Conventional and Real-Time PCR-Based Assay for Detecting Pathogenic *Alternaria brassicae* in Cruciferous Seed

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ABSTRACT

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Alternaria brassicae is an important seedborne pathogenic fungus responsible for the black spot disease of crucifers. Sanitary control of commercial seed is necessary to limit the spread of this pathogen. Current detection methods, based on culture and morphological identification of the fungus, are time consuming, laborious, and not always reliable. Therefore, a polymerase chain reaction (PCR)-based assay was developed with *A. brassicae*-specific primers designed on the basis of the sequence of two clustered genes potentially involved in pathogenicity. Two sets of primers were selected for conventional and real-time PCR, respectively. In both cases, *A. brassicae* was specifically detected using DNA extracted from seed. The real-time PCR-based method presented here can be automated easily and preliminary results indicate that it is efficient for quantitative estimation of seed infection.

Additional keywords: ABC transporter, molecular diagnostic, nonribosomal peptide synthase

The genus *Alternaria* comprises saprophytic and pathogenic species of filamentous fungi. Plant pathogens of this genus have a distinct and limited host range. It has been strongly suggested that both virulence and host specificity are partly dependent on the production of host-specific toxins (HSTs; 18,27). A complex of three species, *A. brassicae*, *A. brassicicola*, and *A. japonica* (formerly named *A. raphani*), is responsible for the black spot disease of crucifers and is transmitted by seed of many species belonging to the genera *Brassica* and *Raphanus*. The disease is of major importance in cultivated crucifers worldwide, with most commercial cultivars being susceptible (25). It occurs at all host plant growth stages, on aerial parts, and is identifiable by black lesions, often surrounded by chlorotic zones (26). The disease also can be destructive for seed producers because infection often leads to premature pod shatter and shriveled seed, with altered germination efficiency, as noted in various crucifer hosts such as *Brassica juncea*, *B. campestris*, and *B. rapa* (20,21). Black spot disease results in

a serious reduction of crop yields, such as reduced market quality of cauliflower heads and oil quality in family *Brassicaceae* oilseed species. The fungi overwinter on infected crop residues, seed, and any related cruciferous weed species. They can be seedborne via mycelia within the seed or transitory spores on the seed surface. Spores are readily windborne and can be dispersed great distances throughout the growing season.

Common practices to prevent the disease do not always provide satisfactory control of infection. Genetic control could be the most effective strategy, but most commercially available cultivars show little resistance to the pathogen. Black spot disease could be controlled with fungicides, but field isolates of *A. brassicicola* expressing cross-resistance to common broad-spectrum fungicides recently were identified in France (7). Rotations with noncruciferous crops, crop residue destruction, and weed control also can help to reduce the incidence of this disease. Moreover, the use of pathogen-free seed is essential to limit the spread and incidence of the disease. The current routine technique used to assess levels of pathogenic *Alternaria* spp. contamination in cruciferous seed is based on the culture of the seedborne fungi on nutritive media and further morphological characterization. This is a time-consuming process, whereby it takes at least 1 week to obtain a diagnostic result. Accurate diagnosis is not easy because numerous saprophytic *Alternaria* spp. commonly are found on cruciferous

seed (1), thus complicating pathogen identification. Some isolates sporulate sparsely or produce sterile mycelium under laboratory conditions. Morphological identification is impossible when this occurs.

Molecular approaches, mainly the polymerase chain reaction (PCR), frequently have been used as tools for the detection of numerous fungal pathogens (4,14,17). A sensitive and rapid PCR assay, based on amplification of sequences of the internal transcribed spacer (ITS) regions of the ribosomal DNA, recently was developed to detect *A. brassicicola* or *A. japonica* infection of cruciferous seed (8). Unfortunately, this method did not generate a reliable diagnosis when seed were contaminated with *A. brassicae* because cross-reactions with other fungal species sometimes were observed.

