

Evaluation of apple replant problems based on different soil disinfection treatments—links to soil microbial community structure?

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Abstract

Background and aims Replant problems or soil sickness are known phenomena but still unsolved. The aims of this study were (i) to set up a test system for detecting replant problems using *in vitro* propagated apple rootstocks (M26) based on different soil disinfection treatments and (ii) to explore the treatment effects on root morphology and soil microbial community structure.

Methods The bio-test involved soil with apple replant problems (apple sick) and healthy soil from an adjacent plot, both either untreated, or submitted to treatments of 50 and 100 °C, or the chemical soil disinfectant Basamid. Histological analyses of roots and denaturing gradient gel electrophoresis (DGGE)

fingerprints in rhizosphere soil collected at the final evaluation were performed.

Results After 10 weeks, shoot dry mass on apple sick soil was 79, 108 and 124 % higher for soil treated at 50 °C, 100 °C and with Basamid, respectively, compared to the untreated soil. Roots in untreated apple sick soil showed destroyed epidermal and cortical layers. DGGE fingerprints revealed treatment dependent differences in community composition and relative abundance of total bacteria, *Bacillus*, *Pseudomonas* and total fungi.

Conclusions The clear differences detected in soil microbial communities are the first steps towards a better understanding of the causes for apple replant problems.

Keywords Apple replant disease · Apple replant problem · Denaturing gradient gel electrophoresis (DGGE) · *Malus domestica* · Microbial community profiling · Root morphology · Specific soil sickness

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Abbreviations

DGGE denaturing gradient gel electrophoresis
EDTA ethylene diamine tetraacetic acid
ITS internal transcribed spacer

Introduction

It is a well-known fact that after repeatedly growing the same crops in the same field the soil loses the ability to serve further on as substrate or growing

medium for these crops. This is called replant problems or soil sickness. The problem is specific to a plant genus or species, it is resident for the place where it appeared, it is immobile, and persists even for 20 to 30 years (Klaus 1939). Some plant species show severe damages when grown repeatedly in the same field, like for instance apple and rose. This cannot be avoided even by having a long interval before returning to the same crop, especially when species of the *Rosaceae* are concerned. The problems were assumed to be species-specific for apples, roses, etc. (Spethmann and Otto 2003), but cross reactions were also observed, for example cherry plants suffering when grown in apple sick soil (Mai and Abawi 1978).

Since it is often difficult to find healthy soil of a similar structure, an approach to detect replant problems is to compare plant growth in sick soil subjected to different treatments and untreated soil (Savory 1966; Hoestra 1968; Gilles 1974; Mai and Abawi 1978; Jackson 1979; Scotto La Massese et al. 1988; Aldea 1998; Hoestra 1994; Mazzola 1998; Manici et al. 2003; Utkhede 2006). The plants showed to improve growth on treated soil, e.g. the study by Mai and Abawi (1978) on apple, cherry and pear replant problems revealed that the fresh mass obtained in steam-treated soil and soil treated with chloropicrin, 1.2 dichloropropane and 1.3 dichloropropene were higher than those in untreated soil. The growth responses of apple rootstocks M9, M26, M7, MM106 and M111 showed an increment in the first year of up to 400 % in soil fumigated with chloropicrin compared to untreated soil (Jackson 1979).

The etiology of replant problems is unclear; however, the possible causes might include both biotic and abiotic factors (Politycka and Adamska 2003). In apple orchards or nurseries, different biological agents have been implicated in disease developments, e.g. nematodes like *Pratylenchus penetrans* were found to attack the roots of every size and age, and were difficult to diagnose by above ground symptoms (Mai et al. 1994). The infected apple roots showed discolored, stunted and sometimes ‘witches-broom’ symptoms (Hoestra 1994). Fungi and oomycetes belonging to the well-known root rot complex, *Rhizoctonia solani*, *Phytophthora* spp., *Cylindrocarpon* spp. and *Pythium* spp. were also shown to be an important factor of replant problems (Mazzola 1998; Manici et al. 2003; Manici and Caputo 2010; Kelderer et al. 2012). Recently, molecular analysis using quantitative

real-time PCR (qPCR) of replant fungal and oomycete pathogens associated with apple replant problems in South Africa also identified oomycetes (*Phytophthora* spp. and *Pythium* spp.) and *Cylindrocarpon* as the causal agents for reduction of the plant growth (Tewoldemedhin et al. 2011). For bacteria, *Bacillus subtilis* and *Pseudomonas putida* were described to be pathogenic components of the well-known root rot complex on apple seedlings (Utkhede 2006).

Molecular fingerprinting has recently become more popular to study soil microbial community using PCR-amplification of 16S rRNA, 18S rRNA gene or ITS fragments from community DNA prior to denaturing gradient gel electrophoresis (DGGE) analysis after different soil treatments (Smalla et al. 2001; Heuer et al. 1997, 2001; Costa et al. 2006a; Weinert et al. 2009; Garbeva et al. 2003). Replant problems especially occur in rose and apple rootstock nurseries where young plants are cultivated very densely and are replanted very frequently. The chemical soil disinfection will no longer be possible in future due to the discontinued registration of the chemical Basamid at least in Germany. Therefore, interest in research on the complex phenomenon of replant problems focusing on possible causes and measures to overcome them is increasing. The aims of this study were (i) to set up a test system for the detection of apple replant problems by using *in vitro* propagated M26 apple rootstock plants under greenhouse conditions, and (ii) to investigate the effects of soil disinfection treatments on root morphology and microbial community structure changes. The effects of the different soil treatments on bacterial and fungal communities were assessed by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene or ITS fragments amplified from total community DNA.

Materials and methods

Plant materials

In vitro cultures of the apple rootstock (M26) kindly provided by Dr. Lihua Zhu, SLU Alnarp, Sweden, were propagated on MS basal medium (macro- and microelements and vitamins according to Murashige and Skoog (1962) full strength, 3 % sucrose, 0.5 μ M IBA (indole-3-butyric acid), 4.4 μ M BAP (benzylaminopurine), 0.8 % Plant Agar

(Duchefa, The Netherlands) pH at 5.8). For rooting, shoots of 2 cm length were transferred to rooting medium of the same basal composition, but without BAP and containing only 1.48 μM IBA 3–4 weeks before acclimatization.

The bio-test was carried out twice, in two subsequent years (2010 and 2011) with independently collected soil in both years. In 2010, acclimatized plantlets were used 28 days after transfer to soil which were about 4 cm in height, whereas in 2011 due to the late sampling of the soil the plants were submitted to the experiments 74 days after transfer to soil, they were less homogeneous, and about 7–18 cm in height.

