Effect of *Debaryomyces hansenii* and the antifungal PgAFP protein on *Alternaria* spp. growth, toxin production, and *RHO1* gene expression in a tomato-based medium

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Abstract

Tomato fruit is susceptible to *Alternaria* spp. spoilage, which poses a health risk due to their mycotoxin production. Biopreservation relies on the use of whole microorganisms or their metabolites to manage spoilage microorganisms including filamentous fungi. However, the use of treatments at fungistatic level might activate intracellular pathways, which can cause an increment in mycotoxin accumulation. The objective of this work was to evaluate the effect of two strains of *Debaryomyces hansenii* and the antifungal protein PgAFP at 10 and 40 µg/mL. Both growth and production of two of the most common mycotoxins (tenuazonic acid and alternariol monomethyl ether) by *Alternaria tenuissima* sp.-grp. and *Alternaria arborescens* sp.-grp. on a tomato-based matrix, were analysed at 12 °C. Additionally, the impact of these biocontrol agents on the stress-related *RHO1* gene expression was assessed. All treatments reduced mycotoxin accumulation (from 27 to 92 % of inhibition). Their mode of action against *Alternaria* spp. in tomato seems unrelated to damages to fungal cell wall integrity at the genomic level. Therefore, the two *D. hansenii* strains (CECT 10352 and CECT 10353) and the antifungal protein PgAFP at 10 µg/mL are suggested as biocontrol strategies in tomato fruit at postharvest stage.

Keywords: *Alternaria*, mycotoxin, tomato fruit, yeasts, antifungal protein, food safety

1. Introduction

Small-spored *Alternaria* species belonging to section *Alternaria* are frequent contaminants isolated during the storage of fresh fruit and vegetables, notably tomatoes. Fruit decay due to *Alternaria* infection causes economic losses for producers. In addition, isolates from *Alternaria* section *Alternaria* can synthesise several mycotoxins, constituting a health risk. *A. tenuissima* and *A. arborescens* sp.-grp. have been reported as the most frequent contaminants of tomato fruit from this genus in recent studies (Andersen et al., 2015; Patriarca et al., 2019). Among *Alternaria* spp. toxins, alternariol (AOH) and its monomethyl ether derivative (AME) have been reported to be cytotoxic, mutagenic and genotoxic *in vitro* and associated to high levels of human oesophageal cancer in China (Fraeyman et al., 2017; Liu et al., 1992; Ostry, 2008; Solhaug et al., 2016). On the other hand, tenuazonic acid (TeA) exerts its toxic effect by inhibiting the release of new proteins from the ribosomes, and has been related to the human haematological disorder Onyalai (Fraeyman et al., 2017; Ostry, 2008; Steyn and Rabie, 1976). Currently, the presence of these chemical contaminants in food is a matter of concern for international health authorities. The European Food Safety Authority (EFSA) highlighted the relevance of the ingestion of these compounds to human health and has identified fresh tomatoes and tomato-based products as major sources of dietary exposure (EFSA, 2011; 2016). In addition, it has been demonstrated that the usual temperature in storage chambers for industrialisation (12 ºC) seems to favour the synthesis of TeA and AME by *Alternaria* spp. in tomato pulp agar (da Cruz Cabral et al., 2019b). Both mycotoxins, but particularly AME, were produced in higher amounts at 12 ºC than at any other temperatures related to tomato postharvest stages (storage or ripening).

Mould contamination has been traditionally controlled in the field through the application of synthetic fungicides. However, the use of these synthetic compounds presents several drawbacks, including the presence of toxic residues in food, soils, or water, and the development of microorganisms' resistance, which entails the utilisation of higher fungicide doses (da Cruz Cabral et al., 2013). Growing concern about the disadvantages of synthetic fungicides has led to the investigation of alternative control strategies. Biocontrol strategies using whole microorganisms or their metabolites have gained attention as a promising alternative (Prendes et al., 2018). Both moulds and yeasts and their metabolites have shown biopreservation potential, alone or in combination with chemical fungicides, contributing to reduce the pesticide dose necessary to control fungal growth (Gil-Serna et al., 2011; Zhu et al., 2016).

Yeasts are endowed with important characteristics to be applied as biopreservatives due to their non-toxic properties and their faster growth rates and simpler nutritional requirements compared to moulds (Grzegorczyk et al., 2017; Medina-Córdova et al., 2016; Pimenta et al., 2009). In particular, *Debaryomyces hansenii* has been granted QPS (Qualified Presumption of Safety) status by EFSA, so it can be applied in the food industry with no need to undergo a full safety assessment (EFSA, 2017). Its inhibitory activity against moulds causing fruit and vegetables decay, including species of *Aspergillus, Penicillium, Rhizopus, Mucor, Fusarium,* and *Monilinia,* has been described (Grzegorczyk et al., 2017; Hernandez-Montiel et al., 2010; Medina-Cordova et al., 2016; Sharma et al., 2009). However, there are few reports about its effect on *Alternaria* spp. growth and their mycotoxin synthesis. Çorbacı and Uçar (2018) stated that two *D. hansenii* strains significantly reduced *Alternaria brassicicola* lesions on tomato fruit. Additionally, *Alternaria alternata* mycelial growth on Potato Dextrose

Agar (PDA) was reduced up to 49 % in the presence of *D. hansenii* (Al-Qaysi et al., 2017).