Johnson et al. (10) recently described the use of a PCR-based technique using primers designed from a gene involved in pathogenicity (synthesis of the host-specific AM-toxin) to specifically detect *A. alternata* apple pathotypes among *Alternaria* field isolates. Host specificity—typical of several pathogenic *Alternaria* spp.—is dependent mainly on toxin production; therefore, effective molecular probes could be developed for diagnostic techniques by cloning genes involved in virulence.

In the present study, we developed PCR assays for detecting *A. brassicae* in seed batches. These methods used oligonucleotides complementary to sequences located on a cluster of genes that may be required for the pathogenicity of the fungus. The specificity and suitability of the designed primers were tested using standard and real-time PCR.

MATERIALS AND METHODS

Fungal strains and culture conditions. Isolates of *Alternaria* spp. and other genera (Table 1) were recovered from seed. Cultures were grown and maintained in petri dishes on potato dextrose agar. *Alternaria* isolates were identified on the basis of morphology criteria (11,16). Identifications then were confirmed by PCR using specific ITS primer pairs (8) for *Alternaria* spp. pathogenic to crucifers.

Preparation of seed samples. Seed samples were prepared from contaminated seed of radish and cabbage as described by Iacomî-Vasilescu et al. (8). For artificial contaminations, seed were disinfected with

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1% sodium hypochlorite, rinsed with sterile water, soaked for 1 h in a calibrated spore suspension (10^6 spores/ml), and dried at room temperature on sterile filter paper. Disinfection and inoculation accuracy was confirmed by placing 20 seed on the surface of malt-agar plates for 1 week at 25°C. Seed batches showing 0% (disinfected seed) or 100% (inoculated seed) infection were selected and mixed together to prepare seed samples with different levels of contamination (0, 5, 10, 50, and 100%). Samples were produced by placing 20 seed (artificially contaminated) or 100 seed (naturally contaminated) in a sterile tube and covering them with 0.5 and 3 ml, respectively, of liquid culture MDP medium (2% malt extract, 2% dextrose, 0.1% peptone). The tubes were incubated at 25°C for 48 h with occasional shaking. This incubation step is an enrichment phase that allows an optimal increase of the fungal biomass from seed. The tubes then were vortexed briefly to separate mycelia and conidia from seed. The seed were discarded and the fungal structures were collected on microcentrifuge filters (pore size: 0.45 μ m; Ultrafree-Mc Millipore, Bedford, MA) by centrifugation at $5,000 \times g$ for 10 min.

DNA manipulation. DNA was extracted from fungal mycelia and conidia harvested by scraping the surface of petri plate cultures (extraction from fungal cultures) or collected on microcentrifuge filters (extraction from seed samples). Nucleic acids were isolated according to the microwave miniprep procedure described by Goodwin and Lee (6). Alternatively, DNA was extracted using the Nucleospin Food kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

PCR-based assay. Specific oligonucleotides ABCsens (5'-CTGGTGAAA-AGGTTGCGATCGT-3') and ABCrev (5'-GTGACTTTCATGAAATGACATTGATG-3'), complementary to the 3' end of open reading frame (ORF)2, and 115sens (5'-AACCCTATAGACCCACGTCGACTA-3') and 115rev (5'-GATGGTACGCAAGGCTTGGT-3'), complementary to a portion of ORF1, were designed for the standard and real-time PCR assays, respectively, using the appropriate software (Primer 3 online and ABI PRISM Primer Express). The two ORFs were deduced from the nucleotide sequence of an *A. brassicae* genomic DNA fragment identified after screening a cos-

