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Potential effects of environmental conditions on the efficiency of the antifungal tebuconazole controlling *Fusarium verticillioides* and *Fusarium proliferatum* growth rate and fumonisin biosynthesis

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ABSTRACT

Fusarium verticillioides and *Fusarium proliferatum* are important phytopathogens which contaminate cereals in the Mediterranean climatic region with fumonisins. In this study we examined the interaction between the fungicide efficacy of tebuconazole and water potential (Ψ_w) (−0.7–7.0 MPa) × temperature (20–35 °C) on growth and *FUM1* gene expression by real time RT-PCR (an indicator of fumonisin biosynthesis) in strains of both *Fusarium* species. Concentrations of tebuconazole required to reduce growth by 50 and 90% (ED_{50} and ED_{90} values) were determined. Growth of strains of both species was largely reduced by tebuconazole, with similar efficacy profiles in the interacting water potential × temperature conditions. In contrast, *FUM1* expression was not generally reduced by tebuconazole. Moreover, sub-lethal doses in combination with mild water stress and temperatures less than 35 °C significantly induced *FUM1* expression with slight differences in both species. These results suggest that the efficacy of antifungal compounds to reduce mycotoxin risk would be more effective if consideration is given to both growth rate and toxin biosynthesis in relation to interacting environmental conditions. This is the first study linking fungicide efficacy of tebuconazole with environmental factor effects on control of growth and *FUM1* gene expression of *F. verticillioides* and *F. proliferatum*.

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). More than ten types of fumonisins have been isolated and characterized. Of these, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin 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). Additionally, these toxins are cataloged as group 2B carcinogens by the International Agency for Research on Cancer (IARC, 1993). Because of the health risk associated with the consumption of contaminated commodities, their occurrence is currently under regulation in many countries including the European Union (Commission Regulation EC No 1126/2007).

Fumonisins are mainly produced by the fungi *Fusarium verticillioides* (*Gibberella moniliformis*, *Gibberella fujikuroi* mating population A) and *Fusarium proliferatum* (*Gibberella intermedia*, *Gibberella fujikuroi* mating population D) during colonization of agricultural commodities in the field or during storage. They are closely related and important pathogens of maize often occurring together. However, they show several differences. *F. verticillioides* is pathogenic to maize only, where it causes ear and stalk rot, although recent reports demonstrated its occurrence in other dietary crops such as wheat and barley (Chehri et al., 2010; González-Jaén et al., 2008; Tančić et al., 2012). *F. proliferatum* colonizes a wider range of hosts (as diverse as pine trees, asparagus or palm trees) in addition to maize, wheat and barley, where it causes black point symptoms (Conner et al., 1996; Desjardins et al., 2007). They have a wide distribution in temperate regions (Aliakbari et al., 2007; Cavaglieri et al., 2009; Desjardins et al., 2000; De Souza and Formento, 2004). Previous studies showed that they have largely similar growth patterns in response to temperature and water stress (Jurado et al., 2008; Marín et al., 1999, 2004, 2010a) in agreement with their frequent occurrence in Mediterranean regions (Gil-Serna et al., 2012; Jurado et al., 2006; Logrieco et al., 2002; Medina et al., 2006; Soldevilla et al., 2005). However, the effects of these environmental factors on fumonisin biosynthesis, in particular water stress, suggested that regulation of fumonisin biosynthesis might differ in these two species (Marín et al., 2010a). Although stress factors may only be transient, in a situation of changing climatic conditions, they may become more

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permanent in certain regions (Miraglia et al., 2009). Mediterranean climate regions have been identified as climate change hot spots where extreme changes in temperature, CO_2 and rainfall patterns are predicted. In particular, the higher temperatures and drought expected might significantly contribute to changes in mycotoxigenic fungal populations of agro-food products and their associated mycotoxin risk but also affect control strategies (Magan et al., 2011).

Control of these pathogens in the field relies on integrated strategies which include the use of antifungal compounds. These compounds have many different target sites such as fungal cell membrane components and result in a reduction in fungal growth. However, increasing evidence suggests that they might not be as efficient at reducing toxin production. In fact, in certain conditions, they may act as stress factors resulting in the induction of toxin biosynthesis (Edwards and Godley, 2010; Haidukowski et al., 2005; Ioos et al., 2005; Mateo et al., 2011; Ramirez et al., 2004). Studies by Schmidt-Heydt et al. (2007) showed that intermediate concentrations of food grade preservatives stimulated the gene expression of the ochratoxin A biosynthetic regulatory gene which correlated with phenotypic toxin production under different environmental conditions.

It is thus important to understand the relationship between antifungal compounds, efficacy at a molecular level on toxin gene expression and the impact of environmental factors on such interactions. New approaches have been developed to examine these aspects based on real time PCR and microarrays to perform a rapid and accurate quantification of the effect of diverse stress conditions (including osmotic stress, pH and the use the diverse compounds) on mycotoxin biosynthetic genes (Jurado et al., 2008; Magan et al., 2011; Marín et al., 2010b; Schmidt-Heydt and Geisen, 2007; Schmidt-Heydt et al., 2008), particularly in *F. verticillioides* and *F. proliferatum* (Marín et al., 2010a). In these species, the key gene involved in fumonisin biosynthesis, *FUM1* gene, was demonstrated to be positively correlated with fumonisin production (Jurado et al., 2010; López-Errasquín et al., 2007; Proctor et al., 1999).

