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Fungal antagonists of the plant pathogen *Rhizoctonia solani*: selection, control efficacy and influence on the indigenous microbial community

Rita GROSCH[a], *Katja SCHERWINSKI*[a,b], *Jana LOTTMANN*[b], *Gabriele BERG*[b,c,*]

[a]*Institute of Vegetable and Ornamental Crops (IGZ) Großbeeren/Erfurt e.V., Echtermeyer Weg 1, D-14979 Großbeeren, Germany*
[b]*University of Rostock, Institute of Life Sciences, Microbiology, Albert-Einstein-Str. 3, D-18051 Rostock, Germany*
[c]*Graz Technical University, Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria*

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ABSTRACT

A broad spectrum of fungal antagonists was evaluated as potential biocontrol agents (BCAs) against the soil-borne pathogen *Rhizoctonia solani* using a new combination of *in vitro* and *in vivo* assays. The *in vitro* characterisation of diverse parameters including the ability to parasitise mycelium and to inhibit the germination *of Rhizoctonia* sclerotia at different temperatures resulted in the selection of six potential fungal antagonists. These were genotypically characterised by their BOX-PCR fingerprints, and identified as *Trichoderma reesei* and *T. viride* by partial 18S rDNA sequencing. When potato sprouts were treated with *Trichoderma*, all isolates significantly reduced the incidence of *Rhizoctonia* symptoms. Evaluated under growth chamber conditions, the selected *Trichoderma* isolates either partly or completely controlled the dry mass loss of lettuce caused by *R. solani*. Furthermore, the antagonistic *Trichoderma* strains were active under field conditions. To analyse the effect of *Trichoderma* treatment on indigenous root-associated microbial communities, we performed a DNA-dependent SSCP (Single-Strand Conformation Polymorphism) analysis of 16S rDNA/ITS sequences. In this first assessment study for *Trichoderma* it was shown that the pathogen and the vegetation time had much more influence on the composition of the microbiota than the BCA treatment. After evaluation of all results, three *Trichoderma* strains originally isolated from *Rhizoctonia* sclerotia were selected as promising BCAs.

Introduction

The widespread soil-borne pathogen *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) is responsible for serious damage to many economically important agricultural and horticultural crops as well as trees worldwide (Adams 1988). Over the last 15 y, the significance of *R. solani* has increased in Europe, where the pathogen causes important diseases such as black scurf on potato, late sugar beet rot, bottom rot on lettuce and damping-off diseases on various vegetable crops (Wolf & Verreet 1999). High yield losses were reported, up to 50 % for sugar beet (Kiewnick *et al.* 2001), up to 70 % for field-grown lettuce (Davis *et al.* 1997), and about 20 % for potato (Grosch *et al.* 2005b). *R. solani* strains are ubiquitous and cosmopolitan as saprophytes in soil and as plant pathogens attacking over 500 host species (Ogoshi 1996). The pathogen

* Corresponding author.
E-mail address: gabriele.berg@tugraz.at
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is a species complex composed of different genetic or anastomosis groups (AGs) with a distinct degree of host specificity (Schneider *et al.* 1997; Carling *et al.* 2002). Strategies to control *Rhizoctonia* diseases are limited because cultivars with complete resistance are not available at present (Li *et al.* 1995). Control of the pathogen is difficult because of its ecological behaviour, its extremely broad host range and the high survival rate of sclerotia under various environmental conditions. For this reason, efficient strategies to control the pathogen are urgently required.

Although broad spectrum fungicides as well as *Rhizoctonia*-specific compounds are available, these are not registered for many crops, especially in horticulture and organic farming. Numerous studies have shown that biological control offers an environmentally friendly alternative to protect plants from soil-borne pathogens (Emmert & Handelsman 1999; Whipps 2001; Weller *et al.* 2002). Interestingly, the combination of fungicides with the biocontrol fungus *Verticillium biguttatum* showed additive effects on black scurf control on potato (van den Boogert & Luttikholt 2004). Although the number of biocontrol products is increasing, these products still represent only a very small proportion of fungicides (Fravel 2005). In recent years, both bacterial and fungal antagonists against *R. solani* have been described (Harman 2000; Carisse *et al.* 2001; Howell 2003; Faltin *et al.* 2004). However, many of these showed inconsistent *in vitro* effects and only very few antagonists were analysed under field conditions (Grosch *et al.* 2005a).

Although originating from the terrestrial habitats themselves, antagonists might pose a threat to the indigenous microbiota and their functions if applied inundatively. Consequently, the impact of applied microorganisms on the non-target root-associated microorganisms should be assessed. At present only a minority of 0.1 to 3.0 % of the total microbiota can be analysed using culture-dependent methods (Smalla 2004). Over the last decade, several molecular fingerprint techniques on the basis of 16S/18S rRNA and related genes have been developed, e.g. single-strand conformation polymorphism analysis (Schwieger & Tebbe 1998) which facilitate an investigation of the whole microbial community. Using these methods, the ecological behaviour of a BCA and its interaction with or effect on other organisms can be monitored (Scherwinski *et al.* 2006). Based on this knowledge the biocontrol effect can possibly be stabilised or enhanced (Kubicek *et al.* 2001; Lu *et al.* 2004).

The objective of this work was to select fungal antagonists to control *Rhizoctonia* diseases on the host plants potato and lettuce. We focussed on fungi because of their low degree of host specificity (Berg *et al.* 2005b). Therefore, 390 previously characterised fungal antagonists obtained from different habitats such as bulk soil, the rhizosphere of *Rhizoctonia* host plants as well as tuber-borne *Rhizoctonia* sclerotia of potato (Berg *et al.* 2005b), were screened against different *R. solani* isolates. To select the most efficient isolates, strains were hierarchically assessed according to their antagonistic activity and characterisation *in vitro*. In addition, different *in vivo* trials were carried out including: (1) inhibition of the germination rate of tuber-borne *Rhizoctonia* sclerotia; (2) suppression of *R. solani* on potato sprouts; and (3) suppression of *R. solani* on lettuce. The most promising candidates were applied in field trials. Additionally, their impact on the indigenous microbial communities was analysed by using SSCP (single-stranded conformation polymorphism). The use of molecular tools offers new possibilities to assess the environmental impact of biocontrol agents and is presented for the first time for antagonistic *Trichoderma* strains.

