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Specific detection of the toxigenic species *Fusarium proliferatum* and *F. oxysporum* from asparagus plants using primers based on calmodulin gene sequences

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Abstract

Fusarium proliferatum and *Fusarium oxysporum* are the causal agents of a destructive disease of asparagus called *Fusarium* crown and root rot. *F. proliferatum* from asparagus produces fumonisin B_1 and B_2, which have been detected as natural contaminants in infected asparagus plants. Polymerase chain reaction (PCR) assays were developed for the rapid identification of *F. proliferatum* and *F. oxysporum* in asparagus plants. The primer pairs are based on calmodulin gene sequences. The PCR products from *F. proliferatum* and *F. oxysporum* were 526 and 534 bp long, respectively. The assays were successfully applied to identify both species from the vegetative part of the plants.

Keywords: *Fusarium proliferatum*; *Fusarium oxysporum*; Asparagus; Calmodulin gene

1. Introduction

Fusarium oxysporum f. sp. *asparagi* and *Fusarium proliferatum* (Matsushima) Nirenberg (*Gibberella intermedia* Kuhlman) are the most severe pathogens of asparagus (*Asparagus officinalis* L.) causing *Fusarium* crown and root rot [1]. These pathogens can be transmitted by seed and can colonize both vascular and epidermal tissues at the same time [2]. Moreover, both *F. proliferatum* and *F. oxysporum* can produce toxins, although with a different profile. This variability could lead to the accumulation of different toxins in infected asparagus tissues, according to the specific degree of contamination by each species [3,4]. In particular, fumonisins, potent carcinogenic toxins associated with both human and animal diseases [5], and fusaproliferin, reported to be a teratogenic metabolite[6], are produced only by *F. proliferatum* [7,8], while beauvericin, a metabolite that induces apoptosis in human lymphocyte cells [9], and moniliformin, a cardiotoxic metabolite causing cardiac failure in broiler chickens [10], are produced by both *F. proliferatum* and *F. oxysporum* [8,11,12]. Therefore, correct identification on infected asparagus plants is of great importance, because each species poses different toxicological risks. Moreover, it is quite common for symptomless parts of the asparagus plant destined for human consumption to be contaminated by toxigenic *Fusarium* species [1]. Consequently, early identification of these infectious species could provide important specific diagnostic tools for the prevention of pathogenic and toxigenic risks arising from *Fusarium* infection. Standard methods for the analysis of *Fusarium* contamination in asparagus comprise microbiological investigation of samples on selective substrates and require specialists in order to distinguish between the two species. However, this procedure is time-consuming and only viable mycelia can be detected.

Alternative molecular diagnostic tools have been developed in the last decade. Polymerase chain reaction (PCR)-based techniques can provide species-specific primers, and are a powerful diagnostic method for distinguishing fungal taxa at a species level [13], both from pure fungal cultures and from plants or food. Moreover, PCR techniques reduce detection times drastically, are accurate and sensitive, and do not require high expertise or expensive equipment. PCR assays have been reported to detect several pathogenic and mycotoxigenic fungi, including *Fusarium* species.

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Species-specific primers for use in PCR assays have been generated from sequences of diverse origin: RAPDs [14], the internal transcribed spacer regions of rDNA [15], mycotoxin biosynthetic genes (trichothecenes) [16,17]. Other gene sequences such as the calmodulin gene, used by O'Donnell et al. [18] for their molecular phylogenetic analysis of *Gibberella fujikuroi* [Sawada] Ito in Ito and K. Kimura complex and related *Fusarium* species, could be used to develop specific primers for the detection of several phytopathogenic fungi. Moreover, while several reports on the development of species-specific primers successfully applied to pure fungal cultures have been published, little work has been done on detecting fungi in food or plant parts using PCR-based techniques, with most of it being devoted to the detection of the main toxigenic fungal contaminants in cereals [19–21].

