International Journal of Food Microbiology 292 (2019) 48–55

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Differential response to synthetic and natural antifungals by *Alternaria tenuissima* in wheat simulating media: Growth, mycotoxin production and expression of a gene related to cell wall integrity

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ARTICLE INFO

Keywords:
Alternariols
Tenuazonic acid
CWI
Antifungal protein
Control
Cereals

ABSTRACT

Alternaria spp. are major contaminants of wheat crops, causing both economic losses for producers and health risk for consumers due to the accumulation of toxic metabolites. The application of synthetic fungicides in the field may trigger mycotoxin accumulation, since fungistatic levels of those compounds might cause fungal responses to stress. Hence, new alternatives are needed for its control. The aim of this work was to compare the effects of a natural antifungal compound, the antifungal protein PgAFP, and a synthetic commercial one, on *Alternaria tenuissima* sp.-grp. growth, mycotoxin biosynthesis (tenuazonic acid and alternariols) and the expression of a stress-related gene associated with cell wall integrity (CWI) pathway, in a wheat-based medium at two water activities (a_w; 0.95 and 0.98 a_w) conditions associated with the ripening of this grain. The application of both antifungals produced comparable fungistatic effects on *Alternaria* spp. growth. However, the presence of PgAFP produced a significant reduction in mycotoxins accumulation, whereas this effect was not observed with the commercial antifungal. To our knowledge, this is the first study on the influence of fungicides on the expression of a key gene involved in CWI stress-related pathway in relation to *Alternaria* mycotoxins accumulation. This information is useful when developing new antifungal methods for foods. The application of PgAFP would be a promising natural strategy for its application in wheat for the control of *Alternaria* spp.

1. Introduction

Wheat (*Triticum aestivum*) is one of the most important cereal crops worldwide, being among the ten most produced commodities (FAOSTAT, 2016). *Alternaria* spp. has been reported as a major contaminant in this crop (Kosiak et al., 2004; Logrieco et al., 2009; Mercado Vergnes et al., 2006; Mónaco et al., 2004; Vučković et al., 2012), causing yield decrease and severe economic losses for producers globally. In particular, ripening wheat is highly susceptible to *Alternaria* spp. contamination, and is mainly associated to small-spored species-groups. *A. alternata* and *A. infectoria* are the most common species isolated worldwide (Logrieco et al., 2009; Patriarca et al., 2014). In Argentina, *A. tenuissima* has been reported as the predominant species in wheat mycobiota (Broggi et al., 2007; González et al., 1998; 1999; Patriarca et al., 2007). These species are associated with the development of the "black point" disease, which is characterised by dark brown to black dots of the germ and the seed due to mycelial and conidial masses. This disease is more frequent when there are persistent rainfalls, intense dews or irrigation during kernel development, although a high incidence has also been observed in relatively dry weather (Logrieco et al., 2003; Patriarca et al., 2007).

Alternaria spp. are also well known for their ability to synthesise a wide spectrum of secondary metabolites, including mycotoxins and phytotoxins. Among them, alternariol (AOH), its derivative alternariol monomethyl ether (AME), and tenuazonic acid (TeA) are considered some of the main *Alternaria* toxins (EFSA, 2016). AOH and AME are mutagenic and genotoxic and their presence in cereal grains have been associated with high levels of human oesophageal cancer in China (Liu et al., 1992; Ostry, 2008; Solhaug et al., 2016). TeA has been reported to be acutely toxic for several animals, and it has been associated with human haematological disorders like Onyalai (Fraeyman et al., 2017; Ostry, 2008; Steyn and Rabie, 1976).

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https://doi.org/10.1016/j.ijfoodmicro.2018.12.005
Received 25 August 2018; Received in revised form 2 December 2018; Accepted 6 December 2018
Available online 07 December 2018
0168-1605/

In wheat, several studies reported the presence of *Alternaria* mycotoxins in grains worldwide, including Germany, Serbia, Australia, China, Poland, Italy, and Egypt (Grabarkiewicz-Szczęsna et al., 1989; Janić Hajnal et al., 2015; Juan et al., 2016; Li and Yoshizawa, 2000; Müller and Korn, 2013; Scott, 2001; Webley et al., 1997). In Argentina, the only survey available about this contamination concluded that AME was the predominant toxin (15 contaminated samples out of 64), but TeA was detected in higher concentration (up to 8814 ppb) (Azcárate et al., 2008). Although natural occurrence in food was demonstrated with the consequent potential risk to consumers' health, there are no international regulations for any of the *Alternaria* mycotoxins in food and feed up to now. However, the European Food Safety Authority (EFSA) recently highlighted that cereal-based foods for infants and young children are one of the main contributors of *Alternaria* spp. toxins to the diet (EFSA, 2011, 2016).

