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# PCR-based strategy to detect contamination with mycotoxigenic *Fusarium* species in maize

Miguel Jurado[a], Covadonga Vázquez[b], Sonia Marín[c], Vicente Sanchis[c], M. Teresa González-Jaén[a,*]

[a]*Faculty of Biology, Department of Genetics, University Complutense of Madrid, José Antonio Nováis 2, 28040 Madrid, Spain*
[b]*Faculty of Biology, Department of Microbiology III, University Complutense of Madrid, José Antonio Nováis 2, 28040 Madrid, Spain*
[c]*Food Technology Department, Lleida University, CeRTA-UTPV, Rovira Roure 191, 25198 Lleida, Spain*

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## Abstract

Contamination of cereals with mycotoxigenic species of *Fusarium* is an important source of trichothecenes, fumonisins and other mycotoxins which cause serious diseases in human and animals. In addition, these species are phytopathogenic and produce severe losses in cereal yield. Methods for early detection of these *Fusarium* species are crucial to prevent toxins entering the food chain and are a useful tool in disease management practices. We have developed an integrated protocol for diagnosis of mycotoxigenic *Fusarium* contamination in maize which can also be used for other cereals. The protocol consisted in an easy and rapid DNA extraction from maize samples (grain and germ), and subsequent group-specific polymerase chain reaction (PCR) assays for genus *Fusarium*, *Gibberella fujikuroi* complex, and trichothecene-producing species of *Fusarium,* that orientate the search of the critical species. We have additionally developed a PCR assay for the identification of *F. proliferatum*. The primers were designed on the basis of IGS sequence (Intergenic Spacer of rDNA), a multi-copy region in the genome that permits to enhance the sensitivity of the assay in comparison with PCR assays based on single-copy sequences. The suitability of the protocol and the relative efficacy of single and multi-copy sequence-based PCR assays have been tested in a wide range of fumonisin-contaminated maize samples.


***Keywords:*** Mycotoxins; Trichothecenes; Fumonisins; *Fusarium*; *Fusarium proliferatum*; *Gibberella fujikuroi*; Cereals; Maize; PCR

## Introduction

*Fusarium* is a phytopathogenic fungus with a global distribution, capable of infecting a wide range of crop plants, including cereals such as maize, wheat or barley. *Fusarium* contamination is an agricultural major problem since quality and yield of the grain can be reduced. In addition, many species of the genus *Fusarium* produce mycotoxins, responsible for serious chronic and acute diseases in human and animals.

The most prevalent *Fusarium* mycotoxins in cereals are trichothecenes and fumonisins. *F. graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. poae* and *F. equiseti* are the most important trichothecene-producing species in cereals. The fumonisin producing species of *Fusarium* associated with cereals are *F. verticillioides* and

*Corresponding author. Tel.: +34 913 944 830; fax: +34 913 944 844.
*E-mail address:* tegonja@bio.ucm.es (M. Teresa González-Jaén).

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*F. proliferatum*, both belonging to the *Gibberella fujikuroi* species complex.

Detection and control of *Fusarium* species is crucial to prevent toxins entering the food chain. Conventional methods to assess mold presence in cereal crops are time consuming, since they comprise microbiological investigation of samples on suitable agar media. These procedures may be especially problematic in the case of *Fusarium* because viability of this fungus can be reduced in seeds after harvest [18]. Furthermore, the identification is based on morphological characteristics and requires considerable expertise in *Fusarium* taxonomy. Since the different *Fusarium* species have also different mycotoxin profiles, the accurate determination of the *Fusarium* species present is critical to predict the potential risk of the *Fusarium* isolate. Therefore, there is a need for developing tools which permit a rapid, sensitive and specific diagnostic of *Fusarium* species in contaminated samples.

Diagnostic methods based on the polymerase chain reaction (PCR) are rapid, as there is no need to culture organisms prior to their identification. They are specific, since identification of species is made on the basis of genotypic differences, and are highly sensitive, detecting target DNA molecules in complex mixtures even when the mycelia are no longer viable. Various PCR assays have been developed for the identification of mycotoxigenic species of *Fusarium*, some of them based on single-copy genes directly involved in mycotoxin biosynthesis [1,6,9,13–16]. Species-specific PCR assays have been developed in our laboratory to detect the main trichothecene-producing species of *Fusarium* associated with cereals, e.g. *F. graminearum*, *F. culmorum*, *F. poae*, *F. sporotrichioides* and *F. equiseti* [7] and the fumonisin-producing *F. verticillioides* [17]. These PCR assays were based on IGS sequence (intergenic spacer of rDNA), a multi-copy region in the eukaryotic genome that permits to enhance the sensitivity of the assay in comparison with PCR assays based on single-copy sequences. This factor is crucial in order to develop diagnostic protocols for infected plant tissue material, where increasing sensitivity means to decrease the percentage of false negatives.

