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Development of specific primers for detection and identification of *Alternaria* spp. in carrot material by PCR and comparison with blotter and plating assays

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Alternaria alternata, *A. radicina* and *A. dauci* are important seed-borne fungi on carrot, with the first two species having a high toxigenic potential, for which a specific and sensitive detection method is required. Because both the traditional deep-freeze-blotter method and plating on selective medium are time consuming and laborious, a PCR-based assay was developed. Sequences of the internal transcribed spacer regions of the ribosomal gene repeat (rDNA) from 45 different *Alternaria* isolates were determined, a restriction fragment length polymorphism analysis was performed and a phylogenetic tree was constructed. Based on the sequences, specific primers for detection and identification of the three *Alternaria* species on carrot seeds and roots were designed. The primers were highly sensitive and were shown to be able to differentiate between the three *Alternaria* species. *A. alternata* and *A. radicina* could be detected in DNA isolated from carrot material applying the specific primers, even at low infection levels. The PCR-assay was compared to the deep-freeze-blotter method (DFBM) and plating on *Alternaria radicina* Selective Agar (ARSA, for *A. radicina*) by testing naturally infected seed samples and root material. Results of the PCR-assay were similar to those of the blotter method and plating on ARSA for the detection of *A. alternata* and *A. radicina*. A positive correlation was found between the percentage of seed infection established by the blotter method and the intensity of the amplified, specific product. The PCR-assay based on the specific primers developed seems to be a good alternative for the deep-freeze-blotter method and plating on ARSA, especially when time is an important issue.

INTRODUCTION

The genus *Alternaria* is ubiquitous and includes both plant pathogenic and saprophytic species that may damage crops in the field or cause post harvest decay (Logrieco *et al.* 1990). Certain species are also capable of producing mycotoxins (King & Schade 1984, Woody & Chu 1992) which can contaminate plant products (Schroeder & Cole 1977, Stinson *et al.* 1981) and are considered as a potential cause of cancer (Blunden *et al.* 1991, Chu 1991, Gui-ting *et al.* 1992, Pohland 1993). Several species of the anamorphic genus *Alternaria* are commonly found on carrot (*Daucus carota*) worldwide (Soteros 1979). *A. alternata* is commonly isolated from carrot seeds and roots; *A. radicina* and *A. dauci* are specific for carrot with some of the cultivars being very susceptible (Tylkowska 1992). Whereas *A. alternata* is usually regarded as a saprophyte or a weak pathogen (Scheffer 1992), *A. radicina* and *A. dauci* are considered the most important seed-borne pathogens on carrot (Meier *et al.* 1922, Soteros 1979, Tylkowska 1992), causing black rot of roots and leaf blight, respectively. Both *A. alternata* and *A. radicina* are reported as producers of harmful mycotoxins (Griffin & Chu 1987, Bottalico & Logrieco 1992, Montemurro & Visconti 1992).

The taxonomy of *Alternaria* is based primarily on the morphology and development of conidia and conidiophores, and to a lesser degree on host plant association and colony morphology (Elliot 1917, Simmons 1967). The conidia of *A. alternata* are commonly found in long branched chains of 10 or more, polymorphous, with 1–9 transverse and several longitudinal or oblique septa. The conidia of *A. dauci* are usually solitary, rarely in chains of 2, with 5–11 transverse and 1 to several longitudinal or oblique septa. The conidia of *A. radicina* are usually solitary, rarely in short chains of 2–3, ovoid, ellipsoid, rounded

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at both ends, with 1–10 transverse and 1 to several longitudinal or oblique septa. The current methods for detection and identification of the three *Alternaria* species mostly rely on the traditional deep-freeze-blotter method (ISTA 1996) which is based on morphological characters, or on agar plating techniques (ISTA 1996) such as plating on the ARSA medium (*Alternaria radicina* Selective Agar, Pryor, Davis & Gilbertson 1994). Both methodologies need incubation times from 7 up to 14 days, before the results can be scored. Because observations are made visually, small amounts of fungal material may be overlooked. Furthermore, although each *Alternaria* species which occurs on carrot has a distinct spore morphology, misidentifications are known to occur, for instance due to the presence of *Stemphylium* spp. For these reasons there is a demand for a rapid and reliable method for detecting and identifying the three *Alternaria* species that is not based on the use of morphological characters.

The toxigenic potential of *A. alternata* and *A. radicina* underlines the need for accurate detection tools. Among the most sensitive methods available is the polymerase chain reaction (PCR). Sequences of the ribosomal DNA genes, in particular the internal transcribed spacer regions ITS1 and ITS2 surrounding the 5.8S gene, have been used to develop specific primers for detection of several phytopathogenic fungi (Nazar *et al*. 1991, Blakemore *et al*. 1994, Ward 1994, Bonants *et al*. 1997). Variations in the ITS regions were used to study the phylogeny of host-specific toxin producing *Alternaria* species (Kusaba & Tsuge 1995, Seung-Jo *et al*. 1997) and the population genetic structure of *Alternaria spp*. causing brown spot of minneola tangelo and rough lemon (Peever *et al*. 1999). Comparison between the rDNA sequences of *A. linicola* strains (McKay *et al*. 1999) or *Alternaria* species pathogenic to crucifers (Jasalavich *et al*. 1995) revealed variation in the ITS sequences both in base composition and in length, although there is a high degree of homology among species and strains of *Alternaria* (Jasalavich *et al*. 1995, Kusaba & Tsuge 1995). By analysis of the ITS regions in common airborne allergenic fungi, including *A. alternata*, *A. brassicicola*, *A. brassicae*, *A. infectoria*, *A. tenuissima* and *A. raphani*, Gaskell *et al*. (1997) concluded that the interspecies similarity in ITS1 and ITS2 regions will make the design of probes to distinguish between these genera relatively straightforward. *A. alternata* could be distinguished from other genera present in tomato products by PCR, but the specificity of the primers was not tested against other *Alternaria* species and closely related genera like *Stemphylium* and *Ulocladium* (Zur *et al*. 1999). Recently Pryor & Gilbertson (2000) studied the phylogenetic relationships among *A. radicina* and other members of the cheiranthi species group based upon rDNA sequence analysis. The *Alternaria* spp. clustered into several distinct species-clades, most of which correlated with species-groups previously established based upon morphological characteristics.

