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Impact of Plant Species and Site on Rhizosphere-Associated Fungi Antagonistic to *Verticillium dahliae* Kleb.

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Fungi with antagonistic activity toward plant pathogens play an essential role in plant growth and health. To analyze the effects of the plant species and the site on the abundance and composition of fungi with antagonistic activity toward *Verticillium dahliae,* fungi were isolated from oilseed rape and strawberry rhizosphere and bulk soil from three different locations in Germany over two growing seasons. A total of 4,320 microfungi screened for in vitro antagonism toward *Verticillium* resulted in 911 active isolates. This high proportion of fungi antagonistic toward the pathogen *V. dahliae* was found for bulk and rhizosphere soil at all sites. A plant- and site-dependent specificity of the composition of antagonistic morphotypes and their genotypic diversity was found. The strawberry rhizosphere was characterized by preferential occurrence of *Penicillium* and *Paecilomyces* isolates and low numbers of morphotypes ($n = 31$) and species ($n = 13$), while *Monographella* isolates were most frequently obtained from the rhizosphere of oilseed rape, for which higher numbers of morphotypes ($n = 41$) and species ($n = 17$) were found. *Trichoderma* strains displayed high diversity in all soils, but a high degree of plant specificity was shown by BOX-PCR fingerprints. The diversity of rhizosphere-associated antagonists was lower than that of antagonists in bulk soil, suggesting that some fungi were specifically enriched in each rhizosphere. A broad spectrum of new *Verticillium* antagonists was identified, and the implications of the data for biocontrol applications are discussed.

One of the most important soilborne pathogens is *Verticillium dahliae* Kleb., causing *Verticillium* wilt, which is responsible for high yield losses in a wide variety of host plants, including many important crops such as strawberry and oilseed rape (33). With the impending phase-out of the fumigant methyl bromide worldwide, there is no possibility of suppressing the pathogen, and therefore alternative management strategies are required (21). One of these alternatives is the use of beneficial or antagonistic microorganisms which can suppress soilborne pathogens in the rhizosphere (2, 34, 38, 39). The rhizosphere, a term introduced by Lorenz Hiltner in 1904, is defined as the layer of soil influenced by root metabolism. In comparison to root-free soil, the rhizosphere forms a nutrient-rich niche for microorganisms as a result of exudation of compounds (32). Antagonistic activities of numerous microbial populations in the rhizosphere influence plant growth and health (38, 39).

Antagonists are naturally occurring organisms with traits enabling them to interfere with a pathogen's growth, survival, infection, or plant attack (6). Mechanisms responsible for antagonistic activity include (i) antibiosis via inhibition of the pathogen by antibiotics, toxins, and surface-active compounds called biosurfactants, (ii) competition for colonization sites, nutrients, and minerals, and (iii) parasitism, which may involve production of extracellular cell wall-degrading enzymes such as chitinase and β-1,3-glucanase (5, 8, 11, 40). Each plant species is colonized by its autochthonous antagonists, bacteria as well as fungi, but it is also possible to enhance the antagonistic potential by introducing allochthonous microorganisms working as biological control agents (2, 10, 34).

Fungi play an important role in the rhizosphere; they mediate many ecological processes and are responsible for plant growth and health 15. Although in recent years molecular tools have been developed to analyze the structures of the rhizosphere-associated fungal communities of several crops (13, 19, 31, 36, 37), little is known about the function and possible role of the observed fungal diversity associated with plant roots, especially their antagonistic potential (18, 36).

In a previous 3-year field study, we analyzed rhizosphere-associated bacterial communities and bacterial antagonists from *Verticillium* host plants in comparison to bulk soil. The proportion and composition of bacterial antagonists on potato, oilseed rape, and strawberry plants were shown to be influenced by the plant species and growth stage (3). The strawberry rhizosphere was characterized by a high proportion and a low diversity of antagonists. In contrast, a low proportion and a high diversity of bacterial *Verticillium* antagonists were observed for the rhizosphere of oilseed rape and potato. Plant specificity of the rhizosphere-associated bacterial communities was also shown using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes amplified from community DNA (30). DGGE patterns of oilseed rape and potato rhizosphere communities were more similar to each other than to the strawberry pattern. Furthermore, several studies have also analyzed the effects of soil and plant type on bacteria (reviewed in reference 12). However, knowledge about the effects of both factors on the fungal community is lacking.

The objectives of this work were (i) to analyze the diversity

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of rhizosphere-associated antagonistic fungi of two different *Verticillium* host plants, strawberry and oilseed rape, in comparison to that in bulk soil, (ii) to determine the effects of the plant species and the site on the proportion and composition of antagonistic fungi, (iii) to find new potential biocontrol agents, and (iv) to assess the data in terms of their implications for biocontrol. Therefore, a total of 4,320 fungi were isolated from the rhizosphere and bulk soil over a 2-year period (2002 and 2003) at three different growth stages of both plants (young, flowering, and senescent) at three different locations in Germany (Berlin, Braunschweig, and Rostock). Fungal isolates obtained after plating on synthetic low-nutrient agar (SNA) were screened by dual culture for antagonistic activity against *Verticillium dahliae*. For all active isolates, a comprehensive phenotypic and genotypic characterization based on morphology and BOX-PCR fingerprints, respectively, was carried out. In addition, the 18S rRNA genes of representative isolates were sequenced. This is the first report on plant- and site-dependent diversity of fungal *Verticillium* antagonists.

MATERIALS AND METHODS

Experimental design. Two crop plants, oilseed rape (*Brassica napus* L. [family: Brassicaceae] cv. Licosmos) and strawberry (*Fragaria* × *ananassa* [Duchense] Decaisne & Naudin [family: Rosaceae] cv. Elsanta), were grown in a randomized block design with four replicates per crop plant during two vegetation periods (2002 and 2003). The fields for the trials were located in Berlin (52°31′N, 13°24′E), Braunschweig (52°16′N, 10°31′E), and Rostock (54°05′N, 12°07′E). Soil parameters at all locations were analyzed by the Institute for Agricultural Analysis and Research (LUFA, Rostock, Germany). In Berlin, the soil texture was sand with a pH of 6.4, 2.3% organic matter, 5% clay, and the following nutrient composition (mg 100 g^{-1} soil): P_2O_5, 51; K_2O, 12; Mg, 7. In Braunschweig, the soil texture was weakly loamy sand with a pH of 5.6, 1.6% organic matter, 6% clay, and the following nutrient composition (mg 100 g^{-1} soil): P_2O_5, 35; K_2O, 19; Mg, 7. In Rostock, the soil texture was weakly loamy sandy loam with a pH of 6.0, 2.6% organic matter, 10% clay, and the following nutrient composition (mg 100 g^{-1} soil): P_2O_5, 41; K_2O, 12; Mg, 5.

