Applied Soil Ecology 48 (2011) 193–200

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Impact of biotic and a-biotic parameters on structure and function of microbial communities living on sclerotia of the soil-borne pathogenic fungus *Rhizoctonia solani*

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ARTICLE INFO

Article history:
Received 5 October 2010
Received in revised form 11 March 2011
Accepted 13 March 2011

Key words:
Sclerotia
Antagonists
Microbial fingerprints
Biocontrol

ABSTRACT

The plant pathogen *Rhizoctonia solani* is very difficult to control due to its persistent, long-living sclerotial structures in soil. Sclerotia are the main source of infection for *Rhizoctonia* diseases, which cause high yield losses on a broad host range world-wide. Little is known about micro-organisms associated with sclerotia in soil. Therefore, microbial communities of greenhouse and field incubated *Rhizoctonia* sclerotia were analysed by a multiphasic approach. Using microbial fingerprints performed by PCR-SSCP, sclerotia-associated bacterial communities showed a high diversity, whereas only a few fungi could be detected. Statistical analysis of fingerprints revealed the influence of soil types, incubation conditions (greenhouse, field), and incubation time (5 and 12 weeks) on the bacterial as well as fungal community. No significant differences were found for the microbial community associated with different *Rhizoctonia* anastomosis sub-groups (AG 1-IB and AG 1-IC). *Rhizoctonia* sclerotia are an interesting bio-resource: high proportions of fungal cell-wall degrading isolates as well as those with antagonistic activity towards *R. solani* were found. While a fraction of 28.4% of sclerotia-associated bacteria (=40 isolates) with antagonistic properties was determined, only 4.4% (=6 isolates) of the fungal isolates were antagonistic. We identified strong antagonists of the genera *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Stenotrophomonas*, which can be used as biological control agents incorporated in soil or applied to *Rhizoctonia* host plants.

1. Introduction

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* [Frank] Donk) is a widely distributed soil-borne plant pathogen responsible for economic yield losses on a high diversity of crops (Sneh et al., 1996; Kiewnick et al., 2001; Büttner et al., 2003). The life cycle of *Rhizoctonia* includes parasitic and saprophytic periods as well as sexual and asexual reproduction. In soil, *R. solani* exists as vegetative mycelium or sclerotia, which are asexually produced. Sclerotia are known as the major source of infection for *Rhizoctonia* diseases (Anderson, 1982). The small (1–3 mm diameter), irregular-shaped brown structures consist of dense masses of monilioid cells. Due to this compact structure and the presence of melanin in all cell walls, sclerotia of *R. solani* are protected against various environmental conditions (Sneh et al., 1996). In addition, sclerotia of *R. solani* exude liquid brown droplets that are complex mixtures composed of phenolics, carboxylic acids, carbohydrates, fatty acids, and amino acids, which contributed to their antifungal and phytotoxic activities (Aliferis and Jabaji, 2010). *R. solani* is characterised by a high intra-specific diversity and comprises both multinucleate and binucleate species, which are further taxonomically divided according to their hyphal anastomosis into anastomosis groups (AGs; Anderson, 1982; Carling et al., 2002; Grosch et al., 2007). Both factors – persistent sclerotia and high diversity – contribute to the fact that *Rhizoctonia* diseases are difficult to control.

In the past, *Rhizoctonia* diseases were managed by soil fumigation using the ozone-depleting chemical methyl bromide, which was completely banned in 2006. Today, available fungicides to control *Rhizoctonia* diseases are limited, and fungicide spraying may prevent symptoms but does not kill the survival structures. Furthermore, other *Rhizoctonia* diseases like black scurf on potato are managed by crop rotation to reduce the pathogen inoculums in soil. However, *Rhizoctonia* sp. can use volunteers or weeds to build-up inoculums that can serve as a "green bridge" to the next crop (Keijer et al., 1997). These backgrounds together with increased public concerns about environmental problems and human health in industrial agriculture (Horrigan et al., 2002), emphasize the need for biological control strategies against *Rhizoctonia*.

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doi:10.1016/j.apsoil.2011.03.006

Table 1
Soil characteristics.

Soil number	Location	Soil type	pH	Nt[a] (mg/100 g)	Ct[b] (%)	P lact.[c] (mg/100 g)	Po lact.[c] (mg/100 g)
Soil 1 (S1)	Golzow	Alluvial loam	7.6	151.0	1.81	46.0	24.4
Soil 2 (S2)	Plattling	Sandy loam	7.1	151.0	1.18	30.0	38.0
Soil 3 (S3)	Großbeeren	Diluvial sand	6.6	70.5	0.79	22.5	17.3
Soil 4 (S4)	Großbeeren	Diluvial sand	3.6	229.0	2.83	8.8	8.9

[a] Nt – total nitrogen.
[b] Ct – total carbon content.
[c] Phosphorous (P) and potassium (Po) were measured using the lactat method according to VDLUFA-Methodenbuch (1991).

