Arch Microbiol
DOI 10.1007/s00203-006-0199-0

ORIGINAL PAPER

Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*

Marco Kai · Uta Effmert · Gabriele Berg · Birgit Piechulla

Received: 6 April 2006 / Revised: 16 October 2006 / Accepted: 14 November 2006

Abstract Bacterial antagonists are bacteria that negatively affect the growth of other organisms. Many antagonists inhibit the growth of fungi by various mechanisms, e.g., secretion of lytic enzymes, siderophores and antibiotics. Such inhibition of fungal growth may indirectly support plant growth. Here, we demonstrate that small organic volatile compounds (VOCs) emitted from bacterial antagonists negatively influence the mycelial growth of the soil-borne phytopathogenic fungus *Rhizoctonia solani* Kühn. Strong inhibitions (99–80%) under the test conditions were observed with *Stenotrophomonas maltophilia* R3089, *Serratia plymuthica* HRO-C48, *Stenotrophomonas rhizophila* P69, *Serratia odorifera* 4Rx13, *Pseudomonas trivialis* 3Re2-7, *S. plymuthica* 3Re4-18 and *Bacillus subtilis* B2g. *Pseudomonas fluorescens* L13-6-12 and *Burkholderia cepacia* 1S18 achieved 30% growth reduction. The VOC profiles of these antagonists, obtained through headspace collection and analysis on GC-MS, show different compositions and complexities ranging from 1 to almost 30 compounds. Most volatiles are species-specific, but overlapping volatile patterns were found for *Serratia* spp. and *Pseudomonas* spp. Many of the bacterial VOCs could not be identified for lack of match with mass-spectra of volatiles in the databases.

M. Kai · U. Effmert · B. Piechulla (✉)
Department of Biological Sciences,
University of Rostock, Albert-Einstein-Str. 3,
18059 Rostock, Germany
e-mail: birgit.piechulla@uni-rostock.de

G. Berg
Environmental Biotechnology,
Graz Technical University, Petersgasse 12,
8010 Graz, Austria

Keywords Bacterial antagonists · Volatile organic compounds · *Serratia* spp. · *Stenotrophomonas* spp. · *Pseudomonas* spp. · *Staphylococcus epidermidis* · *Burkholderia cepacia* · *Bacillis subtilis* · *Rhizoctonia solani*

Introduction

Plants have to cope with a number of biotic and abiotic environmental stresses during their growth and development. For example, the widespread soil-borne pathogen *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* [A.B. Frank] Donk) is responsible for serious damage to many economically important agricultural and horticultural crops as well as to trees worldwide (Anderson 1982; Sneh et al. 1996). In the last few years, the importance of this pathogen has increased dramatically in Europe (Grosch et al. 2005). *R. solani* strains occur ubiquitously and are both saprophytic as well pathogenic to more than 500 plant hosts. Sclerotia-dormant forms of the fungi-are resistant to environmental extremes and allow the fungus to survive adverse conditions. Unfortunately, although the pathogen causes serious economically loss through reduced yield in many crops, no effective strategy to control the pathogen is currently available.

Bacterial antagonists are bacteria that negatively affect the growth of other organisms, including plant pathogens. They play an important role in the suppression of soil-borne plant diseases (Cook et al. 1995; Gupta et al. 2000; Whipps 2001; Weller et al. 2002). Therefore, the knowledge of the mode of action of antagonistic bacteria could help in devising successful and reproducible biological control methods. Mechanisms

responsible for antagonistic activity include (1) inhibition of the pathogen, (2) competition for colonisation sites, nutrients and minerals, (3) parasitism, and (4) mycophagy (Handelsman and Stabb 1996; Bloemberg and Lugtenberg 2001; Bais et al. 2004, 2006; Walker et al. 2003; Haas and Defago 2005). It is a common strategy of bacterial antagonists to inhibit the plant pathogens by excretion of antifungal metabolites (AFMs). Well-known AFMs are antibiotics, toxins and bio-surfactants (Raaijmakers et al. 2002). Recently, it was demonstrated that volatile organic compounds of soil bacteria can influence growth of fungi (Alstrom 2001; Wheatley 2002). Such low molecular weight organic volatiles (VOCs), e.g. terpenoids, phenylpropanoids, fatty acid derivatives, have been shown to serve as inter- and intra-organismic communication signals in general (Stotzky and Schenk 1976; Schöller et al. 2002; Wheatley 2002; Pare and Tumlinson 1999; Piechulla and Pott 2003).

Such compounds can have either harmful or positive effects on the organisms perceiving the signal. For example, a complex mixture of volatile lactones and terpenoids (including geosmin) that exhibits antibiotic properties has been identified from a marine *Streptomyces* species (Dickschat et al. 2005). Schöller et al. (2002) screened 26 *Streptomyces* species and identified a total of 120 different VOCs, comprising alkanes, alkenes, alcohols, esters, ketones, sulfur-containing compounds and terpenoids, of which 10% remained unidentified. However, the VOCs were emitted in different combinations from the different *Streptomyces* species (Schöller et al. 2002). Only recently the first enzymes/genes involved in the biosynthesis of geosmin of *Streptomyces* spp. have been isolated (Gust et al. 2003; Cane and Watt 2003).

