Development of a DNA Microarray-Based Assay for the Detection of Sugar Beet Root Rot Pathogens

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ABSTRACT

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Sugar beet root rot diseases that occur during the cropping season or in storage are accompanied by high yield losses and a severe reduction of processing quality. The vast diversity of microorganism species involved in rot development requires molecular tools allowing simultaneous identification of many different targets. Therefore, a new microarray technology (ArrayTube) was applied in this study to improve diagnosis of sugar beet root rot diseases. Based on three marker genes (internal transcribed spacer, *translation elongation factor 1 alpha*, and 16S ribosomal

DNA), 42 well-performing probes enabled the identification of prevalent field pathogens (e.g., *Aphanomyces cochlioides*), storage pathogens (e.g., *Botrytis cinerea*), and ubiquitous spoilage fungi (e.g., *Penicillium expansum*). All probes were proven for specificity with pure cultures from 73 microorganism species as well as for in planta detection of their target species using inoculated sugar beet tissue. Microarray-based identification of root rot pathogens in diseased field beets was successfully confirmed by classical detection methods. The high discriminatory potential was proven by *Fusarium* species differentiation based on a single nucleotide polymorphism. The results demonstrate that the ArrayTube constitute an innovative tool allowing a rapid and reliable detection of plant pathogens particularly when multiple microorganism species are present.

The sugar beet root represents the main storage organ for the assimilated sucrose, which is used as raw material for sugar manufacturing. During the cropping season, the occurrence of root rot diseases has a great economic impact by reducing the root and white sugar yield (Jacobsen 2006; Karadimos and Karaoglanidis 2006). In general, the sugar beet root is exposed to various root rot causing microorganisms including species such as *Aphanomyces cochlioides*, *Pythium* spp., *Phytophthora* spp., *Rhizoctonia crocorum*, and *Rhizoctonia solani* anastomosis group (AG) 2-2 (Asher and Hanson 2006; Jacobsen 2006; Karadimos and Karaoglanidis 2006). Plant losses of up to 60% accompanied by a substantial yield decline have been reported for *Rhizoctonia* crown and root rot (Allen et al. 1985). Although most root rots are associated with fungal and oomycete species, they can also be caused by bacteria like *Pectobacterium betavasculorum* and *Leuconostoc mesenteroides* subsp. *dextranicum* (Strausbaugh and Gillen 2008; Thomson et al. 1977). As sugar beet roots are subjected to long-term storage after harvest for up to 80 days (Jaggard et al. 1997), the occurrence of postharvest root rots is also of high economic impact. In a 128-day survey conducted at the Moorhead sugar factory in the United States, it was estimated that 1.22% of the total amount of processed beets were affected by storage rots causing a sugar loss of approximately 1,314 t (Bugbee and Cole 1976). Additionally, processing of deteriorated beets causes severe problems in sugar factories due to the microbial induced accumulation of invert sugar and polysaccharide gums (Fugate and Campbell 2009). Species most often associated with storage rots are *Botrytis cinerea*, *Fusarium* spp., *Penicillium* spp., and *Phoma betae* (Bosch and

Mirocha 1992; Bugbee 1975; Bugbee and Cole 1976; Christ et al. 2011).

The correct identification of root rot diseases is crucial to allow a proper handling of infected beets and to develop management strategies. For example, adjusting to an earlier harvest time in fields severely infested with *Aphanomyces cochlioides* or *Rhizoctonia solani* may decrease storage rot issues (Bruhns et al. 2004; Campbell and Klotz 2006). Furthermore, a clear knowledge about microbial species causing root rots during storage is essential for resistance breeding. However, a major problem during disease diagnosis is the vast species diversity that needs to be considered. In vitro isolation, however, is time and labor intensive as specific isolation media and growing conditions are required. Furthermore, the presence of secondary invaders complicates the identification of the causal agent (Strausbaugh et al. 2013). Molecular methods based on polymerase chain reaction (PCR) have been successfully developed for multiplex detection of fungal pathogens including *Aphanomyces cochlioides*, *Cercospora beticola*, *Pythium ultimum*, *Phoma betae*, *F. oxysporum*, and *Rhizoctonia solani* AG 2-2 (Weiland and Sundsbak 2000). Nevertheless, the establishment of PCR-based multiplex assays is technically challenging (Henegariu et al. 1997) and the number of targets is restricted by primer interactions (Wei et al. 2008). Moreover, routine analysis with PCR can become rapidly overwhelming when there is no indication for the causal agent making a screening for many different species necessary.

With the development of DNA microarray technology, high-throughput multiplex detection has been greatly improved for various phytopathogenic microorganisms including bacteria (Fessehaie et al. 2003; Pelludat et al. 2009), fungi (Chen et al. 2009; Lievens et al. 2003, 2007; Zhang et al. 2008), oomycetes (Chen et al. 2013; Tambong et al. 2006), and viruses (Agindotan and Perry 2007, 2008). A DNA array consists of oligonucleotide probes which are either immobilized on a nylon filter (macroarray) or on a glass slide (microarray). Although microarrays generally possess a higher spot density, the detection principle of both array types is based on

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the hybridization between species-specific oligonucleotide probes and labeled PCR amplicons obtained from genomic sequences providing sufficient variability for species discrimination (Chen et al. 2013; Kristensen et al. 2006; Pelludat et al. 2009). The high discriminatory potential even allows the differentiation of a single nucleotide polymorphism (SNP) in two amplicons (Lievens et al. 2006; Zhang et al. 2008); making it suitable for the discrimination of closely related species like *Fusarium* spp. (Kristensen et al. 2006).

In this study, a new low density DNA microarray technique (ArrayTube [AT]) was used to improve the simultaneous detection of the most prevalent sugar beet pathogens causing root rots during the cropping season and storage period. The AT system has been applied in various studies on human and animal pathogenic microorganisms (Borel et al. 2008; Korczak et al. 2005; Monecke and Ehrlich 2005; Sachse et al. 2005, 2008). Recently, Dobnik et al. (2014) utilized this platform for the detection of two important quarantine plant pathogens, namely *Potato spindle tuber viroid* and *Ralstonia solanacearum*. In contrast to previously applied array technologies (Chen et al. 2009, 2013; Fessehaie et al. 2003; Lievens et al. 2003; Tambong et al. 2006; Zhang et al. 2007), all working steps are conducted in one vessel using standard laboratory equipment, and can be completed in less than 3 h including signal analysis (Tomlinson et al. 2014). The workflow of the developed microarray comprised two main steps: (i) exponential amplification of different marker genes followed by (ii) a hybridization between species-specific oligonucleotide probes and target amplicons enabling multiplex detection. The developed assay was validated by (i) testing specificity and sensitivity with pure cultures of reference isolates, (ii) the detection of target organisms in inoculated sugar beet tissue, and (iii) the analysis of diseased field beets for presence of target organisms by microarray as well as by in vitro isolation and PCR detection.