Primary control of fungal contamination in grains relays mainly on the use of fungicides to prevent mould development in pre-harvest stage. Although most commercial antifungal agents have fungicidal effects against certain species, in some cases their effectiveness is reduced to a partial inhibition of fungal growth (fungistatic effect). The latter is relevant in terms of food safety, as it could cause an increase in mycotoxins accumulation as a fungal response to the external stress. Fungi have different intracellular pathways that help them coping with challenging external conditions. Among them, the cell wall integrity (CWI) pathway is responsible for the maintenance of the cell wall by detecting cell wall changes and responding mediating its biosynthesis, actin organization, and other events (Fuchs and Mylonakis, 2009; Hayes et al., 2014). This pathway is particularly important in the resistance of fungi to antifungal compounds, since the cell wall is a common target for these treatments due to its unique structure, different from other eukaryotes (Jabes et al., 2016).

During the last years, natural strategies have gained importance since the indiscriminate use of synthetic antifungals has led to the development of resistant strains, requiring higher doses of fungicides, with the consequent increase in toxic residues in food products (da Cruz Cabral et al., 2013). The application of antifungal proteins (AFPs), secreted naturally by filamentous fungi and yeasts, presumably to provide themselves with an advantage over competing species has been explored as an alternative control strategy (Van der Weerden et al., 2013). AFPs have the benefits of acting specifically against fungi and minimising toxicity to the host. Its mechanism of action has not been completely elucidated, but it is known that its activity is mediated by the interaction with different molecules or receptors in the cell wall or plasma membrane (Binder et al., 2011; Hayes et al., 2014).

The objectives of the present study were to compare the effects of a natural antifungal and a synthetic one on *Alternaria* spp. growth, mycotoxin production and a stress-related gene expression in a wheat based medium at two water activities (a_w) conditions related to the ripening of this grain.

2. Materials and methods

2.1. Fungal strain

One small-spored *Alternaria* strain obtained from Argentinean wheat kernels was used in this study. This isolate has been morphologically identified as *A. tenuissima* sp.-grp. and its ability of synthesising AOH, AME and TeA in vitro has been previously confirmed (da Cruz Cabral et al., 2017; Patriarca et al., 2014).

2.2. Culture medium

Assays were conducted in a 2% milled wheat agar medium (WA), prepared as described in Patriarca et al. (2014). The medium was adjusted at two different a_w levels by adding glycerol. These conditions were measured in a Novasina Lab Master (Novasina AG, Switzerland). Final a_w were 0.981 ± 0.003 (high water availability) and 0.953 ± 0.003 (intermediate water stress). WA was poured into 54 mm Petri plates.

2.3. Antifungals

Two kinds of antifungal treatments were evaluated for each a_w level in WA. On one side, a commercial synthetic fungicide containing two active compounds, fludioxonil 2.5% and metalaxyl-M 1% (F + M), was tested at 0.5 μg/mL WA. Its efficacy on reducing *Alternaria* spp. growth has been previously reported (da Cruz Cabral et al., 2012). On the other side, the antifungal protein PgAFP, produced by *Penicillium chrysogenum* CECT 20922 (Rodríguez-Martín et al., 2010), was evaluated at 10 μg/mL WA.

2.4. Experimental settings

Alternaria spore suspensions were prepared in Phosphate Buffered Saline (PBS; pH 7.2; prepared as follows: 0.32 g NaH_2PO_4, 1.0 g Na_2HPO_4; 9 g NaCl; 1 L H_2O) from 7-day-old Potato Carrot Agar plates (Samson et al., 2010). The suspensions were quantified using a Thoma counting chamber Blaubrand® (Brand, Germany). WA plates were inoculated in the centre with 2 μL of a 10^6 spores/mL suspension and incubated at 25 °C in the dark for a maximum of 14 days. Each treatment (antifungal × a_w) was performed in triplicate.

2.5. Growth assessment

Radial growth was recorded daily by measuring two right-angled diameters. Colony diameter (mm) was plotted against the incubation time (days). Data plots showed, after a lag phase and before a stationary phase, a linear trend with time. Only the linear parts were used for calculations. Data was fitted using a linear model obtained by plotting the results against time. Lag period prior to growth (λ, days) was determined as the abscissa in the origin from this plot and maximum growth rate (μ_{max}, mm/day) as the slope from the linear growth phase (Garcia et al., 2009).

2.6. Mycotoxins extraction and quantification

Samples were taken at the beginning of the stationary phase of growth. Mycotoxins were extracted according to the QuEChERS method proposed by Peromingo et al. (2018), with the following modifications. Nine agar plugs (7 mm diameter each) were cut from the edge of the colony and weighed into a 50 mL conic tube. Metabolite separation was performed by adding 2 mL of milli-Q water acidified with formic acid MS-grade 1% (v/v) and mixed for 30 s with a vortex. Then, 2 mL of acetonitrile MS-grade acidified with formic acid 1% (v/v) were added and mixed for 1 min with a vortex. Phase partitioning was performed by adding 0.40 ± 0.01 g of NaCl and 1.60 ± 0.01 g of anhydrous $MgSO_4$ followed by vigorous shaking by hand after each addition. The mixture was centrifuged for 5 min at 5300 rpm at 4 °C (Diglicen 21R, Ortoalresa, Spain). An aliquot of 1 mL of the organic layer was taken and filtered through 0.22 μm acetate cellulose filters (Jet Bio-Filtration Co., China) for subsequent analyses.