The main objective of this work was to develop an integrated protocol for diagnosis of mycotoxigenic *Fusarium* contamination in cereals. The protocol consisted in an easy and rapid DNA extraction from plant material, and subsequent PCR assays, based on multi-copy sequences. The strategy developed in this work consisted of newly developed group-specific PCR assays for the genus *Fusarium*, *Gibberella fujikuroi* complex, and trichothecene-producing species of *Fusarium*, that orientate the search of the critical species. The single species-specific PCR assays had been previously developed in our laboratory [7,17], except for *F. proliferatum.* In this case, a new species-specific PCR assay was developed. The suitability of single and group-specific PCR assays has been tested in a wide range of fumonisin-contaminated maize samples. The relative efficacy of single and multi-copy sequence-based PCR assays was also analyzed.

## Materials and methods

### Fungal isolates

The fungal strains used in this study are described in Table 1. Cultures were maintained on potato dextrose agar medium (PDA) (Scharlau Chemie, Barcelona, Spain) at 4 °C and stored as spore suspensions in 15% glycerol at −80 °C.

### DNA extractions of fungal cultures

For DNA extraction of fungal cultures, three mycelial disks were excised from the margin of a 5–7-day-old PDA plate cultures and crushed against the wall of a 1.5-mL Eppendorf tube using a sterile pipette tip. DNA extraction was then carried out as previously described [20].

### Development and evaluation of specific PCR primers

Since the range of species of *Fusarium* associated with cereals is quite wide, we have developed three PCR assays based on IGS sequences that orientate the search of the critical species, and identify: (i) genus *Fusarium*, (ii) *Gibberella fujikuroi* complex and (iii) trichothecene-producing species of *Fusarium*.

IGS sequences from different mycotoxigenic species of *Fusarium* obtained in our laboratory and from EMBL database were analyzed and aligned by Clustal method using Dnastar (Lasergene, Madison, WI, USA). Primers were designed on the basis of these DNA alignments. The specificity of the PCR assays was tested in a wide range of *Fusarium* species and other fungal genera associated with cereal contamination, as well as DNA from different cereals (Table 1).

In the case of *Fusarium* spp. assay, the reverse primer Fus-R was designed (5′ GGCGAAGGACGGCTTAC 3′) and combined with the forward primer Fps-F (5′ CGCACGTATAGATGGACAAG 3′) described in Jurado et al. [7]. The size of the amplicon was ∼200 bp. In the case of *Gibberella fujikuroi* complex, the primer Gib2-F (5′ GAGGCGCGGTGTCGGTGTGCTTG 3′) was designed and then combined with the reverse primer Fgc-R (5′ CTCTCATATACCCTCCG 3′), described in Jurado et al. [7]. The size of the amplicon was ∼1000 bp. A pair of primers was designed to detect *Fusarium* trichotecene-producing species, Tct-F

**Table 1.** Fungal isolates analyzed indicating origin, species and the occurrence of PCR amplification with PCR assays for *Fusarium* spp (Fus), trichothecene-producing *Fusarium* species (Tct), *Gibberella fujikuroi* species complex (Gib) and *F. proliferatum* (Fpr) and cereal species.