MATERIALS AND METHODS

Microorganisms. A complete list of fungal, oomycete and bacterial isolates used in this study can be found in Supplementary Table S1. To simplify species designation, only the anamorphic name is provided. Isolates were obtained from the American Type Culture Collection (ATCC, Manassas), the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), and the Agricultural Research Service Culture Collection (ARS/NRRL, Peoria). Additionally, isolates from field grown sugar beet roots and deposited at the culture collection of the Institute of Sugar Beet Research (IfZ, Göttingen, Germany) were included. One reference isolate of each species used for the microarray development was deposited at the DSMZ if not available in the above mentioned official culture collections. For preservation, fungal isolates were stored on Spezieller Nährstoffarmer Agar (SNA) (Nirenberg 1976) at 10°C; oomycetes were kept as water cultures at room temperature (RT) (Zhang et al. 2008); and bacterial isolates were stored in 10% DMSO at -80°C. Before use, fungal and oomycete isolates were grown on potato dextrose agar (PDA) at 25°C in the dark. For DNA extraction, mycelium was grown in potato dextrose broth for 5 days at RT in the dark and dried on sterile filter paper. Bacterial cultures were incubated aerobically at 30°C on de Man–Rogosa–Sharpe agar (*Leuconostoc mesenteroides* subsp. *dextranicum*), tryptic soy agar (*Pectobacterium betavascularum*), and nutrient agar (*Rhizoctonia aquatilis*) as recommended by the DSMZ.

DNA extraction. A silica based method was used for the isolation of fungal, oomycete and bacterial DNA from pure cultures as well as sugar beet root tissue. All extraction steps were performed according to the protocol of Rott and Jelkmann (2001) with a few exceptions. The grinding buffer was prepared with 6 M guanidine hydrochloride instead of 4 M guanidine thiocyanate as described by

Menzel et al. (2002). Approximately 200 mg of sample material was ground to a fine powder in liquid nitrogen prior to homogenization in the grinding buffer. In case of bacterial cultures, 5-day-old colonies were harvested and transferred directly to the grinding buffer. Integrity of DNA was checked by gel electrophoresis (1%) and quantified in a BioPhotometer (Eppendorf, Hamburg, Germany).

Amplification and sequencing of ITS, *tef1*, and 16S rDNA. Primers used for amplification and sequencing of target genes for species identification and probe design were ITS1/ITS4 (White et al. 1990) for the internal transcribed spacer (ITS) of fungi and oomycetes, EF1/EF2 (O'Donnell et al. 1998) for the *translation elongation factor 1 alpha* (*tef1*) of *Fusarium* spp., and 8F/1492R (Turner et al. 1999) for the 16S rDNA of bacteria. All PCR reactions were carried out in 50 µl volume containing 1 mM MgCl₂, 1× Fermentas DreamTaq buffer (ThermoScientific, Schwerte, Germany), 0.25 mM dNTP mix, 200 mM of each primer, 1 U of Fermentas DreamTaq DNA polymerase (ThermoScientific), and 1 µl of DNA (10 to 100 ng). The PCR cycle conditions (ITS and *tef1*) were as follows: 95°C for 5 min; 35 cycles of 95°C for 45 s, 59°C (*tef1*) or 55°C (ITS) for 45 s, 72°C for 45s; and a final elongation step at 72°C for 5 min. Cycle conditions for amplification of 16S rDNA were derived from Turner et al. (1999). PCR products were checked by gel electrophoresis (1%) and purified with SureClean Plus reagent (Bioline, Luckenwalde, Germany) according to the manufacturer's protocol. Purified products were sequenced in both orientations by Eurofins MWG Operon (Ebersberg, Germany). Sequences were checked for similarity against the databases of the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990) and *FUSARIUM*-ID (Geiser et al. 2004).

Design of oligonucleotide probes. In total, three different genes were exploited for the design of species- and genus-specific probes, namely ITS for fungi and oomycetes, *tef1* for *Fusarium* spp., and 16S rDNA for bacteria. Additionally, previously published probes for the same target species were also included in this study if they matched the required probe parameters.

For probe design, multiple alignments were either built using the alignment browser MEGA 5 (Tamura et al. 2011) or the in-house software package Clondiag ArrayDesign (Alere Technologies, Jena, Germany) which selected the best discriminating oligonucleotide probes automatically. Each alignment consisted of sequences from target species and their closest relatives obtained during this study as well as sequences retrieved from NCBI and *FUSARIUM*-ID to cover intra- and interspecific variability. All alignments were inspected for polymorphic sites and probes were placed manually. Mismatches to nontarget species were placed in the center of the oligonucleotide sequence if possible. Finally, probe sequences were checked against the NCBI database and *FUSARIUM*-ID to prove their specificity for the respective target species and to identify possible unintended targets.

All probes were designed to match the following criteria: length (20 to 30 nt), melting temperature (55 to 65°C), and GC content (40 to 50%). The BioMath T_m-calculator (<http://www.promega.com/a/apps/biomath/?calc=tm>) was used to predict the melting temperature, taking into account the exact sequence and the base-stacking effects according to Borer et al. (1974), Rychlik and Rhoads (1989), and SantaLucia (1998).

Amplification and biotinylation of target amplicons for hybridization. Consensus primers were used to amplify the respective target genes carrying probe binding sites for bacteria, fungi, *Fusarium* spp., and oomycetes (Supplementary Table S2). For primer design, an alignment for each target sequence was assembled in MEGA 5 (ClustalW, default options), NCBI sequences of other species to cover a wide range of different genera and sugar beet sequences. The alignments were inspected for conserved sites visually and primers were designed manually. To avoid amplification of sugar beet DNA, mismatches to the sequence of sugar beet were placed close to the 3'-end of the primers. Manually designed primers were proven with Vector NTI for their

compatibility (Invitrogen, Carlsbad, CA). The optimal annealing temperature was determined empirically and all primers were tested for amplification with all isolates used in this study.

Amplicons for hybridization were labeled by incorporation of 5'-biotin-modified forward and reverse primers purchased from Eurofins MWG Operon. PCR for all target genes was carried out as described above, except that 40 cycles were performed using primer specific annealing temperatures. PCR products were checked by gel electrophoresis (1% agarose).

Microarray production. The AT was obtained from Alere Technologies and manufactured as previously described (Monecke and Ehrlich 2005). In brief, 3'-amino-modified probes were purchased from Metabion (Martinsried, Germany) and spotted on the microarray at a final concentration of 15 µM. Additionally, a 3'-amino- and 5'-biotin-modified probe was supplied as positive

control for the staining reaction. All probes were spotted in threefold replication with a spot distance of 200 µm.

Microarray hybridization. All incubation steps were carried out under constant shaking (550 rpm) on the Eppendorf Thermo-mixer comfort (Cologne, Germany). Except for the washing buffer (20× SSC [pH 7.0]: 3 M NaCl, 0.3 M NaCHO), all hybridization reagents were used from the hybridization kit (Alere Technologies). Washing buffers were prewarmed to the required temperature before use. Initially, the ATs were washed with 500 µl of high performance liquid chromatography HPLC grade water (50°C, 5 min), followed by a conditioning step with 200 µl of hybridization buffer (50°C, 5 min). For sample preparation, 15 µl of biotinylated PCR product was mixed with 85 µl of hybridization buffer in a separate tube, incubated at 95°C for 5 min, and cooled down on ice for 1 min. The final volume of the hybridization mix was adjusted to

TABLE 1. Signal intensity of oligonucleotide probes after hybridization of biotin labeled polymerase chain reaction products from pure cultures of target/nontarget species and from sugar beet tissue inoculated with target species

