectrum antagonists were chosen for further analysis: one representative for *Pseudomonas*, *Paenibacillus* and *Serratia* and two representatives from the *Lysobacter* cluster. Partial sequencing of 16S rRNA genes with subsequent BLAST analysis (Altschul et al., 1997) was performed for their identification and the following species could be affiliated to respective strains: *Pseudomonas chlororaphis* P34, *Paenibacillus polymyxa* PB71, *Serratia plymuthica* S13, *Lysobacter antibioticus* L175 and *L. gummosus* L101. To learn more about the mode of antagonism of chosen broad-spectrum antagonists against *D. bryoniae*, dual culture assays in which growth inhibition of *D. bryoniae* A-220-2b by either soluble or volatile antimicrobial compounds secreted by the five test strains was assessed were performed. Results suggest a high capability of broad-spectrum antagonists to synthesize bioactive compounds: sterile culture supernatants from *P. chlororaphis* P34, *L. gummosus* L101 and *P. polymyxa* PB71 as well as volatile organic compounds (VOCs) excreted from these bacteria and *S. plymuthica* S13 as well suppressed growth of the fungus significantly compared to control treatments (ANOVA; LSD, $p < 0.05$; data not shown).

Performances of broad-spectrum antagonists in terms of promoting plant growth and health will facilitate the selection of bacterial strains that will be analysed for the production of a biological strengthener for Styrian oil pumpkin. Studies with the model organism *C. elegans* (Zachow et al., 2009) will give insight into the potential pathogenicity of remaining test strains. The manufacture of the final product will further depend on the finding of an appropriate formulation procedure that guarantees a high stability of the ultimate BCAs/PGPRs.

5. Conclusion

Advanced ecological knowledge about plant-associated microorganisms and interactions of the biocontrol agent(s) with abiotic and biotic factors support the development of efficient biocontrol strategies. As shown in three examples, specific strategies have to be developed adapted to the life cycle of the pathogen and the autochthonous microbial communities in the target habitat. The latter varied strongly dependent on the plant species, microenvironment and climate.

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Biocontrol strategies and next generation sequencing: organic desert agriculture in Egypt

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1 Introduction

Biological control of plant pathogens as well as plant growth promotion with microorganisms has been studied intensively over the past decades and is becoming a realistic alternative to chemical pesticides and fertilisers in sustainable agriculture (Weller, 2007; Berg, 2009). Presently, numerous microbial inoculants have been successfully commercialised and are currently marketed, dependent on their mode of action and effects, as biopesticides, plant strengtheners, biofertilisers, and phytostimulators (Berg, 2009). In screenings for new biological control agents (BCAs), additionally to cultivation and selection of strains, molecular methods are often included to analyse target habitats of biocontrol studies. Usually, "classical" community fingerprints performed by single-strand conformation polymorphism (SSCP), denaturing/temperature gradient gel electrophoresis (D/TGGE) or terminal restriction fragment length polymorphism (T-RFLP) are performed which base on particular nucleotide sequences PCR-amplified from total community DNA extracted from plant microenvironments (Köberl *et al*., 2011b). However, detection limits of these techniques prevent the coverage of the overall microbial diversity within one habitat. Especially the rhizosphere, which is often targeted by biocontrol studies, is colonised by extremely high abundant and diverse microbial populations (van Loon, 2007; Raaijmakers *et al*., 2009).

New molecular tools for metagenomic approaches, such as next generation high-throughput DNA sequencing (454 pyrosequencing or Illumina sequencing), revolutionised microbial detection by providing a greater depth and detection of low abundant and rare species (Hudson, 2008). Deep-sequencing techniques allow new insights into microbial community composition in different environments and extend our understanding of co-occurrence and interaction within the examined microbiome. This knowledge can be implemented in biological control strategies to improve screening strategies for finding potential BCAs. The knowledge about taxonomic and/or functional microbial patterns in target habitats can be used for comparative analyses between different conditions or treatments. For instance, how the behaviour of a specific microbial community is being affected by the presence of pathogens or different field treatments.

In the following chapter, the preparation of samples for amplicon sequencing and step by step data processing is explained from raw data to taxonomic community compositions displayed by principal coordinate analyses (PCoA) and profile clustering networks. Different databases, pipelines and software packages are employed during the data analysis. To assess influencing factors on ecosystem functions of microbial communities, three deep-sequencing studies are presented, which were included in a project develop of a specific biocontrol strategy for sustainable agriculture in desert ecosystems:

1. Influence of 30 years of organic agriculture on the total bacterial community of native desert soil in Egypt.
2. Phylogenetic diversity of bacteria within the order Bacillales in desert soil from Sinai.
3. Assessing the influence of introduced BCAs on indigenous microbial communities.

2 A Biocontrol Strategy for Sustainable Agriculture in Desert Ecosystems

On Sekem farms, which are located in the North-eastern desert region of Egypt, sandy desert was converted into cultivated, arable land. Organic/biodynamic agriculture has been operated since more than 30 years (www.sekem.com). Nowadays, Sekem is carrying out organic agriculture on more than 6,000 hectares and has the largest market for organic products outside Europe and North America. The Egyptian fair trade company produces organic food, herbal teas, phyto-pharmaceuticals as well as non-edible products such as cotton textiles. However, within recent years, soil-borne phytopathogens caused significant yield losses, mainly in medical plants. Major pathogens comprise different taxonomic groups, e.g. fungi (*Verticillium dahliae* Kleb., *Rhizoctonia solani* Kühn, *Fusarium culmorum* [Wm. G. Sm.] Sacc.), bacteria (*Ralstonia solanacearum*) and nematodes (*Meloidogyne incognita* [Kofoid and White] Chitwood) (Faltin *et al*., 2004; Klosterman *et al*., 2009; Messiha *et al*., 2009; Neher, 2010; Tunali *et al*., 2012). Therefore, the objective of the study was to develop a specific biocontrol strategy against these soil-borne pathogens for desert farming on the basis of ecological knowledge (Köberl *et al*., 2011b).

2.1 Influence of 30 Years of Organic Agriculture on the Total Bacterial Community of Native Desert Soil in Egypt

Finding out whether and how the highly specialised natural microbial communities of the desert soil are affected by agriculture and watering was an important issue within this study. To examine the impact of organic agriculture on bacterial diversity and community compositions in desert soil, soil from a Sekem farm and surrounding desert soil was sampled. Differences were assessed by a pyrosequencing-based analysis of partial 16S rRNA gene sequences. To analyse the taxonomic composition of the soil bacterial community, the hypervariable V4-V5 region of the 16S rRNA gene (*Escherichia coli* positions 515 to 927) was amplified in a nested PCR approach. Fragments encompassing this region provide estimates comparable to those obtained with the nearly complete gene fragment (Youssef *et al*., 2009; Will *et al*., 2010). The first PCR with the primer pair 27f/1492r (Lane, 1991) was followed by the second round of amplification using the Unibac-II-515f and Unibac-II-927r primers (Zachow *et al*., 2008) which contained the 454 pyrosequencing adaptors, linkers and sample specific tags (Table 1). The reaction mixture for the first PCR (20 µl) contained 1× Taq-&Go™ Ready-to-use PCR Mix (MP Biomedicals, Solon, USA), 0.25 µM of each primer and 1 µl of template DNA (95 °C, 5 min; 30 cycles of 95 °C, 30 s; 57 °C, 30 s; 72 °C, 90 s; and elongation at 72 °C, 5 min). The second PCR was performed by using 1× Taq-&Go™ Ready-to-use PCR Mix, 1.5 mM $MgCl_2$, 0.4 µM of each primer and 2 µl of template DNA (95 °C, 5 min; 32 cycles of 95 °C, 20 s; 54 °C, 15 s; 72 °C, 30 s; and elongation at 72 °C, 10 min). Respective PCR products from four samples each habitat were pooled in equal volumes and purified by employing the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) (Köberl *et al*., 2011a & 2011b). Pyrosequencing libraries were generated by LGC Genomics (Berlin, Germany) using the Roche 454 GS-FLX+ Titanium™ sequencing platform.

Name	Primer sequence
Unibac-II-515f_MID13	**CGTATCGCCTCCCTCGCGCCA***TCAG*CATAGTAGTGGTGCCAGCAGCCGC
Unibac-II-515f_MID14	**CGTATCGCCTCCCTCGCGCCA***TCAG*CGAGAGATACGTGCCAGCAGCCGC
Unibac-II-927r_454	**CTATGCGCCTTGCCAGCCCGC***TCAG*CCCGTCAATTYMTTTGAGTT

Table 1: Universal primers including 454 pyrosequencing adaptors (bold), linkers (italic) and sample specific tags (underlined).

Primer sequences were cropped and reads shorter than 150 bp and low quality reads (quality threshold 20) were removed from the pyrosequencing-derived data sets. In desert soil 19,244 and in agricultural soil 33,384 quality sequences with a read length of ≥150 bp were recovered. Data were adjusted considering the same number of sequences per sample using the open source software package QIIME (http://qiime.sourceforge.net), which allows analysis of high-throughput community sequencing data (Caporaso *et al*., 2010). From the equalised data set an operational taxonomic unit (OTU) table at a sequence divergence of 3%, corresponding to species level (Schloss & Handelsman, 2006; Will *et al*., 2010), was constructed using the data processing steps aligner and complete linkage clustering of the ribosomal database project (RDP) pyrosequencing pipeline (http://pyro.cme.msu.edu) (Cole *et al*., 2009). Rarefaction curves as well as Shannon (Shannon, 1997) and Chao1 (Chao & Bunge, 2002) indices were calculated based on data of the complete linkage clustering (data not shown) (Köberl *et al*., 2011a). A profile clustering network analysis was performed in order to highlight single OTUs with considerable differences between desert and agricultural soil. The network analysis was carried out with OTUs exhibiting a read change between the soils of more than 0.5% of the normalised data set. If the ratio of OTU read numbers for desert and agricultural soil exceeded 2, the OTUs were regarded as considerably altered and assigned to the respective clusters (abundant in desert soil or agricultural soil). Representative sequences of the OTUs were aligned with reference RNA sequences from NCBI (National Center for Biotechnology Information) database (http://blast.ncbi.nlm.nih.gov) using the nucleotide basic local alignment and search tool (blastn). Visualisation of the network was carried out using Cytoscape 2.8.2 (http://cytoscape.org).

According to the network analysis, the highest alterations were found for OTUs identified as *Ochrobactrum* sp. (closest database match *O. grignonense*, 99% similarity to NR_028901) and *Rhodococcus* sp. (closest database match *R. qingshengii*, 99% similarity to NR_043535). Both genera were much more abundant in desert than in agricultural soil (Fig. 1). Bacteria of these genera are known for their ambivalent interaction with eukaryotes. While showing plant growth promotion effects on plants (Trivedi *et al*., 2007; Chakraborty *et al*., 2009), they can cause opportunistic infections in humans. Several studies provided evidence that similar or even identical functions are responsible for the beneficial interactions with plants and virulence in other eukaryotic hosts (Berg *et al*., 2005 & 2011). For instance, *Rhodococcus qingshengii*, found as closest database match highly enriched in desert soil, is known to survive under extremely harsh conditions and moreover is capable of degrading carbendazim efficiently (Xu *et al*., 2007). Carbendazim is one of the most widely used benzimidazole fungicides in the control of agricultural and forestry diseases. This fungicide can harm the liver and endocrine system of animals. Even at low concentrations it shows mutagenic and teratogenic effects (Mazellier *et al*., 2003). Up to date, only a few bacterial strains with the ability to degrade carbendazim have been documented (Holtman & Kobayashi, 1997; Zhang *et al*., 2005; Xu *et al*., 2006 & 2007). Microbial metabolism has been

reported as the main mechanism of eliminating or minimising contamination by carbendazim (WHO, 1993). However, *R. qingshengii* is also known as opportunistic pathogen for Atlantic salmon (*Salmo salar*) causing appearance of pseudo-membranes on internal organs, i.e. spleen, liver, heart and others (Avendaño-Herrera *et al*., 2011).

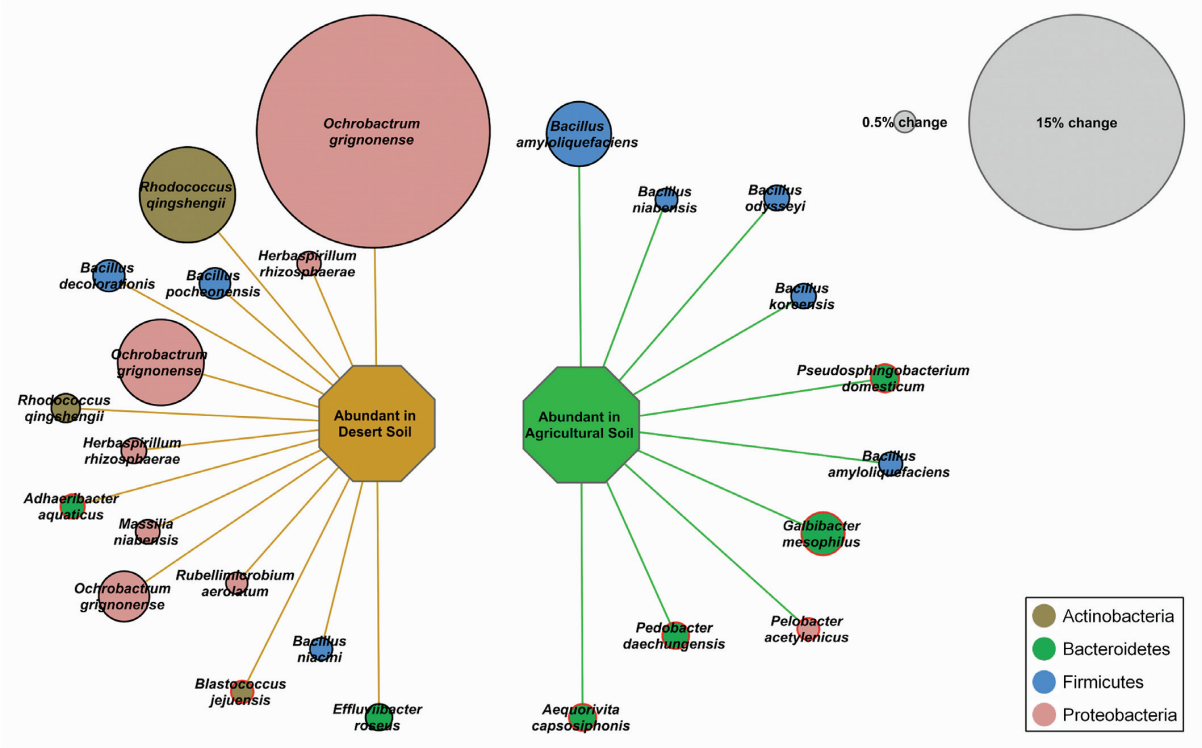

Figure 1: Profile clustering network analysis of bacterial OTUs at a dissimilarity level of 3%. The abundance values for OTUs with a read change between soils of more than 0.5% of the normalised data set were used. If the ratio of OTU read numbers for desert and agricultural soil exceeded 2, the OTUs were regarded as considerably altered and assigned to the respective profile. Each OTU is connected to either an "abundant in desert soil" or "abundant in agricultural soil" node in the network. Node sizes of OTUs correspond to the relative abundance change between the states. Nodes matching to changes of 0.5% and 15% of the data set were added as reference points. Black node borders indicate a similarity to the taxonomic node label (closest reference RNA sequences of the NCBI database) of ≥97%, whereas nodes with red borders have a similarity <97%.

In the network analysis presented in Fig. 1 *Bacillus amyloliquefaciens* (99% similarity to NR_041455; belonging to the *Bacillus subtilis* group), which is known for its high biological control properties (Emmert & Handelsman, 1999; Arguelles-Arias *et al*., 2009; Yuan *et al*., 2012), appear more distinct in agricultural soil. Several genotypically different isolates of this group were also obtained by a parallel cultivation dependent approach (Köberl *et al*., 2011a). All of them showed activity towards the soil-borne pathogenic fungi *Verticillium dahliae* V25, *Rhizoctonia solani* AG4, and *Fusarium culmorum*

E1. Some of them exhibited an additional activity towards the soil-borne pathogenic bacterium *Ralstonia solanacearum* and a nematicidal activity towards *Meloidogyne incognita*. Surprisingly, among the *Bacillus subtilis* group, isolates with identical BOX PCR fingerprints (similarity in the dendrogram >80%) could be detected in desert soil as well as in the agricultural soil, and also in rhizosphere and endorhiza of the medical plants. Some other OTUs identified as *Bacillus* spp. were also found in higher abundance in the agricultural than in desert soil (closest database matches *B. odysseyi*, *B. koreensis* and *B. niabensis*). On the other hand *Bacillus* spp. nearest related to *B. decolorationis*, *B. pocheonensis* and *B. niacini* were mainly found in the native desert soil. A shift among the *Bacillus* community due the agricultural use of the desert was observed. In literature, *B. decolorationis* could be found in damaged parts of mural paintings and was discussed to cause the biodeteriorated discoloration. Genotypically identical isolates were found in mural painting sites of the Servilia tomb of the Roman necropolis at Carmona in Spain and the Saint-Catherine chapel of Castle Herberstein in Austria (Heyrman *et al*., 2003). Sequencing reads closely related to *B. pocheonensis* were recovered in 8.5 fold higher abundance in desert soil than in agricultural soil from the Sekem farm. *B. pocheonensis* was described to live in moderately acidic to neutral soils (pH 6.3-7) (Ten *et al*., 2007; Yadav *et al*., 2011). Agricultural soil at the Sekem farm is alkaline (pH 8.4) (Luske & van der Kamp, 2009). By indirect correspondence analysis (CA biplot) based on bacterial SSCP fingerprints, pH was determined besides water supply as mainly influencing environmental factor shaping the bacterial soil communities (Köberl *et al*., 2011a). *B. pocheonensis* and also *B. niacini* were recently identified as plant growth-promoting rhizobacteria (PGPRs) of rice grown in moderately acidic soils (Yadav *et al*., 2011). In the desert, plants have a very short period of time to develop. Assumedly, plants need PGPRs for germination and growth.