| Isolates | Origin | Species | Fus | Tct | Gib | Fpr |
|---|---|---|---|---|---|---|
| Fg2 | Central Italy | *F. graminearum* | + | + | – | – |
| Fg4 | North Italy | *F. graminearum* | + | + | – | – |
| Fg5 | Central Italy | *F. graminearum* | + | + | – | – |
| CECT 2150[a] | USA | *F. graminearum* | + | + | – | – |
| NRRL 28585[b] | Venezuela | *F. graminearum* (lineage 1) | + | + | – | – |
| NRRL 28436[b] | New Caledonia | *F. graminearum* (lineage 2) | + | + | – | – |
| NRRL 29020[b] | South Africa | *F. graminearum* (lineage 3) | + | + | – | – |
| NRRL 29148[b] | Pennsylvania – USA | *F. graminearum* (lineage 4) | + | + | – | – |
| NRRL 26755[b] | South Africa | *F. graminearum* (lineage 5) | + | + | – | – |
| NRRL 13818[b] | Japan | *F. graminearum* (lineage 6) | + | + | – | – |
| NRRL 29169[b] | Kansas – USA | *F. graminearum* (lineage 7) | + | + | – | – |
| NRRL 29306[b] | New Zealand | *F. graminearum* (lineage 8) | + | + | – | – |
| Fps6 | South Italy | *F. pseudograminearum* | + | + | – | – |
| Fps3 | South Italy | *F. pseudograminearum* | + | + | – | – |
| CECT 2148[a] | | *F. culmorum* | + | + | – | – |
| ITEM 6717[c] | Hungary | *F. culmorum* | + | + | – | – |
| ITEM 6718[c] | Hungary | *F. culmorum* | + | + | – | – |
| ITEM 628[c] | Yugoslavia | *F. culmorum* | + | + | – | – |
| ITEM 4335[c] | | *F. culmorum* | + | + | – | – |
| MUCL 42823[d] | Belgium | *F. culmorum* | + | + | – | – |
| MUCL 42826[d] | Belgium | *F. culmorum* | + | + | – | – |
| ITEM 6606[c] | England | *F. poae* | + | + | – | – |
| ITEM 6607[c] | England | *F. poae* | + | + | – | – |
| MUCL 7555[d] | Belgium | *F. poae* | + | + | – | – |
| MUCL 6114[d] | Belgium | *F. poae* | + | + | – | – |
| MUCL 42824[d] | Belgium | *F. poae* | + | + | – | – |
| CECT 20166[a] | Russia | *F. sporotrichioides* | + | + | – | – |
| ITEM 695[c] | USA | *F. sporotrichioides* | + | + | – | – |
| ITEM 707[c] | Poland | *F. sporotrichioides* | + | + | – | – |
| ITEM 4596[c] | Russia | *F. sporotrichioides* | + | + | – | – |
| ITEM 4597[c] | Russia | *F. sporotrichioides* | + | + | – | – |
| ITEM 550[c] | Poland | *F. sporotrichioides* | + | + | – | – |
| U1-5/1 | South Spain | *F. sambucinum* | + | + | – | – |
| L3-5/3C | South Spain | *F. sambucinum* | + | + | – | – |
| Eq-U6 | South Spain | *F. equiseti* | + | – | – | – |
| L1-2 | South Spain | *F. equiseti* | + | – | – | – |
| Av-U3 | South Spain | *F. avenaceum* | + | – | – | – |
| H3-1/1G | South Spain | *F. avenaceum* | + | – | – | – |
| MPA 0999 | USA | *F. verticillioides* | + | – | + | – |
| MPB 3853 | | *F. sacchari* | + | – | + | – |
| MPC 1995 | Taiwan | *F. fujikuroi* | + | – | + | – |
| MPD 4853 | | *F. proliferatum* | + | – | + | + |
| ITEM 4293[c] | Spain | *F. proliferatum* | + | – | + | + |
| ITEM 2298[c] | Italy | *F. proliferatum* | + | – | + | + |
| ITEM 2191[c] | Italy | *F. proliferatum* | + | – | + | + |
| ITEM 2620[c] | Slovaquia | *F. proliferatum* | + | – | + | + |
| ITEM 2644[c] | Slovaquia | *F. proliferatum* | + | – | + | + |
| ITEM 1486[c] | Italy | *F. proliferatum* | + | – | + | + |
| ITEM 1451[c] | Italy | *F. proliferatum* | + | – | + | + |
| ITEM 2343[c] | Saudi Arabia | *F. proliferatum* | + | – | + | + |
| ITEM 2341[c] | Saudi Arabia | *F. proliferatum* | + | – | + | + |
| ITEM 4291[c] | Spain | *F. proliferatum* | + | – | + | + |
| Gf37 | Ecuator | *F. proliferatum* | + | – | + | + |
| FpFT10 | France | *F. proliferatum* | + | – | + | + |
| MPE 2192 | USA | *F. subglutinans* | + | – | + | – |

**Table 1. (*continued*)**

| Isolates | Origin | Species | Fus | Tct | Gib | Fpr |
|---|---|---|---|---|---|---|
| MPF 4093 | | *F. thapsinum* | + | – | + | – |
| MPG 05111 | | *F. nygamai* | + | – | + | – |
| MPH 69722 | South Africa | *F. circinatum* | + | – | + | – |
| ITEM 6013[c] | | *F. globosum* | + | – | + | – |
| R6 | | *F. oxysporum* | + | – | + | – |
| FOL8 | | *F. oxysporum* | + | – | + | – |
| MUCL 42821[d] | Belgium | *F. tricinctum* | + | – | – | – |
| CECT 2969[a] | | *Aspergillus ochraceus* | – | – | – | – |
| CECT 2906[a] | | *Penicillium verrucosum* | – | – | – | – |
| RH1 | | *Rhizopus* sp. | – | – | – | – |
| CL.1 | Spain | *Cladosporium* sp. | – | – | – | – |
| UCO.1 | Spain | *Alternaria consortiale* | – | – | – | – |
| Wheat | | | – | – | – | – |
| Maize | | | – | – | – | – |
| Barley | | | – | – | – | – |
| Rye | | | – | – | – | – |

