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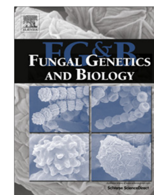

Impact of temperature stress and validamycin A on compatible solutes and fumonisin production in *F. verticillioides*: Role of trehalose-6-phosphate synthase

Beth A. Boudreau [a], Troy M. Larson [b], Daren W. Brown [b], Mark Busman [b], Ethan S. Roberts [a], David F. Kendra [b], Kristi L. McQuade [a,*]

[a] *Mund-Lagowski Department of Chemistry and Biochemistry, Bradley University, 1501 West Bradley Avenue, Peoria, IL 61625, USA*
[b] *Bacterial Foodborne Pathogens and Mycology Research, USDA-ARS-NCAUR, 1815 N. University St., Peoria, IL 61604, USA*

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A B S T R A C T

Fusarium verticillioides is a pathogen of maize that causes root, stalk and ear rot and produces fumonisins, toxic secondary metabolites associated with disease in livestock and humans. Environmental stresses such as heat and drought influence disease severity and toxin production, but the effects of abiotic stress on compatible solute production by *F. verticillioides* have not been fully characterized. We found that decreasing the growth temperature leads to a long-term reduction in polyol levels, whereas increasing the temperature leads to a transient increase in polyols. The effects of temperature shifts on trehalose levels are opposite the effects on polyols and more dramatic. Treatment with validamycin A, a trehalose analog with antifungal activity, leads to a rapid reduction in trehalose levels, despite its known role as a trehalase inhibitor. Mutant strains lacking *TPS1*, which encodes a putative trehalose-6-phosphate synthase, have altered growth characteristics, do not produce detectable amounts of trehalose under any condition tested, and accumulate glycogen at levels significantly higher than wild-type *F. verticillioides*. *TPS1* mutants also produce significantly less fumonisin than wild type and are also less pathogenic than wild type on maize. These data link trehalose biosynthesis, secondary metabolism, and disease, and suggest that trehalose metabolic pathways may be a viable target for the control of *Fusarium* diseases and fumonisin contamination of maize.

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1. Introduction

Fusarium verticillioides (teleomorph *Gibberella moniliformis*) is a filamentous fungus and plant pathogen that causes ear and stalk rots of maize and can contaminate infected grain and silage with secondary metabolites such as fumonisin mycotoxins. This family of toxins is linked to disease in both livestock and humans (Gelderblom et al., 1988; Marasas et al., 2004; Miller, 2008). While the development of resistant cultivars and biocontrol methods appears promising (Cleveland et al., 2003), their potential to limit toxin contamination has not yet been fully realized. Improvements in pest management have had the ancillary benefit of lowering *F. verticillioides* prevalence, presumably because reducing the number of insect wounds reduces access points for fungal invasion (Dowd, 1998). A seed treatment that includes thiabendazole, a fungicide with demonstrated effectiveness against *F. verticillioides* (Goulart, 1993; Goulart and Fialho, 1999; Pinto, 1997), recently became commercially available under the trade name Maxim Quattro (Syngenta), but its large-scale effectiveness under field conditions has yet to be demonstrated.

Both fungal growth and toxin production are strongly influenced by environmental conditions. *F. verticillioides* is prevalent in warm climates, and both disease severity and fumonisin production are increased by conditions of drought (Miller, 2001). The fungus can continue to grow and produce toxins during storage of harvested grain, although post-harvest disease progression is curtailed by the proper control of temperature and moisture conditions (Magan and Aldred, 2007). In laboratory cultures, changes in pH, water availability and temperature have been shown to affect germination, growth, and fumonisin production of *F. verticillioides* (Jurado et al., 2008; Marin et al., 2004). Nutritional factors also influence toxin production. Specifically, fumonisin production is favored under the stress of nitrogen limitation and is suppressed when nitrogen levels are high (Keller and Sullivan, 1996; Shim and Woloshuk, 1999). Interestingly, exposure to some fungicides has been demonstrated to significantly increase toxin production by *F. verticillioides* (Falcao et al., 2011), an effect that may be modulated by temperature and water availability (Magan et al., 2002). A complete accounting of the molecular response to abiotic

* Corresponding author. Fax: +1 309 677 3023.
E-mail address: mcquade@bradley.edu (K.L. McQuade).

