Fungal endophytes in potato roots studied by traditional isolation and cultivation-independent DNA-based methods

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Abstract

The composition and relative abundance of endophytic fungi in roots of field-grown transgenic T4-lysozyme producing potatoes and the parental line were assessed by classical isolation from root segments and cultivation-independent techniques to test the hypothesis that endophytic fungi are affected by T4-lysozyme. Fungi were isolated from the majority of root segments of both lines and at least 63 morphological groups were obtained with *Verticillium dahliae*, *Cylindrocarpon destructans*, *Colletotrichum coccodes* and *Plectosporium tabacinum* as the most frequently isolated species. Dominant bands in the fungal fingerprints obtained by denaturing gradient gel electrophoresis analysis of 18S rRNA gene fragments amplified from total community DNA corresponded to the electrophoretic mobility of the 18S rRNA gene fragments of the three most abundant fungal isolates, *V. dahliae*, *C. destructans* and *Col. coccodes*, but not to *P. tabacinum*. The assignment of the bands to these isolates was confirmed for *V. dahliae* and *Col. coccodes* by sequencing of clones. *Verticillium dahliae* was the most abundant endophytic fungus in the roots of healthy potato plants. Differences in the relative abundance of endophytic fungi colonizing the roots of T4-lysozyme producing potatoes and the parental line could be detected by both methods.

Introduction

Endophytic fungi have been isolated from nearly all plants studied (Petrini, 1991; Schulz *et al.*, 1993; Stone *et al.*, 2000; Vandenkoornhuyse *et al.*, 2003). Fungal endophytes can colonize plants in a local or systemic manner and their growth can be inter- or intracellular (Boyle *et al.*, 2001; Schulz & Boyle, 2005). In general, they colonize the plants without causing visible disease symptoms (Petrini, 1991; Stone *et al.*, 2000). Their function for the host plant is not yet clear and is supposed to depend on the organ–fungus interaction of each plant (Boyle *et al.*, 2001; Faeth, 2002). Schulz & Boyle (2005) hypothesize that there are no neutral interactions but a balanced antagonism between the plant and the endophytic fungi. In several investigations positive effects of fungal endophytes such as enhanced growth (Newsham, 1999) or stress tolerance of the host plants (Bacon & Hill, 1996; Römmert *et al.*, 2002) were found. White *et al.* (2000) reported on a protection against invading epiphytic organisms, and Araújo *et al.* (2001) found inhibitory as well as stimulatory effects of endophytic fungi on endophytic bacteria when tested *in vitro*. Moreover, endophytic fungi have been considered to mediate induced resistance (Bultmann & Murphy, 2000), and some can be latent pathogens (Schulz & Boyle, 2005). In the context of this paper we define endophytic fungi as fungi which were isolated from the tissue of surface-sterilized symptomless root material of potato plants or which were detected in the total community DNA extracted from these roots.

Traditionally, cultivation-dependent methods have been used for the analysis of the community structure of endophytic fungi (Arnold *et al.*, 2001; Bussaban *et al.*, 2001; Photita *et al.*, 2001; Wilberforce *et al.*, 2003). These methods are rather laborious and time intensive and are not suitable to compare large numbers of samples. Moreover, fungi in a latent/quiescent stage or with special growth requirements are often not retrieved.

DNA-based methods allow researchers also to study endophytic fungi which are difficult to grow. Nowadays, molecular fingerprinting methods are well-established and have been successfully applied to assess the fungal communities of habitats such as bulk and rhizosphere soil (Kowalchuk, 1999; van Elsas *et al.*, 2000; Gomes *et al.*, 2003; Anderson & Cairney, 2004), compost (Koschinski *et al.*, 2000), wood (Pennanen *et al.*, 2001) or different water bodies (Lord *et al.*, 2002). Despite the successful use of cultivation-independent fingerprinting techniques for the assessment of microbial communities of several complex habitats, comparably few studies have been performed using them to assess the community structure of endophytic bacteria (Araújo *et al.*, 2002; Krechel *et al.*, 2002; Sessitsch *et al.*, 2004; Berg *et al.*, 2005). To our knowledge no cultivation-independent studies on endophytic fungal communities in roots of crop plants have been published to date.

In the course of a 2-year monitoring field study, the bacterial and fungal rhizosphere communities of nontransgenic and transgenic T4-lysozyme producing potato lines were evaluated using 16S and 18S rRNA gene fragment denaturing gradient gel electrophoresis (DGGE) fingerprints. No significant differences could be detected between the transgenic T4-lysozyme expressing lines and the parental lines (M. Götz *et al.*, unpublished).

As the T4-lysozyme is secreted into the apoplastic space (Düring *et al.*, 1993) and has antifungal activity (Düring *et al.*, 1999), we hypothesized that its activity could affect endophytic fungi. The aim of this study was to explore the potential of traditional isolation techniques and DNA-based, cultivation-independent techniques (18S rRNA gene fragments amplified from total DNA analyzed by DGGE and by cloning and sequencing) to study the endophytic fungi in potato roots and to test the hypothesis that endophytic fungi are affected by T4-lysozyme.

