Strategy to select and assess antagonistic bacteria for biological control of *Rhizoctonia solani* Kühn

Franziska Faltin, Jana Lottmann, Rita Grosch, and Gabriele Berg

Abstract: A screening strategy was developed to assess the potential of plant-associated bacteria to control diseases caused by *Rhizoctonia solani* Kühn. About 434 already characterized antagonistic bacterial strains isolated from diverse plant species and microenvironments were evaluated for biocontrol and plant growth promotion by a hierarchical combination of assays. Analyzing in vitro antagonism towards different *Rhizoctonia* isolates resulted in a selection of 20 potential biocontrol agents. The strains were characterized by their antagonistic mechanisms in vitro as well as their production of the plant growth hormone indole-3-acetic acid. The plant growth promoting effect by antagonistic bacteria was determined using a microtiter plate assay on the basis of lettuce seedlings. Lettuce and sugar beet as host plant were included in the biocontrol experiments in which the antagonistic effect of 17 bacterial isolates could be confirmed in vivo. Sequencing of the 16S rDNA gene and (or) fatty acid methyl ester gas chromatography was used to identify the antagonistic isolates. Molecular fingerprints of isolates obtained by BOX – polymerase chain reaction were compared to avoid further investigation with genetically very similar strains and to obtain unique molecular fingerprints for quality control and patent licensing. According to our strategy, an assessment scheme was developed and four interesting biological control agents, *Pseudomonas reactans* B3, *Pseudomonas fluorescens* B1, *Serratia plymuthica* B4, and *Serratia odorifera* B6, were found. While *S. plymuthica* B4 was the best candidate to biologically control *Rhizoctonia* in lettuce, *P. reactans* B3 was the best candidate to suppress the pathogen in sugar beet.

Key words: biocontrol, *Rhizoctonia solani*, lettuce, sugar beet, antagonistic bacteria.

Résumé : Nous avons élaboré une stratégie de criblage afin d'évaluer le potentiel bactéries associées aux plantes de contrôler des maladies causées par *Rhizoctonia solani* Kühn. Environ 434 souches de bactéries antagonistes déjà caractérisées ont été isolées à partir de diverses espèces végétales et de microenvironnements et leurs capacités de stimulation de la croissance végétale ainsi que de biocontrôle ont été évaluées par une combinaison hiérarchique d'analyses. Les analyses d'antagonisme in vitro envers divers isolats de *Rhizoctonia* ont permis la sélection de 20 agents potentiels de biocontrôle. Les mécanismes d'antagonisme in vitro de ces souches ont été caractérisés de même que leur production de l'hormone de croissance végétale, l'acide indole-3-acétique. La stimulation de la croissance végétale par les bactéries antagonistes a été déterminée à l'aide d'un test sur microplaque basé sur des pousses de laitue. La laitue et la betterave à sucre ont été utilisée comme plantes-hôtes dans les expériences de biocontrôle, dans lesquelles l'antagonisme de 17 isolats bactériens a pu être confirmé in vivo. Le séquençage de l'ADNr 16S et/ou le FAME–GC ont été utilisé afin d'identifier les isolats antagonistes. Les empreintes moléculaires des isolats obtenus par BOX–PCR ont été comparées afin d'éviter des analyses subséquentes de souches très proches au plan génétique et d'obtenir des empreintes moléculaires uniques pour le contrôle de la qualité et la concession de licences de brevet. Un schéma d'évaluation a été conçu à partir de notre stratégie et quatre agents de biocontrôle intéressants ont été trouvés: *Pseudomonas reactans* B3, *P. fluorescens* B1, *Serratia plymuthica* B4 et *S. odorifera* B6. Bien que *S. plymuthica* B4 étaient le meilleur candidat pour combattre *Rhizoctonia* dans la laitue, *P. reactans* B3 étaient le meilleur candidat pour supprimer le pathogène dans la betterave à sucre.

Mots clés : biocontrôle, *Rhizoctonia solani*, laitue, betterave à sucre, bactéries antagonistes.

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Introduction

The very common soil-borne pathogen *Rhizoctonia solani* Kühn is responsible for yield losses on an extremely wide host range of economically important crops as well as in horticulture and agriculture worldwide. The species complex *R. solani* is grouped into 12 genetic groups called anastomosis groups (AGs) according to hyphal anastomosis reaction between isolates of the same group (Anderson 1982; Carling et al. 2002). The AGs showed a distinct degree of host specificity (Schneider et al. 1997; Keijer et al. 1997). The control of *R. solani* is difficult because of the ability to survive as sclerotia under adverse soil environmental conditions for many years in soil, its ability of saprophytic activity, and its extremely wide host range. Particularly in horticulture, no fungicides are registered, and in organic farming, no control methods are currently available. That is why new alternative strategies to control the pathogen are urgently necessary.