Random amplified polymorphic DNA (RAPD) PCR has also been used to study the genetic variation in the genus *Alternaria* (Cooke *et al*. 1998, Roberts *et al*. 2000), among *A. solani* and *A. alternata* isolates from potato and tomato (Weir *et al*. 1998, Morris *et al*. 2000), of *Alternaria* species pathogenic to crucifers (Sharma & Tewari 1998) and of *A. brassicae* (Sharma & Tewari 1995). Application of RAPD-PCR for detection of *A. radicina* has recently been reported (Pryor & Gilbertson 2001). Nonetheless, no reports are known to have studied the three species *A. dauci*, *A. radicina*, and *A. alternata*, which all may occur, even simultaneously, on carrot material.

The objectives of this research were to develop specific primers for detection of the filamentous fungi *A. alternata*, *A. radicina* and *A. dauci* in carrot material (seeds and roots) and to compare the PCR test with the traditional deep-freeze-blotter-method and with plating on ARSA (*A. radicina* only). Initially carrot seed samples were tested by the deep-freeze-blotter-method and plating on ARSA and the mean percentage of infection was calculated. The ITS regions of the rDNA of all isolates obtained from carrot material and other sources were amplified and the PCR products were sequenced, analysed and specific primers were designed for each species. The specificity and suitability of the primers were tested on various *Alternaria* species and isolates, as well as on all other important fungal genera reported on carrot material. PCR with the *Alternaria* specific primers was performed on DNA isolated from carrot seeds and roots and the detection level of the three assays was compared.

MATERIALS AND METHODS

Fungal isolates

Isolates of *Alternaria* spp. and other genera (Table 1) were obtained from collaborative researchers and from CBS (Centraalbureau voor Schimmelcultures, Utrecht). Pure single-spore cultures of all isolates were stored at 4 °C on potato dextrose agar (PDA-Difco) under sterile mineral oil. For use in the present study, cultures were grown on PDA (Difco) or potato-carrot agar (PCA) containing streptomycin sulphate (50 mg ml^{-1}) at 25 °C under 12 h NUV/12 h dark photoperiod to promote sporulation. Cultures were observed at 25–50 × with a stereo microscope and fungal species were identified according to Ellis (1971, 1976), Simmons (1992, 1993) and Simmons & Roberts (1993). Where necessary, additional observations of isolates were made using a compound microscope.

Seed samples

Naturally infected carrot seed samples were obtained from Poznań Agricultural University, Poland, and Rijk Zwaan, The Netherlands. Four experimental carrot seed lots (named 1, 6, 10 and B) with different levels of

Table 1. Names and sources of species and isolates used in the present study.

Species	Isolate no.	Origin	Source	Year of isolation
Alternaria alternata	1/333	Carrot, cv. 4435, Bejo	The Netherlands	2000
A. alternata	2/102*+	Carrot, cv. DC-2	The Netherlands	1999
A. alternata	3/175*	Carrot, cv. DC8	The Netherlands	1998
A. alternata	4/101*	Carrot, cv. DC-2	The Netherlands	1998
A. alternata	5/108*	Carrot, cv. DC-1	The Netherlands	1998
A. radicina	6/332	Carrot, cv. DC-1	The Netherlands	2000
A. radicina	7/321	Carrot, cv. Ebro	The Netherlands	1999
A. radicina	8/329	Carrot, cv. 4435, Bejo	The Netherlands	2000
A. radicina	9/320+	Carrot, cv. Valor	The Netherlands	1998
A. radicina	10/109*	Carrot, cv. Valor	The Netherlands	2000
A. dauci	11/104*+	Carrot, cv. DC-1	The Netherlands	1998
A. dauci	12/322	Wild carrot-Blauwe Kamer	The Netherlands	1998
A. dauci	13/358	Carrot, cv. 4435, Bejo	The Netherlands	1999
A. dauci	14/328	Carrot, cv. RBNs, Bejo	The Netherlands	2000
A. alternata	15/334	Carrot, cv. RANs, Bejo	The Netherlands	2000
A. radicina	16/330	Carrot, cv. RANs, Bejo	The Netherlands	2000
A. radicina	17/331	Carrot, cv. RBNs, Bejo	The Netherlands	2000
A. alternata	18/335+	CBS 154.31	The Netherlands	2000
A. radicina	19/336+	CBS 245.67	The Netherlands	2000
A. radicina	20/338+	Carrot, 75/1L IIRa	Poland	1999
A. radicina	21/339+	Carrot, 76/1a IVRa	Poland	1999
A. dauci	22/337+	CBS 106.48	The Netherlands	2000
A. dauci	23/340	Carrot, 4NL 2/1C	Poland	1999
A. radicina	24/341	Carrot, cv. B289 1D	Denmark	2000
A. citri	25/342+	CBS 106.27	The Netherlands	2000
A. dianthi	26/343+	CBS 100.40	The Netherlands	2000
A. porri	27/344+	CBS 114.38	The Netherlands	2000
A. longipes	28/345+	CBS 113.35	The Netherlands	2000
A. cheiranthi	29/346+	CBS 108.38	The Netherlands	2000
A. solani	30/347+	CBS 110.41	The Netherlands	2000
A. bataticola	31/348+	CBS 531.63	The Netherlands	2000
A. lini	32	CBS 106.34	The Netherlands	2000
Thielavopsis basicola	33/349	Carrot, PAV	The Netherlands	2000
Mycocentrospora acerina	34	Carrot, PAV	The Netherlands	2000
Chalaropsis thielavioides	35/350	Carrot, PAV	The Netherlands	2000
Alternaria alternata	36/351+	Barley, cv. Aheloj-2	Bulgaria	1999
A. alternata	37/352	Radish seeds	The Netherlands	2000
A. alternata	38/354	Tomato seeds	Indonesia	2000
A. alternata	39/355+	Strawberry	The Netherlands	2000
A. brassicicola	40/177+	Brassica	The Netherlands	1998
A. brassicae	41/164	Brassica	The Netherlands	1998
Stemphylium botryosum	42/111	Carrot	The Netherlands	1998
S. botryosum	43/147*	Carrot	The Netherlands	1998
Ulocladium sp.	44/165*	Carrot seed, cv. DC-5	The Netherlands	1999
Fusarium culmorum	45/356	Carrot seeds	The Netherlands	2000
F. nivale	46/357	Carrot seeds	The Netherlands	2000
Epicoccum purpurascens	47/160	Carrot seeds	The Netherlands	1998
Alternaria infectoria	48/351	Barley seeds, cv. Fink	Bulgaria	1999
Cladosporium herbarum	49/132	Carrot seeds	The Netherlands	1999
Alternaria dauci	50	Carrot seeds	France	2000

* Monospore culture.
+ Used in sequence analysis; accession numbers from GenBank are AF397041–AF397058.