Isolation of fungal strains and determination of CFU. Plant roots with adhering soil taken from five or more plants per plot were pooled into sterile stomacher bags and treated as one sample. Prior to cell extraction, 5 g of each pooled sample was transferred to a new stomacher bag. Samples were treated in a stomacher laboratory blender (BagMixer; Interscience, St. Nom, France). Twenty-five milliliters of demineralized water was added to 5 g rhizosphere sample. After a 1-min treatment (BagMixer), the supernatant was decanted into a 50-ml tube. Twenty milliliters of demineralized water was added to the sample, and after a second 1-min treatment, the supernatant was decanted into 50-ml tubes. For each sample, the suspensions (45 ml) were serially diluted and plated onto SNA containing 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 1 liter demineralized water, 0.6 ml 1 N NaOH, and 22 g agar, pH 6.5. After autoclaving for 20 min, the following antibiotics were added: 10 mg $liter^{-1}$ chlorotetracycline, 50 mg $liter^{-1}$ dihydrostreptomycin sulfate, 100 mg $liter^{-1}$ penicillin G (23). Plates were incubated for 3 to 5 days at 20°C, and colony-forming fungi were counted to calculate the mean $\log_{10}$ CFU of colonies based on fresh weight. In two consecutive years, a total of 240 isolates per site and sampling time were randomly selected and subcultured on malt agar (MA; Merck, Darmstadt, Germany), resulting in 4,320 fungal isolates which were screened for antagonistic activity. After incubation for 7 days at 20°C, the MA plates were stored at 4°C. Isolated fungi were encoded by a combination of numbers and letters indicating (i) location (B, Berlin; BS, Braunschweig; R, Rostock), (ii) microenvironment (B, bulk soil; E, rhizosphere of strawberry; R, rhizosphere of oilseed rape), (iii) sampling time (1, young plants 2002; 2, flowering plants 2002; 3, early senescent plants 2002; 4, young plants 2003; 5, flowering plants 2003; 6, early senescent plants 2003), (iv) number of the plot, and (v) consecutive number of the isolate per plant.

Screening for fungi antagonistic to *Verticillium dahliae*. The in vitro inhibition of *Verticillium dahliae* Kleb. was determined by a dual-culture assay on Waksman agar (WA) according to Berg et al. (3). All strains were tested in three independent replicates with *Verticillium dahliae* V25 (isolated from *Brassica napus* L.) and *Verticillium dahliae* V35 (isolated from *Fragaria* × *ananassa* [Duchense] Decaisne & Naudin). Zones of inhibition were measured after 3 to 7 days of incubation at 20°C.

Morphological characterization of antagonistic fungi. Prior to the molecular characterization, all isolates were grouped by their morphology on SNA and MA. Morphological characteristics such as colony morphology, production of pigments, conidiophores, or other morphological organs which could be seen by light microscopy according to Domsch (9) were used for grouping the isolates.

Extraction of DNA from mycelia and/or spores. Mycelia, grown on MA for 1 week, were soaked with TE buffer (10 mM Tris–1 mM EDTA) for 5 min. After the TE buffer was discarded, equal amounts of sterile glass beads and 300 μl of extraction buffer (200 mM Tris, 200 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate) were added to the mycelium. The mycelium was treated with a sterile micropestle or biovortexer (Roth, Karlsruhe, Germany) for about 1 to 2 min. To this suspension 150 μl of 3 M sodium acetate was added, and the sample was vortexed. Samples were frozen for about 30 min and centrifuged for 10 min at 13,000 × *g*, followed by phenol-chloroform extraction and isopropanol precipitation. The resulting pellet was resuspended in 50 μl TE buffer and stored at −20°C.

BOX-PCR fingerprints. BOX-PCR was done as described by Rademaker and De Bruijn (25) 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, followed by 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, with a final extension at 65°C for 16 min. A 10-μl aliquot of the amplified PCR product was separated by electrophoresis on 1.5% agarose gels in 0.5× Tris-borate-EDTA buffer for 6 h, stained with ethidium bromide, and photographed under UV transillumination. The variability of BOX-PCR patterns was assessed by fingerprinting five strains in three independent experiments. Based on this analysis, isolates with more than 70% similarity were considered to be members of the same genotypic group. The genotypic diversity of the bacterial communities was measured by the Shannon information theory function (28). According to the formula, the coefficient of the number of genotype groups (instead of species, the category originally used) and the number of isolates indicates the diversity in a sample. The diversity index ⟨H′⟩ is expressed on an unlimited scale where high numbers represent high diversity.

Identification of fungal isolates by 18S rRNA gene sequencing. PCR amplification of the 18S rRNA gene was performed using the fungus-specific primers NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and FR1 (5′-AIC CAT TCA ATC GGT AIT-3′) (35). The 25-μl PCR mixture contained at least 23 μl PCR SuperMIX Hi Fidelity, 0.5 μl of each primer, and 1 μl of the template (50 ng). PCR was performed in a TGradient thermocycler (Biometra, Göttingen, Germany) by using the following cycles: 1 initial cycle at 95°C for 8 min; 35 cycles of denaturation (30 s at 94°C), annealing (45 s at 48°C), and extension (3 min at 72°C); and a single final extension cycle at 72°C for 10 min, followed by a final soak at 4°C. The PCR products were purified with the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) as recommended by the manufacturer. DNA templates were sequenced by GATC Biotech AG (Konstanz, Germany). The 18S rRNA gene sequences were aligned with sequences of the NCBI sequence databases using the BLAST algorithm (1).

Statistics. All data (CFU, percentages of *Verticillium* antagonists, diversity indices) were analyzed for significance using the Mann-Whitney U test ($P \leq 0.05$) and studied by two-factor analysis of variance by use of Statistical Product and Service Solutions for Windows, Rel. 9.0.1. (SPSS Inc., Chicago, Ill.). BOX-PCR-generated fingerprints were evaluated with the GelCompar program (version 4.1; Applied Maths, Kortrijk, Belgium). The cluster analysis was performed with a Pearson correlation matrix and the UPGMA (unweighted-pair group method using arithmetic averages) algorithm.