The objectives of this study were (i) to develop species-specific primers for *F. proliferatum* and *F. oxysporum* members isolated from asparagus using a partial calmodulin gene sequence and (ii) to adapt a PCR assay to detect the DNA belonging to either species from different parts of asparagus plants.

2. Materials and methods

2.1. Fungal strains

The fungal strains sequenced were [species, strain number(s), accession number(s)] as follows: *F. proliferatum* ITEM 1475 (AJ560771), ITEM 1476 (AJ560772), ITEM 1477, ITEM 1478, ITEM 1480, ITEM 2365, ITEM 2366, ITEM 2368, ITEM 2369 from various localities in the Southern Italian region of Apulia, ITEM 1484 (AJ560773) from the nearby Basilicata region; *F. oxysporum* f. sp. *asparagi* ITEM 3351 (IPO 97/14) from The Netherlands, ITEM 3353 (NRRL 28378) from France, ITEM 3358 (NRRL 28398) from Northern Italy, ITEM 3361 (NRRL 28372) from Maryland, USA, ITEM 3362 (NRRL 28384) from New Jersey, USA, ITEM 3364 (NRRL 28379) from Massachusetts; *F. oxysporum* (these strains were isolated from asparagus plants but not tested for their pathogenicity) ITEM 1461, ITEM 1462, ITEM 1464 from Basilicata, and ITEM 2367 (AJ560774) from Apulia. The strains were from Plant Research International, Wageningen, The Netherlands (IPO); North Regional Research Laboratory, Peoria, USA (NRRL), Institute of Sciences of Food Productions, Bari, Italy (ITEM). Further information on the strains (year of isolation, depositor, toxin production, etc.) are available from the ITEM web site: http://www.ispa.cnr.it/Collection.

2.2. Mycological analyses

Ten asparagus plants with no symptoms of *Fusarium* wilt were surface sterilized in 0.5% NaOCl for 3 min, rinsed with sterile distilled water and placed on the surface of Petri plates (five 3-cm-long pieces per plate) with a *Fusarium*-selective agar medium containing pentachloronitrobenzene [22]. The plates were incubated in the dark at 25°C for 1 week. The *Fusarium* colonies were transferred to plates of potato dextrose agar and were incubated at 25°C for 10 days, under fluorescent and black light lamps (2700 lx; 12 h photoperiod). The colonies of *Fusarium* species were identified according to the criteria and synoptic keys of Nelson et al.'s [22].

2.3. DNA extraction from fungal cultures

For the extraction of genomic DNA, fungi were grown in liquid Wikerman media (40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract and water up to 1 l) and shaken at 150 rpm. Mycelia were harvested by filtration onto paper filters, frozen, and lyophilized. Total genomic DNA of fungal strains was isolated from dried mycelium (~40 mg) using the 'E.Z.N.A. Fungal DNA Miniprep Kit (Omega Bio-tek, Doraville, USA)', according to the manufacturer's protocol. DNA was recovered and dissolved in sterile water. Concentrations of DNA were determined by gel electrophoresis, by measuring the ultraviolet-induced fluorescence emitted by ethidium bromide molecules intercalated into DNA, and comparing the fluorescent yield of the samples with a standard.

2.4. Amplification and sequencing of fungal DNA and primer design

Amplifications of partial calmodulin genes were set up with 2.5 U of *Taq Gold* DNA polymerase (Applied Biosystems) in 100 μl reaction mixtures, containing 30 pmol of each outside primer, 12.5 μM of each dNTP (Applied Biosystems), and 1 μl (approximately 10 ng) of fungal template DNA. All isolates were amplified using primers CL1 and CL2A [18]. PCR conditions were: 5 min at 94°C; 50s at 94°C, 50 s at 55°C, 1 min at 72°C for 35 cycles; 7 min at 72°C followed by cooling at 4°C. PCR products were purified by agarose gel electrophoresis; DNA bands were excised from agarose gel and loaded onto spin columns (Gene Elute Agarose spin columns; Sigma). Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All of the sequencing reactions were purified by gel filtration through columns containing Sephadex G-50 (Pharmacia) equilibrated in double-distilled water and were analyzed on the 310 Genetic Analyzer (Applied Biosystems). The resulting calmodulin region sequences of all the isolates were aligned by the cluster method with the DNAMAN program (Lynnon BioSoft). In the alignment, two sequences from the EMBL Data Bank: AF158365 and AF346507 were also used. Species-specific primers were designed using the