Material and methods

Fungal strains and production of fungal inoculum

The *Rhizoctonia solani* isolates used in this study were isolated from lettuce with bottom rot symptoms (RS 7/3, RS 3/3, AG 1-IB), diseased cabbage (RS W4, AG-2) and potato (RS 3, AG-3). They were routinely grown on potato dextrose agar (PDA; VWR International, Berlin, Germany) and maintained on infested, dried barley seeds stored at −20 °C. Antagonistic fungi investigated in this study ($n = 390$) were isolated in previous studies on the ecology of rhizosphere-associated fungal communities of potential host plants of *Verticillium* and *Rhizoctonia* (Berg *et al.* 2005b). The *in vitro* antagonistic properties of these isolates were determined in a dual culture assay on Waksman agar (Berg *et al.* 2005b). The fungal isolates were cultivated on Sabouraud dextrose agar (SDA; Difco, Detroit) prior to further testing. All isolates were grown in sterile liquid medium (60 % v/v glycerine, 10 % w/v glucose, 2 % w/v pancreatic peptone from casein, 1 % w/v yeast extract) and stored at −80 °C. The fungal isolates used in this study are being maintained in the Strain Collection of Antagonistic Microorganisms (SCAM) at Graz University of Technology (Austria).

For the production of pathogen inoculum, sterilised barley kernels were inoculated with five mycelial discs from a 7 d old *R. solani* culture grown on PDA (Schneider *et al.* 1997). Inoculum of the fungal antagonists was prepared in a mixture of 1:1 (v/v) corn meal and substrate ('Fruhsdorfer Einheitserde' type P; chemical analysis per 100 g: 75 mg N, 75 mg P, and 125 mg K; pH 5.9). Petri dishes (15 cm in diameter) were filled with the sterilised mixture and inoculated with 26 ml (10^6 conidia ml^{-1}) spore suspension of a fungal antagonist produced on PDA. Both pathogen and antagonist inoculum was incubated for 3 wk at 20 °C.

In vitro *assays*

Effect of fungal antagonists on viability of Rhizoctonia solani *mycelium (*in vitro *assay I)*

All antagonistic isolates were tested in a dual culture assay against pathogenic fungi (RS 3/3 and RS W4) on PDA. Agar plugs of *Rhizoctonia solani* and the fungal isolate to be tested were arranged 7 cm apart on agar plates. Inhibition zones and zones of overlapping were assessed after 7 d incubation at 12 and 20 °C. Where an antagonist overgrew mycelium of *R. solani*, the zone of hyphal interaction between both was investigated microscopically (100×). Fungal strains without a microscopically visible effect on mycelium of *R. solani* were excluded from further experiments. Furthermore, the viability of *R. solani* in the region of interaction was tested by transfer of mycelial discs onto water agar plates 5 d after first contact. The *R. solani* mycelium was assessed as viable when the growth of typical hyphae was observed microscopically

(100×). Each experiment was repeated three times with three samples per replicate.

Effect of fungal antagonists on germination of Rhizoctonia solani *sclerotia produced* in vitro *(*in vitro *assay II)*
Effects on the germination of *R. solani* sclerotia were determined according to Mukherjee *et al.* (1999). Briefly, sclerotia of *R. solani* (RS 7/3) of uniform size were placed on a 6 d old culture (PDA, 20 °C) of the fungal antagonist. After incubation for 14, 28 and 35 d at 20 °C, eight sclerotia per replicate (three replicates per antagonist) were transferred from the agar plate onto water agar. Mycelial growth from these sclerotia was assessed under a light microscope (100×).

*Production of cell wall degrading enzymes (*in vitro *assay III)*
The cell wall of the basidiomycete *R. solani* contains mainly chitin and glucan. For this reason, the production of β-glucanases, chitinases and proteases was examined using a synthetic low-nutrient medium as the basis for all substrates (Berg *et al.* 2002). β-glucanase production was tested using a chromogenic glucan (azurine dyed, cross-linked) (AZCL substrates; Megazyme, Bray, Ireland) and chitinase production (β-1,4-glucosamine polymer degradation) was tested on basic medium containing 0.2 % colloidal chitin (Sigma, Deisenhofen, Germany). Protease activity was determined on agar containing 5 % skimmed milk. The formation of blue haloes or clearing zones was detected after 7 d incubation at 20 °C.

In vivo *assays*

Effect of fungal antagonists on germination of tuber-borne Rhizoctonia solani *sclerotia (*in vivo *assay I)*
Potato tubers infected with sclerotia of *R. solani* AG3 (>5 % infestation of tuber surface) were treated with a spore suspension of the fungal antagonist (10^8 conidia ml^{-1}, 25 ml per 15 tubers). The effect of higher inoculum density (10^{10} conidia ml^{-1}) on the viability of tuber-borne sclerotia was also evaluated for two isolates with the highest antagonistic activity at 12 °C and 20 °C. In the control treatment tap water was used instead of conidial suspension. Treated tubers were stored in plastic bags and maintained at 20 °C. Treatments consisted of three replicates with five tubers each. The germination of sclerotia was evaluated 14, 28, 42 and 62 d after treatment with antagonistic strains. Sclerotia (eight per replicate) were harvested from the surface of potato tubers at each time-point and placed onto water agar. Growth of *R. solani* hyphae was observed microscopically (100×).

