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REGULAR ARTICLE

Biocontrol of *Rhizoctonia solani*: complex interaction of biocontrol strains, pathogen and indigenous microbial community in the rhizosphere of lettuce shown by molecular methods

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Abstract
Background and aims Co-inoculation of biocontrol agents with different modes of action is assumed to improve biocontrol activity. The present study aimed to investigate the effects of single or co-inoculation of *Trichoderma viride* strain GB7 and *Serratia plymuthica* strain 3Re4-18 on microbial communities in the rhizosphere of lettuce and their ability to suppress *Rhizoctonia solani* AG1-IB.
Methods Growth chamber experiments with two different application modes were performed with single or co-inoculation of GB7 and 3Re4-18 in the presence or absence of *R. solani.* Biocontrol efficacy and plant growth parameters were assessed. Bacterial and fungal communities were analyzed by 16S rRNA gene and ITS fragments PCR-amplified from total community DNA of rhizosphere samples and analyzed by denaturing gradient gel electrophoresis.
Results Compared to the single application, the co-inoculation of 3Re4-18 and GB7 resulted in an improved biocontrol efficacy. DGGE analysis revealed more pronounced effect on microbial community in co-inoculation treatment. The abundance of 3Re4-18 in the rhizosphere seemed to be increased in the presence of *R. solani*.
Conclusions The applied cultivation-independent methods provided insights into the complex interaction in response to the pathogen and the antagonists. Co-inoculation resulted in an improved biocontrol efficacy and an increased evenness of the microbial communities.

Keywords Biocontrol · Co-inoculation · Rhizosphere community · 16S rRNA gene and ITS-based fingerprints

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Introduction

The heterogenous basidiomycete species *Rhizoctonia solani* Kühn [anamorph, teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is a widely distributed soil-borne pathogen that causes economically important diseases on a wide range of plant species (González-García et al. 2006). Strategies to control *Rhizoctonia* diseases are limited and intensive management increased the disease incidence and severity worldwide (Chellemi 2002). *R. solani* AG1-IB is known to cause bottom rot on lettuce (Blancard et al. 2006). In Germany only the fungicide Signum® (boscalid and pyraclostrobin the active agents, BASF) is registered in particular for controlling *Rhizoctonia* disease in lettuce (Grosch et al. 2006). Lettuce growth usually takes about 6 weeks only. Therefore, fungicide application in the field after planting is critical with respect to pesticide residues in the final food product. The lack of alternative control methods and the public demand for food that is less contaminated with pesticide residues have stimulated research for alternatives to combat soil-borne pathogens by means of biocontrol (Martin 2003). Bacterial and fungal antagonists could be an environmentally friendly alternative for control of *R. solani*. Various bacterial and fungal antagonists, including *Bacillus subtilis* CA32 in eggplants (Abeysinghe 2009), *Pseudomonas fluorescens* In5 (Michelsen and Stougaard 2011), *Burkholderia cepacia* T1A-2B and *Pseudomonas* sp. T4B-2A in tomato (De Curtis et al. 2010), *B. cepacia* BC-1 and *Serratia marcescens* N1-14 in cucumber (Roberts et al. 2005), *Verticillium biguttatum* (Van den Boogert and Luttikholt 2004), *Trichoderma artroviride* (Lahlali and Hijri 2010) and *T. harzianum* T22 in potato (Wilson et al. 2008) have been reported as effective biological control agents against *R. solani*. However, no specific biocontrol agents are available for control of *R. solani* AG1-IB in lettuce. In previous studies based on hierarchical screening schemes (Faltin et al. 2004) the interesting candidates *Serratia plymuthica* 3Re4-18 (Berg et al. 2005; Grosch et al. 2005) and *Trichoderma viride* GB7 (Grosch et al. 2007) with efficient disease suppression effects against *R. solani* AG1-IB in lettuce were selected. Because of the persistence of sclerotia, soil-borne pathogens like *R. solani* are hard to control and the application of single biocontrol agents has often resulted in non-consistent performances in commercial crop growing. The co-inoculation of biocontrol agents with different modes of action might broaden the spectrum of biocontrol activity and help to improve their performance under a wide range of environmental conditions (Pierson and Weller 1994; Marimuthu et al. 2002). The growing interest in improved biocontrol strategies has stimulated research on new combinations of microbial inoculants.

In the present study we aimed to explore whether biocontrol efficacy could be increased by combining antagonists that potentially use different niches in the rhizosphere and supposedly target *R. solani* via different mechanisms. The *T. viride* strain GB7 is able to inhibit the germination of sclerotia and to parasitize the mycelia of *R. solani* AG1-IB (Grosch et al. 2007), whereas *S. plymuthica* 3Re4-18 is a competent root colonizer (Zachow et al. 2010) that produces antifungal metabolites such as chitinase, glucanase and protease (Faltin et al. 2004). Moreover, volatile organic compounds are also involved in antifungal activity of *S. plymuthica* (Müller et al. 2009). The bacterial strain 3Re4-18 showed no inhibition against the fungal strain GB7 *in vitro*. However, strain characteristics of 3Re4-18 such as root colonization ability could be affected by co-inoculation with the fungal antagonist. Therefore, the impact of strain GB7 on root colonization competence of 3Re4-18 was studied in presence and absence of *R. solani* in addition to the effect of co-inoculation of both strains on plant growth and biocontrol efficacy. The effects of co-inoculation of both strains were compared to effects of single application. Total community DNA based analysis was done to improve the understanding of the ecology of the antagonists in the rhizosphere and the interaction with the indigenous bacterial and fungal community. Two growth chamber experiments with different inoculation methods of GB7 and 3Re4-18 were performed. At two time points during plant development lettuce plants were destructively sampled and total community DNA extracted from the microbial pellets obtained from composite samples of roots with adhering soil were analyzed with 16S rRNA gene and ITS-based fingerprinting methods. Biocontrol efficacy and plant growth was assessed by determining shoot dry weight of lettuce 4 weeks after planting.

Material and methods

Fungal and bacterial strains used in this study

The fungal strain GB7 (*Trichoderma viride*, AJ810426) was obtained from the rhizosphere of *Sphagneticola*

trilobata grown in Pernambuco, North-eastern Brazil (Grosch et al. 2007). Stock cultures were stored at -80 °C in 2 ml Eppendorf tubes with 1 ml of liquid storage medium containing 0.6 ml glycerol, 0.2 ml glucose (50 %w/v), 0.1 ml peptone (20 %w/v) and 10 ml yeast extract (10 %w/v).

The bacterial strain *Serratia plymuthica* 3Re4-18 was isolated from the endorhiza of potato (Berg et al. 2005). To facilitate re-isolation of the bacterial inoculant from the rhizosphere, spontaneous rifampicin-resistant mutants of the bacterial antagonist were generated as described by Adesina et al. (2009). The mutant strains were stored at -80 °C in Luria–Bertani broth (ROTH, Karlsruhe, Germany) containing 20 % glycerol supplemented with rifampicin (75 μg ml^{-1}).

Rhizoctonia solani AG1-IB (isolate 7/3; host plant, lettuce) was obtained from the strain collection of the Leibniz Institute of Vegetable and Ornamental Crops (Großbeeren, Germany) and maintained on barley kernels at -20 °C.