Another interesting fact is that members of the *Herbaspirillum* group, some of them known as N-fixing PGPRs, only occurred in desert soil. Owing to compost treatment, agricultural soils are saturated with nitrogen. These facts could explain that this important functional group had a lower abundance in field soil (Köberl *et al*., 2011a). However, the *Herbaspirillum* species found as closest database match (*H. rhizosphaerae*, 99% similarity to NR_043621) do not possess the ability to fix atmospheric nitrogen (Jung *et al*., 2007). In general, several extremophilic bacterial groups, e.g. *Rubellimicrobium* (closest database match *R. aerolatum*, 97% similarity to NR_044469) were found to disappear from the desert soil by agricultural use. But on the other hand, bacterial communities in agricultural soil showed a higher diversity and a better ecosystem function for plant health (Köberl *et al*., 2011a).

Data from the pyrosequencing analysis exhibited substantial congruence with results obtained from classical molecular techniques. The two highly altered genera, *Ochrobactrum* and *Rhodococcus,* were also identified as dominant inhabitants of the desert soil from SSCP gels of 16S rRNA genes (Fig. 2). Interestingly, both genera were also present in plant samples, especially in the endorhiza of three different medical plants *Matricaria recutita* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. & Thonn., which were cultivated under organic farming conditions on a Sekem farm. These results show that bacteria from soil were enriched via plant roots and were taken up within the inner tissue of the roots. Root exudates and the response of microorganisms to the latter as well as to root morphology were shown to shape the microbial communities of the root (Berg & Smalla, 2009). In this study, a clear plant specificity of the bacterial as well as fungal (data not shown) communities was found. In comparison to the desert soil, owing to cultivation and compost treatment, agricultural soil exhibited an impressive diversity of bacteria (Köberl *et al*., 2011a & 2011b). However, plants selected some strains already present in native desert soil.

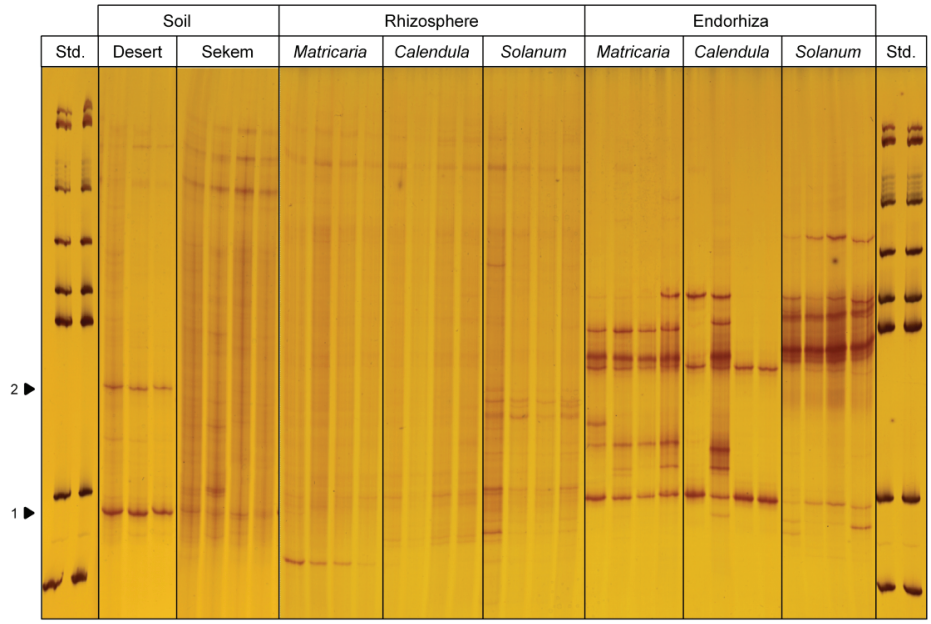

Figure 2: 16S rRNA PCR-SSCP profiles of the bacterial communities in desert and agricultural soil, and additionally in rhizosphere and endorhiza of three different species of medical plant (*Matricaria recutita* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. & Thonn.) grown under organic farming conditions on a Sekem farm. Std.: 1 kb DNA ladder. The following bands were identified as: 1. *Ochrobactrum grignonense,* 99% similarity to NR_028901 and 2. *Rhodococcus qingshengii*, 99% similarity to NR_043535.

2.2 Phylogenetic Diversity of Bacteria within the Order Bacillales in Desert Soil from Sinai

According to the results obtained by network analysis of the total bacterial community (Figure 1), bacteria of the order Bacillales within the phylum Firmicutes were identified as interesting group, shifting their community structure in case of agricultural use of the desert. For deep analysis of the Bacillales taxonomic composition in native desert soil, amplification of the 16S rRNA genes was performed using a nested PCR approach with the universal primer pair 27f/1492r (Lane, 1991) and the Bacillales specific primers BLS342F (Blackwood *et al*., 2005) and BACr833 (Fu *et al*., 2011). Primers for the second PCR carried the 454 pyrosequencing adaptors, linkers and sample specific tags (Table 2). The reaction mixture for the first PCR (10 µL) contained 1× Taq-&Go™ Ready-to-use PCR Mix (MP Biomedicals, Solon, USA), 0.1 µM of each primer and 1 µL of template DNA (95 °C, 5 min; 30 cycles of 95 °C, 30 s; 57 °C, 30 s; 72 °C, 90 s; and elongation at 72 °C, 5 min). The second PCR (30 µL) was performed by using 1× Taq-&Go™ Ready-to-use PCR Mix, 1.5 mM $MgCl_2$, 0.2 µM of each primer and 1 µL of template DNA (95 °C, 5 min; 28 cycles of 95 °C, 45 s; 60 °C, 60 s; 72 °C, 45 s; and elongation at 72 °C, 5 min). PCR products of three independent replicate samples from the Sinai desert were pooled in equal volumes and purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA). Pyrosequencing was performed by Eurofins MWG (Ebersberg, Germany) using the Roche 454 GS-FLX+ Titanium™ platform.

Name	Primer sequence
BLS342F_MID26	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACATACGCGTCAGCAGTAGGGAATCTTC
BACr833_454	**CTATGCGCCTTGCCAGCCCGC***TCAG*CTAACACTTAGCACTCAT

Table 2: Bacillales specific primers including 454 pyrosequencing adaptors (bold), linkers (italic) and sample specific tags (underlined).

Primers were cropped from obtained sequencing reads, quality filtering was performed (minimum average exponential quality score 20) and sequences were trimmed by length (≥200 bp). To construct a phylogenetic tree, filtered amplicon sequences (7,041 quality reads) were aligned and clustered into OTUs at a dissimilarity level of 3% (Schloss & Handelsman, 2006; Will *et al*., 2010) by employing the tools aligner and complete linkage clustering of the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu) (Cole *et al*., 2009). Rarefaction, Shannon (Shannon, 1997) and Chao1 (Chao & Bunge, 2002) indices were calculated based on the complete linkage clustering data. Taxonomic affiliation of representative sequences of OTUs was defined by alignment with reference RNA sequences from NCBI database using blastn algorithm (http://blast.ncbi.nlm.nih.gov). Clusters containing <10 sequences were not phylogenetically designated. Neighbour-joining phylogenetic tree was constructed using PHYLIP software package version 3.69 (http://evolution.genetics.washington.edu/phylip/getme.html) (Felsenstein, 1989) and visualised in MEGA4 (http://megasoftware.net) (Tamura *et al*., 2007). A heat map showing the number of sequences for each OTU was added.

Quality sequences were arranged into 197 OTUs with 3% dissimilarity cut-off. Comparison of observed OTUs with the number of OTUs estimated by the Chao1 index revealed that 79% of estimated richness was recovered at this genetic level. Clustering at genus and family level (5% and 10% dissimilarity) (Schloss & Handelsman, 2006; Will *et al*., 2010) reached 94% and 100%, respectively. At species level (3% dissimilarity), computed Shannon diversity index (H') was 3.27.

Neglecting clusters containing less than 10 sequences, mainly Bacillales of the families Bacillaceae (genera *Bacillus* and *Lysinibacillus*) and Planococcaceae (genera *Planomicrobium* and *Sporosarcina*) were encountered (Fig. 3). Additionally, genus *Macrococcus* of the family Staphylococcaceae was found to a small extent. The largest cluster in the analysed Bacillales community comprised 2,144 sequences (30% of the total data set). For this cluster, *Bacillus pocheonensis* (99% similarity to NR_041377) was identified as the closest database match. *B. pocheonensis* was already found as highly abundant inhabitant of desert soil by the network analysis of the total bacterial community (Fig. 1). In general, soil from the Sinai desert revealed a high diversity within the genus *Bacillus*. Besides *B. pocheonensis*, sequences closely related to *B. benzoevorans* (98% similarity to NR_044828), *B. odysseyi* (97% similarity to NR_025258) and *B. niacini* (99% similarity to NR_024695) were found in high abundance. *B. niacini* is an ubiquitous soil bacterium. Previous culture-independent studies demonstrated that ~15% of all *Bacillus* 16S rRNA gene sequences directly extracted from soils worldwide were affiliated to this species (Felske *et al*., 2004). Spores of *B. niacini* were already isolated from various extreme environments: the interior of granite rocks collected in the Sonoran desert in Arizona (Fajardo-Cavazos & Nicholson, 2006) or from spacecraft assembly facilities (SAFs), clean rooms considered as extreme selective environments with controlled air circulation, strict hygienic practices and a number of sterilants, such as H_2O_2 vapor and UV radiation, applied (Link *et al*., 2004; Kempf *et al*., 2005). Thus, *B. niacini* and also *B. odysseyi*

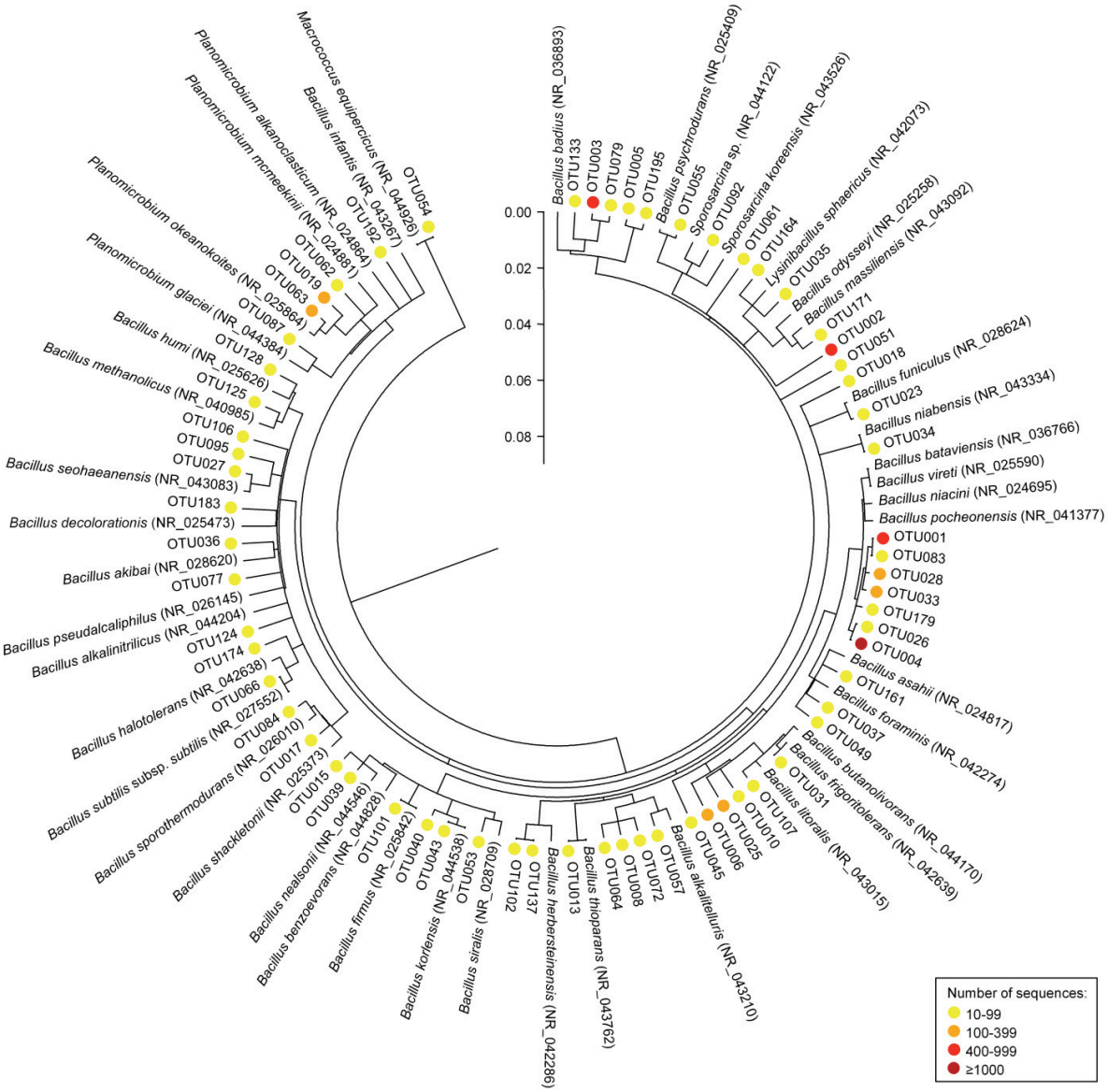

Figure 3: Phylogenetic composition of the Bacillales community in soil from the Sinai desert. Analysed amplicon library comprised 7,041 high quality 16S rRNA gene sequences clustered at 3% dissimilarity. Neighbour-joining phylogenetic tree was constructed with both one representative sequence per each OTU and the nearest reference sequences (accession numbers in brackets). *Lactobacillus acidophilus* (NR_043182) was used as out-group. Reliability of the tree topology was evaluated by bootstrap analysis with 100 re-samplings (bootstrap values not shown). Numbers of sequences in each cluster are indicated in a heat map. Scale bar: substitutions per site.

are discussed to play a potential role in interplanetary transfer of life. *B. odysseyi* was firstly isolated from the surface of the Mars Odyssey spacecraft. La Duc *et al*. (2004) noticed a remarkable resistance of these spores to harsh environmental conditions, such as desiccation, H_2O_2, UV and gamma radiation. Considering the phylogenetic tree based on 16S rRNA sequences, *B. odysseyi* and also the most closely related species *B. massiliensis* are assigned to the same cluster as the genus *Lysinibacillus*. Recently, Jung *et al*. (2012) proposed that *B. odysseyi* and also *B. massiliensis* should be transferred to the genus *Lysinibacillus*.