Suppression of Rhizoctonia solani *on potato sprouts in pot experiments (*in vivo *assay II)*
The ability of antagonistic fungal isolates to control *R. solani* on potato was determined on potato sprouts growing from seed tubers 'Exquisa' in a sand-substrate mixture (SSM). Quartz sand was mixed with the substrate 'Fruhsdorfer Einheitserde' type P at a ratio of 1:1 (v/v). Seed tubers naturally infected with *R. solani* sclerotia (1-5 % infestation) were placed on a 10 cm layer of SSM near the bottom in 12 × 12 × 20 cm plastic pots and covered with approximately 9 cm of SSM. For inoculation of fungal antagonists the SSM was mixed at a ratio of 10:1 (v/v) with the inoculum of fungal antagonist (as described below) 2 d before planting the seed tubers. For control treatments the SSM was mixed with a sterile cornmeal substrate mixture. The tubers were also treated with a spore suspension of the fungal antagonist (10^8 conidia ml^{-1}, 10 ml per six tubers) 2 wk before planting, and stored at 20 °C. The experiments were repeated twice. In another experiment using surface-sterilised tubers (1 % NaOCl for 1 min), only the substrate was inoculated with fungal antagonists at a ratio of 10:1 (v/v) 2 d before planting, and six barley kernels infested with *R. solani* (isolate RS 3) were individually placed 2 cm above each seed tuber and covered with 7 cm of SSM at planting time. The untreated control consisted of sterilised, non-infested barley kernels. In both experiments the potato tubers were incubated for 3 wk after planting at 20 °C in the dark in a phyto-chamber (Kelvitron®kl, Kendro GmbH, Langenselbold, Germany). Water was applied to the sand-substrate mixture as required. Each treatment included six replicates in a randomised design. The disease severity (DS) was assessed on a scale from 1 to 5 (1 = no symptoms, 2 = lesions <5 mm, 3 = lesions >5 mm, 4 = severe damage on whole sprout, 5 = all sprouts dead).

Suppression of Rhizoctonia solani *on potato in the field (*in vivo *assay III)*
Antagonists G1/8 and G3/2 were tested against black scurf disease on potato 'Exquisa' in a field showing natural *R. solani* infestation (Großbeeren near Berlin, 52°31′N, 13°24′E; loamy sand). In addition, for our experiment parts of the field were artificially infested with *R. solani* (isolate Ben3). Each treatment included six replicates with 21 potato plants each in a randomised design. For each antagonist, 126 seed tubers were treated with 130 ml spore suspension (10^9 conidia ml^{-1}) at planting time. Four *R. solani* infested barley kernels were placed on seed tubers that were previously covered with a 4 cm soil layer. Tuber yield and the degree of tuber infestation with *Rhizoctonia* sclerotia were assessed after a cultivation time of 17 wk (July to November), using scores of 1 (no infested tubers), 2 (<1 % infested), 3 (1-5 %) and 4 (>5 %). The average DS was calculated from 120 randomly selected potato tubers (20 tubers per replicate).

Suppression of Rhizoctonia solani *on lettuce in a growth chamber (*in vivo *assay IV)*
The effect of selected antagonists on plant growth and disease incidence (DI) caused by *R. solani* was investigated in three repeated growth chamber experiments. Lettuce seedlings 'Daguan' (S 5601, Syngenta, Bad Salzuflen, Germany) were cultivated in the greenhouse to the 2-3 leaf growth stage at 20/15 °C (16/8 h day/night) in a plant container with 92 seedlings and then maintained in pots (500 ml) filled with substrate ('Fruhsdorfer Einheitserde' type P) in a growth chamber (20/15 °C, 60/80 % relative humidity, 16/8 h day/night cycle, 500 $\mu mol\ m^{-2}\ s^{-1}$; York, Mannheim, Germany). In the plant container, lettuce seedlings were treated with a spore suspension (5 ml per plant, 10^3 conidia ml^{-1}) of the fungal antagonist one week prior to transplanting. Also at planting time the substrate in the pots was mixed with inoculum (as described below) of the fungal antagonist at a ratio of 10:1 (v/v). Three barley kernels per seedling, infested with *R. solani*,

were used as pathogen inoculum at planting time. The control treatment consisted of sterilised, non-infested barley kernels. The kernels were placed 1 cm deep at a distance of 2 cm from each plant. The pots were watered every day to maintain the substrate moisture. Each treatment included six replicates with four plants each, arranged in a randomised design. The DI was assessed at weekly intervals by recording the number of diseased lettuce plants based on visual symptoms. Four weeks after inoculation, the dry mass (DM) of each lettuce plant was measured.

Molecular characterisation and identification of antagonistic strains

Prior to molecular characterisation, all isolates were grouped by their appearance on SDA or Malt Agar (MA, Merck, Darmstadt, Germany) on the basis of colony morphology, production of pigments, conidiophores or other microscopic features according to Domsch *et al.* (1980). Fungal DNA was isolated from mycelium grown on SDA for one week (Berg *et al.* 2005b). BOX-PCR was carried out as described by Rademaker & De Bruijn (1997) using the BOXA1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. PCR amplification was performed with a TGradient thermocycler (Biometra, Germany) using an initial denaturation step at 95 °C for 6 min, and subsequently 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 65 °C for 8 min followed by final extension at 65 °C for 16 min. A 12 µl aliquot of amplified PCR product was separated by gel electrophoresis on 1.5 % agarose gels in 0.5 × TBE buffer for 4 h at 90 V, stained with ethidium bromide, and photographed under UV transillumination. The reproducibility of the results was verified in two independent experiments. Fungal DNA was amplified by PCR using the fungus-specific primers NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and reverse primer FR1 (5′-AIC CAT TCA ATC GGT AIT-3′) for amplification of the 18S rRNA gene (Vainio & Hantula 2000). The 25 µl reaction mixture contained at least 5 µl Taq-&GO PCR Mastermix (Qbiogene), 18 µl sterile distilled water, 0.5 µl of each primer and 1 µl template (ca. 50 ng). PCR reactions were performed in a TGradient thermocycler using one initial cycle at 95 °C for 8 min; 35 cycles of denaturation (30 sec at 94 °C), annealing (45 sec at 48 °C), extension (3 min at 72 °C); and a single final extension cycle at 72 °C for 10 min followed by a final soak at 4 °C. The PCR products were purified with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). DNA fragments were sequenced by GATC Biotech AG (Konstanz, Germany). The 18S rRNA gene sequences were aligned with sequences of the NCBI sequence databases using the BLAST algorithm (Altschul *et al.* 1997). Accession numbers for sequences of antagonistic *Trichoderma* isolates submitted to the EMBL database are AJ810269 and AJ810422–AJ810427.

