Food Microbiology 65 (2017) 193–204

Contents lists available at ScienceDirect

Food Microbiology

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

Exploring polyamine metabolism of *Alternaria alternata* to target new substances to control the fungal infection

N. Estiarte, A. Crespo-Sempere [1], S. Marín, V. Sanchis, A.J. Ramos [*]

[a] Applied Mycology Unit, Food Technology Department, University of Lleida, UTPV-XaRTA, Agrotecnio Center, Lleida, Spain

ARTICLE INFO

Article history:
Received 1 February 2016
Received in revised form
18 January 2017
Accepted 1 February 2017
Available online 3 February 2017

Keywords:
Alternaria alternata
Alternariol
Alternariol monomethyl ether
Polyamines
DFMO
Polyamine transport inhibitor

ABSTRACT

Polyamines are essential for all living organisms as they are involved in several vital cell functions. The biosynthetic pathway of polyamines and its regulation is well established and, in this sense, the ornithine descarboxylase (ODC) enzyme acts as one of the controlling factors of the entire pathway. In this work we assessed the inhibition of the ODC with D, L-α-difluoromethylornithine (DFMO) on *Alternaria alternata* and we observed that fungal growth and mycotoxin production were reduced. This inhibition was not completely restored by the addition of exogenous putrescine. Actually, increasing concentrations of putrescine on the growth media negatively affected mycotoxin production, which was corroborated by the downregulation of *pksJ* and *altR*, both genes involved in mycotoxin biosynthesis. We also studied the polyamine metabolism of *A. alternata* with the goal of finding new targets that compromise its growth and its mycotoxin production capacity. In this sense, we tested two different polyamine analogs, AMXT-2455 and AMXT-3016, and we observed that they partially controlled *A. alternata*'s viability *in vitro* and *in vivo* using tomato plants. Finding strategies to design new fungicide substances is becoming a matter of interest as resistance problems are emerging.

1. Introduction

Alternaria is a common genus of ascomycete fungi that contains numerous species that are both saprophytic on organic materials and pathogenic on many plants. *Alternaria* spp. can contaminate a wide variety of crops in the fields and can cause the spoilage of various fruits, grains, and vegetables during post-harvest and transport, which causes important economic and material losses to the food industry and growers (Bottalico and Logrieco, 1992; Pitt and Hocking, 1997). During pathogenesis, several *Alternaria* species are capable of producing toxic secondary metabolites, some of which are phytotoxins that are involved in fungal pathogenicity, and some others are mycotoxins that elicit adverse effects in humans and animals. The most common group of mycotoxins associated with *Alternaria* contamination includes alternariol (AOH), alternariol monomethyl ether (AME), tentoxin, tenuazonic acid, altenuene and altertoxins. *Alternaria alternata* is one of the most common species and it has been described as the major mycotoxin-producing species of this genus (EFSA, 2011; Logrieco et al., 2009; Ostry, 2008).

Polyamines are small polycationic compounds present in all living organisms. They are essential for growth and development as they regulate several biological processes (Tabor and Tabor, 1983). In higher eukaryotic organisms, including fungi, the most common polyamines are putrescine (PUT), spermidine, and spermine. However, a large number of fungal species do not contain spermine (Pegg and McCann, 1982; Valdés-Santiago et al., 2012; Walters, 1995). Polyamines have been frequently associated with plant stress and defense responses as it has been observed that under these situations, plants significantly accumulate free and conjugated levels of putrescine, spermidine and spermine (Alcázar et al., 2006; Richards and Coleman, 1952). This increase has been seen to go along with an upregulation of two polyamine biosynthetic enzymes, the ornithine decarboxylase (ODC) and the polyamine oxidase (Haggag and Abd-El-Kareem, 2009; Walters et al., 2002). Gardiner et al. (2009) proposed that products of the arginine-polyamine biosynthetic pathway in plants play a role in the induction of trichothecene biosynthesis during fungal infection. Thus, the pathogen would exploit the generic host stress response of