Alternaria infection were tested by each method (DFBM, ARSA, PCR) and the assays were repeated three times.

DNA isolation

Three to five agar discs with mycelium were placed in 50 ml V8 liquid medium or in 50 ml potato dextrose broth (PDB, Difco). For the V8 medium 125 ml of V8 (vegetable juice) and 11.5 g of oatmeal were added to 500 ml demineralized water, boiled for 5 min, and clarified by centrifugation for 15 min at 1600 rpm. Subsequently, 0.94 g $CaCO_3$ was added and the volume was adjusted to 625 ml. The medium was autoclaved for 30 min at 121 °. After incubation on a gyrotary shaker at 25 ° for 5–7 d at 100 rpm, mycelia were collected aseptically by vacuum filtration through a Bchner funnel covered with a sterile filter paper (Whatman No. 1), washed with two volumes of sterile MilliQ and lyophilized. Freeze-dried material was stored at −80 ° until use. Twenty mg lyophilized mycelium or 200 carrot seeds, incubated for 24 h as

described before, were ground in Eppendorf tubes with sterile sand and a pestle. DNA was isolated from fungal mycelium and from carrot seeds with the Puregene kit (Gentra/Biozym, Landgraaf) according to the manufacturer's instructions. For DNA isolation from the seeds 300 µl Cell Suspension Solution was added, the samples were vortexed for 30 s and centrifuged for 30 s at 2000 ***g***. The supernatant was pipetted in a fresh tube and the procedure was continued according to manufacturer's instructions. For some of the isolates an alternative protocol was used: 100 mg fresh mycelium was scraped from a 10-day-old culture grown on PDA, and placed in a sterile 2 ml Eppendorf tube. Genomic DNA was isolated from the fresh mycelium according to Lee & Taylor (1990), including an RNAse A (Sigma) treatment at 10 mg ml^{-1} for 10 min at 37 °. DNA from 100 mg lyophilized healthy and infected carrot roots was extracted according to Kamaly *et al*. (1990) without the chloroform:methanol extraction step. All crude DNA preparations were purified through a polyvinylpolypyrrolidon (PVPP) column to remove polysaccharides, phenolic compounds and pigments that could inhibit the PCR reaction. Three hundred mg insoluble PVPP was loaded in a micro bio-spin chromatography column (Bio–Rad, Veenendaal), 300 µl sterile MilliQ water was added and the columns were centrifuged for 3 min at 4000 ***g***. Columns were transferred to a fresh tube and the DNA extract was loaded in the centre of the PVPP profile. DNA was collected in an Eppendorf tube by centrifuging at 5000 rpm for 4 min. Where necessary, the procedure was repeated. DNA samples were stored at 4 ° for further use. DNA concentrations were estimated by electrophoresis using 0.8 % agarose gels by comparison with DNA standards. Gels were viewed under UV light at 600 nm on a Lumi Imager F1 system and analyzed using the Lumi Analist 3.0 software (both from Roche Mannheim).

DNA sequence analysis

The ITS regions of all the isolates listed in Table 1 were amplified by the polymerase chain reaction (PCR) using primers ITS1 and ITS4 (White *et al*. 1990). Amplification reactions were performed in 25 µl containing 2.5 µl 10 × reaction buffer [100 mM Tris–HCl pH 8.3, 15 mM $MgCl_2$, 500 mM KCl, 0.01 % (w/v) gelatin], 200 µM of each deoxyribonucleotidetriphosphate (dNTP), 1.5 µM of each primer, 0.5 units of Taq2000-polymerase (Stratagene, USA) and 10–100 ng DNA. PCR cycling conditions consisted of denaturation at 94 ° for 2 min, annealing at 57 ° for 30 s, extension at 72 ° for 1 min for 35 cycles (for amplification of fungal DNA) or for 40 cycles (for amplification of carrot DNA) and a final extension at 72 ° for 10 min, followed by cooling at 4 ° until recovery of the samples. 5 µl of the amplification product was separated in a 1 % agarose gel by electrophoresis in 1 × TAE buffer (40 mM Tris–HCl and 2 mM EDTA pH 8.0) and visualized as described above. Some of the PCR products were cloned into the pCR 2.1 vector and transformed in competent TOP10F′ cells from the TOPO-cloning kit (Invitrogen, USA). Positive clones were selected by PCR amplification of the inserts with M13 universal primers and checked by restriction analysis with *Tai*I, *Eco47*I and *Vsp*I (Fermentas, Lithuania). Plasmid DNA was isolated with the Wizard® Miniprep DNA Purification System (Promega, USA). Sequence analysis was performed with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI310 automatic sequencer (both Perkin–Elmer Applied Biosystems, Nieuwerkerk a/d IJssel) with the M13 forward or reverse primer. A part of the purified PCR products were directly sequenced in both directions with the ITS1 or ITS4 primer. The resulting ITS sequences were aligned by the clustal method with the program MegAlign from the DnaStar® software package (Dnastar, USA) and a dendrogram was constructed including sequences of the ITS regions obtained from the database from the GeneBank, as well. The sequence alignment was deposited in TreeBASE under number SN909.

PCR–RFLP analysis

Aliquots (5 µl) of the amplified ITS regions (with the ITS1 and ITS4 primers) of all the isolates tested were digested with 10 U restriction enzymes *Tai*I, *Eco47*I, *Vsp*I (Fermentas, Germany), *Mae*I (Roche Mannheim) or *Tru*9I (Roche, USA) according to the manufacturer's instructions. Restriction fragments were separated by 2 % agarose gel electrophoresis in 1 × TAE buffer, and visualized and analyzed as described above.

Specific PCR amplification

Based on the full-length sequence, two specific primers for each *Alternaria* species were designed (Table 2). The specificity of the primers was tested against isolates of a range of *Alternaria* species, other fungi associated with carrot material and against carrot seeds and roots. The programme parameters for thermal cycling for the three primer sets were as described above, with the exception that annealing took place at 70 ° for 40 s and a final extension for 5 min at 72 °. For PCR with the specific primers, 1.5 units of Taq polymerase (Roche, USA) was used. PCR reactions were performed in a PTC200 thermocycler (MJ Research/Biozym, Landgraaf), Mastercycler Gradient (Merk Eurolab, The Netherlands) and a Robocycler® Gradient 96 (Stratagene, USA).