Molecular analysis of total community DNA by Single-Strand Conformation Polymorphism analysis (SSCP)

To assess the potential impact of antagonist treatments on indigenous potato-associated microorganisms the total microbial community of the potato rhizosphere and the endorhiza was evaluated 4 wk and 17 wk after the BCA treatment. For this, 5 g fresh roots with adhering soil were taken from three replicates per treatment (G1/8, G3/2, artificially infected control, naturally infested control). The DNA of microbial cell consortia was extracted according to Scherwinski *et al.* (2006). Fingerprinting of potato-associated communities by SSCP was carried out as described by Schwieger & Tebbe (1998). Briefly, bacterial communities were analysed using the universal eubacterial primer pair Unibac-II-515f/Unibac-II-927rP (Lieber *et al.* 2002). A nested PCR was applied to obtain genetic fingerprints of fungal communities. In a first PCR the fungus-specific primer pair ITS1f/ITS4rP (White *et al.* 1990) was used, whereas the primer pair ITS1f/ITS2rP (White *et al.* 1990) was used for the second PCR. The amplicons were separated at 400 V and 26 °C in 8 % acrylamide gels for bacterial DNA and 9 % acrylamide gels for fungal DNA, using the TGGE Maxi System (Biometra, Göttingen, Germany). Silver staining was used to detect DNA in SSCP gels (Bassam *et al.* 2001). Three independent samples were analysed to ensure the reproducibility of the results.

Statistical analysis

The STATISTICA programme (StatSoft, Tulsa, OK) was used for the statistical analysis of the biocontrol data. The lettuce DM, the DI on lettuce in the pot experiments, the potato tuber mass (TM) and the frequency of DS categories on potato tubers were compared with the control after ANOVA using the LSD test with $P = 0.05$. The DS on both potato sprouts and tubers and the assessed number of germinated sclerotia were analysed using the non-parametric Kruskal-Wallis test with $P < 0.05$.

The computer-assisted evaluation of BOX-PCR generated fingerprints was carried out using the GelCompar® software (Applied Maths, Version 4.1, Kortrijk, Belgium). The silver-stained SSCP gels were scanned transmissively (Casba™4 scanner, Spiral Biotech Inc., USA) to obtain digitised gel images that could be processed using the GelCompar® software. DNA-standards (Fermentas GmbH, St. Leon-Rot, Germany) were added onto each gel to admit post-run correction of possible gel-specific differences in band separation using the normalisation function of the GelCompar® software. After gel normalisation and background subtraction, similarity matrices were calculated using the band-based Dice similarity coefficient (Dice 1945). Dendrograms were constructed with the hierarchical cluster method of unweighted pair group method using average linkages (UPGMA).

Results

Antagonistic in vitro activity and pathogen-antagonist interaction

Altogether 390 fungal strains were tested against *R. solani* isolates in dual culture using *in vitro* assay I. For the initial screening two *Rhizoctonia* strains (RS 3/3, RS W4) were used, differing in their sensitivity towards antagonistic microorganisms (Faltin *et al.* 2004). Among the 35 strains showing antifungal activity towards both *R. solani* strains, inhibition zones due

to direct antibiosis were observed for 17 fungal strains. Due to problems with antibiotic-producing BCAs in application and registration, these isolates were excluded from further studies. The remaining 18 fungal strains were able to overgrow the mycelium of both *R. solani* isolates and were selected and characterised in further detail.

The effect of these 18 strains on the growth and survival of *R. solani* hyphae was analysed at 20 °C (optimum temperature) and 12 °C (soil-relevant temperature) using *in vitro* assay I. Of eight strains able to completely inhibit the growth of both *R. solani* isolates at 20 °C (data not shown), four strains (RE1-3-4, RB2-3-13, G1/8 and G3/2) achieved complete inhibition also at 12 °C (Table 1).

All eight selected BCA strains showed strong glucanolytic as well as proteolytic activity, but only strains PR26-12-6 and RB1-2-18 also produced chitinases (data not shown).

Using *in vitro* assay II, all fungal strains except RB2-3-9 inhibited the germination of sclerotia within 14 d (Fig 1), with G1/8 and RB2-3-13 showing the strongest inhibition. All eight antagonists displayed complete inhibition of sclerotial germination after 28 d (data not shown).

Due to their weak antifungal activity in *in vitro* assays I and II, strains RB2-3-9 and RB1-2-18 were excluded from further investigations. The remaining six strains were originally isolated from the rhizosphere of the two *Rhizoctonia* host plants potato (PR26-12-6) and strawberry (RE1-3-4), from bulk soil (RB2-3-13) or directly from *Rhizoctonia* sclerotia (G1/8, G1/9, and G3/2).

Molecular characterisation and identification of fungal strains

All six selected antagonists belonged to the genus *Trichoderma*, partial sequencing of the 18S rDNA genes (Table 1) identifying them as *T. viride* (PR26-12-6, RE1-3-4, G1/9, G3/2) and *T. reesei* (RB2-3-13, G1/8).

BOX-PCR pattern of the isolates were compared to obtain unique molecular fingerprints for quality control during this study and potential patent licensing. Although similarities in the BOX-pattern between strains G1/8, G3/2 and RE1-3-4 were observed, a unique fingerprint could be obtained for each strain (Fig 2).

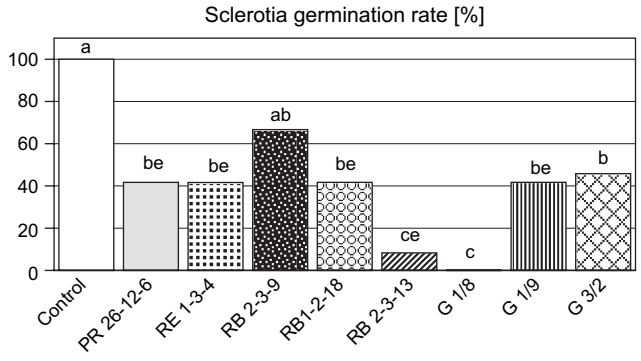

Fig 1 – Effect of *Rhizoctonia* antagonists on the germination of in vitro produced *Rhizoctonia* sclerotia after 14 d at 20 °C on PDA. Letters represent significant differences compared with the untreated control according to the Kruskal-Wallis test ($P < 0.05$).