Experimental design of growth chamber experiments

Two experiments were carried out in a growth chamber (York, Mannheim, Germany; 16 h/8 h day/night cycle, 500 μmol m^{-2} s^{-1}, 20/15 °C and 60 %/80 % relative humidity) under favorable conditions for the pathogen *R. solani* AG1-IB on lettuce plants (cultivar 'Tizian', Syngenta, Bad Salzuflen, Germany). In both experiments lettuce seeds were germinated in a seedling tray containing 92 holes filled with a non sterile mixture of quartz sand and substrate [Fruhstorfer Einheitserde Typ P, Vechta, Germany; chemical analysis (mg per l): *N*=120, *P*=120, *K*=170, Mg=120, *S*=100, KCl=1, organic substance=167, peat=309; pH 5.9] at a 1 : 1 ratio (v/v) at 18 °C. After germination of the seeds the temperature was changed to 20/15 °C mentioned above. Lettuce plantlets were transferred at the two-leaf stage to pots (500 ml) filled with the same soil mixture with one plant per pot. The pots were watered daily to maintain the substrate moisture and fertilized weekly (0.2 % Wuxal TOP N, Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany). Each treatment consisted of five replicates (four pots per replicate) arranged in a randomized design. Six additional pots (three plants per time point and treatment) were included for microbial community analysis.

Each treatment was carried out with and without pathogen inoculation. Visual symptoms and the number of dead plants (DP) caused by the pathogen were evaluated weekly in all treatments. At the end of the experiments the shoot dry weight (SDW) was assessed in all treatments 4 weeks after pathogen inoculation or planting of lettuce into the pots.

Single and co-inoculations of the fungal strain GB7 and bacterial strain 3Re4-18 with and without *R. solani* were tested in two experiments with the inoculation methods described below.

Inoculation of R. solani

Inoculation of the pathogen was done as described by Adesina et al. (2009). Briefly, barley kernels (two per plant) infested with *R. solani* (strain 7/3) were used as pathogen inoculum. The kernels were placed 1 cm deep at a distance of 2 cm from each lettuce plant 1 day after planting. Non-infested barley kernels were used in the control treatments.

Preparation of the bacterial inoculant 3Re4-18

The strain *S. plymuthica* 3Re4-18 was grown overnight in nutrient broth (NB II, SIFIN GmbH, Berlin, Germany) supplemented with rifampicin at 75 μg ml^{-1} on a rotary shaker (90 rpm) at 29 °C. After a centrifugation step, the bacterial suspension was washed twice and re-suspended in 0.3 % NaCl solution. The concentration was adjusted in a spectrophotometer to a density corresponding to 10^8 CFU ml^{-1} for the seed treatment or 10^7 CFU ml^{-1} for young plant treatment.

Preparation of fungal inoculant GB7

The strain *T. viride* GB7 was cultured on potato dextrose agar (PDA, VWR International GmbH, Berlin, Germany) for 3 weeks at room temperature. The conidia were harvested from the Petri dishes and suspended in 10 ml sterile 0.3 % NaCl solution. The number of conidia were counted in a haemicytometer and adjusted by dilution to a density corresponding to 10^3 conidia ml^{-1} for young plant treatment or 10^7 conidia ml^{-1} for substrate treatment.

Application mode I: substrate application with GB7 and plant application with 3Re4-18

Strain GB7 was applied to the substrate (substrate application with fungal inoculant, SAF) that was filled into the pots 1 week before planting of lettuce

seedlings. A mixture of substrate and quartz sand (250 ml) per pot was mixed with 50 ml conidia suspension (10^7 conidia ml^{-1}). The strain 3Re4-18 was applied by watering lettuce plants (plant application with bacterial inoculant, PAB) with 20 ml bacterial suspension (10^7 CFU ml^{-1}) 1 day after planting of lettuce at the two-leaf stage. The effect of both antagonists was tested in single (GB7 or 3Re4-18) and co-inoculation (GB7/3Re4-18) treatments with (+*Rs*) and without pathogen inoculation.

Application mode II: seed and plant application with 3Re4-18, substrate and plant application with GB7

Strain GB7 was applied to the lettuce seedlings (plant application with fungal inoculant, PAF) and to the substrate (SAF) 1 week prior to planting. At the one-leaf stage each seedling in plant containers was watered with 5 ml conidia suspension of 10^3 conidia ml^{-1} (PAF). The substrate application (SAF) was done as described for application mode I. A seed (seed coating with bacterial inoculant, SCB) and young plant treatment (PAB) was carried out with the bacterial strain 3Re4-18. First the seeds were coated with a bacterial suspension directly before sowing. For coating, 100 seeds were transmitted in a glass tube (5 ml) and moved on a Vortex Mixer (MS1 Minishaker, IKA-Werke GmbH, Staufen, Germany). The bacterial suspension (250 μl, 10^8 CFU ml^{-1}) was pipetted on the seeds. A second treatment was carried out as described for application mode I by watering lettuce plants with 20 ml bacterial suspension (10^7 CFU ml^{-1}) 24 h after planting. The effects of the antagonists were tested in single (GB7 or 3Re4-18) and co-inoculation (GB7/3Re4-18) treatments with (+*Rs*) or without pathogen inoculation.

Root colonization by the bacterial inoculant 3Re4-18

The survival and root colonization efficiency of 3Re4-18 was evaluated only for the experiment with application mode II. Rhizosphere samples were collected 2 and 4 weeks after planting. For each treatment with 3Re4-18 and sampling time, three plants were used for root colonization analysis. Loosely adhering soil was removed from the roots before 5 g of roots with adhering soil were suspended in 20 ml of sterile saline. The rhizosphere samples were then shaken vigorously in a sterile glass flask containing six glass beads (0.6 mm in diameter) on a rotary shaker for 1 h at 307 rpm. Aliquots of the rhizosphere suspension were immediately processed for enumeration of the inoculated strains. Colony forming units (CFU) of the inoculant were determined by plating serial dilutions of the rhizosphere suspensions on R2A medium (Difco) supplemented with rifampicin (75 μg ml^{-1}) and cycloheximide (100 μg ml^{-1}). After 2 days of incubation at 28 °C, the CFU counts were determined. For microbial rhizosphere community analysis the remaining rhizosphere microbial fraction was harvested by centrifugation at 13, 000*g* for 5 min. After discarding the supernatants, the cell pellets were stored frozen at -20 °C.

Analysis of indigenous bacterial and fungal rhizosphere communities

DNA extraction from rhizosphere samples

Total community (TC)-DNA was extracted from the previously prepared rhizosphere pellets using the Bio101 extraction kit (Qbiogene, Carlsbad, CA, USA) after a harsh lysis step. The TC-DNA was purified by the GENECLEAN Spin kit (Qbiogene). DNA yields were estimated after electrophoresis in 1 % agarose gel stained with ethidium bromide under UV light in comparison with the 1-kb gene-ruler™ DNA ladder (Fermentas, St Leon-Rot, Germany) run on the same agarose gel. The TC-DNA was diluted with sterile milliQ water between 1:50 and 1:100 (c. 1–5 ng) depending on the extraction yield and was then used as a template for PCR amplification of bacterial 16 S rRNA gene fragments. Undiluted TC-DNA, or 1:10 dilutions (c. 20 ng DNA) served as a template for amplification of the fungal internal transcribed spacer (ITS) regions by PCR-reaction.