Soil treatments

Two types of soils were used: (1) healthy or virgin soil, meaning soil on which no *Rosaceae* had been grown before and (2) apple sick soil on which apple rootstocks had been grown for 50 years; all of which were loamy sand and came from adjacent sites in the area around Pinneberg, Schleswig Holstein, Germany. Each soil type was subjected to four treatments: untreated (control), two thermal treatments at 50 °C and at 100 °C and a chemical treatment with Basamid (Tetrahydro-3,5-dimethyl-2 H-1,3,5-thiadiazine-2-thione) (COMPO 2000). The following abbreviations were used: AH - healthy soil untreated, AA—apple sick soil untreated, BH—healthy soil 50 °C treatment, BA - apple sick soil 50 °C treatment, CH - healthy soil 100 °C treatment, CA - apple sick soil 100 °C treatment, DH - healthy soil Basamid treatment, and DA - apple sick soil Basamid treatment.

Air dry heat in an oven at 50 °C was applied to destroy the nematodes mainly while the 100 °C treatment served to guarantee full sterilization and to kill all living organisms (Spethmann and Otto 2003). Fifteen-liter-quantities of moist soil were filled into autoclavable bags and exposed to the temperatures for 1 h when the aspired temperature was reached in the centre of the bags. For chemical disinfection the soil was filled into plastic bags together with 200 mg of Basamid for 1 L of soil and small amounts of water were added to activate the chemical before incubation at room temperature (about 22 °C) for 2 weeks. Thereafter, it took 3 weeks to ensure the chemical was completely released, which was verified by a germination test with cress seeds.

Experimental set-up for bio-tests

Osmocote-Exact 3–4 M (slow release fertilizer, 16+9+12+2MgO, www.scottspprofessional.com), 2 g L^{-1} soil, were mixed with the soil prior to potting. Mypex, a garden polypropylene was spread at the bottom of each container to prevent the media from dropping off. In 2010, one plant was cultured in a 5 L container with five replicates per treatment. The container volume was reduced to 2 L in 2011, but the number of replicates was increased to 10 per treatment. The containers were placed randomly in a greenhouse chamber with a setting point for heating at 17–18 °C and relative humidity of 65–70 %. Irrigation was applied on a daily basis by hand, and plant protection was carried out according to horticultural practice with spraying against insect pests mainly.

The height of the main shoot per plant was measured weekly from the soil surface to the base of the terminal leaves. At the final evaluation after 10 weeks, the shoots and roots were harvested for dry mass determination at 70 °C for 1 week.

The measured parameters in terms of height and dry mass compared between control and treatments were subjected to the analyses with the statistic program R 2.11.1 (R Development Core Team 2008). The homogeneity of variance within treatments was checked prior to the analysis of variance “ANOVA” and “Tukey” test at $p \leq 0.05$ to test the differences of the treatments against untreated soil.

Histological analyses

The root samples were collected from the plants grown in 2011 in apple sick soil, untreated and treated at 50 and 100 °C, as well as from the plants grown in untreated healthy soil. Therefore, 20 representative roots of 0.4–0.8 mm in diameter from different parts of the root system of different plants were selected and cut into pieces of 5 mm in length. The samples were fixed in 1.5 ml AFA (alcohol-formalin-acetic acid) solution containing 70 % ethanol (96 %), 20 % distilled water, 5 % formaldehyde and 5 % glacial acetic acid for at least 24 h. Dehydration was realized in a graded concentration row of ethanol, and subsequently the root segments were embedded in paraffin. Five replicates were prepared for each treatment with 3 to 4 root sections in one embedding. Then, 4.5–6 μm

thick sections were cut using a dry blade of a rotary microtome. Thereafter, they were stained with FCA (fuchsin-chrysoidin-astrablau) solution. The basic fuchsin stains the secondary cell walls (lignin) orange to brownish red and nuclei in light purple, the chrysoidine stains suberin and cutin yellow and the astrablau primary cell walls (cellulose) in blue (Hoenemann et al. 2010). Pictures were taken under a light microscope (Zeiss axioscope) using the Zeiss AxioVision 4.7.2.0 software.

Microbial community analyses in soils

Soil samples

Healthy and apple sick soils were taken at the end of culture from both bio-tests in 2010 and 2011 to analyze the microbial populations by DGGE. For healthy soil, the samples were taken from the untreated variant (AH), and for apple sick soil the samples were untreated (AA), the treatment at 50 °C (BA) and 100 °C (CA). The sample in 2010 was collected from the mixture of all replicates and was denoted as AH0, AA0, BA0 and CA0. In 2011, only rhizosphere soils (soil attached to the root obtained after vigorous shaking) were collected from two plants and combined to form one replicate. A total of four replicates per treatment was used, after thorough sieving to remove root parts, for DNA extraction.

Extraction of DNA from soil and purification of soil DNA extracts

The soil DNA was extracted from 500 mg from soil per replicate using the FastDNA[®] SPIN Kit for soil after a harsh cell lysis by means of the FastPrep[®] instrument from mpbio (MP Biomedicals, Santa Ana, CA) as described by the manufacturer. Then, the DNA extracts were purified using the GENECLEAN[®] SPIN Kit from Qbiogene following the manufacturer's instructions (Qbiogene, Inc., Carlsbad, CA).

Quantitative PCR targeting 16S rRNA genes

Quantification of 16S rRNA genes was conducted by 5'-nuclease assays in real-time PCR as previously described by Suzuki et al. (2000) and Heuer et al. (2012).

Amplification of bacterial 16S rRNA gene fragments (GC-PCR)

PCR was carried out with 10x PCR buffer (Applied Biosystems), 0.2 μM dNTPs, 3.75 mM MgCl₂, 4 % acetamide, F984GC as forward primer with GC-clamp and R1378 as reverse primer (0.2 μM each) (Table 1), 1.25 U Stoffel fragment (Applied Biosystems), and 1 μl purified DNA extracts diluted 10 fold. The amplification was carried out at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min and finally 72 °C for 10 min (Heuer et al. 1997).

Amplification of Bacillus gene fragments (GC-PCR)

A semi-nested PCR was performed using two sets of primers, Bac F and R1378 (Table 1), in a reaction mixture as was described for the bacterial 16S rRNA gene fragments, except that 0.05 μg BSA (Bovine Serum Albumin) was used instead of acetamide and 2.5 U Stoffel fragment (Applied Biosystems). The thermal cycling program was performed as described by Garbeva et al. (2003). Then, 1 μl of the 10 fold diluted PCR product was used as template for the GC-PCR but with 25 cycles of the thermal cycling program.