Probe name	Target species	Target species ^a				Nontarget species ^a		
		Pure culture		Inoculated sugar beet tissue		Pure culture ^b		
		Isolates code	Signal intensity	Isolates code ^c	Signal intensity	Species	Isolate code	Signal intensity
16S rDNA								
Bacteria_G	<i>Leuconostoc mesenteroides</i>							
	subsp. <i>dextranicum</i>	DSM20484	0.70	DSM20484	0.50	—	—	—
	<i>Pectobacterium betavascularum</i>	DSM18076	0.68	DSM18076	0.80			
	<i>Rahnella aquatilis</i>	DSM4594	0.79					
Leu_G	<i>L. mesenteroides</i> subsp. <i>dextranicum</i>	DSM20484	0.59	DSM20484	0.53	—	—	—
Internal transcribed spacer 1								
Alt_G_1	<i>Alternaria alternata</i>	DSM28977	0.68	DSM28977	0.54	—	—	—
Bot_G_1	<i>Botrytis cinerea</i>	DSM28931	0.70	DSM28931	0.34	—	—	—
Col_G	<i>Colletotrichum coccodes</i>	DSM28929	0.71	DSM28929	0.69	—	—	—
M_pir_S	<i>Mucor piriformis</i>	CBS 169.25	0.53	CBS 169.25	0.28	—	—	—
Pen_G	<i>Penicillium expansum</i>	CBS 325.48	0.76	CBS 325.48	0.79	—	—	—
	<i>Penicillium expansum</i>	PM168	0.76			—	—	—
Phy_G	<i>Phytophthora cryptogea</i>	DSM28934	0.73	DSM28934	0.80	—	—	—
	<i>Phytophthora drechsleri</i>	CBS 292.35	0.74					
	<i>Phytophthora megasperma</i>	CBS 305.36	0.70					
R_arr_S_1	<i>Rhizopus arrhizus</i>	CBS 126971	0.83	CBS 126971	0.7	—	—	—
	<i>Rhizopus arrhizus</i>	PM122	0.85			—	—	—
R_sol_AG_2-2_S	<i>Rhizoctonia solani</i> AG 2-2 IIIB	CBS 101765	0.43	CBS 101765	0.53	—	—	—
	<i>Rhizoctonia solani</i> AG 2-2 IV	CBS 101767	0.40			—	—	—
R_sto_S	<i>Rhizopus stolonifer</i>	CBS 609.82	0.78	CBS 609.82	0.80	—	—	—
V_dah_S	<i>Verticillium dahliae</i>	DSM28973	0.75	DSM28973	0.70	—	—	—
Internal transcribed spacer 2								
Alt_G_2	<i>Alternaria alternata</i>	DSM28977	0.76	DSM28977	0.32	—	—	—
A_coc_S	<i>Aphanomyces cochlioides</i>	DSM28926	0.85	DSM28926	0.82	—	—	—
Asp_G_1	<i>Aspergillus flavus</i>	CBS 100927	0.79	*	*	—	—	—
Asp_G_2	<i>Aspergillus niger</i>	CBS 120.49	0.77	CBS 120.49	0.78	—	—	—
Bot_G_2	<i>B. cinerea</i>	DSM28931	0.76	DSM28931	0.69	<i>S. sclerotiorum</i>	DSM28976	0.64
M pha_S	<i>Macrophomina phaseolina</i>	DSM28933	0.74	DSM28933	0.81	—	—	—
M_hie_S	<i>M. hiemalis</i>	CBS 201.65	0.84	CBS 201.65	0.75	—	—	—
	<i>M. hiemalis</i>	PM121	0.82			—	—	—
Muc_G_1	<i>M. circinelloides</i>	CBS 195.68	0.83	CBS 195.68	0.82	—	—	—
Muc_G_2	<i>M. plumbeus</i>	CBS 634.74	0.69	CBS 634.74	0.71	—	—	—
	<i>M. racemosus</i>	DSM28932	0.68			—	—	—
	<i>M. racemosus</i>	PM139	0.79			—	—	—
P_vul_S	<i>Penicillium vulpinum</i>	CBS 110772	0.73	CBS 110772	0.77	—	—	—
P_bet_S	<i>Phoma betae</i>	DSM28928	0.77	DSM28928	0.80	—	—	—
P_aph_S	<i>Pythium aphanidermatum</i>	CBS 118.80	0.73	CBS 118.80	0.71	—	—	—
P_del_S	<i>Pythium deliense</i>	CBS 314.33	0.80	CBS 314.33	0.80	<i>Pythium aphanidermatum</i>	CBS118.80	0.13
P_int_S	<i>Pythium intermedium</i>	DSM28974	0.60	DSM28974	0.65	—	—	—
P_ult_S	<i>Pythium ultimum</i>	DSM28975	0.83	DSM28975	0.81	—	—	—

(continued on next page)

^a Nontarget species possess at least one mismatch in their sequence complementary to the probe sequences.

^b — Indicates no cross reactions observed.

^c * Indicates isolate CBS 100927 detected by the probe 1_Asp_spp was only available as DNA, and therefore not tested with inoculated sugar beet tissue.

100 µl with HPLC grade water if less than 15 µl of biotinylated PCR product was used. The hybridization solution was then transferred to the ATs and incubated at 50°C for 60 min. After hybridization, supernatants were discarded and the ATs were washed with 500 µl of 0.2× SSC/0.01% Triton X-100 (50°C, 5 min) and twice with 500 µl of 0.2× SSC (50°C, 5 min). Subsequently, 100 µl of peroxidase-streptavidin conjugate (1:100 diluted in conjugate buffer) was added to the ATs and incubated at 30°C for 10 min. The supernatants were then discarded and the ATs washed with 500 µl of 2× SSC/0.01% Triton X-100 (30°C, 5 min) and 500 µl of 2× SSC (30°C, 5 min). Finally, 100 µl of peroxidase substrate was added to the ATs for the staining reaction (30°C, 5 min, without shaking). Hybridization signals were measured at 22°C using the

CCD-based transmission reader ATR 03 (Alere Technologies) and processed with the Iconoclust software version 3.6r0 (Alere Technologies). Based on raw values, normalized signal intensities were calculated automatically for each spot as described by Sachse et al. (2008).

Assay specificity. Biotinylated PCR products of all isolates were obtained as described above. Functionality and specificity of oligonucleotide probes was tested using 2.5 µl of biotinylated PCR product for hybridization. The test comprised the following amplicons: ITS1 and ITS2 of all fungal and oomycete isolates, *tef1* of *Fusarium* spp. isolates, and 16S rDNA of bacterial isolates. In order to determine the optimal experimental parameters allowing species discrimination based on SNP, preliminary experiments with

TABLE 1. (continued from preceding page)