The inoculation of the pathogen *R. solani* resulted in a high number of dead plants in the pathogen control (*R. solani*). However, rhizosphere samples were taken from survived plants.

PCR amplification of 16S rRNA gene and fungal ITS (internal transcribed spacer) fragments for denaturing gradient gel electrophoresis (DGGE) analysis

The 16S rRNA gene fragments were amplified by PCR reaction with a Tgradient thermal cycler (Biometra, Göttingen, Germany) from rhizosphere DNA extracts with the bacterial primers F984-GC and

R1378 according to the PCR conditions described by Heuer et al. (1997). Amplification of fungal ITS regions and the following DGGE were done according to Weinert et al. (2009). The DGGE gels were silver stained and analyzed according to Heuer et al. (2001).

Fungal and bacterial DGGE community fingerprints were analyzed using the software package GELCOMPAR II version 5.6 (Applied Maths, Kortrijk, Belgium). The lane images were normalized and the background was subtracted using a rolling disk method with an intensity of 10 (relative units). A dendrogram was constructed by the hierarchical cluster method un-weighted pair group method using arithmetic averages (UPGMA) and by the Pearson correlation index for each pair of lanes within a gel.

Statistical analysis

Statistical analysis was performed with the STATISTICA program (StatSoft Inc., Tulsa, OK, USA). The counted CFU data per gram root dry weight of the bacterial inoculant were logarithmically (log10) transformed before statistical analysis. The data on inoculant densities were analyzed using three way ANOVA and data on plant shoot dry weight using one way ANOVA and Dunnett's test procedure with $P=0.05$.

Results

Effects of single and co-inoculation treatments on lettuce growth and biocontrol efficiency

Four weeks after planting a total of 20 plants per treatment (five replicates and four plants per replicate) were used to evaluate the effect of single (GB7 or 3Re4-18) and co-inoculation (GB7/3Re4-18) on lettuce growth and the ability to suppress bottom rot disease caused by *R. solani*.

In both experiments the bacterial and fungal inoculants had no effect on lettuce growth in treatments without the pathogen (Table 1) except for the co-inoculation of GB7 and 3Re4-18 in the experiment with application mode II, although the increase in shoot dry weight was not significant. No disease symptoms were observed in all treatments without *R. solani* inoculation. The pathogen had a significant negative effect on lettuce growth in both experiments. In the first experiment (application mode I) a significant increase in ($P=0.05$) shoot dry weight was observed in treatments with 3Re4-18 and with GB7/3Re4-18 in comparison to the pathogen control (Table 1). However, in all treatments with antagonists the number of dead plants did not decrease. In contrast, in the experiment with application mode II an efficient control of *R. solani* on lettuce was achieved

Table 1 Effect of single and co-inoculant treatment of bacterial (*Serratia plymuthica*, 3Re4-18) and fungal (*Trichoderma viride*, GB7) inoculants on shoot dry weight (SDW) of lettuce (cv. Tizian) without and with *Rhizoctonia solani* (*Rs*) inoculation and on number of dead plants (DP from 20) under consideration of the inoculants application mode (AM) 4 weeks after planting of lettuce seedlings (two-leaf stage) into the pots and cultivation at 20/15 °C. Numbers followed by an asterisk in one column are significantly different from the control (SDW) and *Rs* control (DP) respectively, according to Dunnett's test procedure $P=0.05$

Treatments	First experiment			Second experiment		
	AM	SDW [g/plant]	DP [%]	AM	SDW [g/plant]	DP [%]
Control	-	3.73	-	-	5.06 a	-
3Re4-18	PAB	3.75	-	SCB/PAB	4.66 a	-
GB7	SAF	3.65	-	PAF/SAF	5.08 a	-
GB7/3Re4-18	SAF/PAB	3.66	-	PAF/SAF/SCB/PAB	5.92 a	-
Rs conrol	-	2.30*	25		1.60 b	68
3Re4-18 + *Rs*	PAB	3.04	25	SCB/PAB	3.88 a	24*
GB7 + *Rs*	SAF	2.33*	30	PAF/SAF	3.92 a	32*
GB7/3Re4-18 + *Rs*	SAF/PAB	2.92	35	PAF/SAF/SCB/PAB	6.01 a	8*

SCB – seed coating with bacterial inoculant, PAB – plant application with bacterial inoculant, PAF – plant application with fungal inoculant, SAF – substrate application with fungal inoculant

in single and in co-inoculation treatments which showed comparable shoot dry weights to the untreated control (Table 1). The number of dead plants was significantly reduced in all treatments with inoculants compared to the pathogen control (*Rs*). But the best biocontrol effect with the lowest number of dead plants compared to single inoculant treatments was observed in the co-inoculation treatment with GB7 and 3Re4-18. Also the highest shoot dry weight was determined in these treatments with and without pathogen inoculation (Table 1).

Colonization of the lettuce rhizosphere with *S. plymuthica* 3Re4-18

In the experiment with application mode II, the CFU counts of 3Re4-18 were determined in the rhizosphere of inoculated plants 2 and 4 weeks after planting of lettuce into pots. In all treatments with 3Re4-18 inoculant an active colonization of the lettuce rhizosphere without significant differences in the density between the treatments was observed (Table 2). Two weeks after planting, the log CFU counts of 3Re4-18 per gram root dry weight ranged between 5 and 6 after single and co-inoculation with GB7 without and with *R. solani* inoculation. The CFU counts decreased significantly in all treatments 4 weeks after planting with the exception of the co-inoculation treatment (GB7/3Re4-18) (Table 2).

Three way ANOVA revealed that the time (P=0.011) and the pathogen *R. solani* (P=0.010) significantly affected the density of the bacterial antagonist 3Re4-18 in the rhizosphere of lettuce whereas for GB7 (P=0.911) no effect on the density could be observed (Table 2).

Effect of the treatments on the indigenous rhizosphere bacterial community

The effect of the inoculants 3Re4-18 and GB7 after single or co-inoculation without or with inoculation of *R. solani* on the relative abundance of dominant bacterial populations in the rhizosphere of lettuce plants was investigated using DGGE analysis of 16S rRNA gene fragments amplified from total community DNA.