Amplification of Pseudomonas gene fragments (GC-PCR)

A nested PCR approach was used to amplify *Pseudomonas* 16S rRNA gene fragments for DGGE fingerprint. The first taxon-specific PCR amplification was carried out as was described by Costa et al. (2006b), except that the PCR mix contained 2.5 μl 10x PCR buffer (Applied Biosystems), 2.5 mM MgCl₂, 4 % acetamide, 2.5 μg BSA, no DMSO (Dimethyl sulfoxide), 1.25 U of Amplitaq Gold (Applied Biosystems) and 25 cycles were performed. The GC-PCR was done as for *Bacillus*.

Amplification of fungal internal transcribed spacer (ITS) regions

A semi-nested PCR approach was performed for fungal ITS fragments as described by Weinert et al. (2009), except that 2.5 μl 10x Stoffel buffer (Applied Biosystems), 0.16 μM dNTPs, 2 % DMSO, 0.2 μM of

Table 1 Primers used in this study targeting bacteria and fungi

Primer	Sequence 5'—3'	Specificity	Annealing temp (°C)	Reference
F984	AACGCGAAGAACCTTAC	Bacteria	53	(Heuer et al. 1997)
GC-clamp	CGCCCGGGGCGCGCCCGGGCG GGGCGGGGGCACGGGGGG	-	53	(Heuer et al. 1997)
R1378	CGGTGTGTACAAGGCCCGGGAACG	Bacteria	53	(Heuer et al. 1997)
Bac F	GGGAAACCGGGGCTAATACCGGAT	<i>Bacillus</i>	65	(Garbeva et al. 2003)
F311Ps	CTGGTCTGAGAGGATGATCAGT	<i>Pseudomonas</i>	63	(Costa et al. 2006b)
R1459Ps	AATCACTCCGTGGTAACCGT	<i>Pseudomonas</i>	63	(Costa et al. 2006b)
ITS-1 F	CTTGGTCATTTAGAGGAAGTAA	Fungi	55	(Weinert et al. 2009)
ITS4	TCCTCCGCTTATTGATATGC	Fungi	55	(Weinert et al. 2009)
ITS2	GCTGCGTTCTTCATCGATGC	Fungi	55	(Weinert et al. 2009)

both primers and 1.25 U AmpliTaq Gold (Applied Biosystems) were used in the reaction mixture.

DGGE analysis

The gradient for total bacterial community, *Bacillus* and *Pseudomonas* was applied as described in Weinert et al. (2009). Equal volume of 6 µl of DGGE loading buffer (60 % glycerol, 1 M EDTA, 0.5 % bromophenol blue, 0.5 % xylene cyanol) and PCR-products (5–8 µl depending on band intensity in the agarose gels), were mixed before loading on the gel. A mixture of DGGE-PCR products from 11 bacterial species was applied to each DGGE gel as a marker (Heuer et al. 1997). The gels were silver stained as described by Heuer et al. (2001).

Statistical analysis of the DGGE fingerprints

Air dried gels were scanned transmissively by pdi 420oe Scanner, MWG Biotech, Germany and by Gel-Compar II 6.5 (Applied Math, Sint-Martens-Latern, Belgium) the fingerprints were analyzed. The pairwise similarities of lanes were calculated by using Pearson correlation. The Pearson correlation coefficient for each pair of lanes within a gel was calculated as a measure of similarity between the fingerprints, and clustering of the pattern was achieved using the unweighted pair group method using arithmetic averages (UPGMA). The matrix data from the GelCompar analysis were taken to test the significant variability between groups, e.g. untreated vs. treated soil, using a permutation test performed at 10,000 times according to Kropf et al. (2004) and to determine the d-values (% dissimilarity).

Results

Bio-tests for detecting replant problems

Results in 2010

On apple sick soil, a significant reduction of plant height in untreated soil compared to the treated variants was observed after 5 weeks of culture. These differences increased during plant growth and even became more pronounced at harvest (Table 2). After 10 weeks, the increase in plant height on treated soil compared to untreated soil was 64, 83 and 86 % for soil treated at 50 °C, 100 °C and with Basamid, respectively. On healthy soil, the height was only slightly increased by temperature treatments, but decreased by Basamid treatment (Table 2). However, for healthy soil, no significant differences were observed between the treatments and untreated soil until the termination of the experiment.

Shoot biomass revealed similar results as plant height: No significant differences were found for healthy soil, but for apple sick soil, the thermal treatments 50 °C, 100 °C as well as Basamid treatment led to significant increases in shoot dry mass of 79, 108 and 124 % compared to the untreated apple sick soil, respectively (Table 3). Again, the Basamid treatment of healthy soils caused a trend towards biomass reduction of plants (Table 3).

The plants grown in untreated apple sick soil possessed relatively smaller root systems with an abundance of dark brown and necrotic or decayed fibrous roots compared with the plants grown in the treated soils. However, root dry mass did not differ significantly within the soil variants; only a trend of an increase was

Table 2 Plant height of apple rootstocks M26 in 2010 and 2011 grown in soils with or without treatment from planting until harvest