Nucleotide sequence accession numbers. Accession numbers for sequences submitted to the EMBL nucleotide sequence database are AJ745088, AJ745089, AJ748459 to AJ748463, AJ746347 to AJ746356, AJ748271 to AJ748275, AJ781423 to AJ781425, AJ781112 to AJ781116, AJ783407 to AJ783417, AJ783920 to AJ783949, and AJ784284 to AJ784287.

RESULTS

Isolation of fungi from the rhizospheres and from soil. Altogether, CFU counts on SNA were in the range of 3.9 to 5.9 $\log_{10}$ CFU g^{-1} of root (fresh weight) (rfw). The CFU determined for rhizosphere and bulk soil samples were rather similar: for strawberry, 4.98 ± 0.18 $\log_{10}$ CFU g^{-1} rfw; for oilseed rape, 4.73 ± 0.14 $\log_{10}$ CFU g^{-1} rfw; for bulk soil, 4.58 ± 0.18

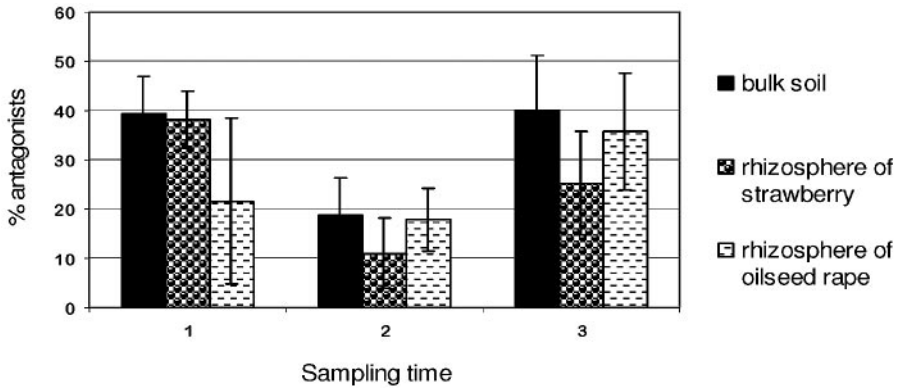

FIG. 1. Proportions of *Verticillium* antagonists determined by dual-culture assay in the rhizosphere of strawberry and oilseed rape and bulk soil during two vegetation periods (2002 and 2003). Sampling times: 1, young; 2, flowering; 3, senescent plants.

$\log_{10}$ CFU g^{-1} rfw) and showed no significant seasonal changes. CFU counts were observed to be lower on average in Berlin (4.50 ± 0.14 $\log_{10}$ CFU g^{-1} rfw) than in Rostock (4.85 ± 0.08 $\log_{10}$ CFU g^{-1} rfw) and Braunschweig (4.9 ± 0.23 $\log_{10}$ CFU g^{-1} rfw). A total of 240 isolates per sampling time and site were used in the initial screening of antagonistic activity toward *Verticillium dahliae* Kleb. The number varied slightly because of the rapid growth of zygomycetes on petri dishes, which sometimes made it difficult to obtain the full number of isolates.

Screening for isolates antagonistic to *Verticillium dahliae*. Altogether, 4,320 fungal isolates were screened for their ability to suppress *V. dahliae* in an in vitro dual-culture assay. Initially 911 isolates that were active against *V. dahliae* were found. Antagonism could be observed in two ways: (i) by the detection of clear inhibition zones up to 25 mm without *Verticillium* mycelium or (ii) by hyperparasitism resulting in the destruction and discoloration of the *Verticillium* mycelium and microsclerotia. Both phenomena were found for a large proportion of fungal isolates. More isolates that induced clear inhibition zones (666 [73%]) than hyperparasites (245 [27%]) were detected. Interestingly, 51 of the active isolates showed inhibition of *Verticillium* microsclerotium formation. Although similar numbers of isolates per site were tested, the proportions of isolates with antagonistic activity were slightly different. The proportion was highest on average for Braunschweig (27%), followed by Rostock (25%) and Berlin (21%). The proportion of isolates with antifungal activity was higher for bulk soil (27%) than for both rhizospheres (22%). However, differences between the sites and the microenvironments are not statistically significant. Additionally, a seasonal shift was observed for the proportion of antagonists. While their number was significantly reduced during flowering time, the highest proportion of antagonists was found at the end of the vegetation period, except for the strawberry rhizosphere (Fig. 1). Overall, in the second year of the study, the percentage of antagonistic isolates was higher (26%) than in the first year (23%), but the difference was not statistically significant.

Phenotypic diversity of antagonistic fungal isolates. In a first step, all fungal isolates for which antagonistic activity was shown were grouped based on phenotype. An impressive diversity of fungal colonies was obtained. Different mycelium colors such as white, grey, black, green, yellow, pinkish, or brown, and sometimes some red or black color diffusing into the agar, were observed. Additionally, some colonies with a typical odor (e.g., fruity, aromatic [like coco], or like perfume or solvents such as acetone) were found. Most of the fungi produced anamorphs of different types, while sexual stages (teleomorphs) were not detected. The morphological characterization of 911 *Verticillium* antagonists resulted in 26 morphological groups. In addition, 76 morphotypes were represented by single isolates. The highest number of morphotypes was found in Rostock (54 clusters, comprising 15 groups and 39 single isolates) and in bulk soil (53 clusters, comprising 19 groups and 34 single isolates) (Table 1). *Penicillium*, *Trichoderma*, *Monographella*, and *Paecilomyces* were the dominant genera. In both years these groups constituted the majority of isolates: 74% of the isolates in 2002 and 65% in 2003. All of them were isolated from bulk and rhizosphere soils of the different sites. The highest number of *Verticillium* antagonists belonging to *Penicillium* was found in Rostock (Table 2). *Penicillium* isolates were more frequently recovered from oilseed rape and strawberry rhizosphere soil than from bulk soils in general. *Verticillium* antagonists belonging to the genus *Trichoderma* were most abundant in Braunschweig and in bulk soil in general. *Paecilomyces* isolates were most frequently recovered from Rostock rhizosphere and bulk soil. However, the proportion was higher in the rhizosphere of strawberry than in the rhizosphere of oilseed rape in general. In contrast, *Monograph-*

TABLE 1. Phenotypic characterization of *Verticillium* antagonists

Parameter	No. of fungi[a]	No. of morphotypes[b]	
		Group	Single
Locations			
Berlin	250	17	17
Braunschweig	288	10	20
Rostock	373	15	39
Microenvironments			
Bulk soil	317	19	34
Strawberry	297	12	19
Oilseed rape	297	18	23

[a] Fungal isolates from all morphological groups.
[b] Morphological grouping according to phenotype.

