

Characterization of *Fusarium* spp. isolates by PCR-RFLP analysis of the intergenic spacer region of the rRNA gene (rDNA)

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Abstract

In the present study, 44 *Fusarium* spp. isolates (5 *Fusarium culmorum*, 7 *Fusarium graminearum*, 1 *Fusarium cerealis*, 1 *Fusarium poae*, 26 *Fusarium oxysporum*, and 4 *Gibberella fujikuroi* species complex) were characterized morphologically, physiologically and genetically. All except one (Dutch Collection: CBS 620.72) were isolated from different hosts grown in various Spanish localizations. Morphological characterization was made according to macroscopic and microscopic aspects. Physiological characterization was based on their ability to produce zearalenone (ZEA) and type B trichothecenes (deoxynivalenol, nivalenol and 3-acetyldeoxynivalenol). ZEA was determined by liquid chromatography and trichothecenes by gas chromatography. Confirmation was carried out by liquid chromatography-ion trap-mass spectrometry (ZEA) or gas chromatography-mass spectrometry (trichothecenes). Molecular characterization of isolates was performed using an optimized, simple and low-cost method for isolation of DNA from filamentous fungi and polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) of the intergenic spacer region (IGS) of the rRNA gene (rDNA).

The results indicate that *F. graminearum*, *F. culmorum* and *F. cerealis* isolates were high ZEA and type B trichothecene producers, the *F. poae* isolate produced very low level of nivalenol while *F. oxysporum* and the *G. fujikuroi* complex isolates did not show this ability. Restriction patterns of the IGS region did not show any relationship with the host, geographic origin of the isolate and mycotoxin-producing capacity. However, the haplotypes obtained with six restriction enzymes (*Cfo*I, *Alu*I, *Hap*II, *Xho*I, *Eco*RI and *Pst*I) permitted to discern the six assayed *Fusarium* species. Therefore, this is a rapid and suitable methodology that allows closely related strains to group and to estimate the genetic relationships between the groups.

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1. Introduction

Complex communities of fungal pathogens are involved in foot rot and head blight diseases of small grain cereals and grasses as well as ear and stalk rot of corn. In temperate regions, *Fusarium* species, including *Fusarium culmorum* W.G. Smith and *Fusarium graminearum* Schwabe, are the most abundant and aggressive plant pathogens (Nelson et al.,

1981). *Fusarium oxysporum* Schlechtend, a common inhabitant of soil with worldwide distribution, is also responsible for vascular wilt diseases on many plants (Nelson et al., 1981; Edel et al., 1995). Devastating epidemics that occurred in Europe, USA, China, and South America resulted in yield losses due to reduced yields and negative impact on the quality of grain crops (Charmley et al., 1994; McMullen et al., 1997).

In addition to plant pathogenicity and yield losses, infection by *F. graminearum* and *F. culmorum* can lead to grain contamination by mycotoxins among which nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON) (known as type B

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trichothecenes) and zearalenone (ZEA) (an estrogenic secondary metabolite) belong to those most frequently isolated around the world (Marasas et al., 1984; Joffe, 1986; FAO/OPS, 1992; McMullen et al., 1997). The high stability of these compounds during storage and processing (Widenstrand and Pettersson, 2001) and their occurrence in a wide range of agricultural commodities lead to the fact that harmful mycotoxins are regularly found in animal feed and in human foodstuff (IPCS, 1990). With respect to health hazard and consumer protection, uniform regulations of these mycotoxin limits in the European Union and several countries are expected within the near future.

Fusarium is one of the most heterogeneous fungal genera and classification of species within this genus is very difficult. Currently, the differentiation of *Fusarium* spp. is based on physiological and morphological characteristics such as the shape and size of the macroconidia, the presence or absence of microconidia and chlamydospores, and colony morphology. Subtle differences in a single characteristic may delineate species. These observations need some practice and are difficult for the non-specialist (Windels, 1992; Bluhm et al., 2002). Therefore, a rapid and reliable assay for the routine identification of toxigenic *Fusarium* spp. would benefit the food-processing industry.