Additionally to the endospore-forming Bacillaceae (*Bacillus* and *Lysinibacillus*), endospore-forming Planococcaceae of the genus *Sporosarcina* were found in the Sinai desert soil. Several *Sporosarcin*a spp. are known as inhabitants of contaminated soils. For instance, *Sporosarcina ureae* prefers to live in alkaline soils contaminated with urine (Chomarat *et al*., 1990) or *Sporosarcina ginsengisoli* not only tolerates even very high concentrations of arsenic, it is also capable for biomineralisation based remediation of As(III) contaminated soils (Achal *et al*., 2012). Among the Planococcaceae also the non-spore forming genus *Planomicrobium* was found. Nevertheless, this genus was frequently isolated from extreme environments, especially from habitats with low temperatures like cold deserts (Mayilraj *et al*., 2005), Antarctic sea ice (Junge *et al*., 1998) or glaciers (Zhang *et al*., 2009), but have been also found in marine mud and intertidal beach sediments (Engelhardt *et al*., 2001; Dai *et al*., 2005; Jung *et al*., 2009). *P. alkanoclasticum* is able to degrade hydrocarbons, which result from activities related to the petrochemical industry (Engelhardt *et al*., 2001). The genus *Macrococcus* belongs to the family of Staphylococcaceae and members of this genus were formerly falsely classified as *Staphylococcus* spp. Beside the 16S rRNA gene sequence differences, *Macrococcus* spp. feature generally larger cells (2.5-4 times larger in diameter compared to *S. aureus*) mostly without cell wall teichoic acids (except *M. caseolyticus*) and a smaller genome (approximately 1,500-1,800 bp) with a higher GC-content than *Staphylococcus*. In general, macrococci are known to be associated with animals and animal products such as milk and meat. *Macrococcus equipercicus* was firstly isolated from the skin of horses and ponies (Kloos *et al*., 1998; Kwok & Chow, 2003). Unlike staphylococcal species, macrococci do not cause human or animal diseases, but recently a multidrug-resistant *Macrococcus caseolyticus* was isolated from chicken skin. The strain harboured a plasmid (pMCCL2), which carries a transposon containing a probable primordial form of a *Macrococcus* methicillin resistance gene complex (*mecIRA*$_m$) of the notorious human pathogen methicillin-resistant *Staphylococcus aureus* (MRSA). This was the first finding of a plasmid-encoding methicillin resistance gene (Baba *et al*., 2009; Tsubakishita *et al*., 2010).

2.3 Assessing the Influence of Introduced BCAs on Indigenous Microbial Communities

In the ecological study, based on genomic fingerprints and antagonistic potential, 45 unique strains were selected of which 89% belonged to the *Bacillus/Paenibacillus* cluster (Köberl *et al*., 2011a). *Bacillus subtilis* was the main cultivated species from farm samples. In contrast, efficient antagonists from the surrounding desert soil were mainly *Streptomyces* strains. Under arid conditions, Gram-positive, spore-forming bacteria dominated the indigenous antagonistic potential. This is in contrast to humid areas, where Gram-negative bacteria like *Pseudomonas* are responsible for the indigenous antagonistic potential of plants (Haas & Défago, 2005). From a hierarchical screening, three promising strains were selected for evaluation on the field: (1) *Streptomyces subrutilus* Wb2n-11 isolated from desert soil from Sinai, (2) *Bacillus subtilis* subsp. *subtilis* Co1-6 obtained from the rhizosphere of *Calendula officinalis*, and (3) *Paenibacillus polymyxa* Mc5Re-14 isolated from the endorhiza of *Matricaria recutita*. These Gram-

positive strains were tested for their plant growth promoting effect *ad planta* in comparison to two Gram-negative strains which are already described as biocontrol agents (Wolf *et al*., 2002; Kai *et al*., 2007; Zachow *et al*., 2010): (4) *Stenotrophomonas rhizophila* P69 isolated from the rhizosphere of *Brassica napus*, and (5) *Serratia plymuthia* 3Re4-18 from the endorhiza of *Solanum tuberosum*. Although originating from plant-associated microenvironments, BCAs, if applied to plant roots in sufficient numbers, may perturb indigenous microbial populations (Scherwinski *et al*., 2006 & 2008). Therefore, unwanted, unspecific actions of the introduced beneficial microorganisms against non-target organisms have to be assessed (Berg *et al*., 2011). To this end, knowledge concerning the microbial ecology of the target habitats is necessary for reasonable risk assessment studies relating to the release of beneficial microorganisms. As only a small proportion of the microorganisms can be analysed by common cultivation techniques, DNA-based, pyrosequencing techniques can be used to overcome the limitations of cultivation techniques.

To analyse the impact of the selected BCAs on the indigenous microbial communities of field-grown chamomile (*Matricaria recutita* L.), one-month old seedlings were root-dipped into bacterial suspensions and planted to a field at Adleya farm/Sekem in a randomised plot trial. A barcoded pyrosequencing approach was used for characterisation of soil bacterial communities associated with *Matricaria recutita*. The V4-V5 hypervariable region of the bacterial 16S rRNA gene was amplified with the set of eubacterial primers Unibac-II-515f/Unibac-II-927r (Zachow *et al*., 2008) containing the 454 pyrosequencing adaptors, linkers and sample specific tags (Table 3). Amplification was performed in 20 µl reactions containing 5-100 ng DNA template, 1× Taq-&Go™ Ready-to-use PCR Mix (MP Biomedicals, Solon, USA), 3 mM $MgCl_2$ and 0.5 µM of forward and reverse primer. Reaction conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 34 cycles of 95 °C for 20 s, 65 °C for 15 s, and 72 °C for 29 s, with a final extension of 72 °C for 10 min. PCR products from four samples of the same treatment were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and quantified using a NanoDrop™ photometer (Peqlab, Erlangen, Germany). Subsequently, amplicons of each treatment were pooled together in an equimolar ratio and subjected to pyrosequencing using Roche 454 GS-FLX+ Titanium™ platform executed by Eurofins MWG (Ebersberg, Germany).

Name	Primer sequence
Unibac-II-515f_MID27	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACGCGAGTATGTGCCAGCAGCCGC
Unibac-II-515f_MID28	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACTACTATGTGTGCCAGCAGCCGC
Unibac-II-515f_MID29	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACTGTACAGTGTGCCAGCAGCCGC
Unibac-II-515f_MID30	**CGTATCGCCTCCCTCGCGCCA***TCAG*AGACTATACTGTGCCAGCAGCCGC
Unibac-II-515f_MID31	**CGTATCGCCTCCCTCGCGCCA***TCAG*AGCGTCGTCTGTGCCAGCAGCCGC
Unibac-II-927r_454	**CTATGCGCCTTGCCAGCCCGC***TCAG*CCCGTCAATTYMTTTGAGTT

Table 3: Custom primers including 454 pyrosequencing adaptors (bold), linkers (italic) and sample specific tags (underlined).

Raw sequencing reads were demultiplexed, quality and length filtered using RDP's pyrosequencing pipeline (Cole *et al*., 2009). Primers were cropped and all sequence reads shorter than 150 bp, with a minimum average quality score <20 and with any ambiguous characters were discarded. A total of 16,007 quality-filtered sequences were recovered. Due to different numbers of sequences among the

samples, data were normalised to the same number of sequences (1,858 quality reads) using an in-house developed Perl script (10 times random re-samplings followed by subset formation). Further downstream analysis of normalised data was achieved using the QIIME (Quantitative Insights Into Microbial Ecology) toolkit (Caporaso *et al*., 2010). Bacterial sequences were clustered into OTUs using 3%, 5% and 20% dissimilarity thresholds with UCLAST (Edgar *et al*., 2010) and the most abundant sequence from each OTU was selected as a representative sequence for that OTU. Taxonomy was assigned by using a QIIME-based wrapper of the RDP classifier program (Wang *et al*., 2007) against the RDP core set (Cole *et al*., 2007 & 2009) using an 80% confidence threshold for taxonomic assignment. Rarefaction analysis and estimation of alpha-diversity was performed using Chao1 (Chao & Bunge, 2002), Shannon (Shannon, 1997) and observed OTU metrics at 3%, 5% and 20% dissimilarity. Beta-diversity was examined using weighted UniFrac distances (Lozupone & Knight, 2005) between samples sub-sampled 20 times, with replacement, at a depth of 100 sequences per sample. This method takes phylogenetic relationships between community members in account, incorporating the abundances of phylotypes into the pairwise community comparisons (Eilers *et al*., 2010). The compositional similarity of all samples was visualised in a three-dimensional principal coordinate system (PCoA) based on previously calculated jackknifed principal coordinates. To reveal the most abundant taxa in different areas of the PCoA plot, taxonomic classification of 20% genetic distance was included.

Rarefaction analysis was performed to an extent of diversity coverage (Fig. 4). Assessment of richness revealed that pyrosequencing effort attained 35.8-46.5% of estimated richness at a genetic distance of 3% (Table 4). At the genetic distances of 5% and 20% amplicon libraries covered 41.7-49.7% and 56.6-88.8% of estimated richness, respectively. Taxonomic composition of bacterial communities (Fig. 5) was similar at phylum level comprising Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria as the most dominant phyla. However, the phylum Verrucomicrobia was only present in the sample from the treatment with *Stenotrophomonas rhizophila* P69 (= DSM14405^T), considering only taxa covering more than 1% of quality sequences. In other *ad planta* studies, this strain was found to have an indirect positive interaction with their host plants by altering fungal communities (Schmidt *et al*., 2012). Acidobacteria were observed in samples treated with *Bacillus subtilis* Co1-6, *Stenotrophomonas rhizophila* P69 and *Serratia plymuthica* 3Re4-18. At genus level, *Rhizobium* (phylum Proteobacteria), *Pseudoxanthomonas* (phylum Proteobacteria), *Pseudomonas* (phylum Proteobacteria), *Flavobacterium* (phylum Bacteroidetes) and *Arthrobacter* (phylum Actinobacteria) represented the most abundant genera showing a slightly different composition according to different treatments. Alpha-diversity of the amplicon libraries was characterised by Shannon index (H') for 3%, 5% and 20% dissimilarity levels. Slight differences between treatments were revealed by the comparison of the index values (Table 4).

At genus level, beta-diversity showed a higher similarity between samples treated with *Bacillus subtilis* Co1-6 and *Serratia plymuthica* 3Re4-18 compared to the other treatments (dendrogram not shown). A jackknifed UniFrac three-dimensional PCoA biplot was constructed in order to visualise relationships among samples based on differences in taxonomic diversity (Fig. 6). Samples were clearly separated implying a difference in bacterial community composition. Results of the pyrosequencing analysis revealed that the treatments with potential BCAs had an influence on the native communities inhabiting the rhizosphere of plants. These community shifts may have positive effects on plant growth and health, harvest yield as well as on the composition of secondary metabolites of medical plants.

Genetic distance	Treatment sample	No. of OTUs	Chao1 (OTUs)	Coverage (%)	Shannon (H')
3%	Wb2n-11	557	1,443	38.6	5.14
	Co1-6	596	1,280	46.5	5.32
	Mc5Re-14	729	1,928	37.8	5.66
	P69	559	1,209	46.2	5.23
	3Re4-18	546	1,525	35.8	5.37
5%	Wb2n-11	433	871	49.7	4.80
	Co1-6	477	998	47.8	5.00
	Mc5Re-14	560	1,342	41.7	5.26
	P69	435	930	46.8	4.90
	3Re4-18	419	973	43.1	5.01
20%	Wb2n-11	81	143	56.6	2.85
	Co1-6	76	86	88.8	3.05
	Mc5Re-14	90	134	67.4	2.87
	P69	92	120	76.9	3.11
	3Re4-18	89	100	88.6	3.20

Table 4: Richness estimates and diversity indices for 16S rRNA gene amplicon libraries of rhizospere samples of *Matricaria recutita* L. The number of sequences of each sample was normalised to 1,858. Genetic distances represent the taxonomic levels of species (3%), genera (5%) and phyla (20%).

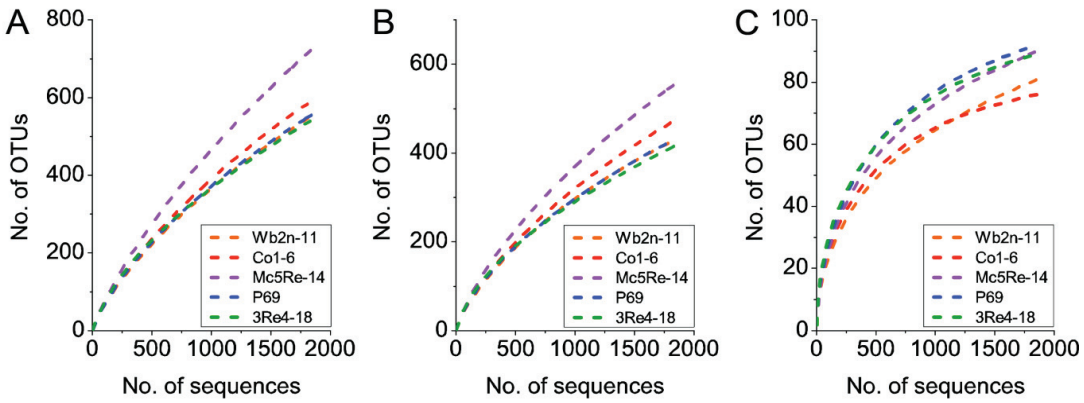

Figure 4: Rarefaction analysis of the 16S rRNA gene amplicon libraries of rhizosphere samples of *Matricaria recutita* L. Rarefaction curves were calculated at (A) 3%, (B) 5% and (C) 20% genetic distance levels, corresponding to the taxonomic levels of species, genera and phyla, respectively. Different treatments are depicted by coloured lines.

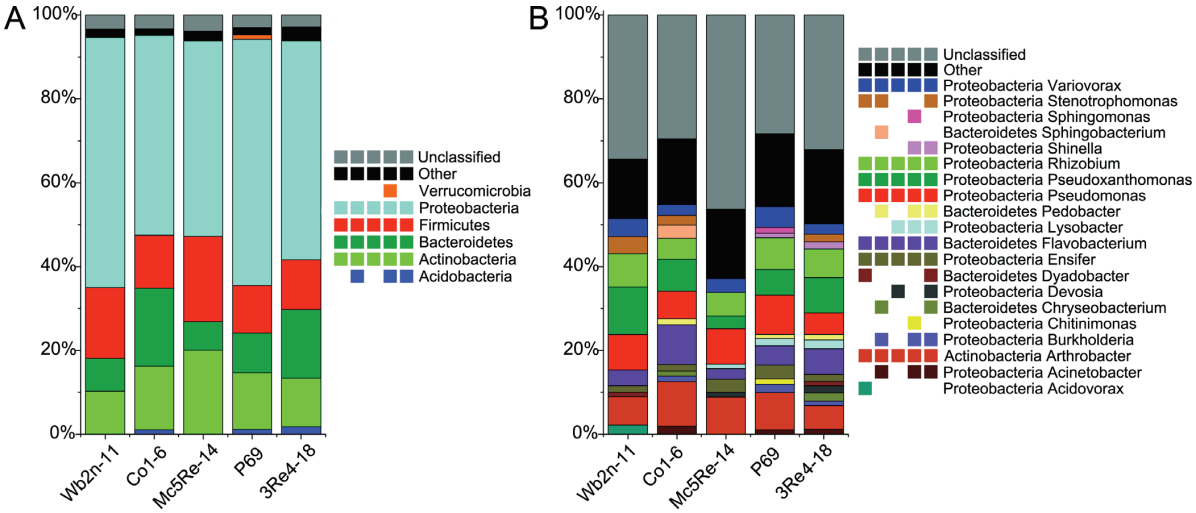

Figure 5: Taxonomic classification of bacterial communities associated with *Matricaria recutita* L. Pyrosequencing reads were classified at phylum (A) and genus (B) level against RDP core set within QIIME pipeline with an 80% confidence threshold. Taxa below 1% of relative abundance are included in "Other". Multi-coloured charts at the legend are shown for each sample correspondingly.

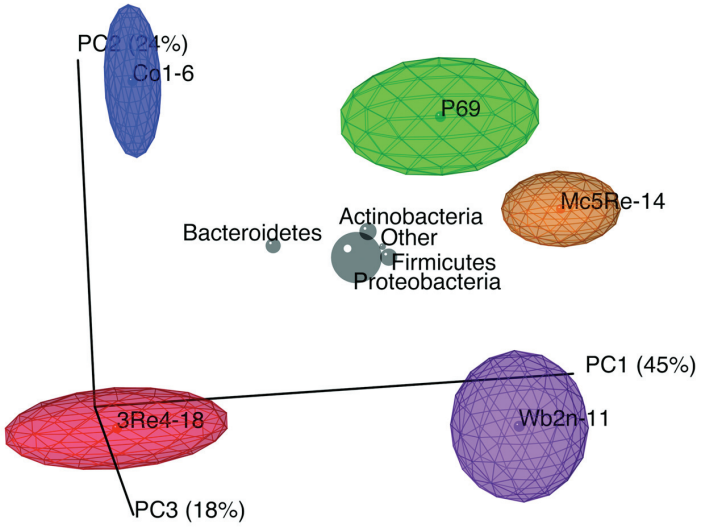

Figure 6: Comparison of the microbial communities of *Matricaria recutita* L. rhizosphere by jackknifed principal coordinate analysis. The biplot illustrates the compositional similarity between samples based on weighted UniFrac. Taxa coordinates are indicated by grey orbs with size as a function of relative abundance. To confine the biplot, the number of the displayed taxa was restricted to 5. The positions of the points are the average for the jackknifed replicates generated by QIIME and are shown with ellipses representing the interquartile range (IQR) in each axis. Larger ellipses represent more diverse communities. Colours correspond to the different treatments.