The use of naturally occurring antagonistic microorganisms to control soil-borne pathogens offers sustainable alternatives to chemical pesticides (Lugtenberg and Kamilova, 2009; Berg, 2009). In biocontrol studies, introduced fungal antagonists like *Trichoderma harzianum* and *Gliocladium virens* successfully suppressed *R. solani* on several hosts (Elad et al., 1980; Grosch et al., 2006; Lahlali and Hijri, 2010). In addition, bacterial antagonists like *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pseudomonas jessenii*, *Serratia plymuthica* and *Lysobacter capsici* (Chernin et al., 1995; Scherwinski et al., 2008; Postma et al., 2010) have shown potential to control *R. solani*. However, the majority of reported strains were only able to antagonise hyphae of the pathogen and not the survival structures (Marcello et al., 2010). Only two specific mycoparasites of *Rhizoctonia* sclerotia were reported: *Verticillium biguttatum* (Jager et al., 1991) and *Trichoderma virens* (Liu et al., 2010). Therefore, analysis of sclerotia-associated microorganisms is not only interesting to understand fungal ecology; results can be used to develop new biocontrol strategies. During their growth, sclerotia accumulate relatively high concentrations of carbohydrates, fats, and proteins, which provide good nutritional niches for microorganisms in soil (Willetts, 1971). In the mycospheres of different basidiomycetous fungi, species-specific bacterial 'fungiphiles' were detected (Warmink et al., 2009), and many fungal structures are colonised by bacteria (rev. in Grube and Berg, 2009). We hypothesize that sclerotia can be a source for adapted antagonistic microorganisms with negative effect on longevity of sclerotia in soil.

Therefore, the objective of our study was to analyse sclerotia-associated microbial communities in detail. In a cultivation-independent approach, we characterised the diversity of bacterial and fungal communities on sclerotia depending on: (i) different soil types, (ii) different incubation conditions in greenhouse and field trials, (iii) under different incubation times, and (iv) on different anastomosis groups of the fungus. Furthermore, by a cultivation-dependent approach, we analysed: (i) the antagonistic potential of bacterial and fungal isolates towards *R. solani* and (ii) the antagonistic mechanisms of the sclerotia-associated bacteria.

2. Materials and methods

2.1. Incubation of R. solani sclerotia in soil

For formation of sclerotia the *R. solani* isolates of different anastomosis sub-groups, AG 1-IB; and, AG 1-IC were cultivated for three weeks on potato dextrose agar (PDA, VWR International GmbH, Berlin, Germany) at 20 °C. Fifteen sclerotia of similar size were wrapped into nylon fabric parcels and 5 parcels were buried in 500 ml pots (10 cm × 10 cm × 10 cm) at 5 cm depth. The pots were filled with different soil types obtained from bulk soil (10 cm) of various locations (Table 1). In these pots, the sclerotia were incubated for 5 weeks (experiment 1) and for 12 weeks (experiment 2) in the dark at 15 °C/10 °C (16 h/8 h, day/night). A third experiment was carried out by incubation of sclerotia in the various soil types under field conditions from end of November 2006 to beginning of April 2007. In this case the pots were buried in top soil in Großbeeren (latitude: 52°N, 13°E). The average temperature during the incubation time was 3.9 °C (minimum −3.1 °C, maximum 14.8 °C) in 5 cm bulk soil. In all experiments each treatment included five replicates with 15 sclerotia each. After incubation, sclerotia were unwrapped under semi-sterile conditions and either used for cultivation of associated fungi and bacteria or for DNA-extraction. Samples were characterised based on soil type (S1–S4), cultivation conditions (F, field trial; GH, green house trial), anastomosis sub-group of *R. solani* AG 1-IB or AG 1-IC (B or C), sample repetition (1–5), and incubation time (5 weeks or 22 weeks = *); e.g. S1_F_B_1.

2.2. Structural analysis of sclerotia-associated microorganisms by Single Strand Conformational Polymorphism Analysis (SSCP)

DNA of *R. solani* sclerotia-associated bacterial and fungal cell consortia was extracted using the FastDNA Spin Kit for Soil (BIO 101, Carlsbad, USA) according to the manufacturer's protocol. Fingerprints of bacterial and fungal communities were obtained by SSCP analysis carried out as described by Schwieger and Tebbe (1998). Briefly, bacterial and fungal gene sequences were amplified by using the primers Unibac-II-515f (5′-GTG CCA GCA GCC GC-3′) and Unibac-II-927rP (5′-CCC GTC AAT TYM TTT GAG TT-3′) primers or ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS2R (5′-GCT GCG TTC TTC ATC GAT GC-3′) primers. The amplicons were separated by using the TGGE Maxi system (Biometra, Göttingen, Germany) at 400 V and 26 °C. Silver staining was used to detect DNA in SSCP gels (Bassam et al., 1991). The band patterns representing the microbial community composition of the different treatments were hierarchically clustered to produce dendrograms using the unweighted pair group method with arithmetic averages (UPGMA). The actual distances calculated and the distances shown on the dendrogram were compared by Pearson product-moment correlation (cophenic correlation) to ensure that the dendrogram gave an accurate representation of the data.

2.3. Isolation of sclerotia-associated microorganisms

For isolation of bacterial strains, sclerotia of each replicate were cultivated in nutrient broth (NB, VWR International GmbH, Germany) for 24 h at 28 °C. Three aliquots (500 μl) were taken from each generated culture suspension and further plated onto nutrient agar (NB amended with 15 g l^{-1} agar to) to isolate associated bacteria. Plates were incubated for five days at 20 °C. Bacteria visually distinguishable based on colony morphology were selected from the plates and transferred to nutrient agar. Colonisation of sclerotia with fungi were investigated by incubation of sclerotia on water agar (1.2%) amended with 50 μg ml^{-1} streptomycin and 50 μg ml^{-1} penicillin. The agar plates were incubated in the dark at 20 °C. After 1–3 days, hyphal tips from colonies were isolated and transferred to PDA. Cultures were stored at 10 °C until further use.