It is well documented that an environmentally friendly alternative to protect plants against soil-borne pathogens is bacteria-mediated biological control (Weller 1988; Emmert and Handelsman 1999). However, only a few biocontrol products are currently on the market (Whipps 1997). Most biological control agents (BCAs) for *Rhizoctonia* described previously belong to fungi, e.g., to the genus *Trichoderma* (Elad et al. 1980; Harman 2000; Lewis et al. 1998; Ahmed et al. 2003), and only a few bacteria have been reported to control *Rhizoctonia*, e.g., *Bacillus licheniformi*s (Ahmed et al. 2003), *Burkholderia cepacia* (Cartwright and Benson 1996), *Pseudomonas fluorescens* (Howell and Stipanovic 1979), *Chryseobacterium gleum* (Krause et al. 2001), and *Lysobacter enzymogenes* (Kilic-Ekici and Yuen 2003). Biological control using endophytes antagonistic to plant pathogenic fungi is potentially a powerful biofungicide control strategy. This was also demonstrated in studies that used endophytically living bacteria to control *R. solani* (Nowak et al. 1995; Pleban et al. 1995; Cho et al. 2003).

The problem of developing an effective biological control of *Rhizoctonia* lies in the screening method used against the pathogen because of its diversity and special ecological behavior. We have recently reported the successful selection of rhizobacteria antagonistic to *Verticillium dahliae* Kleb. (Berg et al. 2001), which is also a soil-borne pathogen with a similar life cycle (Scholte and Jacob 1989). Several studies showed discrepancies between the antagonistic effects under in vitro conditions and the corresponding in situ efficacy (Weller and Cook 1983; Reddy et al. 1993). Ideally, the candidate organisms should be screened on the plants rather than in vitro (Weller 1988). However, it is expensive and time consuming to conduct large-scale screening trials on whole plants. As a compromise, it is necessary to develop new in vitro screening methods that allow more efficient selection of potential antagonists for plant greenhouse and field experiments.

The objective of our study was to develop a screening method to evaluate plant-associated bacteria for their antagonistic activity against *R. solani*. For this reason, different screening methods were used or developed and hierarchically combined. In a first step, the traditional dual-culture assay was used to find out the antagonistic potential against two different *Rhizoctonia* strains. In a next step, the antagonistic mechanisms were investigated in vitro with a special focus on fungal cell wall degrading enzymes (chitinase, β-glucanase, and protease). Parallel to this, the ability to promote plant growth was analyzed in a newly developed assay in microtiter plates using lettuce seedlings in phytochamber and by the production of indole-3-acetic acid. Efficient isolates were identified to avoid a further work with potentially pathogenic species. After the in vitro tests, climate chamber experiments in vivo were conducted. Molecular fingerprints of isolates were performed using BOX–PCR. Based on all assays, an assessment scale was proposed that resulted in the selection of efficient BCAs to control *R. solani*.

Material and methods

Bacterial and fungal strains used in this study

A total of eight *R. solani* isolates of different AGs were used in this study. They belong to AG 1-IB (RHI SO 7/3/14 and RHI SO H5, both from lettuce), AG 2 (RHI SO W4 and RHI SO W8, both from white cabbage), AG 2-2IIIB (RHI SO K, from sugar beet), AG 3 (RHI SO 119 and RHI SO 325, both from potato), and AG 4 (RHI Ben 4 from sugar beet). The fungi were routinely grown on potato dextrose agar (Merck, Darmstadt, Germany). Isolates of *Rhizoctonia* were maintained on infested, dried barley seeds, which are stored at –20 °C.

Bacterial isolates (n = 434) were assessed as potential biocontrol agents in this study and were isolated during previous studies analyzing the ecology of plant-associated microbial communities and derived from the phyllosphere, endosphere, endorhiza, and rhizosphere of potato (Krechel et al. 2002, 2003), the rhizosphere and geocaulosphere of potato (Lottmann et al. 1999; Lottmann and Berg 2001), bryophytes (Opelt and Berg 2004), and the rhizosphere of oilseed rape, potato, and strawberry (Berg et al. 2001) (Table 1). They are available from the Strain Collection of Antagonistic Microorganisms at Rostock University, which harbors more than 4000 antagonistic strains. The bacterial isolates were cultivated in nutrient broth (Sifin, Berlin, Germany) and stored in broth containing 15% glycerol at –70 °C. They were harbored in the Culture Collection of Antagonistic Microorganisms.

Screening for antifungal in vitro activity

Bacterial isolates were tested in a dual-culture assay with pathogenic fungi on Waksman agar containing 5 g of proteose–peptone (Merck, Darmstadt, Germany), 10 g of glucose (Merck), 3 g of meat extract (Chemex, München, Germany), 5 g of NaCl (Merck), 20 g of agar (Difco, Detroit, Michigan), and distilled water (to 1 L), pH 6.8. Zones of inhibition were measured after 4, 7, and 10 days of incubation according to Berg et al. (2002). All 434 bacterial strains were tested in two independent replicates at 20 °C.

Screening for antifungal in vitro mechanisms

The in vitro production of fungal cell wall degrading enzymes such as chitinases, β-glucanases, and proteases was analyzed. Chitinase activity (β-1,4-glucosamine polymer degradation) was tested in chitin minimal medium according to Chernin et al. (1995). Clearing zones were detected 5 days after incubation at 30 °C. β-Glucanase activity was

Table 1. Proportion of *Rhizoctonia* antagonists from different groups of plant-associated bacteria.

