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PCR detection and identification of *Alternaria* species-groups in processed foods based on the genetic marker *Alt a 1*

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ABSTRACT

In this study, a PCR method based on oligonucleotide primers targeting the *Alt a 1* gene has been developed for the rapid detection of DNA from *Alternaria* spp. and for identification of *Alternaria alternata, Alternaria porri, Alternaria radicina,* and *Alternaria infectoria* species-groups in raw materials and processed food products. The assay design consists of two steps. First, a duplex PCR using primers Dir5cAlta1–Inv4Alta1 (that amplifies a specific DNA fragment of approximately 195 bp in all *Alternaria* spp.) and 18Sfweu–18Srveu (that amplifies a conserved 99 bp fragment on all the eukaryotic species), allows detection of *Alternaria* spp. DNA in foodstuffs with a high sensitivity and specificity. As a second step, identification of *Alternaria* species-groups is obtained through a seminested PCR method, without the need for sequencing PCR products. The specificity of the primer pairs designed was verified by PCR analysis of DNA from various *Alternaria* cultures, and also from several non target species. The detection limit of the method was approximately 10^2 CFU/ml, either in viable culture, heat inactivated culture or inoculated tomato pulp. Nevertheless, a sensitivity of 10^3 CFU/ml was obtained for tomato pulp inoculated with *A. alternata* or *A. porri* cultures heat inactivated at 90 °C for 5 min. PCR analysis of commercial foodstuff samples demonstrated the presence of DNA from *A. alternata* species-group in 100% of spoiled tomato samples, and 8% of tomato products, whilst 36.4% of cereal based infant food samples analyzed contained DNA from *A. infectoria* species-group.

1. 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, Bottalico, Mulé, Moretti, & Perrone, 2003; Peever, Su, Carpenter-Boggs, & Timmer, 2004). Certain species are also capable of producing mycotoxins and pose a health hazard as they can contaminate raw or manufactured plant products like juices, sauces and preserves, thereby entering the human food chain (Ostry, 2008). Moreover, the genus *Alternaria* is considered to be one of the most prolific producers of fungal allergens. In particular, Alt a 1, the major allergen produced by *Alternaria* spp., has been associated with asthma, and recently, sensitivity to fungal allergens was shown to be a risk factor for life-threatening asthma (Cramer & Lawrence, 2003; Hong, Cramer, Lawrence, & Pryor, 2005; Hong, Maccaroni, Figuli, Pryor, & Belisario, 2006).

The classification of *Alternaria* species is very complex. Phylogenetic studies support grouping *Alternaria* species in several species-groups (Chou & Wu, 2002; Hong et al., 2005): 1) *A. alternata* species-group includes species like *A. alternata, Alternaria arborescens, A. tenuissima, A. gaisen, A. citri*, and *A. longipes*. They produce mycotoxins like alternariol (AOH), alternariol methyl ether (AME) and tenuazonic acid (TeA) (Andersen, Kroger, & Roberts, 2001, 2002) and are responsible for extensive deterioration of plants and plant products, leading to considerable economic losses to growers and the food processing industry; 2) *A. porri* species-group includes species like *A. solani, A. porri, Alternaria dauci*, and *A. tomatophila*, that are responsible of foliar blights of carrots, onions, potatoes, and tomatoes and produce mycotoxins as AOH, altertoxins and alternariol (Andersen, Dongo, & Pryor, 2008); 3) *Alternaria radicina* species-group is considered one of the most important seed-borne pathogen on carrot and produce phytotoxic compounds as radicinin and radicinol (Konstantinova, Bonants, van Gent-Pelzer, van der Zouwen, & van den Bula, 2002; Solfrizzo et al., 2005; Tylkowska, 1992); and 4) *Alternaria infectoria* species-group affects cereal crops in the field and during storage, and produce metabolites like infectopyrones and novae-zelandins that are not found in other *Alternaria* species-groups (Andersen, Sorensen, Nielsen, van den Ende, & de Hoog, 2009; Christensen et al., 2005).

The current routine technique for detection and identification of *Alternaria* spp. often requires culture isolation and further morphological and physiological characterization (Simmons, 2007).

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This process is tedious and time-consuming, whereby it takes at least days to weeks to obtain a diagnostic result. Moreover, because of the heat treatments used in food processing, viable microflora counts in the processed foodstuffs are extremely low, and traditional plating methods for the detection of microorganisms cannot be used (Zur, Shimoni, Hallerman, & Kashi, 2002). DNA methods, mainly based on the polymerase chain reaction (PCR), offer alternative approaches for detection of viable and not viable pathogenic and spoilage microorganisms in food. These methods have the advantages that the structure of DNA remains stable with physiological stages, and its composition does not depend on culture conditions. Moreover, DNA has regions with different degrees of variability that allow species-specific detection or the identification of wider taxonomic groups. A crucial requirement for successfully detecting specific microorganisms with a PCR assay is to choose adequate genetic markers during the primer design process that allow a high degree of specificity (Scheu, Berghof, & Stahl, 1998). The genetic markers used with this purpose are the internal transcribed spacers, ITS1 and ITS2 (Chou & Wu, 2002; Konstantinova et al., 2002; Zur et al., 2002), and protein-coding genes such as glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and *Alt a 1* (Hong et al., 2005).

Alt a 1 gene encodes the major allergen produced by *A. alternata,* and *Alt a 1* gene homologs have been described in other species of the genus *Alternaria* (Hong et al., 2005). A comparison between *Alt a 1* homologous of several *Alternaria* species revealed greater sequence divergence than that found in similar comparisons of other ribosomal and protein-coding genes, such as the internal transcribed spacer (ITS), mitochondrial small subunit (mt SSU) rDNA, and glyceraldehyde-3-phosphate dehydrogenase (*gdp*) (Cramer & Lawrence, 2003; Hong et al., 2005; Pryor & Bigelow, 2003). The objective of this work was to develop PCR methods, based on the genetic marker *Alt a 1* for detection and identification of *Alternaria* species-groups in raw and processed foodstuffs.