For both application modes complex bacterial community patterns of the 16S rRNA gene fragments were generated at all sampling times. The DGGE profiles indicated similar patterns in all treatments without strong differences compared with the control treatments for application mode I (data not shown). Although several bands showed treatment-dependent changes in intensity most bands were detected in all samples. A band with the same electrophoretic mobility as the 16S rRNA gene fragment of the inoculant 3Re4-18 was present in all treatments inoculated with 3Re4-18 2 weeks after planting (data not shown). More pronounced differences in the DGGE profiles of the bacterial community between the treatments were observed at all sampling times in the experiment with application mode II (Figs. 1a and 2a). In contrast to the experiment with application mode I the relative abundance of several bacterial

Table 2 Cell density of *Serratia plymuthica* 3Re4-18 in the rhizosphere of lettuce (cv. Tizian) in single and co-inoculation treatment with the fungal inoculant *Trichoderma viride* GB7 in absence and presence of the pathogen *Rhizoctonia solani* (*Rs*) under consideration of the inoculants application mode (AM) 2 and 4 weeks after planting of lettuce seedlings (two-leaf stage) into the pots and cultivation at 20/15 °C. Three way ANOVA revealed that the time (P=0.011) and the pathogen *R. solani* (P=0.010) significantly affected the density of the bacterial antagonist 3Re4-18 in the rhizosphere of lettuce whereas for GB7 no effect on the density respectively could be observed

Treatments	AM	$\log_{10}$ CFU g^{-1} root dry weight	
		at 2 weeks	at 4 weeks sampling times
3Re4-18	SCB/PAB	5.5	5.0
GB7/3Re4-18	PAF/SAF/SCB/PAB	5.2	5.3
3Re4-18 + *Rs*	SCB/PAB	6.0	5.4
GB7/3Re4-18 + *Rs*	PAF/SAF/SCB/PAB	6.2	5.4

SCB – seed coating with bacterial inoculant, PAB – plant application with bacterial inoculant, PAF – plant application with fungal inoculant, SAF – substrate application with fungal inoculant

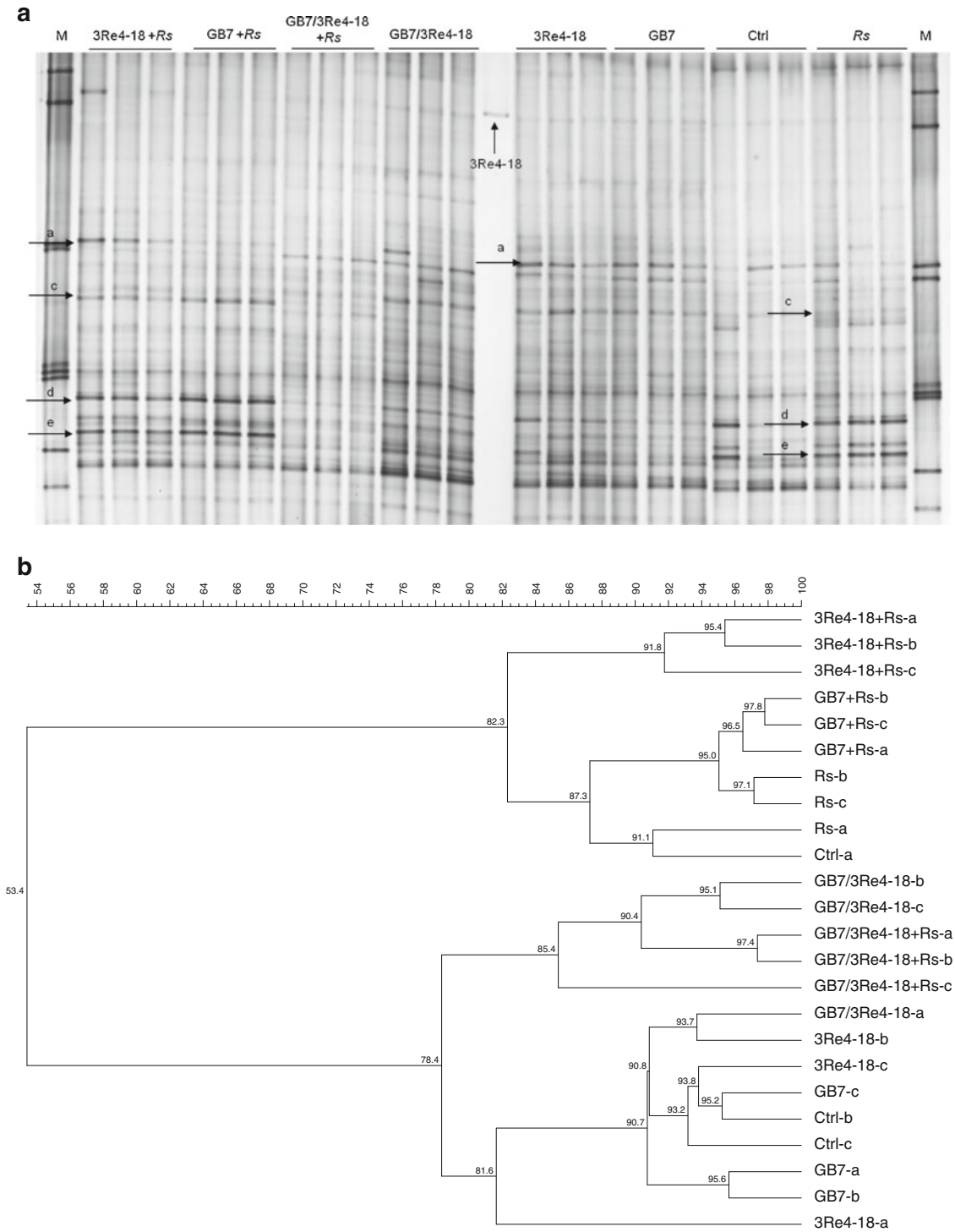
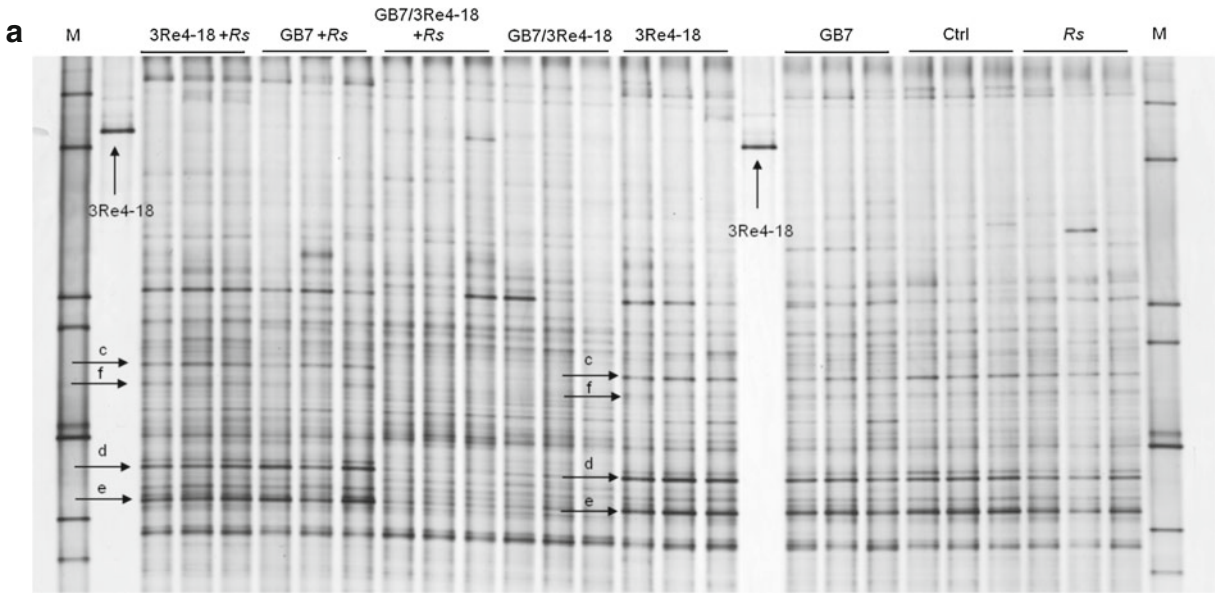
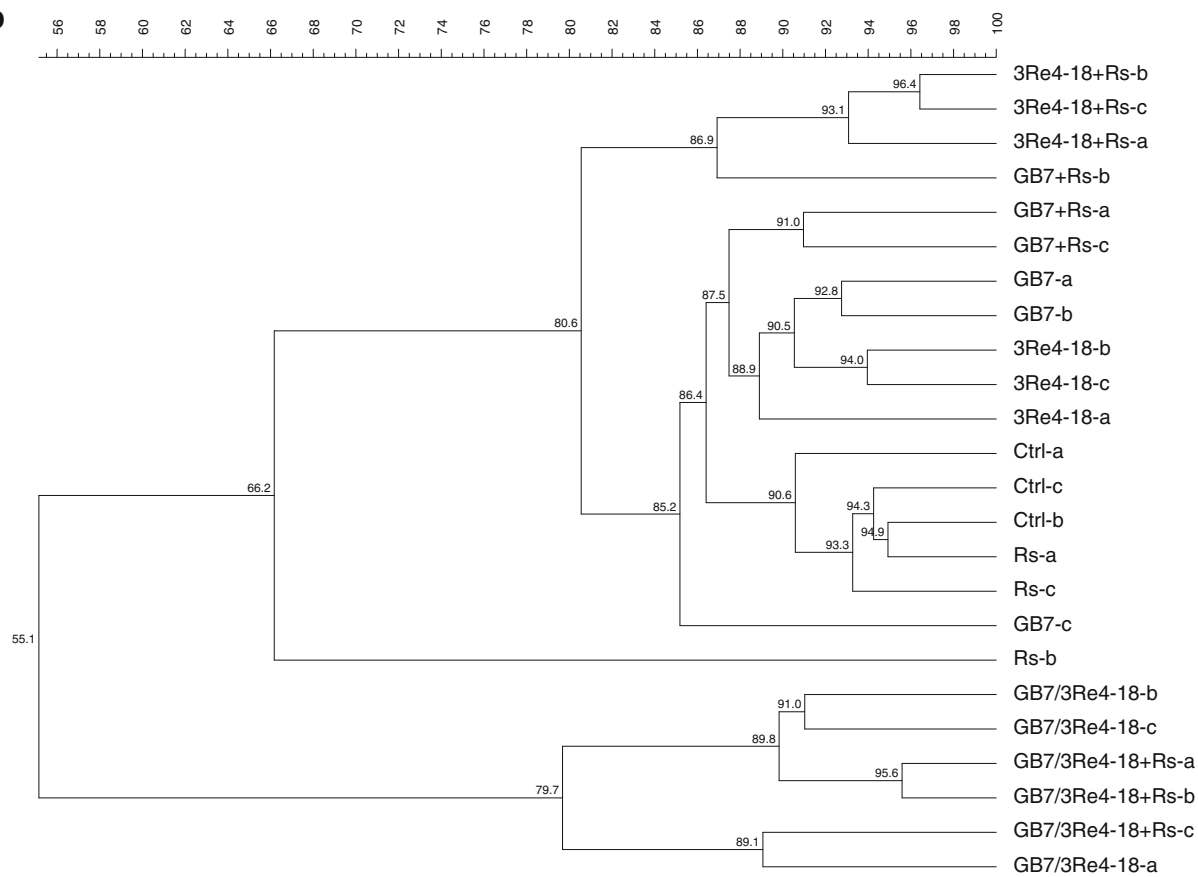