Bacterial VOCs apparently also have the potential to act as antifungal compounds (Alstrom 2001; Wheatley 2002). We therefore began a survey to study the emission of volatiles from bacterial strains, that have been demonstrated to be antagonists of *R. solani*, and are sometimes commercially used as 'biological control agents'. The genus *Serratia* comprises Gram negative gamma-proteobacteria, which are associated with plant roots. *S. plymuthica* has antifungal activities against a wide spectrum of phytopathogenic fungi, e.g. *Fusarium culmorum*, *Pythium* spp., *R. solani* and *Verticillium dahliae* (Ström and Gerhardson 1988; Kurze et al. 2001). Some *S. plymuthica* isolates produce antibiotics such as prodigiosin or pyrrolnitrin (Kalbe et al. 1996); others secrete lytic enzymes (chitinases, glucanases). *S. plymuthica* HRO C48 was isolated from the *Brassica napus* rhizosphere and is a phytopathogen against *V. dahliae* Kleb, *R. solani* Kühn and *Sclerotinia sclerotiorum* De Bary but exhibits growth promoting effects on strawberry plants via IAA production (Kalbe et al. 1996; Kurze et al. 2001). The antifungal activities of *S. odorifera* and *S. plymuthica* 3RE4-18 were characterized by Berg et al. (2002, 2005, respectively). Pseudomonads are highly abundant in many rhizo- and endospheres of *Solanum tuberosum* and exhibit antifungal activity against *V. dahliae* and *R. solani* by producing antibiotics, proteases and siderophores (Lottmann and Berg 2001; Berg et al. 2005). The *S. maltophilia* strain, originally isolated from the rhizosphere of oilseed rape, produces the antibiotic maltophilin which acts against a broad range of fungi (Jakobi et al. 1996; Denton and Kerr 1998). *S. rhizophila* P69 was also isolated from roots of *Brassica napus* and has antifungal activity against *V. dahliae*, *R. solani* and *S. sclerotiorum* (Minkwitz and Berg 2001; Wolf et al. 2002). *Burkholderia cepacia* (β-proteobacteria, Neisseriae) isolated from the sporophyte of the moss *Sphagnum rubellum* has antifungal activity against *V. dahliae*. For this strain antibiotic activity and siderophore secretion was shown (Opelt and Berg 2004). Finally, two Gram positive bacteria were also investigated. *S. epidermidis* 2P3-18 was isolated from the phyllosphere of potato and characterized as antagonist against soil-borne pathogens (Krechel et al. 2002). Although there are many reports of this bacterium found in association with plants, the bacterium is also known as a nosocomial microorganism found to be responsible for infections in immune suppressive humans (Zhang et al. 2003). *B. subtilis* B2g is the 'biological control agent' of the plant protection product RhizoVit (Marten et al. 2000).

In the work presented here, our goals were to determine whether bacterial antagonists emit small organic compounds, and if so, whether these bacterial compounds have the potential to affect the growth and development of the phytopathogenic fungus *R. solani*.

Materials and methods

Bacteria cultures

Bacteria were obtained from the Strain Collection of Antagonistic Microorganisms (SCAM; University of Rostock, Microbiology). Eight strains were isolated from the rhizosphere/endorhiza, one strain derived from the phyllosphere and one from the sporophyte of a bryophyte. A complete overview of the origin of the strains is given in Table 1. An airborne indoor bacterium (not identified) was used as a control. For short-term storage, bacteria were streaked onto nutrient agar II plates (NA II; peptone from casein 3.5 g l^{-1}, peptone

Table 1 Selected rhizobacterial isolates

Isolates	Origin	References
Bacillus subtilis B2g	Rhizosphere of oilseed rape	Marten et al. (2000)
Burkholderia cepacia 1S18	Sporophyte of *Sphagnum rubellum*	Opelt and Berg (2004)
Pseudomonas fluorescens L13-6-12	Rhizosphere of potato	Lottmann and Berg (2001)
Pseudomonas trivialis 3Re2-7	Endorhiza of potato	Krechel et al. (2002)
Serratia odorifera 4Rx13	Rhizosphere of potato	Faltin et al. (2004)
Serratia plymuthica 3Re4-18	Endorhiza of potato	Grosch et al. (2005)
Serratia plymuthica HRO-C48	Rhizosphere of oilseed rape	Kalbe et al. (1996) Kurze et al. (2001)
Staphylococcus epidermidis 2P3-18	Phyllosphere of potato	Berg et al. (2005)
Stenotrophomonas maltophilia R3089	Rhizosphere of oilseed rape	Berg and Ballin (1994)
Stenotrophomonas rhizophila P69	Rhizosphere of oilseed rape	Wolf et al. (2002)

from meat 2.5 g l^{-1}, peptone from gelatine 2.5 g l^{-1}, yeast extract 1.5 g l^{-1}, NaCl 5 g l^{-1}, agar–agar 15 g l^{-1}, pH 7.2), incubated for 12–24 h at 30°C under constant dim light (1.5 μEm^{-2}s^{-1}) and finally stored at 4°C. For long-term storage, bacteria were maintained in nutrient broth II (NB II; peptone from casein 3.5 g l^{-1}, peptone from meat 2.5 g l^{-1}, peptone from gelatine 2.5 g l^{-1}, yeast extract 1.5 g l^{-1}, NaCl 5 g l^{-1}, pH 7.2) supplemented with 20% glycerol at −70°C.

Fungal isolate

Rhizoctonia solani Kühn (RHI S0 WE) was originally isolated from cabbage (Rita Grosch, Institute of Vegetable and Ornamental Crops, Großbeeren/Erfurt e.V.). This isolate belongs to the anastomosis group 2. For long-term storage, tiny pieces of an actively growing colony were brought into fungi preservation medium (peptone from casein 20 g l^{-1}, yeast extract 10 g l^{-1}, glucose 80 g l^{-1}, glycerol 60%) and stored at −70°C. For short-term maintenance, the fungus was cultivated on a Waksman-agar plate (WA; tryptone 5 g l^{-1}, yeast extract 3 g l^{-1}, glucose 10 g l^{-1}, NaCl 5 g l^{-1}, agar-agar 20 g l^{-1}, pH 6.8) at 20°C under darkness. Every 10th day, a 6 mm agar plug with mycelium was taken from the margin of the colony using a sterile cork borer. The plug was placed onto a fresh agar plate and incubated as described above.

Collection and analysis of bacterial volatiles

Bacteria, maintained at 4°C, were inoculated into 6 ml of NB II and incubated at 30°C under agitation for 12–24 h (160 rpm; Bühler, Tübingen, Germany) and constant dim light. Reaching an OD_{600} of 1.0–1.5, cultures were diluted (~1:10^5) to obtain an initial inoculum of 200–300 cfu/10 μl. Five droplets (each of 10 μl) of the dilution were placed onto a NA II plate (glass Petri dish, 94 × 15 mm). Droplets were distributed to form five thin lines and plates were incubated for 48 h at 30°C under constant dim light. For volatile analysis, the incubated glass Petri dish was placed after 48 h incubation without the lid into a glass Petri dish (143 × 30 mm), which had an in- and outlet (analysis chamber). Charcoal-purified, sterile, and humidified air entered the chamber through the inlet with a constant flow of 2 l min^{-1}, which was provided by a membrane pump (Riechle, Puchheim, Germany). A collection trap, which contained 100 mg Super-Q (Alltech Associates, Deerfield, IL, USA) was connected to the outlet and a second pump sucked the volatile-enriched air over the trap with a constant flow of 1 l min^{-1}. The excess air (in: 2 l min^{-1}, out: 1 l min^{-1}) escaped through the space between dish and lid of the analysis chamber. During the following incubation period (24 h = 3rd day, 30°C, 1.5 μEm^{-2}s^{-1}), the volatiles were collected in two intervals, each 12 h. Nonyl acetate as internal standard (1.5 μg) was added to the column, the collected volatiles and nonyl acetate were consecutively eluted with 200 and 100 μl dichloromethane.