A range of antifungal proteins produced by moulds have been described. Biocontrol strategies based on the use of purified antifungal proteins instead of viable microorganisms offer several advantages, including stability, safety, economic viability, and ease of use (Zhu et al., 2016). The antifungal protein PgAFP, produced by the strain *Penicillium chrysogenum* CECT 20922, has been shown to inhibit mould contaminants on different food matrices (Acosta et al., 2009; da Cruz Cabral et al., 2019a; Delgado et al., 2015a; Fodil et al., 2018; Rodríguez-Martín et al., 2010).

Either synthetic or natural antifungals can be applied at levels that inhibit growth without inactivating target moulds (fungistatic level). Exposure to sub-lethal concentrations of antifungals can trigger the activation of stress response pathways which could be linked to 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). The cell wall integrity (CWI) pathway is responsible for the maintenance of the cell wall by mediating its regeneration in response to stress. It is associated with tolerance to antifungal treatments due to the unique structure of moulds' cell wall. This fact makes CWI a common target for antifungal treatments (Jabes et al., 2016). The relative amount of the RHO1 protein, the master regulator within this route, fluctuates in response to different antifungal compounds (Hayes et al., 2014; Levin, 2011). In addition, previous studies have shown the existence of a relationship between changes in *RHO1* gene expression and mycotoxin production by *Alternaria* spp. and *Aspergillus carbonarius*, when growing under different environmental and nutritional conditions and in the presence of antifungal compounds (da Cruz Cabral et al., 2019a, 2019b; Fodil et al., 2018). This connection between *RHO1* gene expression and mycotoxin production has

not been clearly elucidated yet, and could be studied for the development of new antifungal treatments.

The objectives of this work were to evaluate the effect of two strains of *D. hansenii* and the antifungal protein PgAFP at two levels on the growth and mycotoxin production by *Alternaria tenuissima* sp.-grp. and *Alternaria arborescens* sp.-grp. on a tomato based-medium at 12 ºC, a usual postharvest storage temperature. Additionally, the impact of both biocontrol agents on the expression of the stress-related *RHO1* gene was assessed.

2. Materials and Methods

2.1. Culture media

Experiments were performed on a Tomato Pulp Agar (TPA) prepared as described by da Cruz Cabral et al. (2019b). *Alternaria* spp. strains were maintained in Potato Carrot Agar (PCA), while Yeast Extract Sucrose medium (YES) was used for yeasts (Samson et al., 2010).

2.2. Microorganisms

Two small-spored *Alternaria* strains obtained from tomato fruit were used in the present study. One of them belonged to *A. arborescens* sp.-grp. (Id. T_2_L) and was a TeA producer. The other one was classified as *A. tenuissima* sp.-grp. (Id. T_50_H) and was able to produce AME. These two toxins are synthesised by different biosynthetic pathways. Morphological classification at species-group level according to Simmons (2007) had been performed in a previous study (da Cruz Cabral et al., 2017). Their ability to grow and produce mycotoxins under the conditions of the present study had been assessed in a previous work (da Cruz Cabral et al., 2019b). Both strains are

maintained in the Food Mycology Group Culture Collection from the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires.

Alternaria spp. spore suspensions to be used as inoculum were prepared in Phosphate Buffer Saline (PBS; pH 7.2) from 7-day-old PCA plates and adjusted to a concentration of 10^6 spores/mL using a Thoma counting chamber Blaubrand® (Brand, Germany) in a Nikon SE (Tokyo, Japan) microscope.

Two *Debaryomyces hansenii* strains from the Spanish Type Culture Collection (CECT 10352, and CECT 10353) were used in this study. They were formerly isolated from tomato fruit. A 10^4 cells/mL suspension was prepared for each yeast strain in PBS.

The strain *P. chrysogenum* CECT 20922 producer of the antifungal protein PgAFP (Rodríguez-Martín et al., 2010) was also used in this work.

2.3. Antifungal protein

P. chrysogenum CECT 20922 was inoculated into malt extract broth (20 g/L malt extract, 20 g/L glucose, and 1 g/L peptone; MEB), pH 4.5, and incubated for 21 days at 25 °C. PgAFP was primarily obtained from the cell-free culture medium by fast protein liquid chromatography with a cationic exchange column HiTrap SP HP (Amersham Biosciences, Sweden). The antifungal protein was further purified with a HiLoad 26/60 Superdex 75 gel filtration column for FPLC (Amersham Biosciences), and concentrated in 50mM sodium phosphate buffer (pH 7.0) containing 0.15M NaCl, as previously described (Rodriguez-Martin et al., 2010).

