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The development of a rapid PCR assay for detection of *Fusarium moniliforme*

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Abstract

The fungus *Fusarium moniliforme* infects a wide range of crops throughout the world. In maize (*Zea mays L.*) it causes seedling blight and root, stalk, and ear rots. A simple procedure that can be used to detect infection by *F. moliliforme* from infected plant tissues has been developed. A *F. moniliforme* genomic library was prepared and used to identify the recombinant clones containing fungal DNA sequences not hybridizing with the DNA of the host plant, maize. Based on the nucleotide sequence information obtained from the *F. moniliforme* pUCF2 genomic clone, specific oligonucleotides were designed and used as primers for *in vitro* DNA amplification by the polymerase chain reaction. An amplification product was obtained with *F. moniliforme* DNA preparations whereas no amplified DNA was detected with DNAs from other fungal pathogens, including various *Fusarium* species, or from the host plant. This PCR analysis was successfully employed to identify *F. moniliforme* directly from the mycelia that develop from naturally infected maize seeds, with no need to obtain pure fungal cultures for reliable diagnosis. The protocol can be used for the diagnosis of infected plants and soils in epidemiological studies of Fusarium diseases, for seed health testing, and for evaluation of susceptibility to colonization in commercial maize hybrids.

Abbreviations: AP – alkaline phosphatase; PCR – polymerase chain reaction.

Introduction

Species of the genus *Fusarium* are important pathogens of almost all cultivated crops (Shidu, 1988; Damicone et al., 1988; Logrieco et al., 1990). In cereals, several *Fusarium* species are widespread disease agents, causing root-, stem-, and ear-rot. Depending on geographical location and environmental conditions different *Fusarium* species may be associated with Fusarium diseases (Martin et al., 1991). In maize, *Fusarium moniliforme* is one of the fungi most commonly responsible for stalk and ear rots (King and Scott, 1981; Calvert et al., 1985; McGee, 1988; Holley et al. 1989). This is a seed-transmitted pathogen which reduces germination through seed decay, damping-off, and seedling blight. Chemical treatment (i.e. benomyl or thiram treatment) has been used to improve the germinability of infected maize seeds. In addition to their agricultural importance, *Fusarium* species can cause serious animal and human diseases by producing harmful mycotoxins (Marasas et al., 1984; Logrieco et al., 1990). In particular, *F. moniliforme* produces significant quantities of the toxin moniliformin, which adversely affects human and animal health (Burmeister et al., 1979; Marasas et al., 1984).

In maize, differences in susceptibility to infection have been recognized but resistant varieties have not been developed (King and Scott, 1981). Studies on resistance to colonization by *F. moniliforme* in seeds of a wide range of maize hybrids indicated that commercial hybrids appeared to be more susceptible to pathogen development than the topcross hybrids (Holley et al., 1989). It has been suggested that the increasing problems with contamination of maize seeds may

have arisen from the extensive use of highly susceptible hybrids. Related to this, studies on the incidence of internal fungi isolated from seeds of commercial corn hybrids indicate that *F. moniliforme* is the predominant fungus in *Z. mays* (Calvert et al., 1985). The extent to which maize seed contamination can be reduced is dependent upon the development of an efficient screening system. Such a screening system can also be of great utility in research programs aimed at expanding our knowledge of *Fusarium* disease epidemiology, in the precocious selection of resistant maize genotypes, and in the evaluation of the effect of fungicides under laboratory and field conditions.

The current method for identification of *F. moniliforme* and for differentiation between *Fusarium* species mostly relies on morphological traits. Fungi are identified visually by their mycelial and/or reproductive morphology. Another method involves host inoculation for characteristic disease symptoms. These methods are relatively simple and inexpensive with regard to materials but they can be labor-intensive and results may not be obtained for weeks. Besides, such methods depend to some extent on subjective assessments and consequently considerable experience is necessary for reliable diagnosis. Consistently, there is demand for a simple, rapid and reliable method for the detection and differentiation of *Fusarium* species that does not use morphological characters. Considerable potential exists for the use of certain recombinant DNA techniques, such as nucleic acid hybridization, non-isotopic labelling of DNAs, or Polymerase Chain Reaction (PCR) technology, in studies of plant pathology (Henson and French, 1993). The advantages of DNA techniques, especially of the PCR, are high sensitivity and specificity. However, to be effective in these applications, different assays must be first developed and their ability to detect an organism must be evaluated. Related to this, PCR-based assays have been developed for the detection of fungal pathogens in several plant species (Blakemore et al., 1994; Roob et al., 1994; Nicholson et al., 1996). At present, all the existing methods rely on DNA extractions from infected plant tissues. This approach, however, has some important limitations, the most important being the presence of natural inhibitors of the PCR reaction in plant tissues (Hu et al., 1993; De Boer et al., 1995). Furthermore, depending on the source of tissue under analysis, problems are often associated with plant DNA isolation. This is particularly troublesome with seed tissues where the presence of high levels of polysaccharides significantly affect the efficiency of DNA extraction procedures.