Samples were analyzed using a GC/MS-QP5000 from Shimadzu (70 eV; Kyoto, Japan) equipped with a DB5-MS column (60 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA). Splitless liquid injection of 1 μl sample was performed at 200°C with a sample time of 2 min using a CTC autosampler (CTC Analytics, Zwingen, Switzerland). The initial column temperature was set at 35°C, followed by a ramp of 10°C min^{-1} up to 280°C with a final hold for 15 min at 280°C. Helium was used as carrier gas. The column flow was set at 1.1 ml min^{-1} with a linear velocity of 28 cm s^{-1}.

Mass spectra were obtained using the scan modus (total ion count, 40–280 m/z). The confirmation of compound identity was done by comparison of mass spectra and retention times with those of available

standards, by comparison of the obtained spectra with spectra in the library of the National Institute of Standards and Technology (NIST147), and by comparison of Kovats indices. Experiments were replicated twice unless otherwise noted. The resulting profiles were reproducible with some variations of the peak heights.

Dual culture tests

Bacteria were inoculated into 6 ml of NB II and incubated at 30°C under agitation (160 rpm; Bühler, Tübingen, Germany) under constant dim light up to an OD_{600} of 1.0–1.5. Two-compartment plastic plates (92 × 16 mm; Sarstedt AG, Nürnbrecht, Germany) were loaded with NA II on one and WA on the other side. The NA II side was plated with 50 µl of the prepared bacterial strain and incubated at 30°C under dim light. After 48 h, an agar plug (Ø 6 mm) was taken from the margin of a 5–7 days old *R. solani* mycelium and placed onto the WA side. Sterile distilled water or NB II (each 50 µl) instead of the bacterial culture served as controls. The two-compartment plates were incubated at 20°C for 5 days under darkness. Images of the mycelium were then recorded using a digital camera C-3030 Zoom (Camedia, Olympus, Tokyo, Japan). The area of the grown hyphae was assessed by adapting the Image Gauge software of the Image analyser LAS-1000 (Fujifilm, Tokyo, Japan). Experiments were replicated three times and each experiment comprised 3–7 replicates of each bacterial strain.

Results

Volatile emission from bacterial antagonists

Ten isolates from the strain collection of antagonistic microorganisms (SCAM) were selected, comprising *Bacillus*, *Burkholderia*, *Pseudomonas*, *Serratia*, *Staphylococcus* and *Stenotrophomonas* species (Table 1). The criteria for selection of these strains were: (1) the strains should be of different phylogenetic origin, (2) they should possess antagonistic activities, (3) they had been isolated from different plant species, (4) they should act by different modes of action and (5) they should release volatiles that can be recognizable by olfaction.

Preliminary investigations indicated that growth phase and growth conditions could influence bacterial VOC profiles. It was observed that a plateau in total VOC emission was reached between the 3rd and 4th day after inoculation. Therefore, VOC collection was standardized by growing the bacteria on solid media (NBII), and collecting VOCs for 12 h on the 3rd day. Two repetitions under the same experimental conditions were performed. Analysis of the VOC profiles of ten isolates indicated that they were very diverse. No VOCs were detected by GC-MS in the headspace of isolates of *B. subtilis* B2g, *B. cepacia* 1S18 and *S. maltophilia* R3089. The other isolates had VOC profiles differing in numbers and compositions (Fig. 1). Rich profiles of ten to thirty major compounds are found in *S. plymuthica*, *S. odorifera*, and *P. trivialis* isolates, fewer than ten VOCs are present in *S. epidermidis*, *P. fluorescens* and *S. rhizophila* (Table 2). The analysis of the VOC spectra revealed that some compounds are isolate-specific while others are emitted by several antagonists. For example, β-phenylethanol (RI 1129) could be detected in *S. rhizophila*, *S. epidermidis*, *S. plymuthica* and *S. odorifera* (Table 3), while other compounds with the retention time RI 1090, 1094 and 1502 are only emitted from both tested pseudomonads, and compounds RI 1195 and 1287 are present in the volatile products of *P. trivialis* and *S. rhizophila*. *S. rhizophila* and *S. epidermidis* share compound 1419, and the three tested *Serratia* isolates commonly emit a number of compounds (RI 1154, 1366, 1383, 1423, 1440, 1445, 1455, 1460, 1497, 1555, 1574, 1576, 1594, 1725), which were not detected in the VOC products of any of the other investigated bacteria.

Mass spectroscopic analysis of the compounds and comparison with the 147,000 compounds comprising NIST library allowed the identification (probability set at >90%) of only a few compounds, such as β-phenylethanol, trans-9-hexadecene-1-ol, undecene, undecadiene, dodecanal, benzylnitrile, benzyloxybenzonitrile, and dimethyl trisulfide. Many VOCs could not be identified, neither with the quadrupole MS (Shimadzu QP5000) nor with the highly sensitive sector field GCMS (Vacuum Generators), using the NIST library as mass spectroscopy reference (Prof. Dr. Wittko Francke, University of Hamburg). Volatile terpenoids, phenylpropanoids and fatty acid derivatives such as those found in plants are apparently not present in the VOC spectrum of these antagonists. The fact that many bacterial VOCs could yet not be identified is intriguing because this provides a possible source of new, yet unknown small molecular mass chemicals. Structural determinations of these compounds will be necessary to elucidate these compounds.