Table 1
Sequences of oligonucleotide primers designed within the calmodulin gene

Primer name	Primer sequence	Species specificity
CLPRO1	5′-TGCATCAGACCACTCAAATCCT-3′	*F. proliferatum*
CLPRO2	5′-GCGAGACCGCCACTAGAT-3′	
CLOX1	5′-CAGCAAAGCATCAGACCACTATAACTC-3′	*F. oxysporum*
CLOX2	5′-CTTGTCAGTAACTGGACGTTGGTACT-3′	

Primer Express software (Applied Biosystems). Primers were chosen from divergent regions, within the calmodulin-encoding sequence, resulting from the multiple alignment of sequences obtained in this study. The species-specific primers designed are listed in Table 1. PCR reactions for the two species were performed in different tubes, because specific primer pairs amplify fragments similar in length, i.e. 526 bp (*F. proliferatum*) and 534 bp (*F. oxysporum*). PCR products were resolved in 2% TAE-agarose gel and were visualized with EtBr and ultraviolet illumination. Images were captured and stored using an EasyShare DX3215 Zoom Digital Camera (Kodak, Rochester, NY, USA). The PCR containing species-specific primers were set up with 1.25 U of *Taq Gold* DNA polymerase (Applied Biosystems) in 50μl reaction mixtures, containing 15 pmol of each outside primer, 12.5 μM of each dNTP (Applied Biosystems), and 1 μl (approximately 10 ng) of fungal template DNA. PCR conditions were: 5 min at 94°C; 50s at 94°C, 50s at 60°C, 1 min at 72°C for 35 cycles; 7 min at 72°C followed by cooling at 4°C. Amplification products were checked on 2% agarose gel stained with ethidium bromide.

2.5. DNA extraction from asparagus plants

Total genomic DNA from asparagus potentially infected by *Fusarium* was isolated from small pieces of asparagus turions, efficiently ground according to the protocol used by Simpson et al. [20] for cereal grains. Briefly, the turions were cut, lyophilized, and ground in a small mortar and 4 g of powder was extracted for the total genomic DNA. The PCR assays containing species-specific primers were set up with 1.25 U of *Taq Gold* DNA polymerase (Applied Biosystems) in 50μl reaction mixtures, containing 15 pmol of each outside primer, 12.5 μM of each dNTP (Applied Biosystems), and 10 μl of total DNA (from possibly contaminated plants). Reactions were performed as above for species-specific primers. Amplification products were checked on 2% agarose gel stained with ethidium bromide.