The purpose of this research was the development of a method suitable for detection of infection by the fungus *F. moniliforme* by using nucleic acid techniques. Towards this end, nucleic acid hybridization with non-radioactive DNA probes and *in vitro* DNA amplification (PCR) of *F. moniliforme* DNA sequences have been investigated. Initially *F. moniliforme* genomic DNA sequences were cloned. One sequence, the *F. moniliforme* DNA fragment cloned into the pUCF2 clone was studied in more detail. Based on the nucleotide sequence obtained for this *F. moniliforme* cloned DNA, specific oligonucleotides were designed to amplify a DNA fragment from the *F. moniliforme* genome. The specificity and suitability of the PCR technique has been tested with different plant fungal pathogens, including various *Fusarium* species and *F. moniliforme* isolates. When compared with the alternative method based on the use of cloned DNA sequences as hybridization probes for detection of *F. moniliforme*, the use of the PCR technique has definitive advantages in terms of specificity and technical simplicity.

Materials and methods

General methods

All enzymatic DNA manipulations were performed following standard protocols (Sambrook et al., 1989) using modifications recommended by the enzyme manufacturers.

Fungal isolates and plant material

The fungal isolates included in this study were obtained from different sources. Species names, isolate designations, and locations of collections are listed in Table 1. Fungi were mantained on PDA (Potato Dextrose Agar, Difco Laboratories, Detroit, MI) plates, at 25 °C. *Phytophtora infestans* was grown on 'V8' vegetable juice agar.

Maize seeds purchased from the greenhouse (CID, CSIC, Barcelona) were examined for the presence of fungi associated with them. Seeds were placed on PDA plates and the fungi that were naturally present in them were allowed to grow into the medium. The predominant fungi originating from the seeds were independently transferred to new PDA plates for purification.

Table 1. Fungal species and isolates used in this study

Isolates	Geographical origin	Source	PCR
Fusarium moniliforme 1501	Bary, Italy	2	+
Fusarium moniliforme 1758	Bary, Italy	2	+
Fusarium moniliforme 1512	Bary, Italy	2	+
Fusarium moniliforme 1777	Bary, Italy	2	+
Fusarium moniliforme 1774	Bary, Italy	2	+
Fusarium moniliforme	Barcelona, Spain	1	+
Fusarium moniliforme ant.	Torino, Italy	3	+
Fusarium culmorum	Torino, Italy	2	–
Fusarium equiseti	Tarragona, Spain	4	–
Fusarium graminearum	Tarragona, Spain	4	–
Fusarium lateritium	Girona, Spain	4	–
Fusarium merismoide	Tarragona, Spain	4	–
Fusarium oxysporum f.sp. *dianthi*	Barcelona, Spain	4	–
Fusarium tabacinum (1)	Torino, Italy	3	–
Fusarium tabacinum (2)	Torino, Italy	3	–
Fusarium tabacinum (3)	Torino, Italy	3	–
Microdochium nivale	Torino, Italy	3	–
Colletotrichum spp.	Barcelona, Spain	4	–
Gliocladium roseum	Barcelona, Spain	4	–
Penicillium spp.	Barcelona, Spain	1	–
Phytophtora infestans	Madrid, Spain	5	–
Rhizoctonia spp.	Barcelona, Spain	4	–
Thielaviopsis basicola	Torino, Italy	3	–
Trichoderma spp.	Barcelona, Spain	1	–

Sources
1. Centro de Investigación y Desarrollo de Barcelona (CID-CSIC). Spain.
2. Istituto Tossine e Micotossine da Parassiti Vegetali (ITEM- CNR). Bari, Italy.
3. Istituto di Patologia Vegetale, Facolta' di Agraria, Universita' di Torino. Italy.
4. Institut de Recerca Tecno-Alimentària (IRTA). Cabrils, Spain.
5. Departamento de Patología Vegetal, Escuela Técnica Superior de Ingenieros Agrónomos (ETSIA). Madrid, Spain.

Identification of the fungi was carried out at the 'Servei de Protecció del Vegetals, Departament d'Agricultura, Ramaderia y Pesca' Generalitat de Catalunya.

Fungal and plant DNA isolation

Agar plugs containing fungal mycelia were used to inoculate 1L of PDB (Potato Dextrose Broth) medium. After 20 days at 25 °C, mycelia were harvested by filtering through Miracloth, lyophilized and stored at −20 °C.

To extract fungal DNA, 2 g of lyophilized mycelia were ground to a fine powder with a mortar and pestle in liquid nitrogen and suspended in 15 ml of extraction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 0.05 M EDTA, 0.1% 2-mercaptoethanol (v:v)). To this supension, 1 ml of 20% SDS was added, and the suspension was incubated at 65 °C for 10 min. Next, 5 ml of 5M potassium acetate were added and the suspension was kept on ice for 20 min. After centrifugation for 20 min at 10,000 g at 4 °C, the supernatant was filtered through Miracloth (Calbiochem, 20 μm) and 10 ml of isopropanol were added. The DNA was allowed to precipitate for 30 min at −20 °C, centrifuged at 10,000 g for 15 min at 4 °C. The precipitate was redissolved in 1.5 ml of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The DNA was precipitated again with one tenth volume of 3 m sodium acetate (pH 5.2) and two volumes of cold (−20 °C) ethanol. The DNA was then incubated for 30 min at 37 °C with DNase free RNase A (final concentration 10 μg.ml^{-1}). Ammonium acetate (final concentration of 2 M) and ethidium bromide (final concentration of 40 μl.ml^{-1}) were added to the DNA solution. The DNA preparation was extracted twice with phenol:chloroform:isoamylalcohol (50:48:2; v:v:v), precipitated with ethanol, dissolved in TE and stored at 4 °C.