mid library with a probe corresponding to a portion of the nonribosomal peptide synthase (NRPS) gene. In order to test the specificity of the primer pairs against *A. brassicae* isolates, amplifications were performed using DNA extracted from pure fungal cultures from a range of *Alternaria* spp. and other fungi isolated from seed. Then the PCR procedure was applied to detect seed contamination. The universal primer pair ITS1/ITS2 (28) was used as a positive control to assess the quality of the extracted DNA. Conventional PCR was performed using 2 μ l of undiluted DNA preparations under the following conditions: Tris-HCl, pH 9.0, 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01% (wt/vol) Tween 20, 1.5 mM MgCl_2 , 200 μ M each deoxyribonucleotide triphosphate, and 1 unit of thermostable DNA polymerase (Goldstar Red DNA polymerase, Eurogentec, Seraing, Belgium). A Thermojet thermocycler (Equibio, Seraing, Belgium) was used with an initial step of 3 min at 95°C; followed by 35 cycles of 30 s at 95°C, 50 s at 60°C, and 1 min at 72°C; and a final step of 10 min at 72°C. The amplification results were visualized after electrophoresis of an aliquot (10 μ l) of the reaction mixture on a

Fig. 1. Gel electrophoresis of polymerase chain reaction products obtained with internal transcribed spacer (ITS) 1/2 universal primers and with ABCsens/rev diagnostic primers. DNA was extracted from pure *Alternaria brassicae* cultures or from radish seed infected with *A. brassicae*, using the Nucleospin Food kit. Lanes M were loaded with a DNA ladder (SmartLadder Classic, Eurogentec, Seraing, Belgium).

1.2% agarose gel. The real-time PCR reactions were carried out in a 25- μ l final volume containing: 2.5 μ l of DNA, 12.5 μ l of SYBR Green PCR Master Mix 2X (Applied Biosystems, Courtaboeuf, France), 300 nM forward and reverse primers and H₂O up to 25 μ l. An ABI PRISM 700 Sequence Detection System (Applied Biosystems) was used with the following steps: 2 min at 50°C, 10 min at 95°C, and 40 cycles consisting in one step at 95°C for 15 s followed by one step at 60°C for 1 min. Nucleic acids were quantified in unknown samples by direct comparison to a standard. The DNA used as standard was extracted from fungal cultures, with the concentration measured by fluorometric assay (Turner Design Inst. 700, Sunnyvale, CA) before dilution.

RESULTS

Selection of the diagnostic PCR primers. Bacterial and fungal genes involved in toxin production have been used successfully to develop molecular diagnostic tools (10). Therefore, primer pairs were designed on the basis of the nucleotide sequence of a potential pathogenic cluster of two *A. brassicae* genes encoding an NRPS and an ATP-binding cassette (ABC) transporter. These genes putatively are involved in the synthesis and excretion of a toxic peptide metabolite produced by *A. brassicae* and, thus, may be suitable targets for PCR-based detection of this pathogen. We

expected to find homologous genes in related pathogenic *Alternaria* spp.; therefore, PCR screening of the *A. japonica* and *A. brassicicola* genomes was performed using primers that span the *A. brassicae* gene cluster. This preliminary approach revealed that the two *A. brassicae* ORFs have counterparts on the genome of other *Alternaria* spp. pathogenic to crucifers (*data not shown*). However, the central part and 3' end of the NRPS and ABC transporter, respectively, were found to be less conserved among the three species. Therefore, we focused on these regions to select primers for specific detection of *A. brassicae*. Two primer pairs were designed: one for standard PCR assays, called ABCsens/ABCrev, located near the 3' end of the ABC transporter gene coding sequence; and one for real-time PCR assays, called 115sens/115rev, located at approximately 10 kb of the NRPS gene start codon.

DNA extraction from seed. DNA was extracted routinely from fungal mycelia in pure cultures or from seed samples using a standard miniprep procedure. This technique involved lysis at high temperature in a detergent-containing buffer, extraction with organic solvents, and nucleic acid precipitation in the presence of alcohol. However, alternative extraction procedures using commercial kits were tested with the aim of designing a simple and safe diagnostic assay that could be automated to

process numerous samples. The Nucleospin Food kit was found to be the most efficient. Irrespective of the method used for isolating nucleic acids (i.e., from pure fungal cultures or seed samples), similar amplification patterns were obtained with both universal and specific primer pairs (Fig. 1). The DNA obtained with the Nucleospin Food kit also was tested successfully as template for real-time PCR (described below).