Antagonistic activity against Rhizoctonia *in different* in vivo *assays*

In experiments using potato tubers that have been naturally infested (experiments I and II) and artificially infected (experiment III) with *Rhizoctonia solani*, in all *Trichoderma* treated variants the DS on potato sprouts was significantly reduced in comparison with the untreated control (Table 2). However, both the DS and the disease suppression effects of *Rhizoctonia* antagonists varied between the experiments. Altogether, none of the antagonists was able to significantly suppress the DS on sprouts in all experiments. Significant suppression effects of 50 % and 43 % relative to the untreated control were achieved by the BCAs G1/8, PR26-12-6 and G1/9, respectively.

In a second *in vivo* assay, potato tubers infected with *R. solani* sclerotia were treated with a conidial suspension of each antagonist. In comparison with water-treated sclerotia (control), all tested fungal BCAs significantly reduced the germination of tuber-borne sclerotia (Table 3). Differences were observed for different BCA treatments. Strains G1/8 and G3/2 were the most strongly inhibitory, reducing sclerotial germination to 50 % and 53 %. In addition, the incubation period had an

Table 1 – *In vitro* characterisation and identification of *Rhizoctonia* antagonists

Strain code	Viability of mycelium (%)[a]				Origin	Species	Closest match with Gen Bank	Homology (%)
	At 12 °C		At 20 °C					
	RS 3/3	RS W4	RS 3/3	RS W4				
PR26-12-6	0	0	2.3[b]	33.3	rhizosphere, potato, Groß Lüsewitz	*Trichoderma viride*	AF525230	100
RE1-3-4	0	0	0	0	rhizosphere, strawberry, Rostock	*Trichoderma viride*	AF164357	99
RB2-3-9	100.0	88.9	0	0	bulk soil, Rostock	*Trichoderma reesei*	AF510497	100
RB1-2-18	88.9	88.9	0	0	bulk soil, Rostock	*Trichoderma viride*	AF525230	100
RB2-3-13	0	0	0	0	bulk soil, Rostock	*Trichoderma reesei*	AF510497	99
G1/8	0	0	0	0	*R. solani* sclerotia on potato tuber	*Trichoderma reesei*	AF548103	98
G1/9	0	0	0	33.3	*R. solani* sclerotia on potato tuber	*Trichoderma viride*	AF164357	99
G3/2	0	0	0	0	*R. solani* sclerotia on potato tuber	*Trichoderma viride*	AF525230	99

a viability of the *Rhizoctonia* mycelia RS 3/3 and RS W4 after contact with the fungal antagonists on water agar plates.
b inhibition zone in mm.

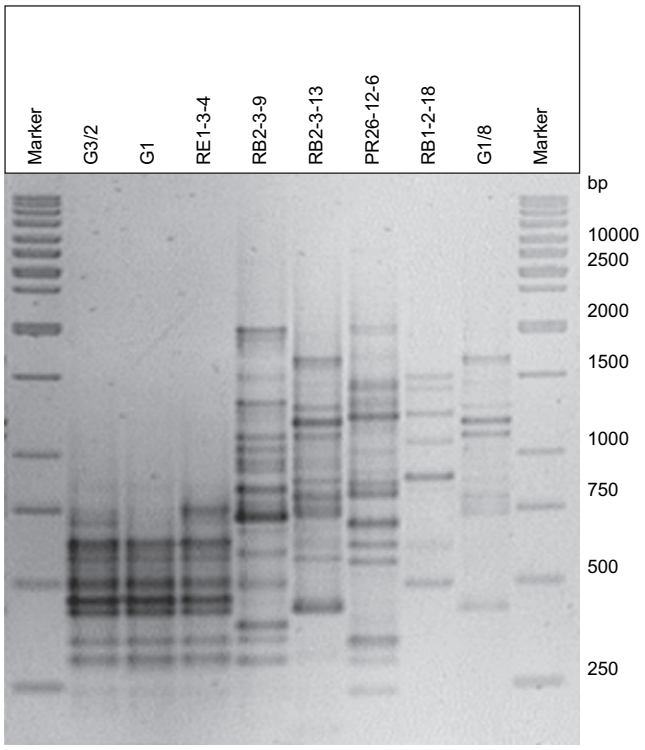

Fig 2 – BOX patterns of antagonistic *Trichoderma* isolates. Marker represents 1 kb ladder.

influence on the effect. Furthermore, inhibition by BCA treatments could be enhanced by higher application densities (Fig 3). A complete inhibition of the germination of sclerotia was observed 56 d after treatment of infected potato tubers with the BCA G1/8 (Fig 3a). With an average germination of 10.4 % at 20 °C and 21.9 % at 12 °C, strain G1/8 was more effective than G3/2 with an average rate of 47.4 % at 20 °C and 26.1 % at 12 °C.

In the *in vivo* assay IV, all fungal antagonists were able to significantly reduce the impact of *R. solani* on lettuce DM in experiment I (Table 4). In contrast, there was no effect of RE1-3-4 and RB2-3-13 in experiment II and of RB2-3-13 and G1/9 in experiment III. Altogether, antagonist G1/8 was the most efficient in all experiments.

Table 3 – Effect of *Rhizoctonia* antagonists on the germination of tuber-borne *Rhizoctonia* sclerotia

Treatment	Germinated sclerotia [%]				
Time	14 d	28 d	42 d	56 d	Mean
Control[a]	93.3	100.0	91.7	91.7	94.2
PR26-12-6	100.0	50.0*	45.8*	87.5	70.8*
RE1-3-4	73.7	69.2*	50.0*	n.d.	64.3*
RB2-3-13	80.0	41.7*	45.8*	62.5	57.5*
G1/8	73.3	62.5*	33.3*	29.1*	49.6*
G1/9	76.4	58.3*	29.2*	66.7	57.6*
G3/2	66.7	41.7*	29.2*	75.0	53.1*

Note: *Significant differences compared with the control according to the Kruskal-Wallis test ($P < 0.05$).
a treatment with water.