* Corresponding author.
E-mail addresses: nestiarte@tecal.udl.cat (N. Estiarte), acrespo@valgenetics.com (A. Crespo-Sempere), smarin@tecal.udl.cat (S. Marín), vsanchis@tecal.udl.cat (V. Sanchis), ajramos@tecal.udl.cat (A.J. Ramos).
[1] Present address: Valgenetics S.L. University of Valencia Science Park, Valencia, Spain.

http://dx.doi.org/10.1016/j.fm.2017.02.001
0740-0020/

polyamine synthesis as a cue for production of trichothecene mycotoxins (Gardiner et al., 2010).

During the last decades, the use of specific inhibitors and the development of mutants has been used to better understand the polyamine metabolism pathway and its regulation. In plants at least two different polyamine pathways involved in polyamine biosynthesis have been described, whereas in fungi there is a unique pathway. In animals and many fungi, putrescine is only synthesized from ornithine by ODC, which is a key enzyme of the entire pathway. This characteristic, makes this metabolic route an ideal target for controlling the growth of pathogenic fungi without altering the plant host as they can use an alternative pathway in which ODC is not involved. In this sense, some researchers have tried to design new strategies to develop new fungicides targeted on the polyamine metabolism (Crespo-Sempere et al., 2015; Gárriz et al., 2003; Mackintosh et al., 2001; Mellon and Moreau, 2004).

Fungicides have been used in agriculture for well over a century, and initially there were no reports of losses of efficacy in the field. Nevertheless, over time it was discovered that plant pathogenic fungi can adapt to fungicide treatments by mutations leading to resistance and loss of efficacy. This is the case of *A. alternata* contaminating nuts, or *Alternata solani* contaminating potatoes, which both achieved resistance against succinate dehydrogenase inhibitors (Avenot and Michailides, 2007; Lucas et al., 2015; Miles et al., 2014). Therefore, it is interesting to explore new targets and new strategies for resistance management. With this aim, we have explored polyamine metabolism of *A. alternata* and we have tried to find out some target, via polyamine synthesis inhibition and polyamine analogs, to achieve the control of *A. alternata* regarding tomato plant diseases. For this purpose, we have analyzed the effect of inhibiting the ODC activity on *A. alternata*. We have also studied the impact of adding exogenous putrescine and the consequences of inhibiting polyamine transport using different polyamine analogs. All these assays have been performed *in vitro* and *in vivo* using tomatoes and tomato plants.

2. Material and methods

2.1. Fungal strain growth conditions

The *A. alternata* strain used in this study was the CBS 116.329 (isolated from apple), provided by Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). To prepare conidial suspensions, *A. alternata* was routinely grown on Potato Dextrose Agar plates (PDA, Biokar Diagnostics, France) and incubated seven days in the dark at 26 °C. Conidia were collected with a scalpel within a sterile solution of phosphate-buffered saline (PBS) with 0.005% (v/v) Tween 80 (J.T. Baker, Deventer, The Netherlands) and filtered through Miracloth (Calbiochem, USA). Conidial suspension concentration was adjusted to 10^6 conidia/mL using a Thoma counting chamber. To study the effect of DFMO (Enzo Life Sciencies, USA), putrescine (Sigma-Aldrich, USA), and polyamine transport inhibitors (PTIs), a medium free of polyamines was prepared and 5 μL of the conidial suspension (10^6 conidia/mL) were centrally inoculated on the plates and incubated under dark conditions at 26 °C for seven days. This medium contained, per liter, 30 g sucrose, 1 g NH_4NO_3, 1 g KH_2PO_4, 20 g agar, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 10 mg $FeSO_4 \cdot 7H_2O$ and 100 μL of trace element solution (per 100 mL, 5 g citric acid, 5 g $ZnSO_4 \cdot 7H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 50 mg $MnSO_4 \cdot H_2O$ and 50 mg H_3BO_3). Medium pH was adjusted to 6.5 with NaOH. Putrescine, DFMO and the polyamine analogs (please change PTIs to polyamine analogs throughout manuscript) were dissolved in water, sterilized by filtering the solution through a 0.45 μm pore size filter and added to the aforementioned autoclaved culture medium. For DFMO experiments, the concentrations tested were 2.5 mM and 5 mM. For putrescine, concentrations were 50, 500, 1000 and 5000 μM, while for the PTIs, the concentrations ranged from 100 to 1200 μM. PTIs, DFMO, and putrescine were stored at −20 °C until needed. We tested seven PTIs, detailed in Table 1, kindly provided by Aminex Therapeutics (USA). The PTIs are lipophilic polyamine analogs, synthesized as polyamine transport inhibitors or antizyme inducing agents (Burns et al., 2001, 2009; Petros et al., 2006). The lipophilic PTIs bind to the lipid membrane of the mammalian cell where the polyamine transport apparatus is blocked and, as a result, the uptake or the excretion of polyamines, or may be both, will be inhibited. The antizyme inducing polyamine analogs induce frameshifting and expression of antizyme, which is a polyamine-feedback biomolecule shown to inhibit polyamine biosynthesis and transport.