TABLE 2. Proportions and numbers of dominant genera in different locations and microenvironments

Fungal group	No. of isolates	% (no.) of isolates in:								
		Berlin			Braunschweig			Rostock		
		Soil	Strawberry	Rape	Soil	Strawberry	Rape	Soil	Strawberry	Rape
Penicillium	275	8.7 (24)	5.8 (16)	9.8 (28)	2.9 (8)	10.2 (28)	5.8 (16)	17.8 (49)	24.4 (67)	14.2 (39)
Trichoderma	210	10.5 (22)	7.6 (16)	1.4 (3)	28.1 (59)	11.4 (24)	23.8 (50)	8.1 (17)	8.1 (17)	1.0 (2)
Monographella	74	8.1 (6)	0 (0)	18.9 (14)	20.3 (15)	4.1 (3)	27.0 (20)	8.1 (6)	2.7 (2)	10.8 (8)
Paecilomyces	63	1.6 (1)	9.5 (6)	1.6 (1)	4.8 (3)	19.0 (12)	9.5 (6)	25.4 (16)	17.5 (11)	11.1 (7)

ella species were most frequently isolated from the rhizosphere of oilseed rape. A clear influence of location was found. While *Trichoderma* and *Monographella* species were the dominant *Verticillium* antagonists isolated at the Braunschweig site, *Penicillium* and *Paecilomyces* were most abundant in Rostock. Additionally, typical genera occurred preferentially in the microenvironments, e.g., *Penicillium* and *Paecilomyces* in all strawberry rhizospheres, *Monographella* in the rhizospheres of oilseed rape, and *Trichoderma* in bulk soil. Interestingly, the number of antagonists belonging to the main morphological groups differed with different sampling times. During flowering, all the main groups, except for group 4, for which the number increased during the whole vegetation period, showed a lower number of isolates. Statistical analysis of these data revealed significant differences in phenotypic diversity between bulk soil and both rhizospheres, between strawberry and oilseed rape, between locations, and between sampling times.

Genotypic diversity of antagonistic fungal isolates. All *Verticillium* antagonists were characterized on the genotypic level using BOX-PCR. BOX-PCR of genomic DNA yielded fingerprints with 7 to 27 amplification products, ranging from 100 to 3,000 bp. Although all of the 911 fungal isolates were characterized and used for calculation of diversity indices (see Table 4), fingerprints of the dominant genera *Penicillium* and *Trichoderma* were selected as examples to show their diversity. BOX patterns of 65 *Penicillium* isolates from strawberry and oilseed rape rhizospheres sampled in 2002 are shown in Fig. 2. Many different BOX patterns were found. At a similarity level of 70%, the strains could be divided into 11 different clusters (P1 to P11) and 8 groups consisting of single isolates. Genotype groups contained 2 to 16 isolates. While 9 of the 11 groups included only isolates from the same location, two groups (P7 and P8) had isolates from different locations. Five groups comprised isolates from the rhizosphere of one plant type (P2, P3, and P4, isolates from oilseed rape; P9 and P10, isolates from strawberry).

A total of 97 *Trichoderma* isolates were isolated from all microenvironments, locations, and sampling times in 2002. GelCompar was used to compare all fingerprints. Figure 3 shows the clustering of *Trichoderma* isolates according to their BOX profiles. At a similarity level of 70%, 13 clusters (genotypes T1 to T13) and 6 single-isolate "groups" were formed. The genotypic groups contained 2 to 16 isolates. Five groups were formed by isolates from one location: T2 (Braunschweig), T4 and T10 (Berlin), and T12 and T13 (Rostock). In all other groups, isolates from different locations were present. Additionally, many groups contained isolates from both rhizospheres as well as from bulk soil. However, groups T2, T4, T8, and T10 contained only isolates from bulk soil, whereas T13 contained only isolates from the strawberry rhizosphere, mainly from one site. The genotypic group T5 contained isolates from different sampling times and all microenvironments and locations (Fig. 4). Interestingly, nearly identical BOX patterns were found for isolates from different locations as well as from different plants. For example, isolates BSE3-1-6 (Fig. 4, lane 1), BSE3-1-5 (lane 2), and BSB3-3-9 (lane 3), obtained from the strawberry rhizosphere and soil in Braunschweig, showed the same pattern. *Trichoderma* isolates with nearly identical fingerprints were obtained from bulk soil in Braunschweig (BSB3-3-9 [lane 3]) and Rostock (RB3-2-14 [lane 4] and RB3-1-20 [lane 5]).

To estimate the key factor determining the diversity of fungal *Verticillium* antagonists, the composition of all genotype groups was analyzed (Table 3). For this comparison only antagonists of the main fungal genera were used, because these genera contain sufficient numbers of isolates to allow appropriate analysis. These BOX profiles formed 60 groups which included 570 isolates. The groups contained 2 to 44 isolates (9 to 10 on average), although the latter number was found only for two clusters, one of *Penicillium* and one of *Trichoderma*. The majority of groups ($n = 33$) consisted of isolates from one location, indicating the high influence of this parameter on clustering. For *Penicillium*, *Monographella*, and *Paecilomyces*, more groups containing only isolates from one location were found. In contrast, *Trichoderma* isolates formed more clusters including isolates from different locations. Regarding the occurrence of the major genera in different microenvironments, clearly more groups containing isolates from more than one microenvironment were found for *Penicillium*, *Paecilomyces*, and *Trichoderma* (Table 3). Regarding the plant species, 29 groups contained only isolates from one plant species, strawberry or oilseed rape. This number was slightly higher than the number of groups formed by isolates from both plant species ($n = 28$). Differences between genera were also found. Isolates of the antagonistic genus *Trichoderma* showed a higher number of groups containing only isolates originating from one plant species than of groups containing isolates from both plants (Table 3). The clustering confirmed the influence of plant species on fungal diversity, although this effect was lower than that of location.

