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Combined effects of benomyl and environmental factors on growth and expression of the fumonisin biosynthetic genes *FUM1* and *FUM19* by *Fusarium verticillioides*

A. Cruz [a], P. Marín [a,*], N. Magan [b], M.T. González-Jaén [a]

[a] Department of Genetics, Faculty of Biology, Complutense University of Madrid (UCM), José Antonio Novais 12, 28040 Madrid, Spain
[b] Applied Mycology Group, Cranfield Soil and AgriFood Institute, Cranfield University, Cranfield, Bedfordshire MK43 OAL, UK

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ABSTRACT

Fusarium verticillioides is predominantly responsible of fumonisin contamination of maize and other cereals in Mediterranean climatic regions. This study examined the interaction of the fungicide benomyl, at ED_{50} and ED_{90} concentrations (effective doses of benomyl to reduce growth by 50% and 90%, respectively), with a range of temperatures (20–35 °C) and water potentials (−0.7, −2.8 and −7.0 MPa) compatible with current and foreseen climate change scenarios for these regions on growth and fumonisin biosynthesis in *in vitro* assays. The expression of fumonisin biosynthetic genes (*FUM1* and *FUM19*) was quantified by real time RT-PCR. *FUM1* encodes a polyketide synthase and *FUM19* an ABC-type transporter, located both in the fumonisin biosynthetic cluster. The ED_{50} and ED_{90} concentrations obtained at 25 °C were 0.93 mg/L and 3.30 mg/L, respectively. Benomyl affected growth and fumonisin gene expression differently but it generally reduced fungal growth and fumonisin biosynthesis and both were significantly affected by temperature and water potential.
This indicated that efficacy of benomyl might be compromised at certain conditions, although at similar or lower levels than other fungicides tested. Both fumonisin biosynthetic genes had similar expression patterns in all treatments and their correlation was positive and significant. The results suggested that Mediterranean climatic scenarios might suffer an additional negative impact of climate change by reducing the efficacy of antifungals used to control pathogens and toxigenic fungi.

1. Introduction

Fumonisins are a family of toxic and carcinogenic mycotoxins that cause serious diseases affecting humans and animals (Marasas et al., 2004) due to their structural similarity to the sphingolipid intermediates sphinganine and sphingosine, which affect sphingolipid metabolism by inhibiting the enzyme ceramide synthase (Wang et al., 1991). Fumonisins B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) are the major fumonisins produced in nature, with FB_1 being the most prevalent and toxic (Musser and Plattner, 1997; Thiel et al., 1992). Due to the health risk associated with the consumption of contaminated commodities their occurrence is regulated in many countries including the European Union (Commission Regulation EC No 1126/2007; Commission Regulation EU No 420/2011). These toxins are also catalogued by the International Agency for Research on Cancer (IARC, 1993) as 2B group carcinogens.

Fumonisins are mainly produced by *Fusarium verticillioides* (*Gibberella moniliformis, Gibberella fujikuroi* mating population A) during colonization of agricultural commodities in the field or during storage. This species is also one of the most important pathogen of maize, where it causes ear and stalk rot. Recent reports demonstrated its occurrence in other dietary crops such as wheat and barley (Chehri et al., 2010; Gil-Serna et al., 2013; Tančić et al., 2012). This fumonisin producer has a wide distribution in temperate regions and occurs frequently in Mediterranean climate regions (Aliakbari et al., 2007; Cavaglieri et al., 2009; Gil-Serna et al., 2013; Jurado et al., 2006). Temperature and water potential are the main factors affecting fungal growth and mycotoxin production (Charmley et al., 1994; Magan, 2007) and produce characteristic patterns of response depending on the fungal species considered. Interactions between these two factors have been shown to limit the ability of this species to grow and produce FB_1 (Jurado et al., 2008; Marín et al., 2010b; Medina et al., 2013). Under changing climatic conditions these environmental factors may be transient or more permanent in certain regions (Miraglia et al., 2009). Scenarios for Mediterranean climate regions foresee extreme changes in temperature and rainfall patterns, in particular, higher temperatures (increase of 4–5 °C) and longer drought periods (European Commission, 2007). These scenarios might significantly contribute to changes in mycotoxigenic fungal populations of agri-food products and changes in their associated mycotoxins. Additionally, these changes might also affect the current control strategies (Magan et al., 2011).