Extracts were analysed by ultra-high-performance liquid chromatography (UHPLC in a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific) coupled to an ion trap mass spectrometer (MS) model Amazon SL (Bruker Daltonics, Germany)). A C_{18} reverse-phase column of 10 cm × 2.1 mm × 1.8 μm particle size (Agilent Technologies, USA) was used as stationary phase. The mobile phases were (A) milli-Q water acidified with formic acid MS-grade 20 mM and (B) acetonitrile acidified with formic acid MS-grade 20 mM. The separation was performed at 0.3 mL/min flow rate using a binary gradient solvent: 0–12 min linear increase from 10 to 85% B, 12–13.5 min 100% B followed by a washing step with 100% B for 1.5 min prior to return to the initial

conditions. MS ionisation was performed in ESI^+. The detection was performed by setting three time stages for scan, depending on the molecular weight and retention time of the toxin: 0–7.50 min, scan range *m/z* 190–200 (TeA); 7.51–9.90 min, scan range *m/z* 255–261 (AOH); 9.91–15.00 min, scan range *m/z* 268–278 (AME). Retention times for TeA, AOH and AME were 7.3 ± 0.2, 7.8 ± 0.2 and 10.1 ± 0.2 min, respectively. Data processing was performed using the software Data Analysis 4.1 (Bruker Daltonics) and peak areas were manually delimited.

Standards of the three toxins were purchased from Sigma-Aldrich Co. (USA). The calibration curves were constructed by plotting peak area versus standard concentrations for each one. Linear response ranges were established from this plot for each toxin ($R^2 > 0.99$): 0.4–75.1 ng for TeA; 0.3–28.0 ng for AOH and 0.2–20.0 ng for AME. The limits of detection (LODs) were calculated from the calibration curve (Long and Winefordner, 1983), being 72.5, 33.3 and 26.7 ppb for TeA, AOH and AME, respectively; while limits of quantification (LOQs) were 220.5, 101.2 and 81.1 ppb for TeA, AOH and AME, respectively. Recovery experiments were performed by spiking WA plates at three levels of addition corresponding to concentrations close to the minimum, medium and maximum points of the calibration curve for each toxin. Three replicates were performed by level. Mean recoveries at the three levels of addition were 114%, 92% and 78% for TeA, AOH and AME, respectively. Quantities reported were corrected by recovery.

2.7. Stress-related gene expression assays

To evaluate the stress-related effect of both antifungal tested on *A. tenuissima* sp.-grp. cells, the expression of one of the main regulators within the CWI pathway, *RHO1* gene, was assessed. Samples were taken at the middle of the linear phase (1st sampling day) and at the beginning of the stationary phase (2nd sampling day) of the mould growth. These days were different for each treatment, given that the growth rates varied in each condition.

2.7.1. RNA extraction and removal of genomic DNA

Whole colonies were collected using a sterile lab spatula for RNA extraction. Mycelia were frozen in liquid nitrogen and ground in a pre-frozen mortar with the aid of a pestle to a fine powder. The extraction was carried out by Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) following the manufacturer's instructions (Protocol A). RNA concentration (ng/μL) and purity (A_{260}/A_{280} ratio) were determined spectrophotometrically using a 1.5 μL aliquot on the NanoDrop™ (Thermo Fisher Scientific, USA). Samples were diluted to a concentration of 0.1 μg/μL and treated with DNAse I (Thermo Fisher Scientific, USA) to remove genomic DNA traces that could be co-extracted with RNA.

2.7.2. Two-step reverse transcription real time PCR

First, reverse transcription (RT) was conducted with PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) by using 5 μL of total RNA (500 ng) as described by the manufacturer (incubation at 37 °C for 15 min and reverse transcriptase inactivation at 85 °C for 5 s). Complementary DNA (cDNA) samples were stored at −20 °C until analysis.

The real-time PCR (qPCR) reactions were performed in a ViiA™ 7 apparatus (Applied Biosystems, Life Technologies, USA) using the SYBR® Green technology. The amplification of the *RHO1* and *β-tubulin* genes was carried out following the methodology described by da Cruz Cabral et al. (2018) and Estiarte et al. (2016), respectively. Briefly, the reaction mixture for amplification of each gene consisted of 6.25 μL of SYBR® Premix Ex Taq™ (Tli RNAseH Plus; Takara Bio Inc., Japan), 0.125 μL of ROX plus (Takara Bio Inc., Japan), 2.5 μL of DNA template and 3.125 μL of Milli-Q water. For the *RHO1* gene the final concentration of the primer pair *Rho1*-F1/R2 was 200 nM each, while for the primers A-BTF and A-BTR that amplify the *β-tubulin* gene, were 300 nM each. The thermal cycling conditions for amplification of both genes included one holding period at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 1 min. After the final PCR cycle, melting curve analyses of the PCR products were performed and the values of the melting temperatures were checked to ensure the truthfulness of the results and specificity of the primers.