Shannon and Weaver (28) indices for fungal antagonists were calculated for different locations and soil fractions based on BOX patterns. In general, genotypic diversity was significantly higher in bulk soil ($\langle H' \rangle = 3.40$) than in both rhizospheres ($\langle H' \rangle = 3.06$) ($P = 0.03$). When the two rhizospheres were compared, the diversity of fungal antagonists for oilseed

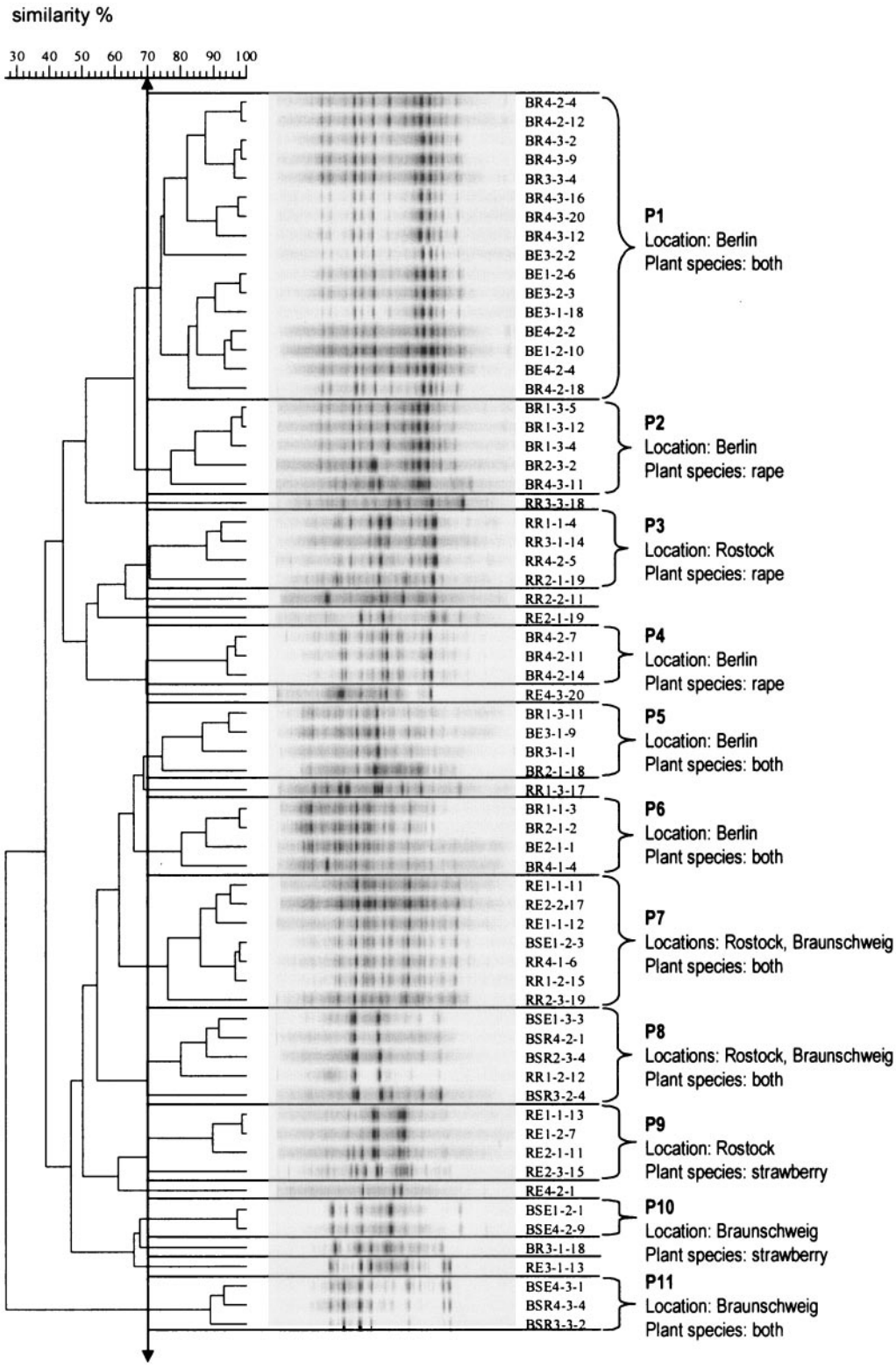

FIG. 2. Dendrogram showing the relationship of *Penicillium* isolates obtained from the rhizosphere of strawberry and oilseed rape in 2002 based on BOX-PCR fingerprints using cluster analysis by UPGMA.