Probe name	Target species	Target species ^a				Nontarget species ^a		
		Pure culture		Inoculated sugar beet tissue		Pure culture ^b		
		Isolates code	Signal intensity	Isolates code ^c	Signal intensity	Species	Isolate code	Signal intensity
Rso_G	<i>Rhizoctonia solani</i> AG 2-1	CBS 101763	0.83	ATCC 76127	0.84	–	–	–
	<i>Rhizoctonia solani</i> AG 2-3	CBS 101769	0.85	CBS 101765	0.80	–	–	–
	<i>Rhizoctonia solani</i> AG 2-2 IIIB	CBS 101765	0.74			–	–	–
	<i>Rhizoctonia solani</i> AG 2-2 IV	CBS 101767	0.75			–	–	–
	<i>Rhizoctonia solani</i> AG 3	CBS 363.82	0.80			–	–	–
	<i>Rhizoctonia solani</i> AG 4 HG I	ATCC 76126	0.79			–	–	–
	<i>Rhizoctonia solani</i> AG 4 HG II	ATCC 76127	0.77			–	–	–
	<i>Rhizoctonia solani</i> AG 5	CBS 212.84	0.85			–	–	–
	<i>Rhizoctonia solani</i> AG 6	CBS 101779	0.82			–	–	–
	<i>Rhizoctonia solani</i> AG 10	ATCC 76107	0.84			–	–	–
R_arr_S_2	<i>Rhizoctonia solani</i> AG 13	ATCC MYA-984	0.84			–	–	–
	<i>Rhizopus oryzae</i>	CBS 126971	0.81	CBS 126971	0.82	–	–	–
R_cro_S	<i>Rhizopus oryzae</i>	PM122	0.80			–	–	–
R_cro_S	<i>Rhizoctonia crocorum</i>	DSM28927	0.57	DSM28927	0.50	–	–	–
S_rol_S	<i>Sclerotinia rolfsii</i>	CBS 115.22	0.78	CBS 115.22	0.79	–	–	–
Ver_G	<i>V. albo-atrum</i>	PM16	0.56	PM16	0.50	<i>V. dahliae</i>	DSM28973	0.12
<i>Translation elongation factor 1 alpha</i>								
F_ave_S	<i>F. avenaceum</i>	DSM23359	0.76	DSM23359	0.78	–	–	–
	<i>F. avenaceum</i>	O91	0.77			–	–	–
	<i>F. avenaceum</i>	PM150	0.79			–	–	–
Fus_G	<i>F. cerealis</i>	DSM28978	0.74	DSM28978	0.69	–	–	–
	<i>F. cerealis</i>	PM48	0.69	DSM23354	0.59	–	–	–
	<i>F. culmorum</i>	DSM23354	0.57			–	–	–
	<i>F. culmorum</i>	O29	0.60			–	–	–
	<i>F. culmorum</i>	PM157	0.70			–	–	–
F_cul_S	<i>F. culmorum</i>	DSM23354	0.70	DSM23354	0.70	<i>F. cerealis</i>	DSM28978	0.13
	<i>F. culmorum</i>	O29	0.66			<i>F. graminearum</i>	DSM23352	0.01
	<i>F. culmorum</i>	PM157	0.79			<i>F. graminearum</i>	O11	0.13
						<i>F. graminearum</i>	O12	0.15
F_equ_S_1	<i>F. equiseti</i>	O19	0.73	O19	0.72	<i>F. graminearum</i>	O80	0.15
	<i>F. equiseti</i>	O73	0.53			<i>F. graminearum</i>	O81	0.09
	<i>F. equiseti</i>	O90	0.67			–	–	–
	<i>F. equiseti</i>	NRRL 13402	0.45	NRRL 13402	0.47	–	–	–
	<i>F. equiseti</i>	DSM13402	0.78	DSM13402	0.76	–	–	–
F_gra_S	<i>F. graminearum</i>	O11	0.85			–	–	–
	<i>F. graminearum</i>	O12	0.85			–	–	–
	<i>F. graminearum</i>	O80	0.83			–	–	–
	<i>F. graminearum</i>	O81	0.76			–	–	–
	<i>F. graminearum</i>	O54	0.82	O54	0.77	<i>F. hostae</i>	CBS 119941	0.22
F_red_S	<i>F. redolens</i>	O54	0.80			<i>F. verticillioides</i>	CBS 218.76	0.09
	<i>F. redolens</i>	DSM23363	0.82			–	–	–
F_tri_S	<i>F. tricinatum</i>	DSM23357	0.86	DSM23357	0.84	–	–	–
	<i>F. tricinatum</i>	O32	0.74			–	–	–
	<i>F. tricinatum</i>	O33	0.82			–	–	–
	<i>F. tricinatum</i>	O34	0.85			–	–	–
	<i>F. tricinatum</i>	PM77	0.82			–	–	–
	<i>F. tricinatum</i>	PM136	0.79			–	–	–
	<i>F. tricinatum</i>	DSM23361	0.70	DMS23361	0.75	–	–	–
F_ven_S_1	<i>F. venenatum</i>	O97	0.61			–	–	–
	<i>F. venenatum</i>	DSM23361	0.71	DMS23361	0.78	–	–	–
F_ven_S_2	<i>F. venenatum</i>	O97	0.72			–	–	–
	<i>F. venenatum</i>	O97	0.72			–	–	–

altered hybridization temperatures were performed. Furthermore, assay specificity was tested by applying different washing stringency conditions following hybridization.

Assay sensitivity. To evaluate the sensitivity of the microarray, six probes and corresponding target species were selected for further experiments, namely *F. redolens* (*tefl*), *Phytophthora cryptogea* (ITS1), *Pythium aphanidermatum* (ITS2), *Rhizopus arrhizus* (ITS1), *Macrophomina phaseolina* (ITS2), and *L. mesenteroides* subsp. *dextranicum* (16S rDNA). Target amplicons of the microorganisms were amplified from a 10-fold serial dilution of microbial DNA (1 ng to 0.1 fg) and 1 µl of biotinylated PCR product was used for hybridization.

Assay validation. The developed assay was validated with two different approaches: (i) detection of target species in inoculated sugar beet tissue and (ii) comparison of the microarray-based detection with species-specific PCR and in vitro isolation using diseased field beets.

(i) For preparation of artificially infected sugar beet tissue, 3-month-old sugar beets of the 'Belinda' (KWS Saat-AG, Einbeck, Germany) were cultivated in the greenhouse as described by Christ et al. (2011). At harvest, beets were manually topped, attached soil was washed off, and two to three 5-mm-thick slices were cut per beet. Slices were surface disinfected (45 s in 0.5% NaClO, rinsed twice in sterile water) and placed in sterile Petri dishes with watered filter paper. For inoculation, an agar plug (diameter ~0.5 cm) from a 5-day-old culture on PDA was placed in the center of each slice. Petri dishes were then incubated at 25°C for 5 to 7 days in the dark. Sample material (diameter ~1 cm) was harvested from the border between diseased and healthy tissue. Each probe was tested with one isolate of the corresponding target organism. Biotinylated amplicons were amplified as described above and 2.5 µl of each amplicon was used for hybridization.

(ii) Field beets displaying symptoms of an infection with root rot pathogens were obtained from different growing regions throughout Germany. Samples (diameter ~1 cm) taken from the border between diseased and healthy tissue were divided into two parts of equal size. One part was subjected to pathogen detection with the developed microarray using 15 µl of biotinylated PCR product (2.5 µl of each target amplicon) for hybridization. The other part was analyzed by species-specific PCR (see below), and additionally by in vitro isolation, depending on the sample quality. For in vitro isolation, the sample tissue was placed on PDA supplemented with three antibiotics (100 ppm penicillin, 10 ppm tetracycline hydrochloride, and 50 ppm streptomycin sulfate). Bacterial microorganisms were excluded from in vitro isolation. Microorganisms were allowed to grow for 3 to 5 days at 20°C in the dark. Pure cultures were obtained by subcultivation on PDA and purification using hyphal tip culture (Leslie and Summerell 2006). Pure isolates were identified by sequencing ITS or *tefl*. Sequences were deposited at the NCBI database (KJ744369 to KJ744381).