Quantification cycle (Cq), the cycle in which fluorescence reaches a defined threshold, was automatically obtained by the instrument using default parameters of the software ViiA7 RUO v.1.2.4 (Applied Biosystems).

2.7.3. Relative expression of the RHO1 gene

Relative quantification of the *RHO1* gene expression was calculated following the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001). The *β-tubulin* gene was used as the endogenous control to normalise the amount of the cDNA target added to each reaction. The fulfilment of the two requirements of the $2^{-\Delta\Delta Ct}$ method was tested before calculations. The calibrator used for calculations was the control samples at each condition, i.e. plates incubated with no antifungal added at each incubation time and a_w condition.

2.8. Statistical analysis

Statistical analyses were performed using the software IBM SPSS v.24 (IBM Corporation, USA). Data sets of growth rates, mycotoxin production and gene expression were tested for normality using the Shapiro-Wilk test. For normal and homoscedastic (according to Levene's test) data, an ANOVA was applied. A post-hoc comparison of means was made using a Dunnet test to compare treatments against the control. When data sets failed the normality test, the analyses were performed using the non-parametric Kruskal-Wallis test. The Mann-Whitney *U* test was then applied to compare the obtained mean values in this case. The statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Effect of antifungals on A. tenuissima sp.-grp. growth

A. tenuissima sp.-grp. growth was severely affected by antifungal treatments at both a_w evaluated (Fig. 1). The a_w had also a strong influence on *Alternaria* growth; being the highest (0.98 a_w) much more favorable for fungal development in WA, as expected.

The effect of the antifungal treatments on λ and μ_{max} at both a_w levels is shown in Fig. 2. At 0.95 a_w, the presence of PgAFP or F + M in the medium produced a significant delay in the onset of growth with respect to the control ($p \leq 0.05$), being the effect of the antifungal protein ($\lambda \approx 6.8$ days) greater than that of the commercial antifungal ($\lambda \approx 3.7$ days). In addition, the μ_{max} in the presence of PgAFP and F + M were slower than the control (29 and 33%, respectively), although no significant differences were observed between both treatments.

At 0.98 a_w, λ were short for the control and the treatments; 1.2, 0.9 and 1.7 days for the control, PgAFP and F + M, respectively. The effect of the antifungals on μ_{max} showed the same tendency as at 0.95 a_w, although it was much more pronounced. The μ_{max} was reduced by 66% in the presence of any of the antifungals with respect to the control.

3.2. Mycotoxin production

The mycotoxin levels synthesised by *A. tenuissima* sp.-grp. with the different treatments at both a_w levels are shown in Table 1. The concentrations of the toxins were strongly influenced by the a_w level. At all conditions assayed, lower concentrations of the three toxins were detected at the lowest a_w evaluated.

TeA accumulation was reduced in the presence of both antifungal compounds. At 0.95 a_w, a significant decrease was detected with both

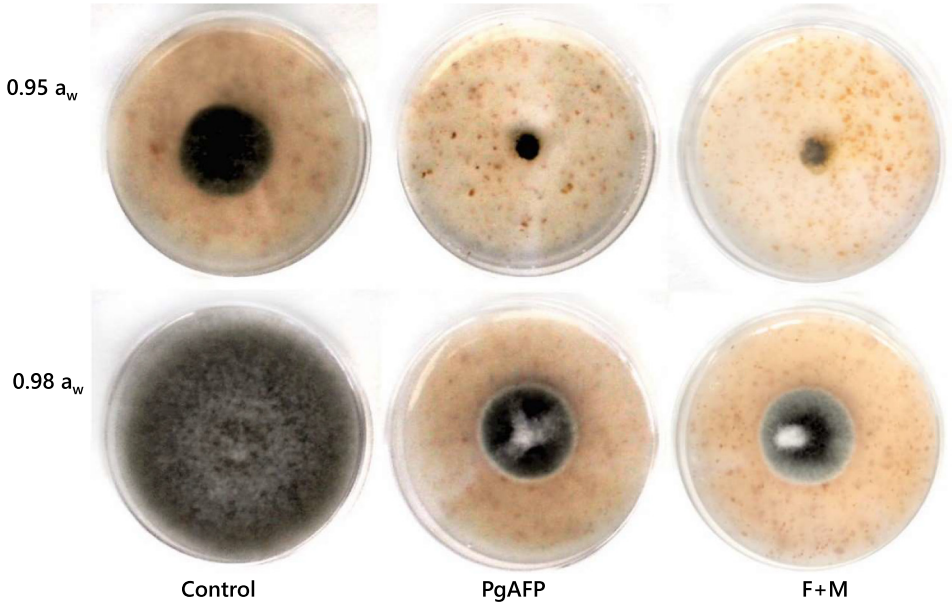

Fig. 1. Colonies of *Alternaria tenuissima* sp.-grp. grown on wheat agar in the presence of two antifungal treatments (PgAFP and F + M) at two water activity (a_w) levels, 0.95 and 0.98 after 6 days of incubation at 25 °C.