Year	Soil variant	Treatment	Plant height (cm) after first week of planting (W1) until harvest (W10)									
			W1	W2	W3	W4	W5	W6	W7	W8	W9	W10
2010	Healthy soil	AH	4.8±1.2 A	6.3±1.0 A	7.6±2.4 A	11.5±4.8 A	18.3±6.8 A	24.0±7.2 A	34.6±8.6 A	44.4±9.2 A	55.8±9.3 A	66.0±9.1 A
		BH	5.1±0.7 A	6.5±1.3 A	8.2±2.1 A	14.2±2.9 A	22.3±4.0 A	28.7±4.4 A	38.6±5.3 A	49.5±5.7 A	60.6±5.9 A	69.8±5.5 A
		CH	4.7±1.0 A	5.8±1.2 A	7.0±1.2 A	11.9±3.4 A	19.9±5.0 A	26.6±6.4 A	36.6±7.2 A	46.5±7.4 A	57.8±8.0 A	67.4±8.3 A
		DH	4.8±1.6 A	5.4±1.3 A	7.1±1.7 A	11.5±4.0 A	19.2±5.9 A	24.7±6.8 A	32.4±8.0 A	40.7±8.8 A	51.0±9.1 A	60.2±9.3 A
Apple sick soil	AA	4.6±0.5 A	6.0±0.8 A	6.3±1.2 A	7.9±1.9 A	9.1±2.7 A	10.8±2.8 A	13.2±3.4 A	19.0±3.1 A	27.2±4.0 A	34.2±5.1 A	
	BA	5.1±0.9 A	6.9±1.6 A	8.5±2.7 A	12.5±4.6 AB	17.8±5.6 B	22.6±6.7 B	29.2±9.0 B	38.0±9.8 B	48.0±10.3 B	56.2±11.0 B	
	CA	4.7±0.4 A	6.3±0.4 A	6.9±0.2 A	11.5±0.8 AB	17.5±1.7 B	23.4±2.7 B	32.4±3.6 B	42.4±4.2 B	53.2±4.3 B	62.6±4.2 B	
	DA	5.8±1.2 A	7.9±1.1 A	9.7±2.8 A	14.7±4.3 B	20.5±6.1 B	25.2±6.8 B	34.0±7.3 B	43.5±8.0 B	54.2±8.9 B	63.6±8.6 B	
2011	Healthy soil	AH	17.7±5.0 A	22.6±5.3 A	28.1±5.8 A	35.6±6.5 A	43.6±7.0 A	52.5±7.2 A	61.1±7.0 A	69.5±7.2 A	78.6±8.1 A	86.3±8.0 A
		BH	17.6±5.1 A	22.9±5.3 A	29.8±5.5 A	39.0±5.1 A	48.2±5.3 A	57.2±5.7 A	65.5±5.8 A	74.4±6.1 A	83.7±6.6 A	91.5±7.3 A
		CH	15.6±5.6 A	20.3±6.2 A	25.6±5.8 A	34.0±5.8 A	43.2±6.0 A	52.5±6.3 A	60.9±6.2 A	69.2±6.6 A	78.9±6.7 A	86.6±6.8 A
		DH	16.7±5.3 A	22.0±6.3 A	28.6±6.7 A	37.1±7.1 A	46.6±7.9 A	55.8±8.3 A	64.0±8.3 A	72.3±8.6 A	81.7±9.2 A	89.8±9.7 A
Apple sick soil	AA	15.9±4.3 A	21.0±4.9 A	26.7±4.8 A	34.2±5.1 A	42.8±5.7 A	51.3±6.2 A	59.6±5.9 A	68.0±5.6 A	77.5±5.7 A	84.5±6.0 A	
	BA	16.5±5.0 A	21.6±5.7 A	28.9±6.1 A	38.5±6.4 A	47.7±6.9 A	57.0±7.2 AB	65.2±7.7 AB	74.4±7.4 AB	84.2±7.9 AB	92.6±8.2 B	
	CA	17.3±5.4 A	22.6±5.6 A	29.5±5.7 A	39.2±5.7 A	49.3±5.7 A	59.3±5.9 B	67.9±6.2 B	77.2±6.4 B	87.9±6.5 B	96.7±6.6 B	
	DA	15.1±4.0 A	19.9±3.3 A	28.1±3.4 A	36.7±3.5 A	46.4±3.8 A	55.2±3.9 AB	63.1±3.9 AB	72.0±4.0 AB	81.7±4.1 AB	89.1±4.0 AB	

Values within columns (Mean±SD) of the same soil variant followed by different letters indicate significant differences between the treatments using Tukey test, $p \leq 0.05$ and $N=5$ for 2010 and $N=10$ for 2011. Treatment abbreviations: first letter: A, B, C, D for untreated, thermal treatments at 50 °C, 100 °C and treatments with Basamid, respectively; second letter: A, H for apple sick soil and healthy soil, respectively

Table 3 Plant biomass response of apple rootstocks M26 grown in soils with or without replant problems that were temperature or Basamid treated compared to the untreated soil

Year		Shoot dry mass				Root dry mass			
		Healthy soil		Apple sick soil		Healthy soil		Apple sick soil	
		Dry mass [g/plant]	Increase [% over untreated]	Dry mass [g/plant]	Increase [% over untreated]	Dry mass [g/plant]	Increase [% over untreated]	Dry mass [g/plant]	Increase [% over untreated]
2010	Untreated	9.6±3.4 a		3.8±0.7 a		1.3±0.3 a		1.0±0.2 a	
	T 50 °C	11.5±2.3 a	20	6.8±1.7 b	79	1.3±0.3 a	3	1.0±0.2 a	0
	T 100 °C	11.4±2.5 a	20	7.9±0.8 b	108	1.5±0.5 a	15	1.1±0.1 ab	16
	Basamid	8.7±2.7 a	-9	8.5±1.9 b	124	1.1±0.4 a	-10	1.3±0.2 b	37
2011	Untreated	18.7±3.8 a		16.9±3.6 a		3.1±1.0 a		3.1±0.7 a	
	T 50 °C	21.5±2.7 a	15	23.0±3.9 b	37	2.9±0.9 a	-6	3.4±0.9 a	11
	T 100 °C	20.3±2.8 a	9	23.3±3.7 b	38	2.7±0.8 a	-12	3.1±0.9 a	2
	Basamid	20.6±4.2 a	10	21.6±3.2 b	28	2.8±0.7 a	-10	3.4±0.8 a	9

Values within columns (Mean±SD) followed by different letters indicate significant differences between the treatments using Tukey test, $p \leq 0.05$ and $N=5$ in 2010, $N=10$ in 2011

determined between plants grown in Basamid treatments and those from untreated apple sick soil. For healthy soil, the Basamid treatment resulted in slight reduction of root growth (Table 3).

Results in 2011

In 2011, less pronounced differences were observed between the different soil treatments. For healthy soil, no significant differences regarding plant height between untreated and treated soil were detected. For apple sick soil, plants grown in soil treated at 100 °C were significantly higher than plants in untreated soil already after 6 weeks of culture, and this trend was extended until the day of harvest, whereas the plants grown in soil treated at 50 °C, presented significant differences in height after 10 weeks only (Table 2). With regard to biomass formation, in apple sick soil the shoot dry mass increased significantly by 37, 38 and 28 % for treatments at 50 °C, 100 °C and Basamid compared to untreated soil, respectively (Table 3). There were no significant differences observed for the root dry mass for the two soil variants (Table 3).