Specific PCR on carrot material

For the PCR analysis, 200 carrot seeds were placed on a wet sterile thick filter paper in 14 × 17 cm plastic trays with a transparent lid and incubated for 24 h at 25 ° to augment the fungi present. The seeds were collected from the blotter with sterile pincers and 100 seeds were placed aseptically in 2 ml Eppendorf tubes in two

Table 2. DNA sequences of internal transcribed spacer (ITS) primers, and specific primers for *Alternaria alternata*, *A. dauci* and *A. radicina* used in this study.

Target sequences	Primer name	Sequence	Fragment size (bp)
Eucaryotic rDNA	ITS1	5′ TCCGTAGGTGAACCTGCGG	500–600
	ITS4	5′- TCCTCCGCTTATTGATATGC	
A. alternata	AAF2	5′- TGCAATCAGCGTCAGTAACAAAT	340
	AAR3	5′- ATGGATGCTAGACCTTTGCTGAT	
A. dauci	ADF2	5′- GCAATCAGCGTCAGTAACAACA	345
	ADR1	5′- CGCAAGGGGAGACAAAAA	
A. radicina	ARF2	5′- AATCAGCGTCAGTAAACAAACG	251
	ARR3	5′- AGAGGCTTTGTGGATGCTG	

replicates. For PCR testing of carrot roots, diseased or healthy fresh carrots were cut in small pieces and lyophilized for 48 h in a Hetosicc (Denmark) lyophylizer. The healthy carrots were peeled with a peeler and rinsed with sterile distilled water before lyophilizing. *Alternaria* infection of the carrot seeds and roots was estimated by PCR with specific primers, as described above, using a scale based upon strength of amplification: −, no amplification; +, weak amplification, ++, moderate amplification; +++, strong amplification. To ensure that the quality of the extracted DNA was adequate for amplification, samples were assayed using universal ITS primers.

The amplification products obtained after PCR with the primers specific for *Alternaria alternata* and *A. radicina* were cloned in pGEM-T EASY (Promega, USA) and transformed in XL-1 BLUE (Stratagene) competent cells of *E. coli* according to the manufacturer's instructions. The positive clones with the right insert were screened with universal M13 forward and reverse primers. Four clones for each primer set were chosen and sequenced in both directions as described above. The obtained sequences were searched for homology with other species in GenBank.

The deep-freeze-blotter method (DFBM)

For the DFBM a total of 200 seeds (4 replicates of 50 seeds) from each sample were placed in 14 × 17 cm plastic trays with transparent lids on a sterile thick filter paper moisturized with 60 ml sterile H_2O. The trays were incubated in the dark at 20 ° for 24 h, to hydrate the seeds, then for 24 h in a freezer at −20 ° to kill the embryo, and subsequently for 7 days at 20 ° under a 12 h near-ultraviolet light (NUV)/12 h dark cycle to stimulate fungal growth and sporulation. The carrot seeds were then examined under a stereo microscope for the presence of *Alternaria alternata*, *A. radicina* and *A. dauci*, which were identified using the classification system of Ellis (1971, 1976). The infection level with the three species was calculated.

Plating on ARSA

The ARSA medium was prepared according to Pryor *et al.* (1994). A total of 200 seeds were placed on ARSA medium in 90 mm plastic Petri dishes, and incubated for 10 days at 28 ° in darkness. The undersurfaces of the dishes were examined using a stereo microscope at 7 × magnification for characteristic vegetative growth of *A. radicina* emerging from infested seeds and infection level was calculated.

RESULTS

rDNA sequence analysis

DNA extracted from all 50 isolates used in this study was amplified by PCR with ITS1 and ITS4 primers resulting in clear 520–550 bp fragments (data not shown). The amplification products of some isolates were sequenced in both directions and searched for homology to those of other species. On the basis of the ITS distances of these sequences and of sequences obtained from the EMBL and GenBank database, a similarity dendrogram was constructed for the ITS-2 region (Fig. 1). The trees of both ITS-1 and ITS-2 regions showed similar branching patterns (data not shown) correlating with morphological characters. All 9 isolates of *Alternaria alternata*, 5 of *A. radicina* and 3 of *A. dauci* clustered together in monophyletic groups with levels of similarity between the isolates of 98.8, 100 and 98.2 %, respectively (Fig. 1). The similarity between *A. alternata* and *A. radicina* isolates varied between 92.4 and 93.6 %, between *A. alternata* and *A. dauci* 82.5–86.0 % and between *A. radicina* and *A. dauci* 84.8–86.5 %. There was 99.4 % similarity in the ITS-2 region between *A. alternata* and *A. lini* and 98.8 % between *A. alternata* and *A. longipes*. *A. citri* was then closest related to *A. alternata* with levels of similarity of 97.1 %.

rDNA sequence analysis of *A. infectoria* showed a large insertion of about 27–30 nucleotides in the ITS-1 region as compared to many other *Alternaria* isolates. The phylogenetic analysis revealed that *A. bataticola*, *A. dauci*, *A. porri*, *A. sesami* and *A. solani*, which all produce large-size conidia, clustered in a monophyletic group.

PCR–RFLP analysis

The ITS sequences (together with the 5.8S sequences) from all the isolates of the three *Alternaria* species were