Fig. 1 DGGE fingerprints (**a**) of bacterial 16S-rRNA gene fragments from community DNA extracts obtained from lettuce rhizosphere after single and co-inoculant treatment (GB7/3Re4-18) with *Serratia plymuthica* (3Re4-18) and *Trichoderma viride* (GB7) without and with *Rhizoctonia solani* (*Rs*) with respect to the control (Ctrl) 2 weeks after planting of lettuce seedlings (two-leaf stage) into the pots and cultivation at 20/15 °C. Lettuce seeds and young plants in two-leaf stage were treated with the bacterial inoculant 3Re4-18. The fungal inoculant GB7 was applied to seedlings and in the substrate 1 week before planting. The corresponding UPGMA dendrogram (**b**) based on Pearson's correlation indices


a
M
3Re4-18 +Rs
GB7 +Rs
GB7/3Re4-18 +Rs
GB7/3Re4-18
3Re4-18
GB7
Ctrl
Rs
M
3Re4-18
3Re4-18
c
f
d
e
c
f
d
e


b
56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100
3Re4-18+Rs-b
3Re4-18+Rs-c
3Re4-18+Rs-a
GB7+Rs-b
GB7+Rs-a
GB7+Rs-c
GB7-a
GB7-b
3Re4-18-b
3Re4-18-c
3Re4-18-a
Ctrl-a
Ctrl-c
Ctrl-b
Rs-a
Rs-c
GB7-c
Rs-b
GB7/3Re4-18-b
GB7/3Re4-18-c
GB7/3Re4-18+Rs-a
GB7/3Re4-18+Rs-b
GB7/3Re4-18+Rs-c
GB7/3Re4-18-a
96.4
93.1
86.9
91.0
87.5
92.8
80.6
90.5
94.0
88.9
86.4
66.2
85.2
90.6
94.3
94.9
93.3
55.1
91.0
89.8
95.6
79.7
89.1

◀ **Fig. 2** DGGE fingerprints (**a**) of bacterial 16S-rRNA gene fragments from community DNA extracts obtained from lettuce rhizosphere after single and co-inoculation treatment (GB7/3Re4-18) with *Serratia plymuthica* (3Re4-18) and *Trichoderma viride* (GB7) without and with *Rhizoctonia solani* (*Rs*) with respect to the control (Ctrl) 4 weeks after planting of lettuce seedlings (two-leaf stage) into the pots and cultivation at 20/15 °C. Lettuce seeds and young plants in two-leaf stage were treated with the bacterial inoculant 3Re4-18. The fungal inoculant GB7 was applied to seedlings and in the substrate 1 week before planting. The corresponding UPGMA dendrogram (**b**) based on Pearson's correlation indices

populations was influenced by the treatments (Fig. 1a). Clear differences in the DGGE fingerprint patterns with the presence or absence of *R. solani* were observed. The bacterial DGGE fingerprint patterns in the co-inoculation treatments (GB7/3Re4-18) without and with *R. solani* inoculation did markedly differ from the banding patterns of all other treatments at both sampling times (Figs. 1a and 2a). Indeed the DGGE profiles of all co-inoculation treatments showed less dominant bands and displayed many equally abundant bands pointing to an increased evenness. In particular the profiles of the pathogen control (*Rs*), of the inoculants 3Re4-18 and of GB7 with the pathogen (+*Rs*) showed dominant bands that were absent or less intense (c, d and e) in both co-inoculation treatments at all sampling times and in all treatments without *R. solani* except for one replicate of the control at 2 weeks sampling time (Figs. 1a and 2a). In addition, one dominant band (a) was typical for all treatments except for both co-inoculation treatments at 2 weeks sampling time and the band (f) at 4 weeks sampling time.