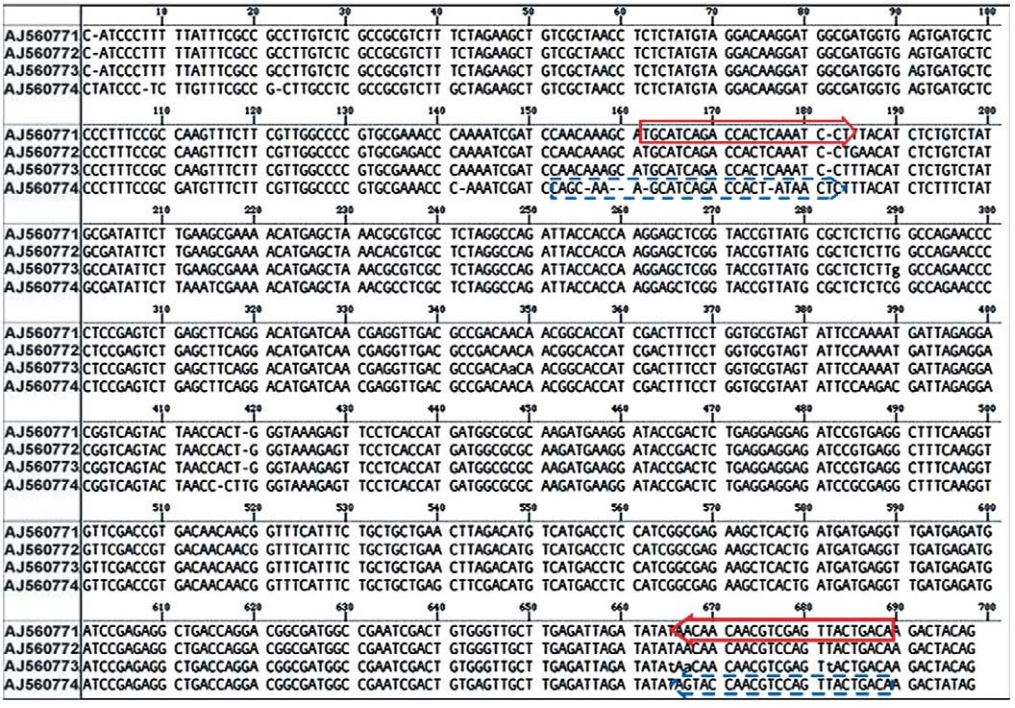

Fig. 1. Full alignment of the partial calmodulin gene from *F. proliferatum* strains (AJ560771, AJ560772, AJ560773) and *F. oxysporum* strains (AJ560774). Unbroken arrows indicate CLPRO1/2 primers specific for *F. proliferatum* species, while broken arrows indicate CLOX1/2 primers specific for *F. oxysporum*. The two primers pairs are designed in the intron regions.

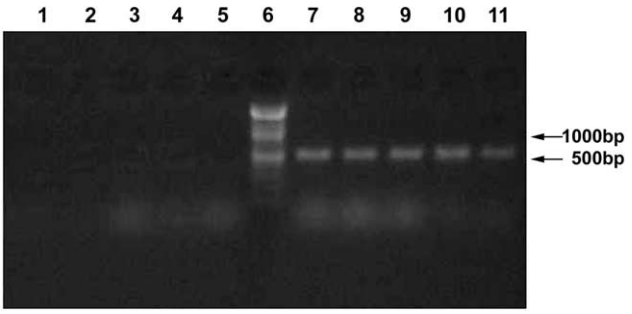

Fig. 2. Lanes 1–5 and lanes 7–11: PCR amplifications of five *F. oxysporum* strains (ITEM 3351, ITEM 3353, ITEM 3358, ITEM 3361, ITEM 3362) with specific primers for *F. proliferatum* and *F. oxysporum* respectively; lane 6: DNA size marker (500 bp multiples).

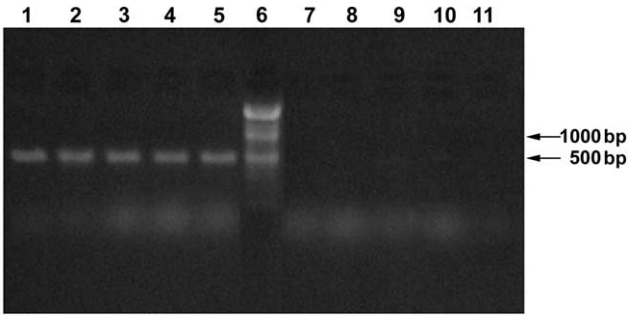

Fig. 3. Lanes 1–5 and lanes 7–11: PCR amplifications of five *F. proliferatum* strains (ITEM 1475, ITEM 1480, ITEM 2365, ITEM 2366, ITEM 2368) with specific primers for *F. proliferatum* and *F. oxysporum* respectively; lane 6: DNA size marker (500 bp multiples).