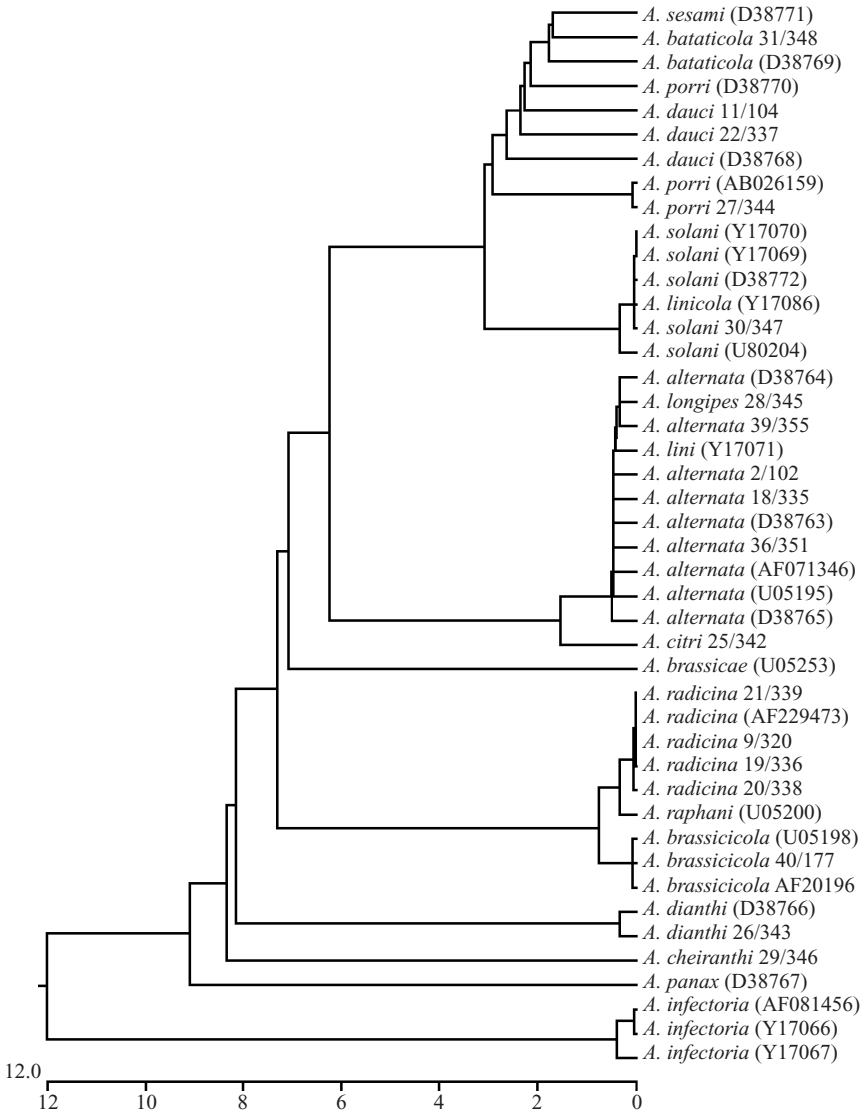

Fig. 1. Similarity dendrogram on distances derived from sequences of the ITS-2 regions of *Alternaria* species. Distances were determined by aligning by clustal method with weighed residue weight table in the programme MegAlign from the DnaStar software package. For the numbers of the isolates, see Table 1. Codes between brackets refer to sequences from GenBank.

analysed with the programme MapDraw from the DnaStar® package and the restriction enzymes *Tai*I, *Eco47*I, *Vsp*I, *Mae*I and *Tru*9I were chosen for a restriction fragment length polymorphism (RFLP) study. All isolates of *A. alternata* and none of *A. radicina* or *A. dauci* were cut with *Vsp*I, *Mae*I and *Tru*9I. All isolates of *A. radicina* were cut with *Tai*I and all of *A. dauci* were digested with *Eco47*I (data not shown). After digestion of amplification products of several isolates with *Mae*I, a specific RFLP-pattern for *A. alternata* was obtained (Fig. 2). All the other species did not have a restriction site for this enzyme with the exception of *A. citri* (25/342), *A. lini* (32) and *A. longipes* (28/345). Digestion of *A. lini* and *A. longipes* with *Mae*I gave the same pattern as *A. alternata* (data not shown). The pattern of *A. citri* was different as compared to *A. alternata*, two fragments, instead of three, with different sizes were obtained. These results suggest that RFLP patterns after digestion of the ITS regions with *Mae*I can be used as a detection marker for *A. alternata*.

Specific PCR amplification

After rDNA sequence analysis of *Alternaria* spp. and closely related genera, a total of fifteen primer sets (five primer sets for each species) were designed and tested for specificity (data not shown). Primer pairs AAF2/AAR3, specific for *A. alternata*, ARF2/ARR3, specific for *A. radicina* and ADF2/ADR1, specific for *A. dauci*, were selected (Table 2). These primers (approximately 25 bases in length) amplified a part of the ITS regions and the 5.8S gene. AAF2/AAR3 specifically amplified a single 341 bp fragment from all 12 *A. alternata* isolates, ARF2/ARR3 a 343 bp fragment from all 11 *A. radicina* isolates and ADF2/ADR1 a 251 bp fragment from the 7 *A. dauci* isolates, irrespective of geographic origin or host. The three

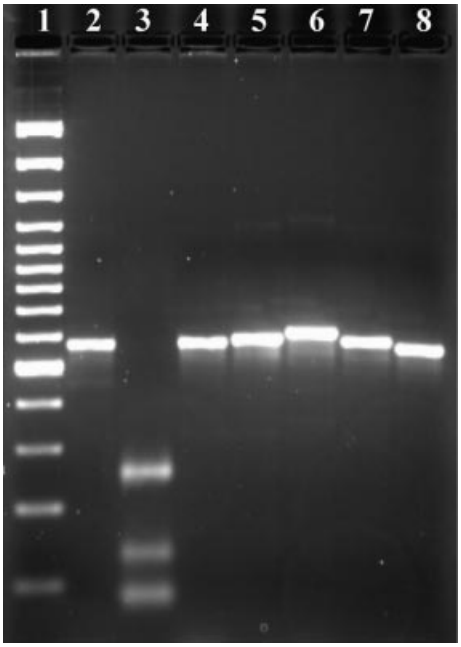

Fig. 2. Gel electrophoresis of PCR products with primers ITS1/ITS4 of DNA from several fungal isolates after digestion with *Mae*I. Lane 1, molecular weight marker (100 bp intervals); lane 2, non-digested PCR product of *Alternaria alternata* 18/335; lane 3, *A. alternata* 18/335; lane 4, *A. radicina* 9/320; lane 5, *A. dauci* 22/337; lane 6, *A. infectoria* 48/351; lane 7, *Stemphylium botryosum* 43/147; and lane 8, *Ulocladium* sp. 44/165. For the numbers of the isolates, see Table 1.