The dendrogram generated by UPGMA analysis revealed the presence of two main clusters at both sampling times (Figs. 1b and 2b). One cluster contained all samples from treatments without *R. solani* inoculation including the samples from both co-inoculation treatments (except one replicate of Ctrl a) 2 weeks after planting (Fig. 1b). Both co-inoculation treatments clustered together in a sub-cluster (except one replicate in absence of *R. solani*) at 2 weeks sampling time and in a main cluster at 4 weeks sampling time (Fig. 2b). The pathogen control (*Rs*) clustered together with the control and single inoculants treatments at 4 weeks sampling time (Fig. 2b). The fingerprints of the co-inoculation treatments (GB7/3Re4-18) without or with *R. solani* inoculation shared a high similarity at both sampling times (Figs. 1b and 2b) and formed separate clusters from the patterns of the treatments with single inoculants (GB7 or 3Re4-18) with and without *R. solani* at 4 weeks sampling time. The treatments with 3Re4-18 or GB7 with or without *R. solani* formed clusters sharing more than 80 % similarity at 4 weeks sampling time (Fig. 2b).

Surprisingly, cluster analysis showed no differences between replicates of untreated control plants (Ctrl) and pathogen control plants (*Rs*). The DGGE fingerprints of these replicates are very similar at the 4 weeks sampling time.

Fungal community fingerprints

Complex fungal DGGE fingerprints were observed in both experiments at all sampling times. In both experiments no band with the electrophoretic mobility of *R. solani* was detected in any fingerprints of treatments with the pathogen. An increased abundance of a band with a similar electrophoretic mobility of *T. viride* GB7 (bands marked with c) was detected in all GB7-treated variants indicating an establishment of the inoculant in the lettuce rhizosphere in both experiments at all sampling times (data shown for the second experiment, application mode II, Figs. 3a and 4a). The fungal profiles were also markedly influenced by the co-inoculation treatment (GB7/3Re4-18) with or without *R. solani* inoculation (Figs. 3a and 4a). The fungal profiles of both co-inoculation treatments showed less dominant bands and were very similar at both sampling times. In particular the bands marked with a, b, e, m, n and p were absent or less intense in both co-inoculation treatments (Fig. 3a and 4A) whereas the bands marked with f, g and w were only present in the co-inoculants treatment at 4 weeks sampling time (Fig. 4a).

The UPGMA analysis revealed also the presence of two main clusters at both sampling times (Figs. 3b and 4b). One cluster contained only the samples from co-inoculation treatments (GB7/3Re4-18) at both sampling times (except two replicates of treatment with GB7 and one replicate of co-inoculation treatment without *R. solani* at 2 weeks sampling time which did not fall in this cluster; Fig. 3b). The samples of the control (Ctrl) were variable at both sampling times. The distance between the main clusters increased at 4 weeks sampling time. The co-inoculation treatments formed a cluster distant from the other treatments at

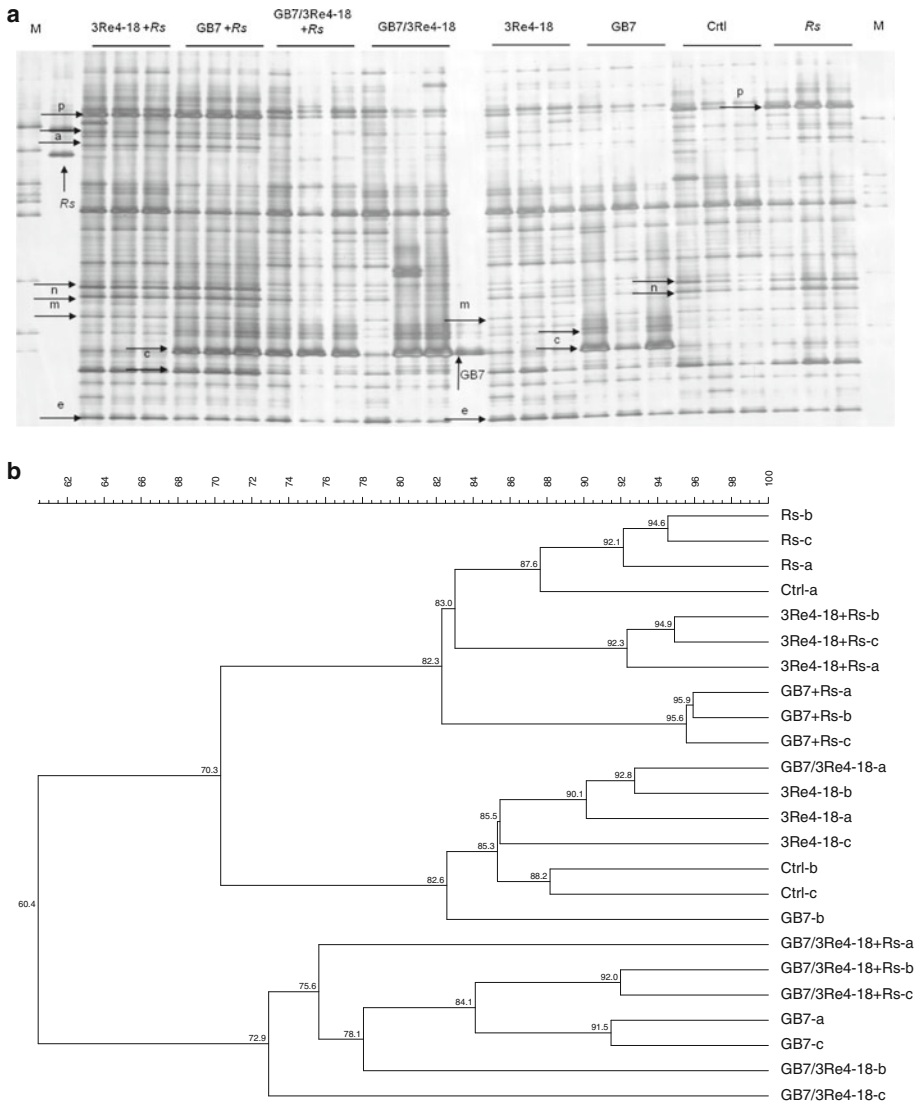


Fig. 3 DGGE fingerprints of fungal ITS gene fragments from community DNA extracts obtained from lettuce rhizosphere after single and co-inoculation treatment (GB7/3Re4-18) with *Serratia plymuthica* (3Re4-18) and *Trichoderma viride* (GB7) without and with *Rhizoctonia solani* (*Rs*) with respect to the control (Ctrl) 2 weeks after transplanting of lettuce seedlings (two-leaf stage) into the pots and cultivation at 20/15 °C. Lettuce seeds and plants in two-leaf stage were treated with the bacterial inoculant 3Re4-18. The fungal inoculant GB7 was applied to seedlings and in the substrate 1 week before planting. The corresponding UPGMA dendrogram (**b**) based on Pearson's correlation indices

both sampling times (Figs. 3b and 4b). The treatments with single inoculants GB7 or 3Re4-18 with *R. solani* (+*Rs*) were grouped in a sub-cluster of the corresponding main cluster at 2 weeks sampling times and shared a high level of similarity (Fig. 3b). All treatments shared more than 70 % similarity at the 4 weeks sampling time except the co-inoculation treatment (Fig. 4b). Compared to the UPGMA analysis of the bacterial DGGE fingerprints the cluster analysis of the fungal communities showed a higher similarity between the replicates of the untreated control (Ctrl) and the pathogen control (*Rs*) at 4 weeks sampling time.