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stresses is likely to facilitate the development of effective methods for reducing disease and toxin production.

Fungi also respond to environmental stresses by accumulating compatible solutes. These small, highly soluble, organic compounds, such as sugars, sugar alcohols (polyols) and amino acids, help maintain osmotic balance and have other more specific protective roles as well (Dijksterhuis and de Vries, 2006; Welsh, 2000). Representatives of the phylum Ascomycota (to which *Fusarium* species belong) respond to heat shock by increasing cytosolic trehalose and inositol and by decreasing mannitol and glycerol, whereas the opposite occurs in response to cold shock (Feofilova et al., 2000). *Fusarium graminearum* has been shown to accumulate glycerol, arabitol and trehalose under osmotic and matric stress (Ramirez et al., 2004). A recent study showed that cytosolic arabitol, mannitol and trehalose levels in *F. verticillioides* vary significantly with culture age and pH and that pH-dependence of compatible solute levels and fumonisin production may share a common regulatory mechanism (Smith and Bluhm, 2011).

Among the best studied compatible solutes is trehalose, a non-reducing disaccharide of glucose that not only acts to prevent water loss, but also stabilizes proteins and cellular membranes against stress-induced denaturation (Crowe et al., 1987) and plays a role in detoxifying radical oxygen species (Benaroudj et al., 2001; Luo et al., 2008). Trehalose metabolism has been extensively characterized in yeast, where the primary synthetic route is a two-step pathway (Paul et al., 2008). In the first step, glucose is transferred from UDP-glucose to glucose-6-phosphate (G6P) to form trehalose-6-phosphate (T6P) via the activity of T6P synthase (Tps1). Dephosphorylation of T6P is catalyzed by T6P phosphatase, yielding trehalose and inorganic phosphate. The hydrolysis of trehalose to glucose in yeast is catalyzed by two distinct trehalases characterized by different pH optima (Jorge et al., 1997; Parrou et al., 2005). Acid trehalase is present at the cell surface, where it can hydrolyze extracellular trehalose to meet growth needs. Neutral trehalase, which is located in the cytosol, is thought to metabolize stored trehalose in response to developmental or stress signals.

Trehalose is known to play critical roles in insects and other invertebrates, but is entirely absent in mammals (Elbein et al., 2003). In plants, the trehalose pathway is thought to be important for regulating development (Eveland and Jackson, 2012), yet only a few desiccation-tolerant species accumulate more than trace levels of trehalose (Paul et al., 2008). The significantly different roles for trehalose in animals, plants and fungi suggest that trehalose metabolism might be a useful target for the control of fungal crop pathogens. This idea has been validated by the use of the trehalose analog validamycin A (VMA) to control rice sheath blight caused by *Rhizoctonia solani*. VMA is a potent inhibitor of trehalases from a variety of organisms (Asano et al., 1987; Kameda et al., 1987; Shigemoto et al., 1989) and is thought to control *R. solani* growth by inhibiting trehalase in hyphal tips, thereby limiting the supply of glucose required for growth (Asano et al., 1987; Shigemoto et al., 1992).

The aim of our study was to examine the effects of heat or cold stress or treatment with VMA on accumulation of compatible solutes and fumonisin production by *F. verticillioides*. We found that both temperature stress and VMA treatment lead to dramatic changes in compatible solute levels. To assess the importance of trehalose in the stress response, we generated a T6P synthase deletion strain of *F. verticillioides*. The *TPS1* mutant was unable to synthesize trehalose, displayed altered growth characteristics, was reduced in its ability to cause disease of maize, and produced negligible fumonisin, suggesting an interplay between the trehalose and fumonisin metabolic pathways and pathogenicity.