* Corresponding author. Tel.: +34 91 394 45 68; fax: +34 91 394 48 44.
E-mail address: patmarin8149@ucm.es (P. Marín).

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Control of this pathogen in the field relies on integrated strategies including the use of anti-fungal compounds to reduce fungal infection, especially during silking of maize. Although they are mostly effective in arresting fungal growth, increasing evidence suggests that control of toxin production might not be so efficient, either *in vitro* or in field situations (Magan et al., 2002; Magan and Olsen, 2004; Mamza et al., 2008; Medina et al., 2007; Ramírez et al., 2004). In certain conditions, they may act as stress factors resulting in the induction of different stress related genes and toxin biosynthesis (Edwards and Godley, 2010; Haidukowski et al., 2005; Mateo et al., 2011; Marín et al., 2013; Ngûyen et al., 2000; Ramírez et al., 2004; Schmidt-Heydt et al., 2007; Tenreiro et al., 2001).

Benomyl and its main degradation compound carbendazim are systemic benzimidazole fungicides that play an important role in plant disease control. Benomyl is one of the most effective fungicides in inhibiting fungal growth and is currently being used in several South American countries to control fungal growth in cereals, vegetables and fruits (Llorent-Martínez et al., 2012; RAP-AL, 2008; Subhani et al., 2011). They are not only used during pre-harvest treatments of plants, but also during post-harvest food storage (Magnucka et al., 2007; Whitehead, 1996). Benomyl and carbendazim are regulated by the European Union, with maximum allowable residue levels (MRL) in maize, millet, rice and sorghum (0.01 mg/kg), on rye and wheat (0.1 mg/kg) and on barley and oats (2 mg/kg) for these fungicides (Commission Regulation EU No 559/2011). These restrictions require more accurate studies to ensure the highest efficiency with the minimal impact on health and environment. Therefore, there is an urgent need for a better understanding of the impact that such control systems have on growth and mycotoxin production under a range of environmental conditions.

Knowledge of the genes involved in fumonisin biosynthesis has been very useful to unravel the factors responsible for the regulation of the production of this toxin. The fumonisin biosynthetic genes are organized in clusters as for other toxins produced by filamentous fungi so far described. At least 15 clustered genes encode enzymatic activities responsible for fumonisins. The expression of these genes seems to be co-regulated (Proctor et al., 1999, 2003). *FUM1* encodes a polyketide synthase (Proctor et al., 1999) which catalyzes the initial step in fumonisin biosynthesis (Bojja et al., 2004) and its expression appears to be positively correlated with fumonisin production in *F. verticillioides* (López-Errasquín et al., 2007; Medina et al., 2013). In the case of *FUM19*, this gene is located about 35 kb downstream of *FUM1* and encodes an ABC (ATP-binding cassette) transporter involved in extracellular export of fumonisins (Proctor et al., 2003). This class of transporters, as well as those included in the MFS family, is involved in the export of a diversity of toxic compounds either of endogenous or exogenous origin reducing their accumulation inside the fungal cell (Bauer et al., 1999). The *FUM19* transporter has not been fully characterized yet, although a previous phylogenetic analysis using the amino acid sequence corresponding to the putative cDNA sequence of *FUM19* gene placed this protein within the MDR (multidrug resistance) subfamily of transporters (López-Errasquín, 2005). It is common for transporters of this group to export diverse compounds. Additionally, one of the mechanisms of multidrug resistance involves an increase in export activity of the compounds by overexpression of the transporter coding gene (Cools and Fraaije, 2012). Therefore, the *FUM19* transporter might also participate in the export of other compounds such as some anti-fungals or, at least, might respond to general or specific toxic or stress challenges. These might have an influence on the expression of the transporter gene and interact with fumonisin production or export. New approaches based on real time RT-PCR and microarrays have been developed using sequence information of the mycotoxin biosynthetic genes to perform the rapid and accurate quantification of gene expression in studies aimed to evaluate the influence of environmental factors or/and the efficiency of antifungals (Jurado et al., 2008; Marín et al., 2010a, b; Medina et al., 2013; Schmidt-Heydt et al., 2008). These strategies permit an evaluation of an antifungal agent (benomyl) simultaneously on growth and fumonisin biosynthesis in order to dissect relevant factors involved in this complex regulation process.