Field trial and effect of fungal antagonists on the indigenous microbial community

The disease-suppressive effect of fungal isolates G1/8 and G3/2 on *Rhizoctonia solani* was tested on potato tubers in a field experiment. No differences in tuber yield were observed between treatments (Table 5). However, the disease severity on harvested potato tubers was significantly reduced by the application of both antagonists in comparison to the artificially *R. solani*-infected control (Table 5).

A culture-independent analysis of potato-associated microbial communities was carried out in order to assess the impact of the BCA treatment on the indigenous microorganisms (Figs 4 and 5). Using diverse primers the SSCP patterns revealed a high microbial diversity in the rhizosphere and endorhiza of potato. In general, bacterial and fungal SSCP patterns showed a strong similarity between replicates, with no specific impact of BCA treatment observed. Changes in microbial community composition were primarily found

Table 2 – Effect of *Rhizoctonia* antagonists on disease severity (DS) on potato sprouts 'Exquisa' growing on tubers that have been naturally (experiments I and II) and artificially (experiment III) infected with *R. solani*

Treatment	Experiment I[b]		Experiment II[b]		Experiment III[c]		Mean	
	DS	SD[a]	DS	SD	DS	SD	DS	SD
R. solani-infested control	2.3	0.3	3.5	0.9	2.7	0.4	3.0	0.8
PR26-12-6	2.0	0.8	1.6*	0.8	1.5*	0.3	1.7*	0.7
RE1-3-4	1.8	0.8	2.4*	1.1	1.9*	0.4	2.1*	0.9
RB2-3-13	1.5*	0.5	2.0*	0.9	2.0	0.5	1.9*	0.8
G1/8	1.8	0.9	1.4*	0.7	1.6*	0.6	1.5*	0.7
G1/9	2.0	0.8	1.7*	0.8	1.5*	0.3	1.7*	0.7
G3/2	1.3*	0.5	1.9*	0.9	2.2	0.5	1.8*	0.8

Note: DS was assessed on a scale from 1 to 5 (1 = no symptoms, 2 = lesions <5 mm, 3 = lesions >5 mm, 4 = strong damages on whole sprout, 5 = all sprouts dead).
*Significant effect according to Kruskal-Wallis test ($P < 0.05$) in comparison with the control.
a Standard deviation.
b Naturally infected seed tubers were used.
c Tubers were artificially inoculated with *R. solani*.

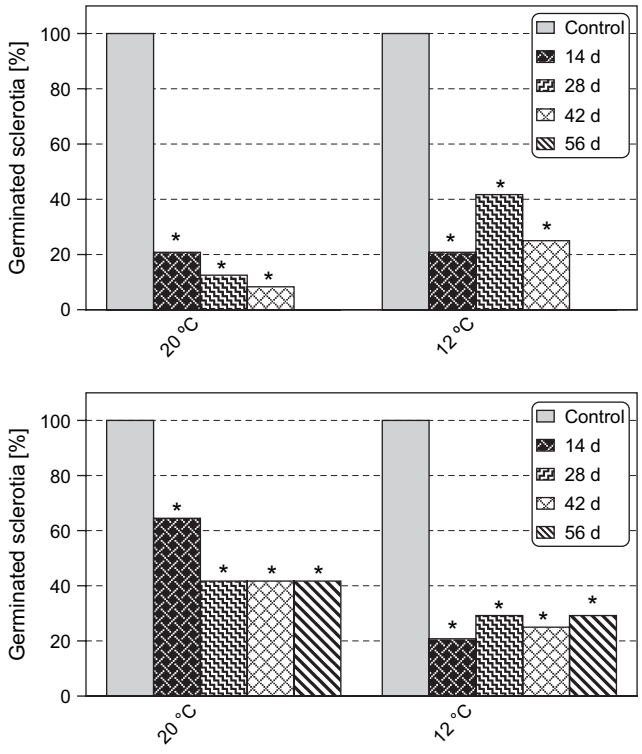

Fig 3 – Effect of fungal antagonists G1/8 (A) and G3/2 (B) on the germination of tuber-born *Rhizoctonia* sclerotia. Potato tubers (cv. Exquisa) were treated with a conidial suspension (10^{10} conidia ml^{-1}) of each antagonist. *Significant differences compared with the untreated control according to the Kruskal-Wallis test ($P < 0.05$).

in correlation with different plant growth stages. Additionally, treatment-dependent compositions of the fungal communities were found 4 wk after BCA application. However, this was only a temporary effect and did not appear in the second sampling after 17 wk. Fig 5 shows a cluster analysis of SSCP fingerprints of the ascomycete communities of treated and untreated plants. Furthermore, SSCP patterns in the rhizosphere and endorhiza showed that the pathogen inoculum had a strong influence on the composition of microbial communities. This influence tended to increase during the vegetation phase. Examples are shown for the total bacterial community of the endorhiza (Fig 4) as well as for the ascomycete community of the rhizosphere (Fig 5), 17 wk after treatment.

Overall assessment of the selected isolates

For a general assessment we used the following *in vivo* results: (1) disease suppression effect on potato sprouts, (2) inhibition of germination rate of tuber-borne *Rhizoctonia* sclerotia; and (3) disease suppression effect on lettuce. The highest possible ranking was 20 points. According to these criteria the most efficient strain was *Trichoderma reesei* G1/8 (20 points) followed by *T. viride* G3/2 (16), *T. viride* G1/9 (12), *T. viride* PR26-12-6 (11), *T. viride* RE1-3-4 (10) and *T. reesei* RB2-3-13 (7).

Discussion

In the present study, fungal isolates from different terrestrial and plant-associated habitats were evaluated to control diseases caused by *Rhizoctonia solani*. The objective was to select fungal antagonists active against the pathogen in soil and/or in the rhizosphere by destroying *R.* sclerotia or mycelium in order to avoid root infection. The present study resulted in the selection of three potential BCAs, *Trichoderma reesei* G1/8 and *T. viride* G3/2 and G1/9, which were able to suppress *Rhizoctonia solani* under different conditions. Interestingly all three strains were isolated from potato tuber-borne sclerotia of *Rhizoctonia*. The potential of previously unexamined ecological niches as sources of antagonists has been discussed by Opelt & Berg (2004).