One of the most common fungicides used to control fungal growth in cereals and other crops in many European countries is tebuconazole which belongs to the family of azoles, particularly triazoles. The increasing concern on the use of chemicals in agriculture has led to imposition of legal limits. Thus the European Union has set the maximum residue level (MRL) on maize, wheat, rye and sorghum at 0.2 μg/kg and on barley, oats and rice at 2 μg/kg for this fungicide (Commission Regulation EU No 524/2011). 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 to evaluate the effects of tebuconazole against *F. verticillioides* and *F. proliferatum* on both growth and fumonisin biosynthesis under a range of environmental conditions. In particular, the conditions considered for those current and predicted for Mediterranean climate regions.

The objectives of this work were (1) to determine the effective doses of tebuconazole to reduce growth by 50% or 90% (ED_{50}; ED_{90}) for strains of both *F. verticillioides* and *F. proliferatum* under different temperature x water potential conditions and (2) their effects on relative growth rate and *FUM1* gene expression under such interacting environmental regimens (20–35 °C; −0.7−7.0 MPa) and fungicide concentrations.

2. Material and methods

2.1. Fungal strains

F. verticillioides FvA (FvMM7-3) and *F. proliferatum* FpA (FpMM1-2) strains were isolated from a maize field in Madrid (Spain), and both were previously reported to produce fumonisins. They are good representative strains of these species previously examined (Jurado et al., 2008, 2010; Marín et al., 2010a). Fungal cultures were maintained on potato dextrose agar medium (Scharlau Chemie, Barcelona, Spain) at 4 °C and stored as spore suspensions 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 fumonisin-inducing solid agar medium used in this study has been previously described (López-Errasquín et al., 2007; Marín et al., 2010a) and contained: malt extract (0.5 g/L), yeast extract (1 g/L), peptone (1 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), KCl (0.3 g/L), $ZnSO_4 \cdot 7H_2O$ (0.05 g/L), $CuSO_4 \cdot 5H_2O$ (0.01 g/L), fructose (20 g/L) and bacteriological agar (15 g/L). This medium was amended with tebuconazole (Folicur®SE, 43.1 g a.i./L tebuconazole, Bayer CropScience, Milan, Italy) in concentrations of 0, 0.5, 1, 2.5, 5, 7.5 and 10 mg/L in order to determine the ED_{50} and ED_{90} values for inhibition of growth of the strains of both species. Thus, appropriate amounts of tebuconazole (based on the concentration of the active ingredient) were added to sterile deionized water and used immediately and homogeneously after preparation and added to the autoclaved medium when temperature reached about 50 °C. Four replicates of each concentration were tested. Each replicate was centrally inoculated with a 3 mm diameter agar disk from the margin of a 7 day old colony incubated at 25 °C from either FvA or FpA. The plates were subsequently incubated at 25 °C for 10 days. Assessment of growth was made daily during the incubation period. Two diameters of the growing colonies were measured at right angles to each other until the colony reached the edge of the plate. The radii of the colonies were plotted against time and a linear regression was applied to obtain the growth rate (mm/day) as the slope of the line. Growth rate (mm/day) was plotted against tebuconazole concentrations (mg/L) in order to determine ED_{50} and ED_{90} values for each *Fusarium* species.

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

The agar medium mentioned above was modified with the non-ionic solute glycerol to obtain the water potentials (Ψ_w) of −2.8 and −7.0 MPa corresponding to water activities (a_w) of 0.982 and 0.95, respectively. The unamended medium had a water potential of −0.7 MPa (=0.995 a_w). Glycerol modified and un-modified media were prepared without fungicide (ED_0), and at the ED_{50} and ED_{90} concentrations for each *Fusarium* species. All agar media (in 9 cm Petri 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. The plates were incubated at 20, 25, 30 and 35 °C for 10 days. The experiment consisted of at least four replicates per treatment. Inoculation of plates with mycelium agar disk, assessment of growth and calculation of growth rates (mm/day) were carried out for all treatments as described previously. Two dimensional growth rate profiles were obtained for each species in relation to temperature x water potential treatments at the ED_{50} and ED_{90} concentrations and compared with the controls.

2.4. RNA isolation and cDNA synthesis

The biomass was removed from the cellophane at the end of the incubation period, and the total RNA was extracted using both the "RNeasy® Plant Mini Kit" and "RNeasy® Mini Kit" (Hilden, Germany), according to the manufacturer's instructions, and stored at −80 °C. First-strand cDNA was synthesized using the "GeneAmp Gold RNA PCR reagent kit" (Applied Biosystems). Each 20 μL reaction mixture contained 500 ng of total RNA, 0.5 μL of oligo $(dT)_{16}$ (50 μM), 10 μL of 5× RT-PCR buffer, 2 μL of $MgCl_2$ (25 mM), 2 μL of deoxynucleoside