Materials and methods

Plants and field design

In 1999 and 2000, three transgenic T4-lysozyme producing plant lines, a transgenic control and the parental line Désirée (*Solanum tuberosum* cv. Désirée) were grown in field trials in Groß Lüsewitz, Germany (Federal Centre for Breeding Research, BAZ). The tubers were planted in a complete randomized block design with six replicates per clone or line. Bacteriophage T4-lysozyme was shown by Düring *et al.* (1993, 1999) to be constitutively expressed and secreted into the intercellular spaces. More details about the transgenic potato lines are given by Düring *et al.* (1993, 1999). All plant lines were provided by Klaus Düring (then MPB Cologne, Cologne, Germany).

Sampling time and sampling

For the analysis of endophytic fungi, root samples of the parental potato line Désirée and the transgenic line DL11 for which the highest amounts of T4-lysozyme were detected by Western blot analysis (A. Mahn, pers. commun.) were taken at growth stage 91–97 (senescent plants; Hack *et al.*, 1993) in 2000. Three to five plants were carefully removed from each plot (six plots per treatment) and their complete roots with tightly adhering soil particles were combined and stored at 4 °C. Surface sterilization and further preparations were performed the following day.

Surface sterilization and isolation of endophytic fungi

Five grams of fresh root material were washed carefully under running tap water. The roots were macroscopically inspected. Areas with lesions and discolorations were discarded so that only healthy root material was used. The sterilization procedure contained a 1 min step in ethanol (70%), 4.5 min sodium hypochlorite (~5% active chlorine) and three washing steps for at least 5 min with sterile water.

After surface sterilization the roots were dried on sterile filter paper and imprinted on biomalt agar 50 g L^{-1} Biomalt (Villa Natura Gesundprodukte GmbH, Kirn, Germany) +20 g L^{-1} Bacto^{TM} Agar (Becton Dickinson and Company, Sparks, France), pH 5.6 with the antibiotics Penicillin G Na 60 mg L^{-1}, Streptomycin sulphate 80 mg L^{-1}, and (Oxy)-tetracycline* HCl 50 mg L^{-1} as a sterility check. These cultures served as control plates to check the surface sterilization procedure. The roots were cut in small pieces and mixed. For the isolation of endophytic fungi, 100 root segments (~2 mm) per plot were incubated on biomalt agar. To inhibit bacterial growth the biomalt agar was supplemented with the above mentioned antibiotics. A total of 600 root segments for each plant line were incubated at 14 °C in the dark. Control plates were incubated at 20 °C in the dark to accelerate the growth of fungi that survived surface sterilization. The remaining root material was kept frozen at −70 °C until total community DNA was extracted.

All cultures were monitored for up to 6 weeks for outgrowth of mycelium, which was then immediately inoculated on fresh biomalt medium. Root segments from which fungi were isolated were discarded so that only one fungus per root segment was obtained. Only one of six control plates from surface-sterilized Désirée roots showed growth of two to five colonies of *V. dahliae*, whereas on all six control plates of DL11 one to three colonies of *V. dahliae* were detected. We suppose that this growth on control plates resulted from small root pieces which remained in the agar after the root material was removed from the agar surface following the imprint and thus we still considered the roots as successfully surface-sterilized.

Characterization of fungal isolates

Classical identification of isolates of the most abundant groups was done based on their macroscopic and microscopic appearance at least to the genus level (Raper & Thom, 1949; Ellis, 1971; Pitt, 1979; Domsch *et al.*, 1980). In addition, genomic DNA was extracted from representative isolates of each morphological group using the Ultra Clean™ Soil DNA Kit (MoBio Laboratories, Solana Beach, CA). The isolates were characterized according to the electrophoretic mobility of the 18S rRNA gene fragment in the gradient gel as well as by means of BOX and ARDRA patterns (see below).

BOX-PCR genomic fingerprints

BOX-PCR was done as described by Rademaker & De Bruijn (1997) using the BOXA1R primer 5′- CTA CGG CAA GGC GAC GCT GAC G -3′. The reaction mixture (25 μL) contained 1 μL template DNA (*c.* 20 ng), Stoffel buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.2 mM dNTP (Roche Diagnostic GmbH, Mannheim, Germany), 3.75 mM $MgCl_2$, 5% DMSO, 0.2 μM of BOXA1R primer and 0.1 U Taq DNA polymerase (Stoffel fragment, Perkin-Elmer Cetus, Norwalk, CT). PCR was performed in an Eppendorf Mastercycler gradient. An initial 7 min denaturation step at 94 °C was followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 65 °C for 8 min subsequently followed by a 16 min extension step at 65 °C. An aliquot of 10 μL of the PCR product was separated by gel electrophoresis on 1.2% agarose gels (Seakem LE agarose, BMA, Rockland, ME) in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) for 4 h (70 V), and after staining with ethidium bromide, the DNA was detected under UV light.