Molecular approaches have been developed for fungal systematic studies, including random amplified polymorphic DNA (RAPD) analysis (Voight et al., 1995), specific diagnostic PCR primers (Nicholson et al., 1998), and DNA sequencing (Appel and Gordon, 1996; O'Donnell et al., 1998). However, the methods more currently used are often based on the analysis of ribosomal RNA (rRNA) gene (or rDNA) sequences that are universal and contain both conserved and variable regions, allowing discrimination at different taxonomic levels (Guadet et al., 1989; Edel et al., 1995). Restriction analysis of PCR-amplified rDNA sequences has been shown to be a suitable method for taxonomic studies in *Fusarium* (Nicholson et al., 1993; Mirete et al., 2003), *Aspergillus* (Moody and Tyler, 1990), *Entomophaga* (Walsh et al., 1990) and other fungi (Cubeta et al., 1991; Chen, 1992).

Variations in rDNA among closely related taxa are found in the intergenic spacer (IGS), which separates the repeated ribosomal units (Avelange, 1994; Fernández et al., 1994). IGS sequences might be good candidates for the differentiation of strains at the intraspecific level (Hillis and Dixon, 1991; Edel et al., 1995) presumably due to relative lack of selective constraints, at least in a large part of its sequence.

The aim of the current study was to evaluate the utility of a DNA-based method, PCR-RFLP (restriction fragment length polymorphism) of the IGS region for characterizing *Fusarium* species.

2. Materials and methods

2.1. Fungal isolates

All isolates were identified morphologically according to Nelson et al. (1983). The *Fusarium* isolates used in this study,

their sources (mainly host plants), and geographic origin are listed in Table 1. Five isolates of *F. culmorum*, seven isolates of *F. graminearum*, one isolate of *Fusarium cerealis*, one isolate of *Fusarium poae*, twenty-six isolates of *F. oxysporum* and four isolates of the *Gibberella fujikuroi* species complex were assayed.

The isolates included in this study are held lyophilized at the fungal collection of the Department of Microbiology and Ecology of the University of Valencia. The *F. cerealis* isolate has also been deposited in the Spanish Collection of Type Cultures (CECT) with the reference 20547.

2.2. Mycotoxin analysis

Single-spore cultures were made of each isolate. Cultures were performed on Potato-Dextrose Agar (PDA). Pieces of these cultures were used to inoculate 500-ml Erlenmeyer flasks containing 100 g of corn. Before inoculation, corn was moistened with about 40 ml of deionized water to obtain a water activity (a_w) of 0.98. It was maintained overnight in a controlled atmosphere having the suitable equilibrium relative humidity. Then, the media were sterilised at 115 °C for 30 min, inoculated and incubated for 3 weeks at different temperatures depending on the particular mycotoxin to be investigated (20 °C for ZEA and NIV, 28 °C for DON, and 15 °C for 3-AcDON). These culture conditions were chosen on the basis of the results of previous assays aimed to optimization of these parameters to maximise the accumulation of these mycotoxins in cultures by producing isolates (Llorens et al., 2004a,b). After incubation, cultures were dried at 40 °C in air stove for 48 h and ground to a powder with a mill. Corn used as a substrate was previously analyzed and found to contain undetectable levels of ZEA or type B trichothecenes.

2.2.1. ZEA analysis

Finely ground cultures were thoroughly homogenized and extracted for analysis of ZEA, following an optimized method developed by Llorens et al. (2002). Ground cultures (4 g) were blended with methanol–1% aqueous NaCl (80:20, vol/vol) (2 × 25 ml). The mixture was filtered, the filtrate was defatted with hexane (2 × 10 ml) and the aqueous phase was extracted with dichloromethane (3 × 15 ml). The combined dichloromethane extracts were dried over anhydrous sodium sulphate and evaporated to dryness. The dry residue was dissolved in 3 ml chloroform–methanol (90:10, vol/vol) for clean-up on Florisil cartridge.

Florisil cartridges were preconditioned with 5 ml chloroform followed by 5 ml chloroform–methanol (90:10, vol/vol). The extract was dissolved in 3 ml chloroform–methanol (90:10, vol/vol) and passed through the cartridge. ZEA was eluted with 20 ml chloroform–methanol (90:10, vol/vol), and then evaporated to dryness under a slow flow of N₂. Finally, the residue was dissolved in 0.5 ml methanol.

ZEA was determined by liquid chromatography (LC). Chromatographic separation and detection were performed using a Waters 600 pump and a Waters 996 photodiode array detector coupled in series with a Waters 474 scanning

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