2. Research materials and methods

2.1. Fungal culture

The *F. verticillioides* wild-type strain M-3125 (Leslie et al., 1992) and *FUM21* deletion mutant GmTCP172 (Brown et al., 2007) were maintained as spore suspensions in 15% glycerol at −80 °C and as mycelia on V8-juice agar at 4 °C. Spores were harvested from 7 to 10 day V8-juice agar cultures grown at 25 °C. Liquid cultures were inoculated at a ratio of 10^5 spores/mL in the fumonisin production medium GYAM (0.24 M glucose, 0.05% yeast extract, 8 mM L-asparagine, 5 mM malic acid, 1.7 mM NaCl, 4.4 mM K_2HPO_4, 2 mM $MgSO_4$, 8.8 mM $CaCl_2$; pH 3.0) in the presence or absence of VMA (Gold Biotechnology, St. Louis, MO). Cultures were grown in siliconized flasks with constant agitation at 130 rpm. Mycelia were harvested by vacuum filtration through Miracloth (EMD Millipore, Darmstadt, Germany), washed with two volumes ice-cold water, snap-frozen in dry ice/methanol, and lyophilized. Spores were obtained from culture filtrates by centrifugation at 3000 rpm, washed twice with water, snap frozen and lyophilized.

Solid cultures of glucose minimal medium (GMM) in 10 cm petri dishes were inoculated in the center with an agar plug from strains grown on V8-juice agar plates at 25 °C. The GMM contained 10 g glucose, 0.52 g KCl, 0.152 g $MgSO_4$, 1.52 g of KH_2PO_4, 0.001% thiamine, 0.1% trace elements, 15 g agar, and either 0.67 g $(NH_4)_2SO_4$ or 0.83 g $NaNO_3$ per liter.

Cracked maize medium was prepared by autoclaving 2.5 g of cracked maize kernels and 1.5 mL distilled water in a four dram vial. Each vial was inoculated with a single mycelial plug obtained from a V8-juice agar culture and grown for 10 days at 25 °C in the dark.

2.2. Fumonisin determination

GYAM culture filtrates were passed through a 0.45 micron syringe filter prior to analysis. Cracked maize cultures were extracted with acetonitrile:water (1:1) (Proctor et al., 1999). Fumonisin B_1 was quantified by reversed phase chromatography coupled to positive mode electrospray ionization – mass spectrometry (LC–MS) by monitoring m/z 722 protonated ([M + H]+) ions as previously described (Plattner et al., 1996). A 0.3 mL/min flow of a 35–95% gradient of aqueous methanol was used to elute analyte from a 3 mm × 150 mm C18 column. The entire flow of the chromatographic separation was directed to the ion source of the mass spectrometer. Quantitation was done by comparison of the FB_1 chromatographic peak areas to those for fumonisin concentration standards.

2.3. Chemical and enzymatic assays

Reagents were purchased from Sigma (St. Louis, MO). For trehalose and glycogen determination, mycelial extracts were prepared by heating freeze-dried mycelia or spores at 95 °C for 2 h in 0.25 M Na_2CO_3 (100 μL/mg dry weight) and clarified by centrifugation at 16000*g* at 4 °C. Trehalose was quantified using a microplate assay adapted from published methods (McBride and Ensign, 1987). Briefly, trehalose was hydrolyzed to glucose using porcine trehalase in citrate/phosphate buffer (pH 5.3), and glucose was measured using a microplate version of the glucose oxidase/peroxidase method. Glycogen content was measured as previously described (Parrou and Francois, 1997). Extracts (83 μL) were adjusted to pH 5.2 by adding 50 μL 1 M acetic acid and 200 μL 0.2 M Na-acetate, pH 5.2. Glycogen was hydrolyzed to glucose by overnight incubation with *Aspergillus niger* amyloglucosidase (1.2 U/mL) at 57 °C under constant agitation. Glycogen-derived

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