Species-specific PCR for root rot pathogens. Species-specific PCRs were developed for the routine diagnosis of fungal plant pathogens causing sugar beet seedling damping-off and root rots. Primers for the target species *Aphanomyces cochlioides*, *Phoma betae*, *Pythium ultimum*, *Rhizoctonia crocorum*, and *Rhizoctonia solani* AG 2-2 are listed in Supplementary Table S3. All primers were tested for specificity and validated with infected field beets from growers throughout Germany. PCR for *Aphanomyces cochlioides*, *Phoma betae*, *Pythium ultimum*, and *Rhizoctonia solani* AG 2-2 were performed in a 50 µl reaction volume, each containing 1× CoreLoad PCR buffer (Qiagen, Hilden, Germany), 0.25 mM dNTP mix, 200 mM of each primer, 0.65 U of HotStarTaq Plus DNA polymerase (Qiagen), and 1.5 µl of DNA (10 to 100 ng). PCR cycle conditions were as follows: 95°C for 15 min; 36 cycles of 94°C for 30 s, 64°C for 45 s, 72°C for 1 min; and a final elongation step at 72°C for 10 min. Species-specific PCR for *Rhizoctonia crocorum* was performed in a single 50 µl reaction volume containing 1 mM MgCl₂, 1× Fermentas DreamTaq buffer, 0.25 mM dNTP mix,

200 mM of each primer, 1 U of Fermentas DreamTaq DNA polymerase, and 1 µl of DNA (10 to 100 ng). The PCR cycle conditions were as follows: 96°C for 5 min; 35 cycles of 96°C for 45 s, 50°C for 45 s, 72°C for 30 s; and a final elongation step at 72°C for 10 min. All PCR products were analyzed by gel electrophoresis (1%).

RESULTS

Design of oligonucleotide probes and functionality testing.

Throughout the study, a total of 69 probes were designed and tested in hybridization experiments for functionality and specificity. Several probes (27) were eliminated as they displayed signal intensities lower than 0.4 or strong cross reactions with nontarget species. As a result, the final microarray layout comprised 42 probes summarized in Supplementary Table S4. Although probes were designed with the aim to detect certain target species, in silico validation against the database of the NCBI for some probes revealed perfect matches to other nontarget species. Such probes were defined as group-specific indicated by an uppercase "G" and the first three letters of the genus (e.g., probe Col_G for *Colletotrichum* spp.). In contrast, species-specific probes are indicated with first letter of the genus, followed by the first three letters of the species name and an uppercase "S" for species-specific (e.g., A_coc_S for *Aphanomyces cochlioides*). In total, the final microarray layout contained 26 species and 16 group-specific probes. On average, probes had a length of 27 nt (range: 24 to 32 nt) with a GC content of 49% (range: 40 to 58%) and a melting temperature of 61°C (range: 58 to 64°C). All probes were tested for specificity with 103 pure cultures representing 73 microorganism species, mainly obtained from sugar beet. All probes included in the final microarray layout produced strong and unambiguous signals with their corresponding perfect match amplicons varying between 0.40 and 0.85 (Table 1). The workflow as well as an example of the hybridization pattern from an environmental sample is given in Figure 1.

A universal probe for bacteria (Bacteria_G) and a group-specific probe for *Leuconostoc* spp. (Leu_G) were designed using the sequence variability of the 16S rDNA. Both probes performed well when isolates of their target species were hybridized. Based on ITS1, five species-specific probes for *M. piriformis* (M_pir_S), *Rhizopus arrhizus* (R_arr_S_1), *Rhizopus stolonifer* (R_sto_S), *Rhizoctonia solani* AG 2-2 (R_sol_AG_2-2_S), and *Verticillium dahliae* (V_dah_S) produced strong hybridization signals without any cross reactions. Similarly, five group-specific probes covering *Alternaria* spp. (Alt_G_1), *Botrytis* spp. (Bot_G_1), *Colletotrichum* spp. (Col_G), *Penicillium* spp. (Pen_G), and *Phytophthora* spp. (Phy_G) strongly hybridized to their respective target amplicons. Furthermore, signal intensities were highly reproducible with little variation (coefficient of variation: 5%) when different isolates of the same species were tested (e.g., detection of *Rhizoctonia solani* isolates by Rso_G). Most of the well-performing probes were designed based on the sequence variability of the ITS2, including 12 species-specific probes for targets like *Aphanomyces cochlioides* (A_coc_S), *Rhizoctonia crocorum* (R_cro_S), *Phoma betae* (P_bet_S) and *Pythium ultimum* (P_ult_S). Apart from that, group-specific probes were designed for species of *Aspergillus* spp. (Asp_G_1, Asp_G_2), *Mucor* spp. (Muc_G_1, Muc_G_2) as well as different anastomosis groups of *Rhizoctonia solani* (Rso_G). Additionally, a second probe was included for species of *Alternaria* spp. (Alt_G_2) and *Botrytis* spp. (Bot_G_2) to increase the detection specificity. For *Fusarium* spp. detection, the sequence variability in the *tefl* gene was exploited for the design of 10 probes which strongly hybridized to the *tefl* amplicons of *F. avenaceum* (F_ave_S), *F. culmorum* (F_cul_S), *F. graminearum* (F_gra_S), *F. redolens* (F_red_S), and *F. tricinctum* (F_tri_S). In case of *F. equiseti*, two probes (F_equ_S_1, F_equ_S_2) were necessary to cover different haplotypes within this species. Furthermore, insufficient sequence variability complicated the design of a

specific probe for *F. cerealis* resulting in a group-specific probe (Fus_G) detecting both *F. cerealis* and *F. culmorum*. In contrast, multiple polymorphic sites in the sequence of *F. venenatum* enabled the design of two specific probes (F_ven_S_1, F_ven_S_2).

Testing of specificity. In general, only a few cross reactions were observed during hybridization experiments (Table 1). Most probes proved to be highly specific and able to discriminate closely related species based on a few nucleotide mismatches. For example, the probes F_ven_S_1 and F_ven_S_2 detecting *F. venenatum* were highly specific and showed no cross reactions in hybridization with PCR products of the other 19 *Fusarium* species tested. The discriminatory potential and occurrence of cross reactions was strongly affected by the number of stringent washing steps conducted with 0.2× SSC buffer after hybridization (Fig. 2). The discrimination of *F. culmorum* from *F. cerealis* is based on a single base substitution (G→A) located close to the 3' end of the probe F_cul_S (sequence: 5'- TCTTCCCACAAACCATTCCTGGGC-3' [mismatch position underlined]). As a result, the signal intensity of this probe was reduced below 0.1 when three consecutive stringent washing steps with 0.2× SSC buffer were performed after hybridization of PCR products from *F. cerealis*. In contrast, the probe Fus_G had a perfect match to *F. cerealis* and therefore hybridized strongly with a signal intensity of 0.6. Reducing the number of stringent washing steps caused a significant increase in the signal intensity of the probe 1_Fcu. When no stringent washing step was performed, the probe 1_Fcu produced a signal as strong as the probe Fus_G designed for the detection of both species.

Even under high stringent washing conditions, a few probes showed cross reactions when amplicons of closely related nontarget species were hybridized. Although two mismatches for *Sclerotinia sclerotiorum* were present in the ITS1 amplicon, the probe Bot_G_1 produced a strong cross reaction. Nevertheless, this probe was not rejected because another probe (Bot_G_2) was designed for *B. cinerea* which successfully discriminated both species based on a single mismatch. Apart from that, the probe F_red_S strongly hybridized with *F. redolens* but also showed weak cross reactions with *F. hostae* and *F. verticillioides tefl* amplicons. Additionally, weak cross reactions were observed between the following species

and probes: *F. graminearum*/F_cul_S, *Pythium aphanidermatum*/P_del_S, and *V. dahliae*/Ver_G. However, signal intensities (ranging between 0.01 to 0.15) were considerably lower compared with the perfect match target species. Moreover, species-specific probes were included in the microarray for the cross reacting nontarget species *Pythium aphanidermatum* (P_aph_S), *F. graminearum* (F_gra_S), and *V. dahliae* (V_dah_S).