F + M and PgAFP (99 and 84% reduction with respect to the control, respectively). At 0.98 a_w, the greatest inhibition was obtained with PgAFP (66% reduction), whereas the treatment with F + M did not significantly affect TeA production.

Regarding AOH production, this mycotoxin was not detected at the lowest a_w tested neither in the presence nor in the absence of the antifungal compounds. At 0.98 a_w, only PgAFP produced a decrease in the accumulation of this toxin with respect to the control (59% reduction), while the use of F + M did not produce a significant effect on the production of this mycotoxin.

With respect to AME accumulation, *A. tenuissima* sp.-grp. only synthesised this mycotoxin in WA plates in the absence of any of the

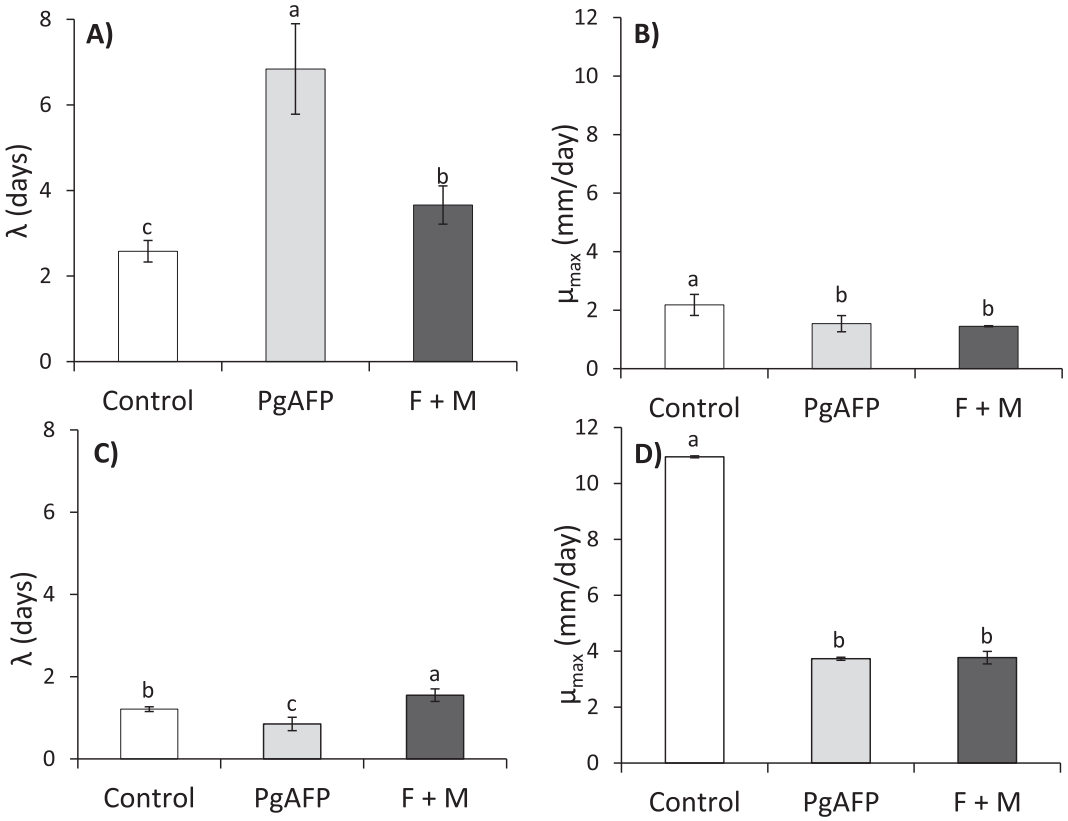

Fig. 2. Lag phase (λ, days) and maximum growth rate (μ_{max}, mm/day) for *Alternaria tenuissima* sp.-grp. on wheat agar at 0.95 and 0.98 water activity (a_w) and 25 °C, with two antifungal treatments (PgAFP and F + M). A) λ at 0.95 a_w; B) μ_{max} at 0.95 a_w; C) λ at 0.98 a_w; D) μ_{max} at 0.98 a_w. The lines indicate the standard deviation and the letters, the groups with significant differences for each parameter at each a_w ($p \leq 0.05$).

Table 1
Mycotoxin concentration (μg toxin/g WA) produced by *Alternaria tenuissima* sp.-grp. in wheat agar, at two water activity (a_w) levels (0.95 and 0.98) and 25 °C in the presence of two antifungal treatments (PgAFP and F + M).

Mycotoxin	a_w	Control	PgAFP	F + M
TeA	0.95	38.0 ± 2.8[a]	6.1 ± 0.5[b]	0.25 ± 0.05[c]
	0.98	162.2 ± 27.4[a]	56.0 ± 4.9[b]	120.4 ± 21.4[a]
AOH	0.95	n.d.	n.d.	n.d.
	0.98	3.0 ± 0.6[a]	1.2 ± 0.5[b]	3.07 ± 0.09[a]
AME	0.95	0.04 ± 0.01*	n.d.	n.d.
	0.98	1.2 ± 0.5[b]	0.5 ± 0.2[c]	3.2 ± 0.2[a]

Values correspond to mean concentration ± standard deviation (SD) of three replicates.
n.d.: not detected (< LOD); *: values between LOD and LOQ.
Values with the same letter in superscript are not significantly different ($p \leq 0.05$).
TeA: Tenuazonic acid; AOH; Alternariol; AME; Alternariol monomethyl ether.

antifungals at 0.95 a_w and in low concentration. At the highest a_w, both antifungals caused an opposite effect in AME accumulation. While the concentration of this toxin in the presence of PgAFP was significantly lower than in the control plates (62% reduction), in the case of F + M a significant increase was detected (57% higher than the control).