[a]Strains supplied by Colección Española de Cultivos Tipo (CECT, Spain).
[b]Strains kindly provided by K. O'Donnell (NCAUR, USA).
[c]Strains kindly provided by A. Moretti (CNR, Italy).
[d]Strains supplied by Belgian Co-ordinated Collections of Micro-organisms (BCCM, Belgium).

(5′ CACTGCGTGCTGATTCACTGG 3′) and Tct-R (5′ GAGACAAGCATATGACTACTGGCAG 3′) which amplified a fragment of ~500 bp. Additionally, a pair of primers specific to *F. proliferatum* was designed: Fp3-F (5′ CGGCCACCAGAGGATGTG 3′) and Fp4-R (5′ CAACACGAATCGCTTCCTGAC 3′), which amplified a fragment of ~230 bp. The PCR assay for *F. verticillioides* described in Patiño et al. [16] has been adapted in order to unify protocols and reduce the number of primers used: reverse primer VERT-2 (5′ CACCCGCAGCAATCCATCAG 3′) has been combined with primer Fps-F. The size of the amplicon was ~700 bp.

The amplification protocol for *Fusarium* spp. PCR assay was 1 cycle of 85 s at 94 °C, 25 cycles of 35 s at 95 °C (denaturalization), 30 s at 67 °C (annealing), 30 s at 72 °C (extension), and 1 cycle of 5 min at 72 °C. The amplification protocol for the rest of the PCR assays only differed in the annealing temperature: 58 °C for *Gibberella fujikuroi*, 62 °C for trichothecene-producing *Fusarium* assay, 69 °C for *F. proliferatum* and 65 °C for the adapted PCR assay for *F. verticillioides*.

The primers used for amplification of *fum1*, F56UP (5′ CCAGGGTCGGGGTGTTG 3′) and PKSex-6R (5′ GCACACGCGCTTCCCAATCTCAT 3′), were previously described [11]. The amplification protocol was as follows: 1 cycle of 85 s at 94 °C, 35 cycles of 35 s at 95 °C (denaturalization), 30 s at 60 °C (annealing), 60 s at 72 °C (extension), and 1 cycle of 5 min at 72 °C. The amplicon was ~1100 bp long.

In all cases, amplification reactions were carried out in volumes of 25 μL containing 200 ng of template DNA in 3 μL, 1.25 μL of each primer (20 μM), 0.2 μL of Taq DNA polymerase (5 U/μL), 2.5 μL of 10XPCR buffer (20 mM $(NH4)_2SO_4$; 75 mM Tris-HCl; 50 mM ClK; ph 9), 1 μL of MgCl2 (50 mM), and 0.25 μL of dNTPs (100 mM) (Ecogen, Barcelona, Spain). PCR was performed in a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany). Amplification products were detected by electrophoresis on 1.5% agarose ethidium bromide gels in 40 mM Tris-acetate and 1.0 mM EDTA 1 × buffer.

## Sensitivity of PCR assays

Serial dilutions containing from 90 ng to 170 fg of DNA from *Fusarium* species were prepared to test the sensitivity of PCR assays described in this work, and the adapted PCR assay for *F. verticillioides*. The sensitivity of the primers was additionally tested in the presence of 200 ng of DNA from maize virtually free of *Fusarium* infection.

Various PCR primers presented in this work have been designed on the basis of conserved regions of IGS sequence, and can be used in more than one PCR assay. For example, primer Fps-F is used as forward primer in specific PCR assays for *F. sporotrichioides*, *F. poae* [7] and *F. verticillioides*, but was also used in genus-specific *Fusarium* spp. PCR assay. Cereals are often contaminated with a complex mixture of fungi, which can include several species of *Fusarium*. This means that primer Fps-F could anneal with DNA from any species of *Fusarium* in a PCR assay carried out with total DNA

from multi-infected plant tissue. Therefore, in order to test if sensitivity of the PCR assays could be affected by template competition, 200 ng of DNA from *F. culmorum* CECT2148 were added to dilutions containing from 90 ng to 170 fg of DNA either from *F. sporotrichioides* CECT 20166 or from *F. graminearum* Fg5, and then subjected to independent PCR assays specific to *F. sporotrichioides* and to *F. graminearum*, because these PCR assays have a common primer with PCR assay for identification of *F. culmorum*.