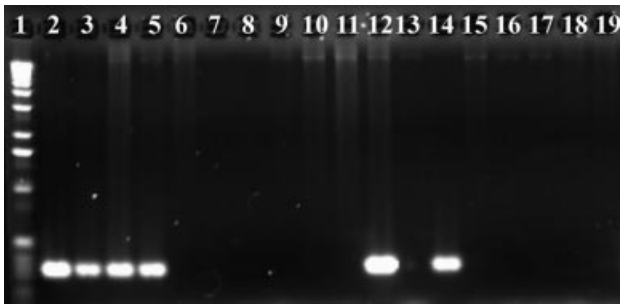

Fig. 3. Gel electrophoresis of PCR products with primers AAF2/AAR3 of DNA from several fungal isolates. Lane 1, molecular weight marker (1 kb ladder); lanes 2–5, *Alternaria alternata* 2/102, *A. alternata* 18/335, *A. alternata* 5/108, *A. alternata* 36/351; lanes 6–7, *A. radicina* 9/320, *A. radicina* 19/336; lanes 8–9, *A. dauci* 22/337, *A. dauci* 50; lane 10, *A. infectoria* 48/351; lane 11, *A. citri* 25/342; lane 12, *A. longipes* 28/345; lane 13, *A. bataticola* 31/348; lane 14, *A. lini* 32; lane 15, *Stemphylium botryosum* 42/111; lane 16, *Ulocladium* sp. 44/165; lane 17, *Fusarium culmorum* 45/356; lane 18, *Epicoccum purpurascens* 47/160; and lane 19, negative control (MilliQ). For the numbers of the isolates, see Table 1.

primer pairs were tested against total genomic DNA of a range of fungi commonly found on infected carrot tissue and a range of *Alternaria* isolates (Figs 3–5). AAF2/AAR3 (specific for *A. alternata*) cross-reacted with the *A. longipes* 28/345 (CBS 113.35) and *A. lini* 32 (CBS 106.34) isolates (Fig. 3). No product was amplified

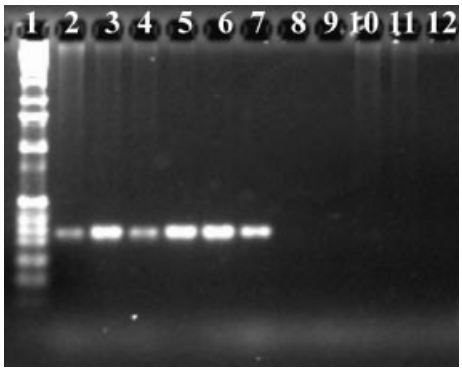

Fig. 4. Gel electrophoresis of PCR products with primers ARF2/ARR3 of DNA from several fungal isolates. Lane 1, molecular weight marker (1 kb ladder); lanes 2–7, *Alternaria radicina* 6/332, *A. radicina* 7/321, *A. radicina* 9/320, *A. radicina* 19/336, *A. radicina* 20/338, *A. radicina* 21/339, lane 8, *A. alternata* 18/335, lane 9, *A. dauci* 22/337; lane 10, *Stemphylium botryosum* 43/147; lane 11, *Ulocladium* sp. 44/165; and lane 12, negative control (MilliQ). For the numbers of the isolates, see Table 1.

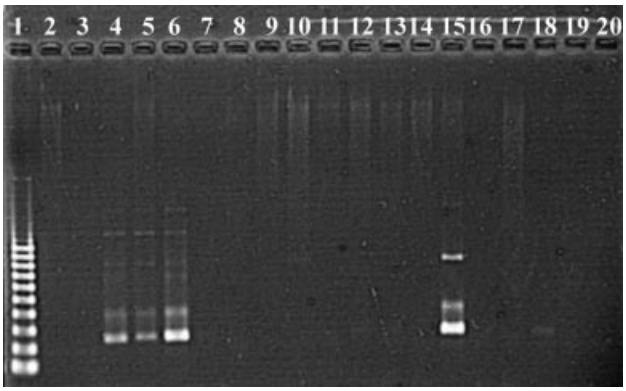

Fig. 5. Gel electrophoresis of PCR products with primers ADF2/ADR1 of DNA from several fungal isolates. Lane 1, molecular weight marker (100 bp intervals); lane 2, *Alternaria alternata* 2/102; lane 3, *A. radicina* 8/329; lanes 4–6, *A. dauci* 11/104, *A. dauci* 13/358, *A. dauci* 14/328; lane 7, *A. alternata* 18/335; lane 8, *A. radicina* 19/336; lane 9, *A. citri* 25/342; lane 10, *A. dianthi* 26/343; lane 11, *A. porri* 27/344; lane 12, *A. longipes* 28/345; lane 13, *A. cheiranthi* 29/346; lane 14, *A. solani* 30/347; lane 15, *A. bataticola* 31/348; lane 16, *A. brassicicola* 40/177; lane 17, *A. brassicae* 41/164; lane 18, *Stemphylium botryosum* 42/111; lane 19, *Ulocladium* sp. 44/165; and lane 20, negative control (MilliQ). For the numbers of the isolates, see Table 1.

from DNA of any other fungal species following PCR with ARF2/ARR3, the primer set which is specific for *A. radicina* (Fig. 4). The primer set ADF2/ADR1, specific for *A. dauci*, gave a cross-reaction with one *A. bataticola* isolate 31/348 (CBS 531.63) (Fig. 5).

Detection of Alternaria alternata species in naturally infected carrot material

DNA extracted from carrot seeds and roots with known levels of infection with the three *Alternaria*