Genomic DNA was also prepared from maize (*Zea mays* pure inbred line W64A, grown in the greenhouse in Barcelona, Spain). DNA was obtained from leaves of 6-week old maize plants following the protocol described by Dellaporta et al. (1983).

Preparation of digoxigenin-labeled DNA probes

This work was carried out using a nonradioactive DNA labelling system. We choose digoxigenin labelling of our probes, followed by immunological detection of the digoxigenin-labelled-DNA probes using an anti-digoxigenin-AP conjugate. Maize and *F. moniliforme* genomic DNAs were digested with SacI and labelled with digoxigenin by random priming following instructions from the manufacturer (Boehringer Mannheim). These digoxigenin-labelled DNAs were used as probes for the differential screening of the *F. moniliforme* genomic library. The selected *F. moniliforme* DNA sequences were also used as probes for dot blot hyabridizations with DNAs from various fungal pathogens. For this, inserts were isolated from the recombinant plasmid DNAs by agarose gel electrophoresis and electroelution (Biotrap system, electroelution buffer 1 × TAE (1 × TAE = 40 mM Tris-acetate, pH 8.0, 2 mM EDTA)). These DNAs were finally labelled with digoxigenin by random priming

and used to probe the Hind III-digested genomic fungal DNAs.

Construction and differential screening of the F. moniliforme *genomic library*

F. moniliforme genomic DNA was digested with SacI. The resulting fragments were ligated to SacI digested pUC19, and used to transform competent cells of *Escherichia coli* (DH5α strain). Replica filters were prepared from the ampicillin-resistant colonies which were then subjected to differential screening with the digoxigenin-labelled maize and *F. moniliforme* genomic DNAs. Prehybridization was carried out at 42 °C for 6 h in 50% formamide, 5 × SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1.0% (w/v) blocking reagent (Boehringer Mannheim). For hybridization, the digoxigenin-labelled DNA probes were denatured at 100 °C and added to the prehybridization solution to a final concentration of 40 ng.ml^{-1}. Filters were hybridized overnight at 42 °C, washed twice at room temperature, and twice at 68 °C in 0.1 × SSC, 0.1% SDS. Positive hybridizations were visualized by incubation with the antidigoxygenin AP conjugate (Boehringer Mannheim) followed by the coloured enzymatic reaction with the NBT/BCIP substrates. Plasmid DNAs were prepared from the *F. moniliforme* genomic clones by the alkaline minipreparation procedure (Sambrook et al., 1989).

Southern blot analysis of fungal DNAs

Genomic DNAs from *F. moniliforme*, *F. oxysporum* f. sp. *dianthi* (race 2), *Penicillium* spp., *Trichoderma* spp., and also from maize plant, were obtained. DNAs were digested with HindIII (Promega, Madison WI), electrophoresed in 0.8% agarose gels, depurinated in 0.25% HCl, and transferred to Hybond N membranes (Amersham International, Aylesbury, Bucks.). Membranes were prehybridized at 65 °C for 6 h in 5 × SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1.0% (w/v) blocking reagent (Boehringer Mannheim). For hybridization, the digoxigenin-labelled DNA probes were added to a final concentration of 20 ng.ml^{-1}. Detection of positive hybridizations was carried out with the anti-digoxigenin-AP conjugate and the chemiluminescent AP substrate Lumigen PPD (Boehringer Mannheim).

DNA sequencing

Nucleotide sequence was determined using the dideoxynucleotide chain termination method (Sanger et al., 1977) on an automated fluorescence (ALF) sequencing apparatus (Pharmacia LKB). The *F. moniliforme* DNA sequence cloned into the pUCF2 recombinant plasmid was sequenced from both ends using the pUC sequencing primers. Internal oligonucleotide primers were designed to obtain the complete nucleotide sequence. Oligonucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer and purified with OPC cartridges (Applied Biosystems) following the manufacturer‘s instructions.

Fungal DNA minipreparation and PCR amplification

The miniprep procedure used to obtain the fungal DNA samples to be directly used as the target DNA in the PCR assay was as follows. Fungal cultures were grown on PDA in a petri dish (mycelium covered approximately 2.5 cm diameter disks). The mycelium was covered with liquid nitrogen, scraped into a precooled mortar and ground in the presence of liquid nitrogen. The resulting powder was suspended in 1 ml of extraction buffer (1% SDS, 10 mM EDTA) by vortexing. After adding 70 mg of sodium chloride and incubating 30 min on ice, the sample was centrifuged in a microcentrifuge at 13,000*g* for 15 min at room temperature. Next, 100 mg of solid PEG 6000 was added to the supernatant which was incubated on ice for 60 min. After centrifugation (13.000*g*, 15 min, at room temperature), the pellet was dissolved in 0.18 ml of TE. The DNA was precipitated again by adding 0.02 ml of 4 M LiCl and 0.2 ml of isopropanol. After washing with 70% ethanol, the DNA was dried and dissolved in 50 μl of TE. Aliquots of these samples (5 μl) were then used for PCR amplification as described above.