### DNA extraction from fumonisin-contaminated maize samples

We have analyzed 136 fumonisin-contaminated maize samples ranging from 0.4 to 72.3 ppm, and consisting of both grain (68 samples) and maize germ (a by-product from dry milling process) (68 samples). The maize samples (5 g) had been previously analyzed with ELISA for determination of total fumonisin contents (RIDASCREEN® Fumonisin, R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's protocol for corn and corn products. The detection limit of the analysis is 0.025 ppm and the recovery rate is approximately 60% with a mean coefficient of variation of approximately 8%.

The seeds (15–20 g) were previously ground with a hammer and sieved by a 0.1 mm screen, to obtain a subsample of 0.08 g that was subsequently subjected to DNA extraction. In the case of germ (15–20 g), a subsample of 0.08 g was directly used for DNA extraction without any previous processing. DNA extractions were carried out as described in Querol et al. [20]. Total DNA (200 ng) was used in PCR assays described in this work following the protocols described above, as well as in PCR assays described in Jurado et al. [7] for *F. graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. poae* and *F. equiseti*, and in Patiño et al. [17] for *F. verticillioides*, in this case with modifications mentioned above.

### Comparison between single and multi-copy-based PCR assays

We have carried out a comparative analysis in order to estimate differences in sensitivity when single- and multi-copy sequences are used as target in PCR diagnostics. For this purpose, we have chosen a PCR assay described in Mirete [11] based on *fum1*, a single-copy gene involved in biosynthesis of fumonisins [19]. This PCR assay amplifies a fragment of ~1100 bp in *F. verticillioides* and *F. proliferatum*, as well as *F. fujikuroi* and *F. nygamai*. Since these two species are associated with rice and sorghum [3] and has not been described in maize, we could assure that a positive signal in a *fum1*-based PCR assay carried out with DNA from maize could only be due to the presence of *F. verticillioides*, *F. proliferatum* or both.

Two separate serial dilutions containing from 90 ng to 170 fg of DNA from *F. verticillioides* and *F. proliferatum* species were prepared and subjected to *fum1* PCR assay to test the sensitivity of the assay. In addition, genomic DNA (200 ng) from fumonisin-contaminated samples of maize indicated above was also used for PCR assay based on *fum1*.

The results obtained in the analysis of fumonisin-contaminated samples of maize with *fum1* PCR assay were compared with those obtained in the analogue IGS-based PCR assays. In this case, amplification in one or both species-specific PCR assays for the fumonisin-producing species *F. verticillioides* and *F. proliferatum* were considered as a positive result. Quartiles of the 136 samples of maize were calculated according to fumonisin contents ($Q_1 = 3.1$ ppm, $Q_2 = 6.5$ ppm and $Q_3 = 12.6$ ppm) and four groups were subsequently obtained using quartiles as delimitating values (group 1:0.4–2.9 ppm and 100% of grain samples; group 2: 3.1–6.4 ppm and 74.28% of grain samples and 25.71% of germ samples; group 3: 6.5–12.3 ppm and 21.22% of grain samples and 78.78% of germ samples; group 4:12.6–72.3 ppm and 5.72% of grain samples and 94.28% of germ samples). Frequencies of positive samples with single- and multi-copy-based PCR assays were then calculated for each group.

## Results

### Specificity of PCR assays

The specific PCR assays developed in this work have been tested on a diverse range of *Fusarium* strains commonly associated with cereals, as well as other fungal genera and plant material, and results are shown in Table 1. Genus-specific primers Fps-F/Fus-R amplified a DNA fragment of ~200 bp only in the isolates of *Fusarium* species. Primers Gib2-F/Fgc-R amplified a DNA fragment of ~1000 bp in the species of *Gibberella fujikuroi* species complex and in the two strains tested of *F. oxysporum*. Primers Tct-F/Tct-R amplified a DNA fragment of ~400 bp in all the trichothecene-producing species of *Fusarium* tested (*F. graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. poae*, *F. sambucinum* and *F. pseudograminearum*), excluding *F. equiseti* strains. Primers Pr3-F/Pr4-R amplified a DNA fragment of ~230 bp only in *F. proliferatum* strains.