Sensitivity of oligonucleotide probes. The sensitivity of the microarray was evaluated exemplarily for six probes using a serial dilution of genomic DNA (gDNA) obtained from pure cultures of different target species (Fig. 3). All probes proved to be highly sensitive by detecting at least 10 pg/μl of gDNA of the corresponding target species. The probe Bacteria_G showed highest sensitivity by detecting as few as 1 fg/μl of *Rahnella aquatilis* gDNA. In contrast, the lowest sensitivity was observed for the probe P_ult_S which produced no signal when less than 10 pg/μl of gDNA of *Pythium ultimum* was used for PCR. For probes F_red_S, M_pha_S, Phy_G, and P_ult_S, no PCR amplicons were visible in agarose gel electrophoresis when the signal intensity decreased below the detection limit. In contrast, the probes Bacteria_G and R_arr_S_1 could even detect their targets when PCR amplicons were not visible by agarose gel electrophoresis (data not shown).

Assay validation with inoculated sugar beet slices. All probes successfully tested for specificity were further evaluated by in planta detection of their corresponding target species. All probes, which proved to be functional and specific in the detection of pure cultures, were also reliably detecting their target species in inoculated sugar beet tissue (Table 1). Amplicons obtained from the different genomic loci hybridized strongly to their capture probes. The signal intensity ranged between 0.28 and 0.84, whereas most probes produced strong signals equal to the values obtained by hybridization of the respective pure culture. For example, species-specific probes like A_coc_S or R_sto_S clearly identified their target species in necrotic tissue. Furthermore, group-specific probes detecting multiple species (e.g., Bacteria_G, Phy_G, Rso_G, and Fus_G) performed well when PCR amplicons obtained from tissue colonized by the different species were hybridized. Equally, probe pairs targeting different polymorphic sites of the same species produced strong and unambiguous hybridization patterns when the target species was assayed from infested sugar beet tissue

Fig. 1. A, Schematic representation of the microarray workflow including B, a hybridization pattern from an environmental sample and C, the calculated gray values after processing by the software.

Fig. 2. Specificity of *Fusarium cerealis* (isolate O43) detection with specific (Fus_G) and unspecific (F_cul_S) probes depending on washing stringency during hybridization. To alter the washing stringency, arrays were washed three times after hybridization and the number of washing steps with 0.2× SSC buffer were stepwise replaced by 2× SSC buffer (A: 3× 0.2× SSC; B: 2× 0.2× SSC + 1× 2× SSC; C: 1× 0.2× SSC + 2× 2× SSC; and D: 3× 2× SSC). Error bars indicate standard deviation.

(Bot_G_1/Bot_G_2, Rso_G/R_sol_AG_2-2_S, R_arr_S_1/R_arr_S_2, and F_ven_S_1/F_ven_S_2).

Assay validation with naturally infected sugar beets. In addition, the microarray based assay was validated with 25 diseased sugar beets displaying varying root rot symptoms. Most sugar beets roots were completely rotten, hampering isolation of the causal agent. After visual assessment, all samples were investigated by species-specific PCR while in vitro isolation was only applied when possible. Results from all three detection methods are summarized in Table 2. A detailed overview of the microarray hybridization pattern for each sample including signal intensity is provided in Supplementary Table S5. To avoid false positive results due to the above described cross reactions, a general cut-off level was applied in all hybridization experiments. The threshold of 0.3 was selected as cross reactions were below this value.

The probes Bacteria_G and Biotin_control always produced strong signals, confirming a successful hybridization and substrate conversion during all hybridization experiments. Based on the results of the microarray approach, the causal agent of the root rot was clearly identified in all samples and detection results were in accordance with disease symptoms observed. Additionally, the infection with root rot pathogens was confirmed in 23 out of 25 samples by at least one of the alternative approaches. *Rhizoctonia solani* AG-2-2 was most frequently identified (eight samples) by the two probes Rso_G and R_sol_AG_2-2_S as well as by PCR. A determination of the AG subgroup was not possible as both methods are not able to discriminate between subgroups of AG-2-2. Equally, root rots caused by *Rhizoctonia crocorum* were identified in seven samples by the probe R_cro_S which was also confirmed by PCR. *Aphanomyces cochlioides* was detected with similar frequency; however, neither PCR nor in vitro isolation was able to confirm the infection in two samples (2012-005, 2012-008). In contrast, in vitro isolation revealed the presence of *M. circinelloides* (2012-005) and *Rhizopus arrhizus* (2012-008). Both pathogens were successfully detected in the samples by the corresponding probes on the microarray (R_arr_S_1/R_arr_S_2; Muc_G_1). Less frequent species identified by microarray and at least by a second method were *M. phaseolina* (2009-045), *Pythium ultimum* (2012-031), *Rhizopus arrhizus* (2013-028), and *Rhizoctonia solani* AG-4 HGII (2012-012).

Besides an infection with the causal agent of the root rot, microarray based identification revealed the presence of multiple

other microorganism species which were neither detected by in vitro isolation nor by PCR. An intensive colonization was observed for the samples 2012-005, 2012-06, 2012-08, and 2012-18 by detecting up to 11 different microorganism species (Table 2). For instance, at least one *Fusarium* species was detected in 19 out of 25 beets. Most frequent species were *F. culmorum* (13 samples) and *F. redolens* (13 samples) followed by *F. graminearum*, *F. tricinctum*, *F. equiseti*, and *F. avenaceum*. As the group-specific probe Fus_G always produced a positive signal when *F. culmorum* was also detected by the probe F_cul_S, colonization with *F. cerealis* could not be excluded. In contrast, the presence of *F. cerealis* was identified in three samples (2012-006, 2012-031, 2012-32) with a positive signal from probe Fus_G and no signal from probe F_cul_S.

DISCUSSION

Root rot diseases on sugar beet are of great economic importance during the growing season and storage, but attempts to develop molecular tools for their detection have hardly been made. This constitutes a great challenge as a large number of species must be considered as causal agents. Therefore, a multiplex assay based on the AT system in combination with consensus PCRs was developed in this study to improve diagnosis of sugar beet root rot disease. Based on hybridization of biotin labeled amplicons to short oligonucleotide probes, the microarray allows the identification of prevalent field pathogens (e.g., *Aphanomyces cochlioides*), storage pathogens (e.g., *Botrytis cinerea*), and ubiquitous spoilage fungi (e.g., *Penicillium expansum*).

In total, 69 probes were designed throughout the study; however some needed to be excluded from the final microarray layout due to low signal intensity or strong cross reactions. Thus, the final AT design contained 42 functional and sensitive probes. To some extent, the signal intensity was improved by extension of probe length, resulting in a higher duplex stability between probe and amplicon (Relógio et al. 2002). In general, DNA-DNA hybridization is a complex process that is difficult to predict (Mueckstein et al. 2010; Pozhitkov et al. 2007). Possible secondary structures of the amplicon represent a critical factor by blocking the probe binding site and therefore should be reduced by minimizing the amplicon length (Ratushna et al. 2005). In this study, the amplicon length was reduced to mainly polymorphic regions, resulting in

Fig. 3. Detection limit of six different microarray probes targeting *Rhizoctonia solani* (Bacteria_G), *Fusarium redolens* (F_red_S), *Macrophomina phaseolina* (M pha_S), *Pythium ultimum* (P ult_S), *Phytophthora cryptogea* (Phy_G), and *Rhizopus oryzae* (R arr_S_1). A tenfold serial dilution (1 ng/μl to 0.1 fg/μl) from genomic DNA of each species was hybridized. The following isolates were used: *Rhizoctonia solani* (DSM4594), *F. redolens* (DSM23351), *M. phaseolina* (DSM28933), *Pythium ultimum* (DSM28975), *Phytophthora cryptogea* (DSM28934), and *Rhizopus oryzae* (CBS126971). Error bars indicate standard deviation.

lengths between 250 and 500 bp. Hybridization signals could be further improved by excluding the competition with the complementary strand via asymmetric PCR. However, Tomlinson et al. (2014) could show that this approach can be less discriminatory when SNP detection is required.