Amplified 18S rRNA gene restriction analysis (ARDRA)

PCR-amplified 18S rRNA gene fragments of fungal isolates and clones were digested with *Hinf*I. A 10 μL aliquot of each PCR product containing *c.* 3 μg DNA was digested in a total volume of 30 μL with 1 μL (10 U) of the restriction enzyme *Hinf*I (New England Biolabs, Beverly, MA) and 3 μL reaction buffer for 2.5 h at 37 °C. The digested fragments were precipitated by adding 3 μL sodium acetate (3 M, pH 5.2) and 75 μL ethanol (absolute) and kept at − 70 °C for at least 1 h. The fragments were separated by gel electrophoresis on 2.5% agarose gels (Seakem LE agarose, BMA) in TBE buffer (see above) for 3 h (100 V), and DNA was detected under UV light after ethidium bromide staining.

DNA extraction

The extraction of DNA of endophytic fungi was performed after grinding the surface-sterilized roots in liquid nitrogen with the Ultra Clean™ Soil DNA Kit (MoBio Laboratories). Surface-sterilized root material (0.25 g) was processed according to the manufacturer's protocol with an additional 2 min bead beating step (Cell Homogeniser MSK, B. Braun Diessel Biotech, Melsungen, Germany) to achieve a harsh lysis. To ensure that no DNA from the root surface was amplified, surface-sterilized root pieces were extracted without prior grinding.

PCR amplification of 18S rRNA gene fragments

PCR amplification of the 18S rRNA gene fragment (1650 bp) was carried out with the primers NS1: 5′- GTA GTC ATA TGC TTG TCT C -3′ and the GC-clamped FR1: 5′- AI CCA TTC AAT CGG TAI T -3′ (I = inosine) described by Vainio & Hantula (2000). The reaction mixture (25 μL) contained 1 μL template DNA (*c.* 20 ng), Stoffel buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.2 mM dNTP (Roche Diagnostic GmbH, Mannheim, Germany), 3.75 mM $MgCl_2$, 2% DMSO, 0.2 μM of each primer (NS1 and FR1-GC) and 2 U taq DNA polymerase (Stoffel fragment, Perkin-Elmer Cetus). The PCR was performed with an initial 8 min denaturation step at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 45 s, and extension at 72 °C for 3 min, subsequently followed by a 10 min extension step at 72 °C.

DGGE analysis

The 18S rRNA gene fragments were analyzed as described by Vainio & Hantula (2000) using a denaturing gradient of 18–43%. DGGE was performed with a DCode™ System (Biorad, Munich, Germany) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 58 °C and a constant voltage of 180 V for 18 h. The DGGE gels were silver stained as described by Heuer *et al.* (2001).

Cloning and sequencing

PCR products were amplified from the DNA extracted from the surface-sterilized roots with primers NS1 and FR1 (without GC-clamp). Aliquots of 50 μL of each plot of Désirée and DL11 were combined and purified using UltraClean™ DNA Purification Kit (MoBio Laboratories). Ligation into the pGEM-T vector and transformation into competent cells (*Escherichia coli*, JM 109; Promega, Madison, WI) was done according to the instructions of the manufacturer (pGEM-T® Vector System II, Promega). White colonies were picked and lysed using a heating and a freezing step. After PCR amplification using the primer set NS1 and FR1-GC the size of the PCR fragments was checked on an agarose gel (1% agarose, TBE buffer for 1.5 h at 100 V) to confirm the presence of the insert in the transformants. PCR fragments of 1.65 kb size were run on a gradient gel to

determine the electrophoretic mobilities of the inserts. Inserts of clones with the same electrophoretic mobility were analyzed by ARDRA. At least one clone representing each ARDRA pattern was sent for sequencing (IIT Biotech Bioservice GmbH, Bielefeld, Germany; AMODIA Bioservice GmbH, Braunschweig, Germany). The 18S sequences were analyzed using the ARB software package (Linux beta vers. 011107) and the ARB database ssujun02 (Ludwig *et al.*, 2004), including additional partial fungal 18S rRNA gene sequences from EMBL. The clone sequences were automatically aligned using the arb sequences editor with the fast-aligner. The resulting alignment was manually corrected if needed. For each clone sequence the similarity to the best database hits was calculated using a distance matrix with the particular sequence itself as a filtre. The initial tree was calculated using neighbour joining and the full sequences from the database. The sequences of the clones were submitted to GenBank (Accession numbers AM161470–AM161498).

Computer-assisted cluster analysis

Evaluation of the fungal community profiles, BOX-PCR generated fingerprints and ARDRA patterns was performed with the software package GELCOMPAR 4.0 (Applied Math, Kortrijk, Belgium). Background was subtracted using a rolling disk method with an intensity of 10 (relative units), and the lanes were normalized. A dendrogram was constructed by Pearson correlation index for each pair of lanes within a gel and cluster analysis by the un-weighted pair group method using arithmetic averages (UPGMA).

Results

Isolation of endophytic fungi

Isolation of fungi from surface-sterilized root segments indicated that the potato roots were highly colonized: 528 fungi were isolated from 600 cultivated root segments from Désirée and 594 isolates were obtained from 600 segments from DL11. The proportion of root segments from which outgrowth of fungi was observed was higher for the roots of the transgenic T4-lysozyme expressing potato than for Désirée. All isolates were grouped in at least 63 morphological groups (Désirée: 40, DL11: 48) based on their macroscopic and microscopic characteristics (Table 1).