3 Conclusion

New molecular tools for metagenomic appraoches have not only a deep impact on human medicine and environmental ecology; they also promote applied research fields like environmental biotechnology. In our study we have shown that pyrosequencing techniques can be integrated at various stages of biocontrol research – from screening to risk assessments. Profiling of 16S rRNA genes is a relatively quick way to get an impression about the taxonomic composition of a metagenomic sample. However, it is still difficult to discover the whole plant-associated microbial diversity, especially rare species. We found out that the depth of an amplicon sequencing analysis and the corresponding detection rate can be drastically improved by the use of more specific primers (like it was shown for the Bacillales community in section 2.2). Nevertheless, one should always take into account potential technical biases, for instance associated with the annealing of PCR primers in metagenomic templates. Beside 454 pyrosequencing, several other platforms for amplicon massive sequencing strategies are available and probably very soon all these powerful platforms will be replaced by new alternatives reaching even much higher levels of sequencing. Amplicon sequencing approaches can also be applied to investigate functional communities, such as the nitrogen-fixing (*nifH* gene) community, to get an insight into the functional interactions between beneficial microorganisms and their host plants. To gain a broader overview about genetic composition and functional capacity of a microbial community, alternatively to amplicon sequencing which is usually based on one gene, metagenomic shotgun sequencing focusing on the whole genome and metatranscriptomic sequencing can be applied and involved in biological control research. An understanding of the microbial ecology of the target habitats of biocontrol as well as of interactions and effects of BCAs will lead to more consistent effects of BCAs. Because currently, in particular theirs inconsistency in the field constitutes the main hurdle for commercialisation.

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Additional Publication I

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Biological control agents for combating soil-borne pathogens in Egypt

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Biological control agents for combating soil-borne pathogens in Egypt

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Abstract: SEKEM is an Egyptian initiative and a Fair Trade company, where biodynamic agriculture is carried out for more than 30 years. Medicine, which is produced from ecologically grown medical plants, was developed and is available world-wide. However, the cultivation of plants is more and more affected by soil-borne pathogens, which leads to significant yield losses. An environmentally friendly and sustainable solution of the problem is the introduction of biological control agents (BCA). Through the introduction of BCAs, the vitality and the natural biodiversity of the soil can be restored. To develop a specific biocontrol strategy for the SEKEM farms, microorganisms were isolated from rhizosphere and endorhiza of medical plants (*Matricaria chamomilla*, *Calendula officinalis* and *Solanum distichum*) as well as from Egyptian soil, and were screened for their antagonistic potential towards the soil-borne phytopathogens. Promising biocontrol strains were genotypically characterized and identified.

Key words: biocontrol, biological control agents, medical plants

Introduction

The major problems in the cultivation of medical plants in SEKEM farms are caused by the soil-borne pathogens *Fusarium culmorum* (Wm.G. Sm.) Sacc., *Rhizoctonia solani* Kühn and *Verticillium dahliae* Kleb. Although grown in organic agriculture, which aims to minimize the impact on the environment by practices such as crop rotation, using pathogen resistant cultivars, and the use of organic manure (compost) instead of synthetic fertilizers, they have an increasing importance. One reason is an intensive growing of a limited number of crops in short rotations. Biocontrol using naturally occurring antagonists as BCAs, which are able to effectively colonize plants and promote growth of healthy plants by various mechanisms, offers a sustainable solution in plant protection (Berg, 2009).

The objective of this project is to develop a biocontrol strategy against soil-borne pathogens. The product will be optimized for Egyptian conditions – regarding soil, weather, pathogen species, etc. For this reason, the microorganisms were isolated from Egyptian sample materials collected from the rhizosphere or endorhiza of plants and characterized for their antagonistic activity.

Material and methods

Sampling strategy

The samples were obtained from the Adleya farm in SEKEM from three different species of medical herbs: *Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum*

Schumach. & Thonn. Furthermore, soil samples from the farm and in the surrounding desert were selected. From each plant and each soil four independent replicates were taken.

Isolation of microorganisms in search for antagonists

To isolate from the rhizosphere and from the soil 5 g of roots/soil and 45 ml of NaCl [0.85 %] were mixed for 5 min on the vortex. After a brief settling, the supernatant was used for dilution and plating on R2A (for bacteria) and TSM (*Trichoderma* selective medium; Elad & Chet, 1983). For the isolation from the endorhiza, 5 g material of roots were surface-sterilized with NaOCl [4 %] for 5 min, then the roots were washed three times with sterile Aqua dest. After 10 ml sterile NaCl [0.85 %] were added the roots were homogenized using mortar and pestle and as previously the supernatant was used for dilution and plating on R2A and TSM. R2A (Roth) is a nutrient-poor medium, where an optimal biodiversity of bacteria can be achieved. The TSM plates specifically are designed for the enrichment of *Trichoderma*. These fungi live as mycoparasites and are known for their good antagonistic potential. All plates were incubated at room temperature.

Analysis of the antagonistic potential of the isolated microorganisms

The isolates obtained were screened in dual-culture *in vitro* assays (Berg et al., 2002) for their antagonistic potential towards *Rhizoctonia solani*, *Verticillium dahliae* and *Fusarium culmorum*. The bacterial isolates were tested on WA (Waksman agar) and the fungal isolates on PDA (potato dextrose agar). Each isolate was tested twice independently.

Genotypic characterization by ARDRA and identification of antagonistic bacteria

From selected isolates bacterial DNA was prepared following the protocol of Berg et al. (2002). ARDRA (amplified rDNA restriction analysis) of the 16S rRNA gene with the restriction endonuclease *Hha*I was used to group isolates at the genus level. The evaluation of the 2 % agarose gel was performed with the software GelCompar II (Applied Maths). Representative isolates of each ARDRA cluster were identified by partial 16S rRNA gene sequence analysis.

Results and discussion

Microbial abundance in rhizosphere, endorhiza and soil

The rhizosphere of all three medical plants was highly colonized by bacteria: the colony-forming units (CFU) per gram fresh weight ranged from $2.4*10^8$ to $3.7*10^8$ g^{-1} fw (Fig. 1).

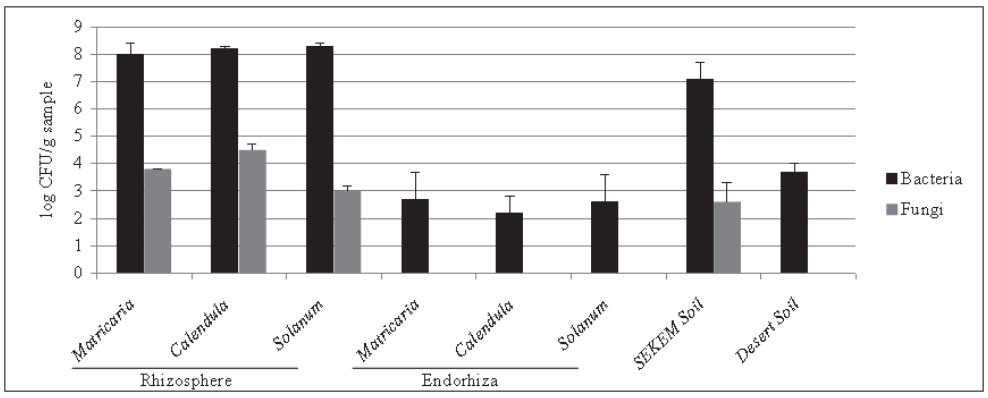

Figure 1. Abundances of bacteria and fungi in the different microhabitats. The bacterial data were ascertained on R2A and the fungal on TSM. Averages of the viable counts per gram of sample as $\log_{10}$ and standard deviations are shown.

In contrast, in the endorhiza statistically significantly lower CFUs were detected. The bacterial abundance was higher in soil from the SEKEM farm than in desert soil. The isolation of fungi was carried out on TSM. There were indeed isolated numerous fungi, which were not *Trichoderma*, but through the antibiotics and fungicides in the medium a strong selection occurred. From the desert soil and the endorhiza of the plants no fungi could be isolated on TSM.

Antagonistic potential towards soil-borne phytopathogens

Altogether 1007 isolates (680 bacterial and 327 fungal) were tested regarding their antagonistic activity. Of the bacterial isolates, 10.6 % showed an antagonistic activity against *V. dahlia*, 9.9 % against *R. solani,* and 14.0 % had an antagonistic potential against *F. culmorum*. The highest proportion of bacterial antagonists was found in the endorhiza from *Matricaria chamomilla*, followed by the soil, which was sampled on the Adleya farm in SEKEM. Among the fungal isolates only a few of them showed antagonistic abilities: 3.1 % against *V. dahliae*, 1.5 % against *R. solani* and only 0.6 % against *F. culmorum*.

Genotypic characterization and identification of antagonistic bacteria

Figure 2. Dendrogram of the strongest bacterial antagonists derived from the ARDRA of the 16S rDNA with *Hha*I. This dendrogram was created with GelCompar II, following settings were used: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); similarity coefficient: band based: dice; position tolerances: optimization: 4 %, position tolerance: 1 %.

A selection of the bacterial antagonists (37 isolates) with high antagonistic activity was characterized genotypically in detail. By ARDRA of the 16S rDNA using the restriction enzyme *Hha*I, the isolates could be clustered into four groups, within them the similarity of the band patterns is to 100 % identical (Fig. 2).

By sequence alignment of the partially sequenced 16S rDNA with the NCBI database, three isolates of the large ARDRA-cluster were identified as *Bacillus* sp. The bacteria in the cluster with only desert soil isolates are *Streptomyces* sp. and the other clusters could be identified as *Paenibacillus* sp. and *Lysobacter* sp. All of the identified antagonists were already described as biocontrol agents. For example, *Bacillus* species are well-known as BCAs, for example with *Bacillus subtilis* several biological pesticides are already in use (Schisler et al., 2004). Also for *Paenibacillus polymyxa* an antagonistic potential against a wide range of mycotoxin-producing fungi such as *Fusarium culmorum* has already been described (Tupinamba et al., 2008). And among the genus *Lysobacter* for example for *Lysobacter capsici* has already been detected a wide antimicrobial activity (Park et al., 2008). However, the biocontrol effect and mode of action is strain-specific (Berg, 2009), therefore the promising isolates obtained from the Egyptian samples will be tested *ad planta*.

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Plant-specific selection of drought-resistant biological control agents against soil-borne pathogens

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Abstract

Desert agriculture is a strongly growing field of land use. However, emerging problems with soil-borne pathogens limit the yield. Biological control agents (BCAs), which are able to suppress soil-borne pathogens, are promising candidates for plant protection but for desert application specific, drought-resistant strains are required. Here we report a stepwise selection procedure of BCAs from one of the most prominent organic desert farms SEKEM in Egypt. In a first step, we characterized the bacterial and fungal communities of the target habitat – the rhizospheres and endorhiza of medical plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. & Thonn.). The bacterial communities were highly different – for the plant species as well as for both investigated microenvironments. The fungal community was less discriminative but characterized by phytopathogens. In a cultivation-dependent approach, isolates from all parts were obtained and characterized by their anti-phytopathogenic potential against fungi (*Fusarium culmorum*, *Rhizoctonia solani*, *Verticillium dahliae*), bacteria (*Ralstonia solanacearum*) and nematodes. In parallel, genotypic diversity was analysed by ARDRA and BOX-PCR. Both procedures resulted in the selection of 46 unique, broad-spectrum antagonists. However, their diversity was low: 89% of the selected strains belonged to the *Bacillus*/*Paenibacillus* cluster. *Bacillus subtilis* (subsp. *subtilis* and *spizizenii*) was the main species identified in cultures and also a dominant band in soil, rhizosphere and endorhiza in microbial fingerprints. Furthermore, using a metagenomic approach, it was shown that Firmicutes and especially *Bacillus* was enriched in SEKEM soil in comparison with the surrounding desert. In contrast, from the original desert soil, diverse antagonistic *Streptomyces* strains were selected.

Key words: desert farming; medical plants; antagonists

Introduction

On the Sekem farms in Egypt, desert land was converted into arable land, and organic (biodynamic) agriculture is operated for over 30 years now (www.sekem.com). Today, Sekem is carrying out organic agriculture on more than 6,000 hectares and has the largest market for organic products outside Europe and North America. They produce organic foods, spices, tea, cotton textiles and natural remedies.

Crop production, especially cultivation of medical plants is more and more affected by soil-borne phytopathogens, which lead to significant yield losses. The major problems are caused by the soil-borne pathogenic fungi *Verticillium dahliae* Kleb., *Rhizoctonia solani* Kühn and *Fusarium culmorum* (Wm.G. Sm.) Sacc. as well as by the soil-borne pathogenic bacterium *Ralstonia solanacearum*. Although grown in organic agriculture, which aims to minimise the impact on the environment by practices such as crop rotation, using pathogen resistant cultivars, and the use of organic manure (compost) instead of synthetic fertilisers (Schmid *et al.*, 2011), they have an increasing importance. One reason is an intensive growing of a limited number of crops in short rotations. Here, BCAs should solve these problems and help to suppress soil-borne pathogens on a natural and sustainable way. The objective of the study was to develop a specific biocontrol product, which will be optimised for desert farming-regarding soil, weather, pathogen species, etc. Thus, autochthonous bacteria isolated from rhizosphere and endorhiza of medical plants as well as from bulk soil collected in Egypt were evaluated for their potential for biocontrol (Köberl *et al.*, 2011).

Beside soil-borne pathogens, contamination and colonisation of plants with *Escherichia coli* pathovars caused infections in humans (van Elsas *et al*., 2011). For example, in 2011 Germany reported one of the largest outbreaks of haemolytic uremic syndrome (HUS) and bloody diarrhoea caused by Shiga toxin-producing *Escherichia coli* (STEC), also commonly referred to as verocytotoxin-producing *E. coli* (VTEC) and enterohaemorrhagic *E. coli* (EHEC) (Frank *et al.*, 2011; Struelens *et al.*, 2011). Between 2nd May and 15th June, 3,351 STEC cases, including 821 cases of HUS, were reported from 13 European Union (EU)/European Economic Area (EEA) Member States and 37 patients have died. Over 97% of STEC cases have been reported from Germany (ECDC, 2011). The search for the source and vehicle of the outbreak has been a long and arduous process. Initial epidemiological findings pointed to raw vegetables and salads as likely vehicles of infection, but extensive investigations identified sprouts produced at an organic farm in Lower Saxony. Also for the target pathogen *E. coli*, biocontrol is a possible and environmentally friendly alternative. To avoid the colonisation of crops and medical plants with *E. coli,* the introduction of naturally occurring microorganisms with antibacterial activity towards

those intestinal bacteria is a promising strategy (van Elsas *et al.*, 2011). For this reason, *E. coli* was additionally included in the selection procedure of drought-resistant BCAs.

Materials and Methods

Experimental design and sampling

Samples from agriculturally used soil were taken at the Sekem farm Adleya, located in the north-eastern desert region of Egypt near Bilbeis (30°22'88"N; 31°39'41"E). Plant growth completely depended on irrigation water coming from the Nile or from local ground water drillings; sprinkler and drip irrigation systems were used. The soil at Sekem was fertilised with compost that was produced on their own composting facility, where rice straw, water hyacinth, wood chips, organic waste, clay, chicken and cow manure was mixed to receive a final C/N ratio between 20 and 30. The compost was applied twice a year (May and September), during the preparation of the fields for the cropping seasons. The soil texture at the Sekem farm was classified by Luske & van der Kamp (2009) as loamy sand (pH 8.5) with an organic carbon content of 0.5% and a clay content of 3%. For microbial analysis, desert soil was collected in the Sinai desert (1st sampling; 30°35'01"N; 32°25'49"E) and in the desert of Saqqara near Cairo (2nd sampling, 35°59'0"N; 41°2'0"E). The soil was characterised by a low moisture level; plants were very scarce (Luske & van der Kamp, 2009). At each site, four composite samples of soil in a horizon of 0～30 cm depth were collected. Regarding agricultural soil, roots with adhering soil were obtained from three different species of medical plants (German chamomile [*Matricaria chamomilla* L.], pot marigold [*Calendula officinalis* L.] and *Solanum distichum* Schumach. & Thonn.) planted on the Adleya farm (30°22'88"N; 31°39'41"E). From each plant four independent composite samples consisting of 5～10 plants were taken. At the first sampling time (October 2009), *Matricaria chamomilla* and *Calendula officinalis* have been in the seedling stage, whereas the samples from the perennial *Solanum distichum* were taken from lignified plants. At the second sampling time (April 2010), all medical plants were in the flowering stage.

Isolation and selection of bacteria

To isolate bacteria from soil and rhizosphere 5 g of soil or roots with adhering soil were added to 45 ml of 0.85% NaCl and vortexed. For isolation from the endorhiza, 5 g of roots were surface-sterilised with 4% NaOCl for 5 min, then the roots were washed three times with sterile Aqua dest. After 10 ml sterile 0.85% NaCl was added the roots were homogenised using mortar and pestle. Cell suspensions were used for dilution and plating on R2A (Roth, Karlsruhe, Germany) in duplicates. Plates were incubated for 4 days at room temperature (RT) and colony forming units were counted to calculate the means of colonies ($\log_{10}$ CFU) based on fresh weight (fw). If

possible, for each replicate 24 bacterial isolates were selected and subcultured on nutrient agar (NA). The isolates were purified and then stored at -70 ℃ in nutrient broth (NB) (Sifin, Berlin, Germany) containing 15% glycerol. Isolates were encoded using a combination of letters and numbers indicating: (1) soil type or plant species (Wb = desert soil; Sb = Sekem soil, Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*), (2) replicate (1-4), (3) microenvironment (Re = endorhiza, rhizosphere and soil have no further designation), and (4) consecutive number of the isolate per replicate.