The genomic loci selected for probe design provided sufficient sequence variability to finally generate 26 species-specific probes. Whenever possible, indels were chosen as target sites as this reduces the probability to obtain cross reactions (Chen et al. 2009). However, lack of variability in the *Pectobacterium betavascularum* sequence prevented the design of a species-specific probe. Similar problems have been reported for fungal species like *Alternaria alternata* and *Penicillium expansum* which display poor interspecies variability within the ITS regions (Sholberg et al. 2005; Zhang et al. 2008). Therefore, only group-specific probes were obtained

for those species. Although the ITS region has been proposed as the primary fungal barcode marker, protein coding genes often display a higher sequence variability for species discrimination (Schoch et al. 2012). The integration of coding target genes in array based detection can increase the discrimination power as shown for *Penicillium* spp. using the cytochrome *c oxidase subunit 1* (Chen et al. 2009). However, the low sequence conservation above genus level can complicate the development of universal primers for higher taxonomic ranks.

Taxonomic relationships within the genus *Fusarium* are under constant revision (Geiser et al. 2013 and references therein) making establishment of detection assays rather difficult, in particular for species belonging to a species complex. Any future changes in taxonomy will certainly affect the species coverage of the probes. Apart from that, large intraspecies variation in the *tef1* sequence

TABLE 2. Microorganism species detected by in vitro isolation, species-specific polymerase chain reaction (PCR), and microarray in naturally diseased sugar beets displaying root rot symptoms

No.	Sample ID	Origin	In vitro isolation ^a	PCR ^b	Microarray ^c
1	2009-045	Moldavia	<i>Macrophomina phaseolina</i>	n.d.	<i>M. phaseolina</i>
2	2011-015	Germany	n.a.	<i>Rhizoctonia solani</i> AG 2-2	<i>Fusarium culmorum</i> , <i>F. equiseti</i> , <i>F. redolens</i> , <i>Rhizoctonia solani</i> AG 2-2
3	2011-016	Germany	n.a.	<i>Rhizoctonia solani</i> AG 2-2	<i>F. redolens</i> , <i>F. tricinatum</i> , <i>Leuconostoc</i> sp., <i>Rhizoctonia solani</i> AG 2-2
4	2011-017	Germany	n.a.	<i>Rhizoctonia solani</i> AG 2-2	<i>Leuconostoc</i> sp., <i>Rhizoctonia solani</i> AG 2-2
5	2011-018	Germany	n.a.	<i>Rhizoctonia solani</i> AG 2-2	<i>F. avenaceum</i> , <i>F. redolens</i> , <i>Leuconostoc</i> sp., <i>Rhizoctonia solani</i> AG 2-2
6	2011-041	Germany	n.a.	<i>Rhizoctonia crocorum</i>	<i>Rhizoctonia crocorum</i> , <i>Penicillium</i> sp.
7	2012-001	Germany	n.a.	<i>Aphanomyces cochlioides</i>	<i>Aphanomyces cochlioides</i> , <i>F. culmorum</i> , <i>F. tricinatum</i>
8	2012-003	Germany	<i>Aphanomyces cochlioides</i> , <i>F. oxysporum</i>	<i>Aphanomyces cochlioides</i>	<i>Aphanomyces cochlioides</i>
9	2012-005	Germany	<i>Mucor circinelloides</i>	n.d.	<i>Aphanomyces cochlioides</i> , <i>Alternaria</i> sp., <i>Botrytis</i> sp., <i>F. culmorum</i> , <i>F. tricinatum</i> , <i>M. hiemalis</i> , <i>Mucor</i> sp., <i>Phytophthora</i> sp.
10	2012-006	Germany	n.a.	<i>Aphanomyces cochlioides</i> , <i>Rhizoctonia solani</i> AG 2-2	<i>Aphanomyces cochlioides</i> , <i>Colletotrichum</i> sp., <i>F. avenaceum</i> , <i>F. cerealis</i> , <i>F. redolens</i> , <i>Leuconostoc</i> sp., <i>Rhizoctonia solani</i> AG 2-2
11	2012-008	Germany	<i>Rhizopus arrhizus</i>	<i>F. oxysporum</i>	<i>Aphanomyces cochlioides</i> , <i>Alternaria</i> sp., <i>F. graminearum</i> , <i>F. equiseti</i> , <i>F. redolens</i> , <i>F. tricinatum</i> , <i>Phytophthora</i> sp., <i>Rhizopus</i> <i>arrhizus</i>
12	2012-012	Germany	<i>Rhizoctonia solani</i> AG-4 HG II	n.d.	<i>Rhizoctonia solani</i>
13	2012-014	Germany	n.a.	<i>Rhizoctonia crocorum</i>	<i>F. culmorum</i> , <i>F. redolens</i> , <i>Rhizoctonia</i> <i>crocorum</i>
14	2012-015	Germany	n.a.	<i>Rhizoctonia solani</i> AG 2-2	<i>F. culmorum</i> , <i>F. redolens</i> , <i>F. tricinatum</i> , <i>Rhizoctonia solani</i> AG 2-2
15	2012-017	Germany	n.a.	<i>Rhizoctonia solani</i> AG 2-2	<i>Colletotrichum</i> sp., <i>Pythium aphanidermatum</i> , <i>Rhizoctonia solani</i> AG 2-2
16	2012-018	Germany	n.a.	<i>Rhizoctonia crocorum</i>	<i>Alternaria</i> sp., <i>Colletotrichum</i> sp., <i>F. culmorum</i> , <i>F. equiseti</i> , <i>F. redolens</i> , <i>F. tricinatum</i> , <i>Rhizoctonia crocorum</i> , <i>M. hiemalis</i> , <i>Penicillium</i> sp., <i>Rhizopus arrhizus</i>
17	2012-019a	Germany	n.a.	<i>Rhizoctonia crocorum</i>	<i>F. culmorum</i> , <i>F. redolens</i> , <i>Rhizoctonia</i> <i>crocorum</i>
18	2012-019b	Germany	n.a.	<i>Rhizoctonia solani</i> AG 2-2	<i>F. culmorum</i> , <i>F. redolens</i> , <i>F. tricinatum</i> , <i>Rhizoctonia solani</i> AG 2-2
19	2012-028	Germany	n.a.	<i>Rhizoctonia crocorum</i>	<i>F. culmorum</i> , <i>F. graminearum</i> , <i>F. redolens</i> , <i>Rhizoctonia crocorum</i>
20	2012-029	Germany	n.a.	<i>Rhizoctonia crocorum</i>	<i>F. culmorum</i> , <i>F. graminearum</i> , <i>F. redolens</i> , <i>F. tricinatum</i> , <i>Rhizoctonia crocorum</i>
21	2012-031	Slovakia	n.a.	<i>Pythium ultimum</i>	<i>F. cerealis</i> , <i>F. graminearum</i> , <i>Pythium ultimum</i>
22	2012-032	Germany	n.a.	<i>Rhizoctonia crocorum</i>	<i>F. cerealis</i> , <i>F. graminearum</i> , <i>Rhizoctonia</i> <i>crocorum</i> , <i>Penicillium</i> sp.
23	2013-003	Moldavia	<i>Aphanomyces cochlioides</i> , <i>F. oxysporum</i>	<i>Aphanomyces cochlioides</i>	<i>Aphanomyces cochlioides</i> , <i>Alternaria</i> sp., <i>F. culmorum</i> , <i>F. graminearum</i> , <i>F. tricinatum</i>
24	2013-016	Germany	<i>F. redolens</i> , <i>F. oxysporum</i>	<i>Aphanomyces cochlioides</i>	<i>Aphanomyces cochlioides</i> , <i>Alternaria</i> sp., <i>F. culmorum</i> , <i>F. graminearum</i> , <i>F. redolens</i>
25	2013-028	Germany	<i>Rhizopus arrhizus</i>	n.d.	<i>Alternaria</i> sp., <i>F. culmorum</i> , <i>F. graminearum</i> , <i>F. redolens</i> , <i>Rhizopus arrhizus</i>