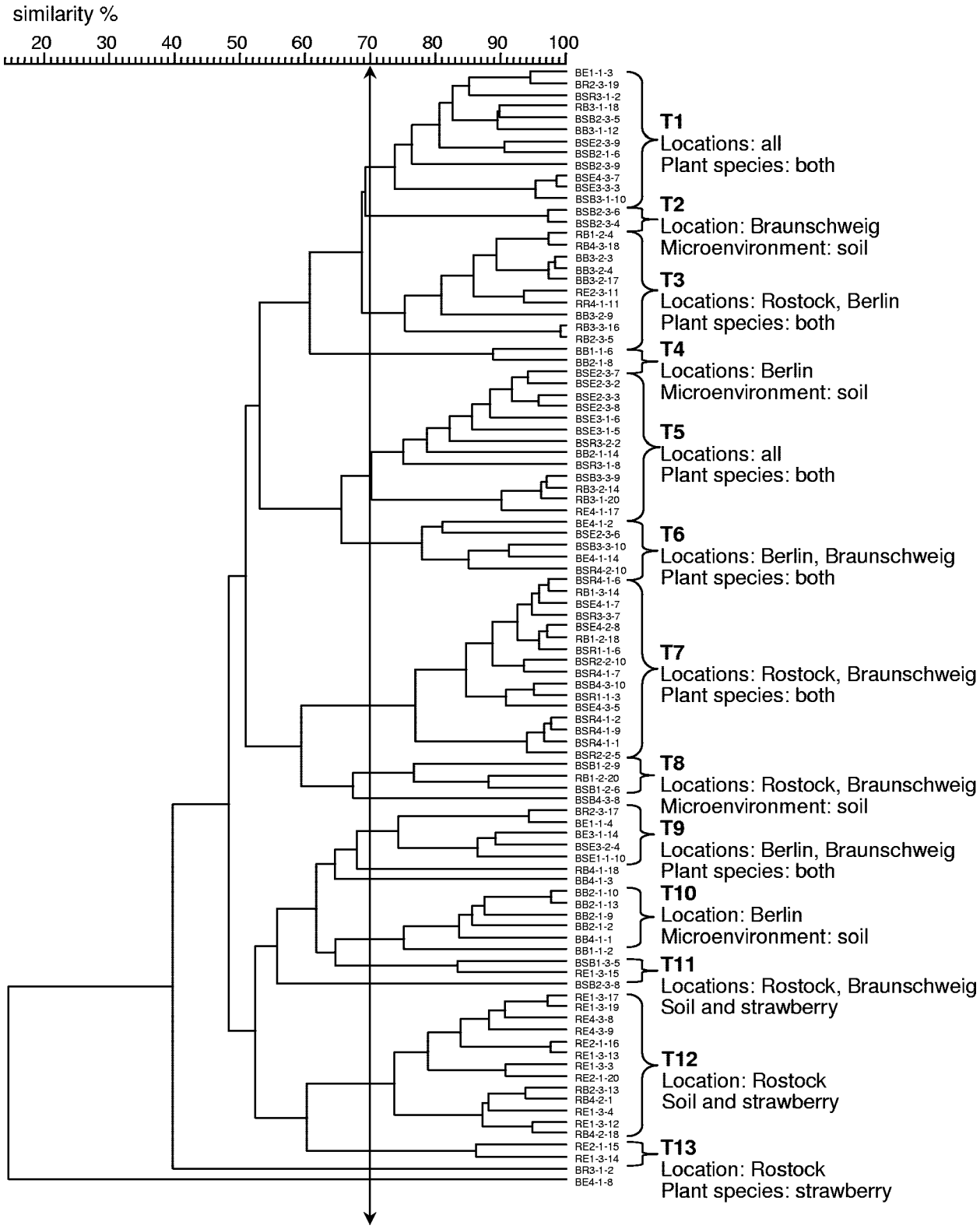

FIG. 3. Dendrogram showing the relationship of *Trichoderma* isolates obtained in 2002 based on BOX-PCR fingerprints using cluster analysis by UPGMA.

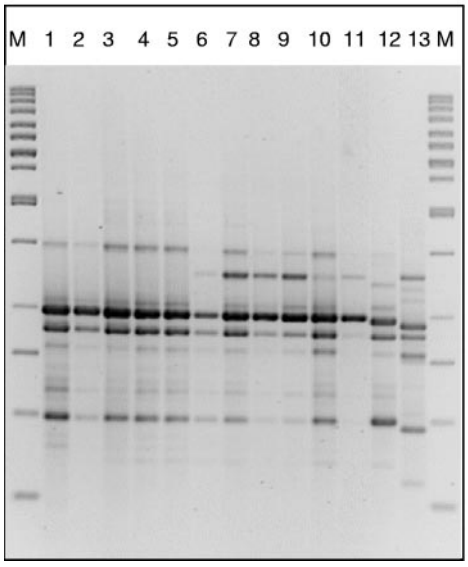

FIG. 4. BOX patterns of *Trichoderma* isolates belonging to genotype group T5. 3. Lanes: 1, BSE3-1-6; 2, BSE3-1-5; 3, BSB3-3-9; 4, RB3-2-14; 5, RB3-1-20; 6, BSR3-1-8; 7, RE4-1-17; 8, BSE2-3-3; 9, BSE2-3-8; 10, BSE2-3-7; 11, BSE2-3-2; 12, BSR3-2-2; 13, BB2-1-14. Designations are explained in Materials and Methods.

rape ($\langle H' \rangle = 3.07$) was only slightly lower than that for strawberry ($\langle H' \rangle = 3.09$). Additionally, diversity was influenced by location. The indices were highest in Rostock, followed by Braunschweig and Berlin, as shown in Table 4. The diversity indices calculated for bulk soil and the rhizospheres were highest in Rostock. In most cases the highest diversity was found at the end of the vegetation period ($\langle H' \rangle = 3.77$) and the lowest during flowering ($\langle H' \rangle = 3.51$). Diversity indices were also calculated separately for the four dominant fungal genera (Table 4). The intrageneric diversity of *Verticillium* antagonists was highest for the genus *Penicillium* ($\langle H' \rangle = 2.65$), followed by *Trichoderma* ($\langle H' \rangle = 2.47$), *Monographella* ($\langle H' \rangle = 1.62$), and *Paecilomyces* ($\langle H' \rangle = 1.46$). Antagonistic isolates of *Penicillium* showed the highest diversity in Rostock, while *Paecilomyces* strains were highly diverse in Berlin. A high diversity of *Trichoderma* and *Monographella* species was observed for antagonistic isolates from all microenvironments (Table 4). Seasonal shifts found for all fungi could be confirmed only by analysis of the dominant genera.

Identification of antagonistic fungi by sequencing of 18S rRNA gene fragments. A total of 75 isolates representing all the main groups as well as single isolates were selected according to their BOX-PCR patterns and identified by sequencing of approximately 500 bp of their 18S rRNA genes (Table 5). Sequencing analysis resulted in the identification of 22 fungal species belonging to 16 *Ascomycota* and 2 *Basidiomycota* genera. Ascomycete isolates belonged to six different orders and therefore showed immense phylogenetic diversity. The richness of antagonistic species was microenvironment dependent. Bulk soil was characterized by the occurrence of *Trichoderma viride*, *Penicillium italicum*, and *Paecilomyces marquandii*, which represent the main morphological groups. While in the strawberry rhizosphere two species (*P. italicum* and *Eladia saccula*) were characteristic, *Trichoderma*, *Paecilomyces carneus*, and *P. marquandii* additionally occurred in the rhizosphere of oilseed rape. However, a large proportion of species (17/29) were isolated only once. A comparison with the dendrogram constructed according to the BOX pattern of isolates showed that these are often representatives of groups with similar genotypes. For example, *Trichosporon laibachii* BSR3-5-9 is representative of a cluster group which contains 33 strains originating from all sites and microenvironments. In contrast, *Trichosporon multisporum* BSR1-5-12 represents a group which consists of four isolates from the rhizosphere of oilseed rape in Braunschweig (data not shown).

DISCUSSION

Several studies have identified different biotic and abiotic factors influencing the structural and functional diversity of bacterial communities: (i) the plant species or genotype, (ii) the plant development stage or vegetation time, (iii) colonization with pathogens as well as the physiological state of the plant, (iv) the quality of the soil, (v) crop rotation, (vi) treatment with pesticides or fertilizers, and (vii) the geographical region (12, 14, 16, 20, 26, 27, 29). However, relatively little is known about how these factors influence fungal communities in rhizosphere and bulk soil. The aim of this study was to analyze the influence of the plant species and the site on the proportion and composition of fungi antagonistic toward *Verticillium* and to evaluate these data in terms of their implications for biocontrol.

