



Evaluation of the activity of the antifungal PgAFP protein and its producer mould against *Penicillium* spp postharvest pathogens of citrus and pome fruits

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ABSTRACT

Postharvest fungal diseases are among the main causes of fresh fruit losses. Chemical control is against claims for “natural” or “chemical-free” products. Biocontrol agents, such as antifungal proteins or their producing moulds, may serve to combat unwanted pathogens. Since the effectiveness of these bioprotective agents depends on the food substrate, their effect must be tested on fruits. The objective of this work was to study the effect of the antifungal protein PgAFP and its producer, *Penicillium chrysogenum*, against *Penicillium expansum* and *Penicillium digitatum* growth on apple and oranges respectively, and the PgAFP effect on eleven *P. expansum*, *Penicillium italicum*, and *P. digitatum* strains *in vitro*, and on patulin production on apple substrate. The sensitivity upon PgAFP was *P. digitatum* > *P. expansum* > *P. italicum*. In oranges, broadly, no inhibitory effect was obtained. PgAFP and *P. chrysogenum* did not inhibit the *P. expansum* CMP-1 growth on Golden Delicious apples, however, a successful effect was achieved on Royal Gala apples. On apple substrate, patulin production by *P. expansum* CMP-1 rose in parallel to PgAFP concentrations, linked with high reactive oxygen species levels. PgAFP cannot be proposed as a bioprotective agent on apple. However, *P. chrysogenum* is a promising agent to be used on Royal Gala apples.

1. Introduction

Postharvest fungal diseases are among the main causes of fresh fruits losses during their distribution worldwide (FAO, 2011). Chemical control of these postharvest diseases is efficient and cost-effective, but they are against claims for “natural” or “chemical-free” products. Biocontrol using antifungal proteins or their producer moulds provide a valuable strategy against unwanted moulds.

Some moulds are able to produce antifungal proteins, which have been considered as an ecological advantage in order to colonise a given niche (Marx, 2004). PgAFP is an antifungal protein produced by *Penicillium chrysogenum* CECT 20922 (formerly RP42C) isolated from dry-cured ham (Acosta et al., 2009; Rodríguez-Martín et al., 2010). This protein efficiently retarded the growth of a wide variety of reference moulds *in vitro* as well as reduced the counts of *Aspergillus flavus* and *Penicillium restrictum* in dry-fermented sausages (Delgado et al., 2015a).

Moreover, *P. chrysogenum* CECT 20922 used as protective culture limited the growth of aflatoxin-producing *A. flavus* and ochratoxigenic moulds on dry-cured ham (Bernáldez et al., 2014; Rodríguez et al., 2015). However, PgAFP did not inhibit *A. flavus* growth in cheese due to the calcium content (Delgado et al., 2017).

The antifungal proteins produced by moulds have some common structural properties such as being small, cationic and cysteine-rich. They cause an inhibitory effect on sensitive moulds in a dose-dependent manner (Delgado et al., 2015a; Kaiserer et al., 2003; Skouri-Gargouri et al., 2009). The mechanism of action of PgAFP has been partially elucidated, being involved reactive oxygen species (ROS) induction, cell wall integrity disturbance, membrane permeabilization, inhibition of metabolic activity, and necrosis and apoptosis phenomena (Delgado et al., 2016). However, ROS can activate mycotoxin production (Reverberi et al., 2012; Schmidt-Heydt et al., 2014), and the oxidative stress is considered a prerequisite for aflatoxin production (Jayashree

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and Subramanian, 2000). On the other hand, mycotoxin production can be reduced by antioxidants, such as butylated hydroxyanisole (Reverberi et al., 2006), β -glucans (Reverberi et al., 2005) or caffeic acid (Kim et al., 2008). In this sense, antioxidants such as phenolics naturally present on apples (Rana and Bhushan, 2016) could decrease the adverse effects of ROS in patulin synthesis. Thus, patulin production by *P. expansum* treated with PgAFP should be studied in apple substrate.