Trial	Plant	Microenvironment	Reference	Number of isolates tested	Antagonistic number[a]	Isolates (%)	Selected for further investigations[b]
I	Oilseed rape	Rhizosphere	Berg 2000	1	1	100	0
II	Potato	Phyllosphere	Krechel et al. 2002	35	2	5.7	1
		Endosphere	Krechel et al. 2003	26	2	7.7	1
		Endorhiza		41	13	31.7	2
		Rhizosphere		55	19	34.5	6
III	Potato	Rhizosphere	Lottmann et al. 1999	98	31	31.6	1
		Geocaulosphere	Lottmann and Berg 2001	52	9	17.3	1
IV	Bryophytes: *Tortula*, *Aulacomnium*, *Sphagnum*	Phyllosphere	Opelt and Berg 2004	63	5	7.9	0
		Rhizosphere					
V	Potato, oilseed rape	Rhizosphere	Berg et al. 2001	10	8	80	8
	Strawberry						
VI	Potato	Rhizosphere	K. Smalla, personal communication	53	2	3.8	0
Summary				434	91	21	20 (4.6%)

[a]Dual-culture assay against *R. solani*.
[b]According to antagonistic activity.

tested using chromogenic substrates (azurine dyed, cross-linked) (AZCL substrates (Megazyme)). Formation of blue haloes was recorded until 5 days after incubation. Protease activity (casein degradation) was determined from clearing zones in skim milk agar (50 mL of sterilized skimmed milk mixed at 55 °C with 50 mL 1/5 trypticase soy agar and 4% agar) after 5 days of incubation at 30 °C.

The ability of bacterial isolates to produce indole-3-acetic acid was checked using the microplate method developed by Sawar and Kremer (1995). To analyze the ability of bacterial isolates to produce siderophores, we used the plate assay according to Schwyn and Neilands (1987).

Screening for plant growth promotion on lettuce seedlings

Surface-sterilized (1% NaOCl, 5 min) lettuce seeds 'Daguan' (S 5601, Syngenta Seeds GmbH) were pregerminated in moist chambers at 20 °C for 2 days. Sterility of seeds was proved by a print on nutrient agar. Three standard 24-well microplates (Roth, Karlsruhe, Germany) were filled with 1 mL of water agar containing 12 g of agar (Difco) made up to 1 L with distilled water (pH 6.8). One pregerminated seed followed by 10 µL of bacterial suspension (nutrient broth 2 (Sifin), grown for 18 h) was added to each well after centrifugation and resuspension in physiological salt solution (0.85%). In a preliminary experiment, different bacterial strains were evaluated at 10^3, 10^5, 10^7, and 10^9 CFU·mL^{-1} and compared with a control of 10 µL of distilled water. A dose-dependent effect was found with an optimal effect at a concentration of 10^5 CFU·mL^{-1}. Thus, bacterial isolates were evaluated at 10^5 CFU·mL^{-1} and compared with a control of 10 µL of physiological salt solution (0.85%). Two weeks after incubation (22/16 °C, 16/8 h day/night, and artificial lights) in a chamber (Percival Scientific, Boone, N.C.), the number of leaves was counted and the length of the whole plant was measured to determine the effects of bacterial treatment on plant growth. Each strain was tested in 24 replicates.

Molecular fingerprinting using BOX–PCR

Bacterial DNA was prepared following the protocol of Anderson and McKay (1983) modified for genomic DNA. BOX–PCR was done as described by Rademaker and De Bruijn (1997) using the BOXA1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. PCR amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hessisch Oldendorf, 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 a final extension at 65 °C for 16 min. A 10-µL aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5× Tris–borate–EDTA buffer for 4 h, stained with ethidium bromide, and photographed under UV transillumination. The reproducibility of the results was verified in three independent experiments.

Identification of bacterial antagonists

All isolates were identified based on their cellular fatty acids following fatty acid methyl ester gas chromatography (FAME–GC) as described by Sasser (1990) and character-

ized by the MIDI system (Microbial Identification System, Inc., Newark, N.J.). Antagonistic isolates were additionally identified by 16S rDNA sequencing aligned with the reference gene sequence 16S rRNA using the BLAST algorithm according to Altschul et al. (1997).

Screening for biocontrol activity

The effect of the bacteria to inhibit symptom expression by *R. solani* on lettuce leaf discs (cv. Daguan) was tested according to Fiddaman et al. (2000). Plastic boxes (18 cm × 28 cm) were filled with a 5-mm layer of sterilized and moisturized sand (SDW). Fifteen mycelia discs (5 mm) from a 7-day-old *R. solani* potato dextrose agar culture (RHI SO 7/3/14, AG 1-IB) were placed in a 5 cm × 3 cm matrix on top of the sand. The pathogen inocula were covered with a 10-mm humid sterilized sand layer. The sand surface was drenched with 20 mL of bacterial suspension (10^8 CFU·mL^{-1}) or SDW in the control treatments. Leaf discs (3 cm × 3 cm) were cut from leaves of 7-week-old lettuce'Daguan' plants that had been propagated under controlled conditions (70%/90% relative humidity, 20/15 °C, 16/8 h day/night cycle, light 500 µmol·m^{-2}·s^{-1}). The leaf discs (n = 15) were placed on the sand surface directly over each pathogen plug. Evaluation of symptoms was carried out 6 days after incubation of the boxes at 20 °C. The percentage of infected leaf disc or the lesion size was assessed on a scale from 0 to 5 (0 = no symptoms, 1 = <20%, 2 = 21%–40%, 3 = 41%–60%, 4 = 61%–80%, 5 = 100%).