2.4. In vitro screening for antifungal and hydrolytic activity and production of secondary metabolites

Bacterial isolates were tested in a dual-culture assay with *R. solani* AG 1-IB on Waksman agar containing per liter 5 g of peptone (Carl-Roth, Karlsruhe, Germany), 10 g of glucose (Carl-Roth), 5 g of NaCl (Carl-Roth), 3 g of yeast extract (Carl-Roth) and 20 g of agar (Carl-Roth). Zones of inhibition were measured and evaluated according to Berg et al. (2002).

Chitinase activity (beta-1,4-glucosamine polymer degradation) was tested in chitin minimal medium by the method of Chernin et al. (1995). Clearing zones were detected five days after incubation at 20 °C. Beta-glucanase activity was tested by using chromogenic (azurine-dyed, cross-linked [AZCL]) substrates (Megazyme, Bray, Ireland). Formation of blue halos was recorded until 5 days after incubation. Protease activity (casein degradation) was determined from clearing zones in skim milk agar (50 ml of sterilized skim milk mixed at 55 °C with 50 ml of 1/5 tryptic soy agar and 4% agar) after 5 days of incubation at 20 °C.

Antibiosis against *R. solani* by the bacterial strains was assayed on Waksman agar plates (15 ml, pH 7–8, 9 cm diameter) containing 5 ml of sterile culture filtrate (24-h culture, nutrient broth II (Sifin), sterile filtration using 0.2 μm filters). An actively growing *R. solani* mycelial disk from the margin of a 7 day old PDA-culture (0.8 cm diameter) was placed in the centre of a Waksman agar plate. The growth of *R. solani* inoculant was compared to its growth on a control plate using sterile water instead of culture filtrate.

The ability to produce indole-3-acetic acid (IAA) was tested for selected bacterial strains in a plate test according to Sawar and Kremer (1995). The test was modified by using an Immobilion™-P Transfer Membrane (Millipore, Billerica, USA) instead of a Nitrocellulose membrane.

2.5. Identification of antagonistic isolates

Initial genotypic characterisation of selected bacterial antagonists ARDRA (amplified rDNA restriction analysis) was performed. For amplification of bacterial 16S RNA genes Eub 1F primer (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) were used. Thereby obtained, 1400 bp long PCR fragments were digested at 37 °C over a period of at least 3 h, using 0.25 μl restriction endonuclease *Hha*I (New England Biolabs), 2 μl of NEB-4 puffer (New England Biolabs), 0.2 μl of BSA (10 mg ml^{-1}) (New England Biolabs), 2.55 μl of water and 15 μl of clean PCR product. A 10 μl aliquot of restriction product was separated by gel electrophoresis on 2.0% agarose (Carl-Roth) gels in 0.5× Tris–borate–EDTA buffer for 3 h. Representative antagonists of selected and characterised antagonistic bacteria were identified by partial 16S rRNA gene sequencing and subsequent alignment using the BLAST algorithm (Altschul et al., 1997).

2.6. Nucleotide sequence accession numbers

Sequence accession numbers for sequences of the partial 16S rRNA gene (FR752798 to FR752814 and FR752817 to FR752818) of the bacterial strains were submitted to the NCBI nucleotide sequence database.

2.7. Statistical analysis

To compare SSCP fingerprints of the microbial communities, a computer-assisted cluster analysis was carried out using the GelCompar II® software (Applied Math, Kortrijk, Belgium). Computer-assisted pattern analyses of molecular fingerprints of the communities resulted in correlation matrices, which were subjected to significance test by applying permutation tests with 10^4 random permutations of sample elements (Kropf et al., 2004). The relationships between samples taken from different soil types, at different incubation times and conditions as well as from anastomosis groups were investigated.

3. Results

3.1. Molecular fingerprinting of sclerotia-associated bacterial and fungal communities

The cultivation-independent analysis of sclerotia-associated microbial populations was performed to get insights into the impact of environmental factors on their structure. In general, SSCP (Single Strand Conformational Polymorphism Analysis) fingerprints of bacterial and fungal sclerotia-associated communities revealed differences in abundances and diversity. The bacterial community was much more diverse than the fungal community (data not shown). In fungal community fingerprints with less than 10 different bands were observed; especially very few bands were found for the fungal community obtained from sclerotia incubated in the field. Differences between field and greenhouse incubated communities were not found in this extent for the bacterial communities; the bacterial population was always highly diverse and consisted of numerous populations.

On the basis of microbial fingerprints performed by PCR-SSCP, dendrograms and statistical analyses were performed to analyse the impact of biotic and a-biotic parameters on the structure of microbial communities living on sclerotia. According to these analyses, bacterial as well as fungal fingerprints revealed the influence of soil type, incubation conditions (greenhouse and field) and incubation time (5 and 12 weeks). All factors affected significantly the microbial community ($P \leq 0.05$). In contrast, no significant differences were found for the structure of microbial communities associated with different anastomosis AG 1 sub-groups (IB and IC) of *R. solani* (Figs. 1 and 2 and Table 2).

3.2. Antagonistic potential of sclerotia-associated microorganisms

Bacterial as well as fungal strains were isolated from the incubated *Rhizoctonia* sclerotia. While the bacterial isolates showed a high diversity based on colony morphology, a limited morphologically visual diversity was observed for fungal isolates associated with sclerotia (<7 genera, like *Mucor* spp., *Fusarium* spp., *Trichoderma* spp., *Cylindrocarpon* spp., and *Penicillium* spp. isolates with sterile mycelium). Altogether, 141 bacterial and 270 fungal isolates were obtained and analysed for *in vitro* activity towards *R. solani*. A proportion of 28.4% (40 isolates) from the bacterial isolates and of 4.4% (6 isolates) of the fungal isolates was able to antagonise *R. solani*. In addition, to estimate lytic properties on the fungal cell wall, hydrolytic enzymes were investigated: 82.4% of selected antagonistic isolates showed protease activity, 57.6% had β-1,4-glucanase activity and 35.3% showed chitinases. Furthermore, a high proportion of isolates was able to produce antifungal metabolites; 87.5% of all tested strains were able to produce such compounds *in vitro*. Using these data to compare the activity of sclerotia-associated bacteria at the two sampling times, we found that the antagonistic population of sclerotia-associated bacteria increased after a prolonged period of incubation (Fig. 3). The proportion of bacteria producing all three hydrolytic enzymes was more than doubled (increased from 14.3% to 31.6%).