Altogether, 390 fungal strains with antagonistic properties were screened against *R. solani in vitro*. Only 35 of them were active against different isolates of the pathogen *in vitro*. Species- or strain-specific mechanisms of pathogen-BCA interaction could explain the low number of effective antagonists. In a study analysing autochthonous antagonists of sugar beet, an extremely high specificity of antagonists against several fungal pathogens of the crop was determined, and only a very low number (1.6 %) of antagonists with a broad host range were found (unpubl.). Surprisingly, all active strains belonged to the genus *Trichoderma*, even though a broad spectrum of naturally occurring fungal antagonists, including genera like

Table 4 – Effect of *Rhizoctonia* antagonists on dry mass (DM) of lettuce 'Daguan' and the disease incidence (DI)

Treatment	Experiment I		Experiment II		Experiment III	
	DM [g/plant]	DI [%]	DM [g/plant]	DI [%]	DM [g/plant]	DI [%]
Untreated control	2.1 a	-	5.5 a	-	6.9 a	-
R. solani-infested control	1.4 b	100 a	3.1 b	100 a	4.1 b	83.3 a
PR26-12-6	2.1 a	41.5 b	6.0 a	95.7 a	5.3 c	83.3 a
RE1-3-4	2.0 a	62.5 b	4.0 bc	100 a	6.6 ad	79.0 a
RB2-3-13	2.3 a	70.7 a	3.3 b	100 a	5.0 bc	79.0 a
G1/8	2.4 a	87.5 a	6.1 a	100 a	7.4 d	70.7 a
G1/9	2.3 a	50.0 b	4.8 ac	100 a	4.0 b	87.5 a
G3/2	2.0 a	37.5 b	5.1 ac	83.2 b	5.2 c	75.0 a

Note: DM and percentage DI followed by the same letter are not significantly different according to the LSD test ($P = 0.05$).

Table 5 – Effect of *Rhizoctonia* antagonists on tuber mass (TM) of potato 'Exquisa' and disease severity (DS) of harvested tubers with *R. solani* (R.s.) sclerotia and frequency of assessed DS categories after artificial inoculation with *R. solani* (isolate RS 7/3) at planting time

Treatment	TM [kg/plant]	DS [%]	Frequency of DS categories [%]			
			DS-1	DS-2	DS-3	DS-4
R. solani-infested control	0.59 a	2.5 a	5.8 a	43.3 a	45.8 a	4.9
G1/8 + R.s.	0.53 a	1.7 b	41.6 b	53.2 b	5.2 b	-
G3/2 + R.s.	0.47 a	1.8 b	34.4 b	54.2 b	11.5 b	-

Note: The means in the TM and frequency of DS categories followed by the same letter are not significantly different in the LSD test ($P = 0.05$), and means in the DS column are not significantly different according to Kruskal-Wallis test ($P < 0.05$). DS was assessed as 1 (no sclerotia) 2 (<1 % sclerotial infestation), 3 (1-5 %), and 4 (>5 %).

Monographella, *Paecilomyces* and *Penicillium* (described in Berg *et al.* 2005b), were included in our study. The genus *Trichoderma* comprises a large species complex, and biocontrol strains uniformly called *T. harzianum* in the past are probably genetically diverse species (Druzhinina & Kubicek 2005). The potential of *Trichoderma* strains as biocontrol agents against plant diseases, including those caused by *R. solani*, has a long history (Weindling 1932; Elad *et al.* 1982; Henis *et al.* 1984; Harman *et al.* 2004), and clearly dominates the literature on antagonistic fungi (Whipps 2001). *Trichoderma* isolates are able to parasitise hyphae, sclerotia and other fungal structures, and many of these observations are linked with biocontrol. Additionally, many *Trichoderma* strains produce antifungal metabolites known to enhance competitiveness, and are able to induce resistance in their host plant (Yedida *et al.* 1999; Howell 2003). However, *Trichoderma* isolates show a strain-specific mode of action and their interaction with fungal pathogens as well as their host plants has been characterised only partially (Harman *et al.* 2004). The *Trichoderma* isolates examined in the present study belong to the group of efficient hyperparasites. According to our preliminary observations, they are also able to interact with their host plants, and this aspect warrants further examination.

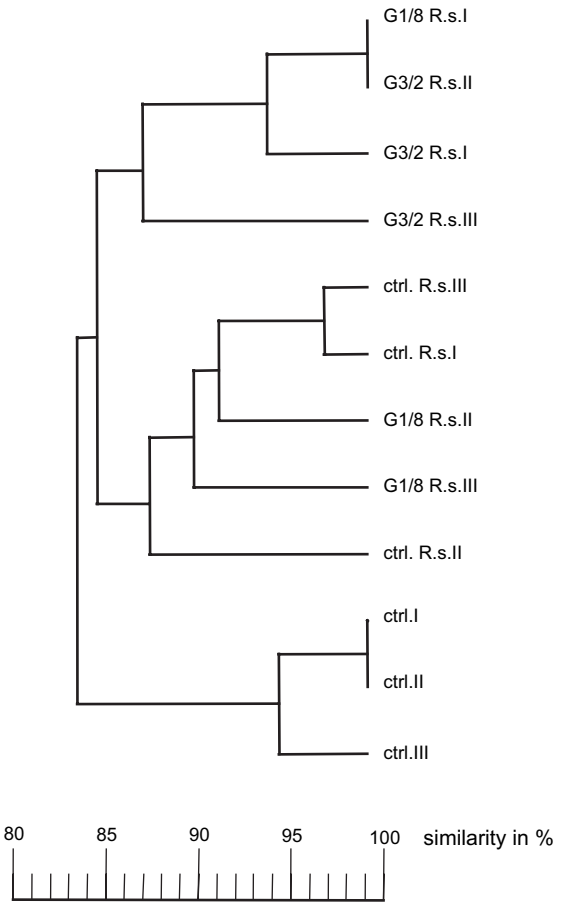

Fig 4 – Cluster analysis of SSCP fingerprints of endorhiza bacterial communities, 17 wk after addition of antagonists analysed in three independent replicates (I-III). Potatoes treated with fungal antagonists (G1/8; G3/2) and infected with *Rhizoctonia solani* (R.s.). Clustering was carried out using the UPGMA method following the calculation of the Dice similarity coefficent.