For the four most abundant fungi the number of isolates per single plot was calculated (Figs 1a–d). Except for *V. dahliae* in the plots of Désirée and *P. tabacinum* in the plots of DL11, the distribution of *V. dahliae*, *C. destructans*, *Col. coccodes* and *P. tabacinum* in the six plots was relatively homogeneous. No significant differences were found comparing the number of isolates of *Col. coccodes* and *P. tabacinum* per plant line. In contrast to this, the colonization of the roots of Désirée with *V. dahliae* was higher and with *C. destructans* significantly lower than that of DL11 (Student's *t*-test, $P < 0.001$).

Table 1. Fourteen most abundant fungal isolates obtained from surface-sterilized roots of Désirée and of the transgenic T4-lysozyme producing potato line DL11 (growth stage 91–97); 600 root segments for Désirée and DL11 each

Genus/species	Total isolates	Désirée	DL11
Verticillium dahliae	175	129	46
Cylindrocarpon destructans	163	50	113
Colletotrichum coccodes	100	51	49
Plectosporium tabacinum	92	46	46
Alternaria ssp.*	73	34	39
'Oomycete'	48	25	23
Fusarium sp.	47	16	31
Acremonium sp.	28	10	18
Mycelium sterile	24	11	13
White yeast	22	12	10
Fusarium sp.	20	0	20
Microdochium sp.	13	1	12
Trichosporon sp.	13	13	0
Ulocladium sp.	11	0	11

*Containing *Alternaria infectoria*, *Alternaria tenuissima* and no longer sporulating *Alternaria* species.

Molecular characterization of fungal isolates

A selection of isolates of the three most abundant endophytic fungi was characterized by DGGE of their 18S rRNA gene fragment and by BOX-PCR. The molecular characterization of 20 isolates of *V. dahliae* obtained from root segments of Désirée and DL11 by means of PCR-DGGE showed the same electrophoretic mobility for all *V. dahliae* isolates tested. BOX-PCR genomic fingerprints also showed only small differences (Fig. 2a).

For *C. destructans* and *Col. coccodes*, eight isolates obtained from root segments of Désirée and DL11 were evaluated regarding their DGGE mobility and BOX-PCR fingerprints. Whereas the DGGE mobility was the same for all isolates of the same species, seven different BOX-PCR patterns were observed for *C. destructans* (Fig. 2b) and four BOX-PCR patterns for *Col. coccodes* (Fig. 2c).

18S rRNA gene fragment DGGE fingerprints of endophytic fungal communities

The molecular fingerprints of the endophytic fungal communities of the roots of Désirée and DL11 are shown in Fig. 3. The DGGE patterns indicated a low degree of variability between replicates of the same line. Five to six dominant bands and several faint bands could be detected in all replicates of endophytic fungal DGGE patterns. However,

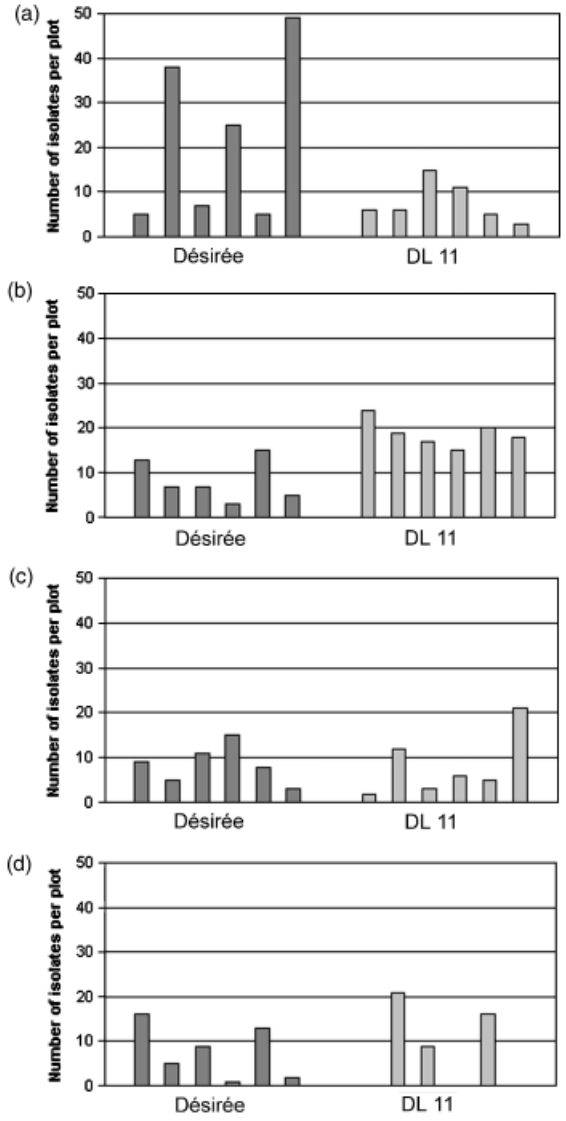

Fig. 1. Distribution of *Verticillium dahliae* (a), *Cylindrocarpon destructans* (b), *Colletotrichum coccodes* (c) and *Plectosporium tabacinum* (d) isolated from root segments obtained from surface-sterilized roots of the transgenic T4-lysozyme producing line DL11 (100 per plot; total: 600) and the parental line Désirée (100 per plot; total: 600) in the six different plots.