Additionally, the ability of bacterial isolates to inhibit damping-off disease caused by *R. solani* (RHI Ben 4, AG 4) on sugar beet seedlings (cv. Dorena) was tested after seed bacterization. Seventy surface-sterilized seeds (1% NaOCl, 2 min) were moistened with 200 µL of bacterial suspension (10^8 CFU·mL^{-1}). Twelve bacterized seeds were placed in a pot (10 cm × 10 cm × 10 cm) 1 cm below the surface. The pots were filled before with substrate, Fruhstorfer Einheitserde Typ P. For pathogen inoculation, five oat kernels colonized with *R. solani* were used for each pot. The kernels were placed in the center of the pot. The pots were watered lightly every day to maintain the substrate moisture. Each treatment included six replications in a randomized design. The sugar beet seeds were cultivated in a phytochamber (60%/80% relative humidity, 20/15 °C 16/8 h day/night cycle, light 500 µmol·m^{-2}·s^{-1}) for 12 days. The germinated seeds and the number of seedlings with damping-off symptoms were counted at the end of the experiment. The germination rate and the disease incidence were calculated.

For both experimental types, the bacterial isolates for each experiment were cultivated for 24 h in nutrient broth (Merck) at 30 °C. Each bacterial number was adjusted by measuring optical density. For the preparation of *R. solani* inocula, sterilized oat kernels were inoculated with agar discs of 7-day-old *R. solani* potato dextrose agar culture. The inoculated oat kernels were incubated for 2 weeks at 25 °C.

Statistical analysis

Data on plant growth promotion were analyzed for significance using the Mann–Whitney U test ($P \leq 0.05$) by Statistical Product and Service Solutions for Windows, release 9.0.1 (SPSS Inc., Chicago, Ill.). Computer-assisted evaluation of BOX–PCR-generated fingerprints was made using the GelCompar program, version 4.1 (Kortrijk, Belgium). The cluster analysis was performed with Ward's unweighted pair-group average (UPGMA) algorithm. The STATISTICA program (StatSoft Inc., Tulsa, Oklahoma) was used for the other statistical analysis. The number of diseased sugar beet seedlings was compared with the control after ANOVA using the Tukey's procedure (HSD) with $P = 0.05$. The disease severity on lettuce leaf discs in comparison with the pathogen control was tested after nonparametric analysis using a Kruskal–Wallis test with $P < 0.05$.

Results

The strategy used to characterize and assess plant-associated bacteria for biocontrol against *R. solani* is shown in Fig. 1. Altogether, the strategy consisted of six different steps. First, the antagonistic activity against two *Rhizoctonia* strains was evaluated. In a second step, antagonistic mechanisms as well as mechanisms of plant growth promotion were analyzed in vitro followed by the identification of isolates. The biocontrol activity *ad planta* was investigated using lettuce and sugar beet as host plants. All selected isolates were genotypically characterized by a molecular fingerprint. Finally, the best candidates were selected according to their efficiency in all assays.

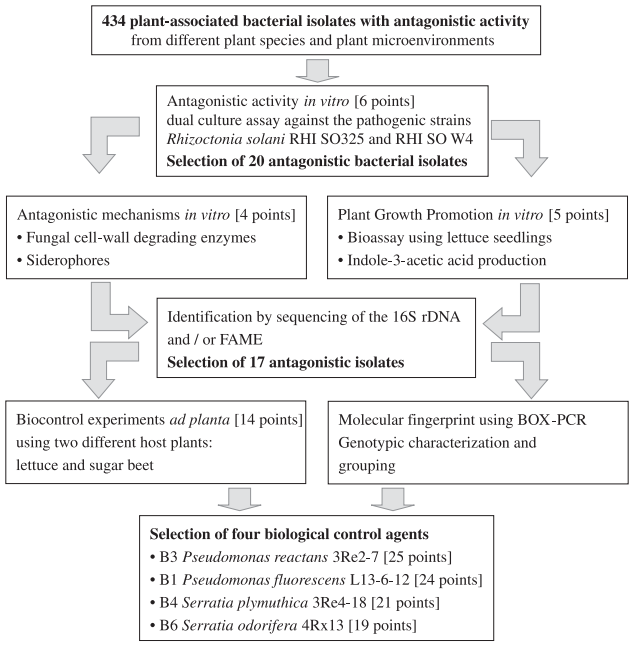
Proportion of *Rhizoctonia* antagonists from different groups of plant-associated bacteria

A total of 434 bacterial isolates, which were obtained from different plant species and plant-associated microenvironments, were analyzed for in vitro antagonism against *R. solani*. Table 1 shows the result of the first step of in vitro screening. In preliminary experiments, the effect of four defined BCAs, *Bacillus subtilis* B2g, *Serratia plymuthica* C48, *Pseudomonas putida* RRE 109, and *Streptomyces setonii* RP 87 (Berg 2003), on eight different *Rhizoctonia* isolates were analyzed. The *Rhizoctonia* isolates showed a different sensitivity against BCAs, RHI SO 325 the lowest sensitivity and RHI SO W4 the highest. Thus, these two isolates were applied in the screening procedure for all bacterial isolates. Only minor differences in antagonistic activity could be found between the different temperatures, 15 and 20 °C. Finally, all 434 bacterial strains were tested in two independent replicates at 20 °C. In a dual-culture assay on Waksman agar, the fungi grew as well as the bacterial isolates. Inhibition was clearly discerned by limited growth or by the complete absence of fungal mycelium in the inhibition zone surrounding a bacterial colony. All in all, 91 (21%) bacterial isolates with antifungal activity against *Rhizoctonia* were found. The highest antagonistic potential was found analyzing rhizosphere-associated isolates already described as *Verticillium* antagonists, e.g., in trials I, II, and V (Table 1).