Therefore, these proteins and their producing moulds could be proposed to control some unwanted moulds in foods achieving products free of synthetic chemicals. The use of compounds produced by *P. chrysogenum* fulfills the food safety requirement, given that this species is used to obtain generally recognized as safe (GRAS) compounds. Thereby the application of these proteins as a biocontrol strategy deserves to be studied. However, given that the effectiveness of these bioprotective agents depends on the food substrate, considering antifungal effect must be tested on fruit matrix and fruits. There are also recent reports indicating the potential application of antifungal peptides and proteins to control postharvest pathogens (Garrigues et al., 2018; Wang et al., 2018).

Given the increasing interest in the use of alternative preservation methods based on natural products and the already proven control ability of both the antifungal PgAFP proteins as well as its producing fungus (Bernáldez et al., 2014; Delgado et al., 2015a; Rodríguez et al., 2015), we sought to evaluate the inhibitory activity of PgAFP and the producer *P. chrysogenum* against *P. expansum* and *P. digitatum* growth on apple and oranges, respectively. In addition, the antifungal effect of PgAFP on *P. expansum*, *P. italicum*, and *P. digitatum* *in vitro* and on patulin production on apple substrate by *P. expansum* was assessed.

2. Materials and methods

2.1. Microorganisms and culture conditions

Penicillium chrysogenum CECT 20922 (formerly *P. chrysogenum* RP42C), isolated from dry-cured ham (Acosta et al., 2009), was used to produce PgAFP. Antimicrobial assays were conducted with eleven strains with sequenced genomes from the three major postharvest pathogenic species of citrus and apple fruits from the genus *Penicillium*. *P. digitatum* (Pers.:Fr.) Sacc. and *P. italicum* Wehmer are pathogens of citrus fruit and *P. expansum* Link is a pathogen of pome fruit. Details on these strains are given in Table 1. All strains were grown on potato dextrose agar (PDA, Difco-BD Diagnostics, Sparks, MD, USA) plates. Cultures were incubated at 24 °C for 7–10 days. Conidia were collected with sterile water, filtered through a nylon mesh and titrated with a haemocytometer.

2.2. PgAFP purification

The PgAFP-producing *P. chrysogenum* CECT 20922 was inoculated

Table 1
Fungal strains used in the PgAFP *in vitro* susceptibility analyses.

Species	Strain	Origin	GenBank acc. No.	Reference
<i>P. digitatum</i>	Pd1 (CECT 20795)	Spain	AKCU00000000.1	Marcet-Houben et al. (2012)
<i>P. digitatum</i>	PHI26 (CECT 20796)	Spain	AKCT00000000.1	Marcet-Houben et al. (2012)
<i>P. digitatum</i>	Pd01-ZJU	China	ANGJ00000000.1	Sun et al. (2013)
<i>P. digitatum</i>	PDC 102	Israel	LHSL01000000.1	Julca et al. (2016)
<i>P. expansum</i>	d1 (CECT 20907)	Israel	JQFY00000000.1	Ballester et al. (2015)
<i>P. expansum</i>	CMP-1 (CECT 20906)	Spain	JQFX00000000.1	Ballester et al. (2015)
<i>P. expansum</i>	MD-8 (CECT 20908)	USA	JQFZ00000000.1	Ballester et al. (2015)
<i>P. expansum</i>	T01	China	AYHP00000000.1	Li et al. (2015)
<i>P. expansum</i>	NRRL 62431 ^a	USA	ALJY00000000.1	Yang et al. (2014)
<i>P. italicum</i>	PHI-1 (CECT 20909)	Spain	JQGA00000000.1	Ballester et al. (2015)
<i>P. italicum</i>	B3	China	JMDK00000000.1	Li et al. (2015)
<i>P. chrysogenum</i>	RPC42 (CECT 20922)	Spain	Not sequenced	Rodríguez-Martín et al. (2010)

^a This strain was previously classified as *P. aurantiogriseum*.

into malt extract broth (20 g/L malt extract, 20 g/L glucose, and 1 g/L peptone; MEB), pH was adjusted at 4.5, and incubated up to 21 days at 25 °C. PgAFP was obtained from the cell-free medium by fast protein liquid chromatography with a cationic exchange column HiTrap SP HP (Amersham Biosciences, Uppsala, Sweden), further purified with a HiLoad 26/60 Superdex 75 gel filtration column for FPLC (Amersham Biosciences), and concentrated in 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl, as previously described (Acosta et al., 2009; Rodríguez-Martín et al., 2010).