### Sensitivity of PCR assays

The detection limit estimated was >5.5 pg of DNA template in the PCR reactions carried out with specific

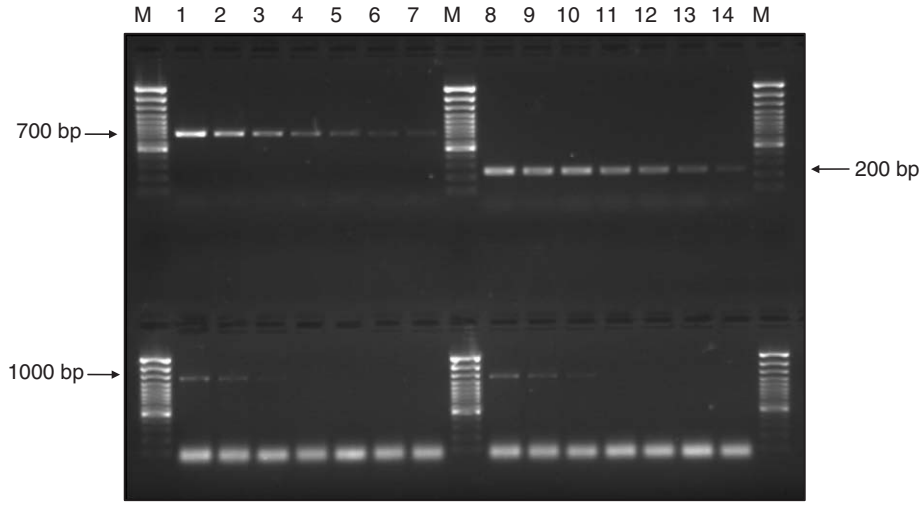


**Fig. 1.** Sensitivity of single- and multi-copy-based PCR assays. Above: PCR assay specific to *F. verticillioides* (lanes 1–7) and *F. proliferatum* (lanes 8–14) carried out with serial dilutions containing 350, 175, 87.5, 44, 22, 11 and 5.5 pg of DNA from *F. verticillioides* MP A 0999 (lanes 1-7) and *F. proliferatum* MPD 4853 (lanes 8–14). Below: the same dilutions of DNA mentioned above subjected to PCR assay for *fum1*; DNA marker (M).

primers for *Fusarium* spp., *Gibberella fujikuroi* species complex, trichothecene-producing species of *Fusarium, F. proliferatum* and the adapted PCR assay for *F. verticillioides* (Fig. 1). In the case of *fum1* PCR assay the detection limit was estimated as $>87.5$ pg of DNA template (Fig. 1).

The sensitivity of PCR assays was not affected by the addition of 200 ng of genomic DNA from *F. culmorum* CECT2148 to serial dilutions (90 ng–170 fg) of DNA either of *F. sporotrichioides* CECT 20166 or of *F. graminearum* Fg5, in their respective PCR assays (data not shown). Sensitivity was neither affected when PCR assays were carried out in presence of 200 ng of DNA from maize. In this case, 5 pg of *Fusarium* DNA in 200 ng of DNA from maize would result in a level of sensitivity of 25 ppm (data not shown).

## Analysis of maize samples and comparison between diagnosis with single- and multi-copy sequence-based PCR assays

The results obtained in the analysis of maize samples are shown in Fig. 2. Three *Fusarium* species were detected, *F. verticillioides* (74.26%), *F. graminearum* (23.46%) and *F. proliferatum* (21.32%). *F. equiseti*, *F. poae*, *F. sporotrichiodes* and *F. culmorum* were not detected. When a positive result was obtained in the *Fusarium* spp. PCR assay, at least one *Fusarium* species was found when species-specific PCR assays were performed. In addition, a positive result in any species-specific PCR assay corresponded with a positive result in the genus-specific PCR assay for *Fusarium* spp.

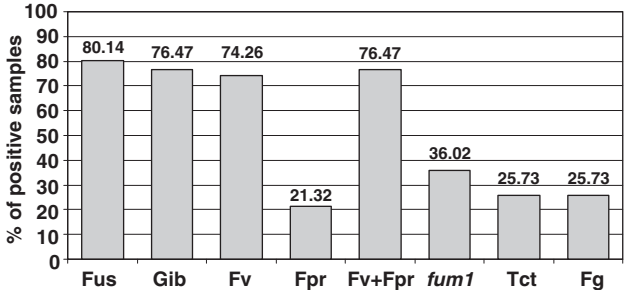


**Fig. 2.** Percentage of maize samples ($n = 136$) positive to PCR assays for *Fusarium* spp. (Fus), *Gibberella fujikuroi* species complex (Gib), *F. verticillioides* (Fv), *F. proliferatum* (Fpr), *F. verticillioides* and/or *F. proliferatum* (Fv + Fpr), *fum1*-based PCR assay (*fum1*), trichothecene producer species of *Fusarium* (Tct) and *F. graminearum* (Fg).