Identification of the bacterial antagonists and risk assessment

Of the 91 bacterial isolates, 20 were selected according to their high efficiency in in vitro antagonism, which was shown as inhibition zones in the dual-culture assay. They were identified at the species level (similarity index > 0.5)

Fig. 1. Strategy and assessment for evaluation of BCAs.



by FAME–GC and (or) sequencing of the 16S rDNA genes (Table 2). Altogether, 13 different species were found that belong to the γ-proteobacteria, to low and high G + C Gram-positives, as well as to the enteric group. The majority of isolates were identified as *Pseudomonas*. According to the results, two bacterial isolates were excluded from further investigation. Strain B19 (trial III) was identified as *Erwinia chrysanthemi*, which is known as a plant pathogenic species. *Micrococcus kristinae*, which was determined for strain B20 (trial V), is known for its facultative pathogenicity to humans. All other species determined are in risk group 1, which means no risk to the environment and to human health. Furthermore, *S. setonii* B18 was excluded at this step of the investigations because this strain was difficult to cultivate.

Antifungal activity and production of antifungal substances

A total of 17 antagonistic strains were characterized in detail. Table 3 shows the results of in vitro tests of antagonism toward both *R. solani* strains and the production of lytic enzymes and biologically active substances in vitro. *Rhizoctonia solani* is a basidiomycete with a chitin–glucan-containing cell wall. Production of chitinases, glucanases, and proteases was analyzed as an important mechanism of fungal inhibition. Two or three out of 17 strains showed fungal cell wall degrading enzyme β-1,3-glucanase activity and chitinolytic activity. In contrast, protease production was shown for all strains. Nearly half of the isolates produced siderophores. The *S. plymuthica* isolate B4 was the only one that produced all lytic enzymes and siderophores.

Plant growth promotion test in a phytochamber

A bioassay to assess the plant growth promoting effect by bacterial strains was developed. To achieve that, lettuce seeds treated with bacteria, or untreated, were cultivated in 24-well microplates in a phytochamber. The effect of bacterial isolates on the growth of seedlings was monitored by measuring leaf and plant length (Table 3). A total of 16 isolates induced statistically significant effects on plant growth compared with the nontreated control at minimum for one parameter. The main influence was seen on the length of leaves. Eleven isolates were able to enhance this parameter to a statistically significant extent. Only one isolate enhanced the length of plants. The isolate that was able to statistically significantly enhance both parameters belong to *P. fluorescens* (B8). In contrast, the isolates *P. putida* B16 and *P. syringae* B17 showed a negative influence on plant length as well as on leaf length. Parallel to the *ad planta* assay, the production of the plant growth hormone indole-3-acetic acid was monitored. Ten isolates were able to produce this compound in vitro.

Biocontrol activity of the antagonistic bacteria

To evaluate the antagonistic activity of the 17 selected bacterial strains *ad planta*, a lettuce leaf disc bioassay infected by *R. solani* was used. Strains of this test bacteria could be selected that are able to inhibit the colonization process by the pathogen on detached lettuce leaf, measured by lesion size. The detached leaves in the control treatment, with no pathogen inoculation, showed no symptoms during the incubation period of 6 days. Within 6 days, *R. solani* colonized approximately 70% of the leaf area on average. Only six bacterial strains B1 and B3–B7, were able to significantly inhibit the colonization of leaf discs by the pathogen (Fig. 2). The highest inhibition effects were observed in *S. plymuthica* B4 and *P. fluorescens* B1 treatments that resulted in a reduction of lesion size by 76% and 70% in comparison with the pathogen control. A significantly higher lesion size was observed in the treatments with the bacterial strains B11 and B17.

The ability of bacterial BCAs to inhibit damping-off disease on sugar beet, caused by *R. solani*, was tested in three independent experiments after a seed application. The germination rate of sugar beet seeds was 87% on average, which was not influenced by the pathogen. The germination rate was 85% in the pathogen control on average. No effect of BCAs on germination rate was observed after seed application either. *P. fluorescens* strains B1 and B2 and the BCA *P. reactans* B3 significantly ($P < 0.05$) decreased the number of seedlings with damping-off symptoms in the means of the experiments (Table 4). Eight further bacteria strains reduced the disease incidence significantly but only in one of the three experiments.

Molecular fingerprinting

BOX-primed PCR was used to differentiate the isolates and to obtain fingerprints. The PCR products obtained with BOX primers yielded DNA profiles with sufficient numbers of DNA bands to differentiate the 17 isolates (Fig. 3*a*). Various specific PCR fingerprints of bacteria were found. Among them, BOX–PCR patterns of the potato-associated isolates B10–B12 were very similar to each other. The different BOX–PCR profiles were compared by numerical methods, and the resulting dendrogram based on percent similarity between isolates showed a high degree of genetic diversity (Fig. 3*b*). Intraspecies diversity of BOX patterns

Table 2. Identification and grouping of selected bacterial strains antagonistic to *R. solani*.