The PCR technique was used to identify *F. moniliforme* from mycelia originating from naturally infected seeds. For this, maize seeds were placed on PDA plates for 5 days. Mycelia grown into the agar medium from maize seeds were scraped off and used directly to extract fungal DNA by the miniprep procedure above indicated. There was no need to prepare subcultures to obtain pure cultures.

For PCR amplification, two pairs of oligonucleotide primers were synthesized on the basis of the sequence of the *F. moniliforme* DNA fragment cloned into the pUCF2 clone. The two *F. moniliforme*-specific primers were the following:

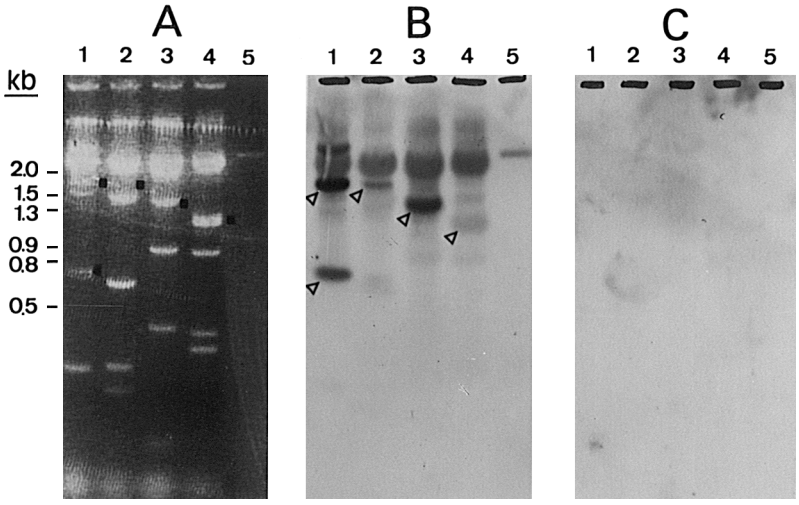

Figure 1. Analysis of the cloned *F. moniliforme* sequences that had been selected by the differential screening of the genomic library. A) Agarose gel electrophoresis containing ethidium bromide (0.5 μg.ml-1). Plasmid DNA minipreps from the pooled *F. moniliforme* clones were digested with Eco RI and Pst I, and subsequently transferred to nylon membranes. As an example, only four plasmid minipreps (lanes 1–4) are shown. Lane 5, pUC19 plasmid DNA digested with Eco RI. Black squares indicate those DNA fragments that were selected for further analysis (also indicated by open arrow heads in B). B) Hybridization of the DNA minipreps shown in A with the digoxigenin-labelled *F. moniliforme* genomic DNA probe. C) Hybridization of the DNA minipreps shown in A with the digoxigenin-labeled genomic maize DNA probe. Detection of positive hybridizations was carried out by incubation with the anti-digoxigenin-AP conjugate. The chemiluminescent substrate was used to detect AP activity (exposure time, 60 min).

FUS1: 23-mer, 5′-CTTGGTCATGGGCCAGTCAAGAC-3′ FUS2: 24-mer, 5′-CACAGTCACATAGCATTGCTAGCC-3′

Location of these primers is indicated in Figure 2A. A preliminary study for obtaining optimal conditions for DNA amplification was carried out by using the pUCF2 plasmid DNA as the target sequence.

PCR reactions were carried out in a thermal cycler (OmniGene, Hybaid) in a volume of 100 μl with 0.2 mM of each dNTP, 1 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3, 1,5 mM $MgCl_2$, 50 mM KCl, 0.1 mg.ml^{-1} gelatin), 0.2 μM of both primers and 2.5 U of Taq polymerase (Promega, Madison WI). DNA amplifications from maize and fungal genomic DNA samples were performed using 100 ng of DNA as template. A program of 32 cycles (96 °C 1 min, 55 °C 90 s., 72 °C 2 min. 30 s.) was performed. Aliquots of 10% of the PCR product mixtures were analyzed on 1% agarose minigels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), containing 0.5 μg.ml^{-1} ethidium bromide.

Results

Maize diseases caused by fungi have been grouped according to the seedborne and seed transmission aspects of the pathogen (McGee, 1988). As an example, *Fusarium* and *Penicillium* rots are seedborne and seed transmitted diseases while *Trichoderma* rot is a seedborne but not seed transmitted maize disease. In this work, infection by *F. moniliforme* in maize seeds was initially evaluated. For this, seeds were placed on PDA medium and any fungi present on them were allowed to grow on the medium. Afterwards, pure cultures were prepared and used for identification of the fungi. All the seeds examined proved to be naturally infected by different fungi, *F. moniliforme* and *Penicillium* being the most predominant. *Trichoderma*, *Aspergillus* spp., and *Mucor* spp. were also found.