2. Material and methods

2.1. Fungal strains and culture conditions

A total of 27 fungal strains were obtained from the CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), the CECT (Coleccion Española de Cultivos Tipo, Valencia, Spain), the BCCM/MUCL (Mycothèque de l'Universite catholique de Louvain, Louvain-la-Neuve, Belgium) and our own collection (Table 1). They were grown on Potato Dextrose Agar medium (PDA, Sigma, St. Louis, MO, USA), Malt Extract Agar (MEA) and Potato Carrot Agar (PCA, Sigma) for 7 days at 25 °C (Pitt & Hocking, 1997). Identification of *Alternaria* cultures was confirmed according to Simmons (2007). To determine the detection limit of the assay, *A. alternata* CBS 117143, *A. porri* CBS 109.41, *A. radicina* CBS 245.67 and *A. infectoria* CBS 210.86 were grown in malt extract broth for 4 days at 25 °C. Serial dilutions were used for DNA extraction and plated on Sabouraud-CAF (Liofilchem s.r.l., Roseto degli Abruzzi, Italy). Twenty milliliters of the *Alternaria* malt extract cultures were heated at 90 °C for 5 min and 20 ml at 60 °C for 30 min, to analyze the influence of thermal treatments on the assay performance.

2.2. DNA extraction

DNA was extracted from the fungal cultures grown in MEA, PDA and malt extract broth using the method described by Marek, Annamalai, and Venkitanarayan (2003). Briefly, the mycelium was resuspended in 500 μl of extraction buffer (Tris–HCl 200 mM pH 7.5, SDS 0.5% w/v, EDTA 25 mM, NaCl 250 mM) and 300 μl 3 M Na acetate. One hundred milligrams of 150–212 μ Glass Beads Acid-Washed (Sigma) was added, and the mixture was vigorously agitated using a Vortex for 10 min. The resultant lysate was treated with 50 μl of 2 mg/ml proteinase K solution (Merck, Damstadt, Germany), and incubated for 30 min at 37 °C in a water bath. Following incubation, 100 μl of 1.5 M NaCl was added to the lysate and mixed. After incubation for 5 min at room temperature, the mixture was centrifuged at 16,000*g* to separate out insoluble cell debris. The supernatant was transferred into a new tube and was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated by adding 0.6 volume of isopropanol and incubating at −20 °C for 1 h. The DNA was pelleted, washed with 70% ethanol, allowed to air dry, and finally resuspended in 100 μl of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Thermo Fisher Scientific, Wilmington, DE).

DNA integrity was checked by agarose gel electrophoresis in a 1.5% low electroendosmosis D1 agarose gel (Pronadisa, Torrejón de Ardoz, Madrid, Spain) containing 1 μg/ml ethidium bromide in Tris–acetate buffer (TAE, 0.04 M Tris–acetate and 0.001 M EDTA, pH 8.0). Electrophoresis was performed at 25 °C, 85 V for 1 h.

Alternatively, DNA extraction was performed using the Wizard DNA Clean-up System (Promega Corp., Madison, WI). Briefly, two hundred milligrams of sample were homogenized with 860 μl of extraction buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% SDS, pH 8.0), 100 μl of 5 M guanidine hydrochloride, and 40 μl of

Table 1
Sources of the fungal isolates used in this study and the accession numbers of their *Alt a 1* gene partial sequences.

Fungal	Strain	Host	GenBank–EMBL accession number
Alternaria alternata	CBS 154.31	*Staphylea trifolia*	
	CBS 117130	Potted strawberry tree (*Arbutus unedo*)	FN689396
	CBS 117143	*Capsicum annuum*, fruits	FN689397
A. arborescens	CBS 109730	Tomato	FN689398
	MAP05[a]	*Triticum*, grain	FN689414
A. citri	CBS 192.81	*Citrus sinensis*, fruit	FN689399
A. dauci	CBS 101592	Carrot seeds	FN689400
A. gaisen	CBS 632.93	Leaf spot in *Pyrus pyrifolia*	FN689401
A. infectoria	CBS 210.86	*Triticum*	FN689402
	MAP03[b]	*Triticum*, grain	FN689415
	MAP04[b]	*Triticum*, grain	FN689416
	MAP08[b]	*Triticum*, grain	FN689417
A. longipes	CBS 917.96	*Nicotiana tabacum*	FN689403
A. porri	CBS 109.41	–	FN689404
A. radicina var. radicina	CBS 245.67	*Daucus carota*	FN689405
A. solani	CBS 347.79	Fruit rot in *Lycopersicon esculentum*	FN689406
A. tenuissima	CBS 880.95	*Fragaria vesca*	FN689407
	MAP01[c]	*Triticum*, grain	FN689408
	MAP02[c]	*Triticum*, grain	FN689409
	MAP06[c]	*Triticum*, grain	FN689410
	MAP07[c]	*Triticum*, grain	FN689411
	MAP09[c]	*Triticum*, grain	FN689412
	MAP10[c]	*Triticum*, grain	FN689413
Aspergillus ochraceus	MUCL 14207	Cotyledon, Coffea robusta	
Fusarium oxysporum	CECT 2866	Tomato	
Penicillium expansum	CECT 2278	Mould-fermented sausage	
Rhizopus stolonifer	CECT 2344	–	

[a] Member of the *A. arboresecens* species-group based on morphology.
[b] Member of the *A. infectoria* species-group based on morphology.
[c] Member of the *A. tenuissima* species-group based on morphology.