For further analysis of the antagonistic microorganisms, only bacteria were considered: the antagonistic fungal isolates were characterised by a slow growth, weak antagonism and none mycoparasitic activity, and are therefore not appropriate as biocontrol

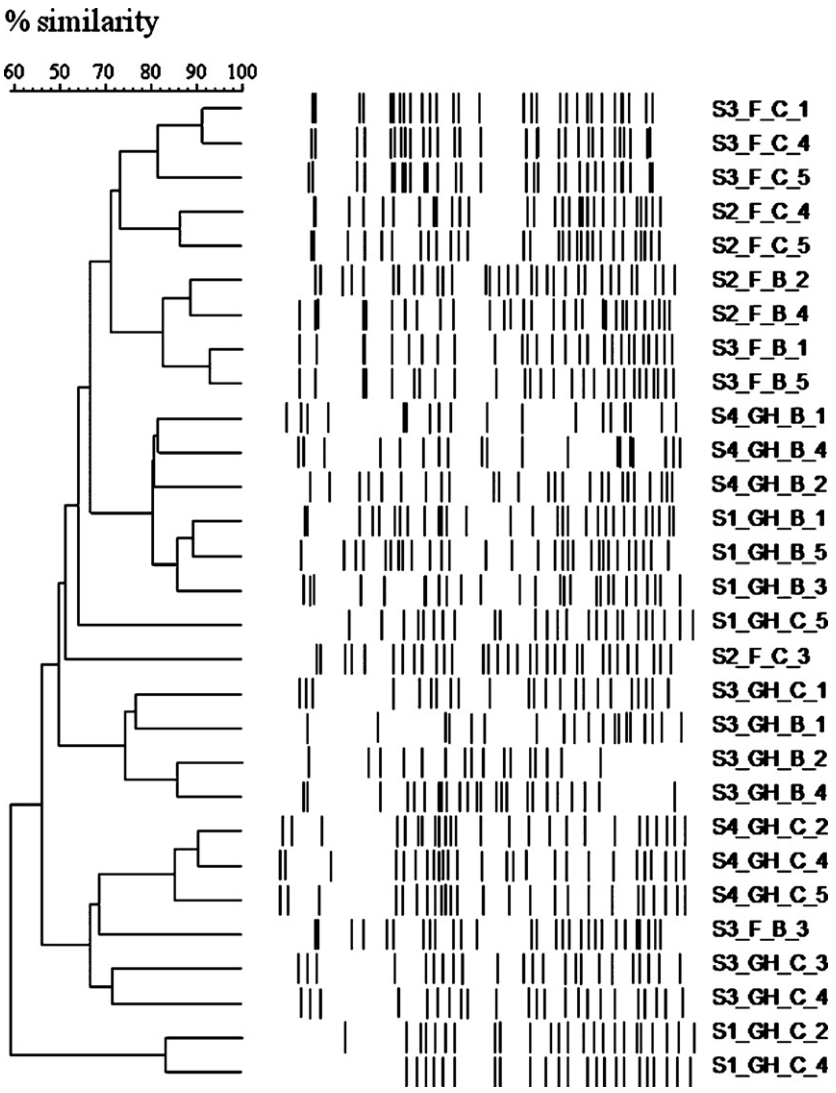

Fig. 1. UPGMA dendrogram of the bacterial communities associated with *Rhizoctonia sclerotia* performed by single strand conformation polymorphism (SSCP). Different location/soil types in Germany (S1 Golzow, S2 Plattling, S3, S4 Großbeeren), with different cultivation conditions (F, field trial; GH, green house trial) and different anastomosis sub-groups of *R. solani* (AG 1-IB or IC). Each location was presented in three replicates (exception S3_F_B, two repetitions). Incubation time of field samples was 22 weeks and for green house samples 5 or 12 weeks (*).

agents. To select bacterial antagonists, the antifungal as well as fungal cell wall-degrading properties were considered (Table 3).

3.3. Identification and characterisation of Rhizoctonia antagonists

For the identification of bacterial antagonists, the isolates were firstly grouped by ARDRA (Table 3). From each ARDRA group, 34 isolates were randomly selected and identified by partial 16S rRNA gene sequencing. Sclerotia-associated bacteria belong to four phylogenetic groups: Enterobactericeae, Pseudomonadaceae, Xanthomonadaceae, and Firmicutes. Altogether, 18 different strains were identified, wich resulted into nine species: *Bacillus cereus*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Enterobacter aerogenes*, *Enterobacter ludwigii*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Stenotrophomonas maltophilia*. Furthermore, selected isolates were characterised regarding their antagonistic and fungal cell wall-degrading activity,

Table 2
Statistical analysis of investigated parameters. Microbial fingerprints obtained by PCR-SSCP were analysed using permutation test (Kropf et al., 2004) $P \leq 0.05$.