Cloning and selection of F. moniliforme *DNA sequences*

A library was prepared from genomic DNA obtained from the fungus *F. moniliforme*. Fungal DNA sequences that did not cross-hybridize with the DNA from the host plant were initially identified. Differential screening of the *F. moniliforme* genomic library (about 2,500 recombinant clones were screened) resulted in identification of 104 Fusarium clones. These clones showed positive hybridization with digoxigenin-labelled Fusarium DNA probe,

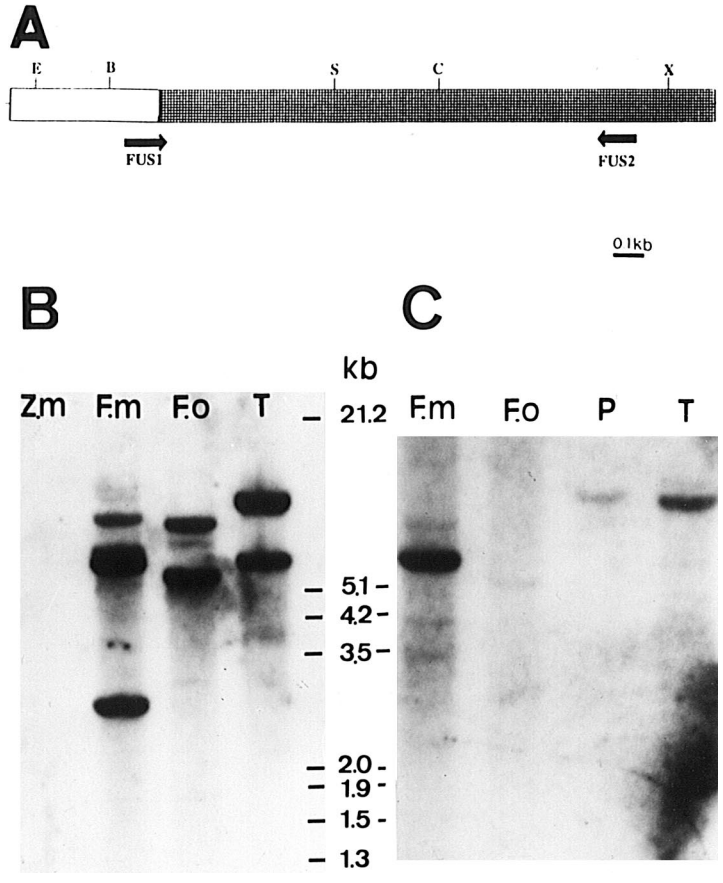

Figure 2. Physical map of the *F. moniliforme* DNA sequence cloned into the pUCF2 genomic clone (A) and Southern blot analysis of genomic DNAs from fungal pathogens (B and C).

A) Partial restriction map for the genomic pUCF2 clone. The white box corresponds to the nucleotide sequence of the *F. moniliforme* genomic sequence containing the open reading frame with similarity to the heat shock proteins from yeast. Arrows denote regions used to design the synthetic oligonucleotides used for PCR. The ClaI site (C) was used for the analysis of the PCR product (as shown in Figure 3). B, BglI; E, EcoRI; S, SnaBI; X, XhoI.

B) Fungal genomic DNAs (10 μg) were digested with the restriction enzyme Hind III and hybridized with the *F. moniliforme* genomic sequence cloned into the pUCF2 plasmid. Positive hybridizations were detected with the anti-digoxigenin-AP conjugate and the chemiluminescent substrate (exposure time, 15 min). Sizes of molecular markers are shown in kilobases. F.m, *F. moniliforme*; F.o, *F. oxysporum* f.sp. *dianthi*, T, *Trichoderma* spp. Genomic DNA from *Z. mays* (Z.m, 10 μg) was also run on the electrophoresis.

C) Fungal genomic DNAs were hybridized with the internal DNA fragment obtained by PCR using the FUS1 and FUS2 oligonucleotides primers. DNAs were from *F. moniliforme* (F.m, 10 μg), *F. oxysporum* f. sp. *dianthi* (F.o, 2 μg), *Penicillium* spp. (P, 2 μg) and *Trichoderma* spp. (T, 10 μg).

whereas the hybridization to the digoxigenin-labelled *Z. mays* DNA was negative. Positive clones detected on the primary screening together with adjacent, non hybridizing colonies, were pooled (1 to 10 colonies each pool). Plasmid DNA minipreps were prepared from each of these colony pools. Inserts were excised by restriction enzyme digestion, separated by agarose gel electrophoresis and transferred to nylon membranes (Figure 1A). Hybridization with the digoxigenin-labelled genomic DNAs from *F. moniliforme* and maize was then carried out (Figures 1B, C). The five genomic sequences which gave the strongest signals with the *Fusarium* probe were cloned individually into the pUC19 plasmid and used for further studies.