No.	Strain	Identification[a]	Similarity index[b]	Origin[c]	Risk group[d]	Fingerprint group[e]	Concluding assessment[f]
B1	L13-6-12	*Pseudomonas fluorescens*	0.785	III, rhizosphere	1	III	24
B2	2Re2-6	*Pseudomonas fluorescens**	98	II, endorhiza	1	III	17
B3	3Re2-7	*Pseudomonas reactans**	98	II, endorhiza	1	II	25
B4	3Re4-18	*Serratia plymuthica**	99	II, endorhiza	1	X	21
B5	3Rc3	*Serratia fonticola**	99	V, rhizosphere	1	IX	18
B6	4Rx13	*Serratia odorifera*	0.735	V, rhizosphere	1	XI	19
B7	1Pe4-13	*Bacillus subtilis**	98	II, endosphere	1	V	17
B8	2R1-7	*Pseudomonas fluorescens**	99	II, rhizosphere	1	III	12
B9	3R1-19	*Pseudomonas putida*	0.965	II, rhizosphere	1	IV	11
B10	5Re4-6	*Pseudomonas corrugata*	0.816	II, endorhiza	1	I	11
B11	5Re4-12	*Pseudomonas corrugata*	0.737	II, endorhiza	1	I	9
B12	5Re4-21	*Pseudomonas corrugata*	0.768	II, endorhiza	1	I	10
B13	4Kc13	*Pseudomonas putida*	0.680	V, rhizosphere	1	I	10
B14	6Kp8	*Pseudomonas putida* B	0.824	V, rhizosphere	1	VI	12
B15	6Kp10	*Pseudomonas chlororaphis*	0.848	V, rhizosphere	1	VII	10
B16	8Rr24	*Pseudomonas putida* A	0.678	V, rhizosphere	1	VIII	12
B17	4Rr41	*Pseudomonas syringae*	0.919	V, rhizosphere	1	II	12
B18	RP87	*Streptomyces setonii*	98	I, phyllosphere	1	nd	nd
B19	LC21-3-3	*Erwinia crysanthemi*	0.496	III, caulosphere	2	nd	nd
B20	4Rr44	*Micrococcus kristinae*	0.871	V, rhizosphere	2	nd	nd

[a]Identification according to 16S rDNA sequencing (asterisk) and FAME–GC.
[b]Similarity indices 0%–100% for 16S rDNA sequencing and 0–1 for FAME–GC.
[c]See Table 1.
[d]According to Anonymous et al. (1990).
[e]According to cluster analysis obtained from BOX–PCR fingerprints at 95% similarity level (nd, not determined).
[f]Antagonistic activity = 6 points, antagonistic mechanisms = 4 points, plant growth promotion in vitro = 5 points, biocontrol activity = 2 × 7 points.

Table 3. Antagonistic activity and mechanisms and their effect on plant growth in vitro of selected bacterial strains antagonistic to *R. solani*.

	Antagonistic activity[a]		Lytic enzymes and metabolites[b]					Effect on plant growth (length)[c]	
No.	RHI SO 325	RHI SO W4	Chitinases	Glucanases	Proteases	Siderophores	Indole-3-acetic acid	Leaves	Plant
B1	++	+++	–	–	+	+	–	p	–
B2	+++	+++	–	–	+	+	–	p	–
B3	+++	+++	–	–	+	+	+	p	–
B4	+++	+++	+	+	+	+	–	p	e
B5	+++	+++	+	–	+	–	+	–	e
B6	+++	+++	–	–	+	–	+	p	e
B7	++	+++	–	+	+	–	–	p	–
B8	++	+++	–	–	+	+	–	p	p
B9	+++	+++	–	–	+	+	+	–	e
B1+	+++	+++	–	–	+	+	+	p	–
B11	+++	+++	–	–	+	+	+	–	–
B12	+++	+++	–	–	+	+	–	–	e
B13	+++	+++	–	–	+	–	+	p	–
B14	+++	+++	–	–	+	–	+	p	e
B15	+++	+++	–	–	+	–	+	p	–
B16	+++	++	–	–	+	–	+	e	e
B17	+++	+++	–	–	+	–	+	e	e

[a]Antagonism towards *R. solani* isolate RHI SO 325 (AG 3) and RHI SO W4 (AG 2) using a dual-culture assay. +, 0- to 5-mm-wide zone; ++, 5- to 10-mm-wide zone; +++, 10- to 15-mm-wide zone.
[b]Plate assays. +, hydrolysis; –, no hydrolysis.
[c]p, significant plant growth promoting effect; e, statistically significant effect on plant growth; –, no effect.

analyzed in three independent replicates of one isolate, B1, was shown at 97% similarity. Isolates of BOX patterns with more than 95% similarity were nearly identical. At the 90% similarity level, which is indicated in Fig. 3*b* by a dotted line, three groups and eight single isolates were defined. Group I (G I) consists of the three isolates from potato

Fig. 2. Effect of bacterial BCAs on lesion size (0 = without symptoms, 1 = <20%, 2 = 21%–40%, 3 = 41%–60%, 4 = 61%–80%, 5 = >80% colonization of leaf) on lettuce leaf discs (cv. Daguan) by *R. solani* (R.s.) (isolate 7/3). *Significant differences in comparison with the pathogen control according to a Kruskal–Wallis test ($P < 0.05$).