Specificity of the selected PCR primers. To test the specificity toward *A. brassicae* isolates, ABCsens/rev primers were first used in a PCR reaction with genomic DNA extracted from mycelial pure cultures of different fungal isolates (Table 1). These were selected to represent a range of fungi commonly found on cruciferous seed, including the three pathogenic *Alternaria* spp. *A. brassicicola* (five isolates), *A. japonica* (five isolates), and *A. brassicae* (five isolates), as well as saprophytic *A. alternata* isolates (two isolates), and isolates of *Fusarium* spp. (two isolates), *Penicillium* spp., *Aspergillus* spp., and *Phoma* spp. (one isolate each). Another seedborne *Alternaria* sp. (*A. dauci*) and isolates from the closely related genera *Ulocladium* and *Stemphylium* also were tested, along with one *Botrytis cinerea* strain isolated from sunflower seeds. A representative sample gel is shown in Figure 2. After optimisation of the cycling parameters, PCR products of the expected size (780 bp) were obtained from *A. brassicae* isolates only. No cross hybridization was observed with other cruciferous pathogenic *Alternaria* spp. (*A. brassicicola* or *A. japonica*; Fig. 2A and 2B), with nonpathogenic *A. alternata* or other tested fungi (Fig. 2C). Signals were obtained with all tested fungi when the universal ITS1-ITS2 primer pair was used as a control of DNA quality (*data not shown*).

The specificity of the real-time PCR primers was checked using 100 pg of DNA extracted from pure cultures of fungi listed in Table 1. All of the selected fungi originated from cruciferous seed. No increasing fluorescent signal exceeding the background fluorescence (baseline threshold) was observed except when *A. brassicae* DNA was used as template. In that case, standard fluorescent amplification curves representing an exponential growth of PCR products were recorded and a mean 26.65 threshold cycle (C_T) was obtained. Melting temperature profiles of the PCR products at the end of the cycling reactions were determined with samples containing *A. brassicae* DNA. A single dissociation peak of increased fluorescence was obtained for the specific 115sens-115rev primer at a melting temperature of 83 to 84°C (*data not shown*). This indicated that the specific amplification obtained did not involve cross hybridization with other *A. brassicae*-related sequences or the formation of primer dimers.

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

Species	Isolate no.	Origin	Detection by	
			conv-PCR ^a	Q-PCR ^b
<i>Alternaria brassicae</i>	Bre14	Radish seed	+	+
<i>A. brassicae</i>	Bre16	Radish seed	+	+
<i>A. brassicae</i>	Bre39	Radish seed	+	+
<i>A. brassicae</i>	Bre103	Radish seed	+	+
<i>A. brassicae</i>	Bre112	Radish seed	+	+
<i>A. brassicicola</i>	Bra3	Radish seed	–	–
<i>A. brassicicola</i>	Bra18	Radish seed	–	–
<i>A. brassicicola</i>	Bra40	Radish seed	–	–
<i>A. brassicicola</i>	Bra41	Radish seed	–	–
<i>A. brassicicola</i>	Bra43	Radish seed	–	–
<i>A. japonica</i>	Jap4	Radish seed	–	–
<i>A. japonica</i>	Jap19	Radish seed	–	–
<i>A. japonica</i>	Jap52	Radish seed	–	–
<i>A. japonica</i>	Jap63	Radish seed	–	–
<i>A. japonica</i>	Jap108	Radish seed	–	–
<i>A. alternata</i>	Alt62	Radish seed	–	–
<i>A. alternata</i>	R7404	Cabbage seed	–	–
<i>A. dauci</i>	R9228	Cress seed	–	nt
<i>Ulocladium chartarum</i>	Ulo75	Radish seed	–	–
<i>Stemphylium botryosum</i>	C27381	Cabbage seed	–	–
<i>S. botryosum</i>	R7410	Cabbage seed	–	–
<i>Botrytis cinerea</i>	BC40	Sunflower seed	–	nt
<i>Fusarium</i> sp.	Fu6	Radish seed	–	–
<i>Fusarium</i> sp.	Fu7	Radish seed	–	–
<i>Phoma</i> sp.	Pho1	Radish seed	–	–
<i>Penicillium</i> sp.	P1	Cabbage seed	–	–
<i>Aspergillus</i> sp.	A2	Cabbage seed	–	–
<i>Verticillium</i> sp.	V1	Tomato seed	–	nt