The next step in our selection strategy was to consider the specificity of the selected *F. moniliforme* sequences not only against the host DNA but also against other fungal DNAs. This search centered on the fungi that were previously found to naturally infect

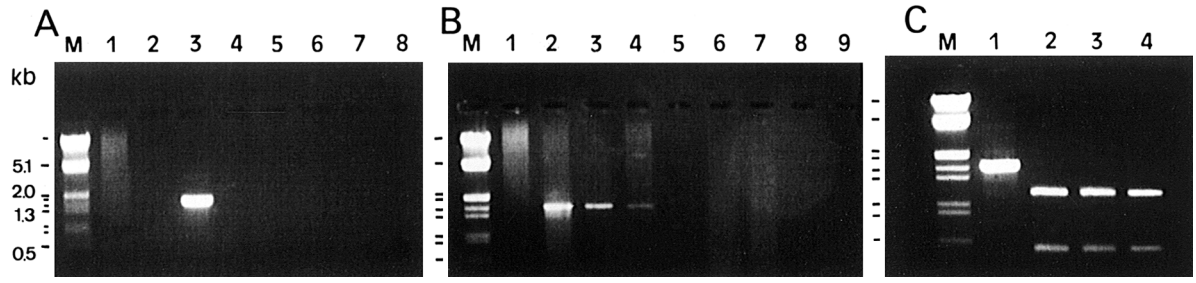

Figure 3. PCR amplification of the *F. moniliforme* DNA sequence with the FUS1 and FUS2 primers. Ethidium bromide-stained 1% agarose gels of PCR products. 10% of each assay was loaded.
A) Pure genomic DNAs were used as targets for the PCR amplification. Lane 1, negative control with all reagents except template DNA. DNAs from *Z. mays* (lane 2), *F. moniliforme* (lane 3), *F. culmorum* (lane 4), *Microdochium nivale* (lane 5), *F. oxysporum* f. sp. *dianthi* (lane 6), *Trichoderma* spp. (lane 7), and *Penicillium* spp. (lane 8).
B) DNAs extracted by the miniprep procedure from fungal mycelium grown in agar (pure cultures) were used as targets for PCR amplification. Lane 1, negative control with all reagents except DNA template. Lane 2, positive control, using purified genomic DNA from *F. moniliforme*. Lane 3, *F. moniliforme* mycelium. Lane 4, same DNA as in lane 3 but in this case the PCR reaction was carried out using one hundredth of the target DNA. Lanes 5 to 9 contain PCR reactions with the DNAs extracted from pure mycelium of *F. culmorum*, *Microdochium nivale*, *F. oxysporum* f. sp. *dianthi*, *Penicillium* spp., and *Trichoderma* spp.
C) Lane 1, PCR product obtained by using the miniprep procedure to extract DNA from the total mycelia grown into agar medium from naturally infected maize seeds. Cla I digestion of the PCR products obtained using purified *F. moniliforme* genomic DNA (lane 2), DNA extracted from *F. moniliforme* mycellium (lane 3) or DNA extracted from the total mycellia grown into the agar media from naturally infected maize seeds (lane 4) as targets for PCR amplification. M, DNA molecular weight markers, lambda DNA digested with Hind III and Eco RI.

maize seeds, such as *Penicillium* and *Trichoderma* and also on another *Fusarium* species (*Fusarium oxysporum*). The analysis of cross-hybridization of the selected *F. moniliforme* sequences with the DNAs of these fungi (dot blot analysis, results not shown) revealed that the cloned *F. moniliforme* sequences hybridized with DNAs of the fungi above mentioned. Thus, the specificity of the *F. moniliforme* cloned sequences, which did not hybridize with the host DNA, turned out to be unsatisfactory under field conditions because of the possible presence of other fungi whose DNA would cross-hybridize with the probe. In view of these results, we reasoned that the PCR technique would have the specificity needed for our purposes and would overcome the limitations observed with the use of DNA probes. The recombinant plasmid pUCF2 was chosen for nucleotide sequence analysis.

A schematic representation of the *F. moniliforme* genomic sequence cloned into the pUCF2 recombinant plasmid is given in Figure 2A. Sequence analysis of this clone revealed an open reading frame, from nucleotide 1 to nucleotide 540, that showed homology to the C-terminal region of heat shock proteins from yeast (Parsell et al., 1991; Leonhard et al., 1993) (results not shown).

The organization of the cloned sequence in the *F. moniliforme* genome, and its presence in the genome of other fungal pathogens was investigated. Southern blot hybridization of the genomic DNA from *F. moniliforme*, *F. oxysporum* f. sp. *dianthi* (a fungus for which maize is not a host), and *Trichoderma* spp. (a fungus with a broad host range), was conducted (Figure 2B). With the *F. moniliforme* DNA, few hybridizing bands were observed. Furthermore, a similar number of hybridizing sequences were detected when genomic DNAs from the other fungi were probed under stringent conditions with the *F. moniliforme* DNA probe. As expected, there was no hybridization between the fungal and plant DNAs. Thus, this hybridization revealed the presence of related sequences in the genome of the various fungal pathogens here tested, the genomic organization being similar in all the fungi analyzed.