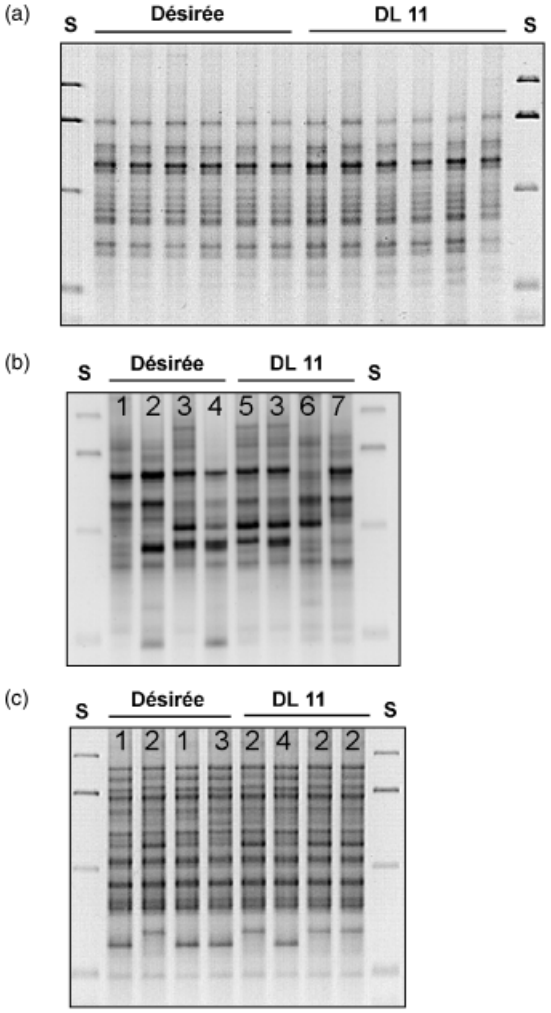

Fig. 2. BOX-PCR genomic fingerprints of *Verticillium dahliae* (a), *Cylindrocarpon destructans* (b) and *Colletotrichum coccodes* (c) isolated root segments of surface-sterilized roots of the transgenic T4-lysozyme producing line DL11 and the parental line Désirée (growth stage 91–97). Isolates with the same lane number show the same BOX-patterns. S, 1 kb ladder.

slight differences between the DGGE patterns were observed not only for the same line from different plots but also when DNA was extracted twice from root material of the same plot (Fig. 3).

The strongest band which occurred in all samples showed the same electrophoretic mobility as *V. dahliae* isolates (Fig. 3, band 'c'). Another dominant band which was found in most replicates showed the same mobility as *Col. coccodes* (Fig. 3, band 'b'). The main band possessing the same mobility as *C. destructans* occurred more often in samples of DL11 (Fig. 3, band 'a') than of Désirée. A double band (Fig. 3, bands 'f' and 'g') was also more pronounced in the samples of DL11. A band in the lower part of the gel was much stronger in the patterns of the roots of Désirée than of DL11 (Fig. 3, band 'h'). Several bands were observed with electrophoretic mobilities below the band of *Penicillium kapuscinskii* (Fig. 3, band 'f') in the DGGE standard but fungal isolates with corresponding mobilities to these bands were never obtained.

Cloning and sequence analysis

After cloning 18S rRNA gene fragments PCR-amplified from DNA extracted from surface-sterilized roots of Désirée and DL11 (growth stage 91–97), 96 white colonies from Désirée