3. Results

3.1. Primer design

The first experiments sought to analyze 20 strains of two *Fusarium* species isolated from asparagus: 10 each of *F. proliferatum* and *F. oxysporum*, from different geographical areas. The first step in the development of a species-specific PCR assay was to select a suitable species-typical variability in nucleotide sequences from *F. proliferatum* and *F. oxysporum* occurring on asparagus. To this purpose, we sequenced the calmodulin-encoding gene, which is able to resolve phylogenetically distinct species in the *G. fujikuroi* species complex [18]. DNA extracted from all 20 strains used in this study was amplified by PCR with CL1 and CL2A primers, resulting in fragments of approximately 700 bp (data not shown). The amplification products of all isolates were sequenced in both directions. Visual inspection of the aligned calmodulin partial gene sequences readily identified unique regions for the species analyzed (Fig. 1). These differences were then used to design two species-specific primer pairs for identifying and distinguishing isolates belonging to these asparagus pathogens. In the calmodulin sequences, we observed 99.71% identity for strains of *F. proliferatum*, and 100% identity for the *F. oxysporum* strains. Using the genetic sequence variation found in this region, we derived two sets of primers, one for each species analyzed. All primers were designed to operate at high annealing temperatures (60°C), thereby preventing the co-amplification of non-specific DNA targets. Species-specific primers were chosen with mismatches located in the 3′ extremity. Primer sequences were compared against existing sequences of calmodulin gene available in GenBank, EMBL, DDBJ and PDB. Single bands of correct size were obtained with species-specific primers from all strains belonging to the two species, 10 of *F. proliferatum* and 10 of *F. oxysporum*. PCR products were 526 bp and 534 bp in length from *F. proliferatum* and *F. oxysporum*, respectively (Figs. 2 and 3). In order to confirm primer specificity, fragments derived from the species studied were sequenced. Comparison of the sequences obtained previously with calmodulin sequences, used for primer design, confirmed the species specificity of the primers, since the sequences were 100% homologous. In order to validate the primers, the specificity of the primer pairs was tested against 50 strains each of the two species. The strains were obtained from the ITEM culture collection. All *F. oxysporum* strains were amplified by CLOX1/2 species-specific primers, and no cross-reactions were observed with *F. proliferatum* strains (Fig. 2). In the same way, all *F. proliferatum* strains were amplified by CLPRO1/2 species-specific primers and no cross-reactions were observed with *F. oxysporum* strains (Fig. 3). In order to avoid false-negative results, some PCR assays were performed at least three times. The primers were also tested against other main pathogens of Asparagus, such as *F. culmorum*, *F. solani* and *Sclerotinia minor*, and against the phylogenetically related species *F. verticillioides* (syn. *F. monili-*

Table 2
F. proliferatum and *F. oxysporum* detection from samples of asparagus plant tissues by morphological and PCR analysis

Plant sample	Morphological analysis	PCR analysis
1	*F. proliferatum*	*F. proliferatum*
2	*F. proliferatum*+*F. oxysporum*	*F. proliferatum*+*F. oxysporum*
3	*F. proliferatum*	*F. proliferatum*
4	*F. oxysporum*	*F. proliferatum*+*F. oxysporum*
5	*F. oxysporum*	*F. oxysporum*
6	–	*F. proliferatum*
7	*F. proliferatum*	*F. proliferatum*+*F. oxysporum*
8	–	*F. proliferatum*

forme) and *F. subglutinans*, also belonging to the *G. fujikuroi* species complex [18]. None of these tests showed positive results. Finally, the calmodulin sequences were deposited and the relevant accession numbers obtained for the following strains: *F. proliferatum* ITEM 1475 (AJ560771), as a representative strain of ITEM 1477, ITEM 1478, ITEM 2368, ITEM 2369, because they share 100% identity; *F. proliferatum* ITEM 1484 (AJ560773) as a representative strain of ITEM 1480, ITEM 2365, ITEM 2366, which share 100% identity; *F. proliferatum* ITEM 1476 (AJ560772), which had unique sequences. For *F. oxysporum*, the calmodulin sequences deposited and the relative accession number obtained regarded only strain ITEM 2367 (AJ560774), since all strains of *F. oxysporum* examined in this study showed 100% identity.