Screening for in vitro activity towards pathogens

Altogether, 1,212 bacterial isolates obtained were screened in dual-culture *in vitro* assays on Waksman agar (WA) (Berg *et al.*, 2002) for their antagonistic potential towards the phytopathogenic fungi *Verticillium dahliae* Kleb. V25, *Rhizoctonia solani* Kühn AG4, and *Fusarium culmorum* (Wm.G. Sm.) Sacc. E1. For *R. solani* and *F. culmorum* agar disks of 5 mm diameter with mycelia were directly cut out from PDA plates (Roth, Karlsruhe, Germany) and placed between the streaks of four bacterial isolates. *V. dahliae* was grown in liquid culture in Czapek Dox broth (Duchefa, Haarlem, Netherlands) at 20℃. 200 µl of the suspension containing hyphal fragments were plated onto WA and after surface drying the bacterial isolates were placed on the same plate. Inhibition zones were measured after 4～7 days of incubation at 20℃. Each isolate was tested twice independently.

A selection of 45 promising biocontrol strains with antagonistic activity towards pathogenic fungi was tested for antibacterial activity towards *Escherichia coli* OP50 and *Ralstonia solanacearum* 1609 and B3B. The activity of all isolates against both *R. solanacearum* strains was identical. *E. coli* was grown in tryptic soy broth (TSB) (Roth, Karlsruhe, Germany) at 30℃ and the bacterial suspension (OD_{600} = 0.6～0.8) was mixed with 1.5% LB agar (Roth, Karlsruhe, Germany) at a ratio 1:10. Bacterial antagonists were streaked onto those plates and after an incubation time of 3 days at RT presence or absence of inhibition zones surrounding the antagonists were assessed. For the screening towards *R. solanacearum* yeast peptone glucose (YPG) medium was used and Tetrazolium Violet (Sigma-Aldrich, Saint Louis, USA) was added to the medium prior pouring (Adesina *et al.*, 2007).

In vitro screenings for direct plant growth promoting abilities

The 45 selected bacterial *in vitro* antagonists were tested for nitrogen fixation, ACC deaminase activity and phosphate solubilisation. For detection of N_2 fixation and ACC deaminase activity bacteria were transferred to Brown & Dilworth (BD) minimal medium (Brown & Dilworth, 1975) with and without the addition of 0.07% ACC solution as unique nitrogen source. After 2 weeks at RT their extent of growth was assessed as described by Fürnkranz *et al.* (2009). As positive control for the

bacterial growth BD medium with 0.07% NH_4Cl solution was used. P solubilisation was tested on National Botanical Research Institute's phosphate growth agar (NBRIP) (Fürnkranz *et al.*, 2009). Formation of clear halos was recorded 5 days after incubation at 20℃.

Results and Discussion

Cultivation and selection of strains

The rhizosphere of all three investigated medical plants was highly colonised by bacteria: $\log_{10}$ CFU ranged from 7.8 ± 0.3 to 8.0 ± 0.2 g^{-1} fw. In contrast, in the endorhiza significantly lower CFUs were detected: $\log_{10}$ 2.0 ± 0.2 to 3.7 ± 0.8 g^{-1} fw. The highest colonised rhizosphere as well as endorhiza was detected for *S. distichum*, followed by *M. chamomilla,* and the lowest population density in both microenvironments was found for *C. officinalis*. The determined bacterial abundance was significantly higher in agricultural soil ($\log_{10}$ 7.7 ± 0.4 g^{-1}) in comparison to desert soil ($\log_{10}$ 4.6 ± 0.6 g^{-1}). From each of the four replicates per plant and microenvironment, 24 bacterial colonies were randomly selected for further characterisation.

Characterisation of the indigenous antifungal potential in vitro

All 1,212 selected bacterial strains were screened by dual testing regarding their antagonistic activity towards *Verticillium dahliae*, *Rhizoctonia solani* and *Fusarium culmorum*, which are known as main soil-borne fungal pathogens. In general, isolates obtained from *M. chamomilla* and *S. distichum* showed a higher *in vitro* antagonistic potential towards soil-borne phytopathogenic fungi than those from *C. officinalis* (Table1). Especially isolates from the endorhiza from *M. chamomilla* harboured a high proportion of antagonists. Whereas in the soil and in the rhizosphere bacterial antagonism was mainly directed towards *F. culmorum*, in the endorhiza of the medical plants antagonism was mainly found towards *V. dahliae*. Bacterial isolates obtained from the soil of the farm exhibited a higher antagonistic potential in comparison to the bacteria isolated from the desert soil (agricultural soil 21.6% ± 0.8%; desert soil 12.4 %± 0.7%).

Detection of antibacterial activity

According to unique genotypic patterns ascertained by employing amplified ribosomal RNA gene restriction analysis (ARDRA) with the restriction endonucleases *Hha*I and *Pst*I and BOX-PCR genomic fingerprinting (data not shown), 45 genotypically different strains with antagonism towards fungal pathogens were selected to test them regarding their antibacterial activity towards *Escherichia coli* and *Ralstonia solanacearum*. Of these isolates 15.6% were able to inhibit *in vitro* the growth of *E. coli*. These were all isolates of the genus *Paenibacillus* (identified as *P.*

brasilensis, *P. polymyxa* and *P. kribbensis*). The growth of *R. solanacearum* was inhibited by 33.3% of the selected antagonists: most isolates of *Streptomyces* (3 of 4 isolates) and some strains of the *Bacillus subtilis* group (12 of 30 isolates). Interestingly, the antibacterial activity was very specific, i.e. there was no isolate with suppression of both bacterial species (Fig.1).

Table 1 Proportions of bacterial isolates antagonistic towards the soil-borne fungal pathogens *Verticillium dahliae*, *Rhizoctonia solani* and *Fusarium culmorum*

		Relative abundance of antagonists (%)[a]		
Origin	Microenvironment	*V. dahliae*	*R. solani*	*F. culmorum*
Matricaria chamomilla	Rhizosphere	12.5± 2.9	8.3 ± 0.7	13.0 ± 1.8
	Endorhiza	19.9± 1.8	16.4± 2.3	18.8 ± 2.6
Calendula officinalis	Rhizosphere	9.0 ± 0.5	7.1 ± 0.1	10.1 ± 3.1
	Endorhiza	4.2 ± 2.9	0.0 ± 0.0	1.4 ± 1.0
Solanum distichum	Rhizosphere	13.7± 2.3	13.8± 3.8	15.7 ± 0.0
	Endorhiza	13.5± 5.1	10.4± 5.8	12.5 ± 5.8
Soil	Agricultural Soil	20.0± 1.6	21.9± 2.2	22.6 ± 1.4
	Desert Soil	11.1± 1.8	12.8± 0.6	13.4 ± 0.1

[a]Data are averages of 1st and 2nd sampling ± confidences.

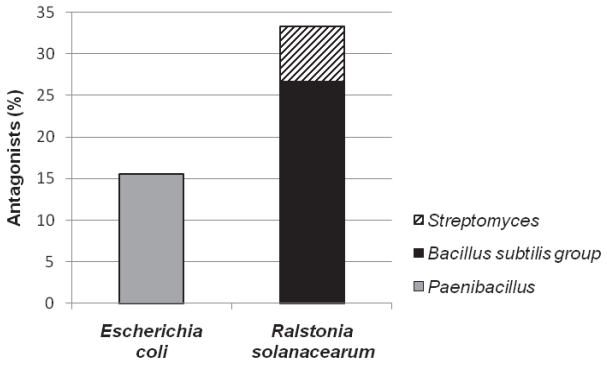

Figure1 Relative abundance and diversity of antagonists with additional antibacterial activity towards *E. coli* and *R. solanacearum*. Out of all strains, 45 genotypically different isolates with antifungal properties were tested and identified by partial 16S rRNA gene sequencing.

Determination of plant growth promoting abilities

The selected 45 genotypically different antagonists were screened for direct PGP properties. Among all tested strains, 24.4% were declared as diazotrophs, all *Paenibacillus* spp. and *Streptomyces* spp. were able to grow on N-free medium. ACC degradation could be shown only for 4.4% strains (two isolates of *Bacillus subtilis*

subsp. *spizizenii*). No isolate of the selected antagonists was able to solubilise phosphate.

Assessment of antagonistic strains

From 1,212 isolates, 45 selected antifungal strains were characterised more in detail. For half of the strains additional antibacterial activity was detected. Whereas the majority of strains showed antifungal activity towards all three target pathogens, antibacterial activity was exclusively found against one of the pathogens. According to the results, specific antagonists against pathogens but also broad spectrum antagonists were found. Two outstanding specific antagonists were identified towards the main soil-borne fungal pathogen *Verticillium dahliae*: *Paenibacillus kribbensis* Sb3-1 and *Brevibacillus limnophilus* Mc6-4. Activity against *E. coli* was exclusively found by *Paenibacillus* isolates from various origins, whereas *Streptomyces* isolates from desert soil but also *Bacillus* strains were active against the phytopathogen *R. solanacearum.* Beside some broad spectrum antagonists, which could be isolated from plant-associated environments from different plant species, also plant-specific BCAs were found. Surprisingly, isolates selected from desert soil belong to the most active isolates. One isolate of this group – *Streptomyces subrutilus* Wb2n-11 – was the most promising biocontrol candidate by rating all antagonistic properties. Further interesting candidates were *Bacillus subtilis* subsp. *subtilis* Co1-6 and *Streptomyces peucetius* Wb2n-2.

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Indigenous PGPR and below-ground microbial communities of an organically managed desert agro-ecosystem

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Indigenous PGPR and below-ground microbial communities of an organically managed desert agro-ecosystem

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Abstract

Microbial inoculants such as plant growth promoting rhizobacteria (PGPR) with biocontrol activity provide an environmentally friendly and promising strategy for plant and stress protection. For application in desert ecosystems, specific, drought-resistant strains are required. Therefore, we selected promising candidates from one of the most popular organic desert farms Sekem in Egypt by a hierarchical screening procedure. Isolates were characterised by their anti-phytopathogenic potential towards fungi (*Verticillium dahliae*, *Rhizoctonia solani*, *Fusarium culmorum*), bacteria (*Ralstonia solanacearum*) and nematodes (*Meloidogyne incognita*). In general, the indigenous antagonistic potential was highly dominated by Gram-positive, spore-forming bacteria. Three most promising strains (*Streptomyces subrutilus* Wb2n-11, *Bacillus subtilis* Co1-6, *Paenibacillus polymyxa* Mc5Re-14) were selected for *ad planta* field applications on German chamomile (*Matricaria chamomilla* L.) in comparison to three Gram-negative strains (*Pseudomonas fluorescens* L13-6-12, *Stenotrophomonas rhizophila* P69, *Serratia plymuthia* 3Re4-18), which were already evaluated under humid conditions. The influence of bacterial inoculants on plant growth promoting effects was evaluated based on blossom harvest yield. Secondary metabolites of chamomile blossoms as well as structures of rhizosphere microbial communities were compared between different treatments. In parallel to the selection and evaluation of strains, bacterial and fungal communities of the target habitat – the rhizosphere and endorhiza of medicinal plants (*Matricaria chamomilla* L., *Calendula officinalis* L., *Solanum distichum* Schumach. & Thonn.) – were characterised in comparison to the surrounding field and desert soil. To get an insight into the community of the nitrogen-fixing PGPR, the *nifH* gene communities were deeply assessed by a pyrosequencing-based approach. Specificity could be observed for all investigated communities of each medicinal plant.

Keywords: desert farming; medicinal plants; soil-borne pathogens

Introduction

The organically managed Sekem farms extend over an area of 6,000 hectares in the North-eastern desert region of Egypt and have the largest market for organic products outside Europe and North America. The Egyptian fair trade company produces organic food, herbal teas, phyto-pharmaceuticals as well as non-edible products such as cotton textiles. However, within recent years, soil-borne phytopathogens caused significant yield losses. These pathogens comprise different taxonomic groups, e.g. fungi (*Verticillium dahliae* Kleb., *Rhizoctonia solani* Kühn, *Fusarium culmorum* (Wm. G. Sm.) Sacc.), bacteria (*Ralstonia solanacearum*) and nematodes (*Meloidogyne incognita* (Kofoid and White) Chitwood). Therefore, the objective of this project in general is to develop a biological control strategy against these soil-borne pathogens suitable for the arid conditions of desert farming (Köberl et al., 2013b).

A polyphasic ecological study revealed a high abundance of Gram-positive, spore-forming bacteria under arid conditions in general and an overwhelming dominance among the indigenous antagonistic potential (Köberl et al., 2011). Based on genomic fingerprints and antagonistic potential, 45 unique strains were selected of which 89% belonged to the *Bacillus/Paenibacillus* cluster. *Bacillus subtilis* was the main cultivated species from farm samples. In contrast, efficient antagonists from the surrounding desert soil belong mainly to *Streptomyces*. Details about their antagonistic activities against soil-borne phytopathogens were described in Köberl et al. (2013a). From this hierarchical screening, three promising antagonists were selected for evaluation in the field: *Streptomyces subrutilus* Wb2n-11 isolated from desert soil from Sinai, *Bacillus subtilis* subsp. *subtilis* Co1-6 obtained from the

rhizosphere of *Calendula officinalis*, and *Paenibacillus polymyxa* Mc5Re-14 isolated from the endorhiza of *Matricaria chamomilla*. These Gram-positive strains were tested for their plant growth promoting effect *ad planta* in comparison to three Gram-negative strains, which are already known for their beneficial plant-microbe interactions in humid soils (Lottmann and Berg, 2001; Wolf et al., 2002; Kai et al., 2007; Zachow et al., 2010): *Pseudomonas fluorescens* L13-6-12 isolated from the rhizosphere of potato (*Solanum tuberosum*), *Stenotrophomonas rhizophila* P69 from the oilseed rape (*Brassica napus*) rhizosphere, and *Serratia plymuthia* 3Re4-18 from the endorhiza of potato. First results about the impact of applied BCAs on the indigenous bacterial communities associated with roots of chamomile plants grown under organic management in Egypt were shown in Köberl et al. (2013b).

Within the scope of this paper, we present direct and indirect *in vitro* plant growth promoting abilities of all 45 genotypically different broad-spectrum antagonists isolated from arid areas and their tolerance to abiotic stresses of desert habitats.

Materials and Methods

Belowground communities were studied at the organic desert farm Sekem (www.sekem.com) in Egypt (30°22'88"N, 31°39'41"E) in comparison to surrounding desert soil (30°35'01"N, 32°25'49"E; 35°59'0"N, 41°2'0"E). Physico-chemical data of the soil is provided in Luske and van der Kamp (2009). Sampling strategy and isolation of bacteria are described in detail in Köberl et al. (2011). Based on *in vitro* antifungal potential and genomic diversity, 45 strains were selected (Köberl et al., 2011) and screened for their plant growth promoting abilities and tolerance to abiotic stress. Their affiliation to bacterial genera and isolation sources are summarised in Table 1.

Table 1. Genotypically unique antifungal isolates and their isolation sources.

Phylum	Genus	Rhizosphere			Endorhiza			Soil		
		Mc	Co	Sd	Mc	Co	Sd	Sekem	Desert	
Firmicutes	*Bacillus*	4	3	4	8		4	7	2	32
	Paenibacillus	2			2		1	1	1	7
	Brevibacillus	1								1
Actinobacteria	*Streptomyces*								4	4
Proteobacteria	*Lysobacter*	1								1
		8	3	4	10	0	5	8	7	45

Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*.

Protease activity (casein degradation) was determined from clearing zones on skim milk agar (Berg et al., 2002). β-1,3-glucanase activity was tested by chromogenic azurine-dyed, cross-linked (AZCL) substrates (Megazyme, Bray, Ireland). Formation of blue halos was recorded until 5 days after incubation at 20 °C. Chitinase activity (β-1,4-glucosamine polymer degradation) was tested on chitin minimal medium (Berg et al., 2002). Clearing zones were detected 7 days after incubation at 20 °C. The production of siderophores under Fe^{3+}-limited conditions was analysed using the plate assay developed by Schwyn and Neilands (1987). The size of orange haloes formed around the streaks was measured after 3 days of incubation at 20 °C. Phosphate solubilisation was tested on National Botanical Research Institute's phosphate growth agar (NBRIP) (Fürnkranz et al., 2009). Formation of clear halos was recorded 5 days after incubation at 20 °C. Tolerance to abiotic stress was tested according to Marasco et al. (2012). Salt resistance was assessed by their growth on nutrient agar (NA) containing additionally 5%, 8% and 10% of NaCl. Tolerance to osmotic stress was evaluated by adding 10%, 15% and 20% of Poly-Ethylen-Glycol (PEG) to nutrient broth (Sifin, Berlin, Germany).