3.3. *RHO1* gene expression

The results of the effect of PgAFP and F + M on the *RHO1* gene expression by *A. tenuissima* sp.-grp. in WA at both growth phases (linear and stationary) and two a_w levels (0.95 and 0.98) at 25 °C are shown in Fig. 3. At 0.95 a_w, the presence of any of the antifungal treatments in the medium caused a repression of the *RHO1* gene expression during the linear growth phase (Fig. 3A). At the beginning of the stationary phase of growth, an inhibition of the expression of the stress-related gene was also observed in the presence of F + M; however, no significant differences were found between PgAFP and control plates (Fig. 3B).

At 0.98 a_w, the presence of both antifungals evaluated caused a subexpression of the *RHO1* gene regarding to the control during the linear growth phase (Fig. 3C). Nevertheless, in the onset of the stationary phase of growth, the presence of PgAFP did not significantly modify the relative gene expression from the control, while the presence of F + M caused an overexpression of *RHO1* gene (Fig. 3D).

4. Discussion

In the present study, two different antifungal treatments, one of natural origin and the other a synthetic commercial one, were compared in an attempt to manage *Alternaria* spp. contamination in wheat, using a model medium. The utilisation of model media based on wheat grains is a first simplified step for this kind of studies, that allows discerning the implied mechanisms under highly controlled conditions, which could be hidden in natural systems (Crowther et al., 2017).

The natural antifungal evaluated was the protein PgAFP produced by *P. chrysogenum* CECT 20922 (Rodríguez-Martín et al., 2010). This compound presents a mechanism of action that involves changes in proteins associated with the cell wall structure (da Cruz Cabral et al., 2018; Delgado et al., 2015b, 2016). A previous study evaluated its effect on the growth of several mycotoxigenic fungal species, commonly found in food, showing different levels of sensitivity to this treatment (Delgado et al., 2015a). However, the effect of this protein on *Alternaria* spp. growth and mycotoxin accumulation had not been investigated yet. The PgAFP treatment was applied in wheat agar at 10 μg/mL, since it showed fungistatic effect at this level on other fungal species in accordance with previous studies (da Cruz Cabral et al., 2018).

The commercial antifungal used was a broad spectrum fungicide containing two active compounds, fludioxonil (phenylpirrole) and metalaxyl-M (phenylamide), with different mechanisms of action. Fludioxonil inhibits spore germination, elongation of the germ tube, mycelial growth, and also induces germ tube distortions and cell bursting (Avenot and Michailides, 2015; Kanetis et al., 2008; Rosslenbroich and Stuebler, 2000); whereas the primary mechanism of action of metalaxyl-M involves alteration of RNA biosynthesis, with a concomitant effect on mitosis (Barak et al., 1984; Fisher and Hayes, 1982). Its effectiveness in reducing small-spored *Alternaria* growth has been previously described (Barak et al., 1984; da Cruz Cabral et al., 2012; Malandrakis et al., 2015), although Avenot and Michailides (2015) reported the resistance to fludioxonil in *A. brassicicola*. In the present work, the applied level of this treatment (0.5 μg/mL) was the maximum at which fungal development was observed, higher levels were fungicidal.

The a_w levels selected for the present study correspond to different steps during wheat grain ripening, which includes stages of intermediate humidity, such as flowering, phase in which fungal infection, mainly by *Alternaria* and *Fusarium* spp., can occur. A former work regarding the ecophysiology of *A. tenuissima* sp.-grp. in wheat based medium (Patriarca et al., 2014) showed its ability to grow at both 0.98 and 0.95 a_w, and pointed out optimum temperatures for its development between 25 and 30 °C for both a_w levels. The evaluation of the effectiveness of the antifungal treatments was carried out at 25 °C, which is the worst scenario in terms of the risk of colonisation. Both fungal growth and mycotoxin production by *A. tenuissima* sp.-grp. were influenced by the a_w level. As previously reported, the two parameters decreased with a_w reduction (Magan et al., 1984; Sanchis and Magan, 2004; Vaquera et al., 2014, 2016; Young et al., 1980).