Growth development of *R. solani* co-cultivated with bacterial antagonists

To investigate whether VOCs of bacterial antagonists could negatively affect the growth of *R. solani*, a dual

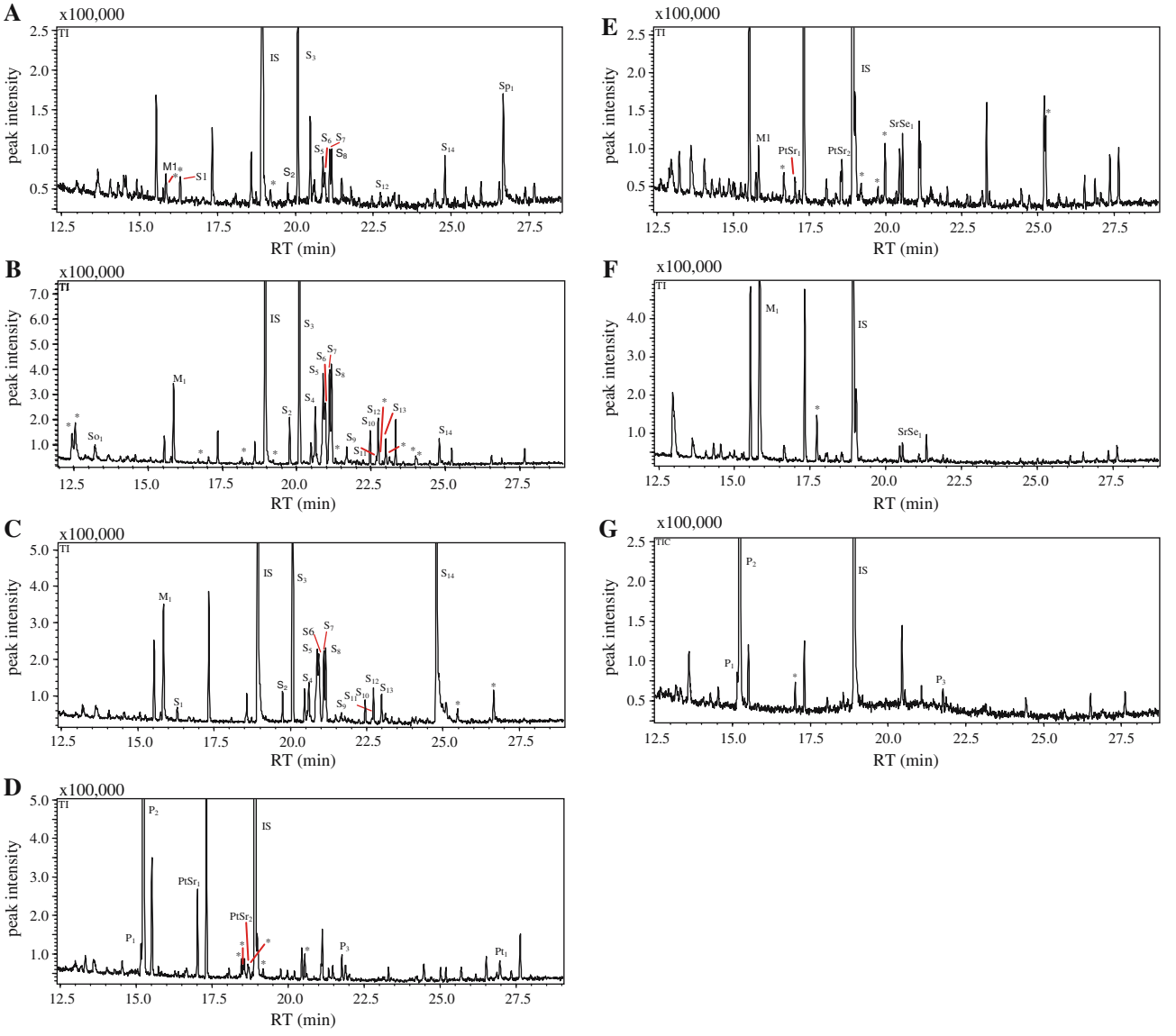

Fig. 1 GC profile of bacterial volatile analysis. Bacterial antagonists were grown on agar plates for 2 days and then transferred to the VOC analysis chamber, VOCs were collected in two intervals (each 12 h) on Super-Q absorbance material, eluted and analysed with a GC-MS (Shimadzu QP5000). Typical GC profiles of bacterial VOCs of *Serratia plymuthica* HRO-C48 (**a**), *S. odorifera* 4Rx13 (**b**), *S. plymuthica* 3Re4-18 (**c**), *Pseudomonas trivialis* 3Re2-7 (**d**), *Stenotrophomonas rhizophila* (**e**), *Staphylococcus epidermidis* (**f**), *P. fluorescens* (**g**) are shown. Compounds that are emitted from bacteria are either indicated by *asterisk* or a *letter code* (S1-14, So etc.), other peaks result from the headspace system. VOCs were identified with a minimum of 90% mass spectrum identity. S1-14: VOCs from *Serratia* spp. (S1: benzyl nitrile). So: VOCs from *S. odorifera* (So1: dimethyl trisulfide). Sp: VOCs from *S. plymuthica* (Sp1: trans-9-hexadecene-1-ol). P1-P3: VOCs from *Pseudomonas* spp. (P1: undecadiene; P2: undecene). Pt1: VOCs from *P. trivialis* (benzyloxybenzonitrile). PtSr1, PtSr2: VOCs from *P. trivialis* and *S. rhizophila*. SrSe1: VOC from *S. rhizophila* and *S. epidermidis*, dodecanal. M1: VOC from several bacterial isolates, β-phenylethanol

culture system was developed whereby the two organisms grew on the same plate but divided by a physical barrier so that only volatiles but no solutes of the bacteria could reach the fungus. After 5 days of co-cultivation, the amount of mycelium growth of *R. solani* was measured (Fig. 2). Mycelium growth was inhibited by more than 80% when *P. fluorescens*, *P. trivialis*, *S. maltophilia*, *S. rhizophila*, *S. odorifera*, both isolates of *S. plymuthica*, and *B. subtilis* were cultivated next to *R. solani* (Fig. 3). *B. cepacia* inhibits *R. solani* growth by 30% and *S. epidermidis* negatively influences the mycelial growth of *R. solani* by only 8%. *S. maltophilia* R3089 caused the strongest inhibition, 99%, under these test conditions, followed by *S. plymuthica* HRO-C48 (96%), *S. rhizophila* P69 (94%), *S. odorifera* 4Rx13 (92%), *P. trivialis* 3Re2-7 (89%), *S. plymuthica*