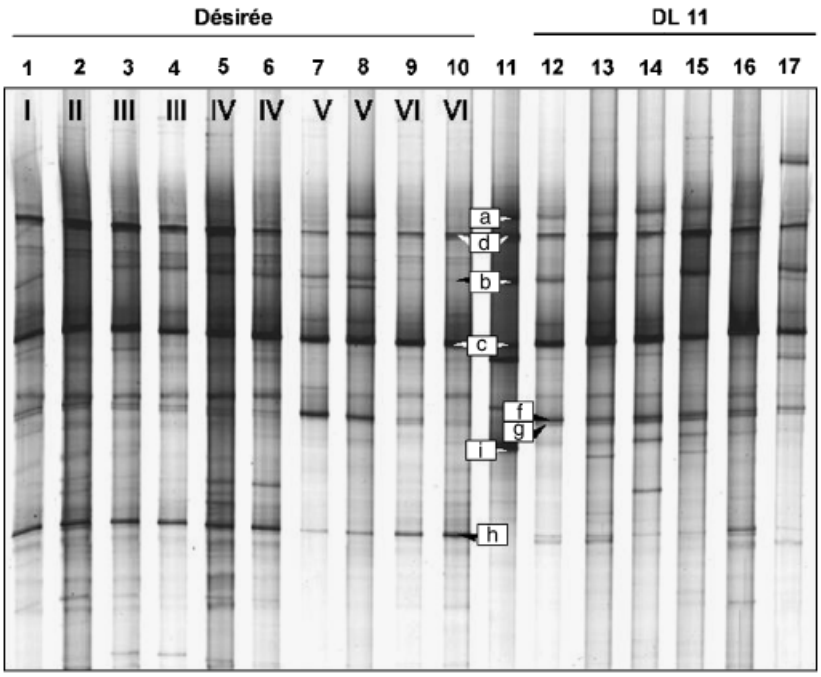

Fig. 3. Comparison of community patterns of endophytic fungi after surface sterilization of roots of the parental line Désirée (lanes 1–10) and the transgenic T4-lysozyme producing line DL11 (lanes 12–17) by PCR-denaturing gradient gel electrophoresis of 18S rRNA fragment (growth stage 91–97). Lanes with the same Roman numbers represent the patterns of independent repeats of DNA extraction and amplification of roots of the same plot. Lane 11 contains a mixture of 18S rDNA fragments of different fungal isolates: a, *Cylindrocarpon destructans*; b, *Colletotrichum coccodes*; c, *Verticillium dahliae; i, Penicillium kapuscinskii*. Band d is referred to single strand DNA, e–h, see text.

and 97 from DL11 were picked. The presence and correct size of the cloned fragment (*c.* 1.65 kb) was verified by reamplifying the cloned fragment with primer NS1/FR1-GC. A high proportion of the clones screened contained inserts of the correct size (90/96 for Désirée and 93/97 for DL11). DGGE analysis of the amplicons revealed that a large proportion of the cloned inserts comigrated with dominant bands in the community patterns (47% from Désirée and 65% from DL11). The majority of the cloned 18S rRNA gene inserts showed the same electrophoretic mobility as band 'c' in the fungal community patterns (Désirée: 37/42; DL11: 45/60), which corresponded to electrophoretic mobility of the 18S rRNA gene fragment generated from *V. dahliae* isolates. Only two clones from Désirée and four from DL11 comigrated with band 'b' in the community patterns, which corresponded to the electrophoretic mobility of the PCR product generated from *Col. coccodes*. While four clones from DL11 showed the same electrophoretic mobility as *C. destructans* isolates and comigrated with band 'a' in the DGGE community patterns, none of the clones from Désirée with this electrophoretic mobility was found. Interestingly, band 'a' was detected in five of six replicates of the community patterns of DL11 but in only one from Désirée. None of the clones displayed the electrophoretic mobility of *P. tabacinum* isolates.

The diversity of the clones was further characterized by ARDRA analysis. For Désirée 37 and for DL11, 45 cloned 18S rRNA gene fragments had the same mobility in the DGGE as the isolates of *V. dahliae* but a total of nine ARDRA patterns were obtained. The ARDRA patterns of 62 clones (ARDRA pattern 1) were identical to those obtained for the *V. dahliae* isolates. Nine clones representing ARDRA pattern 2, one clone representing each of the patterns 3–10 were sent for sequence analysis. Based on the partial sequence (*c.* 300–1500 bp) all sequences were assigned to *V. dahliae* with a high similarity (Table 2), except for one chimera and one sequence, which was assigned to *Colletotrichum* sp. However, when the 18S rRNA gene fragment of this clone was reanalyzed on a gel with a gradient from 18% to 38% an electrophoretic mobility different from that of the other *V. dahliae* clones was observed.

All cloned 18S rRNA gene fragments matching the DGGE electrophoretic mobility of *Col. coccodes* isolates (band 'b'; two for Désirée and four for DL11) had the same ARDRA patterns which corresponded also to the ARDRA patterns of the *Col. coccodes* isolates. The partial sequence determined from two clones showed more than 99% similarity with a sequence of *Colletotrichum trifolii* in the database (no *Col. coccodes* is included in GenBank). All the four clones with the same electrophoretic mobility as the isolates determined as *C. destructans* had the same ARDRA patterns as these isolates. However, the closest hit (above 99%) of the partial 18S rRNA gene sequence obtained for two of the clones was *Volutella ciliata*, which might be explained by the absence of *Cylindrocarpon* sp. sequences in GenBank.

Although no amplification products were obtained with NS1/FR1-GC from DNA extracted from sterile potato plants, the sequence of three cloned 18S rRNA gene fragments (Desi 10, Desi 15, Desi 41) revealed chimeric sequences of fungal as well as plant origin. Although the

Table 2. Analysis of partial 18S rRNA gene sequences of clones of the endophytic fungal communities of roots from the parental Désirée (Desi) and the transgenic T4-lysozyme producing DL11 (growth stage 91–97) matching dominant bands in the community fingerprints (Fig. 3)

