Fusarium proliferatum – Causal agent of garlic bulb rot in Spain: Genetic variability and mycotoxin production

Laura Gálvez [a], Monika Urbaniak [b], Agnieszka Waśkiewicz [c], Łukasz Stępień [b], Daniel Palmero [a],[*]

[a] Department of Agricultural Production, Plant Protection Laboratory, School of Agricultural, Food and Biosystems Engineering (ETSIAAB), Technical University of Madrid, Avda. Puerta de Hierro 4, 28040, Madrid, Spain
[b] Department of Pathogen Genetics and Plant Resistance, Institute of Plant Genetics of the Polish Academy of Sciences, Strzeszyńska 34, 60-479, Poznań, Poland
[c] Department of Chemistry, Poznań University of Life Sciences, Wojska Polskiego 75, 60-625, Poznań, Poland

ARTICLE INFO

ABSTRACT

Fusarium proliferatum is a world-wide occurring fungal pathogen affecting several crops included garlic bulbs. In Spain, this is the most frequent pathogenic fungus associated with garlic rot during storage. Moreover, *F. proliferatum* is an important mycotoxigenic species, producing a broad range of toxins, which may pose a risk for food safety. The aim of this study is to assess the intraspecific variability of the garlic pathogen in Spain implied by analyses of translation elongation factor (*tef-1α*) and *FUM1* gene sequences as well as the differences in growth rates. Phylogenetic characterization has been complemented with the characterization of mating type alleles as well as the species potential as a toxin producer.

Phylogenetic trees based on the sequence of the translation elongation factor and *FUM1* genes from seventy nine isolates from garlic revealed a considerable intraspecific variability as well as high level of diversity in growth speed. Based on the *MAT* alleles amplified by PCR, *F. proliferatum* isolates were separated into different groups on both trees. All isolates collected from garlic in Spain proved to be fumonisin B_1, B_2, and B_3 producers. Quantitative analyses of fumonisins, beauvericin and moniliformin (common secondary metabolites of *F. proliferatum*) showed no correlation with phylogenetic analysis neither mycelial growth. This pathogen presents a high intraspecific variability within the same geographical region and host, which is necessary to be considered in the management of the disease.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Fusarium proliferatum belongs to the *Liseola* section within the *Fusarium* genus. Its teleomorph, *Gibberella intermedia*, belongs to the *G. fujikuroi* species complex, which comprises of at least 12 reproductively different biological species (mating populations) (Lima et al., 2012). *F. proliferatum* is a world-wide occurring fungal pathogen affecting several relevant crops including maize (Logrieco et al., 1995), wheat (Conner et al., 1996), rice (Desjardins et al., 1997), asparagus (Elmer, 1990), date palm (Abdalla et al., 2000), garlic (Dugan et al., 2003), onion (Toit et al., 2003), ornamental palms (Armengol et al., 2005), and Chinese chive (Yamazaki et al., 2013). In particular, *F. proliferatum* has been reported as a pathogen of garlic bulb rot in USA (Dugan et al., 2003), Serbia (Stankovic et al., 2007), Spain (Palmero et al., 2010), Italy (Tonti et al., 2012), India (Sankar and Babu, 2013), Egypt (Moharam et al., 2013) and Argentina (Salvalaggio and Ridao, 2013). The disease develops during the drying process and can affect almost 30% of the stored bulbs (Tonti et al., 2012). In Spain, *F. proliferatum* is the most frequent pathogenic fungus associated with garlic rot during storage (Palmero Llamas et al., 2013). Spain is the largest garlic producer within the European Union with 169,468 tons in 2014 (FAOSTAT, 2014), which represents 10.77% of total world marketed production. Spain exports around 269,462 tons in 2015 (TRADEMAP, 2015).

Fusarium rot symptoms consist of superficial dry brown necrotic spots that progress toward the clove and, in some cases, the presence of white mycelium and water-soaked symptoms can be

observed. *Fusarium proliferatum* is able to colonize garlic roots during the crop, remain as a latent infection and develop rot during storage. Moreover, *F. proliferatum* is an important mycotoxigenic species, producing a broad range of toxins, such as fumonisins (FB$_1$, FB$_2$ and FB$_3$), moniliformin (MON), beauvericin (BEA), fusaric acid (FA), and fusaproliferin (FUP), which pose a high risk for food safety (Desjardins, 2006).

Contamination with fumonisins has been reported in garlic, both for bulbs and powder (Boonzaaijer et al., 2008; Seefelder et al., 2004; Waśkiewicz et al., 2013a,b). Seefelder et al. (2002) detected FB$_1$ in the garlic tissue after artificial inoculation. Stankovic et al. (2007) demonstrated that *F. proliferatum* isolated from garlic can produce fumonisin B$_1$, BEA, fusaric acid, fusaproliferin and MON. Stępień et al. (2011b) also detected the production for FB$_2$ and FB$_3$ together FB$_1$ by *F. proliferatum* strains isolated from garlic. Palmero et al. (2012) working with *F. proliferatum* isolated from Spanish garlic, indicated that it can produce fusaric acid.

Regarding the phylogenetic studies, the analysis of the translation elongation factor (*tef-1α*) gene sequences appear to be the most useful in taxonomic studies of fungi among all markers used for phylogeny reconstruction (e.g. internal transcribed spacers region, β-tubulin, calmodulin, and H3 histone genes), especially in the *Gibberella fujikuroi* species complex, as well as in other members of the *Fusarium* genus (Geiser et al., 2004; Kristensen et al., 2005).