PgAFP concentration in a pooled stock solution was measured by Lowry method (Lowry et al., 1951), sterilised through 0.22 μ m acetate cellulose filters (Fisher Scientific, United Kingdom), and stored at –20 °C until use.

2.3. *In vitro* antimicrobial assays

Antimicrobial activity of PgAFP against a collection of different isolates from three *Penicillium* species was conducted in sterile 96-well flat-bottom microtiter plates (Nunc, Thermo Scientific, United Kingdom). Fungi were grown at 24 °C for seven days in a final volume of 100 μ L of potato dextrose broth (PDB; Scharlau Chemie, S.A., Spain) containing 10⁴ conidia per well and different amounts of PgAFP protein, in two-fold serial dilutions ranging from 75 to 1.17 μ g/mL (11.6–0.2 μ M). In order to avoid evaporation, plates were incubated inside a box on top of water-saturated filter paper. Growth was followed by measuring the absorbance at 600 nm (A_{600}) with a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). To minimize the effect of non-homogeneous fungal growth within the well, each A_{600} value was the average of nine measurements regularly distributed within the well. Each treatment was conducted in triplicate within the same plate. Values given represent the mean \pm standard deviation of the mean, after background subtraction, of these three replicates. IC₅₀ (the concentration required to obtain 50% inhibition of growth) values were determined at 4 days by adjustment of growth data to a four-parameter sigmoid curve. The minimum inhibitory concentration (MIC) is defined as the minimum concentration at which no growth was observed at the end of the incubation period.

2.4. Fruit pathogenicity tests

Experiments were conducted either with freshly harvested oranges (cv Navelina and Lanelate) or with apples (cv Golden Delicious and Royal Gala) obtained from a local grocery. Fruits were submerged for 5 min in a 5% commercial bleach solution, rinsed extensively with tap water and allowed to dry. Each fruit was wounded at four places across the equatorial axis with a nail (2 mm wide x 4 mm depth) and each wound was inoculated with a freshly made mixture containing 10 μ L of a spore suspension (10⁴ conidia/mL for Navelina and Lanelate oranges and Golden Delicious apples, or 10⁵ conidia/mL in the case of Royal

Gala apple fruits) and PgAFP at either 50 (Navelina oranges and Golden Delicious and Royal Gala apples) or 100 µg/mL (Lanelate oranges). We have also tested a pre-incubation of PgAFP at 100 µg/mL with *P. digitatum* spores for 5 h before inoculation of Lanelate oranges.

The biocontrol capability of *P. chrysogenum* CECT 20922 was assayed by co-inoculation of 100-fold more spores than the pathogen: 10⁶ conidia/mL in Navelina and Lanelate oranges and Golden Delicious apples, and 10⁷ conidia/mL in Royal Gala apples. Fruits were placed in plastic boxes and incubated at 20 °C and 90% relative humidity. Decay incidence (percentage of infected wounds) and severity (macerated lesion diameter) were evaluated periodically. Five fruits constituted a replicate and there were three replicates per experiment. Infection experiments were conducted at least twice at different times.

2.5. Patulin production

For assays in solid media, apple agar was prepared using lyophilized apple powder (30 g/L) and bacteriological agar (20 g/L). Apple powder was achieved by cutting apples in pieces, froze them at -80 °C for at least 3 h and lyophilizing the pieces in a Bulk Tray Dryer with a 6-Port Manifold coupled to a FreeZone 6L Console Freeze Dry System (Labconco, USA). The lyophilized pieces were then pulverized in a crusher. After dissolving these ingredients by stirring, the medium was sterilised at 121 °C for 16 min. To obtain the three batches with different PgAFP concentrations 0, 10 and 40 µg/mL, melted apple agar (45 °C) was supplemented with a sterile solution of PgAFP prior to being poured into Petri plates. They were stored at room temperature until agar solidification. Then, plates were centrally inoculated with 2 µL from a spore suspension of *P. expansum* CMP-1 (10⁶ conidia/mL) and incubated for 15 days at 25 °C.