Both antifungals assayed were effective in reducing *A. tenuissima* sp.-grp. growth, while their effect on mycotoxin production varied depending on a_w and the mycotoxin evaluated. The effect of PgAFP on *A. tenuissima* gr.-esp. development was different at each a_w level; at 0.95 a_w it caused a significant lengthening of the lag phase, while at 0.98 a_w, the most noticeable effect was observed in the slowdown of the growth rate. In addition, a repression of the *RHO1* gene was observed in the linear growth phase in the presence of the protein at both a_w, indicating that PgAFP would cause an inhibition of the CWI pathway. It has been reported that some compounds that inhibit fungal development, such as thioridazine and curcumin, are able to interfere with the response of the CWI pathway, causing lower levels of the expression of the stress-related genes (Jabes et al., 2016; Kumar et al., 2014). Concerning mycotoxins biosynthesis, several authors have described that stress-response intracellular pathways could be related to the regulation of mycotoxin biosynthesis (Geisen et al., 2017; Graf et al., 2012; Kohut et al., 2009; Malavazi et al., 2014; Ochiai et al., 2007; Stoll et al., 2013). At both a_w evaluated in this study, the presence of PgAFP caused significant reductions in the concentration of the three studied toxins regarding to the control. Even though AOH was not produced at 0.95 a_w, an inhibitory activity of PgAFP was evidenced on alternariols biosynthetic pathway, since AME was detected in the control, but not in the presence of the protein. A recent study has also demonstrated that PgAFP is able to control OTA biosynthesis by *Aspergillus carbonarius* (Fodil et al., 2018).

Although the influence of the commercial antifungal on *Alternaria* growth was similar to that found with PgAFP; its effect on mycotoxin biosynthesis was quite different. Even though it caused a decrease in growth rates in WA with respect to the control at both a_w evaluated, it could not reduce the accumulation of any of the three mycotoxins studied at 0.98 a_w. Moreover, at this condition, while TeA and AOH did not show significant differences with respect to the control, AME was detected at higher concentrations in the presence of the antifungal agent. With respect to the *RHO1* gene expression in the linear growth phase, an inhibition of the expression was found in the presence of the commercial antifungal at both a_w conditions, indicating that the fungus was not able to activate the CWI pathway to overcome the stress caused