The PCR technique specifically amplifies a DNA fragment from the F. moniliforme *genome*

Oligonucleotide primers, FUS1 and FUS2, were synthesized on the basis of the nucleotide sequence obtained from the *F. moniliforme* pUCF2 DNA fragment (Figure 2A). In order to set up the experimental conditions for the PCR assay, genomic DNAs were prepared from pure cultures obtained from the different fungi and then used as template in PCR experiments. Results are shown in Figure 3A. Agarose gel electrophoresis of the PCR amplification products revealed a band representing the expected 1.6 kb DNA fragment

when the reaction mixture contained DNA extracted from *F. moniliforme* (Figure 3A, lane 3). No PCR product was obtained when DNA from any of the other fungi was used as template, not even with the DNAs from other *Fusarium* species (Figure 3A, lanes 4–8). Definitive proof of the specificity of the PCR amplification was obtained by restriction enzyme analysis of the PCR product. Digestion with the enzyme ClaI divides the PCR fragment into two DNA fragments of 1130 and 510 nucleotides (see Figure 2A). As can be seen in Figure 3C (lane 2), the expected DNA fragments were obtained when the PCR product obtained from the *F. moniliforme* genomic DNA was subjected to ClaI digestion.

During the course of this work, the DNA fragment obtained by PCR was also used as hybridization probe for Southern blot hybridization with the fungal genomic DNAs. Contrary to what was found when the entire pUCF2 *F. moniliforme* DNA sequence was used as hybridization probe (Figure 2B), only a single fragment hybridized to the PCR fragment in all the fungal genomic DNAs (Figure 2C). These results indicated that even though the PCR product is recognizing a single DNA fragment in the *F. moniliforme* genome, it is not able to specifically detect the *F. moniliforme* genome from the other fungal genomes.

Detection and identification of F. moniliforme *from mycelium directly grown from infected tissues*

It was of interest to assess the utility of the PCR technique for detection and identification of the fungus *F. moniliforme* from the fungal mycelium grown in agar cultures. Towards this end, various minipreparation methods for template DNA extraction from the fungal mycelium grown in agar cultures were assayed. The best results were obtained by using the DNA miniprep procedure from frozen fungal mycelium described in Materials and methods. This procedure was assayed with *F. moniliforme* isolates of different origins, and also with other fungal species (see Table 1 for isolates used in this work). The DNAs prepared by this miniprep procedure were directly used in the PCR amplification reaction. PCR testing of the different fungal DNAs yielded positive results when the DNAs extracted from mycelia of the *F. moniliforme* isolates were used for the PCR reaction (Figure 3B, lane 3). The same DNA fragment was amplified using the pure genomic DNA (Figure 3B, lane 2), or the DNA prepared by the miniprep procedure from *F. moniliforme* mycelium grown on agar (Figure 3B, lane 3). Digestion with the restriction enzyme ClaI confirmed the nature of the DNA fragment obtained by PCR (Figure 3C, lane 3). Furthermore, the yield of the PCR product was dependent on the amount of DNA added to the PCR reaction mixture (Figure 3B, lanes 3 and 4). No amplification product was observed when the DNAs prepared from mycelium of the other fungal species were used for the PCR reaction (some examples are shown in Figure 3B, lanes 5-9).

The PCR technique was assayed with different *F. moniliforme* isolates and Fusarium species as well as with other fungi. Results are presented in Table 1. In conclusion, these results indicated that *F. moniliforme* could be detected by the PCR protocol here described using a simple and rapid miniprep procedure for the extraction of the DNA from the fungal mycellium.

These findings prompted us to assess the possibility of detection and identification of this fungus from the mycelia directly grown from infected plant tissues. Accordingly, naturally infected maize seeds were placed on PDA plates and the fungal mycelia that originated from them were allowed to grow into the medium. No subcultures for purification of the different fungi grown from the seeds into the medium were made before using the DNA miniprep procedure to extract DNA from the total mycelia grown into the agar medium. The presence of *F. moniliforme* was however subsequently confirmed by its purification from the mixed culture grown into the agar medium. As can be seen in Figure 3C (lane 1), the expected DNA fragment was obtained from the PCR reaction. Digestion of this PCR product with the enzyme ClaI also yielded the expected bands (Figure 3C, lane 4). These results proved that the PCR reaction positively identified *F. moniliforme* from the mixture of fungi present in naturally infected maize seeds. The method works to detect the presence of infection by this fungus in a single maize seed, and differentiates *F. moniliforme* from other fungi which are known to be pathogens of maize and other cereal crops, and that are known to occur in the natural habitat of *F. moniliforme*.

Discussion

The genus *Fusarium* comprises a wide and heterogeneous group of fungi, many of considerable importance as pathogens in agriculture. The traditional methods of diagnosis, i.e. visual inspection of the plant, relies on the presence of clear symptoms in the infected plants. On the other hand, identification and classi-

fication of *Fusarium* spp. based on the morphology of fungal structures, which are typically examined in pure culture, is quite laborious and time-consuming. Both methods require the skills of a trained plant pathologist, and they are not always satisfactory for species identification. Several alternative approaches have been worked out in order to classify and differentiate *Fusarium* species on the basis of soluble protein electrophoretic patterns (Glyn and Reid, 1969), zymograms (Scala et al., 1981), immunoelectrograms (Abd-el-Rehim and Fadel, 1980), monoclonal antibody reactions (Iannelli et al., 1983), and restriction fragment length polymorphisms (RFLPs) (Manicon et al., 1987; Edel et al., 1996). The use of the RAPD (random amplified polymorphic DNA)-PCR technique for identification of Fusarium species, and in particular of *F. moniliforme*, has also been reported (Blakemore et al., 1994; Migheli and Cavallarin, 1994; Amoah et al., 1996). However, the efficiency of the RAPD-PCR analysis, which is based on the use of nonamer or decamer random primers, depends on the reproducibility of the technique. In many cases, differences are observed depending on the procedure used for DNA isolation, which is one major source of irreproducibility of RAPD patterns. In other cases, complex patterns of amplified products may cause difficulties in interpreting the results.