Table 2 Volatiles released from bacterial antagonists

Isolates	Number of VOCs	Identified VOCs (minimum of 90% identity)
Bacillus subtilis B2g	0	–
Burkholderia cepacia 1S18	0	–
Pseudomonas fluorescens L13-6-12	4	Undecene[a] (RI 1094)
Pseudomonas trivialis 3Re2-7	12	Undecadiene[a] (RI1091) Undecene[a] (RI 1094) Benzyloxybenzonitrile (RI1906)
Serratia odorifera 4Rx13	25	Dimethyl trisulfide (RI 990) β-Phenylethanol (RI 1130)
Serratia plymuthica HRO-C48	14	β-Phenylethanol (RI 1124) Trans-9-hexadecene-1-ol (RI 1890)
Serratia plymuthica 3Re4-18	17	β-Phenylethanol (RI 1129) Benzylnitrile (RI 1156)
Staphylococcus epidermidis 2P3-18	3	β-Phenylethanol (RI 1129) Dedecanal (RI 1419)
Stenotrophomonas maltophilia R3089	0	-
Stenotrophomonas rhizophila P69	9	β-Phenylethanol (RI 1129) Dodecanal (RI 1419)

Bacterial isolates from the rhizosphere and phyllosphere of different plants, see Table 1; volatiles where collected on a super Q column, eluted and analysed on a DB5 column in a GCMS Shimadzu QP5000, RI retention index

[a] Position and geometry of double bonds unknown

Table 3 Overlapping presence of VOCs in various antagonists

Pseudomonas fluorescens L13-6-12	*Pseudomonas trivialis* 3Re2-7	*Stenotrophomonas rhizophila*	*Staphylococcus epidermidis*	*Serratia plymuthica* HRO-C48	*Serratia plymuthica* 3Re4-18	*Serratia odorifera*
–	–	1129	1129	1124	1129	1130
1090	1091	–	–	–	–	–
1094	1094	–	–	–	–	–
1502	1503	–	–	–	–	–
–	1195	1196	–	–	–	–
–	1287	1288	–	–	–	–
–	1329	1331	–	1328	–	–
–	–	1419	1419	–	–	–
–	–	–	–	1154	1156	–
–	–	–	–	1366	1366	1367
–	–	–	–	1383	1387	1388
–	–	–	–	–	1423	1424
–	–	–	–	1440	1442	1442
–	–	–	–	1445	1445	1448
–	–	–	–	1455	1457	1458
–	–	–	–	1460	1461	1462
–	–	–	–	–	1497	1497
–	–	–	–	–	1555	1556
–	–	–	–	1574	1571	1572
–	–	–	–	–	1576	1577
–	–	–	–	–	1594	1596
–	–	–	–	1725	1734	1735

Numbers present retention index

3Re4-18 (85%), *B. subtilis* B2g (82%) and *P. fluorescens* L13-6-12 (79%).

Discussion

Secondary metabolites have been shown to function in aboveground communication between living organisms, and recently it has also been demonstrated that they are also important for underground communication (Rasmann et al. 2005). Here we selectively focussed on small organic molecules (whose molecular mass is usually <300) that characteristically have a high vapour pressure and therefore easily volatilize. Such volatile organic compounds (VOCs) are ideal infochemicals because they can act over a wide range of distances and

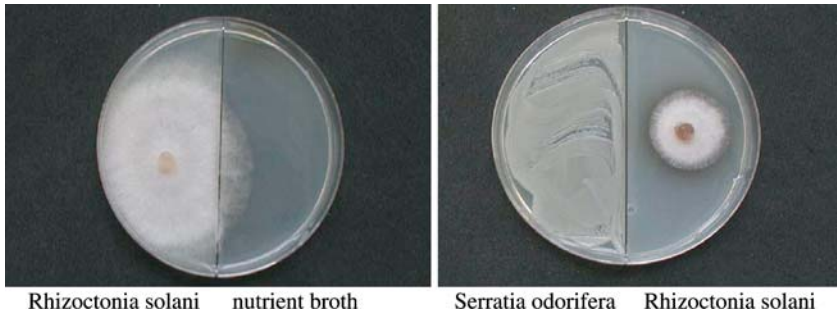

Fig. 2 Dual culture of *Serratia odorifera* and *Rhizoctonia solani*. *S. odorifera* was grown for 2 days at 30°C before a plug of *R. solani* mycelium was placed on the other side of the two-chamber Petri dish, the co-cultivation was performed for 5 days at 30°C

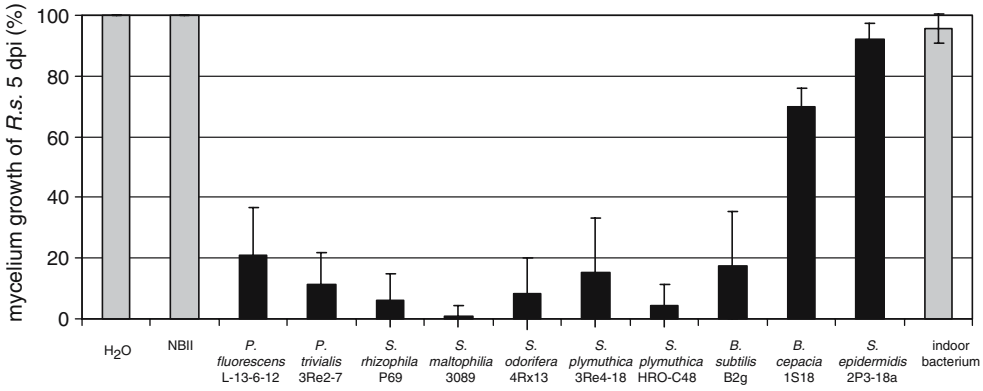

Fig. 3 Radial mycelium growth of *Rhizoctonia solani* in dual culture. Rhizobacterial isolates were co-cultivated with *R. solani* in a two-chamber Petri dish for 5 days. The radial growth was determined and the percent inhibition compared to the control growth with water or nutrient broth (*NB*) was calculated. The experiment was repeated three times and each experiment comprised 5–7 plates of each rhizobacterial isolate. *Error bars* indicate ±SE. *Pseudomonas fluorescens* L-13-6-12, *Pseudomonas trivialis* 3Re2-7, *Stenotrophomonas rhizophila* P69, *Stenotrophomonas maltophilia* 3089, *Serratia odorifera* 4Rx13, *Serratia plymuthica* 3Re4-18, *S. plymuthica* HRO-C48, *Burkholderia cepacia* 1S18, *Staphylococcus epidermidis* 2P318a, and an unidentified airborne indoor bacterium

their spheres of activity will extend from proximal interactions, due to aqueous diffusion, to greater distances via diffusion in air, including in soil pores (Wheatley 2002). In this paper, volatile emission from antagonistic acting bacteria was investigated and respective VOCs were used as a source of antifungal metabolites.