2.6. Patulin extraction

Around 1 g of apple agar where *P. expansum* CMP-1 was grown was placed into a test tube prior to ethyl acetate addition (2 mL). The test tube was vortexed for 2 min and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to a new tube and evaporated to dryness under a gentle stream of nitrogen. Samples were kept at -20 °C until resuspension to be analysed.

2.7. Patulin detection

Patulin was analysed by an uHPLC system, Thermo Scientific (United Kingdom) Dionex UltiMate 3000 coupled to an Ion Trap Mass Spectrometer System amaZon SL (Bruker Daltonics Inc., Germany). The column was a reversed-phase column C18 (100 mm × 2.1 mm, 2 µm; Agilent Technologies, USA). The analysis was done in a linear gradient from 2 to 98%. The mobile phase consisted of 0.1% acetic acid-10 mM ammonium acetate (solvent A) and methanol (solvent B). The injection volume was 10 µL and the flow rate was set at 0.3 mL/min. MS detection of patulin was performed using the ions 153 in ESI⁻ ionization mode. The run time was 15 min, being the patulin detected at 3.0 ± 0.2 min. Signals were processed by Hystar 3.2 software (Bruker Daltonics Inc.).

2.8. ROS detection

Apple broth was prepared similarly to apple agar (section 2.5), except for the no addition of bacteriological agar. After sterilization, tubes containing the broth were centrifuged to discard any solid particle, which could interfere in the absorbance or fluorescence assays. This broth was poured into a 96-well plate and supplemented with PgAFP to obtain 0, 10 and 40 µg/mL. *P. expansum* CMP-1 was inoculated at 10⁵ conidia per well and incubated at 25 °C for 48 h. A parallel assay was performed using PDB instead of apple broth. Both assays were carried out in three biological replicates. Each well was supplemented with

2',7'-dichlorofluorescein diacetate (DCFDA, Molecular Probes, Eugene, OR, USA) to get 25 µM. The DCFDA is not fluorescent, but when ROS are generated, DCFDA is oxidized to 2',7'-dichlorofluorescein (DCF), generating green fluorescence. The amount of DCF formed is proportional to cellular oxidant production (Chen and Dickman, 2004). The samples were incubated in darkness for 45 min at 25 °C. Then, measures of absorbance to obtain growth data, and fluorescence (Ex 485/Em 535) to obtain total ROS content, were carried out in a Varioskan LUX (Thermo Scientific, United Kingdom). Assays were carried out with six biological replicates.

2.9. Statistical analyses

Statistical analyses were performed with IBM SPSS v.22 (<https://www.ibm.com/products/spss-statistics>). Data were tested for normality (Kolmogorov-Smirnov with Lilliefors correction) and homoscedasticity (Levene's test). Given that patulin concentration and ROS data were non-normally distributed, median values were compared using the non-parametric Kruskal-Wallis test. Then, to compare treatments in pairs, the Mann-Whitney *U* test was applied (*p* < 0.05). To study the relationship between patulin production and intracellular ROS, Spearman correlation was applied (*p* < 0.05). Statistical analyses of pathogenicity assays were conducted by one-way ANOVA. Variance normality was checked according to Levene's test. Data means were compared among treatments (F-test) and those showing differences were further categorized according to Fisher's least significant difference test (*p* < 0.05).

3. Results

3.1. PgAFP purification

PgAFP was purified from the supernatant of a twenty-one-day culture of *P. chrysogenum* CECT 20922 in MEB medium, following the procedure described in section 2.2. The yield of the purified protein was 585 µg/mL, very close to the 678 µg/mL concentration obtained in previous work (Delgado et al., 2015a).