^a conv-PCR refers to conventional polymerase chain reaction (PCR) with the ABCsens-ABCrev primer set.

^b Q-PCR refers to real-time PCR with the 115sens-115rev primer set; + = amplification, – = no amplification, and nt = not tested.

PCR detection in seed samples. In a preliminary experiment, the PCR detection test was applied to radish and cabbage seed batches artificially contaminated with *A. brassicae* (isolate Bre112). DNA was extracted from the different seed samples and used as a template in PCR experiments with either the ABCsens-ABCrev primer pair (standard assays) or the 115sens-115rev primer pair (real-time PCR). In the latter case, parallel experiments with serial dilutions of a calibrated *A. brassicae* DNA extract were carried out. *A. brassicae* was detected using the conventional PCR procedure in all contaminated seed batches (Fig. 3), even at contamination levels as low as 5% (Fig. 3B). As expected, a signal also was observed with the positive control (DNA extracted from a mycelial pure culture of the *A. brassicae* isolate) but not with the negative control (disinfected seed). Amplifications using the universal ITS1/ITS2 primer pair were successful with all samples, indicating that the extracted DNA was suitable for PCR (Fig. 3A). No fungal growth was observed with disinfected seed plated on solid growth medium; therefore, the signal obtained with these seed after amplification with the nonspecific primers probably originated from co-extracted host plant DNA.

When real-time PCR was used with serial dilutions of an *A. brassicae* DNA calibrated extract as template, a standard curve was plotted using known amounts of *A. brassicae* DNA against the C_T calculated with ABI Prism 7000 sequence detection system (SDS) software (Fig. 4). This curve revealed that the primer set used in this experiment was quite accurate over a linear range of at least 3.5 orders of magnitude and that the correlations between C_T and DNA quantities were high ($R^2 = 0.986$). The mean C_T values obtained with 100 and 10% artificially contaminated radish seed batches were 25.56 and 32.55, respectively, corresponding to approximately 250 and 3.5 pg of *A. brassicae* DNA, respectively.

These results were obtained with artificially contaminated seed free of other fungal or bacterial contaminations. It was then necessary to check this molecular diagnosis approach with cabbage and radish seed naturally contaminated with pathogenic *Alternaria* spp. Six different seed lots were tested (Table 2). Infection levels were determined on 200 seed using the standard plating technique. Amplifications using the nonspecific ITS1-ITS2 primer pair were successful with all DNA extracted from samples, indicating that this DNA was suitable for further amplifications (Fig. 5B). When the ABCsens/rev primer pair was used, *A. brassicae* contaminations up to 3% (seed lot D) were detected (Fig. 5A). No amplification signal was obtained with DNA extracted from the A, B, and C seed lots.

Real-time PCR was carried out with samples prepared with the six radish seed lots. Amplification curves were obtained only with DNA extracted from lots C to F. Similar C_T values (mean: 32.54) were noted for seed lots E and F (Fig. 4). This mean value is close to that obtained with the 10% artificially contaminated seed batch. The C_T value for seed lot C, with an *A. brassicae* infection level of 2%, was 34.28.