This report present results from an initial screening of genomic DNA sequences from *F. moniliforme*, as a basis for the design of a specific test for the direct detection and identification of infection by this fungus in plant tissues. *F. moniliforme*, a seed borne and seed transmitted pathogen, is the fungus most frequently found in maize seeds. Even when only a small proportion of infected seeds might produce diseased plants, large numbers of infected seeds in a seed lot can still produce sufficient foci to initiate serious disease epidemics thus increasing the chances of introducing this plant pathogen to new areas via imported seeds. For appropiate and cost-effective control measures to operate it is necessary to know whether this seed borne pathogen is present and it is also important to ascertain the quantity of infected seeds in a sample. Accordingly, we have investigated the use of the PCR technique to detect infection by this fungus in maize seeds, and also to differentiate among this pathogen and other common fungal pathogens of maize. The PCR method here developed works with crude DNA samples which can be easily prepared from fungal mycelia by a rapid DNA miniprep procedure. Most importantly, this method can be employed to identify *F. moniliforme* grown into the agar medium from any infected plant material.

In the literature, cloned DNA fragments have been used as probes to identify species of fungi, such as *Fusarium oxysporum* (Manicon et al., 1987) or *Phytophthora parasitica* (Goodwin et al., 1989). An assay based on Southern hybridization with species-specific DNA probes has allowed the differentiation between two *Fusarium* species, *F. culmorum* and *F. graminearum* (Koopmann et al., 1994). In the present work, several cloned *F. moniliforme* genomic DNA sequences have been isolated and tested for sensitivity and specificity when used as hybridization probes. These experiments indicated that although the cloned *F. moniliforme* DNA probes did not hybridize with the genome of the host plant, they failed to distinguish between different fungal DNAs. On the other hand, the use of DNA fragments as hybridization probes requires larger amounts of pure fungal DNA to allow restriction enzyme digestion and includes time-consuming operations which constitute important limitations for routine use in diagnosis of infection in plant tissues. Although *F. moniliforme* was not specifically detected by hybridization with the cloned DNA fragment, the use of this nucleotide sequence to design specific primers for DNA amplification has allowed us to develop an assay that detect the presence of this fungus in infected plant tissues. In addition to being technically simpler, the PCR technique enables faster results to be obtained than by hybridization with DNA probes.

The value of PCR techniques in diagnostic work depends on designing good specific primers and developing the appropriate experimental conditions for the DNA amplification process (selection of times, temperatures, number of cycles, etc). The high sensitivity of the PCR technique might enable detection of *F. moniliforme* DNA in preparations containing both plant host DNA and fungal DNA. However, it has been reported that some plant tissues contain natural inhibitors of the PCR reaction (Hu et al., 1993; De Boer et al., 1995). The levels of inhibition tend to vary from tissue to tissue and plant to plant and must be determined empirically in each sample. The main problems associated with plant nucleic acid isolation have been attributed primarily to the presence of high levels of phenolic compounds and polysaccharides (Rogers and Bendich, 1994). The presence of polyphenols that are coextracted with plant DNA might inhibit PCR by sequestering Mg^{++} and false negatives may be obtained in these analysis. For this reason, whenever plant extracts are used, control reactions using internal controls must

be carried out. As a consequence of these drawbacks, the rationale of the procedure developed here was to favor the growth of the fungi, if present, and allow the mycelia to come out of the infected tissue. In this way there is no need either to extract DNA from plant tissues. DNA minipreps can be easily prepared from fungal mycelium growing from the infected plant tissue, i.e. from a single seed, and directly analyzed by PCR.

In conclusion, our results indicate the potential of PCR as an important method useful for the detection and identification of *F. moniliforme*. The method offers several advantages, the most important of which are the following. First, its symptom-independence as the assay can be useful for fungal detection at population levels below those causing yield loss. Second, its applicability to infected plant tissues without the need of subculturing to obtain pure cultures of the pathogen. Third, speed of execution, which allows many samples to be processed. Forth, the diagnosis can be carried out by laboratory technicians. Equally, this methodology can be successfully used in epidemiological screening programs, and can be adapted to detect colonies of *F. moniliforme* developing from infected soil samples. It can be also used for the screening of maize genotypes and commercial hybrids on the basis of their susceptibility to colonization by *F. moniliforme*. Related to this, it is worthwhile to mention that the extensive use of highly susceptible hybrids might have contributed to the increasing problems with contamination of maize seeds observed in these days throughout the world.

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