Food Microbiology 31 (2012) 17–24

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Characterization of *Fusarium verticillioides* strains isolated from maize in Italy: Fumonisin production, pathogenicity and genetic variability

Lorenzo Covarelli [a,*], Simonetta Stifano [a], Giovanni Beccari [a], Lorenzo Raggi [b], Veronica Maria Teresa Lattanzio [c], Emidio Albertini [b]

[a] Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
[b] Dipartimento di Biologia Applicata, Università degli Studi di Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
[c] National Research Council of Italy, Institute of Sciences of Food Production, Via Amendola 122/O, 70126 Bari, Italy

ARTICLE INFO

Article history:
Received 11 August 2011
Received in revised form
18 January 2012
Accepted 7 February 2012
Available online 13 February 2012

Keywords:
Fusarium verticillioides
Maize
Fumonisins
Pathogenicity
AFLP

ABSTRACT

Fusarium verticillioides (teleomorph *Gibberella moniliformis*) is the main fungal agent of ear and kernel rot of maize (*Zea mays* L.) worldwide, including Italy. *F. verticillioides* is a highly toxigenic species since it is able to produce the carcinogenic mycotoxins fumonisins. In this study, 25 *F. verticillioides* strains, isolated from maize in different regions of Italy were analyzed for their ability to produce fumonisins, their pathogenicity and their genetic variability. A further referenced strain of *G. moniliformis* isolated from maize in USA was also used as outgroup. The fumonisins B_1, B_2, and B_3 were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Pathogenicity tests were carried out by symptom observation and determination of growth parameters after inoculation of maize seeds, seedlings and wounded detached leaves. Total genomic DNA was used for Amplified Fragment Length Polymorphism (AFLP) analysis. About 20% of the analyzed strains were unable to produce fumonisins in *in vitro* experiments on inoculated maize flour, while, among fumonisin producers, a great variability was observed, with values ranging from 1 to 115 mg kg^{-1}. The different analyzed strains showed a wide range of pathogenicity in terms of effect on seed germination, seedling development and of symptoms produced on detached leaves, which were not correlated with the different *in vitro* fumonisin production. AFLP analysis indicated the presence of genetic diversity not only between the Italian strains and the American reference but also among the Italian isolates.

1. Introduction

Fusarium verticillioides (teleomorph *Gibberella moniliformis*) is the most common pathogen causing *Fusarium* ear rot of maize. This disease is widespread in temperate and semi-tropical areas, including all European maize-growing areas (Logrieco et al., 2002). In particular, ear rot caused by *F. verticillioides* prevails in Mediterranean areas characterized by dry and warm conditions like those present in Italy. This fungus is able to produce fumonisins, dangerous mycotoxins which can accumulate in the kernels and that can be toxic for humans and animals when used for food or feed (Desjardins et al., 1998; Munkvold and Desjardins, 1997).

Production of fumonisins by *F. verticillioides* is dependent on a biosynthetic gene cluster (*FUM*) constituted by 16 contiguous and co-expressed genes (Brown et al., 2007; Glenn et al., 2008; Proctor et al., 2003; Xu and Leslie, 1996). Proctor et al. (1999) found that the *FUM1* gene is relevant in fumonisin biosynthesis because its expression precedes toxin accumulation, and that deletion of this gene disrupts 99% of FB_1 production. Moreover, molecular genetic analysis indicated that phenotypes with altered fumonisin biosynthesis can result from mutations in genes within the *FUM* cluster (Proctor et al., 2006).

Different toxicological profiles of *F. verticillioides* strains could reflect important differences in the risk for mycotoxin contaminations, with potential implications for human and animal health, on international trade, on biological control of mycotoxin contamination and on understanding mycotoxin biosynthesis (Proctor et al., 2006).

Therefore, a good knowledge of the potentiality for fumonisin biosynthesis by fungal strains coming from different geographic regions could help predict the risk of contamination by mycotoxins in the surveyed areas.

In addition to the role of fumonisins as food and feed contaminants, the potential role of these mycotoxins in the development of

* Corresponding author. Tel.: +39 075 585 6464.
E-mail address: lorenzo.covarelli@unipg.it (L. Covarelli).

0740-0020/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2012.02.002

maize *Fusarium* diseases has been investigated but it has not been completely clarified (Abbas and Boyette, 1992; Desjardins and Hohn, 1997; Desjardins et al., 2007; Doehlert et al., 1994; Lamprecht et al., 1994; van Asch et al., 1992; Williams et al., 2006, 2007).

Fumonisin B_1 (FB_1), the most important fumonisin together with FB_2 and FB_3, is structurally similar to the AAL toxin, a compound produced by *Alternaria alternata* f. sp. *lycopersici* (Gilchrist et al., 1992) which inhibits the acyl CoA-dependant ceramide synthase in tomato plants, and is essential for the fungus to cause stem cankers on susceptible tomato plants (Abbas et al., 1994). For this reason, a possible role of fumonisins as virulence factor during the infection process has been hypothesized. With regard to maize seedling blight, it has been shown that fumonisins increased the virulence of *F. verticillioides* but are not necessary or sufficient for disease development (Desjardins et al., 1995). In addition, it has been demonstrated that fumonisin non-producing strains were able to cause maize ear infection and ear rot following silk-channel application and seed application at planting like fumonisin producing strains (Desjardins and Plattner, 2000). Conversely, Williams et al. (2006, 2007) reported a significant positive correlation between leaf lesion development on maize seedlings and the production of FB_1 by *F. verticillioides*. They also showed a significant inverse correlation between root weight and stalk height and the amount of FB_1 associated with seedling roots. Fumonisin non-producing strains did not cause leaf lesions and had significantly less effect on root weight and stalk height.