Different PgAFP stocks were pooled in a stock solution, which was measured by Lowry method (Lowry et al., 1951), sterilised through 0.22 µm acetate cellulose filters (Fisher Scientific, United Kingdom), and stored at −20 °C until use.

2.4.Experimental settings

To evaluate the efficacy of *D. hansenii* strains as biocontrol agents, an aliquot of 100 µL of the yeast suspension was uniformly distributed onto TPA plates and allowed to absorb for 1 h at room temperature. In parallel, other TPA plates were supplemented with PgAFP after sterilisation, at 50 °C, prior to solidification, to reach final concentrations of 10 and 40 µg/mL. These levels were formerly selected based on optical density measurements at λ=595 nm, following the methodology described by Fodil et al. (2018) (Supplementary Figure S1). Additionally, non-supplemented TPA plates were used as controls. All TPA plates were centrally inoculated with 2 µL spore suspensions of *A. arborescens* sp.-grp or *A. tenuissima* sp.-grp., and incubated in the dark for a maximum of 18 days at 12 °C, a temperature commonly used in tomato storage chambers. Plates were enclosed in separate polyethylene bags which were placed in a temperature chamber together with four 500 mL beakers containing water to maintain relative humidity close to 100 % during the incubation. Each treatment 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 growth rate 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. Mycotoxin extraction and quantification

The levels of TeA and AME were studied from TPA plates at the end of the incubation period (18 days). The extraction and quantification were performed as described by da Cruz Cabral et al. (2019b). Briefly, metabolites were extracted from nine plugs cut from the edge to the centre across one of the diameters of the fungal colony and dissolved in MS-grade acetonitrile acidified with formic acid 1 % (v/v) after phase partitioning with an aqueous salted solution. Extracts were analysed by ultra-high-performance liquid chromatography (UHPLC in a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, USA) coupled to an ion trap mass spectrometer (MS) model Amazon SL (Bruker Daltonics, Germany). The limits of detection (LODs) were 72.5 ng/g, and 26.7 ng/g for TeA and AME, respectively; while the limits of quantification (LOQs) were 220.5 ng/g for TeA, and 81.1 ng/g for AME.

2.7. Stress-related gene expression assays

To evaluate the stress-related effect of both biocontrol strategies on *Alternaria* spp., the expression of the *RHO1* gene was assessed. Sampling was performed at 12 and 18 days of incubation, corresponding to the middle of the linear phase and the beginning of the stationary phase of the mould growth, respectively, in accordance with a previous study (da Cruz Cabral et al., 2019b). After each incubation time, the mycelium was carefully taken and scratched from the plates and placed into a 2 mL tube under sterile conditions, quickly frozen in liquid nitrogen, and stored at −80 °C. A sample of 50 mg of frozen biomass was used for RNA extraction. Isolation of RNA and two-step reverse transcription real-time PCR (RT-qPCR) were performed as described in da Cruz Cabral et al. (2019a). The primers used to conduct RT-qPCR reactions are shown in Table 1, according to the methodology optimised by da Cruz Cabral et al. (2018) and Estiarte et al. (2016). Relative quantification of the *RHO1* gene expression was calculated

following the $2^{-\Delta\Delta C_T}$ 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 calibrator used for calculations was the non-treated control sample (*A. arborescens* sp.-grp. or *A. tenuissima* sp.-grp.) at each sampling time.

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 versus 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 median values in this case. The statistical significance was set at $p<0.05$.

3. Results and Discussion

The present investigation evaluates the use of two different strategies in order to manage *Alternaria* spp. spoilage in tomato fruit, focusing on mycotoxin accumulation since this is the most worrying hazard regarding fungal contamination from a food safety point of view.

The study was performed in a model matrix based on food (tomato fruit), which is a first step before the direct application in the storage chambers. This approach allows discerning the implied mechanisms under highly controlled conditions that could be hidden in natural systems (Crowther et al., 2018). The relative humidity is considered a

key factor for fungal development and burden. The assays were performed at humidity *c.a.* 100 % with the aim to set the worst-case scenario. The working temperature was selected because high growth rates and mycotoxin production levels were detected in a previous study (da Cruz Cabral et al., 2019b) for both *Alternaria* strains at temperatures close to 12 °C, which indicates the need of an efficient control under this environmental condition. Finally, it is important to point out that a stimulation of the expression of the key gene of the CWI pathway led to increase AME accumulation by *A. tenuissima* sp.-grp. at 12 ºC (da Cruz Cabral et al., 2019b). Therefore, it is of great importance to find out the impact of both biocontrol strategies on the regulation of the CWI pathway to efficiently counteract AME production.