In our survey, of the ten bacterial antagonists that were investigated, VOCs from seven bacterial isolates were detectable with a GC-MS. These results confirm that volatile compounds could be of bacterial origin (Stotzky and Schenk 1976). The number of compounds detected in the headspaces of the bacteria varied for the different bacterial isolates. A rich VOC profile was obtained from all *Serratia* isolates, and *P. trivialis* 3Re2-7, a less complex spectrum was found in *S. epidermidis* 2P3-18, *P. fluorescens* L13-6-12 and *S. rhizophila* P69, while no volatiles were detected for *B. subtilis* B2g, *B. cepacia* 1S18 and *S. maltophilia* R3089.

The investigation of the bacterial antagonists in respect to the emission of volatile metabolites brought new and interesting insights concerning the physiology and features of these microorganisms. Of the approximately 80 VOCs that were emitted from seven of the bacterial isolates tested here, as many as 60 could not be unidentified. It appears that these compounds are not the common VOCs previously identified in plants, animals and bacteria and present in the NIST library (comprising 147,000 compounds).

It has recently been shown that two very well known, small organic compounds (2,3-butanediol and acetoin) emitted by *Bacillus subtilis* stimulate plant growth (Ryu et al. 2003, 2004). Interestingly, *B. subtilis* also emit approximately 20 other VOCs that have no effect on plant growth. Butanediol and acetoin are typically synthesized and excreted by bacilli upon growth on e.g. glucose. After glucose consumption bacilli are able to take up butanediol and acetoin to further metabolize these compounds. Butanediol and acetoin, therefore, serve as external nutrient sources, which are advantageous for bacilli in their ecological niche. That such released compounds can have additional effects, for example on other organisms such as plants (e.g. growth promotion) is a new and very interesting aspect.

The large number of compounds emitted by the bacterial antagonists may influence the organisms of the rhizosphere community, e.g. fungi, plants and other bacteria. Particularly interesting in this context is the wealth of VOCs emitted from *Serratia* spp., of which many appear to be specific for this genus (Table 3). Beside β-phenylethanol, which can have cytotoxic effects (Etschmann et al. 2002), it is very likely that the *Serratia* spp. also emit trans-9-hexadecen-1-ol, dimethyl trisulfide and benzyl nitrile. Dimethyl trisulfide is also emitted from *Phallus impudicus* to attract flies to support spore dispersal, from many *Streptomyces* spp., and from Gram negative bacteria growing on beef meat (Borg-Karlson et al. 1994; Schöller et al. 2002; Dainty et al. 1989). The compound with the retention index RI 1386, which is emitted from the *Serratia* spp. is most likely an unknown sesquiterpene (W. Francke, personal communication). It will be of significant interest if it can be unequivocally demonstrated that VOC products of bacterial antagonists contain terpenoids, since terpenoids are well known deterrent agents (Gershenzon and Kreis 1999; Arimura et al. 2004). Such a role could also be performed by β-phenylethanol, which is emitted from other tested antagonists (*Serratia* spp., *S. epidermidis*, *S. rhizophila*). β-phenylethanol is also known as a typical floral fragrance compound (rose-like odor) emitted from roses, hyacinths, jasmines, daffodiles and lilies, but also from bacteria (*Mycobacterium* spp., *Brevibacterium linens*, *Streptomyces* spp.) and fungi (*Saccharomyces* spp., *Phellinus* spp., *Ischnoderma benzonium*) (Etschmann et al. 2002; Schöller et al. 2002). Antimicrobial effects of β-phenylethanol due to permeability alterations at the plasmamembrane, alterations of the amino acid and sugar transport system, and inhibition of macromolecule synthesis (Ingram and Buttke 1984; Lucchini et al. 1993; Etschmann et al. 2002).

The VOCs identified to date from both Gram negative pseudomonads comprise primarily aliphatic unsaturated hydrocarbons or ketones, however many volatiles released from *P. trivialis* 3Re2-7 remained to be identified. Interestingly, the VOC profiles of both *Stenotrophomonas* spp. do not share common compounds. Although both strains can strongly inhibit the growth of *Rhizoctonia* via VOCs, the number of the VOCs detected in their headspaces is different. The lack of detected volatiles in the headspace of *S. maltophilia* R3089 was however unexpected, because the culture of *S. maltophilia* had a distinct smell as determined by the human nose. This discrepancy is most likely due to the fact that the GC-MS analysis started at a retention time of 10 min, and small compounds might elute earlier. Only a few volatiles are emitted from *S. epidermidis*, while no compounds were detected from *B. subtilis*.

It should be noted that the volatile patterns presented in Fig. 1 and summarized in Table 2 do not reflect the complete spectrum of volatiles emitted from these bacterial isolates. It is more likely that the methods used allowed the detection of only some of the components of the headspace. This is due to the following facts (a) the volatile compounds were dissolved in a solvent before injection into the GC. The solvent came out as a large peak extending to RI 837, and therefore small compounds with a retention index smaller than 837 could not be analysed during GC, (b) volatiles were only collected from the 3rd to the 6th day after inoculation, (c) the open headspace system allows the outflow of approximately 50% of the Petri dish atmosphere, and (d) the GCMS analysis was only performed with one column type (DB5) and it can be expected that other analytical columns, e.g. hydrophobic columns, separate other compounds and reveal other profiles. Together these points may explain the unexpected results that although for *S. maltophilia* and *B. subtilis* no VOCs could be detected, both bacterial isolates exhibit inhibitory activity against *R. solani*. Furthermore, discrepancies with previously published bacterial volatile patterns may be the result of the use of different bacterial isolates, the use of different growth media/growth conditions, or the growth phase of the bacterial population (Ryu et al. 2003, 2004; Fernando et al. 2005; Fiddaman and Rossall 1994; M. Kai, personal observation).