Discussion

In the present study the effects of the single and co-inoculation of *S. plymuthica* 3Re4-18 and *T. viride* GB7 on biocontrol, plant growth and interaction with the indigenous rhizosphere community were investigated simultaneously. Different application modes of the inoculant strains were used in experiments performed which might account for differences in the effects on biocontrol efficiencies observed. Although the pathogen was applied with barley kernels in both experiments in the same way, the disease severity was much lower in the first experiment. In both

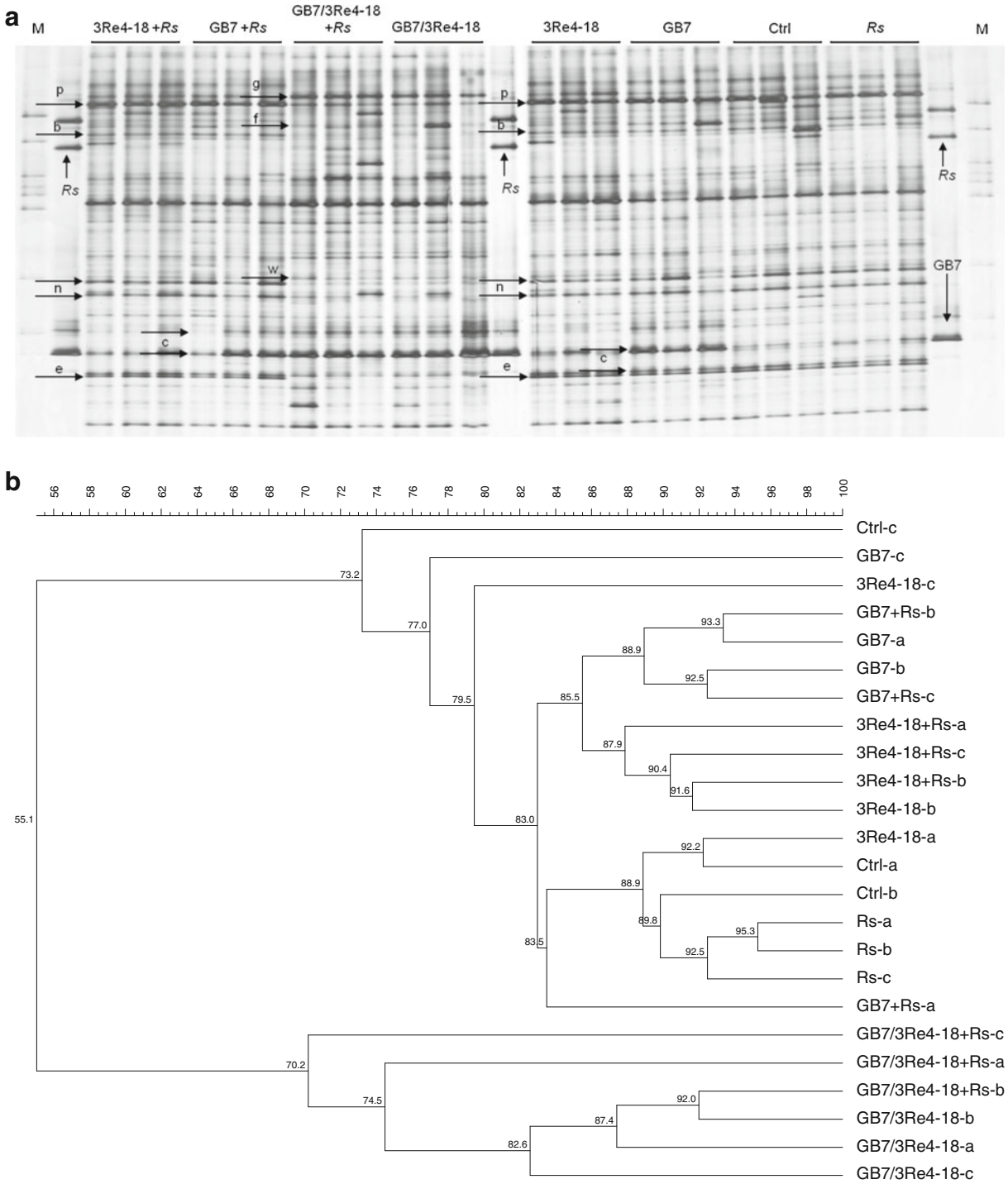


Fig. 4 DGGE fingerprints of fungal ITS gene fragments from community DNA extracts obtained from lettuce rhizosphere after single and co-inoculation treatment (GB7/3Re4-18) with *Serratia plymuthica* (3Re4-18) and *Trichoderma viride* (GB7) without and with *Rhizoctonia solani* (*Rs*) with respect to the control (Ctrl) 4 weeks after transplanting of lettuce seedlings (two-leaf stage) into the pots and cultivation at 20/15 °C. Lettuce seeds and young plants in two-leaf stage were treated with the bacterial inoculant 3Re4-18. The fungal inoculant GB7 was applied to seedlings and in the substrate 1 week before planting. The corresponding UPGMA dendrogram (**b**) based on Pearson's correlation indices

experiments in the absence of *R. solani* lettuce growth was not affected by inoculants treatment independent of the application mode neither in single nor in co-inoculation treatment. These findings accorded with previous results for single applications of 3Re4-18 and GB7 (Grosch et al. 2005, 2007). However, the highest shoot dry weight (SDW) was observed in co-inoculation treatment in the second experiment. In the experiment with application mode I the single inoculation of 3Re4-18 and the co-inoculation treatment (GB7/3Re4-18) resulted in increased SDW compared to the pathogen control but similar numbers of dead plants were recorded. No biocontrol effect was observed in single inoculation of GB7. The differences achieved due to the changes in application mode were quite remarkable. The most likely reason for the lack of biocontrol in the first experiments was insufficient colonization of the lettuce rhizosphere in particular for GB7 and 3Re4-18. In contrast, a successful biocontrol effect was given in all variants in the experiment with application mode II. In this experiment the seeds were inoculated with 3Re4-18 and young plants were additionally treated with a conidia suspension of GB7 one week before planting as done in previous experiments showing successful biocontrol effects (Grosch et al. 2007). Hence, the results support that the fungal antagonist should be established in the rhizosphere before the attack of the pathogen in order to achieve successful disease suppression.