3.1. Effect of D. hansenii *on* Alternaria *spp.*

The first strategy evaluated for the control of *Alternaria* spp. was the application of two strains of *D. hansenii* (CECT 10352 and CECT 10353). These yeasts were originally isolated from tomato fruit, which gives them an additional advantage since they are probably pre-adapted to that environment as previously stated (Cebrián et al., 2019; Graf et al., 2012; Petersen et al., 2002).

Their effect on *Alternaria* spp. growth parameters (λ and μ_{max}) on TPA plates at 12 °C is shown in Table 2. The presence of any of the *D. hansenii* strains did not significantly affect λ of both *Alternaria* spp. ($p \geq 0.05$). Regarding μ_{max} values, they were lowered in the presence of *D. hansenii* for both *Alternaria* strains (between 15 and 35 %), although this value was only significantly different ($p<0.05$) from the corresponding control in the case of *A. arborescens* sp.-grp. with CECT 10352.

The mycotoxin levels synthesised by *Alternaria* spp. treated with the different yeast strains are shown in Table 3. The accumulation of TeA produced by *A. arborescens* sp.-

grp. significantly decreased (p<0.05) in the presence of *D. hansenii* with respect to the control (86 and 88 % of reduction with CECT 10352 and CECT 10353, respectively). On the other hand, AME was not detected in the TPA plates of *A. tenuissima* sp.-grp. in the presence of any of the yeast strains, while it was detected (4.8 μg/g) in their absence. The effect of *D. hansenii* strains on controlling the accumulation of other mycotoxins has been previously achieved, such as fumonisins by *Fusarium subglutinans*, ochratoxin A by *Aspergillus westerdijkiae* or *Penicillium verrucosum*, and aflatoxins by *Aspergillus parasiticus* (Gil-Serna et al., 2011, Medina-Córdova et al., 2016; Peromingo et al., 2018; 2019). Nevertheless, to the best of our knowledge, this is the first study in which their effect is evaluated against *Alternaria* spp. toxins. Several modes of action for *D. hansenii* have been described, against both fungal growth and mycotoxin production, including nutrient and space competition, specific enzymes and antimicrobial substances secretion, and volatile compounds production, among others (Andrade et al., 2014; Medina-Cordova et al., 2016; Núñez et al., 2015). In addition, the ability of yeasts to reduce mycotoxin accumulation without affecting fungal growth has also been reported, throughout mechanisms that involve cell wall adsorption, repression in the activity of mycotoxin biosynthetic genes at transcriptional level, and the production of enzymes that biotransform those metabolites (Binder, 2007; Gil-Serna et al., 2011; Peromingo et al., 2018; 2019; Pfliegler et al., 2015).

Since the application of *D. hansenii* might trigger the activation of metabolic pathways on *Alternaria* spp. to cope with the antagonist, the effect of both strains of *D. hansenii* on *RHO1* gene expression from *Alternaria* spp. was evaluated at both linear and stationary growth phases. These results are shown in Figure 1. The presence of the yeasts did not have a pronounced influence on the expression of the *RHO1* gene with respect to the control; significant differences were detected only in two cases. During

the linear growth phase, *A. arborescens* sp.-grp. showed stimulation of the *RHO1* gene expression with respect to the control in the presence of CECT 10353 (Figure 1A). This result may indicate that the CWI pathway is activated, since RHO1 protein is a key regulator of this route. Although the activation of CWI pathway could be postulated as a mechanism for this mould to overcome the stress and keep growing normally, more evidence is necessary to confirm it. On the other hand, in the presence of CECT 10352, no significant differences in *RHO1* gene expression were observed with respect to the control, coincidently with a reduction in growth rate for *A. arborescens* sp.-grp.

At the stationary phase, a significant inhibition of this gene expression in *A. tenuissima* sp.-grp. was observed in the presence of the strain CECT 10352, indicating that the CWI pathway was repressed under these conditions. This matches with a decline in AME quantities synthesised by this fungus. In previous research a positive correlation between the expression of the *RHO1* gene and alternariols accumulation (da Cruz Cabral et al., 2019b) was found in relation with incubation temperature. However, although both yeast strains were able to inhibit AME production by *A. tenuissima* sp.-grp., no evident relation was found between this inhibition and the CWI pathway. This may indicate that the influence on this mycotoxin biosynthesis by the *D. hansenii* strains might involve other mechanisms and further studies should be carried out to find out their intracellular mode of action.