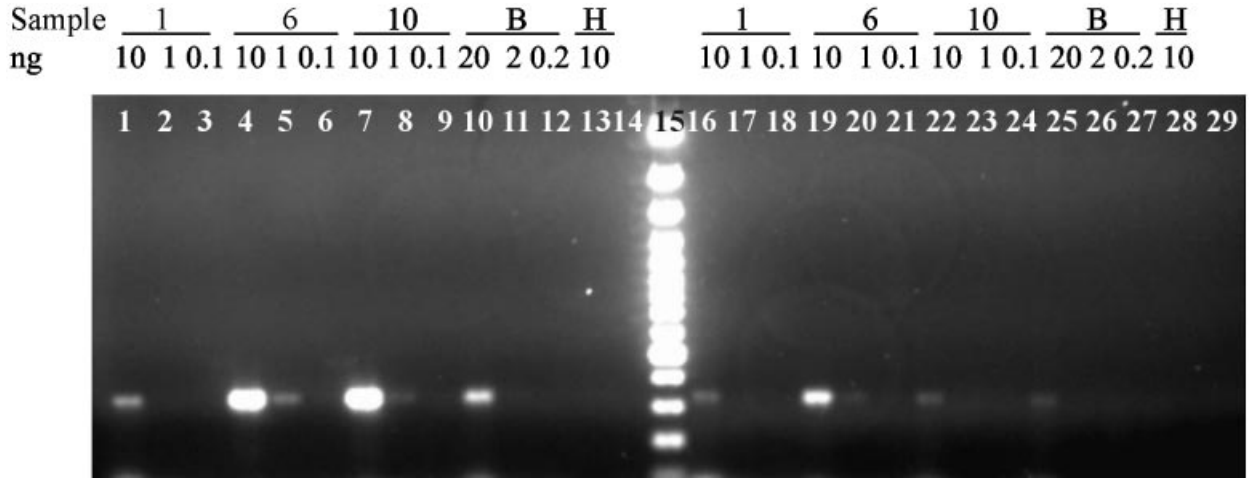

Fig. 6. Gel electrophoresis of PCR products of carrot seed DNA with primers AAF2/AAR3 (lanes 1–14) and ARF2/ARR3 (lanes 16–29). Different amounts of template DNA from several carrot samples (1, 6, 10 and B) and H (healthy carrot tissue) are amplified; lanes 14 and 29, negative control (MilliQ); lane 15, 100 bp molecular weight marker.

species, determined by the deep-freeze-blotter method and plating on ARSA (Table 2), was amplified with the three sets of specific primers. The sensitivity of AAF2/AAR3 and ARF2/ARR3 was greatly improved after adding 1 ng μl^{-1} BSA, and *A. alternata* and *A. radicina* were clearly detectable in infected carrot seeds (Fig. 6) and roots (data not shown). ADF2/ADR1 failed to amplify DNA from infected carrot seeds. Since it was impossible to obtain completely uninfected carrot seeds, total DNA from a healthy carrot tissue was used as a negative control to check for cross-reactions with the plant genome. No amplification product was observed for any primer pair with DNA extracted from healthy carrot tissue (Fig. 6).

The obtained sequences from PCR products of DNA extracted from carrot seeds with AAF2/AAR3 (specific for *A. alternata*) or with ARF2/ARR3 (specific for *A. radicina*) showed 99–100 % homology with the corresponding species after a BLAST search in the GenBank database and after alignment with sequences from ITS1/ITS4 PCR from pure fungal cultures.

Table 3. Infection level (%) of carrot seed lots and roots by *Alternaria alternata*, *A. dauci* and *A. radicina* as detected by the deep-freeze-blotter method, plating on ARSA and PCR.

		Infection level		
Sample no.	Species	DFBM (%)	ARSA (%)	Detection by PCR[1]
1	*A. alternata*	3.38	−	+
	A. dauci	0	−	−
	A. radicina	2.63	0.75	+
6	*A. alternata*	99.63	−	+++
	A. dauci	2.5	−	−
	A. radicina	22.75	38.25	++
10	*A. alternata*	99.13	−	+++
	A. dauci	0.75	−	−
	A. radicina	5.75	26.5	+
B	*A. alternata*	4.75	−	++
	A. dauci	0	−	−
	A. radicina	1.75	2.00	+
Rotten carrot tissue	*A. alternata*	100	−	+++
	A. dauci	0	−	−
	A. radicina	100	100	+++
Healthy carrot tissue	*A. alternata*	0	−	−
	A. dauci	0	−	−
	A. radicina	0	0	−

[1] −, no amplification; +, weak amplification, ++, moderate amplification; +++, strong amplification.

Comparison between the deep-freeze-blotter method, plating on ARSA and PCR

The infection percentages of the seed-lots used in this study were determined with the deep-freeze-blotter method, plating on ARSA and PCR (Table 3). A higher percentage of *Alternaria radicina* infection was generally detected after plating on ARSA, than by the deep-freeze-blotter method in two of the four seed-lots tested.

Significant relationships were found between the levels of seed infection with *A. alternata* and *A. radicina* for the four samples as defined by the deep-freeze-blotter method or plating on ARSA (for *A. radicina*) and PCR with the specific primers (Table 3). Although PCR is not a quantitative test, the differences between the infection levels of the seed samples were clearly indicated by the intensity of the amplification products on gel (Fig. 6). PCR amplification with AAF2/AAR3 on 10 ng seed DNA gave intense bands for samples 6 and 10 (with *A. alternata* infection of 99.63 % and 99.13 %, respectively according to the DFBM), a weak band for sample 1 (with 3.38 % *A. alternata* infection) and an intermediate band for sample B (with 4.75 % *A. alternata* infection). ARF2/ARR3 strongly amplified 10 ng seed DNA from sample 6 (with *A. radicina* infection of 22.75 % and 38.25 %, respectively, according to the DFBM or plating on ARSA), and a weak product for samples 1, 10 and B (with lower *A. radicina* infection percentages). PCR with AAF2/AAR3 pri-

mers, specific for *A. alternata*, and ARF2/ARR3, specific for *A. radicina*, was performed on dilution series over the range of 20 ng to 2 fg of DNA extracted from carrot seeds (data not shown). When DNA extracted from the seeds with high *A. alternata* infection levels (samples 6 and 10) was diluted to concentrations of 1 ng, the target DNA could be reproducibly detected by PCR with the specific primers (Fig. 6). Similar results were obtained after amplification of 25 or 10 ng DNA extracted from the seed samples with the *A. radicina* specific primers (Fig. 6). Due to the lower infection level of the samples with *A. radicina* compared to *A. alternata*, as defined by the deep-freeze-blotter method, there was no amplification product following PCR of 1 ng DNA isolated from carrot seeds with ARF2/ARR3 (specific for *A. radicina*; Fig. 6).

DISCUSSION

Detection of phytopathogenic fungi in plant material and seeds can be difficult, especially at low infection levels. A suitable diagnostic assay needs to be both sensitive and specific in order to avoid problems such as cross-reactions with other fungi. The most commonly used method for assaying carrot seed for fungal infestation involves incubation of seed on moistened filter paper for 10 days (the deep-freeze-blotter method). Plating on selective ARSA medium is an alternative for the detection of *Alternaria radicina*. Both methods are relatively simple, easily and quickly set up, inexpensive with regard to materials, require little equipment and are quite sensitive. However, they can be labor intensive and results may not be obtained for weeks. If the pathogen grows but does not produce conidia, identification is impossible by the deep-freeze-blotter method. The ARSA test is based on the formation of characteristic black thick mycelium of *A. radicina* (Pryor *et al.* 1994) and in case of heavy infections (like in samples 6 and 10) is much more sensitive than the deep-freeze-blotter method. The use of the ARSA medium, however, is limited to detection of *A. radicina*. A PCR-based assay for detection of the three *Alternaria* spp on carrot may therefore be an interesting alternative.