Parameter	Compared data set	*P* value for	
		Bacterial communities	Fungal communities
Soil type	S1 + S2 + S3 + S4[a]	0.0000	0.0001
Incubation condition	GH + F[b]	0.0000	0.0001
Incubation time	5 + 12 weeks	0.0000	0.0000
Anastomosis group	IB + IC[c]	0.6235	0.1277

[a] S1 Golzow, S2 Plattling, S3 Großbeeren, S4 Großbeeren (see Table 1).
[b] Greenhouse (GH) trial or field (F) trial.
[c] AG 1-IB or AG 1-IC.

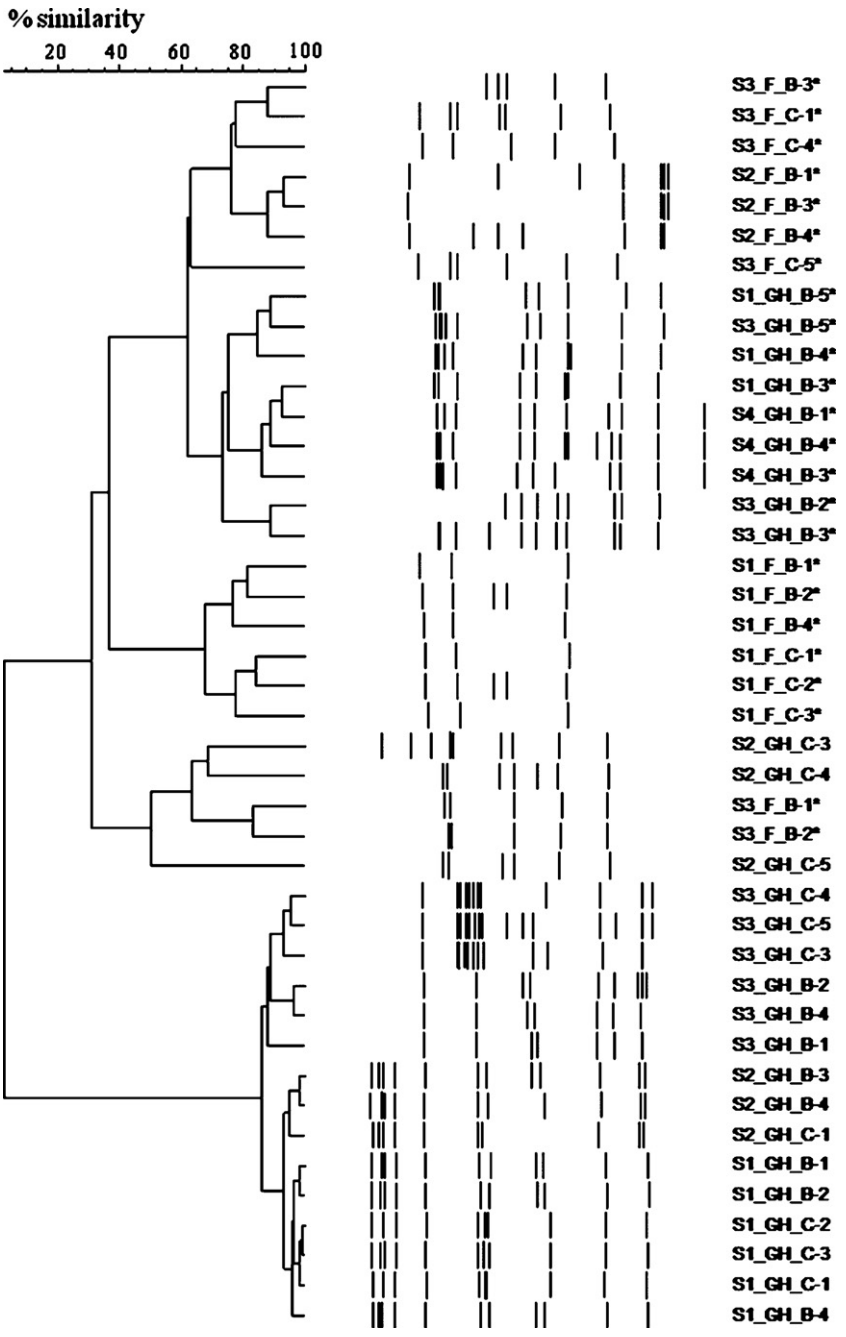

Fig. 2. UPGMA dendrogram of the fungal communities associated with *Rhizoctonia sclerotia* performed by single strand conformation polymorphism (SSCP) profiles. Different location/soil types in Germany (S1 Golzow, S2 Plattling, S3, S4 Großbeeren), with different cultivation conditions (F, field trial; GH, green house trial) and different anastomosis sub-groups of *R. solani* (AG 1-IB or IC). Each location was presented in three replicates. Incubation time of field samples was 22 weeks and for green house samples 5 or 12 weeks (*).

IAA production and antibiosis capacity (Table 3). All strains, which were selected according to their antagonistic activity, produced antifungal antibiotics. In addition, a high proportion was characterised by proteolytic, chitinolytic and glucanolytic properties. Interestingly many strains produced the phytohormone indole-3-acetic acid (IAA), which is not only known to influence growth of plants; it is also a signal molecule and growth factor for fungi (Prusty et al., 2004).

4. Discussion

While sclerotia formation and structure of diverse plant pathogenic fungi are well-investigated, only little is known about sclerotia-inhabiting microorganisms. In this study, we found that sclerotia of the plant pathogenic fungus *R. solani* were colonised by various specific bacterial and fungal isolates. Moreover, especially between the bacterial inhabitants, we found a high proportion, approx. 1/3 of the bacteria, which expressed antagonistic traits against *R. solani*. In addition, a high proportion of sclerotia-associated micro-organisms posses fungal cell wall-degrading abilities. These facts show that sclerotia-associated bacteria are a potential bio-resource to select novel biocontrol agents against sclerotia-forming pathogens.