3.2. Detection from plant tissues

With a view to using the assay to detect these pathogens in infected asparagus, we tested the primers on total genomic DNA (~60 ng) extracted from asparagus plants. To avoid false-positive results by cross-reaction with plant DNA (there are no calmodulin gene nucleotide sequences for Asparagus in the database) we performed PCR assays on plant DNA from the turion's apex. We tested eight plants showing no symptoms of disease by comparing morphological and molecular analyses. The PCR assay generated fragments corresponding to *F. oxysporum* and *F. proliferatum*, and the data generally agreed with the morphological analysis, with the exception of samples 6 and 8 (Table 2), where PCR assays identified *F. proliferatum* and *F. oxysporum*, respectively, which were not detected by morphological analysis.

4. Discussion

Adoption of nucleic acid-based assays enhances the sensitivity and specificity of methods for detecting fungal pathogens in plant tissues and for assessing pathogens [13]. Using specific DNA probes, fungi which are generally not amenable for rapid identification can be detected and identified. The sensitivity of the diagnostic assay needs to be enhanced, especially for the fungal pathogens causing vascular wilts and root rots, since it would be useful to detect them rapidly when their populations are still low. Among the molecular methods, PCR-based assays have proved more sensitive, reliable, and rapid for the detection and differentiation of fungal pathogens.

Sequences of the calmodulin gene carried out by O'Donnell et al. [18] proved to be highly reliable for phylogenetic analysis on the *G. fujikuroi* complex and *Fusarium*-related species.

As far as we are aware, this is the first report on species-specific primers for these two *Fusarium* species obtained from DNA sequences of a calmodulin gene, designed in the intron regions. Although these genetic elements exhibit a very poor level of sequence conservation and mutational changes might lead to false negatives, this study is another important piece of evidence that the intron variation between species is greater than the exon variation [23,24]. In particular, while *F. oxysporum* is considered a morphologically and phylogenetically related species of the so-called *G. fujikuroi* complex [18,22], *F. proliferatum* is one of the anamorph members of this teleomorphic species complex [25]. Identification of *F. oxysporum* and *F. proliferatum* by morphological traits is very difficult due to the similarity of their phenotypic features, such as the shape and size of conidia, conidiophore morphology, and mycelial pigmentation, and could be influenced by media composition and environmental conditions. Therefore, it is important to have reliable molecular markers for both species, in order to correctly identify strains from asparagus plants, since several reports show *F. proliferatum* and *F. oxysporum* to be the main *Fusarium* pathogens of asparagus plants worldwide [26–29], and could be isolated from the same field or even the same plant [30]. The results presented here show that primer pairs CLOX1/2 and CLPRO1/2 are highly specific; therefore PCR assays can be used to identify unknown strains belonging to these important pathogens, without the need for morphological analysis. Moreover, the different toxigenic profiles of these species need very reliable diagnostic detection tools, since early diagnosis would provide important information on the different toxicological risks for asparagus plants. In this respect, the *F. proliferatum* strains used in this study have been shown to produce high levels of fumonisins [3] beauvericin and fusaproliferin [8]. The primers could also be used to investigate the temporal and spatial spreading of the pathogens within the host tissues, providing a better understanding of their epidemiology, in order to evaluate the true role of both pathogens as agents of asparagus wilting. Recently, Moretti et al. [30] showed that these two species were predominant in asparagus fields all over Italy, and their occurrence in samples with different origins was in most cases negatively correlated, while they were rarely detected from asparagus tissues with the same frequency in samples originating from the same field. Therefore, new identification tools could help to improve our understanding of how these diseases evolve on plants. To this end, molecular detection of *Fusarium* species from asparagus tissue would be a particularly powerful tool, especially if combined with the quantification of DNA belonging to the main fungal contaminants. Together, these techniques will provide a key for the evaluation of fusariosis etiology in asparagus, and will constitute the next step in our research.