Results and Discussion

The isolate collection was screened for direct and indirect plant growth promoting abilities and resistance to abiotic stresses occurring in arid soils (Table 2). Because of the promising antifungal properties (Köberl et al., 2011, 2013a), a special focus was given to the fungal cell wall degrading enzymes. Production of chitinases could be detected for 18% of the total antagonist collection, especially *Lysobacter enzymogenes* Mc1-3 showed a high chitinolytic activity and also all *Streptomyces* isolates. Glucanase activity was shown for almost all antagonists (93%), only isolates identified as *Bacillus endophyticus* (Wb1-13 and Mc4-18) and *Brevibacillus limnophilus* Mc6-4 were not able to degrade β-1,3-glucan. Casein degradation by proteases could be shown for 69% of the strains (all isolates of the *Bacillus subtilis* group and *Lysobacter enzymogenes*). The production of siderophores was shown for almost all antagonists (89%) except three isolates of *Paenibacillus* spp. (Wb2-3, Mc5-5 and Mc2Re-16), *Bacillus endophyticus* Wb1-13 and *Streptomyces subrutilus* Wb2n-11. No isolate of the selected antagonists was able to solubilise inorganic phosphate. Concerning the tolerance to abiotic stress, 71% of the antagonist collection exhibited a high resistance to salt stress and 68% showed a remarkable tolerance to low water availability. Isolates of *Bacillus* revealed the predominant role in halotolerance and together with *Brevibacillus* in resistance to drought stress.

Table 2. Distribution of plant growth promoting potential according to bacterial genera.

Genus	**Isolates**	**Direct and indirect PGP activities and tolerance to abiotic stress (%)**										
		Prot	Gluc	Chit	Sid	P sol	5% NaCl	8% NaCl	10% NaCl	10% PEG	15% PEG	20% PEG
Bacillus	32	94	94	9	97	0	100	100	100	100	97	78
Paenibacillus	7	0	100	0	57	0	0	0	0	86	57	29
Brevibacillus	1	0	0	0	100	0	0	0	0	100	100	100
Streptomyces	4	0	100	100	75	0	50	0	0	nd	nd	nd
Lysobacter	1	100	100	100	100	0	0	0	0	0	0	0
total	45	69	93	18	89	0	76	71	71	95	88	68

Prot = protease activity; Gluc = glucanase activity; Chit = chitinase activity; Sid = siderophore production; P sol = phosphate solubilisation; nd = not determined.

While none of the antifungal strains showed all the assayed PGP activities, because no isolate was able to solubilise phosphate, 9% of the antagonists presented four of them. By rating all PGP properties, *Bacillus subtilis* Sb3-24 and *Bacillus atrophaeus* Sb3-13, both isolated from bulk agricultural soil, and *Bacillus subtilis* Mc3Re-13, isolated from the endorhiza of the chamomile, were identified as the most promising PGP antagonists. *Lysobacter enzymogenes* Mc1-3, isolated from the rhizosphere of the chamomile, was also positive tested for four PGP abilities, but in contrary to the *Bacillus* strains, revealed *Lysobacter* a low tolerance to abiotic stresses of desert ecosystems.

The three strains selected for field application based on their outstanding antagonistic potential *in vitro* also revealed promising PGP activities and stress tolerances. *Bacillus subtilis* Co1-6 exhibited, besides high drought and salt resistance, also production of siderophores as well as protease and glucanase activity. *Paenibacillus polymyxa* Mc5Re-14 had a lower tolerance to abiotic stresses in comparison to the *Bacillus* strain, but was also tested positive for siderophores and glucanase activity. *Streptomyces subrutilus* Wb2n-11 showed hydrolytic degradation of chitin and glucan.

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Host-parasite interaction and microbiome response: effects of fungal infections on the bacterial community of the Alpine lichen *Solorina crocea*

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RESEARCH ARTICLE

Host–parasite interaction and microbiome response: effects of fungal infections on the bacterial community of the Alpine lichen *Solorina crocea*

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lichen symbiosis; host–parasite interaction; lichenicolous; microbiomes; pyrosequencing.

Abstract

The lichen symbiosis allows a self-sustained life under harsh environmental conditions, yet symbiotic integrity can be affected by fungal parasites. Nothing is known about the impact of these biologically diverse and often specific infections on the recently detected bacterial community in lichens. To address this question, we studied the arctic–alpine 'chocolate chip lichen' *Solorina crocea*, which is frequently infected by *Rhagadostoma lichenicola*. We sampled healthy and infected lichens at two different sites in the Eastern Alps. High abundances of *Acidobacteria*, *Planctomycetes*, and *Proteobacteria* were identified analyzing 16S rRNA gene regions obtained by barcoded pyrosequencing. At the phylum and genus level, no significant alterations were present among infected and healthy individuals. Yet, evidence for a differentiation of communities emerged, when data were analyzed at the strain level by detrended correspondence analysis. Further, a profile clustering network revealed strain-specific abundance shifts among *Acidobacteria* and other bacteria. Study of stability and change in host-associated bacterial communities requires a fine-grained analysis at strain level. No correlation with the infection was found by analysis of *nifH* genes responsible for nitrogen fixation.

Introduction

Lichen-forming fungi develop long-living thallus structures, which contain the symbiotic photoautotrophic algal partners in a mycelial shelter. The light-exposed joint organisms can be extremely robust to changing conditions in harsh natural environments. In these habitats, however, infections with specialized fungal parasites commonly occur. These lichen-inhabiting fungi have been recognized even before the nature of lichens was discovered to represent a fungal–algal symbiosis. Lichenicolous (= lichen-inhabiting) fungi are both taxonomically and biologically heterogeneous. Today more than 1800 species of lichenicolous fungi have been classified, but numerous lichenicolous fungi are still undescribed or recognized at species level (Lawrey & Diederich, 2003). The lifestyles of lichenicolous fungi range from parasitism to commensalism, and for some pathogens, preferences were shown for either the algal or fungal organism of the host symbiosis (Grube & Hafellner, 1990; De los Rios & Grube, 2000; Grube & de los Rios, 2001). Only few species progressively destroy the fungal structures of the lichens, whereas mild or localized infections are far more common. Interestingly, most of the species expressing mild infections and commensalic lifestyles are also highly specialized for their hosts. They form their reproductive structures only on their genuine host lichens.

Recent studies revealed the abundance of lichen-associated bacterial communities, which are now characterized in detail in their taxonomic and morphological structure (Cardinale *et al.*, 2006, 2008). All studies so far found evidence for host-specific bacterial communities (Grube *et al.*, 2009; Bates *et al.*, 2011; Hodkinson *et al.*, 2012). Yet, recent analyses also reveal effects of habitat parameters (Cardinale *et al.*, 2012), photobiont type, geography (Hodkinson *et al.*, 2012), and age of the thallus (Mushegian *et al.*, 2011; Cardinale *et al.*, 2012) on the composition of the bacterial communities. Fluorescent *in*

situ hybridization combined with confocal laser scanning microscopy suggests a general predominance of Alphaproteobacteria in growing parts of lichens (Cardinale *et al.*, 2012), whereas other bacterial phyla may become more prevalent in older parts. The aging of a lichen thallus is apparent in microscopic sections by decreased vitality of algae and morphological changes, such as in fine structure. Similar effects are sometimes also observed in parts of lichen thalli that are parasitized by lichenicolous fungi. We hypothesized a response of the bacterial community as a consequence to the fungal infection.

The effect of a lichenicolous fungal infection on the lichen microbiome was studied with the common and widespread soil-inhabiting lichen *Solorina crocea* (L.) Ach., also known as chocolate chip lichen (Fig. 1). It is a characteristic species of late snowbeds and found on acidic soils in arctic–alpine habitat above the tree line. The thalli are unique and easy to recognize by the crystallized bright red pigment (solorinic acid) produced on the lower surface. The vernacular name chocolate chip lichen refers to the large brownish fruitbodies (ascomata), which recall the color and appearance of chocolate. *Solorina crocea* is frequently infested by the lichenicolous fungus *Rhagadostoma lichenicola* (de Not.) Keissl. (*Sordariomycetes, Ascomycota*). As a specialized biotroph of *S. crocea*, this parasite develops crowded blackish ascomata on the upper surface of host thallus (see Fig. 1) and a richly branched, dark mycelium below the fruitbodies. The mycelium extends unspecifically and locally in the functional layers of the nearby lichen thallus. No specific infection structures are formed with algal or fungal host cells (M. Grube, unpublished observations). The infections persist on the thalli, yet fertility of the lichen is apparently not affected by the lichenicolous fungus. We used a barcoded pyrosequencing approach with different primers combined with a statistical design to analyze the bacterial community associated with healthy and infected lichens from two different sites in the Eastern Alps.

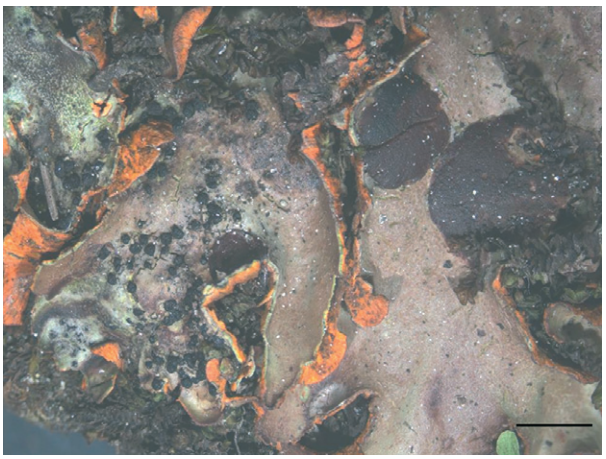

Fig. 1. The chocolate chip lichen *Solorina crocea* and its parasite *Rhagadostoma lichenicola* (black dots in right half of the image represent fruitbodies of the parasite). Zirbitzkogel (Image: W. Obermayer). Bar = 0.5 mm.

Materials and methods

Experimental design and sampling procedure

Lichen samples of *S. crocea* were collected in June 2011 from two different sampling points in the Austrian district Styria: Wölzer Tauern (N47°16.212, E14°22.860, 1892 m) and Zirbitzkogel, Ochsenboden (N47°4.65, E14° 33.783, 2050 m). At each site, three independent composite samples of five thalli were collected from healthy lichens and three from lichens, which were infested with the pathogenic fungus *R. lichenicola*. The typical fruitbodies of the fungal pathogen were detected by visual inspection using a 10× hand lens in the field and then confirmed using a stereomicroscope (Leica, Wetzlar, Germany). Because infections with this fungus occur locally on lobes of the host thalli, we sampled only thalli where multiple lobes were affected by the pathogen.

Total community DNA isolation

Total community DNA isolation was carried out with 2 g of each lichen sample. Samples were washed for 3 min with sterile 0.85% NaCl. The lichens were transferred into whirl packs and homogenized with 2 mL of 0.85% NaCl using mortar and pestle. From the liquid parts, 2 mL was centrifuged at high speed (16 000 ***g***, 4 °C) for 20 min, and resulting microbial pellets were stored at −70 °C. Total community DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon) according to the manufacturer's protocol. Resulting DNA concentrations ranged from 130.4 to 279.0 ng μL^{-1}. Metagenomic DNA samples were encoded using abbreviations indicating: (1) sampling location (W = Wölzer Tauern; Z = Zirbitzkogel), (2) independent replicate (W: A–F; Z: 1–3), and (3) status of infestation with the lichen pathogenic fungus *R. lichenicola* (healthy or infested).

Bacterial community analysis by 454 pyrosequencing of 16S rRNA genes

For the deep sequencing analysis of the lichen-associated bacterial community, the hypervariable V4-V5 region of the 16S rRNA gene (*Escherichia coli* positions 515–902) was amplified for pyrosequencing (Binladen *et al.*, 2007) using the primers Unibac-II-515f (Zachow *et al.*, 2008)

and 902R (Hodkinson & Lutzoni, 2009), containing the 454 pyrosequencing adaptors, linkers, and sample-specific tags (Table 1). The reverse primer 902R was specifically designed by Hodkinson & Lutzoni (2009) to target bacteria, but exclude sequences derived from lichen photobionts (chloroplasts of algae and *Cyanobacteria*). The PCR was performed using a total volume of 30 μL containing 1× Taq&Go (MP Biomedicals, Eschwege, Germany), 1.5 mM of $MgCl_2$, 0.2 μM of each primer, and 1 μL of template DNA (95 °C, 2 min; 35 cycles of 95 °C, 20 s; 64 °C, 15 s; 72 °C, 24 s; and elongation at 72 °C, 10 min). PCR products of two independent PCRs were pooled and purified by employing the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison). Pyrosequencing libraries were generated by GATC Biotech (Konstanz, Germany) using the Roche 454 GS-FLX+ Titanium™ sequencing platform.

A taxonomy-based analysis was carried out with the web server SNOWMAN 1.11 (http://snowman.genome.tugraz.at). Primer sequences were cropped, and sequences shorter than 200 bp in length or of low quality (quality threshold 20) were removed from the pyrosequencing-derived data sets. The following SNOWMAN settings were used: analysis type: BLAT pipeline; reference database: greengenes_24-Mar-2010; rarefaction method: MOTHUR; taxonomy: ribosomal database project (RDP); confidence threshold: 80%; include taxa covering more than: 1%. All analyses were performed with normalized data, considering the same number of sequences to all samples (1213 sequences per sample). For the normalization, STRAWBERRY PERL 5.12.3.0 (http://strawberryperl.com) and the Perl program selector of the PANGEA pipeline (Giongo *et al.*, 2010) were used. To determine rarefaction curves, operational taxonomic units (OTUs) were identified at sequence divergences of 3% (species level), 5% (genus level), and 20% (phylum level) (Schloss & Handelsman, 2006; Will *et al.*, 2010), and curves were calculated using the tools aligner, complete linkage clustering, and rarefaction of the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu) (Cole *et al.*, 2009). Shannon diversity (Shannon, 1997) and Chao1 richness (Chao & Bunge, 2002) indices were calculated based on the complete linkage clustering data.

Functional gene analysis by 454 pyrosequencing of *nifH* genes

Because fixation of atmospheric nitrogen is known as a key function in symbioses, we selected to analyze variation of nitrogenase genes as a functional marker. Nitrogenase genes are present in cyanobacteria, which commonly occur in the analyzed lichen species as a second autotrophic partner. The nitrogenase gene *nifH* was amplified according to a nested PCR protocol with primers designed by Zani *et al.* (2000). The reaction mixture of the first PCR (20 μL) was composed of 1× Taq&Go, 4 mM $MgCl_2$, 2 μM of the primers nifH4 and nifH3 (Zani *et al.*, 2000), and 1 μL of template DNA (95 °C, 5 min; 30 cycles of 95 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; and elongation at 72 °C, 10 min). Amplicons served as templates for the second PCR (30 μL) with the primer pair nifH1 and nifH2 designed by Zehr & McReynolds (1989), which contained the 454 pyrosequencing adaptors, linkers, and sample-specific tags (Table 1). Therefore, 3 μL of DNA was added to 1× Taq&Go, 1.5 mM $MgCl_2$, and 0.2 μM of each primer (95 °C, 5 min; 30 cycles of 95 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min; and elongation at 72 °C, 10 min). Obtained PCR products of the independent replicates were pooled to one healthy and one infested composite sample per location and purified using Wizard® SV Gel and PCR Clean-Up System. Pyrosequencing libraries were created by

Table 1. Custom primers including 454 pyrosequencing adaptors (bold), linkers (italic), and sample-specific tags (underlined)

Name	Primer sequence	References
Unibac-II-515f_MID13	**CGTATCGCCTCCCTCGCGCCA***TCAG*CATAGTAGTGGTGCCAGCAGCCGC	
Unibac-II-515f_MID14	**CGTATCGCCTCCCTCGCGCCA***TCAG*CGAGAGATACGTGCCAGCAGCCGC	
Unibac-II-515f_MID29	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACTGTACAGTGTGCCAGCAGCCGC	
Unibac-II-515f_MID30	**CGTATCGCCTCCCTCGCGCCA***TCAG*AGACTATACTGTGCCAGCAGCCGC	
Unibac-II-515f_MID31	**CGTATCGCCTCCCTCGCGCCA***TCAG*AGCGTCGTCTGTGCCAGCAGCCGC	
Unibac-II-515f_MID59	**CGTATCGCCTCCCTCGCGCCA***TCAG*CGTACTCAGAGTGCCAGCAGCCGC	
Unibac-II-515f_MID61	**CGTATCGCCTCCCTCGCGCCA***TCAG*CTATAGCGTAGTGCCAGCAGCCGC	
Unibac-II-515f_MID62	**CGTATCGCCTCCCTCGCGCCA***TCAG*TACGTCATCAGTGCCAGCAGCCGC	Zachow *et al.* (2008)
902r_454	**CTATGCGCCTTGCCAGCCCGC***TCAG*GTCAATTCITTTGAGTTTYARYC	Hodkinson & Lutzoni (2009)
nifH1_MID23	**CGTATCGCCTCCCTCGCGCCA***TCAG*TACTCTCGTGTGYGAYCCNAARGCNGA	
nifH1_MID24	**CGTATCGCCTCCCTCGCGCCA***TCAG*TAGAGACGAGTGYGAYCCNAARGCNGA	
nifH1_MID25	**CGTATCGCCTCCCTCGCGCCA***TCAG*TCGTCGCTCGTGYGAYCCNAARGCNGA	
nifH1_MID26	**CGTATCGCCTCCCTCGCGCCA***TCAG*ACATACGCGTTGYGAYCCNAARGCNGA	Zehr & McReynolds (1989)
nifH2_454	**CTATGCGCCTTGCCAGCCCGC***TCAG*ADNGCCATCATYTCNCC	Zehr & McReynolds (1989)

GATC Biotech using the Roche 454 GS-FLX+ Titanium system.