3.2. Effect of the antifungal protein PgAFP on Alternaria *spp.*

The antifungal protein PgAFP produced by *P. chrysogenum* was the second strategy evaluated in the present study. Its presence at 10 and 40 µg/mL slightly affected *Alternaria* spp. growth parameters on TPA, as shown in Table 2. Regarding *A. arborescens* sp.-grp, its λ was lengthened 29 % and its μ_{max} lowered (23 %) when the

antifungal protein was applied at 10 µg/mL (p<0.05). Moreover, the application of the protein at the lowest dose tested also caused a significant reduction in TeA amounts (27 % with respect to the control) (Table 3). Surprisingly, when PgAFP was tested at 40 µg/mL, no significant differences, either on growth or TeA production, were observed between the control and PgAFP-treated batch. Similar results were encountered by Fodil et al. (2018) when the efficacy of PgAFP at 10 and 40 µg/mL was tested on *A. carbonarius* growth in a raisin simulating medium, and differences between the two levels used were not found. Besides, the absence of differences between the highest and the lowest PgAFP doses evaluated also matches with the results displayed in Supplementary Figure S1, where it is observed that PgAFP amounts of 9.38 and 37.5 µg/mL had a similar impact on *Alternaria* spp growth when growing on Potato Dextrose Broth. Minor differences were found for *A. arborescens* sp.-grp when the antifungal protein was applied in a more complex matrix, TPA. Thus, it is crucial to establish the dose for each target species, and for every food matrix, when testing new antifungal treatments; given that it is not always true that the higher the antifungal dose, the more effective growth inhibition is achieved.

With respect to *A. tenuissima* sp.-grp., the protein did not have significant influence on the λ or μ_{max} (Table 2). Otherwise, at both PgAFP concentrations, reductions in AME production were detected, with 58 and 92 % of inhibition at 10 and 40 µg/mL of PgAFP, respectively (p<0.05) (Table 3). Some former works evaluated the effect of this antifungal protein on the growth of mycotoxigenic fungal species that commonly contaminate foods, observing different levels of susceptibility to this treatment depending on the species and the food-based matrix (da Cruz Cabral et al., 2019a; Delgado et al., 2015a; Fodil et al., 2018). Furthermore, some of these recent studies have also examined the effect of PgAFP on mycotoxin accumulation, with different

levels of success. On one hand, PgAFP was able to reduce ochratoxin A production by *Aspergillus carbonarius* in raisin-simulating medium (Fodil et al., 2018), whilst its presence provoked an increase of patulin biosynthesis by *Penicillium expansum* on apple-based agar in a dose-dependent manner (Delgado et al., 2019). Additionally, a recent work reported that PgAFP reduced the TeA, AOH and AME amounts synthesised by *A. tenuissima* sp.-grp. in a wheat-based matrix (da Cruz Cabral et al., 2019a).

The mechanism of action of the antifungal protein PgAFP is based on membrane permeability, reactive oxygen species (ROS) induction, apoptotic events, and CWI impairment (da Cruz Cabral et al., 2018; Delgado et al. 2015b). The effect of the protein on the expression of the CWI-related gene in *Alternaria* spp. at linear and stationary growth phases is depicted in Figure 2. Even though significant differences were not detected in the *RHO1* gene relative expression in the presence of PgAFP with respect to the control for *A. arborescens* sp.-grp., at any phase, a trend to increase its expression was observed (Figure 2A-B). In the case of *A. tenuissima* sp.-grp., a significant repression of the expression of this gene was detected in the presence of PgAFP at 10 µg/mL at both linear and stationary growth phases (Figure 2C-D). Definitely, the CWI pathway was not stimulated in *A. tenuissima* sp.-grp. upon PgAFP treatment in the linear growth phase. Given that PgAPF did not affect its growth at any tested concentration, it was not expected that the CWI pathway was activated by the fungus to cope with the antifungal protein at this stage (primary metabolism). It is noteworthy that the repression of the *RHO1* gene relative expression in *A. tenuissima* sp.-grp. in the presence of PgAFP was accompanied by a decrease of AME quantities with regard to the control, as it was observed in the presence of *D. hansenii* CECT 10352 (Figure 1D; Table 3). Although a negative relation was observed between AME accumulation and

RHO1 expression, further studies are needed to establish the relationship between CWI and the alternariol biosynthetic pathway. Delgado et al. (2015a) showed that the activity of PgAFP remained stable over a very wide range of pH (1-12), so that the low effectiveness could not be attributed to the possible degradation of the protein due to the acidic environment of the TPA (pH=4.34). However, it has been formerly reported that the signalling pathway regulated by the transcription factor PacC is directly associated with the activation and/or repression of genes as a function of environmental pH, which influences growth, physiology, processes of differentiation and pathogenicity in filamentous fungi (Peñalva et al., 2008; Soares et al., 2014). Then, it is possible, that this route prevails over CWI and allows the fungus to cope with the stress caused by PgAFP. In addition, other routes may be activated as a fungal response to the presence of the protein in the medium. Some studies reported that the CWI pathway is not the only compensatory mechanism to repair cell wall damage, but some antifungal peptides activate the Ca^{2+}/calcineurin signalling pathway (Fiedler et al., 2014).