The objectives of this work were to (1) determinate effective doses of benomyl to reduce growth of *F. verticillioides* by 50% or 90% (ED_{50} and ED_{90} respectively), (2) examine the effect of different temperature × water potential conditions, simulating climate change scenarios in the Mediterranean climatic regions, on fungal growth rates and (3) quantify the relative expression of the fumonisin biosynthetic genes *FUM1* and *FUM19* in relation to imposed temperature × water potential stress.

2. Material and methods

2.1. Fungal strains

The fumonisin-producing *F. verticillioides* FvA (FvMM7-3) strain used in this study was originally isolated from a maize field in Madrid (Spain) and has been extensively characterized in several previous studies (Jurado et al., 2008; Marín et al., 2010b). The culture was maintained on PDA (potato dextrose agar medium, Scharlau Chemie, Barcelona, Spain) at 4 °C and stored as a spore suspension in 15% glycerol at −80 °C in the Department of Genetics of the Complutense University of Madrid (UCM).

2.2. Inoculation, incubation conditions and growth assessment: Determination of ED_{50} and ED_{90} values

The medium used in this study was a fumonisin-inducing solid agar medium previously described (López-Errasquín et al., 2007). The fungicide benomyl (Benopron(R), Probelte, Murcia, Spain) containing 50% of the active ingredient was added to this medium. Based on the concentration of the active ingredient, appropriate amounts of benomyl were added to sterile deionised water to reach benomyl concentrations of 0, 0.5, 1, 2, 2.5, 3.5 and 4 mg/L. These were added later to the autoclaved molten medium when the temperature was cooled to 50 °C. These concentrations were used to determine the ED_{50} and ED_{90} values, in comparison with the control medium without benomyl (ED_0). Each concentration was tested in four independent experiments. A 3 mm-diameter agar disk from the margin of a 7-day-old growing colony of FvA grown at 25 °C was used to centrally inoculate each replicate. The plates were subsequently incubated at 25 °C for 10 days. Assessment of growth was made once a day during the incubation period. Two diameters of the growing colonies were measured until the colony reached the edge of the plate or the tenth day was reached. The ratio of each colony was plotted against time and a linear regression was applied to obtain the growth rate (mm/day) as the slope of the line. This growth rate was plotted against benomyl concentrations (mg/L) in order to determine ED_{50} and ED_{90} values for the strain FvA.

2.3. Evaluation of ED_{50} and ED_{90} concentrations of benomyl on *F. verticillioides* growth at different temperatures and water potentials

The water potentials ($_w$) of the fumonisin conductive agar medium mentioned above were modified with the non-ionic solute glycerol, to obtain target values of −2.8 and −7.0 MPa which correspond to water activities (a_w) of 0.982 and 0.950 respectively. A non-modified control medium had a water potential of −0.7 MPa (a_w = 0.995). Every medium was prepared either without fungicide (ED_0) or in which the ED_{50} or ED_{90} concentrations of benomyl were added. All plates were overlaid with sterile cellophane sheets (P400; Cannings, Ltd., Bristol, United Kingdom) before inoculation to facilitate removal of the fungal biomass for RNA extractions at the end of the 10-day incubation period. The plates were incubated at 20, 25, 30 and 35 °C for