The antifungal activities of the bacterial antagonists used in our study had been previously documented (Berg et al. 2002, 2005), but these activities were correlated with the secretion of antibiotics, lytic enzymes or siderophores. Here we present an additional mode of action against the soil-borne plant pathogen *R. solani*. Volatiles of the *Pseudomonas* spp., *Serratia* spp., *Stenotrophomonas* spp. drastically inhibited the growth of *R. solani*, while a moderate or no inhibition was observed with the VOCs of *Burkolderia cepacia* and *Staphylococcus epidermidis*, respectively. To our knowledge this is the first documentation that VOCs of these bacterial antagonists inhibit the mycelium growth of *R. solani*. Previously, it was demonstrated that VOCs of randomly selected soil bacteria isolates stimulate or inhibit the growth rate of *Trichoderma viridae*, *Phaenaerochaete magnoliae*, *Phytophthora cryptogea*, *Gaeumannomyces graminis* and *Microdochium nivale* (Wheatley 2002). Alstrom (2001) reported that 21 strains of soil bacteria (e.g. *Serratia proteamaculans*, *Pseudomonas putida*, *P. acidovorans*, *P. chlororaphis*, *Stenotrophomonas* spp. and *Alcaligenes* spp.) isolated

from oil seed rape roots suppressed the pathogen *V. dahliae*, and Fernando et al. (2005) demonstrated inhibitory effects of *Pseudomanas* spp. volatiles on *Sclerotinia sclerotiorum*.

Together, these investigations clearly demonstrate that VOC-mediated interactions between bacteria and fungi occur. Such interactions can be species-specific, but it also appears that VOCs of many microorganisms can have effects on multiple members of the ecological community. In vitro, these interactions range from almost complete mycelium growth inhibition to small growth reduction as well as mycelial and conidial morphological abnormalities (Chaurasia et al. 2005). It has also been recently demonstrated that plant roots produce and emit secondary metabolites (Chen et al. 2004; Steeghs et al. 2004). Sesquiterpene lactones in root exudates of various agricultural crops stimulate germination of the parasitic weeds *Striga* and *Orobanche* and induce hyphal branching of abuscular mycorrhizal fungi at low concentrations (Bouwmeester et al. 2003; Akiyama et al. 2005). (E)-β-caryophyllene was shown to act as a root herbivore-induced soil volatile to attract nematodes to maize roots (Rasmann et al. 2005). The results reported here add to the results of previous investigations in supporting the conclusion that belowground VOCs play a crucial role in the rhizosphere interactions.

Acknowledgment The authors thank Hella Goschke (University of Rostock) for cultivating the antagonists and *R. solani*, Prof. Dr. W. Francke (University of Hamburg) for structural investigations of the *Serratia odorifera* VOC, and Prof. Dr. E. Pichersky (University of Ann Arbor, Michigan, USA) for critical reading the manuscript.

References

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827

Alstrom S (2001) Characteristics of bacteria from oil seed rape in relation to their biocontrol of activity against Verticillium dahliae. J Phytopathol 149:57–64

Anderson NA (1982) The genetics and pathology of *Rhizoctonia solani*. Annu Rev Phytopathol 20:329–374

Arimura G, Ozawa R, Kugimiya S, Takabayashi J, Bohlmann J (2004) Herbivore-induced defense response in a model legume. Two-spotted spider mites induce emission of (E)-β-ocimene and transcript accumulation of (E)-β-ocimene synthase in *Lotus japonicus*. Plant Physiol 135:1976–1983

Aström B, Gerhardson B (1988) Differential reactions of wheat and pea genotypes to root inoculations with growth-affecting rhizobacteria. Plant Soil 109:263–269

Bais HP, Park SW, Weir TL, Callaway RM, Vivanco JM (2004) How plants communicate using the underground information superhighway. Trends Plant Sci 9:26–32

Bais HP, Weir TL, Perry GR, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266

Berg G, Ballin G (1994) Bacterial antagonists to *Verticillium dahliae*. J Phytopathol 141:99–110

Berg G, Roskot N, Steidle A, Eberl L, Zock A, Smalla K (2002) Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl Environ Microbiol 68:3328–3338

Berg G, Krechel A, Ditz M, Faupel A, Ulrich A, Hallmann J (2005) Comparison of endophytic and ectophytic potato-associated bacterial communities and their antagonistic activity against plant pathogenic fungi. FEMS Microbiol Ecol 51:215–229

Bloemberg GV, Lugtenberg BJJ (2001) Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4:343–350

Borg-Karlson AK, Englund FO, Unelius CR (1994) Dimethyl oligosulphide, major volatile released from *Sauromatum guttatum* and *Phallus impudicus*. Phytochemistry 35:2:889–892

Bouwmeester HJ, Matusova R, Sun ZK, Beale MH (2003) Secondary metabolite signalling in host-parasitic plant interactions. Curr Opin Plant Biol 6:358–364

Cane DE, Watt RM (2003) Expression and mechanistic analysis of a germacradienol synthase from *Streptomyces coelicolor* implicated in geosmin biosynthesis. Proc Natl Acad Sci USA 100:1547–1551

Chaurasia B, Pandey A, Palni LMS, Trivedi P, Kumar B, Colvin N (2005) Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi in vitro. Microbiol Res 160:75–81

Chen F, Ro DK, Petri J, Gershenzon J, Bohlmann J, Pichersky E, Tholl D (2004) Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monterpene 1,8-cineole. Plant Physiol 135:1956–1966

Cook RJ, Tomashow LS, Weller DM, Fujimoto D, Mazzola M, Bangera G, Kim DS (1995) Molecular mechanisms of defense by rhizobacteria against root disease. Proc Natl Acad Sci USA 92:4197–4201

Dainty RH, Edwards RA, Hibbard CM, Marnewick JJ (1989) Volatile compounds associated with microbial growth on normal and high pH beef stored at chill temperature. J Appl Bacteriol 66:281–289

Denton M, Kerr KG (1998) Microbiological and clinical aspects of infections associated with *Stenotrophomonas maltophilia*. Clin Microbiol Rev 1:7–80