4. Conclusions

Taking together the results of the present work, there is no question that although both strategies employed were not always efficient in reducing fungal growth, they were effective against counteracting the mycotoxin synthesis by two *Alternaria* spp. strains in a tomato-based medium under the experimental conditions assayed. The toxins studied, TeA and AME, are produced by different biosynthetic pathways, which demonstrates that the mode of action of these treatments is broad. This result is of utmost importance considering that a decrease in the production of mycotoxins, which is a major hazard from a food safety point of view, cannot be directly assured in the presence of an agent with fungistatic effects. Moreover, this inhibition occurred at a temperature used in

tomato storage chambers, which makes these treatments promising alternatives for its application therein. Thus, these biological agents could be applied to prevent mycotoxin accumulation in tomatoes for industrialisation, which are held for longer periods in cold chambers, and whose main hazard resides in the presence of these toxic compounds, which would not be destroyed by subsequent processing.

AME and TeA reductions were observed in the presence of any of the *D. hansenii* strains or the antifungal protein PgAFP at 10 µg/mL, being *D. hansenii* 10352 the one that achieved the best results. Thus, these treatments are suggested as strategies to be applied at postharvest management of tomato fruit. Based on the genomic findings, fungal CWI appears not to be the only pathway involved in the mode of action of both antifungal strategies against *Alternaria*.

Further studies will be carried out to evaluate *in vivo* treatments on tomato fruit for industrialisation, in order to establish the most suitable mode and time of application. The interaction of each agent with the rest of the microbiota present in the food, its effects on the sensory characteristics, and the possible combination with strategies of low environmental impact (hurdle technologies) should be also taken under consideration.

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6. Declarations of interest

None

7. References

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Table 1: Primers used in this study.

Primer	Nucleotide sequences (5′-3′)	Gene	Concentration	References
RHO1-F1	CTTTCCCCGAGGTCTACGTC	*RHO1*	200 nM	da Cruz Cabral et al. (2018)
RHO1-R2	TCGTAATCCTCCTGACCAGC		200 nM	
A-BTF	ACAACTTCGTCTTCGGCCAGT	*β-tubulin*	300 nM	Estiarte et al. (2016)
A-BTR	ACCCTTTGCCCAGTTGTTACCAG		300 nM	

Table 2: Lag phase (λ, d) and maximum growth rate (μ_{max}, mm/d) for *Alternaria arborescens* sp.-grp. and *A. tenuissima* sp.-grp. on Tomato Pulp Agar (TPA) at 12 °C, in the presence of *Debaryomyces hansenii* (CECT 10352 and CECT 10353) and the antifungal protein PgAFP (at 10 and 40 µg/mL).

Treatment	*A. arborescens* sp.-grp.		*A. tenuissima* sp.-grp.	
	λ (d)	μ_{max} (mm/d)	λ (d)	μ_{max} (mm/d)
Control	2.7 ± 0.2	4.4 ± 0.3	4.8 ± 0.3	5.6 ± 0.2
D. hansenii				
CECT 10352	2.1 ± 0.7	2.9 ± 0.5*	7.8 ± 1.3	3.7 ± 0.2
CECT 10353	2.3 ± 1.0	3.7 ± 0.7	5.9 ± 0.4	4.0 ± 0.2
Antifungal protein				
PgAFP 10	3.9 ± 0.6*	3.4 ± 0.3*	4.4 ± 0.9	5.5 ± 0.3
PgAFP 40	2.3 ± 0.2	4.1 ± 0.3	4.7 ± 0.1	5.5 ± 0.2

Values correspond to mean concentration ± standard deviation (SD) of three replicates.

*: values significantly different from the control (p<0.05).

Table 3: Mycotoxin concentration (µg toxin per g agar) produced by *Alternaria arborescens* sp.-grp. and *A. tenuissima* sp.-grp. on Tomato Pulp Agar at 12 °C after 18 days of incubation, in the presence of *Debaryomyces hansenii* (CECT 10352 and CECT 10353) and the antifungal protein PgAFP (at 10 and 40 µg/mL).

Treatment	µg/g TeA (*A. arborescens* sp.-grp.)	µg/g AME (*A. tenuissima* sp.-grp.)
Control	363.6 ± 61.5	4.8 ± 1.0
D. hansenii		
CECT 10352	43.0 ± 7.6*	<LOD*
CECT 10353	52.5 ± 15.4*	<LOD*
Antifungal protein		
PgAFP 10	267.0 ± 32.6*	2.0 ± 0.7*
PgAFP 40	305.8 ± 61.9	0.4 ± 0.5*

Values correspond to mean concentration ± standard deviation (SD) of three replicates.

*: values significantly different from the control (p<0.05).

Figure legends

Figure 1. Relative *RHO1* gene expression in presence of yeasts. Relative expression of the *RHO1* gene by *Alternaria arborescens* sp.-grp. and *A. tenuissima* sp.-grp. in Tomato Pulp Agar at 12 °C, in the presence of *Debaryomyces hansenii* (CECT 10352 and CECT 10353) during two growth phases (linear and stationary). A) *A. arborescens* sp.-grp., linear phase; B) *A. arborescens* sp.-grp., stationary phase; C) *A. tenuissima* sp.-grp., linear phase; D) *A. tenuissima* sp.-grp., stationary phase. Control plates without *D. hansenii* were used as calibrators for calculations (relative expression value≈1). Bars indicate the standard deviation and the asterisks indicate the groups with significant differences with respect to the calibrator (p<0.05).