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References

[1] Elmer, W.E., Johnson, D.A. and Mink, G.I. (1996) Epidemiology and management of the diseases causal to asparagus decline. Plant Dis. 80, 117–125.
[2] LaMondia, J.A. and Elmer, W.H. (1989) Pathogenicity and vegetative compatibility among isolates of *Fusarium oxysporum* and *F. moniliforme* colonizing asparagus tissue. Can. J. Bot. 67, 2420–2424.
[3] Logrieco, A., Doko, B., Moretti, A., Frisullo, S. and Visconti, A. (1998) Occurrence of fumonisin B1 and B2 in *Fusarium proliferatum* infected asparagus plants. J. Agric. Food Chem. 46, 5201–5204.
[4] Nigh Jr., E.L., Guerrero, C. and Stanghellini, M.E. (1999) Evaluation of *Fusarium* spp. infected asparagus spears for fumonisin mycotoxins. Proc. 9th International Asparagus Symposium, pp. 247–252.
[5] Marasas, W.F.O. (1996) Fumonisins: history, worldwide occurrence and impact. Adv. Exp. Med. Biol. 392, 1–17.
[6] Ritieni, A., Monti, S.M., Randazzo, G., Logrieco, A., Moretti, A., Peluso, G., Ferracane, R. and Fogliano, V. (1997) Teratogenic effects of fusaproliferin on chicken embryos. J. Agric. Food Chem. 45, 3039–3043.
[7] Nelson, P.E., Plattner, R.D., Shackelford, D.D. and Desjardins, A. (1992) Fumonisin B_1 production by *Fusarium* species other than *F. moniliforme* in section *Liseola* and by some related species. Appl. Environ. Microbiol. 58, 985–989.
[8] Moretti, A., Logrieco, A., Bottalico, A., Ritieni, A., Fogliano, V. and Randazzo, G. (1996) Diversity in beauvericin and fusaproliferin production by different populations of *Gibberella fujikuroi* (*Fusarium* section *Liseola*). Sydowia 48, 45–56.
[9] Logrieco, A., Moretti, A., Ritieni, A., Caraffa, M.F. and Macchia L. (2002) Beauvericin: chemistry, biology and significance. In: Advances in Microbial Toxin Research and its Biotechnological Exploitation (Upadhyay, R.K., Ed.), pp. 23–30. Kluwer Academic Publishers, New York.
[10] Nagaray, R.Y., Wu, W., Will, J.A. and Vesonder, R.F. (1995) Acute cardiotoxicity of moniliformin in broiler chickens as measured by electrocardiography. Poultry Sci. 74 (Supplement 1), 21.
[11] Moretti, A., Belisario, A., Tafuri, A., Ritieni, A., Corazza, L. and Logrieco, A. (2002) Production of beauvericin by different races of *Fusarium oxysporum* f. sp. *melonis*, the Fusarium wilt agent of muskmelon. Eur. J. Plant Pathol. 108, 661–666.
[12] Schütt, F., Nirenberg, H.I. and Deml, G. (1998) Moniliformin production in the genus *Fusarium*. Mycol. Res. 14, 35–40.
[13] Edwards, S., O' Callaghan, J. and Dobson, A.D.W. (2002) PCR-based detection and quantification of mycotoxigenic fungi. Mycol. Res. 106, 1005–1025.
[14] Turner, A.S., Lees, A.K., Rezanoor, H.N. and Nicholson, P. (1998) Refinement of PCR-detection of *Fusarium avenaceum* and evidence from DNA marker studies for phenetic relatedness to *Fusarium tricinctum*. Plant Pathol. 47, 278–288.
[15] Moukhamedov, R., Hu, X., Nazar, R. and Robb, J. (1994) Use of polymerase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. Phytopathology 84, 256–259.