Dickschat JS, Martens R, Brinkhoff T, Simon M, Schulz S (2005) Volatiles released by a *Streptomyces* species isolated from the North Sea. Chem Biodivers 2:837–865

Etschmann MMW, Bluemke W, Sell D, Schrader J (2002) Biotechnological production of 2-phenylethanol. Appl Microbiol Biotechnol 59:1–8

Faltin F, Lottmann J, Grosch R, Berg G (2004) Strategy to select and assess antagonistic bacteria for biological control of *Rhizoctonia solani* Kühn. Can J Microbiol 50:811–820

Fernando WGD, Ramarathnam R, Krishnamoorthy AS, Savchuk SC (2005) Identification and use of potential bacterial organic antifungal volatiles. Biocontrol 37:955–964

Fiddaman PJ, Rossall S (1994) Effect of substrate on the production of antifungal volatiles from *Bacillus subtilis*. J Appl Bacteriol 76:395–405

Gershenzon J, Kreis W (1999) Biochemistry of terpenoids: monoterpenes, sespuiterpenes, diterpenes, sterols, cardiac glycosides and steroid saponins. In: Wink M (ed) Biochemistry of plant secondary metabolism, annual plant reviews. Academic, Sheffield, pp 222–280

Grosch R, Faltin F, Lottmann J, Kofoet A, Berg G (2005) Effectiveness of three antagonistic bacterial isolates to suppress *Rhizoctonia solani* Kühn on lettuce and potato. Can J Microbiol 51:345–353

Gupta AM, Gopal KVB, Tilak R (2000) Mechanism of plant growth promotion by rhizobacteria. Ind J Exp Biol 38:856–862

Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA 100:1541–1546

Haas D, Defago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 10:1–13

Handelsman J, Stabb EV (1996) Biocontrol of soilborne plant pathogens. Plant Cell 8:1855–1869

Ingram LO, Buttke TM (1984) Effects of alcohols on microorganisms. Adv Microb Physiol 25:253–300

Jakobi M, Winkelmann G, Kaiser D, Kempter C, Jung G, Berg G, Bahl H (1996) Maltophilin: a new antifungal compound produced by *Stenotrophomonas maltophilia* R3089. J Antibiot 49:1101–1104

Kalbe C, Marten P, Berg G (1996) Members of the genus *Serratia* as beneficial rhizobacteria of oilseed rape. Microbiol Res 151:4433–4400

Krechel A, Faupel A, Hallmann J, Ulrich A, Berg G (2002) Potato-associated bacteria and their antagonistic potential towards plant pathogenic fungi and the plant parasitic nematode *Meloidogyne incognita* (Kofoid & White) Chitwood. Can J Microbiol 48:772–786

Kurze S, Bahl H, Dahl R, Berg G (2001) Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. Plant Dis 85:529–534

Lottmann J, Berg G (2001) Phenotypic and genotypic characterization of antagonistic bacteria associated with roots of transgenic and nontransgenic potato plants. Microbiol Res 156:75–82

Lucchini JJ, Bonnaveiro N, Cremieux A, le Goffic F (1993) Mechanism of bactericidal action of phenylethyl alcohol in *Escherichia coli*. Curr Microbiol 27:295–300

Marten P, Smalla K, Berg G (2000) Genotypic and phenotypic differentiation of antifungal biocontrol strains belonging to *Bacillus subtilis*. J Appl Microbiol 89:463–473

Minkwitz A, Berg G (2001) Comparison of antifungal activities and 16S ribosomal DNA sequences of clinical and environmental isolates of *Stenotrophomonas maltophilia*. J Clin Microbiol 39:139–145

Opelt K, Berg G (2004) Diversity and antagonistic potential of bacteria associated with byrophytes from nutrient poor habitats of the Baltic Sea Coast. Appl Environ Microbiol 70:6569–6579

Pare PW, Tumlinson JH (1999) Plant volatiles as a defense against insect herbivores. Plant Physiol 121:325–331

Piechulla B, Pott MB (2003) Plant scents—mediator of inter- and intraorganismic communication. Planta 217:687–689

Raaijmakers JM, Vlami M, de Souza JT (2002) Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek 81:537–47

Rasmann S, Köllner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J (2005) Recruitment of entomophathogenic nematodes by insect-damaged maize roots. Nature 434:732–737

Ryu CM, Farag MA, Hu CH, Reddy MS, Wie HX, Pare PW, Kloepper JW (2003) Bacterial volatiles promote growth in *Arabidopsis*. PNAS 100:4927–4932

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol 134:1017–1026

Schöller CEG, Gürtler H, Petersen R, Molin S, Wilkins K (2002) Volatile metabolites from Actinomycetes. J Agric Food Chem 50:2615–2621

Sneh B, Jabaji-Hare S, Neate SM, Dijst G (1996) *Rhizoctonia* species: taxonomy, molecular biology, ecology; pathology and disease control. Kluwer, Dordrecht

Steeghs M, Bais HP, Gouwe JD, Goldan P, Kuster W, Northway M, Fall R, Vivanco JM (2004) Proton-transfer-reaction mass spectrometry as a new tool for real time analysis of root-secreted volatile organic compounds in *Arabidopsis*. Plant Physiol 135:47–58

Stotzky G, Schenk S (1976) Volatile organic compounds and microorganisms. CRC Crit Rev Microbiol 4:333–382

Walker TS, Bais HP, Grotewold E, Vivanco MJ (2003) Update on root exudation and rhizosphere biology. Plant Physiol 132:44–51

Weller DM, Raaijmakers JM, Gardener BB, Tomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 40:309–48

Wheatley RE (2002) The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie Van Leeuwenhoek 81:357–364

Whipps JM (2001) Microbial interaction and biocontrol in the rhizosphere. J Exp Bot 52:487–511

Wolf A, Fritze A, Hagemann M, Berg G (2002) *Stenotrophomonas rhizophila* sp. Nov., a novel plant associated bacterium with antifungal properties. Int J Syst Evol Microbiol 52:1937–1944

Zhang YQ, Ren SX, Li HL, Wang YX, Fu G, Yang J, Qin ZQ, Miao YG, Wang WY, Chen RS, Shen Y, Chen Z, Yuan ZH, Zhao GP, Qu D, Danchin A, Wen YM (2003) Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). Mol Microbiol 49:1577–1593