Figure 2. Relative *RHO1* gene expression in presence of PgAFP. Relative expression of the *RHO1* gene by *Alternaria arborescens* sp.-grp. and *A. tenuissima* sp.-grp. in Tomato Pulp Agar at 12 °C, in the presence of the antifungal protein PgAFP (at 10 and 40 µg/mL) during two growth phases (linear and stationary). A) *A. arborescens* sp.-grp., linear phase; B) *A. arborescens* sp.-grp., stationary phase; C) *A. tenuissima* sp.-grp., linear phase; D) *A. tenuissima* sp.-grp., stationary phase. Control plates without PgAFP were used as calibrators for calculations (relative expression value≈1). Bars indicate the standard deviation and the asterisks indicate the groups with significant differences with respect to the calibrator (p<0.05).

Supplementary Material

Supplementary Figure S1: Effect of PgAFP concentration (75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17, and 0 μg/mL) on *A. arborescens* sp.-grp. (A) and *A. tenuissima* sp.-grp. (B) growth measured as optical density at 595 nm during 96 h at 25 °C on Potato Dextrose Broth.

Figure 1

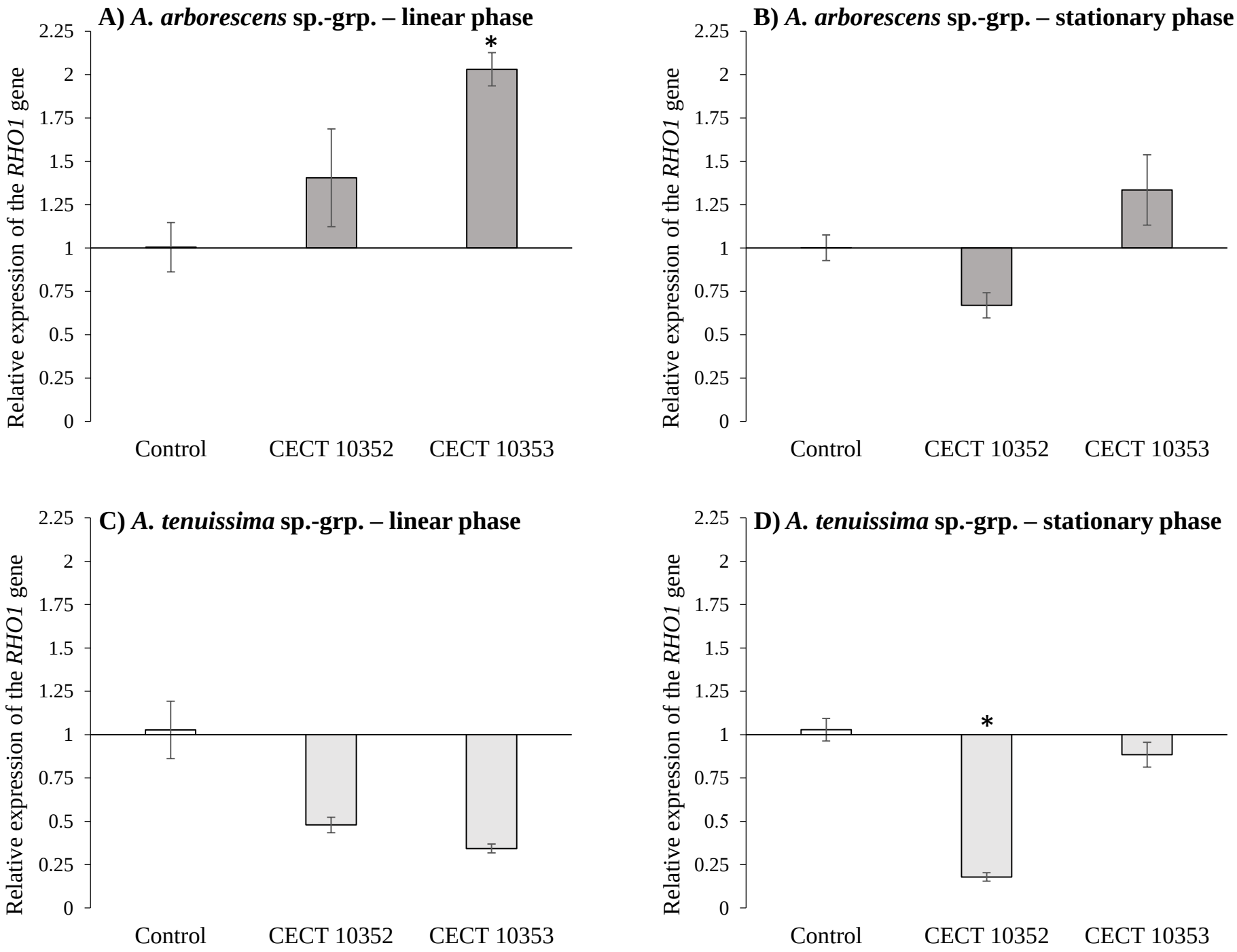
A) A. arborescens sp.-grp. – linear phase
B) A. arborescens sp.-grp. – stationary phase
C) A. tenuissima sp.-grp. – linear phase
D) A. tenuissima sp.-grp. – stationary phase
Relative expression of the RHO1 gene
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
*
Control
CECT 10352
CECT 10353