Our data showed that in all experiments sclerotia were colonised by similar microbial populations. However, several biotic and a-biotic factors were identified, which influenced

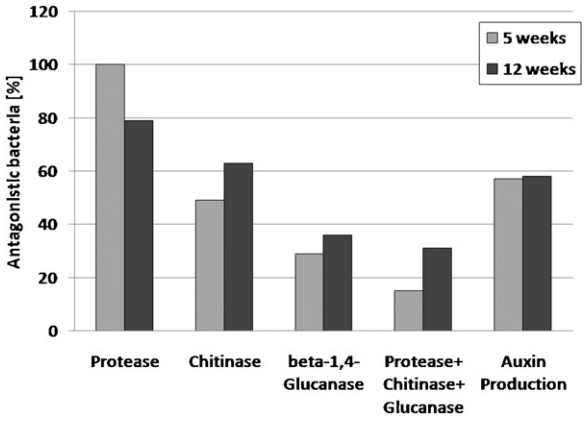

Fig. 3. Summary of antagonistic mechanisms of the sclerotia-associated bacteria to produce proteases, chitinases, beta-1,4-glucanases or the plant growth hormone indole-3-acetic acid (IAA) after different durations of incubation (grey 5 weeks, black 12 weeks).

the structure of the microbial communities. Interestingly, the structure of bacterial communities was influenced by the same environmental factors than the fungal community. The latter includes soil types, incubation conditions, and incubation time. Soil type, especially pH, is an important driver of the microbial communities in soil (Rousk et al., 2010). We investigated three different soil types (alluvial and sandy loam, diluvial sand), which were characterised by extremely different pH values (3.6–7.6). They can explain the high impact of this factor on the structure of sclerotia-associated microorganisms. Furthermore, incubation conditions in greenhouse and field differed strongly in temperature; the difference was more than 20 °C. This could be a key factor here. The time of incubation was also an important impact factor. The microbial fingerprints reflect the impact of these factors. We found that the proportion of associated bacteria producing hydrolytic enzymes was more than doubled after 12 in comparison to 5 weeks. No impact was found on the structure of microbial communities associated with different *R. solani* AG 1 sub-groups (IB and IC). However, the similarity in the ITS region of the isolates from each AG 1 sub-group is, although they can be distinguished (Grosch et al., 2007), very high, whereas isolates of *R. solani* AG 1 were clearly separated from *R. solani* AG 2-1, AG 4, and binucleate *Rhizoctonia* AG-Bb and AG-K (Toda et al., 2011). While the impact of plant species and cultivars on structure and function of plant-associated microbial communities is well-known (rev. in Berg and Smalla, 2009), specificity of fungi–bacteria interaction is only partly understood (Grube and Berg, 2009).

The composition of the fungal community was characterised by low diversity, which was found in cultivation-dependent and -independent experiments. Sclerotia-associated fungi could be influenced by antifungal metabolites produced by sclerotia themselves. Aliferis and Jabaji (2010) identified a high number of antifungal and phytotoxic compounds in the exudates of *R. solani*. Antibiotic properties are widespread amongst *Rhizoctonia* spp.;

Table 3
Results obtained from physiological and molecular characterisation of selected antagonistic sclerotia-associated bacteria. ARDRA, amplified rDNA restriction analysis; n.d., not determined.

Isolate	ARDRA group	Closest database match (16S rRNA gene similarity)	Accession no.	SI[b]	Antagonistic activity[a]	IAA[c]	Protease	Chitinase	Glucanase	Antibiosis[a]
B104	1				+	+	+	+	–	+++
B163	1	*Pseudomonas fluorescens*	GU198122.1	99	++	n.d.	n.d.	n.d.	n.d.	++
B44	2	*Pseudomonas fluorescens*	HQ317182.1	100	+	n.d.	n.d.	n.d.	n.d.	n.d.
B106	3	*Bacillus cereus*	HM771661.1	100	++	–	+	+	+	++
B156	3	*Bacillus thuringiensis*	HQ256544.1	99	+++	–	+	+	–	+++
B32	4	*Pseudomonas chlororaphis*	HM241942.1	97	+++	+	+	–	–	++
B13	4				+	+	+	–	–	+
B50	4				+	+	+	–	–	++
B111	4				++	+	+	–	–	++
B102	5	*Enterobacter aerogenes*	GQ165811.1	99	+	+	–	–	+	+
B2	5	*Enterobacter ludwigii*	AM184285.1	98	++	n.d.	n.d.	n.d.	n.d.	++
B3	5				++	n.d.	n.d.	n.d.	n.d.	++
B31a	6	*Stenotrophomonas maltophilia*	HQ166115.1	98	++	–	+	+	–	+++
B31b	6	*Stenotrophomonas maltophilia*	HQ407233.1	99	+	+	+	+	–	++
B168	6				+	+	+	+	–	+++
B47	7	*Bacillus subtilis*	HQ123476.1	100	+++	+	+	–	+	+++
B48	7	*Bacillus subtilis*	EU627169.1	100	+++	+	+	–	+	+++
B158	8				+	+	+	–	–	++
B142	8	*Pseudomonas* sp.	FJ225200.1	99	++	–	+	+	+	++
B167	8				+++	–	–	+	–	+
B119	8				+	+	+	+	+	+
B164	9				+	–	+	–	–	++
B136	9	*Pseudomonas putida*	HQ658765.1	100	+	+	–	–	–	++
B103	9				+	+	–	–	–	++
B134	9				+	+	–	–	–	n.d.
B21	10	*Bacillus cereus*	HM771661.1	99	+	–	+	+	–	++
B23	10	*Bacillus cereus*	GQ462533.1	98	++	–	+	+	+	++
B40	10				++	–	+	+	+	++
B182	10	*Bacillus cereus*	HQ407233.1	100	+++	–	+	+	+	+++
B107	11				+	+	–	–	–	+
B121	11				+	+	+	+	+	++
B147	11	*Bacillus thuringiensis*	HQ256544.1	99	+	+	+	+	–	++
B169	11				+	n.d.	+	n.d.	–	++
B178	11	*Bacillus thuringiensis*	DQ286343.1	98	++	–	+	+	+	+++