In our study we analyzed the group of fungi with antagonistic activity toward *Verticillium dahliae* Kleb. isolated from the rhi-

TABLE 3. Composition of groups based on genotypic characterization by BOX-PCR

Fungal group	No. of isolates	No. of groups[a]	No. of isolates in clusters	Avg no. of isolates (range) per cluster	No. of groups containing isolates from one or more:					
					Location		Microenvironment		Plant species	
					One	More	One	More	One	Both[b]
Penicillium	275	27	250	9 (2–44)	16	11	8	19	12	14
Trichoderma	210	20	197	10 (2–44)	7	13	3	17	12	7
Monographella	74	7	64	9 (2–33)	5	2	4	3	4	2
Paecilomyces	63	6	59	10 (2–16)	5	1	1	5	1	5
Total	622	60	570		33	27	16	44	29	28

[a] Strains were grouped according to BOX fingerprints using GelCompar (UPGMA) at 70% similarity.
[b] Strawberry and oilseed rape.

TABLE 4. Diversity indices[a] (<H′>) based on genotypic characterization by BOX-PCR

Location or habitat	All fungi		*Penicillium*		*Trichoderma*		*Monographella*		*Paecilomyces*	
	No. of isolates	<H′>	No. of isolates	<H′>	No. of isolates	<H′>	No. of isolates	<H′>	No. of isolates	<H′>
Locations										
Berlin	250	2.34	61	1.16	40	1.72	21	0.64	6	1.33
Braunschweig	288	2.59	49	1.03	60	2.08	45	1.1	38	0.89
Rostock	373	2.83	140	2.32	34	1.56	17	0.84	27	1.28
Habitats										
Bulk soil	317	3.40	77	2.24	82	2.22	22	1.01	21	1.21
Strawberry	297	3.09	103	2.11	53	1.86	5	0.84	25	1.18
Oilseed rape	297	3.07	70	2.19	49	1.39	41	1.11	13	0.58

[a] According to Shannon and Weaver (28) based on clustering of BOX patterns at a similarity level of 70%.

zosphere of two *Verticillium* host plants at three different locations in two consecutive years. A surprisingly high proportion of fungal isolates from all microenvironments and sites showed in vitro antagonistic activity toward *Verticillium dahliae.* In contrast, the proportion of bacteria antagonistic toward *Verticillium* in general was lower (2, 4, 20). In a study performed for 3 years under field conditions in Braunschweig, the proportion of isolates with antagonistic activity was highest for strawberry rhizosphere (9.5%), followed by oilseed rape rhizosphere (6.3%), potato rhizosphere (3.7%), and bulk soil (3.3%) (3). The same trend was observed when samples from the present study were screened for bacterial *Verticillium* antagonists, although the proportion of antagonists on average was higher than that in the previous study (42). While the proportions of bacteria with antagonistic activity toward *Verticillium dahliae* were significantly different for the three microenvironments analyzed in both studies (3, 4), no such differences were found for fungi in the present study. However, the analysis of phenotypic and genotypic characteristics of antagonistic fungi was suitable for detection of clear differences due to the plant species and the site.

An effect of the site on the composition and relative abundance of microfungi was found. The fungal isolates from the Berlin site were characterized by a high number of morphotypes without dominance of the main morphological groups. Furthermore, isolates belonging to the main morphological groups showed low genotypic diversity at the Berlin site. The genus *Trichoderma* with the key species *Trichoderma viride* was frequently found in the collection of *Verticillium* antagonists from Braunschweig and showed high genotypic diversity. Fungal *Verticillium* antagonists from Rostock were dominated by *Penicillium*, for which one species was identified by 18S rRNA gene sequencing and two genotypic groups were found based on BOX-PCR. Interestingly, at the Braunschweig site, the proportion of antagonists was highest for fungi (this study) as well as for bacteria (4). In contrast, a low proportion of antagonists but the highest genotypic diversity was found for bacteria and fungi at the Rostock site. In addition, fungal community fingerprints done for the same samples by DGGE analysis of 18S rRNA gene fragments amplified from bulk and rhizosphere soil DNA revealed a clear influence of the site (7). Different soil parameters can be one reason for soil-dependent microbial-community composition. While the soil types were similar for the sites (sand in Berlin; loamy sand in Braunschweig and Rostock), the organic-matter, clay, and nutrient contents were different. The soil from the Rostock site, which showed the highest diversity of antagonists, contained the highest percentage of organic matter, clay, and a sufficient nutrient content. However, other factors such as weather conditions or the history of the field trial, e.g., crop rotation, could have influenced the results.

Independently of the site, specificity for each of the microenvironments investigated was found. The strawberry rhizosphere at all sites was characterized by a high proportion of *Penicillium* and *Paecilomyces* isolates. The latter were enriched in the strawberry rhizosphere and generally more abundant than in the rhizosphere of oilseed rape. In addition to *Penicillium*, *Monographella* isolates were more frequently found in the rhizosphere of oilseed rape. In bulk soil the proportion of *Verticillium* antagonists belonging to *Trichoderma* was higher, and that of *Penicillium* was lower, than observed in rhizosphere soil. In general, *Trichoderma* strains with antagonistic activity were highly abundant. Although they displayed high diversity in all soils, they showed a high degree of plant specificity based on their BOX patterns. Fungal fingerprints of the same samples obtained by DGGE of the 18S rRNA gene fragments also indicated a plant-dependent composition of the fungal community. Due to a high variability of the fungal DGGE patterns in the first year, plant-dependent diversity of the fungal community was clearly revealed only in the second year of the field trial (7). One reason for plant specificity of root-associated microorganisms is root exudates such as amino acids, sugars, and organic acids, which are an important nutritional source for these microorganisms. The composition of root exudates was shown to differ depending on the plant species and the stage of plant development (17, 22). Additionally, seasonal shifts corresponding to the plant growth stage were found for each of the parameters analyzed except for plate counts, confirming previous findings of an influence of the vegetation time for bacterial communities (16, 30) as well as for whole fungal communities (13).