Figure 2

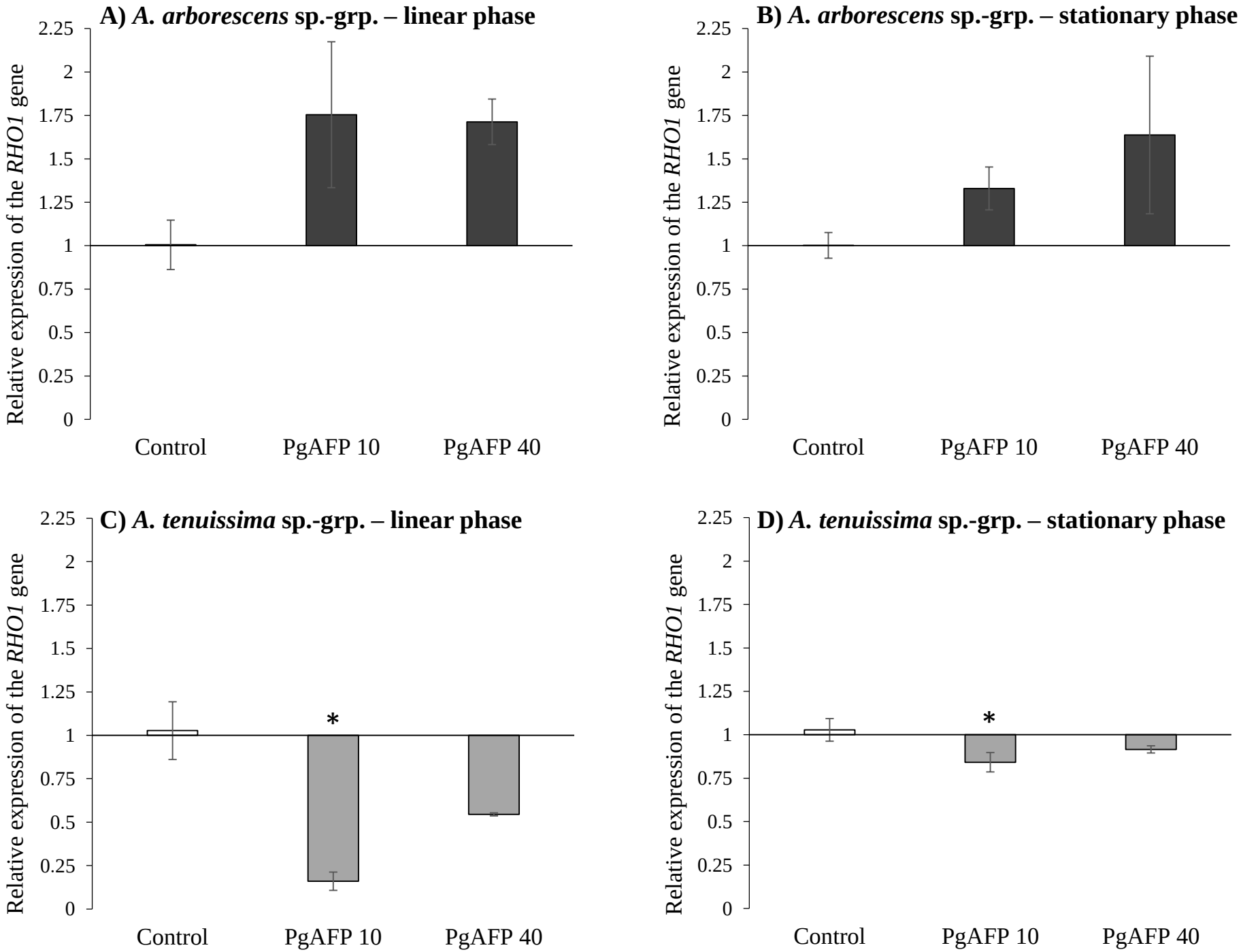
A) A. arborescens sp.-grp. – linear phase
B) A. arborescens sp.-grp. – stationary phase
C) A. tenuissima sp.-grp. – linear phase
D) A. tenuissima sp.-grp. – stationary phase
Relative expression of the RHO1 gene
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
Control
PgAFP 10
PgAFP 40
*

Figure S1 (Supplementary Figure)