2.2. Radial growth rate and sporulation assessment

Radial growth rate was determined by measuring daily, over 4 days, two perpendicular diameters of the growing colonies. Sporulation assessment was carried out by collecting all the mycelia grown on a Petri dish with the help of a scalpel and placing it on a Falcon® tube containing a sterile solution of PBS with 0.005% (v/v) of Tween 80. Tubes were vigorously shaken on the vortex and conidia were recovered by filtration through Miracloth. Conidia concentration was measured by using a Thoma counting chamber and results were expressed as conidia/mm^2 of fungal colony.

2.3. Extraction and detection of AOH and AME from culture

Mycotoxin production (AOH and AME) was quantified in seven day old cultures. To this aim, one agar plug (5 mm in diameter) was removed from the center of the colonies and extracted with 500 μL of acetonitrile-methanol-water (45:10:45 v/v/v), adjusted to pH 3 with *o*-phosphoric acid. After 60 min, samples were filtered (Millex-HV 0.45 μm, 25 mm, Millipore Corporation, USA) into another vial and mycotoxin extracts were dried in a speed vacuum concentrator at room temperature. Samples were stored at −20 °C until HPLC analysis. Prior to analysis, the extracts were resuspended in 500 μL of the mobile phase solution (water-methanol, 50:50 v/v). Separation, detection and quantification of AOH and AME was performed on an HPLC system consisting of a Waters 2695 Alliance Separations Module connected to a UV/Visible dual λ absorbance Detector Waters 2487, using a reverse phase Kinetex PFP column (5 μm, 4.6 × 150 mm, Phenomenex, Torrance, CA, USA) preceded by a Spherisorb guard column (5 μm ODS2, 4.6 × 10 mm, Waters, Millford, MA, USA). Columns were set at a temperature of 35 °C. For chromatographic separation of AOH and AME the flow rate was 0.5 mL/min and the injection volume was 100 μL. Absorption wavelength was set at 258 nm. The mobile phase consisted of a gradient of double distilled miliQ water (MiliQ Academic Millipore, USA) and methanol-water (70:30 v/v) according to the gradient described in Estiarte et al. (2016). Retention times were 24 min for AOH and 32 min for AME. For mycotoxin quantification, working standards were used to perform a ten-point calibration curve for the mycotoxins (1500, 1250, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL). The limit of detection (LOD) for AOH was 0.02 ng/mm^2 for *in vitro* assays and 0.009 μg/g tomato, while for AME the LOD was 0.034 ng/mm^2 *in vitro* and 0.012 μg/g of tomato. The LOD was based on a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was calculated as 3 × LOD. All solvents were HPLC grade and all chemicals were analytical grade. The recoveries and repeatability of AOH and AME on tomatoes are summarized in Estiarte et al. (2016).