Recently, genes and other sequences directly involved in secondary metabolism gained more attention in phylogenetic studies, as those have the advantage of possible usage in combined approaches to the diagnostics of mycotoxigenic abilities (Proctor et al., 2009; Stępień, 2014). Mycotoxigenic genes are organised in clusters that undergo similar regulation. For fumonisin-producing species, a *FUM* cluster has been cloned and its essential role for fumonisin biosynthesis characterised (Proctor et al., 2003). Secondary metabolism is less strictly preserved than basic metabolic processes, therefore, these gene clusters can be more diverse. The genes from the *FUM* cluster merit investigation as a good additional marker for phylogenetic studies of fumonisin-producing *Fusarium* species (Baird et al., 2008; González-Jaén et al., 2004; Stępień et al., 2011a). In particular, the sequence of the *FUM1* gene of *F. proliferatum* was found to be useful in revealing the intraspecific polymorphism, which is, to some extent, specifically correlated with the host plant (Stępień et al., 2011a).

Mycotoxicological and phylogenetic characterization of *F. proliferatum* isolates can be complemented by the characterization of mating type alleles (*MAT-1* and *MAT-2*). The application of subsequently developed polymerase chain reaction (PCR) markers specific for *MAT-1* and *MAT-2* of some *Fusarium* species has substantially shortened the time needed for such analyses. All this can reveal genetic variability within the same species, giving the information about genetic relatedness of fungal isolates as well as possible genetic differences.

Therefore, it seems necessary to study the intraspecific variability of the pathogen to know its possible influence on disease control strategies such as the possibility of sexual reproduction increasing the population's diversity or differential response of individual strains to chemical control. There is little information available on the intraspecific variability of *F. proliferatum*, and only few dealing with isolates from garlic. Previous studies give some information on the high variability of this species on different hosts based on phylogenetic analyses, its potential as a producer of mycotoxins or its growth rate (Jurado et al., 2010; Palacios et al., 2015; Stępień et al., 2011b), but there are not available studies for *F. proliferatum* strains originating from garlic.

The aim of this study was to assess the variability of *F. proliferatum* strains isolated from garlic bulbs of Spain origin on

three levels: (i) determination of the *MAT-1* and *MAT-2* alleles and mycelial growth rates, (ii) phylogenetic relationships implied by *tef-1α* and *FUM1* genes' sequences and, (iii) quantitative analyses of fumonisins, beauverecin and moniliformin produced by *F. proliferatum* isolates on rice cultures.

2. Materials and methods

2.1. Fusarium isolates purification and morphological identification

Seventy nine *F. proliferatum* isolates were collected from stored garlic bulbs with *Fusarium* rot symptoms originating from different cooperatives from Spain in different years. Symptomatic cloves were cut, surface-disinfested for 3 min in a 2% sodium hypochlorite solution, rinsed twice with sterilized distilled water and cultured for 5 days at 25 °C in potato dextrose agar (PDA, Conda). After 5 days, initial fungal isolation was made by transferring the growing mycelia to a Petri plate containing PDA, and then monosporic cultures were obtained. All isolates are summarized with its geographical origin in Table 1. They were maintained on PDA at 4 °C and stored as spore suspensions in 20% glycerol at −80 °C. The morphological identification was performed following the taxonomic criteria of Gerlach and Nirenberg (1982), Nelson et al. (1983) and Leslie and Summerell (2006).

2.2. DNA extraction, primers, PCR assays and DNA sequencing

Mycelia of the isolates studied were grown 7 days on solid PDA medium. Genomic DNAs of all isolates were extracted using a hexadecyltrimethylammonium bromide (CTAB) method according to Stępień et al. (2003), and, based on the Nanodrop® measures, the DNA concentrations were adjusted to 10 ng/μl. Amplification reactions were carried out in volumes of 25 μL containing 0.1 μL of each primer (100 μM), 0.5 μL of Taq DNA polymerase (5 U/μL), 2.5 μL of 10× PCR buffer, 1.5 μL of dNTPs (10 μM) and 10—20 ng of genomic DNA as template.

The PCR amplification of a partial region of the tef-1α gene was carried out with primers Ef728M and TefR1 (Carbone and Kohn, 1999; Samuels et al., 2002) and the PCR conditions were as follows: (5 min at 94 °C, 34 cycles of 45 s at 94 °C, 62.5 s at 45 °C, 1 min at 72 °C) and 5 min at 72 °C. The amplification of a partial regions of the FUM1 was made using primers Fum1F1 and Fum1R2 (Stępień et al., 2011a) and the PCR conditions were the same as above, except the annealing temperature was 65.5 °C.

Amplicons were electrophoresed in 1.5% agarose gels (Invitrogen) with GelRed (Biotium) staining and visualised using UV light.

Fragments of *tef-1α* and *FUM1* genes obtained with the Ef728M/TefR1 and Fum1F1/R2 primers were sequenced. For sequence analysis, PCR-amplified DNA fragments were purified with exonuclease I (Thermo Scientific) and FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) using the following program: 10 min at 37 °C, followed by 15 min at 80 °C. Both strands were labelled using the BigDye Terminator v3.1 kit (Applied Biosystems) and purified by ethanol precipitation. Sequence reading was performed using Applied Biosystems 3130 equipment.

2.3. Mating type determination and colony growth rate measurement

The mating type genes (*MAT-1* and *MAT-2*) of the isolates was identified by PCR using the primers GfMAT1-F/GfMAT1-R for *MAT-1* allele, and GfMAT2-F/GfMAT2-R for *MAT-2* allele as described by Kerényi et al. (2002). The genomic DNA templates as well as equipment and electrophoresis conditions were the same as for the