DISCUSSION

Conventional tests used to detect *A. brassicae* in seed involve a combination of pathogenicity assays and morphological characterization. However, the drawback of such methods is that accurate pathogen identification can be difficult and time-consuming. Hence, there is an urgent need for a sensitive and rapid test that could

detect seedborne *A. brassicae*. With advances in molecular biology, most recently developed techniques for microorganism detection involve PCR analysis. The advantages of PCR-based assays include specificity, sensitivity, and speed. Hence, such assays have been designed for the detection of various plant pathogens, including seedborne fungi (22,24). A few PCR-based methods have already been reported for the detection of *Alternaria* spp. on carrot (12,19), linseed (15), and cruciferous (8) seed, as well as in host tissues (15) or food products (32). The ITS region of nuclear rDNA (8,12,15,32) is the main genomic region targeted for PCR primer development. Recently, Iacomi-Vasilescu et al. (8) reported a sensitive and rapid PCR diagnostic assay for the identification of *A. brassicicola* and *A. japonica* on cruciferous seed. Nevertheless, the ITS

Fig. 2. Gel electrophoresis of polymerase chain reaction products obtained with the ABCsens/rev diagnostic primers and DNA extracted from pure cultures of various *Alternaria brassicae* isolates or from pure cultures of **A**, *A. brassicicola* isolates or **B**, *A. japonica* isolates or **C**, isolates from different fungal species recovered from seed. Numbers above the gels correspond to isolate identification codes as described in Table 1. M lanes were loaded with a DNA ladder (SmartLadder Classic, Eurogentec, Belgium).

primers designed in this study for *A. brassicae* were found to be not very specific because amplification signals were consistently obtained with DNA from some *A. japonica* isolates.

In the present study, we used two primer sets, highly specific to *A. brassicae*, designed from two clustered genes corresponding to an NRPS gene and an ABC transporter gene. These two genes may produce and secrete factors associated with the virulence of the fungal pathogen (i.e., host-specific peptidic toxins or siderophores).

The conventional PCR-based seed assay that we developed specifically detects the presence of *A. brassicae* in cruciferous seed, even at infection levels as low as 3% and after only 2 days of incubation. The first major obstacle that we had to overcome was the low levels of target DNA from the fungi. An incubation of 48 h in MDP liquid nutritive medium was suitable to obtain an optimal increase of the fungal biomass (8); therefore, the amount of fungal genomic DNA recovered generally was sufficient for amplification of the target

Fig. 4. Standard curve of *Alternaria brassicae* DNA concentration standards against the cycle threshold (C_T). C_T s were determined, using real-time PCR and 115sens/rev diagnostic primers, as the cycle at which the fluorescent signal exceeded background fluorescence. C_T s obtained with DNA extracted from 100% (Δ) and 10% ($\square$) artificially contaminated radish seed or 10% naturally infected radish seed ($\blacksquare$) are indicated on the plot.

Fig. 3. Gel electrophoresis of polymerase chain reaction products obtained with internal transcribed spacer (ITS) 1/2 universal primers or ABCsens/rev diagnostic primers and DNA extracted from seed artificially infected with *Alternaria brassicae*. Seed contamination levels are indicated above the gels. Lanes labeled T+ were loaded with DNA extracted from a pure culture of *A. brassicae* and lanes labeled M were loaded with a DNA ladder (SmartLadder Classic, Eurogentec, Seraing, Belgium).

DNA sequences. The nutritive medium used for macerating seed was chosen on the basis of our previous observations. Two other liquid incubation media (i.e., clarified V8 broth and CW; 30), also were tested by Iacomi-Vasilescu et al. (8). Similar results were obtained with MDP or V8 media, but MDP was preferred because of easier handling during a subsequent filtration step, whereas no signal was obtained with the CW medium. PCR inhibitors

represent another obstacle commonly encountered when conducting assays using complex samples (3,9). These inhibitors often have seed component origins (19). In this study, the seed were removed after incubation and prior to DNA extraction and fungal mycelia and conidia were collected using a spin filter unit. For technical reasons (e.g., size of the spin filter), we used seed batches containing only 100 seed. Thus, infection levels lower than 3%

were not detected with good repeatability. In the original PCR diagnostic method described for *A. japonica* and *A. brassicicola* (8), phenol-chloroform extraction followed by an isopropanol precipitation step were sufficient to obtain a crude DNA preparation that subsequently could be used as template for amplification without any further treatment. In the present work, we demonstrated that this DNA isolation procedure could be replaced successfully