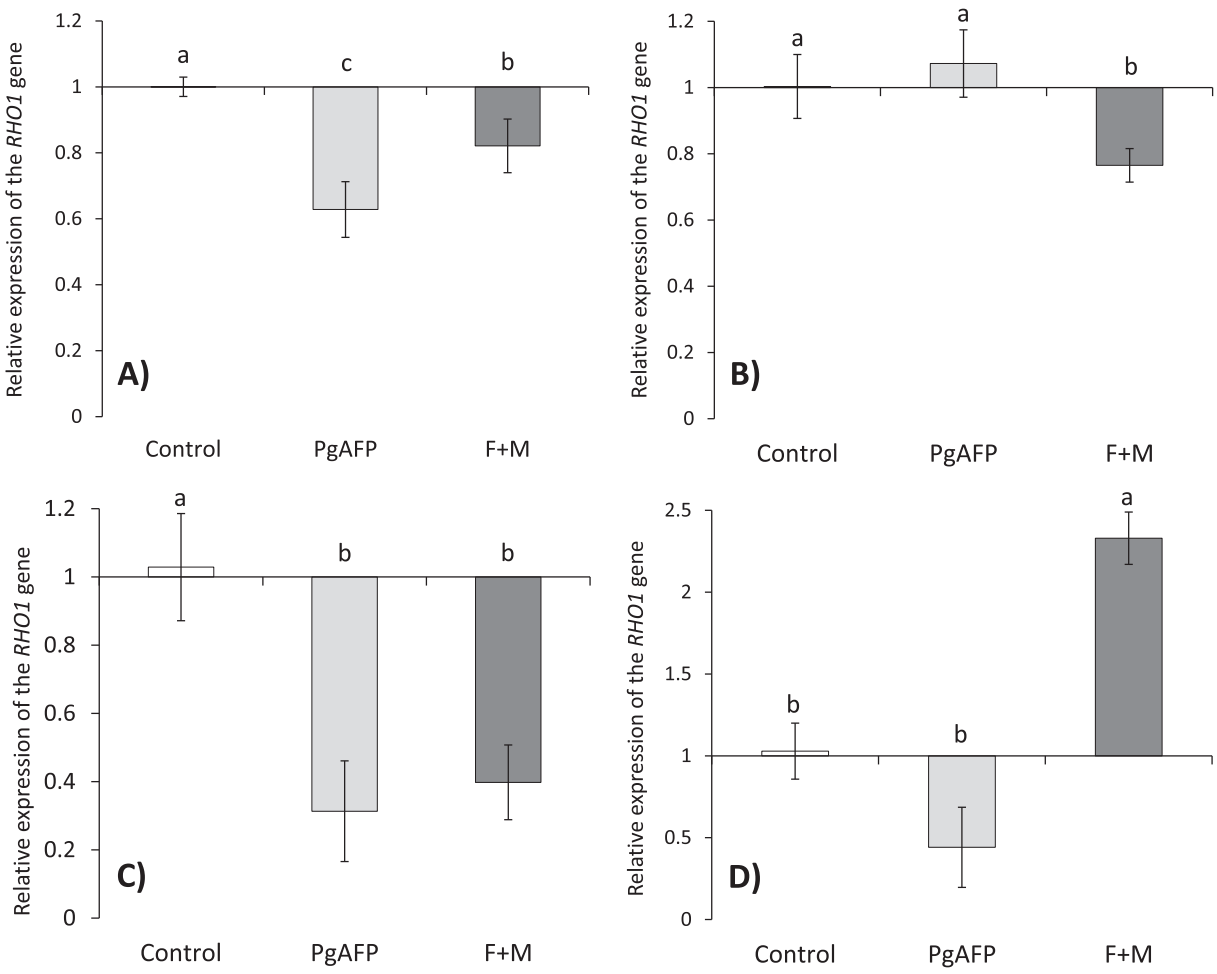

Fig. 3. Relative expression of the *RHO1* gene by *A. tenuissima* sp.-grp. in wheat agar at 0.95 and 0.98 water activity (a_w) and 25 °C, with two antifungal treatments (PgAFP and F + M) during two growth phases (linear and stationary). A) 0.95 a_w, linear phase; B) 0.95 a_w, stationary phase; C) 0.98 a_w, linear phase; D) 0.98 a_w, stationary phase. Control plates at each a_w level were used as calibrators for calculations (relative expression value ≈ 1). The lines indicate the standard deviation and the letters indicate the groups with significant differences ($p \leq 0.05$).

by the presence of antifungal when the fungus is in active growth stage. When reaching the stationary phase, this inhibition of the *RHO1* gene expression was maintained at 0.95 a_w, condition at which TeA was produced in lower concentration than in the control, while AOH and AME were not detected. On the other hand, at 0.98 a_w an increase of the *RHO1* gene expression in the stationary phase was observed. It has been formerly proposed that fludioxonil, one of the active compounds in the commercial antifungal, exerts the capability to activate the CWI pathway in *Cryptococcus neoformans* in an attempt to prevent cell rupture due to swelling and increased pressure caused by this compound (Hayes et al., 2014; Kojima et al., 2006). It should be noted that the increase of this stress-related gene expression linked to the greater accumulation of AME at the highest a_w tested. This fact suggests that the CWI and the alternariols biosynthetic pathway may be related, but this hypothesis should be in-depth evaluated in further studies.

The alternariols biosynthetic route has been previously associated with other stress-related pathways in *Alternaria*, including HOG (related to osmotic stress) and PacC (responses to pH changes) (Geisen et al., 2015; Graf et al., 2012). These authors reported that, under specific environmental conditions, an increase in the transcription levels of these stress-associated pathways corresponded to a higher accumulation of alternariols, indicating that their production is finely regulated in nature. They also stated that this induction of secondary metabolites biosynthesis occurred at transcriptional level, detecting that the expression of the *pksJ* gene, a key gene in the AOH/AME biosynthetic pathway (Saha et al., 2012), paralleled the phenotypic biosynthesis of alternariol. On the other hand, to the best of our knowledge, TeA biosynthetic pathway is not completely elucidated, thus this kind of studies at transcriptional level cannot be performed yet. Additionally, there are no studies about the relationship between stress-related intracellular pathways and TeA accumulation.

The results of the present work constitute the first evidence of relationship between the alternariols accumulation and the CWI pathway when antifungals are applied under different environmental conditions that can be found in the ripening of wheat. This association was not detected with TeA. This is not surprising since response mechanisms are not universal; the same route can act positively for a certain species and mycotoxin, and negatively, either for another species and the same toxin or for another mycotoxin produced by the same species through a different biosynthetic pathway (Geisen et al., 2017).

5. Conclusions

The application of both antifungals evaluated produced comparable fungistatic effects on *Alternaria* spp. growth; however, the presence of PgAFP produced significant reductions in *Alternaria* spp. mycotoxins accumulation, whereas with the commercial antifungal (fludioxonil and metalaxyl-M), this effect was not observed. Moreover, the presence of the last one stimulated the expression of a CWI-related gene, which was not observed with PgAFP. Thus, the CWI and the accumulation of

Alternaria spp. mycotoxins seem to be related. The application of PgAFP would be a promising natural strategy for its application in wheat for the control of *Alternaria* spp. The higher effectiveness of PgAFP than the commercial fungicide supposes an advantage from a food safety point of view, in addition to its lower environmental impact due to its natural origin.

Acknowledgements

This work has been funded by the Spanish Ministry of Economy and Competitiveness, Government of Extremadura, and FEDER (AGL2013-45729-P, AGL2016-80209-P, GR15108). Dr. Lucía da Cruz Cabral was recipient of a pre-doctoral fellowship (BEC.ar call 2015, República Argentina) to do a research placement at the University of Extremadura. Dr. Alicia Rodríguez was supported by a "*Juan de la Cierva*-Incorporación" senior research fellowship (IJCI-2014-20666) from the Spanish Ministry of Economy and Competitiveness. Technical support was provided by Facility of Innovation and Analysis in Animal Source Foodstuffs of SAIUEx (financed by UEX, Government of Extremadura, MICINN, FEDER, and FSE).

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