The use of a cultivation-independent method, the single-strand conformation polymorphism (SSCP) analysis (Schwieger & Tebbe, 1998) offers a simple, inexpensive and sensitive method to analyse the influence of a BCA on the indigenous microflora of host plants. These investigations are important to assess the potential risk of the BCA on the environment and should be included in a pre-commercial phase in biocontrol studies (Scherwinski *et al.* 2006). In contrast to other molecular fingerprinting methods, SSCP avoids heteroduplex formation during electrophoresis and does not require the construction of compound gradient gels. It guarantees the reproducibility and comparability of gels. By analysing both bacterial and fungal communities, similar patterns in treated and untreated plants were obtained in the present study. BCA application therefore did not appear to have a long-lasting impact on the diversity of indigenous microbial communities. Similar results have been obtained in analyses of bacterial rhizosphere communities of transgenic potatoes treated with antagonistic bacterial strains (Lottmann *et al.* 2000), and of bacterial and fungal communities associated with the BCA *Serratia plymuthica* HRO-C48 (Scherwinski *et al.* 2006).

In our experiments reported here, native microbial communities were clearly influenced by pathogen inoculum. This was also observed by Reiter *et al.* (2002) who found that the composition of potato endophytes was strongly affected by the presence of the phytopathogenic bacterium *Erwinia carotovora* subsp. *atroseptica*. Furthermore, we have observed responses of the plant-associated microbiota to the growth stage of the host plant, and these effects are well-known elsewhere. Finally, the existence of microenvironment-specific

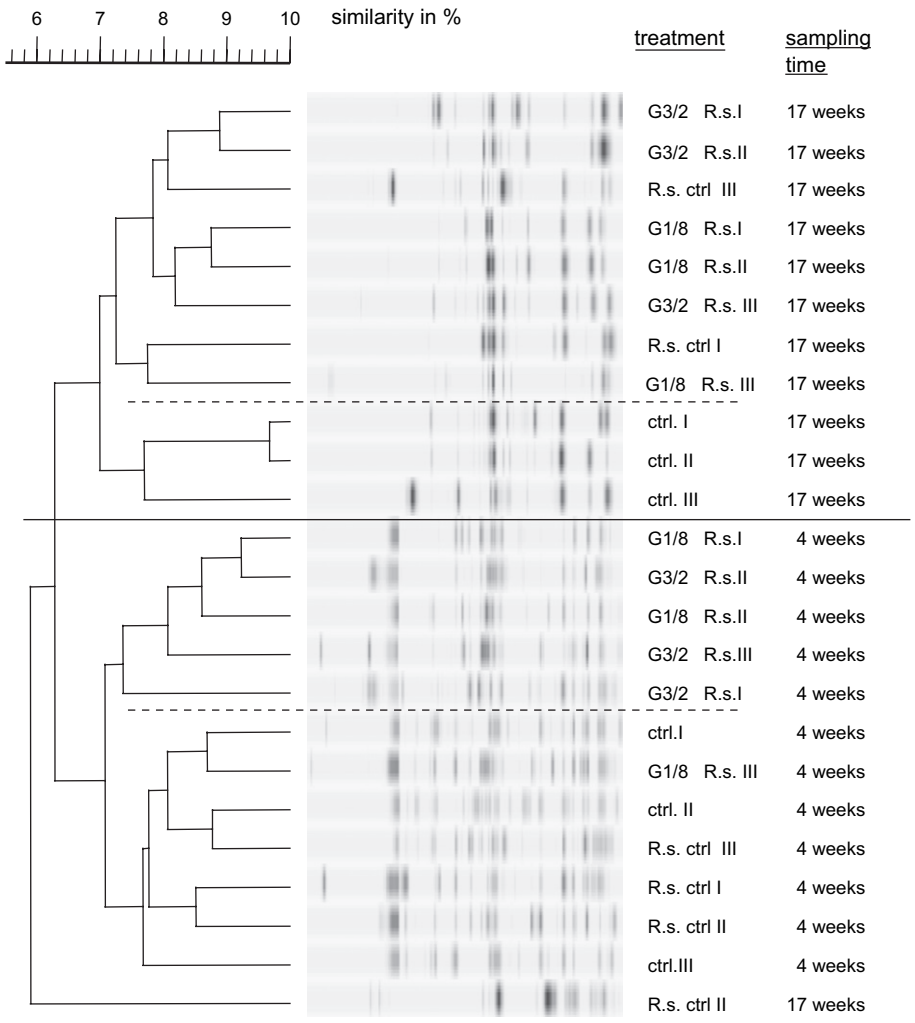

Fig 5 – Cluster analysis of SSCP fingerprints of the ascomycete communities from the rhizosphere 4 and 17 wk after inoculation analysed in three independent replicates (I-III). Potatoes treated with fungal antagonists (G1/8; G3/2) and infected with Rhizoctonia solani (R.s.). The Dice similarity coefficent was calculated before clustering using UPGMA method.

patterns, as found in the present study for the rhizosphere and endosphere, has been described by Berg *et al.* (2005a).

In a previous study we described endo- and ectophytic rhizobacteria as *Rhizoctonia* antagonists and used these to protect roots from *R. solani* infections (Faltin *et al.* 2004; Grosch *et al.* 2005a), whereby the success of biocontrol agents was highly specific for each pathosystem (Grosch *et al.* 2005a). Although in the present study we also detected different levels of antagonism on the two host plants studied, such a BCA-host plant specificity cannot be generalised for fungal antagonists. Rather, we have found evidence of bacterial antagonists possessing a higher degree of plant specificity than fungi (Berg *et al.* 2002; Berg *et al.* 2005b). One of our further objectives is to combine bacterial and fungal antagonists to achieve a higher rate of disease control. Multipartner-biocontrol with synergistic modes of action and a different ecological behaviour, as already suggested by Bakker (1991), might be more effective than the application of only one type of microorganism. This could be a promising alternative for future biocontrol strategies, especially to control pathogens with a high ecological versatility such as *Rhizoctonia*.

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