^a n.a. = not applied.

^b n.d. = not detected.

^c Results from group-specific probes are indicated only by the genus name (e.g., *Leuconostoc* sp.). Only probes displaying signal intensities above 0.3 were considered. Based on symptom expression, the supposed causal agent of the root rot detected by the microarray is highlighted in bold.

(Kristensen et al. 2005) further complicates probe design (Kristensen et al. 2006). In this study, the sequence of the probe F_gra_S is shared only by 9 of the 14 previously identified phylogenetic lineages in the *F. graminearum* species complex (Yli-Mattila et al. 2009). Furthermore, both probes targeting *F. equiseti* (F_equ_S_1, F_equ_S_2) cover only four out of five haplotypes reported by Kristensen et al. (2005). In contrast, several polymorphisms in the sequence of *F. venenatum* enabled the design of two well performing probes (F_ven_S_1, F_ven_S_2). To further improve *Fusarium* spp. detection, other marker genes (e.g., *beta-tubulin*) should be exploited for polymorphic sites that can be used as probe target sites.

Besides the 26 species-specific probes, the final microarray contained 16 group-specific probes targeting additional species that were not included in this study. A positive signal of such a probe gives no species information but detects the presence of at least one member from the genus. For example, the probes Bot_G_1 and Bot_G_2 detect *B. cinerea* as well as other *Botrytis* species. However, the host range of these other species is quite narrow and sugar beet has not been described as a host plant; only *B. cinerea* is a polyphagous species causing rots on eudicotyledons (Staats et al. 2005). Therefore, a positive signal from these probes is almost certainly caused by *B. cinerea* and not by any other species of this genus. Another example is the probe Pen_G which is predicted to detect *Penicillium italicum* even though only *Penicillium expansum* isolates were tested. However, this probe was retained as both species are known to represent destructive food spoilage pathogens (Pitt and Hocking 2009 and references therein). In future work, isolates of *Penicillium italicum* should be tested because a perfect match in silico does not permit performance prediction under hybridization conditions.

Despite low target sequence variability, a single mismatch in principle is sufficient for discrimination of two amplicons as it was demonstrated for the probe Bot_G_2, differentiating *B. cinerea* from *S. sclerotiorum*. However, the success depends on many parameters like the kind of mismatch, mismatch position and probe length (Lievens et al. 2006; Seringhaus et al. 2008; Tomlinson et al. 2014). According to the aforementioned studies, best differentiation can be expected when amplicons are short with staggered positioned mismatches, resulting in purine-purine mispairs. To some extent, these parameters can be adjusted but limitations are often set by sequence variability. Therefore, discrimination power was increased by stringent washing steps during hybridization. For example, increasing the number of stringent washing steps significantly reduced the signal intensity of the mismatch-duplex between probe F_cul_S and the nontarget species *F. cerealis*. This is likely to be explained by the compensating effect of cations in the hybridization buffer on the negative charge of the oligonucleotide backbone, resulting in a more stabilized duplex. Nevertheless, a few probes displayed cross reactions which could not be eliminated. Despite two mismatches, the probe Bot_spp_1 strongly cross reacted with *S. sclerotiorum*, however, a second probe for *B. cinerea* (Bot_G_2) permitted reliable detection of *B. cinerea* when both probes generated positive signals. The probe F_red_S cross reacted with the species *F. hostae* and *F. verticillioides*; however, until now, *F. hostae* has only been isolated from *Hosta* plants (Baayen et al. 2001). In contrast, *F. verticillioides* is known to colonize sugar beets too (Bosch and Mirocha 1992) but the signal intensity for the cross reaction was very weak (0.09). Nevertheless, hybridization signals from cross reacting probes should generally only be considered positive when the signal intensity is higher than the intensity observed for the cross reacting nontarget species. Moreover, the detection specificity for these species should be further improved through the combination of different marker genes as demonstrated by Chen et al. (2013) for *Phytophthora* spp.

In accordance with previous studies (Zhang et al. 2007, 2008), DNA-DNA hybridization was proven to be highly sensitive by detecting at least 10 pg/μl of genomic DNA of their corresponding

target species. Individual detection thresholds of the tested probes displayed large variation (ranging between 10 pg/μl and 0.1 fg/μl of DNA). The amount of gDNA necessary to obtain a hybridization signal is heavily affected by the copy number of PCR products produced during amplification, and therefore limited by the PCR efficiency. Under optimized experimental conditions, a single PCR-amplifiable copy of target DNA is sufficient to obtain an unambiguous hybridization pattern with the AT (Ehrlich et al. 2006). However, the detection threshold of the developed assay was probably limited by the sensitivity of the different PCR reactions as stringent annealing temperatures were applied to avoid amplification of sugar beet DNA.

The microarray based identification of root rot pathogens in diseased field beets was in agreement with the results from in vitro isolation and PCR detection except for two samples in which the presence of *Aphanomyces cochlioides* could not be confirmed by the latter two techniques. In contrast, all species detected by in vitro isolation were also identified by the developed microarray. In addition to the supposed primary pathogen in each sample, a diverse microorganism population indicative for substantial colonization by secondary invaders was detected only by microarray. In case of *Fusarium* spp., the seven different species identified in this study are well known as colonizers of sugar beets (Bosch and Mirocha 1992; Christ et al. 2011). Nevertheless, results of such multiplex detection assays must be carefully analyzed as the detection of a certain species gives no evidence for its pathogenicity on sugar beet (Christ et al. 2011; Strausbaugh and Gillen 2008).

Severe root rot symptoms on sugar beet can be quite unspecific, making identification of the causal agent a challenge. Compared with other diagnostic methods available for sugar beet (Kageyama et al. 1997; Weiland and Sundsbak 2000), the developed microarray allows rapid testing of multiple, replicated samples for the presence of many different microorganism species. Apart from that, the species spectrum detected by the microarray comprises also important postharvest pathogens. Until now, the number of studies focusing on species causing storage rots is restricted to a few ones in which in vitro isolation was solely applied (Bosch and Mirocha 1992; Bugbee and Cole 1976; Christ et al. 2011). Moreover, relatively little is known about the epidemiology of storage pathogens including effects of genotype and environment on the species spectrum. A clear knowledge about species involved in storage rot development is essential for the establishment of management strategies (e.g., resistance breeding). These questions can be now addressed in comprehensive studies as the present microarray enables rapid screening of multiple replicated samples in a few hours.

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