[a] + 0–5 mm, ++ 5–10 mm, +++ >10 mm radius of zone of inhibition in dual culture assay or mycelial growth area of *R. solani* in antibiosis tests, –no suppression.
[b] Plant growth hormone indole-3-acetic acid (IAA).
[c] Similarity index.

antibiotic compounds were also found in the long-living sclerotia of *Rhizoctonia cerealis* and *Rhizoctonia oryzea* (Burton and Coley-Smith, 1993). Furthermore, beside the presence of antibiotic compounds, melanin contributes to longevity and resistance to microbial lysis (Sneh et al., 1996).

In contrast to the results obtained by cultivation-independent methods, cultivation captures only a very low proportion of microorganisms (Schwieger and Tebbe, 1998). But the combination of both assays has advantages: functions can be assessed and isolates can be used for biotechnological applications. Till now, only cultivation-dependent studies about sclerotia-colonising bacteria were already published. For example, Gladders and Coley-Smith (1980) showed a high diversity of *Bacillus* on sclerotia of *Rhizoctonia tuliparum*. They also identified antibacterial pyrone antibiotics produced by the sclerotia and showed that isolates belonging to the *Bacillus* group and *Serratia* sp. were less sensitive against these antibiotics. In our study, it was not possible to calculate the number of bacterial and fungal microorganisms. We selected abundant and culturable microorganisms by incubation of sclerotia in NB or water agar. However, during the incubation of sclerotia in NB media the bacteria propagate already, so the counting of the CFU on agar media reflect not the number of bacteria on the sclerotia surface only the diversity. To assess bacterial abundances on sclerotia, FISH (fluorescence in situ hybridization) in combination with confocal laser scanning microscopy would be a useful method (Grube and Berg, 2009). We used the cultivation approach to isolate strains of *Bacillus*, *Enterobacter*, *Pseudomonas* and *Stenotrophomonas* with antagonistic properties against *R. solani*. All genera are well-known that they harbour potent antagonists against plant pathogens (Haas and Défago, 2005; Ryan et al., 2009). However, strain-specific antagonistic activity was reported (Berg et al., 2002; Zachow et al., 2010), and several of the isolated bacteria were highly active *in vitro* antagonists. Fungal isolates from sclerotia belonging mainly to the genera *Mycor*, *Fusarium*, *Cylindrocarpon* and *Trichoderma*. Fungi of these genera were also observed on *R. solani* sclerotia from potato tubers (Chand and Logan, 1984), and on sclerotia of *R. tuliparum* (Gladders and Coley-Smith, 1980). Our results support previous findings that the outer layers of sclerotia may be invaded by a number of soil fungi but we could not find specific mycoparasites of *R. solani*.

Interestingly, we found a high proportion of bacterial antagonists on sclerotia. In contrast to the rhizosphere of plants, which is a hotspot for antagonists (Berg et al., 2002) the number of antagonistic bacteria was much higher on sclerotia. In our study we could show, that fungal sclerotia are also an interesting reservoir for naturally occurring antagonists. The knowledge about sclerotia-associated microorganisms and molecular mechanisms involved in sclerotial lysis can support biological control strategies against fungi producing long-term survival structures.

Acknowledgements

We thank Martina Gass and Monika Schneider-Trampitsch for valuable technical assistance. The work was supported by a grant of the Austrian Science Foundation FWF to G.B.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Aliferis, K.A., Jabaji, S., 2010. Metabolite composition and bioactivity of *Rhizoctonia solani* sclerotial exudates. Journal of Agricultural and Food Chemistry 58, 7604–7615.

Anderson, N.A., 1982. The genetics and pathology of *Rhizoctonia solani*. Annual Review of Phytopathology 20, 329–347.

Bassam, B.J., Ceatano-Anolles, G., Gresshoff, P.M., 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry 196, 80–83.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., Smalla, K., 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. Applied and Environmental Microbiology 68, 3328–3338.

Berg, G., 2009. Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84, 11–18.

Berg, G., Smalla, K., 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiology Ecology 68, 1–13.

Burton, R.J., Coley-Smith, J.R., 1993. Production and leakage of antibiotics by *Rhizoctonia cerealis*, *R. oryzae-sativae* and *R. tuliparum*. Mycological Research 97, 86–90.

Büttner, G., Pfähler, B., Petersen, J., 2003. *Rhizoctonia* root rot in Europec – incidence, economic importance and concept for integrated control. In: Proceedings of the 1st joint IIRB-ASSBT Congress, San Antonio , pp. 897–901.

Carling, D.E., Kuninaga, S., Brainard, K.A., 2002. Hypal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. Phytopathology 92, 43–50.

Chand, T., Logan, C., 1984. Antagonists and parasites of *Rhizoctonia solani* and their efficacy in reducing stem canker of potato under controlled conditions. Transactions of the British Mycological Society 83, 107–112.