The phenotypic and genotypic diversity of *Verticillium* antagonists isolated in this study offers an enormous resource for biological control. Interestingly, antagonistic *Trichoderma* strains were frequently isolated from all sites and microenvironments, indicating a cosmopolitan occurrence. This aspect might explain why antagonists belonging to *Trichoderma* species are rather successful as biocontrol agents, and several such biocontrol products are available worldwide (40, 41). Many species identified in this study are known for their antagonistic

TABLE 5. Species list according to 18S rRNA gene sequence and distribution in different microenvironments and locations

Species[a]	Taxonomy		No. of isolates[b]						
	Phylum	Order	Total	Bulk soil	Strawberry[c]	Oilseed rape	B	BS	R
Arthrobotrys conoides Drechsler	*Ascomycota*	*Orbiliales*	1			1		1	
Clonostachys rosea (Preuss) Mussat	*Ascomycota*	*Hypocreales*	1		1			1	
Didymella cucurbitacearum A.J. Roy (1968)	*Ascomycota*	*Pleosporales*	1		1		1		
Eladia saccula (E. Dale) G. Sm.	*Ascomycota*	*Hypocreales*	13	2	3	8	1		12
Escovopsis sp. J.J. Muchovej & Della Lucia	*Ascomycota*	*Hypocreales*	2	1	1			2	
Fusarium culmorum (W.G. Sm.) Sacc.	*Ascomycota*	*Hypocreales*	2	1	1			2	
Microdochium nivale (Fr.) Samuels & I.C. Hallett	*Ascomycota*	*Xylariales*	2	1		1	1		1
Monographella cucumerina (Lindf.) Arx	*Ascomycota*	*Phyllachorales*	2	2			1		1
Myrothecium roridum Tode	*Ascomycota*	*Hypocreales*	1			1	1		
Nectria lugdunensis J. Webster	*Ascomycota*	*Hypocreales*	1			1			1
Paecilomyces carneus (Duché & R. Heim) A.H.S. Br. & G. Sm.	*Ascomycota*	*Eurotiales*	2			2	1		1
Paecilomyces javanicus (Friedrichs & Bally) A.H.S. Br. & G. Sm.	*Ascomycota*	*Eurotiales*	1		1		1		
Paecilomyces marquandii (Massee) S. Hughes	*Ascomycota*	*Eurotiales*	6	3	1	2	1	3	2
Penicillium freii Frisvad & Samson	*Ascomycota*	*Eurotiales*	1	1				1	
Penicillium geastrivorus[c]	*Ascomycota*	*Eurotiales*	1		1			1	
Penicillium italicum Stoll	*Ascomycota*	*Eurotiales*	6	3	3		4		2
Penicillium verruculosum Peyronel	*Ascomycota*	*Eurotiales*	1			1	1		
Trichoderma viride Pers.	*Ascomycota*	*Hypocreales*	8	5	1	2	1	4	3
Volutella ciliata	*Ascomycota*	*Hypocreales*	1	1					1
Dothideomycete sp.	*Ascomycota*		1	1					1
Soil ascomycete	*Ascomycota*		1			1	1		
Hypocreales sp.	*Ascomycota*	*Hypocreales*	3	1		2	1	2	
Uncultured *Hypocreales* sp.	*Ascomycota*	*Hypocreales*	3	1	1	1	1		2
Uncultured ascomycete	*Ascomycota*	*Hypocreales*	8	5	1	2		6	2
Acremonium-like hyphomycete	*Ascomycota*		1		1		1		
Apiotrichum porosum Stautz	*Basidiomycota*	*Trichosporonales*	1			1		1	
Trichosporon dulcitum (Berkhout) Weijman	*Basidiomycota*	*Trichosporonales*	1	1					1
Trichosporon laibachii (Windisch) E. Guého & M.T. Sm.	*Basidiomycota*	*Trichosporonales*	1			1		1	
Trichosporon multisporum G. Cochet	*Basidiomycota*	*Trichosporonales*	1			1		1	

[a] According to sequencing of the 18S rRNA gene.
[b] B, Berlin; BS, Braunschweig; R, Rostock.
[c] Not a validly published name.

or biocontrol activity, e.g., *Clonostachys rosea*, *Escovopsis* sp., *Paecilomyces marquandii*, *Penicillium italicum*, and *Monographella* spp. However, we also found a long list of hitherto unknown antagonists belonging to the genera *Arthrobotrys*, *Eladia*, *Paeciloymes*, *Penicillium*, *Trichosporon*, and *Volutella*. Some of the antagonists characterized are known for their phytopathogenic capacity: *Fusarium culmorum*, *Didymella cucurbitacearum*, *Myrothecium roridum*, and *Microdochium nivale*. Apathogenic or low-pathogenicity strains of well-known pathogenic species can also act in biocontrol, as has been shown previously for apathogenic *Fusarium* species (24).

Basic information about bulk and rhizosphere soil fungi with antagonistic activity toward *Verticillium* and factors influencing their abundance and diversity, which was provided by this study, is important for optimization of biocontrol applications. Successful and consistent biological control requires a better understanding of the dynamics and composition of antagonistic rhizosphere communities. We could show that in all soils investigated, an impressive autochthonous antagonistic potential was present, representing approximately one-third of the fungal isolates. Although the proportions of antagonistic isolates were similar for all habitats and locations investigated, their composition was different. This is important because an introduced biocontrol agent should act together with these different antagonistic communities. Some genera with antagonistic activity occurred ubiquitously (*Trichoderma*, *Penicillium*), while others seemed to be more specialized (*Paecilomyces*). The universal distribution of some *Trichoderma* strains at all sites and microenvironments in our study underlined their great potential for *Verticillium* biocontrol. The occurrence and composition of antagonists in bulk soil influenced the composition in the rhizosphere, as shown, for example, by a high abundance of *Trichoderma* in all microenvironments in Braunschweig. Very often the same genotype was found in bulk soil and in the rhizosphere of one location. Each plant species was able to select antagonistic fungi independently of the soil type. However, the influence of plant species on the abundance, distribution, and composition of fungal antagonists was lower than that reported before for bacterial antagonists (3, 4). This might be an advantage for the use of fungal antagonists in biocontrol. Although improving the antagonistic activity in the rhizosphere either directly, by treating soils with biocontrol agents, or indirectly, by organic amendments (e.g., manure, compost), remains a challenge, this study supports the notion that the rhizospheres of different plants might differently support the colonization of biological control strains. This is the first report on the antifungal potential of bulk and rhizosphere soil-associated fungi, and hopefully it will provide a basis for new and innovative concepts in the biological control of fungal pathogens.

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