3.2. In vitro antifungal activity of PgAFP

Antifungal activity of purified PgAFP was tested *in vitro* against a collection of isolates from the postharvest fungal pathogens *P. digitatum*, *P. expansum* and *P. italicum* whose genome sequences have been published (Table 1). This approach was used to determine possible intraspecific differences in antifungal susceptibility. Although growth curves of the different strains of the same species seemed similar (Fig. 1), some differences may be observed, which are reflected in the IC₅₀ and MIC values (Table 2). Thus, the four *P. digitatum* strains showed similar IC₅₀ values, in the range of 1.0–1.7 µg/mL. However, the highest dose of PgAFP (75 µg/mL) was not able to inhibit completely the growth of the strains Pd1 and Pd01 after 7 days of incubation, whereas the strains PHI26 and PDC102 showed MIC values of 9.4 and 37.5 µg/mL, respectively.

The five *P. expansum* strains were less sensitive to PgAFP than the *P. digitatum* strains, ranging their IC₅₀ values between 7.6 and 12.7 µg/mL. Among *P. expansum* strains, isolate d1 showed the lowest MIC value (37.5 µg/mL), followed by strain MD-8 (75 µg/mL). The three remaining *P. expansum* strains were not completely inhibited by PgAFP under the assayed conditions.

The two *P. italicum* strains were even more resistant to PgAFP than any of the *P. digitatum* or *P. expansum* strains, with IC₅₀ values of 12.4 and 39.7 µg/mL and MIC values > 75 µg/mL. Moreover, the addition of PgAFP at 2.3 and 4.7 µg/mL led to a 40% increase in the growth of the strain B3.

The strain PgAFP-producing *P. chrysogenum* CECT 20922 was insensitive to PgAFP under the assay conditions. As noted in the *P.*