Table 2. Infection level (%) of seed lots naturally infected with pathogenic *Alternaria* spp. used in the present study

Fungal species	Infection level (%) of seed lot number					
	A (cabbage)	B (radish)	C (radish)	D (radish)	E (radish)	F (radish)
<i>Alternaria brassicae</i>	0	0	2	3	9.5	10
<i>A. brassicicola</i>	8	0	0	0	0	0
<i>A. japonica</i>	0	1	0	0	0	0
<i>A. alternata</i>	19	39	11	38.5	27	13.5
<i>Fusarium</i> sp.	2	0	0	1	0	0.5
<i>Phoma</i> sp.	1	0	0	1.5	0	0

Fig. 5. Gel electrophoresis of polymerase chain reaction products obtained with **A**, ABCsens/rev diagnostic primers or **B**, internal transcribed spacer (ITS) 1/2 universal primers and DNA extracted from seed. Samples were prepared with seed naturally infected with *Alternaria* spp. from seed lots A to E. Lanes labeled T+ were loaded with DNA extracted from a pure culture of *Alternaria brassicae* and lanes labeled M were loaded with a DNA ladder (SmartLadder Classic, Eurogentec, Seraing, Belgium).

by treating the fungal mycelium with a lysis buffer initially designed for processing food samples, and purifying DNA using an affinity-based method with silica membranes from a commercial kit. This DNA extraction procedure potentially could be used for routine analysis of seed lots and is amenable to automation. In the same vein, routine analyses require an alternative method for electrophoretic separation of the amplification products. Our preliminary results with real-time PCR using SYBR green are encouraging. This fluorescent reporter dye binds double-stranded DNA; therefore, increasing fluorescence may be monitored continuously during the amplification procedure, thus eliminating the need for post-PCR analysis. Real-time PCR assays have been implemented for the detection of fungal plant pathogens in several recent studies (2,5,13,29,31). This technique should soon be useful for seed health testing, and Taylor et al. (23) already have used it for the detection of *Microdochium nivale* in wheat seed. We showed here that rapid and specific detection of *A. brassicae* is possible using real-time PCR in the presence of SYBR green with a primer set designed from an NRPS gene sequence. This primer set gave satisfactory results with a wide range of target DNA concentrations, thus allowing accurate quantification of seed infection levels. We obtained similar C_T values with DNA extracted from naturally contaminated seed (10% infection, as estimated by the standard plating method) or from seed artificially infected with *A. brassicae* at a level of 10%. Real-time PCR was found to be more sensitive than the standard amplification procedure, and a successful amplification was obtained with a seed lot with 2% *A. brassicae* infection. According to the Internal Seed Testing Association, this corresponds to the maximal level of infection of commercial cruciferous seed by pathogenic *Alternaria* spp.

The PCR diagnosis method described here, which combines DNA extraction from seed using the Nucleospin Food kit and real-time PCR in the presence of SYBR green and primers designed from an NRPS gene, is readily amenable to automation. With this method, after approximately 50 h, *A. brassicae* can be accurately and sensitively detected in infected seed. Homologous sequences have been identified in the *A. japonica* and *A. brassicicola* genomes; therefore, the same method also may be applied for the detection of these two seed pathogens, provided that relevant primers are designed. Moreover, although many tests probably will be necessary to accurately correlate the amount of DNA extracted from infected seed and their level of infection (particularly when testing seed lots with very low infection levels), it should be

possible to further develop this method to make it quantitative.

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