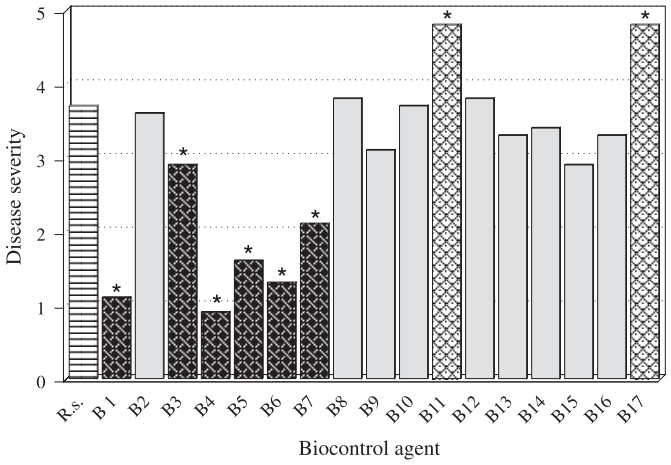


Table 4. Disease incidence of sugar beet seedlings 'Dorena' 12 days after inoculation of *R. solani* (R.s.) dependent on seed treatment with BCAs.

	Disease incidence (%)			
No.	Experiment I	Experiment II	Experiment III	Mean
R.s.	28.9	30.0	30.6	29.5
B1	8.7*	7.5*	nd	8.1*
B2	16.3	5.4*	9.0*	10.2*
B3	1.9*	5.9*	nd	3.9*
B4	30.0	16.4	7.0*	17.8
B5	20.9	21.1	nd	21.0
B6	17.0	1.8*	16.5	11.8
B7	4.1*	30.8	14.9	16.6
B8	28.8	18.2	nd	23.5
B9	16.3	21.3	nd	18.8
B10	7.4*	32.6	20.9	20.3
B11	12.5	42.5	nd	27.5
B12	19.6	17.6	nd	18.6
B13	4.6*	32.6	18.6	18.6
B14	5.8*	14.6	26.7	15.7
B15	5.9*	37.2	24.3	22.5
B16	18.2*	29.2	27.5	25.0
B17	14.0*	42.6	22.5	26.4

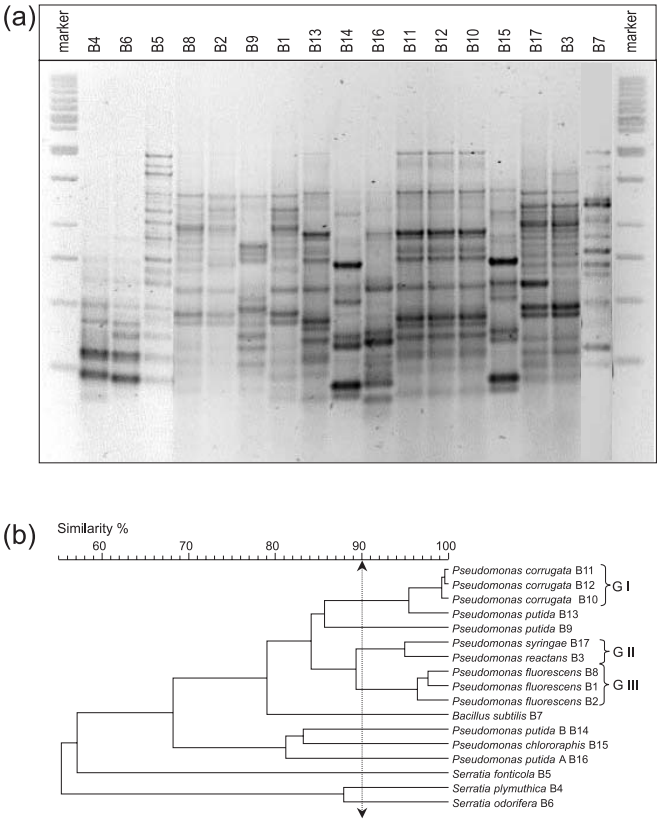
Note: *, Significant differences in comparison with the pathogen control (*R. solani*) according to Tukey's test ($P = 0.05$); nd, not determined.

(B10–B12) with a very similar fingerprint, and *P. putida* B13 was also isolated from potato but from a completely different origin. Group II (G II) includes two *Pseudomonas* isolates, B3 and B17, from different plant species of oilseed rape and potato. The three *P. fluorescens* isolates B1, B2, and B8 belong to group III (G III). For all other isolates, a unique fingerprint could be obtained.

Assessing the best candidates for biological control

In an attempt to better select bacterial isolates with high antagonistic potential, an assessment scheme was developed.

Fig. 3. (*a*) Genomic BOX–PCR profiles of selected bacterial strains B1–B17. Lanes 1 and 19, 1-kbp ladder (marker). (*b*) UPGMA dendrogram generated from the BOX–PCR profiles of selected bacterial strains B1–B17.