				Closest phylogenetic relatives		
Mobility in the DGGE (Fig. 3)/isolate	ARDRA	Clone	Length (bp)	Identity/strain	Accession no.*	%
Band a/*Cylindrocarpon destructans*	1	DL11 2	327	*Volutella ciliata*	AJ301967	99.4
				DL11 26		100
		DL11 26	563	*V. ciliata*		99.4
				DL11 2		100
Band b/*Colletotrichum coccodes*	1	Desi 20	373	*Colletotrichum trifolii*	AJ301941	99.4
		DL11 51	454	*Col. trifolii*		99.3
Band c/*Verticillium dahliae*	2	Desi 1	580	*Verticillium dahliae*	AF104926	100
				Verticillium longisporum	AF153421	100
		Desi 48	503	*V. dahliae*		100
				V. longisporum		100
		Desi 14	1499	*V. dahliae*		99.9
				V. longisporum		99.9
		Desi 17	578	*V. dahliae*		100
				V. longisporum		100
		Desi 35	1479	*V. dahliae*		99.9
		Desi 36	591	*V. dahliae*		99.8
		Desi 63	592	*V. dahliae*		99.7
		DL11 42	289	*V. dahliae*		100
				V. longisporum		100
		DL11 50	565	*V. dahliae*		99.8
	4	Desi 34	1479	*V. dahliae*		99.7
	5	Desi 18	1478	*V. dahliae*		99.8
	8	DL11 33	731	*V. dahliae*		99.9
	3	DL11 38	542	*V. dahliae*		99.8
	9	DL11 62	1486	*V. dahliae*		99.9
	10	DL11 67	1492	*V. dahliae*		98.3
	8	DL11 69	503	*V. dahliae*		99.8
	11	DL11 70	1480	*V. dahliae*		99.9
				V. longisporum		99.9
Band f	12	DL11 64	421	*Septoria nodorum*	U04236	99.8
				Paraphaeosphaeria quadriseptata	AF250826	99.7
		DL11 78	1485	*P. quadriseptata*		99.7
Band g	ND	DL11 54	463	*Acremonium* sp.	AJ278754	100
				Phialophora sp.	AJ278753	100

*GenBank sequence accession numbers of closest fungal sequence.
ND, not determined.

fragments of all three clones melted at high denaturant concentrations, only one clone Desi 41 showed the same electrophoretic mobility in the DGGE as band 'h', which was more intense in the patterns of Désirée than of DL11 (Fig. 3).

Discussion

In this study the diversity of endophytic fungi in the surface-sterilized roots of a transgenic T4-lysozyme producing potato DL11 and of the parental cultivar Désirée was assessed by means of traditional isolation techniques and DNA-based, cultivation-independent techniques. With both approaches we were able to detect differences in the relative abundance of endophytic fungi in the roots of DL11 and Désirée. Endophytic fungi were isolated by plating root segments on nutrient agar, which is a common method for the isolation of endophytic fungi (Hallmann *et al.*, 2006). This method favours fast growing fungi, but the use of very small root segments and long incubation times (up to 6 weeks in this study) also enabled us to isolate slower growing fungi. Although latent or quiescent fungal endophytes are probably not obtained with this approach, the method still provides quantitative information on the composition of fungi accessible to cultivation. In this study the cultivation-dependent approach showed high numbers of morphologically different endophytically, especially fast growing fungi in the roots of senescent potato plants (growth stage 91–97). Schulz & Boyle (2005) mentioned an increasing colonization density and diversity in the course of the vegetation

period as well. Many fungal isolates were retrieved only once or a few times, and therefore a comparison of the frequency of their occurrence in DL11 or Désirée would not have been meaningful. Remarkably, *V. dahliae*, which is known as the causal agent of the Verticillium wilt of potato (Stevenson *et al.*, 2001), was most frequently isolated from root segments of surface-sterilized roots. Nearly three times the number of *V. dahliae* isolates were obtained from root segments of Désirée than from DL11. However, this difference was not significant because the majority of the isolates from Désirée originated only from three of the six plots.

The molecular characterization of several *Verticillium* isolates from both potato lines by means of PCR-DGGE and BOX-PCR of genomic fingerprints showed nearly no differences, suggesting that there was no selection for a different genotype because of the T4-lysozyme expression by DL11. The DGGE band of the *V. dahliae* isolates corresponded to the major band (Fig. 3, band 'c') in the endophytic fungal community patterns. Based on the more frequent isolation of *V. dahliae* from plots 2, 4 and 6, differences in the intensity of band 'c' were expected. However, in contrast to the isolation results, no differences in the intensity of this band were observed for the different plots of DL11 and Désirée. Reamplification of DNA excised from bands of fungal community patterns has not been successful, possibly due to the large size of the amplicon (1.65 kb). Therefore, we generated a clone library to get a better insight into the diversity of fungal taxa contributing to the DGGE bands. The clone library even more impressively revealed the high abundance of *V. dahliae* in healthy potato roots.