Histological analyses of root morphology

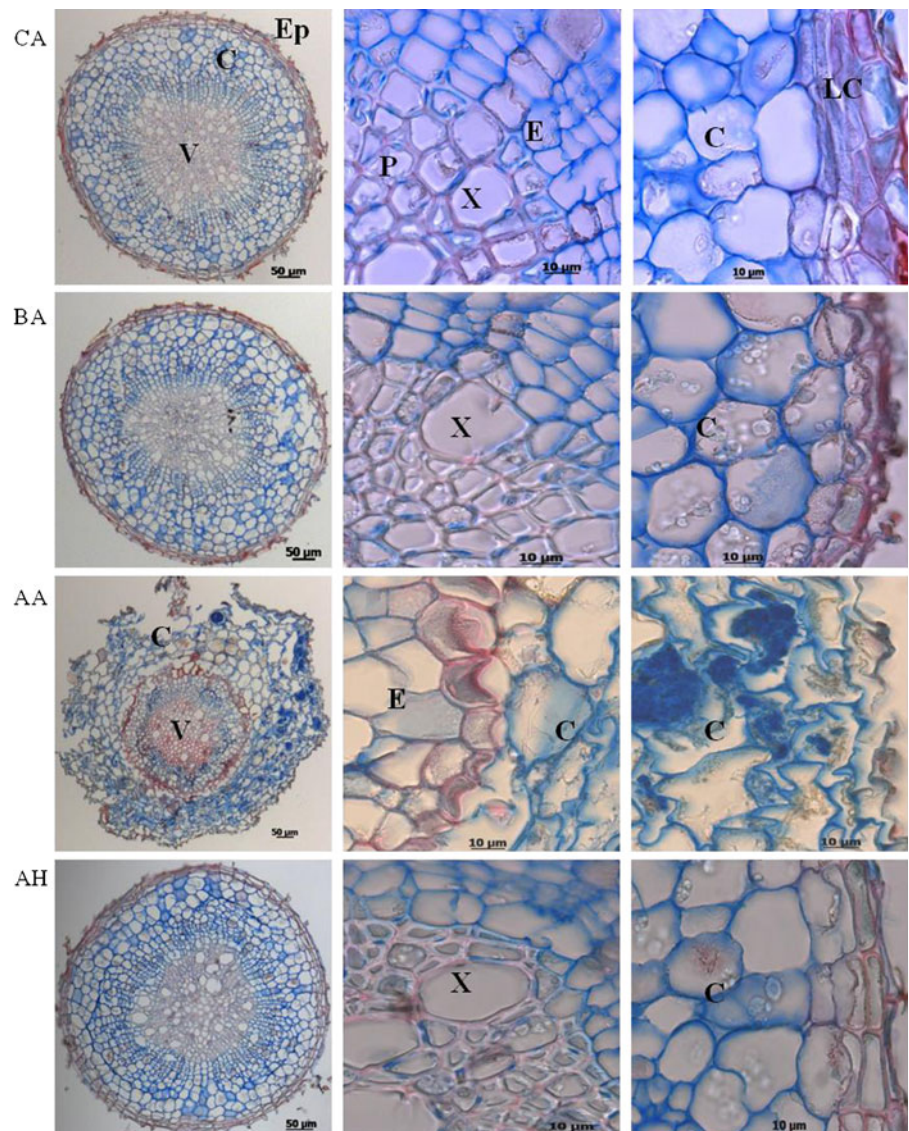
The structure of inspected apple roots grown in untreated healthy soil consisted of a thin layer of epidermal cells surrounding the cortex, and the cell layers

were arranged properly and structurally intact (Fig. 1, row AH). No marked differences were observed in roots grown in apple sick soil treated at 50 and 100 °C (Fig. 1, rows BA and CA). In contrast, almost all inspected root segments that had been grown in untreated apple sick soil showed different cell structures and destruction of the whole outer root layers (Fig. 1, row AA). Some parts of the epidermal and cortical layers had even spread and were no longer attached to the central tissue. It was obvious that the cortical cells possessed significant amounts of densely stained material (Fig. 1, row AA). Once the cortical layer was detached, lignifications of the endodermis and phi layer cells were observed (Fig. 1, row AA). The microscopic pictures corresponded to the visual symptoms of the roots showing a dark brown color, necroses and relative small diameters compared to roots grown on treated sick soil and untreated healthy soil.

Microbial community analyses

Quantitative real time PCR analyses showed that the different treatments did not influence the abundance of bacteria as 16S rRNA gene copy numbers detected in total community DNA from rhizosphere soil samples taken in 2011 were similar and did not significantly differ from each other (Suppl. Fig. S1).

Fig. 1 Cross section of M26 2011 grown in different soils and treatments viewed by light microscope. In each row, from left to right, the figure depicted the whole cross section of roots, area of vascular tissue and cortex, respectively within the soil variant. AH, untreated healthy soil; AA, untreated healthy soil; BA and CA for untreated, thermal treatments at 50 and 100 °C, apple sick soil, respectively. Abbreviations: C=cortex, E=endodermis, Ep=epidermis, LC=lignified cortex, P=phloem, V=vascular tissues and X=xylem