The following point system was applied to each of the bacterial traits: up to six points were given for antagonistic activity against both *R. solani* strains and one point each for production of hydrolytic enzymes (chitinases, cellulases, and proteases) and siderophores. Additionally, five points were given for plant growth promoting effects including indole-3-acetic acid production. The main point was formed by the *ad planta* investigations; therefore, seven points were given for a statistically significant effect in each pathosystem, *R. solani* – lettuce and *R. solani* – sugar beet. A maximum of 29 points could be achieved. The three best isolates received more than 20 points (Table 2). The best candidate of all was *P. reactans* B3, originally isolated from the endorhiza of potato, with 25 points. This strain showed a high in vitro antagonism against both *Rhizoctonia* strains, an enhancement of plant growth in vitro, and a strong biocontrol effect in both pathosystems. The BCA B1 (24 points) was detected as a second candidate that was able to antagonize *R. solani* in vitro and showed significant biocontrol effects against *Rhizoctonia* in lettuce as well as in sugar beet. *Pseudomonas fluorescens* B1 showed high biocontrol efficiency in vivo, although in vitro effects were weak. The strain *S. plymuthica* B4 achieved with 21 points the third position and was the most efficient BCA for the pathosystem *Rhizoctonia* – lettuce. In contrast, no biocontrol effect was observed on sugar beet. The strain was the most efficient one under in vitro conditions. *Serratia odorifera* B6 was with 19 points in the fourth position.

Discussion

Antagonistic plant-associated bacteria are an important functional group of beneficial bacteria responsible for the control of soil-borne pathogens (Weller 1988; Sørensen 1997). To find new biocontrol agents antagonistic to the soil-borne fungus *R. solani*, 434 rhizobacterial isolates were evaluated using a combination of different screening steps.

As a result of the first screening, isolates were found to produce detectable inhibition zones against *Rhizoctonia* on agar. Most isolates that were investigated in this study were screened as *Verticillium* antagonists previously (Lottmann et al. 1999; Berg 2000; Lottmann and Berg 2000; Krechel et al. 2002, 2003; Berg et al. 2002; Opelt and Berg 2004). As a consequence, the percentage of *Rhizoctonia* antagonists was generally very high. The production of clear inhibition zones in dual-culture screens is due to the production of antibiotics, toxic metabolites, or siderophores as mechanisms for biological control (Swadling and Jeffries 1996). Because of the discrepancies in in vitro and in vivo studies, the assay should be combined with other investigations as was done here. In our study, we found examples for a good correlation between in vitro and in vivo results, e.g., B3 and B4, and vice versa. For example, *P. fluorescens* B1 showed high biocontrol efficiency in vivo, although in vitro effects were weak. The identification of an antagonistic bacterium is another important step in the assessment of the BCA. Among identified strains, not only species known for their neutral interaction with eucaryotes were found. *Micrococcus kristinae* is known as facultative pathogenic for humans. In addition, one strain showed high homology to the plant pathogenic bacterium *Erwinia chrysanthemi*. Both groups of bacteria were also found as endophytes of potato by Reiter et al. (2002), showing that the distinction between plant-benefitting bacteria and pathogens can be unclear. Mechanisms of pathogenicity and symbiosis are very similar, and sometimes only the expression of one metabolite determines the effect or the effect dependent on the concentration of bacteria (Suckstorff and Berg 2003). Isolates with potentially pathogenic character were excluded from further investigations.

In a second step, the mode of action of the antagonistic activity as well as of plant growth promotion was included. The ability of antagonistic bacteria to produce proteolytic enzymes was widely distributed between our antagonistic isolates. However, only a low percentage of glucanolytic and chitinolytic isolates was found. In general, the composition of antagonistic activity in the dual-culture assay was specific for each isolate. Recent studies have provided some evidence, however, that biocontrol activity is more than a simple process involving a single metabolite; rather, it involves a series of complex events expressed in a controlled sequence. A good example for such a BCA is *S. plymuthica* B4, which was the best candidate to biologically control *Rhizoctonia* in lettuce. This strain distinguished itself by chitinolytic, glucanolytic, and proteolytic activity and statistically significant plant growth promotion in vitro.

Many plant-associated bacteria have a direct positive influence on plant growth and can indirectly stimulate plant health (Höflich et al. 1994). The newly developed plant growth promotion assay in microtiter plates is an easier *ad planta* test than a whole-plant system in terms of time, plant material, and growth facilities. The microplate assay allowed many repetitions and screening of a large number of bacterial isolates. With the exception of isolate B11, bacterial isolates that were able to enhance growth to a statistically significant extent and extend the length of plants or leaves were also efficient in at least in one pathosystem. Unfortunately, no correlation between the in vitro production of indole-3-acetic acid and plant growth promotion on lettuce was found, although in other studies, indole-3-acetic acid was involved in plant growth promotion (Suckstorff and Berg 2003).

To evaluate selected bacterial isolates with antagonistic properties for approaches in biocontrol, a multistep screening for biocontrol activity against *R. solani* and plant growth promotion combined with its genotypic and phenotypic characterization was performed. All of these data were integrated into an assessment scheme to identify the best antagonists. Based on this assessment scheme, four isolates were considered good biocontrol candidates. Although promising candidates such as *P. reactans* B3 could be found to control *Rhizoctonia* in both pathosystems, special BCAs for each plant were found. While *S. plymuthica* B4 was the best candidate to biologically control *Rhizoctonia* in lettuce, *P. reactans* B3 was the best candidate to suppress the pathogen in sugar beet. Surprisingly, both candidates were originally isolated from the endorhiza. Endophytic bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant are able to interact more closely with the host plant and fungal pathogens living in plant tissues (Hallmann et al. 1997). Our findings confirm that they could be efficient BCAs. The rhizosphere-associated *P. fluorescens* B1 is another interesting candidate for biological control. Although this strain showed low effects in vitro, high biocontrol activity was found in vivo. In further experiments, these four selected BCAs will be evaluated in field trials to select the best candidates for commercialization.

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