The other fungus for which differences in the isolation frequency from DL11 and Désirée were observed was the weak plant pathogen *C. destructans*. Isolates which were assigned to *C. destructans* were obtained more than twice as frequently from DL11 than from Désirée. In this case the isolation-based finding was confirmed by the molecular analysis. A band in the fungal community patterns which corresponds to the DGGE mobility of *C. destructans* isolates was detected in five of six replicates of DL11 but in only one replicate of Désirée. Clones containing inserts with the electrophoretic mobility of *C. destructans* were only isolated from DL11. However, based on the number of *C. destructans* isolates, we would have expected a much more intense band 'a' (Fig. 3) and a higher frequency of clones carrying inserts with the DGGE mobility of *C. destructans*. In contrast to the collection of *V. dahliae* isolates, BOX-PCR fingerprints indicated some degree of genomic diversity of isolates with identical DGGE mobility. *Colletotrichum coccodes*, the third frequently obtained endophytic fungus – a weak pathogen causing the black dot disease of potato – was isolated in equal numbers from DL11 and Désirée, and band 'b' (Fig. 3) corresponding to *Col. coccodes* isolates was detected in most of the replicates in the community patterns of DL11 and Désirée. The ARDRA patterns of the cloned inserts with the DGGE mobility of band 'b' were all identical to the ARDRA patterns of the *Col. coccodes* isolates tested. However, the ARDRA patterns were also the same as for *C. destructans*, although the DGGE mobilities of 18S rRNA gene fragments of both species were clearly different. The limitations of the 18S rRNA gene also became apparent when sequencing the 18S RNA genes from clones (Table 2) or isolates (data not shown). The low degree of variability, in addition to the fact that no 18S rRNA gene sequences have been deposited for some genera or species (e.g. *C. destructans*), presents a major limitation of 18S rRNA gene-based detection techniques.

The numbers of isolates retrieved by cultivation were mirrored in the cultivation-independent approach for only some of the species. Although we easily obtained DGGE-PCR products from isolates of the fourth frequently retrieved fungal endophyte *P. tabacinum*, the corresponding band was not detected in the fungal community patterns. The discrepancies observed between the isolation-based and the cultivation-independent approaches are not too unexpected and have been reported by others (Zuccaro *et al.*, 2003).

An impressive diversity of endophytic fungal isolates colonizing healthy potato roots was revealed in this study. Although only 0.25 g root material was used in this study, most of the dominant bands appeared in the fungal community patterns of all replicates. Some fainter bands were more variable. These results corresponded well to those of the cultivation-dependent approach where the most frequently isolated fungi were abundant in all plots, whereas less frequently or only sporadically isolated fungi were obtained only from few plots. However, the variability between the DGGE patterns of replicates from one plot indicates a heterogeneous distribution of fungi within the composite sample used for direct DNA extraction. Therefore the homogenization step as performed in this study was important to obtain DGGE patterns with relatively little variability, even when using small amounts of plant material for DNA extraction. Amplification products of fungal 18S rRNA gene fragments from DNA directly extracted from surface-sterilized potato roots were easily obtained with the primer system NS1/FR1-GC, although several authors reported on biases of the primer set when using this primer system. Despite the limitations of 18S rRNA gene based analysis of fungi, the primers originally described by Vainio & Hantula (2000) seemed to be suitable to analyze endophytic fungal communities at least from surface-sterilized potato roots. Although we did not obtain PCR products when this primer pair was used to amplify DNA from sterile potato plants, the sequence of one clone which comigrated in the DGGE with a dominant band in the fungal community patterns, and which was more intense in the patterns of Désirée than in the DL11 patterns, turned out to be a

chimeric sequence. Although the PCR-DGGE system used in this study to compare the endophytic fungal communities allowed a rapid screening of many samples, the in-depth interpretation of the patterns, in particular if differences are detected, requires the use of more laborious polyphasic approaches, as done in this study. As the diversity detectable in the DGGE patterns reflects the relative abundance of each ribotype, no information will be obtained for less abundant fungi when universal fungal primers are used. However, an increased resolution and sensitivity could be achieved using primers specific for particular fungal groups – an approach which is widely applied to analyze bacterial communities (Costa *et al.*, 2006).

Several studies on the potential effects of T4-lysozyme expression on the bacterial communities in the rhizosphere of transgenic T4-lysozyme producing potato lines have been published so far (Lottmann *et al.*, 1999, 2000; Heuer *et al.*, 2002). However, the effects of T4-lysozyme expression on the structural and functional diversity observed were negligible relative to environmental factors (Heuer *et al.*, 2002). In this study, significant differences between the parental line Désirée and the transgenic line DL11 were revealed. *Cylindrocarpon destructans* was more abundant in surface-sterilized roots of DL11 than in Désirée as shown by the classical and molecular approach. The higher colonization of roots of Désirée with *V. dahliae* found with the classical approach could not be confirmed by the molecular approach. Despite the experimental limitation discussed before, the combination of traditional isolation techniques and DNA-based, cultivation-independent techniques (18S rRNA gene fragments amplified from total DNA analyzed by DGGE and by cloning and sequencing) used in this study provided useful information on the taxonomy and diversity of fungi present in surface-sterilized potato roots and revealed some differences in their relative abundance when DL11 and Désirée were compared. The finding that two of the endophytic fungi, *V. dahliae* and *Col. coccodes,* most frequently detected in roots of healthy field-grown potato plants were potential potato pathogens (Stevenson *et al.*, 2001) is rather remarkable.

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