Considering the low disease pressure in the experiment with application mode I, a better control effect was anticipated by the inoculant 3Re4-18. Possibly the density of 3Re4-18 was too low for a more efficient control effect. A high rhizosphere competence of a biocontrol agent is a key factor for successful control activity (Lugtenberg and Kamilova 2009). The underlying mechanisms (production of antifungal compounds, induced systemic resistance) for biocontrol activity of 3Re4-18 are influenced by quorum sensing (QS) (Müller et al. 2009). The importance of N-acyl homoserine lactone (AHL)-mediated communication in biocontrol activity was demonstrated for *S. plymuthica* HRO-C48 in the pathosystem *Verticillium dahliae*-oilseed rape, and the characteristics of 3Re4-18 are comparable to the strain HRO-C48 (Müller et al. 2009). Moreover, *S. plymuthica*-mediated (strain IC1270) induced systemic resistance (ISR) response was described within the rice defense network to the necrotrophic pathogen *R. solani* (De Vleesschauwer et al. 2009). An antagonist density of $\log_{10}$ 4.0 CFU g^{-1} was estimated as crucial for successful biocontrol (Scher et al. 1994). Because the CFU of 3Re4-18 were not determined in the experiment with application mode I it can only be assumed that the density of 3Re4-18 was too low. However, a sufficiently high density of 3Re4-18 was achieved in the experiment with application mode II with successful biocontrol effects in all treatments. Moreover, it should be considered that the disease pressure caused by *R. solani* in lettuce was much higher in the second experiment than in the first experiment. Under this condition the best control efficacy was achieved in the co-inoculation treatment of GB7 and 3Re4-18.

Positive results on biocontrol efficacy of co-inoculation treatments with biocontrol agents with various modes of action like *B. subtilis* and *T. viride* against *R. solani* on potato (Brewer and Larkin 2005), *T. virens* and various *Burkholderia* strains against *R. solani* in cucumber (Roberts et al. 2005) and *B. subtilis* and a Pseudomonas strain on eggplant and pepper against *R. solani* (Abeysinghe 2009) support our observation. Moreover, our results confirm the sufficient rhizosphere colonization of microbial inoculants as a key factor for successful biocontrol of soil-borne pathogens (Gravel et al. 2005; Haas and Défago 2005; Raaijmakers and Weller 2001). Interestingly, statistical analysis over both time points revealed an increased density of 3Re4-18 on average in the presence of *R. solani* as indicated by a cultivation-dependent approach, whereas *T. viride* GB7 did not affect 3Re4-18 densities in the rhizosphere of lettuce. This observation suggests an interaction between the fungal pathogen and the bacterial inoculants, but most likely the effect of the pathogen was caused by changes in the root exudation patterns or due to damaged plant tissue which increased the availability of nutrients.

Besides plant growth and biocontrol effects due to single or co-inoculation, another aspect taken into account was the impact on the indigenous rhizosphere microflora. Fingerprints derived by DGGE had been successfully used to study the impact of treatments on soil (Vargas Gil et al. 2011) and rhizosphere microflora (Götz et al. 2006; Haichar et al. 2007; Adesina et al. 2009). In contrast to Adesina et al. (2009), the fungal community analysis was not based on the 18S rRNA gene fragments but on ITS fragments amplified from total community DNA as more complex fingerprints due to the better resolution level. DGGE analysis

showed that the inoculation of single inoculant strains (3Re4-18 and GB7) did not result in pronounced changes in the relative abundance of dominant bacterial and fungal populations in the rhizosphere. These findings were in accord with previous observations for single inoculants on the dominant rhizosphere community like *Pseudomonas jessenii* RU47 in lettuce (Adesina et al. 2009) and *S. plymuthica* HRO-C48 in strawberry (Scherwinski et al. 2007). In contrast, Kozdroj et al. (2004) found an altered balance among the indigenous maize rhizosphere population in response to introduced pseudomonads (Herschkovitz et al. 2005) and in response to *Azospirillum brasilense* inoculation. An impact of single application of *B. subtilis* and *A. brasilense* on the bacterial population in the rhizosphere of tomato plants was also shown by Felici et al. (2008).

DGGE fingerprints demonstrated a strong impact of co-inoculation on bacterial and fungal rhizosphere community contrary to single inoculant application (Figs. 1, 2, 3, 4). Typically less dominant bands were observed in all replicates of the co-inoculation treatments indicating a higher evenness. This effect was not influenced by the presence of the fungal pathogen *R. solani*. This supports that an interaction between the inoculants, the plants and the indigenous microbes had occurred. More in-depth insights into changes of dominant bacterial or fungal populations will be obtained by amplicon pyrosequencing. While this type of analysis will typically provide information on the taxonomic affiliation of dominant bacterial and fungal responders to the treatment, traditional cultivation methods might still be needed to gain information on their functions. The co-inoculation of GB7 and 3Re4-18 showed the best biocontrol effect and it must be supposed that the inoculants inhibited effectively the impact of the pathogen on the lettuce plants. This supports the higher biocontrol efficacy of co-inoculation of biocontrol agents with various modes of action.

Moreover, the pathogen *R. solani* AG1-IB affects some populations of bacteria and fungi inhabiting the rhizosphere of lettuce at high disease pressure. The study by Adesina et al. (2009) done also under growth chamber conditions with artificial *R. solani* inoculation showed also pronounced differences in the relative abundance of bacterial and fungal populations in the rhizosphere, especially in response to the pathogens that were weaker when the antagonist was present. In the present study, no effect of the pathogen on the bacterial rhizosphere community was observed at low disease pressure in the first experiment. Also replicates of the pathogen control clustered together with replicates of the control treatment 4 weeks after planting in the second experiment as only symptomless plants were sampled from the pathogen controls. These findings support the importance of quantification of the pathogen density in the rhizosphere of lettuce with respect to the microbial rhizosphere community in future experiments. The present study and the study of Adesina et al. (2009) indicated that not only the effects of the inoculant, but also the impact of the pathogen on the bacterial and fungal community composition should be considered. The shifts in microbial community structure can affect positively or negatively the plant growth and health and the affected microbial populations should be analyzed in future experiments. The band of *R. solani* was not detected in any of the profiles indicating that this is not a preferred compartment for the pathogen which may be preferred to infect the plant at the root crown.

Various studies showed that co-inoculation of biocontrol agents increased performance in disease suppression (Raupach and Kloepper 1998; Vestberg et al. 2004; Roberts et al. 2005; Abeysinghe 2009). Our results were in accord with these findings. However, other studies have reported decreased disease suppression (Meyer and Roberts 2002; Robinson-Boyer et al. 2009) or no synergistic effects on plant protection and plant growth promoting (Schisler et al. 1997; Schmidt et al. 2004; Felici et al. 2008; Xu et al. 2010) following co-inoculation of microbial inoculants compared to single application of the inoculants. These support that the effects of co-inoculation treatment need to be studied in view of the specific plant and the target pathogen.

The co-inoculation of *S. plymuthica* 3Re4-18 and *T. viride* strain GB7 resulted in an improved biocontrol efficacy only in the experiment with application mode II. The findings of the present study underline that the application mode of inoculants is critical for the success of biocontrol. Our study provided first insights into the complex interaction in response to the pathogen presence and to co-inoculation of a bacterial and fungal biocontrol strain. DGGE analysis revealed an increased evenness of the bacterial and fungal community in the co-inoculation treatments compared to single application.

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