Mycotoxicological and pathogenetic characterization of fungal plant pathogens can be complemented by fingerprinting with Amplified Fragment Polymorphisms Analysis (AFLP). This can reveal genetic variability between different species and within the same species, giving us information about genetic relatedness of fungal strains and about possible genetic differences which could correspond to different mycotoxicological or pathogenetic profiles (Moretti et al., 2004; Reynoso et al., 2009).

Therefore, the aim of the present work was to carry out a comprehensive study to determine i) the ability of selected *F. verticillioides* strains isolated from maize grain in different Italian regions to biosynthesize fumonisins B_1, B_2 and B_3, ii) their pathogenicity on germinating seeds, seedlings and leaves and iii) the genetic variability among them and with respect to an American reference strain.

2. Materials and methods

2.1. Fungal isolates

Thirteen strains of *F. verticillioides* were selected among ~100 strains isolated from maize kernel samples collected in Umbria (Covarelli et al., 2011), central Italy, in order to have representative samples from the whole surveyed region. Additionally, 12 *F. verticillioides* strains isolated from typical maize cultivation areas of Northern Italy (Piedmont, Emilia–Romagna) and Central-Southern Italy (Molise) and one strain isolated from maize in the USA to be used as a reference (Leslie et al., 1992) were received from the ITEM fungal collection of ISPA-CNR Bari, Italy.

2.2. Microscopic and molecular identification of *F. verticillioides* strains and PCR assays for the detection of *FUM1* gene

To confirm the identification of *F. verticillioides* strains, microscopic observation of the morphological characteristics of the isolates, grown for two weeks in Petri dishes containing Potato Dextrose Agar (PDA), was performed according to Leslie et al. (2006). Furthermore, species specific PCR assays were conducted as follows. One-hundred microliters of a microconidial suspension of each isolates were used to inoculate 30 mL of Potato Dextrose Broth (PDB). Cultures were maintained in agitation (Lab-Line Orbital Shaker) for seven days at 22 °C. Mycelia were harvested by filtration through filtering cloth, freeze-dried for 24 h and then stored at −80 °C.

Genomic DNA of strains was obtained using the GenElute Plant Genomic DNA Miniprep kit (Sigma–Aldrich, St. Louis, MO, USA), according to the manufacturer's instruction. PCR assays were carried out by employing two different sets of primers: VER1 and VER2 (Mulè et al., 2004) and VERT1 and VERT2 (Patiño et al., 2004). Moreover, FUM5F and FUM6R primer pair was used to investigate the presence of the *FUM1* gene in all the *F. verticillioides* strains, according to Baird et al. (2008).

PCR amplifications were performed in volumes of 25 μL containing 20 ng of fungal genomic DNA, 0.25 μL of each forward and reverse primer pairs (100 μM), 2 μL of $MgCl_2$ (10 mM), 0.2 μL *Taq* DNA Polymerase (5 U μL^{-1}, Invitrogen, Carlsbad, CA), 2.5 μL of a 10X PCR buffer (Invitrogen) and 2.5 μL of dNTPs (10 mM).

Amplification was performed by using the Thermo Hybaid (USA) thermalcycler. The cycling conditions for the VERT1/VERT2 primers were as follow: 1 cycle for 1′25″ at 94 °C followed by 25 cycles of 35″ at 95 °C, 30″ at 64 °C, 2′ at 72 °C. A final step at 72 °C for 5′ was also performed. The same cycle was used for VER1/VER2 primer pair, with the exception of the annealing temperature which was 54 °C. Fifteen microliters of each PCR were loaded into a 2% agarose gel, stained with ethidium bromide and separated by gel electrophoresis.

For *FUM1* gene determination, PCR cycling conditions were as follows: 1 cycle at 95 °C for 3′ followed by 32 cycles at 95 °C for 1′, 69 °C for 1′ and 72 °C for 3′ with a final extension step at 72 °C for 5′.

2.3. Fumonisin extraction and LC–MS/MS analysis

2.3.1. Fungal cultures and sample preparation

Fifteen grams of autoclaved maize flour were positioned inside 100 mL flasks and 12.5 mL of sterile water were subsequently added in order to have the right moisture to allow fungal development. Three flasks for each fungal strain were then inoculated with 100 μL of a conidial suspension containing 10^6 conidia mL^{-1}. Three flasks were used as controls by adding only 100 μL of sterile water. Flasks were incubated at 21 °C for two weeks in order to allow fungal development and fumonisin production and then harvested and reduced to a fine powder with mortar and pestle and stored at −80 °C.

Each fungal culture and the un-inoculated control were extracted and analyzed in triplicate according to the procedure by Sydenham et al. (1992) with minor modifications. Five grams of ground sample were extracted with 20 mL of methanol/water (75:25, v/v) by 60 min shaking. The extract was filtered through filter paper. Prior to LC–MS/MS analysis the extract was diluted 1:100 with a mixture of methanol/water (60:40), then filtered through 0.45 μm syringe filter. An extract aliquot of 200 μL was added with 100 ng $^{13}CFB_1$ and 40 ng $^{13}CFB_2$. Twenty microliters were injected into the LC–MS/MS apparatus.

2.3.2. Chemicals and reagents

Methanol (HPLC grade) and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Fully isotope labeled ^{13}C-fumonisins solutions were purchased from Biopure Referenzensubstanzen GmbH (Tulln, Austria). Filter papers (Whatman no. 4) were obtained from Whatman International Ltd (Maidstone, UK). HPLC syringe filters (regenerated cellulose, 0.45 μm) were from Alltech (Deerfield, IL, USA).