Chernin, L., Ismailow, Z., Haran, S., Chet, I., 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Applied and Environmental Microbiology 61, 1720–1726.

Elad, Y., Chet, I., Katan, J., 1980. *Trichoderma harzianum*: a biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology 70, 119–121.

Gladders, P., Coley-Smith, J.R., 1980. Interactions between *Rhizoctonia tuliparum* sclerotia and soil microorganisms. Transactions of the British Mycological Society 74, 579–586.

Grosch, R., Scherwinski, K., Lottmann, J., Berg, G., 2006. Fungal antagonists of the plant pathogen *Rhizoctonia solani*: selection, control efficacy and influence on the indigenous microbial community. Mycological Research 110, 1464–1474.

Grosch, R., Schneider, J.H., Peth, A., Waschke, A., Franken, P., Kofoet, A., Jabaji-Hare, S.H., 2007. Development of a specific PCR assay for the detection of *Rhizoctonia solani* AG 1-IB using SCAR primers. Journal Applied Microbiology 102, 806–819.

Grube, M., Berg, G., 2009. Bacterial associations with fungi, focusing on the lichen symbiosis. Fungal Biology Reviews 23, 72–85.

Haas, D., Défago, G., 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology 3, 307–319.

Horrigan, L., Lawrence, R.S., Walker, P., 2002. How sustainable agriculture can address the environmental and human health harms of industrial agriculture. Environmental Health Perspectives 110, 445–456.

Jager, G., Velvis, H., Lamers, A., Mulder, A., Roosjen, J., 1991. Control of *Rhizoctonia solani* by biological, chemical, and integrated control measures. Potato Research 34, 269–284.

Keijer, J., Korsman, M.G., Dullemans, A.M., Houterman, P., De Bree, J., Van Silfhout, C., 1997. In vitro analysis of host plant specificity in *Rhizoctonia solani*. Plant Pathology 46, 659–669.

Kiewnick, S., Jacobsen, B.J., Braun-Kiewnick, A., Eckhoff, J.L.A., Bergman, J.W., 2001. Integrated control of *Rhizoctonia* crown and root rot of sugar beet with fungicides and antagonistic bacteria. Plant Disease 85, 718–722.

Kropf, S., Heuer, H., Grüning, M., Smalla, K., 2004. Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. Journal of Microbiological Methods 57, 187–195.

Lahlali, R., Hijri, M., 2010. Screening, identification and evaluation of potential biocontrol fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. FEMS Microbiology Letters 311, 152–159.

Liu, L.N., Zhang, J.Z., Xu, T., 2010. Histopathological studies of sclerotia of *Rhizoctonia solani* parasitized by the EGFP transformant of *Trichoderma virens*. Letters Applied Microbiology 49, 745–750.

Lugtenberg, B., Kamilova, F., 2009. Plant-growth-promoting rhizobacteria. Annual Review of Microbiology 63, 541–556.

Marcello, C.M., Steindorff, A.S., da Silva, S.P., Silva, R.D., Bataus, L.A.M., Ulhoa, C.J., 2010. Expression analysis of the exo-beta-1,3-glucanase from the mycoparasitic fungus *Trichoderma asperellum*. Microbial Research 165, 75–81.

Postma, J., Nijhuis, E.H., Yassin, A.F., 2010. Genotypic and phenotypic variation among *Lysobacter capsici* strains isolated from *Rhizoctonia* suppressive soils. Systematic Applied Microbiology 33, 232–235.

Prusty, R., Grisafi, P., Fink, G.F., 2004. The plant hormone indole acetic acid induces invasive growth in *Saccharomyces cerevisiae*. PNAS 101, 4153–4157.

Rousk, J., Bååth, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., Fierer, N., 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. ISME Journal 4, 1340–1351.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D., Dow, J.M., 2009. Versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nature Microbiology Reviews 7, 514–525.

Sawar, M., Kremer, R.J., 1995. Determination of bacterially derived auxins using a microplate method. Letters in Applied Microbiology 20, 282–285.

Scherwinski, K., Grosch, R., Berg, G., 2008. Effect of bacterial antagonists on lettuce: active biocontrol of *Rhizoctonia solani* and negligible, short-term effect on non-target microbes. FEMS Microbiology Ecology 64, 106–116.
Schwieger, F., Tebbe, C.C., 1998. A new approach to utilize PCR-single strand conformation polymorphism for 16S rRNA gene-based microbial community analysis. Applied and Environmental Microbiology 64, 4870–4876.
Sneh, B., Jabaji-Hare, S., Neate, S., Dijs, G., 1996. *Rhizoctonia* Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control. Kluwer Academie Publishers, Dordrecht, Netherlands.
Toda, T., Mghalu, J.M., Priyatomojo, A., Hyakumachi, M., 2011. Comparison of sequences for the internal transcribed spacer region in *Rhizoctonia solani* AG 1-ID and other subgroups of AG 1. Journal of General Plant Pathology 70, 270–272.
Warmink, J.R., Nazir, R., van Elsas, J.D., 2009. Universal and species-specific bacterial 'fungiphiles' in the mycospheres of different basidiomycetous fungi. Environmental Microbiology 11, 300–312.
Willetts, H.J., 1971. The survival of fungal sclerotia under adverse environmental conditions. Biological Reviews 46, 387–407.
Zachow, C., Fatehi, J., Cardinale, M., Tilcher, R., Berg, G., 2010. Strain-specific colonisation pattern of *Rhizoctonia* antagonists in the root system of sugar beet. FEMS Microbiology Ecology 74, 124–135.