Similarly, correspondence was observed when PCR assay for the *Gibberella fujikuroi* species complex was performed, where a positive result corresponded to the presence of either *F. verticillioides* or *F. proliferatum*, or both, and vice versa. In the case of PCR for trichothecene-producing species of *Fusarium*, the percentage of positive samples was 25.73, the same percentage was obtained with PCR assay for *F. graminearum*. The percentage of positive samples in one or both PCR assays for *F. verticillioides* and *F. proliferatum* was 76.47, more than twice the percentage of positives in *fum1* PCR assay (36.02).

When percentages of positive samples to one or both PCR assays for *F. verticillioides* and *F. proliferatum* were calculated in the four groups with different fumonisin contents, detection was more sensitive for multi-copy

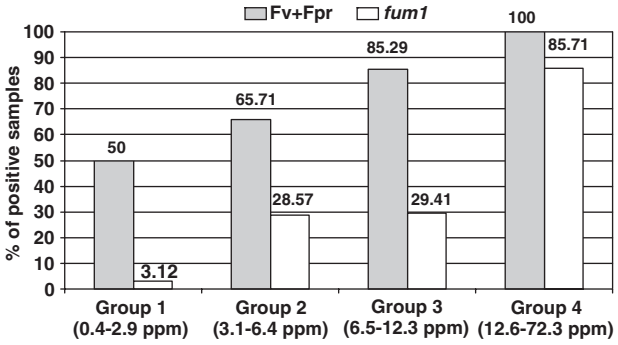


**Fig. 3.** Percentage of maize samples positive to *F. verticillioides* and/or *F. proliferatum* PCR assays (Fv + Fpr) and to *fum1*-based PCR assay in the four groups with different fumonisin contents.

IGS-based PCR assay than for single-copy PCR assay, particularly in groups 1 and 2 (Fig. 3).

## Discussion

In this work, a rapid, sensitive and specific strategy has been developed and tested to evaluate the potential ability of PCR assays to detect the presence of the most common mycotoxigenic species of *Fusarium* in cereals.

The correspondence among group-specific PCR assays and single-specific PCR assays developed in this work was optimal. The PCR assay initially developed to detect species of the *Gibberella fujikuroi* complex also detected the two strains tested of the closely related species *F. oxysporum*, which is considered a minor contaminant in cereals [2,10]. Development of a PCR assay for most of the trichothecene-producing *Fusarium* species was possible since phylogeny and ability to produce trichothecenes are correlated in *Fusarium* species [8,12,22]. The PCR assay developed for this group was able to detect the most important trichothecene-producing species of *Fusarium* (*F. graminearum*, *F. culmorum*, *F. sporotrichioides* and *F. poae*), but also the less frequent *F. pseudograminearum* and *F. sambucinum*. However, the more distantly related *F. equiseti* needed a specific PCR assay.

The set of primers presented in this work allowed to process a high number of samples and to obtain results in a short time in comparison with conventional diagnostics or using a battery of primers for independent PCR assays. We propose a procedure, schematically represented as a flowchart (Fig. 4) that can be followed in diagnosis of *Fusarium*-contaminated cereal samples. The strategy has been optimized to reduce the number of PCR assays and the number of primers used. With the exception of *F. culmorum* and *F. equiseti* PCR assays described in Jurado et al. [7], PCR conditions only differed in annealing temperature, so different PCR assays, and especially the generic ones, could be carried out simultaneously (up to eight samples in a standard gradient thermocycler), reducing drastically the time of analysis to a single day.

The occurrence of *Fusarium* species in the maize samples analyzed agreed with surveys carried out worldwide where *F. verticillioides*, *F. proliferatum* and *F. graminearum* are the most frequently isolated species in maize [10], and *F. equiseti*, *F poae*, *F. sporotrichioides* and *F. culmorum* are considered less frequent [10]. *F. verticillioides* was clearly the dominant species and would probably be the main source of the fumonisins present in the samples.

Since the main objective of this work was to develop an integrated protocol to be used directly in commodities, sensitivity and robustness are critical factors. The PCR assays based on IGS sequences had a sensitivity estimated in 25 ppm of *Fusarium* genomic DNA detectable in total DNA extracted directly from plant material.