Primer sequences were trimmed, amplicon sequences with low quality (average base quality score 20) or a read length shorter than 200 bp were removed, and remaining sequences were translated into their amino acid sequence using the tool FrameBot of RDP's FunGene Pipeline (http://fungene.cme.msu.edu/FunGenePipeline). All subsequent analyses were carried out on amino acid sequences, which were normalized to 7005 sequences per sample using again STRAWBERRY PERL 5.12.3.0 and the Perl program selector of the PANGEA pipeline (Giongo *et al.*, 2010). Amino acid sequences were aligned and clipped at the same alignment reference position (~ 60 amino acids) using CLUSTALX 2.1 (Larkin *et al.*, 2007). Farnelid *et al.* (2011) showed that fragments encompassing the 60 amino acids of the *nifH* gene starting directly downstream from the nifH1 primer provide estimates comparable to those obtained with the full-length fragment of 108 amino acids. OTUs were classified, and rarefaction curves were constructed based on the distance matrices of amino acid sequences at 0%, 4%, and 8% dissimilarity (Farnelid *et al.*, 2011) using the tools mcClust and rarefaction of RDP's FunGene Pipeline. Diversity indices were ascertained based on the clustering data (Shannon, 1997; Chao & Bunge, 2002). Representative sequences at 92% similarity were selected for the following phylogenetic analysis (Farnelid *et al.*, 2011), where clusters with < 10 sequences were not designated. Nearest relatives were retrieved using the search tool TBLASTN of the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov) with an *E*-value cutoff of 0.001.

Detrended correspondence and profile network clustering analysis

Detrended correspondence analysis (DCA) was used to detect differences in bacterial communities in healthy and infected lichen at the strain level. For this analysis, we used CANOCO 4.5 for Windows (Lepš & Smilauer, 2003) and defined OTUs at a dissimilarity level of 3%. The profile network analysis was carried out with those OTUs that showed a cumulative read change ≥ 5 sequences between the states. Average values of OTU read numbers were calculated for healthy and infected states of the two localities in a Microsoft Excel spreadsheet. If the ratio of values for healthy and infected states exceeded 1.5, the OTUs were regarded as altered and assigned to the respective clusters (abundant in healthy or infected). We considered only those OTUs that showed the same pattern in both sampling sites. Visualization of the network was carried out in CYTOSCAPE 2.8.2 (http://www.cytoscape.org).

Results

Pyrosequencing-based 16S rRNA gene profiling of lichen-associated bacteria

Using primers excluding lichen photobionts such as Cyanobacteria and chloroplasts of algae (Hodkinson & Lutzoni, 2009), a deep sequencing study of the bacterial communities associated with healthy and *Rhagadostoma*-infected *S. crocea* has been employed. Between 3221 and 1213 quality sequences per sample with a read length of $\geq$ 200 bp were recovered, which were normalized to the lowest number of sequences to all samples. Of all quality sequences 88.2% could be classified below the domain level. The rarefaction analyses of the amplicon libraries are shown in Supporting Information. Comparisons of the rarefaction analyses with the number of OTUs estimated by the Chao1 index revealed that at phylum level, 69.6–96.0% of estimated richness was recovered (Table 2). The pyrosequencing efforts at genus and species level reached 49.3–80.9% and 46.3–61.8%, respectively. The computed Shannon indices of diversity (H') barely showed differences between samples. At a genetic distance of 3% (species level), Shannon values ranged from 4.22 to 4.86.

Taxonomic analysis (Fig. 2) revealed that all samples of *S. crocea* were dominated by the bacterial phylum *Acidobacteria* (between 42.4% and 66.4% of sequences). Further dominant phyla present in all samples were *Planctomycetes* (7.2–25.2%) and *Proteobacteria* (11.1–30.0%). In lower concentrations but also found in all samples were lichen-associated *Actinobacteria* (1.3–5.2%). Considering only phyla covering more than 1% of quality sequences, *Bacteroidetes* were only detectable in one of the infested samples from Wölzer Tauern (2.0%). Most *Acidobacteria* reads were affiliated with subdivision 1 (93% of classified *Acidobacteria*), 6% were identified as belonging to subdivision 3. *Planctomycetes* sequences were classified as genera *Isosphaera* and *Gemmata* (46% and 34% of classified *Planctomycetes*). Among the *Proteobacteria*, only 19% could be identified to the genus level. *Sphingomonas* and *Novosphingobium* (*Alpha*-) as well as *Dyella* (*Gamma*-) and *Byssovorax* (*Deltaproteobacteria*) were found. The classifiable *Actinobacteria* (only 6%) were identified as *Nakamurella*. From both sampling locations together, infested metagenomes included nine genera, whereas healthy lichens harbored only seven of them. *Dyella* and *Nakamurella* occurred only in some samples infested with the lichen pathogen.

Pyrosequencing-based *nifH* profiling of lichen-associated communities

To analyze whether the fungal infection could have an effect on a key functional gene in the *S. crocea* symbiosis,

Table 2. Richness estimates and diversity indices for 16S rRNA gene and *nifH* gene amplicon libraries of *Solorina crocea* samples

	Quality sequences	Shannon index* (*H'*)			Rarefaction† (no. of OTUs)			Chao1‡ (no. of OTUs)			Coverage (%)		
16S rRNA gene													
Genetic dissimilarity		3%	5%	20%	3%	5%	20%	3%	5%	20%	3%	5%	20%
W_A-healthy	1213	4.22	3.76	1.62	261	190	36	528	336	45	49.5	56.6	80.0
W_F-healthy	1213	4.79	4.25	1.83	311	201	31	504	248	34	61.8	80.9	92.5
W_D-infested	1213	4.86	4.42	2.25	316	234	40	598	372	42	52.8	63.0	96.0
W_E-infested	1213	4.71	4.23	1.72	322	238	38	553	399	47	58.3	59.7	80.6
Z_1-healthy	1213	4.48	4.06	1.99	312	230	40	646	391	55	48.3	58.9	72.7
Z_3-healthy	1213	4.75	4.24	1.96	315	223	42	566	375	60	55.7	59.5	69.6
Z_2-infested	1213	4.78	4.16	1.63	294	206	47	488	372	64	60.2	55.3	73.4
Z_3-infested	1213	4.68	4.06	1.58	275	189	29	593	383	34	46.3	49.3	84.7
nifH gene													
Genetic dissimilarity		0%	4%	8%	0%	4%	8%	0%	4%	8%	0%	4%	8%
W_healthy	7005	3.53	3.06	2.16	1102	400	151	3853	658	285	28.6	60.8	52.9
W_infested	7005	2.78	2.41	1.80	831	310	123	3990	584	266	20.8	53.1	46.3
Z_healthy	7005	3.88	3.37	2.39	1259	432	181	5368	763	348	23.5	56.6	52.0
Z_infested	7005	3.92	3.24	2.55	1298	435	180	4067	782	355	31.9	55.6	50.7

*A higher number indicates more diversity.

†Results from the rarefaction analyses are also depicted in the Supporting Information.

‡Nonparametric richness estimator based on the distribution of singletons and doubletons.

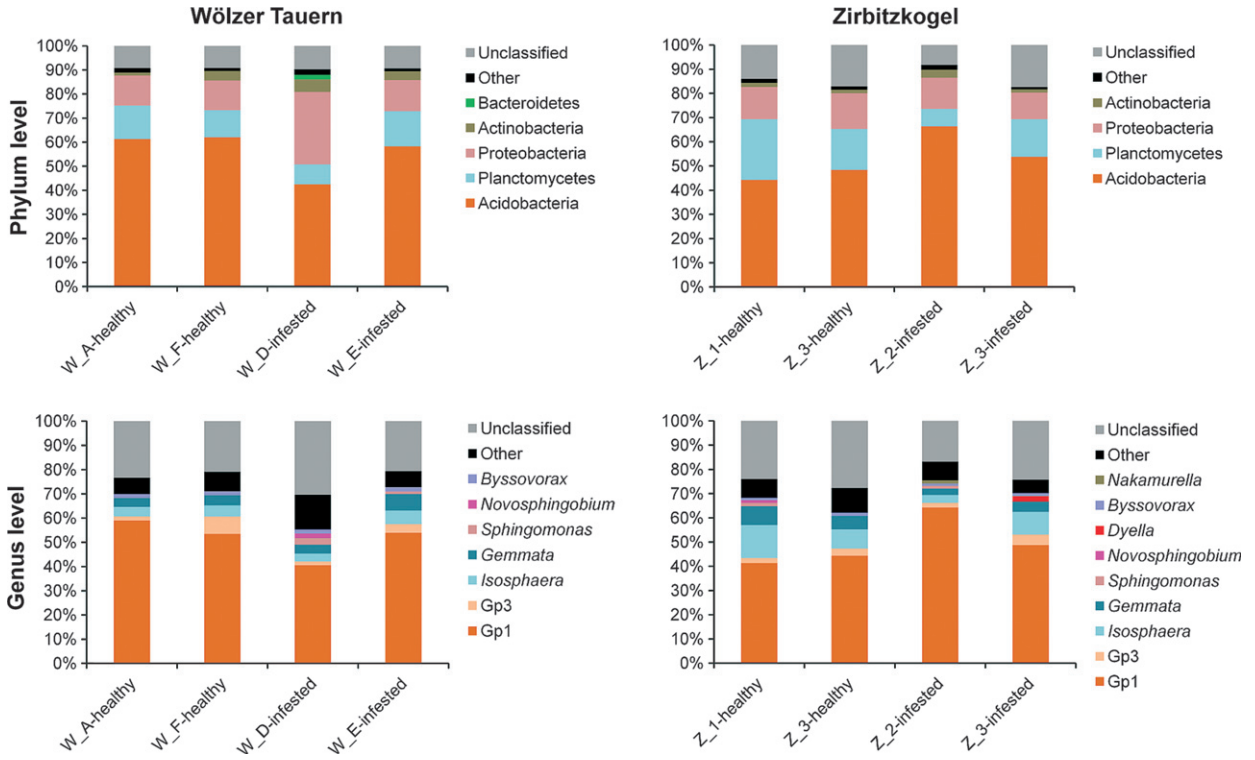

Fig. 2. Nonphotobiont bacterial communities associated with healthy and infested *Solorina crocea* samples from Wölzer Tauern and Zirbitzkogel. Relative clone composition of major phyla and genera was determined by pyrosequencing of 16S rRNA gene from metagenomic DNA. Phylogenetic groups accounting for $\leq$ 1% of all quality sequences are summarized in the artificial group 'Others'.

PCR amplicons of a fragment of the *nifH* gene were deep sequenced by a pyrosequencing approach. The number of quality reads with a read length of $\geq$ 200 bp varied among samples from 7005 to 10 788. To allow comparisons of diversity and richness, samples were normalized to the same number of sequences. The amino acid

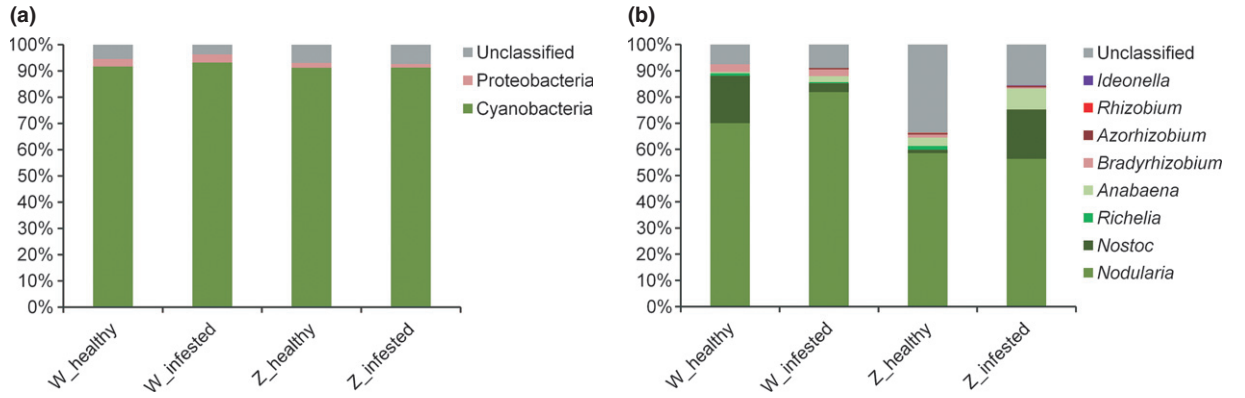

Fig. 3. Taxonomic classification of *nifH* gene communities associated with healthy and infested *Solorina crocea* samples from Wölzer Tauern and Zirbitzkogel. Amino acid sequences of the *nifH* genes were classified at phylum (a) and genus (b) level. Clusters containing < 10 sequences were not phylogenetically designated.

sequences were classified into 123–181 OTUs with a similarity cutoff of 92%. At this genetic similarity level, the coverage of Chao1 estimated richness reached 46.3–52.9% (Table 2). In general, Shannon indices (*H*′) were higher for *S. crocea* samples originating from Zirbitzkogel than for those from Wölzer Tauern. Three cutoff levels (100%, 96%, and 92% amino acid similarity) were used for generating rarefaction curves (Supporting Information).

Of all quality amino acid sequences, 94.1% could be classified to the phylum level. All classifiable sequences were affiliated to the canonical *nifH* cluster I (Chien & Zinder, 1996; Zehr *et al.*, 2003; Farnelid *et al.*, 2011), which includes mainly *Cyanobacteria, Alpha-*, *Beta-*, and *Gammaproteobacteria*. Not surprisingly for lichens, all samples showed an overwhelming dominance of *nifH* amplicons related to *Cyanobacteria* (between 91.2% and 93.2% of sequences) (Fig. 3). The noncyanobacterial diazotrophs were all assigned to *Proteobacteria* (between 1.3% and 3.1% of sequences). Most of the closest hits of cyanobacterial reads revealed the genus *Nodularia* (73%). Further, *Nostoc* (11%), *Anabaena* (4%), and *Richelia* (1%) were found. *Proteobacteria* sequences were classified as *Alpha-* (98%) and *Betaproteobacteria* (2%). Samples from Wölzer Tauern were dominated by *Bradyrhizobium*, whereas *Rhizobium* was found only in samples from Zirbitzkogel. *Ideonella* occurred only in the lichen pathogen-infested composite sample from Zirbitzkogel. However, we did not find a distinct community shift or depletion of cyanobacterial *nifH* genes with the infections.

DCA using CANOCO and profile clustering network analysis

Detrended correspondence analyses using presence/absence data of OTUs with a similarity cutoff of 97% indicate that strains are not randomly distributed in healthy and infected individuals of *S. crocea* (Fig. 4). The differences, however, are neither exclusive nor clear-cut. To gain better insight into the differences of healthy and infected lichens, we applied a profile clustering network analysis. This analysis revealed that several genetically distinct *Acidobacteria* are more abundant in infected samples (all belonging to *Acidobacteria* Gp 1) beside few *Proteobacteria*. *Bryocella elongata* and few other *Acidobacteria*,

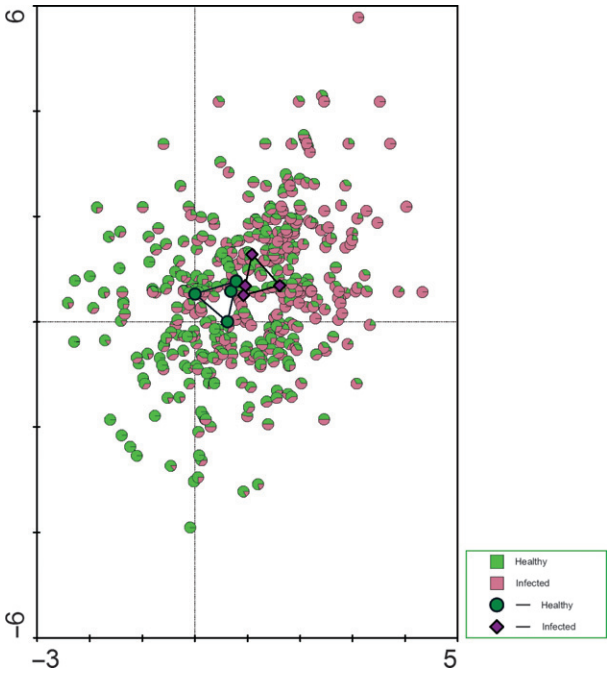

Fig. 4. DCA (indirect unimodal gradient analysis) of OTUs at a dissimilarity level of 3% identified by pyrosequencing. Eigenvalues of first and second axis are 0.325 and 0.150, respectively; sum of all eigenvalues 1.100. Diamonds show the location of the 8 samples and circles the location of the OTUs in the biplot. The colors indicate its preference to healthy (green) and/or infected (red) thalli of *Solorina*.