An understanding of the phenetic relationship between DNA markers in the target species and other host-associated fungi is required to design an efficient PCR-based assay. Phenetic investigations using sequence analysis of the ITS regions of rDNA revealed a close relationship between the *Alternaria* species tested. *A. alternata* was shown to be closest to *A. longipes*, *A. lini* and *A. citri*, although on the basis of RAPD analysis Cooke *et al.* (1998) found the latter species to be closest to *A. radicina*. The phylogenetic analysis of ITS variations showed that *Alternaria* spp. cluster together in monophyletic groups, corresponding to the morphology and size of the conidia as also reported by Kusaba & Tsuge (1995) and recently by Pryor & Gilbertson (2000). The sequence of *A. infectoria*, the only *Alternaria* species, which is known to have a sexual stage (*Lewia infectoria*), has an insertion of 27–30 nucleotides in the ITS-1 region, which corresponds to the results of McKay *et al.* (1999) and of Pryor & Gilbertson (2000). The DNA-based phenetic analyses done in these studies confirm the results of earlier studies on *Alternaria* genetic variability (Kusaba & Tsuge 1995). ITS sequence based profiling, whilst underlining the similarities between the three species, revealed interspecific differences large enough to enable a clear discrimination between the target species.

An ITS-RFLP analysis revealed specific restriction sites of *Tai*I for *A. radicina*, of *Eco47*I for *A. dauci* and of *Vsp*I, *Mae*I and *Tru*9I for *A. alternata*, which can be used as markers for differentiation between the three species. The PCR-RFLP pattern after digestion of *A. alternata* with *Mae*I was the same for *A. lini* and *A. longipes*, due to the high percentage of homology (99.6% for the ITS-2 region) between the three species. The high level of homology in the ITS regions of *A. alternata* and *A. lini* is the same as that described by McKay *et al.* (1999). Nonetheless, the assay can be applied for identifying *A. alternata* in carrot material, which is known not to be infected by *A. lini* or *A. longipes*. The close relationship between *A. dauci* and *A. bataticola*, as shown by the high level of homology between the two species found in this study, was also observed by Kusaba & Tsuge (1995).

In this study primers were designed that were highly specific for *A. alternata*, *A. dauci* and *A. radicina*, and thus were able to differentiate between the three *Alternaria* species. Specific primers, which were derived from RAPD fragments, were recently described for detection of *A. radicina* (Pryor & Gilbertson, 2001). Zur *et al.* (1999) developed a PCR-based assay to detect *A. alternata* in food products, but the specificity of the primers was not tested against other *Alternaria* species and closely related genera such as *Stemphylium* and *Ulocladium*. Sequence differences found between the ITS regions were large enough to prevent the primers exhibiting nonspecific cross-reactions, neither with each other nor with a range of other carrot pathogens or *Alternaria* species. Indeed, *A. alternata* specific primers cross-reacted with one isolate of *A. lini* and one from *A. longipes*, but because *A. lini* and *A. longipes* have never been reported to occur on carrot this does not impose a practical problem. RAPD analysis of small-spored *Alternaria* species showed a small genetic distance between *A. alternata* and *A. longipes*, but the two species were still clearly distinguishable (Roberts *et al.* 2000). On the other hand, RAPD fragment pattern analysis efficiently reveals differences between a set of isolates rather than interspecific variabilities, and complex patterns of amplified products may cause difficulties in interpreting the results. Besides, the efficiency of the RAPD–PCR analysis, which is based upon the use of nonamer or decamer random primers, depends on the reproducibility of the technique. In our study we found that primers specific for *A. dauci* cross-

reacted with *A. bataticola*. Nevertheless, this does not restrict the application of the *A. dauci* primer set, because *A. bataticola* does not occur on carrot material. These results based on DNA sequences suggest that the taxonomy of *Alternaria* spp. may need revision, and is still not definitive (Simmons 1993).

In addition, the ability of the specific primers for *A. alternata* and *A. radicina* to detect each species in an extract from infected plant tissue (seeds or roots) was demonstrated. Sensitivity of the primers reported here indicate a detection limit of approximately 0.5–1 ng pathogen DNA in a background of 10–20 ng of total DNA isolated from plant tissue. PCR analysis of seed lots infected with *A. alternata* and *A. radicina* indicated a sensitivity of about 1 infected seed in 100 seeds (1 % seed infection). Pryor & Gilbertson (2001) obtained positive results for the detection of *A. radicina* for 0.1 % of infestation, but more seeds (1000 seeds) were used and seeds were incubated for a longer period (5 d) compared to our studies (100 seeds and 1 d incubation).

Sequence analysis of the cloned PCR products of DNA extracted from carrot material proved the high specificity of the *A. alternata* and *A. radicina* primers towards the target fungal DNA. The primercombination ADF2/ADR1, specific for *A. dauci*, failed to amplify DNA from infected carrot seeds probably due to the low infection levels of the samples tested. Whilst inhibition problems can never be completely ruled out, it is recommended to use target template DNA as a positive control in the PCR assay. The good reproducibility of the results from the three detection methods confirms the usefulness of such an approach, although an improvement of the PCR assay for *A. dauci* is needed. The rapidity of the PCR assay allows detection of the fungal species in the seeds much faster than the deep-freeze-blotter method or plating on ARSA. Comparison of these methods showed that carrot seeds may be screened for the presence of *Alternaria* species using PCR assay. This will also contribute to the early diagnostics of the potential presence of mycotoxins in the plant material.

A good correlation was observed between the percentage of infection established by the deep-freeze-blotter method and the intensity of the PCR- amplified product. Samples with the heaviest infections gave the strongest bands following PCR. The PCR approach, however, has the disadvantage that the quantification of fungal biomass is not easy. Although some PCR-based quantification methods have been described (Schilling *et al*. 1998, Nicholson *et al*. 1998), they are too laborious for routine analysis. Whatever PCR method is used, it does not distinguish between dead and living fungal cells. Although this may be a disadvantage for the detection of pathogenic micro-organisms in food, it is advantageous in the case of mycotoxigenic fungi, as mycotoxins are usually very stable. A positive PCR can therefore be taken as an indication that the sample potentially contains mycotoxins and should be analysed further.

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