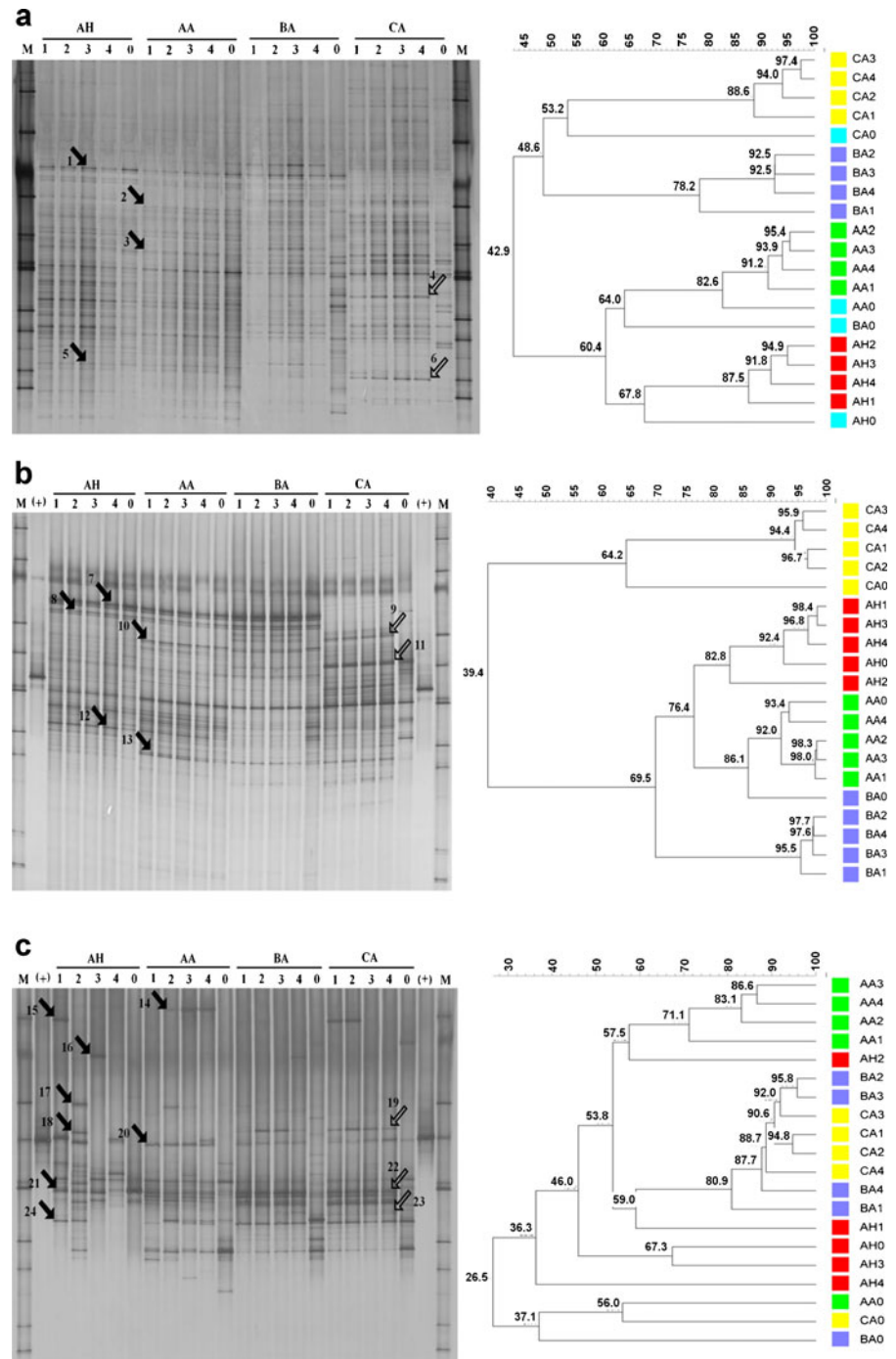


The influence of the soil treatments on the composition and relative abundance of total bacteria, *Bacillus*, *Pseudomonas* and total fungal communities was studied. Therefore, DGGE fingerprints of 16S rRNA gene or ITS fragments amplified from total community DNA were generated for one composite sample per treatment of the 2010 experiment (lanes 0) and four replicates per treatment of the 2011 experiment (lanes 1, 2, 3 & 4). The arrows indicate characteristic individual bands which disappeared or appeared in response to the thermal treatments (Figs. 2 and 3).

The DGGE fingerprints for **total bacterial communities** (Fig. 2a) showed highly complex patterns with 50–56 equally intense bands. The UPGMA

analysis revealed distinct treatment-dependent clusters. The fingerprints of the four replicate rhizosphere samples per treatment shared a high similarity and also the bulk soil fingerprint belonged to the corresponding treatment cluster except for BA0. Two main clusters sharing less than 43 % similarity were formed. One cluster contained the fingerprints of the heat-treated samples (BA, CA) which displayed 48.6 % similarity while the other main cluster was formed by the fingerprints of the healthy (AH) and apple sick soil (AA) which shared 60.4 % similarity. The Pearson similarity matrices of the fingerprints of rhizosphere samples 2011 obtained by GelCompar were used for the permutation test for significant differences between the

Fig. 2 Denaturing gradient gel electrophoresis fingerprints of 16S rRNA gene fragments amplified from soil of bio-test experiments using apple rootstocks M26. Samples from bulk soil 2010 (lanes 0), rhizosphere soil (lanes 1, 2, 3 & 4) and each soil treatment: AH, for untreated healthy soil; AA, BA and CA for untreated, thermal treatments at 50 and 100 °C, apple sick soil, respectively. M, marker and (+), positive control: *Bacillus amyloliquefaciens* in b) and *Pseudomonas sp.* in c). Gels obtained for the samples with their respective dendrogram generated by cluster analysis (UPGMA) are shown: **a** total community bacterial profiles and respective dendrogram, **b** *Bacillus* profiles and respective dendrogram and **c** *Pseudomonas* profiles and respective dendrogram



treatments. Statistical analysis revealed highly significant treatment-dependent differences (<0.05) with d-values of 42.86 % (Table 4). Bands 1 and 5 were present in both untreated healthy (AH) and apple sick (AA) soil and they disappeared by thermal treatment at 100 °C. Bands 2 and 3 were unique to the apple sick

soil, whereas bands 4 and 6 appeared only after heat treatments. Some populations, i.e. band 1, could not be controlled by temperature treatment at 50 °C but at 100 °C (Fig. 2a).

In comparison to the total bacterial communities, the *Bacillus* community fingerprints contained less

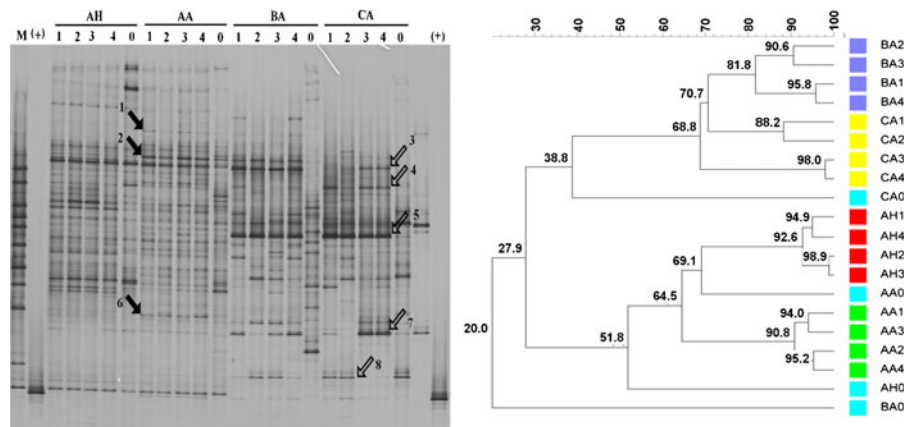


Fig. 3 Denaturing gradient gel electrophoresis fingerprints of Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) amplified from soil of bio-test experiments using apple rootstocks M26. Samples from bulk soil 2010 (lanes 0), rhizosphere soil (lanes 1, 2, 3 & 4) for each soil treatment: AH,

for untreated healthy soil; AA, BA and CA for untreated, thermal treatments at 50 and 100 °C, apple sick soil, respectively; M, marker and (+), positive control (*Trichoderma harzianum*). Gels obtained for the samples with their respective dendrogram generated by cluster analysis (UPGMA)

bands (33–38 bands) (Fig. 2b). Again, the dendrogram showed treatment-dependent clusters and revealed that the replicates within each treatment shared high similarities. Untreated soils (AA and AH) were found in one cluster (76.4 % similarity) together with the 50 °C treated AA soil (69.5 %), while soil treated at 100 °C formed a separate cluster (Fig. 2b). The fingerprints of the bulk soil samples 2010 clustered with the corresponding rhizosphere fingerprints 2011. Statistical analysis showed significant treatment-dependent differences ($p < 0.05$) with d-values of 38.95 % (Table 4). The populations behind the strong bands 7 and 8 detected in the fingerprints of both untreated soil variants could be controlled by a temperature of 100 °C only, whereas band 12 already disappeared at 50 °C. Interestingly, bands 10 and 13 were observed only in untreated AA soil, and these populations were eliminated at 100 °C (Fig. 2b). Intense bands presumably caused by *Bacillus* populations re-colonizing the soils after

heat treatments were observed which were even more intense after 100 °C treatment (bands 9 and 11).