[16] Niessen, M.L. and Vogel, R.F. (1998) Group specific PCR-detection of potential trichothecene-producing *Fusarium*-species in pure cultures and cereal samples. Syst. Appl. Microbiol. 21, 618–631.
[17] Bakan, B., Giraud-Delville, C., Pinson, L., Richard-Molard, D., Fournier, E. and Brygoo, Y. (2002) Identification by PCR of *Fusarium culmorum* strains producing large and small amounts of deoxynivalenol. Appl. Environ. Microbiol. 11, 5472–5479.
[18] O'Donnell, K., Nirenberg, H.I., Aoki, T. and Cigelnik, E. (2000) A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience 41, 61–78.
[19] Moeller, E.M., Chelkowski, J. and Geiger, H.H. (1999) Species-specific PCR assays for the fungal pathogens *Fusarium moniliforme* and *Fusarium subglutinans* and their application to diagnose maize ear rot. J. Phytopathol. 147, 497–508.
[20] Simpson, D.R., Rezanoor, H.N., Parry, D.W. and Nicholson, P. (2000) Evidence for differential host preference in *Microdochium nivale* var. *majus* and *Microdochium nivale* var. *nivale*. Plant Pathol. 49, 261–268.
[21] Knoll, S., Mulfinger, S., Niessen, L. and Vogel, R.F. (2002) Rapid preparation of Fusarium DNA from cereals for diagnostic PCR using sonification and an extraction kit. Plant Pathol. 51, 728–734.
[22] Nelson, P.E., Toussoun, T.A. and Marasas, W.F.O. (1983) Fusarium Species: An Illustrated Manual for Identification, 193 pp. Pennsylvania State University Press, University Park, PA.
[23] Boucher, H., Mercure, S., Montplaisir, S. and Lemay, G. (1996) A novel group I intron in *Candida dubliniensis* is homologous to a *Candida albicans* intron. Gene 180, 189–196.
[24] Donnelly, S.M., Sullivan, D.J., Shanley, D.B. and Coleman, D.C. (1999) Philogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of ACT1 intron and exon sequences. Microbiology 145, 1871–1882.
[25] Leslie, J.F. (1991) Mating populations in *Gibberella fujikuroi* (*Fusarium* section *Liseola*). Phytopathology 81, 1058–1060.
[26] Blok, W.J. and Bollen, G.J. (1995) Fungi on roots and stem bases of asparagus in the Netherlands: species and pathogenicity. Eur. J. Plant Pathol. 101, 15–24.
[27] Elmer, W.E., Summerell, B.A., Burgess, L.W., Backhouse, D. and Abubaker, A.A. (1997) *Fusarium* species associated with asparagus crowns and soil in Australia and New Zealand. Australas. Plant Pathol. 26, 225–261.
[28] Elmer, W.H. (1995) A single mating population of *Gibberella fujikuroi* (*Fusarium proliferatum*) predominates in asparagus fields in Connecticut, Massachusetts, and Michigan. Mycologia 87, 68–71.
[29] Schreuder, W., Lamprecht, S.C., Marasas, W.F.O. and Calitz, F.J. (1995) Pathogenicity of three *Fusarium* species associated with asparagus decline in South Africa. Plant Dis. 79, 177–181.
[30] Moretti, A., Logrieco, A., Doko, B., Frisullo, S. and Visconti A. (1997) *Fusarium proliferatum* from asparagus in Italy: occurrence, fertility and toxigenicity. Proceedings of the 5th European Fusarium Seminar, Szeged, Hungary, August 29th–September 5th. Cereal Res. Commun. 25 (3/1), 785–786.