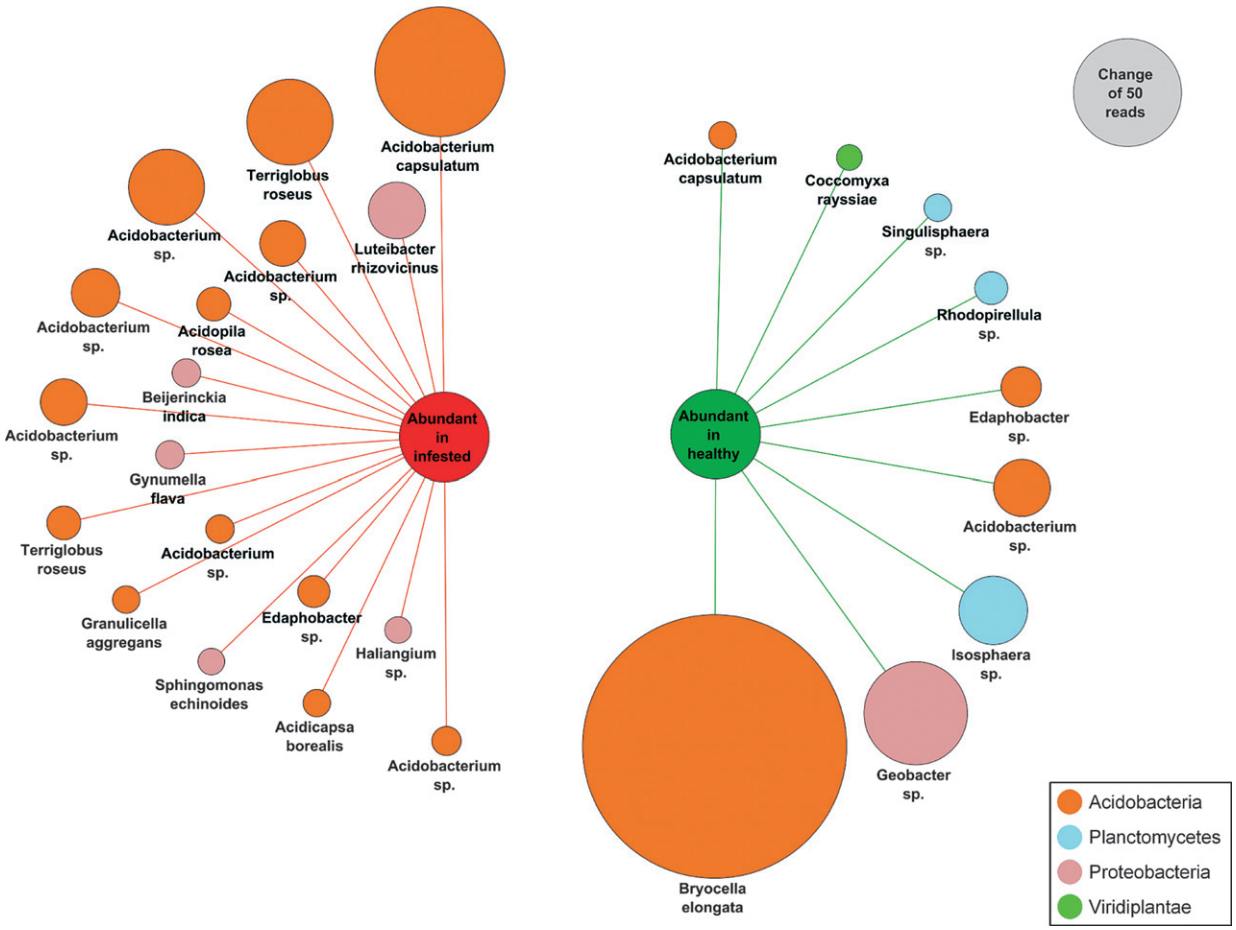

Fig. 5. Profile clustering network analysis of bacterial OTUs at a dissimilarity level of 3%. The relative abundance values for OTUs with a cumulative read change $\geq$ 5 were used; if the ratio of values for healthy and infected states exceeded 1.5 in one sampling site, the OTUs were regarded as altered and assigned to the respective clusters. Only those OTUs that featured the same pattern in both sampling sites are shown. Henceforth, each OTU is connected to either an 'abundant in infested' or 'abundant in healthy' node in the network. Node sizes of OTUs correspond to the mean relative abundance change between the states, and a node matching to a change of 50 reads was added as reference point.

strains of *Planctomycetes*, *Geobacter* (*Proteobacteria*), and the green-algal photobiont *Coccomyxa* appear more distinct in healthy individuals according to the network analysis (Fig. 5).

Discussion

Abundances of bacterial groups in host-associated microbiomes are often indicated as bar charts of phyla, classes, families, or genera. This approach is followed also in studies of lichen-associated microbiomes (Mushegian *et al.*, 2011; Hodkinson *et al.*, 2012). Evaluation at this level gives a general insight into microbial ecology of a microhabitat and agrees well with the notion of ecological coherence at high taxonomic ranks (Philippot *et al.*, 2011). At the phylum level, no clear differences were observed between the bacterial microbiomes of healthy thalli of *S. crocea* and those infected by the specific pathogenic fungus *R. lichenicola*. In both categories, we found a high proportion of *Acidobacteria* (which may exceed 50% of the sequence reads), as well as other bacterial groups such as *Planctomycetes* and *Proteobacteria*. Differences in the composition of bacteria at the genus level were likewise not clearly consistent with the lichenicolous infection status. Only the analysis of raw data revealed more genera in the infected samples, notably in the samples from Wölzer Tauern. This site has a slightly different habitat history than the Zirbitzkogel site. The wind-swept ridge of the Wölzer Tauern site represents a secondary habitat after nearby construction of wind power generators, whereas the Zirbitzkogel site was not affected by recent human activities. These differences, nevertheless, disappear when normalized data are used for the representation of microbiome composition. We were then

looking for possible differences between healthy and infected thalli at the lowermost taxonomic level and analyzed the sequence data as individual OTUs at a dissimilarity level of 3%, using DCA. In contrast to the results from analyses at higher taxonomic levels, we found that strains indeed segregate between healthy or infected samples (Fig. 4). Probably, because fungal-infected thalli also include to some extent externally healthy thallus material, these differences between healthy and infected thalli are not clear-cut. Using network analysis, we assessed which bacterial strains increase their occurrence in healthy or infected thalli. Interestingly, the recently described *Bryocella elongata* (Acidobacteria) appeared to be more typical for healthy thalli. *Bryocella elongata* was originally isolated from a methanotrophic enrichment culture obtained from an acidic *Sphagnum* peat (Dedysh *et al.*, 2012). A strain of *Acidobacterium capsulatum* was more frequent in infected thalli, yet a distinct strain of the same species occurred at slightly higher abundance in healthy lichens. Many others of these potential health-indicator strains could only be classified at the genus level because of insufficient information in public database for the time being. We suppose that these strains likely belong to yet-unculturable and new species of *Planctomycetes* and *Acidobacteria* (Fig. 5).

Our results suggest that the lichen-associated bacterial community reacts to infections of the host lichen by lichenicolous fungi by bacterial shifts at the strain level. Similar results are also found in other studies, for example, on bacterial communities affected by invasive bacteria (Jousset *et al.*, 2011).

The biology of many lineages in these large bacterial phyla *Planctomycetes* and *Acidobacteria* is still poorly understood, but their abundance in lichens and the strain-specific shifts indicate their fine-tuned ecological adaptation to the lichen habitat. The prevalence of *Acidobacteria* in our data set is somehow in contrast with the results of previous microscopic studies where *Alphaproteobacteria* were the prominent bacteria (Cardinale *et al.*, 2008; Grube *et al.*, 2009). These analyses focused mainly on young thallus parts, whereas in this study, we analyzed whole thalli, including the older parts. A recent study by us showed that old parts of thalli differed clearly in the composition with bacterial phyla (Cardinale *et al.*, 2012). A high number of *Acidobacteria* was recently also found in other analyses of lichen-associated microbiomes using whole thalli (Mushegian *et al.*, 2011; Hodkinson *et al.*, 2012). *Acidobacteria* of Gp1 made up a significantly higher proportion in central thallus parts of the rock-inhabiting *Xanthoparmelia* species (Mushegian *et al.*, 2011). The Gp1 lineage of *Acidobacteria* was also prevalent in our data set, irrespective of infection status. The second most abundant lineage of *Acidobacteria* in our data set was Gp3. *Acidobacteria* are well known as soil inhabitants, and their slow growth and tolerance to desiccation are ideal preconditions for a sustained presence in lichen thalli. We therefore argue that *Acidobacteria* are increasingly abundant in aging thallus parts.

At the genus level, the most important groups of the facultatively aerobic *Planctomycetes* were *Isosphaera* and *Gemmata.* The overall ecology of these genera is poorly known, but they are an ubiquitous, abundant aspect in acidic soils and bogs and may have particular mechanisms conferring tolerance to osmotic stress (Jenkins *et al.*, 2002), which is also a requirement to persist in the lichen habitat.

In agreement with the overall similarity of microbial communities at high taxonomic levels in healthy and infected thalli, we did not find a major shift in the phylogenetic spectrum of nitrogenase *nifH* genes, which is a key functional gene of this lichen symbiosis. Most reads belonged to cyanobacteria, which are known to be commonly present as a second autotrophic partner in *S. crocea*. However, closest BLAST hits of cyanobacterial *nifH* genes were with *Nodularia* and not with *Nostoc*, although the latter would be expected from microscopic studies (often seen as a layer below the algal layer in the thalli). A posteriori alignment of the data (not shown) revealed that the difference to the second closest hit (*Nostoc*) is only by one nonsynonymous mutation in the gene. In view of the abundance of *Nostoc* in the lichen thalli compared with the most abundant reads, we argue that the closest assignment to *Nodularia* is arbitrary, and in fact sequences are from *Nostoc*. We suggest that unreflected assignment of coding gene reads to genera, using only the first closest BLAST hits, could be a potential source of error, especially as we cannot exclude determination errors in the databases.

Lichens are a valuable system to study microbiome structure and variation. The light-exposed thalli can usually be regarded as well-delimited miniature ecosystems shaped by the name-giving lichenized fungal species (Grube *et al.*, 2009). We previously optimized analysis of these complex systems using a polyphasic approach (e.g. FISH and confocal laser scanning microscopy, fingerprinting: Cardinale *et al.*, 2008; Grube *et al.*, 2009). With the observation of shifts in lichen-associated bacterial communities at the strain level when exposed to biotic stress conditions, we conclude that functional dependencies in the lichen micro-ecosystem are finely tuned. It is known from other studies that related bacterial strains may differ in their effects on symbiotic relationships (e.g. Lehr *et al.*, 2007). *Rhagadostoma lichenicola* is a highly specific lichenicolous fungus, which relies on the function and integrity of the host thallus. The pathogen does not rapidly degrade the host and apparently persists on the hosts for

years. The observed subtle changes of the associated bacterial microbiome in infected thalli agree well with this ecology.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction analyses for 16S rRNA gene amplicon libraries of *Solorina crocea* samples from Wölzer Tauern (a) and Zirbitzkogel (b).

Fig. S2. Rarefaction analyses for *nifH* sequence libraries of *Solorina crocea* samples.

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Supplemental file

Fig. S1. Rarefaction analyses for 16S rRNA gene amplicon libraries of *Solorina crocea* samples from Wölzer Tauern (A) and Zirbitzkogel (B). Saturation curves are presented for non-photobiont bacteria associated with healthy and *Rhagadostoma lichenicola* infested lichens. OTUs are shown at genetic distance levels of 3%, 5% and 20%, corresponding to the taxonomic levels of species, genera and phyla.

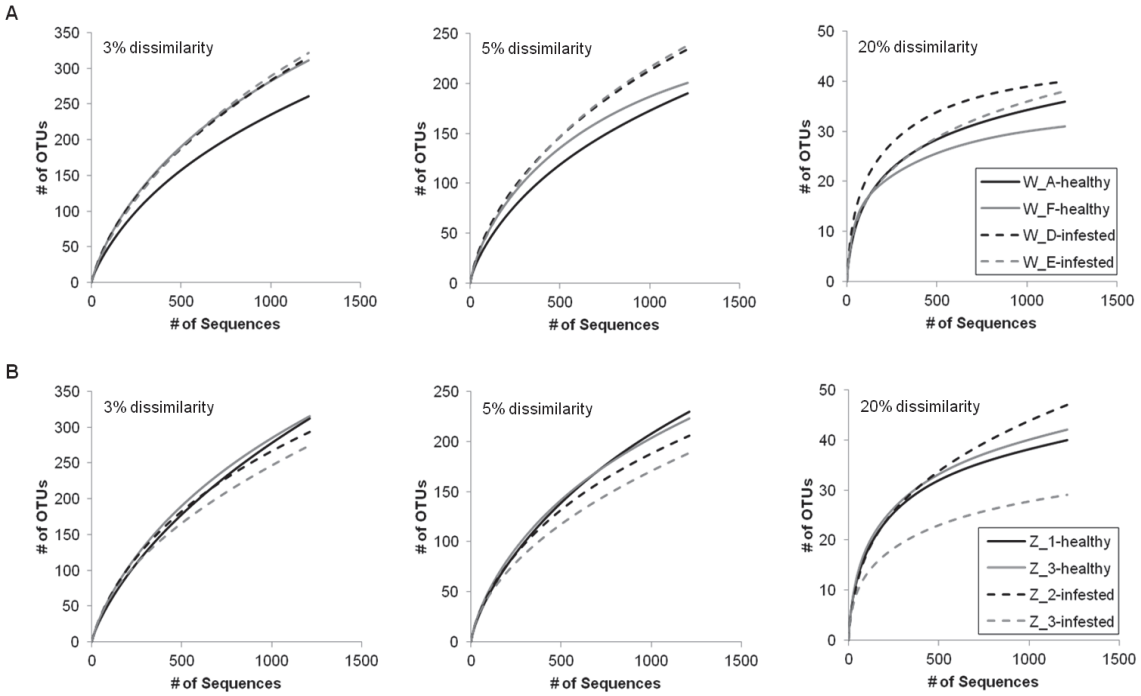
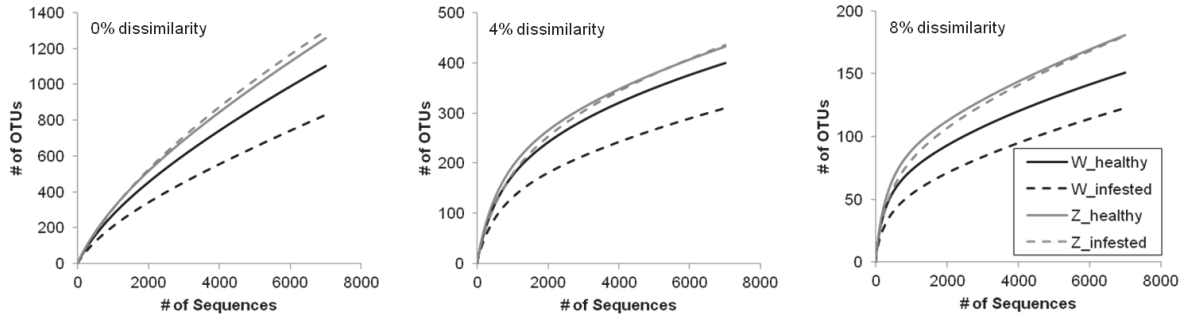


Fig. S2. Rarefaction analyses for *nifH* sequence libraries of *Solorina crocea* samples. Curves are presented for healthy and *Rhagadostoma lichenicola* infested lichens collected at Wölzer Tauern and Zirbitzkogel. Phylotype clusters are identified at genetic dissimilarity of 0%, 4% and 8%.



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Curriculum Vitae

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Publication List

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

09. 09. 2013 ..... Martina Kölbl .....

Date Signature