The efficacy of the PCR assay based on multi-copy IGS sequence to detect the fumonisin producing species *F. verticillioides* and *F. proliferatum* was higher compared to the efficacy of single-copy PCR assay based on *fum1*. The differences in sensitivity were more evident in group 1 (0.4–2.9 ppm), where PCR assay based on single-copy gene *fum1* only detected 3.12% of positive samples in contrast with 50% of positive samples detected with multi-copy-based PCR assays. The strategy proposed and evaluated in this work indicated that conventional PCR assays were able to detect the source of fumonisins in contaminated seeds and derivatives at 50% when fumonisin contents were between 0.4 and 2.9 ppm, with a higher efficacy than single-copy PCR assays, which only reached similar values in samples with fumonisin contents higher than 12.3 ppm.

Fumonisin contents seemed to be correlated at a certain level with the percentage of positive samples to *F. verticillioides* and *F. proliferatum*, since efficiency in detection of these species by PCR assays increased when the fumonisin contents was higher. Chemical analysis has detected fumonisin contamination in samples where PCR assays could not detect the presence of *Fusarium*. This occurred in samples with low content of fumonisins and, particularly, in seed samples (data not shown). Besides the standard error associated with the fumonisin test, in this case, complete homogenization of the samples could be critical since only 0.08 g of the ground seeds was used for PCR assays. Therefore, we suggest that a more efficient method to ground maize seeds would provide a better homogenization and would probably improve the results.

To date, there has been no overall introduction of limits for fumonisins so far [21]. Average contamination value of fumonisins in maize products has been

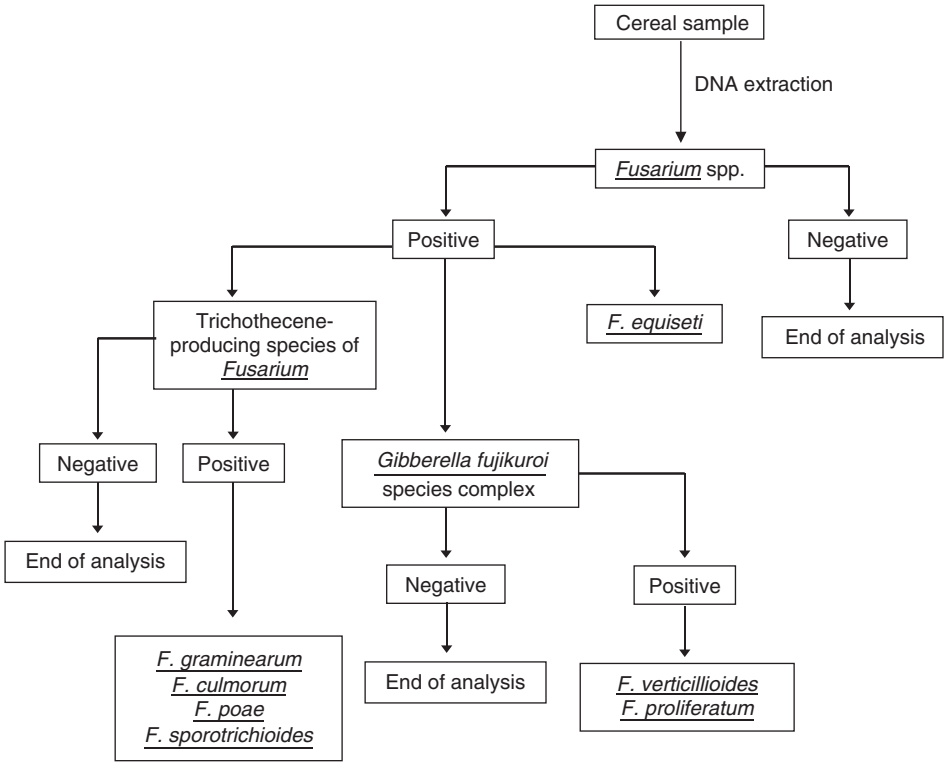


**Fig. 4.** Scheme representing PCR assays as a flowchart that can be followed in diagnostic of *Fusarium*-contaminated samples of cereals.

estimated as 1.4 ppm [23], and the American Food and Drug Administration proposed that the recommended maximum fumonisin levels to protect human and animal health should be between 2 and 4 ppm for human foods and 5–100 ppm for animal feeds [5]. The European Union has established limits for several *Fusarium* toxins, and in the case of fumonisins in cereals the maximum levels will be fixed before 2008 [4].

The method proposed in this work represents a useful tool to evaluate quality of raw material at different critical points of the food chain. It can be used in epidemiological studies and permits to predict potential risk for the presence of a range of mycotoxins. Combination of this approach with the more expensive and laborious conventional chemical analysis of toxins would improve the control of toxins entering the food chain.

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