The *Pseudomonas* community fingerprints were less complex and displayed only 15–20 bands. A higher variability between replicates was obvious, especially in the healthy (AH) but also apple sick soils (AA), as illustrated for example by the bands 15, 16, 17 and 18 being present only in one replicate, or band 21 appearing in three, and band 24 only in two replicates (Fig. 2c). UPGMA analysis revealed that the fingerprints of the four AH replicates mingled into different clusters. Also for the untreated AA soil lower levels of similarity of 71.1 % for the replicates were recorded. All heat-treated soils (BA, CA) shared a cluster with 80.9 % similarity regardless of the temperature (Fig. 2c). Interestingly, the fingerprints of the bulk soil samples 2010 for AA, BA and CA clustered separately. The permutation test revealed significant treatment-dependent differences ($p < 0.05$) with d-

Table 4 Pair-wise comparisons between similarity measures obtained within soil replicates in 2011

DGGE profile	Treatment	Dissimilarity ¹
Community bacteria	AH vs. AA vs. BA vs. CA	42.86*
<i>Bacillus</i>	AH vs. AA vs. BA vs. CA	38.95*
<i>Pseudomonas</i>	AH vs. AA vs. BA vs. CA	17.98*
ITS	AH vs. AA vs. BA vs. CA	41.95*

¹ average dissimilarity of fingerprints between treatments, corrected for the within-treatment dissimilarity

Values indicate differences of similarity among treatments and * indicates significant differences between treatments ($P < 0.05$). AH, healthy soil untreated; AA, apple sick soil untreated; BA, apple sick soil treated at 50 °C and CA, apple sick soil treated at 100 °C.

values of 17.98 % (Table 4). Some bands were detected only for samples of the untreated AA soil (bands 14 and 20), but were neither visible in untreated AH soil nor after heat treatments. As in the case of *Bacillus*, some bands obviously were due to re-colonization after 100 °C treatment (bands 19, 22 and 23).

The **fungal fingerprints** contained depending on the treatment significantly different numbers of bands (31–47) with the lowest numbers observed for the heat-treated soils. The fingerprints of the heat-treated soils showed considerably more variability among replicates of the same treatment than the untreated AH and AA soils. Two main clusters, one containing the fingerprints of the heat-treated soils (BA, CA) and the other comprising the untreated soils (AH, AA) were observed by UPGMA analysis. The fingerprints of the fungal communities of AH and AA soils formed distinct clusters with high similarities (more than 90 %) among the replicates (Fig. 3). The permutation test revealed significant treatment-dependent differences ($p < 0.05$ %) and a d-value of 41.95 % (Table 4). The intense band 5 detected in all replicates was specifically found after heat treatments. Bands 1, 2 and 6 appeared only in untreated AA soil but not in healthy or disinfected sick soil and thus might be interesting candidates contributing to the replant disease (Fig. 3).

Discussion

Bio-test for detecting replant problems

A bio-test for detecting replant problems should be fast, reproducible, and independent of the season and work with small volumes of soil. The indicator plant material should be genetically homogeneous, free of pathogens, available throughout the whole year and inexpensive. Previous studies mainly worked with seedlings which fulfill many of the demands, except genetic homogeneity. Recently bio-tests with rooted cuttings of apple rootstock M9 were reported, but when using this kind of plant material bigger containers and soil volumes (15 L) were applied (Kelderer et al. 2012). Tests involving *in vitro* propagated plant material were reported for *Rosa* ‘The Fairy’ (Strassburger 1992) and peach (Manici and Caputo 2010). In the present study, we could show that *in vitro* propagated *Malus* M26 were suitable material for setting up the bio-test for apple.

As expected, the bio-test showed significantly improved plant growth after disinfection treatments for soil with apple replant problems (Tables 2 and 3). This indicated the importance of biotic factors such as nematodes, bacteria, fungi or other unknown agents. Based on plant growth responses in apple sick soil, soil treatments at 100 °C and with Basamid were more effective than the 50 °C treatment (Table 3). Therefore, nematodes probably were not the only causal agents, further causes were apparently present which were controlled by the treatments at 100 °C and with Basamid, as also described previously (Mai and Abawi 1978; Spethmann and Otto 2003). These observations also correspond to the shifts in microbial populations shown by the DGGE analyses (Figs. 2 and 3, discussed below).

In healthy soil, the plants also showed increased growth when the soil had been disinfected, indicating that even the soil where no *Rosaceae* had been planted before was not completely free of pathogens.

The root dry mass was not as severely affected on untreated apple sick soil compared to treated soil as shoot dry mass was (Table 3). This could have been due to the fact that in untreated soil, the roots needed to grow more to counterbalance the damages that have been clearly demonstrated in the visual inspection of the root systems in the histological analyses (Fig. 1). Since washing out the roots from the soil for the dry mass sometimes was destructive, experimental errors cannot fully be excluded.

Compared with 2010, the reactions were not as clear in 2011, especially when shoot height and dry mass formation are regarded (Tables 2 and 3). The different responses could be due to the different sizes and ages of the plants and/or the container sizes. The bigger plants in 2011 had developed stronger root systems that probably could better resist pathogens. Together with the smaller volume of soil which was assumed to be connected to lesser causing factors of replant problems being present, this might have resulted in less pronounced differences as compared to 2010.

Also Jaffee and Mai (1978) reported that in their experiment employing apple seedlings inoculated with the same number of nematodes *P. penetrans*, the one-week-old seedlings that had been inoculated were more severely affected than seedlings inoculated at an age of 3 or 5 weeks since they contained more *P. penetrans*/g root than the older seedlings. Also, Mai et al. (1994) reported the roots of young apple trees

attacked by *P. penetrans* appeared to be much more vulnerable to damages than older trees. In conclusion, the plant material used in bio-tests for replant problems should be young and exposed to a certain minimum amount of soil volume which needs to be specified in further studies.

Differences in plant height and shoot dry mass in both years were unexpectedly low and not significant when comparing the heat treatments of 50 °C versus 100 °C. That might have been due to the effects of heat sterilization. To reach 50 °C inside the soil packs, the oven was heated up to 70–80 °C, and approximately it took around 7 h to reach 50 °C (temperature inside the bag containing 15 L soil). Therefore, the outer layer of the soil in every autoclavable bag was exposed to higher temperatures for longer time which might have killed not only the nematodes which are destroyed already at 50 °C (Spethmann and Otto 2003), but also other pathogens. Broadbent et al. (1971) reported that aerated steam treatment at 60–70 °C for 30 min is commonly used to destroy root pathogens. Future experiments should employ heating of smaller volumes of soil, e.g. portions of 2 L, for which 50 °C were observed to be reached already within 1 h 15 min (data not shown).

To further improve the sterilization treatments, future tests should involve irradiated soil. By irradiation less structural and chemical changes are caused and so the test results are not negatively affected by these changes (Trevors 1996).

Morphology of M26 roots

Cross section of apple roots aimed at unraveling changes at the cellular level in roots grown in sick soil which was assumed to contain biological agents that have deleterious effects on the plants. Thereby, explanations for the observed decline in plant growth (Tables 2 and 3) and correlations to the changing microbial community profiles (Figs. 2 and 3) could be found. The epidermis as the outermost covering of the root, is involved in protection of the roots and the cortex, a major component of root, mainly functioning as storage tissue of photosynthetic products and being active in uptake of water and minerals (Hendrix and Powell 1970; Gregory 2006; Beeckman 2010). Therefore, the damage of epidermal as well as cortical root cells as observed in sick soil most probably resulted in reduced vigor and shoot growth of the plants. Recently, also

Kelderer et al. (2012) investigating the inter-row planting of apples on sites with replant problems described root health to be strongly correlated with plant growth. Another explanation for the reduction in plant growth in relation to reduced root biomass or damaged roots comes from the fact that cytokinins are synthesized in roots and are responsible for cell division, differentiation and outgrowth of the axillary shoot buds (Le Bris 2003).

The intact structures found for roots grown in untreated healthy and treated apple sick soil confirmed that there was no pathogen causing apple root rot present in these variants (Fig. 1). Severe destruction of root tissues in plants growing in soils with apple replant problems as depicted in Fig. 1 was also observed in previous studies on the macroscopic as well as microscopic (Caruso et al. 1989) level. The infected roots of plants showed different symptoms depending on the pathogens involved. Spethmann and Otto (2003) reported that symptoms in apple roots infected by Actinomycetes were mainly observed in the epidermal cell layer which seems to be quite different from our results (Fig. 1, row AA). Therefore, Actinomycetes were probably not or less important in the soils of this study. Typical symptoms of nematode damage (defined stylets) were also not observed in the investigated cross sections. In the histological study of apple roots affected by replant problems by Caruso et al. (1989), the possible hyphae of *Rhizoctonia*, *Phytophthora* and *Pythium* were found in apple roots of trees grown in soil with replant disease. The densely stained inclusions in cortical layers of the roots that were also detected in Fig. 1 (row AA) were assumed to be tannins resulting from the oxidation of phenols playing a role in the defense mechanism against pathogenic microorganisms.

Once the cortical layer was damaged and removed, the plant roots responded by forming a secondary protective tissue in the phi layer by thickening of radial and transverse walls of the phi-layer cells (Fig. 1) as also described for apple and pear during secondary growth (Weerdenburg and Peterson 1983) and for oak and cherry roots during acclimatization (Soukup et al. 2004).

Microbial community in rhizosphere soil

DGGE analyses revealed significant differences in the composition of the bacterial and fungal communities in the rhizosphere of apple trees grown in

healthy and apple sick soil. Highly similar and reproducible fingerprints of replicate samples were observed in nearly all analyses, except for *Pseudomonas* communities of healthy soil and fungal communities of heat-treated soils (Fig. 2 and 3). Variability among replicates could be due to lower abundance (*Pseudomonas*) or a higher heterogeneity (fungi). Surprisingly the fingerprints of the composite bulk soil samples of the different treatments analyzed from 2010 experiment clustered in most cases with the corresponding treatment-dependent cluster of the rhizosphere samples from 2011 except for *Pseudomonas* communities. This finding indicates that the rhizosphere shaped the *Pseudomonas* community composition more strongly than the treatments. Although the 16S rRNA gene copy numbers detected by qPCR were comparable for both untreated and treated soils 10 weeks after the treatments (Fig. S1), the fingerprints of heat-treated soils were significantly different and formed distinct clusters in the UPGMA analyses (Fig. 2 & 3). This obviously showed that the heat treatment severely affected most microorganisms and that the soils were re-colonized by other microbial populations 10 weeks after the treatment. Thus, the fingerprints showed significant changes in microbial communities and these differences correlated with significant differences in shoot and root growth of apple plants in the bio-tests. Similarly, in a study of the effect of steam disinfection in organic farming soil, steaming at 100 °C at the soil surface significantly decreased the microbial populations, but after 15 to 60 days the bacterial community recovered while the community structures remained very different as compared to those in untreated soil (Roux-Michollet et al. 2008).

The re-colonizing fungi in the soil after treatment at 100 °C of apple sick soil showed high variability between replicates of fungal fingerprints (Fig. 3), confirming the observations of other studies dealing with rhizosphere fungal communities (Girvan et al. 2004; Costa et al. 2006a). Re-colonizing bacteria and fungi in the heat-treated soil could originate from resting stages that survived after the heat treatments, e.g. spores of some *Bacillus* spp. can survive at up to 135 °C, those of *B. subtilis* at 120 °C (Janstova and Lukasova 2001). Other sources of re-colonizing microorganisms would be air, irrigation water and the acclimatized apple plantlets.

Obviously the re-colonizing of both bacteria and fungi in heat-treated soil had either no or beneficial effects on the plants which could be direct or indirect effects via controlling pathogenic organisms.

Several microbial species of root colonizing fungi have been isolated from soils with apple replant diseases: *Fusarium oxysporum*, *Fusarium solani*, *Fusarium acuminatum*, *Cylindrocarpon destructans*, *Pythium* spp., *Phytophthora* spp., *Alternaria* spp. and *Rhizoctonia* spp., out of which only *Cylindrocarpon*, *Rhizoctonia* spp., *Pythium* spp. and *Phytophthora* spp. were found to be pathogenic to plants (Mazzola 1998; Manici et al. 2003; Manici and Caputo 2010; Tewoldemedhin et al. 2011; Kelderer et al. 2012). For bacteria, only two strains of *Bacillus subtilis* and *Pseudomonas putida* (Utkhede 2006) were reported to be pathogenic and contribute to the root rot complex on apple seedling-derived plants. Since the DGGE analyses do not allow a clear assignment of a band to a given organism, the causing agents of apple replant disease cannot be deduced from the present study. Cloning and sequencing of re-amplified DNA fragments from such bands that appear only in the fingerprints of untreated apple sick soil could provide insight into the occurrence of individual microbial species.

In conclusion, thermal soil disinfection of apple sick soil resulted in significantly increased shoot growth and at the same time clear differences in soil microbial communities. In future analyses the bacterial and fungal bands of different abundance should be identified by sequencing and thus allow a better understanding of the phenomenon of apple replant problems. Moreover, studies involving other soil types and test plants should follow.

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