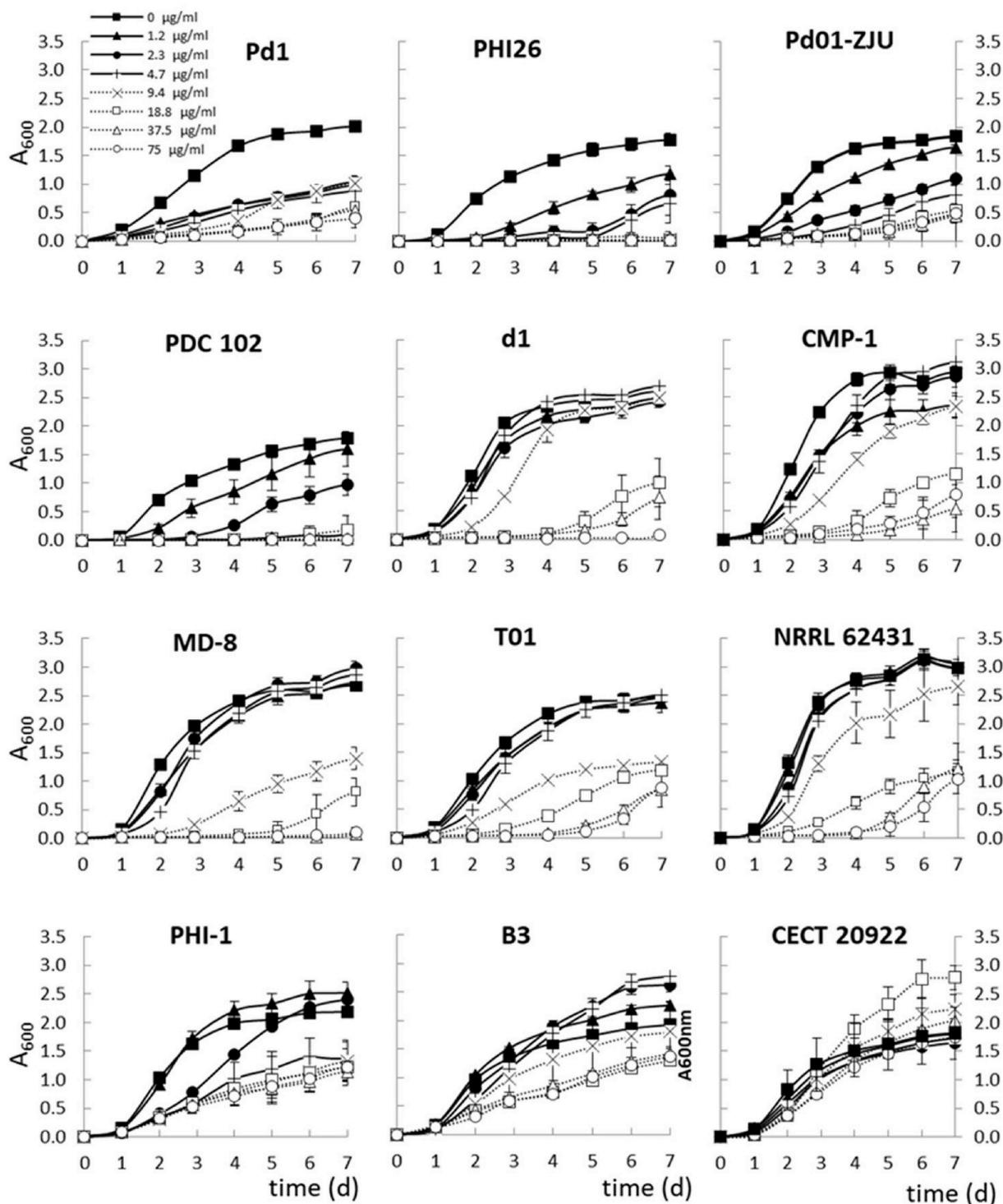


Fig. 1. Growth curves of *P. digitatum* (strains Pd1, PHI26, Pd01-ZJU and PDC 102), *P. expansum* (strains MD-8, CMP-1, d1, T01 and NRRL 62431), *P. italicum* (strains PHI-1 and B3) and *P. chrysogenum* CECT 20922 in medium PDB supplemented with different amounts of PgAFP. Strains were inoculated in 96-well microtiter plates and incubated at 24 °C. Absorbance at 600 nm was recorded daily. Values are the means and standard deviation of three replicates.

italicum B3 strain, some concentrations of PgAFP resulted in up to 50% growth stimulation.

3.3. In vivo antifungal activity of PgAFP

The capability of PgAFP to protect citrus fruit against infection by *P. digitatum* was first assayed. *P. digitatum* Pd1 was chosen because represents the worst scenario since it was the most resistant strain to the

antifungal protein *in vitro* test. In a first assay, Navelina oranges were co-inoculated with *P. digitatum* Pd1 conidia and PgAFP at 50 µg/mL, which is more than 25 fold the calculated *in vitro* IC₅₀ value. This treatment was compared with the co-inoculation of *P. chrysogenum* CECT 20922 at a 100-fold higher concentration than *P. digitatum* conidia (10⁶ vs 10⁴ conidia/mL). None of the treatments resulted in a statistically significant difference with respect to the control inoculation in disease incidence (Fig. 2A) or disease severity (Fig. 2B) by 5 days

Table 2
IC₅₀ and MIC values (µg/ml) of the antimicrobial protein PgAFP against different *Penicillium* spp isolates.

	<i>P. digitatum</i>				<i>P. expansum</i>				<i>P. italicum</i>		<i>P. chrysogenum</i>	
	Pd1	PHI26	Pd01-ZJU	PDC 102	d1	CMP-1	MD-8	T01	NRRL 62431	PHI-1	B3	CECT 20922
IC ₅₀	1.2	1.0	1.7	1.4	7.6	8.2	11.8	9.1	12.7	12.4	39.7	N.I.
MIC	> 75	9.4	> 75	37.5	37.5	> 75	75	> 75	> 75	> 75	> 75	N.I.

N.I. No inhibition at the highest assayed concentration.

post-inoculation (dpi). Then, a second experiment was conducted with Lanelate oranges in which the concentration of PgAFP was increased to 100 µg/mL to see whether this higher dose could have an antifungal effect. This treatment did not alter the development of *P. digitatum* Pd1 within the fruit by 5 dpi. In this second experiment, the co-inoculation with *P. chrysogenum* CECT 20922 resulted in a delay in the development of green mould decay, which was reflected in a lower disease incidence (Fig. 2A), although no difference in the macerated area with respect to control fruits inoculated only with *P. digitatum* was observed (Fig. 2B). A 5 h pre-incubation of PgAFP with *P. digitatum* spores before inoculation of Lanelate oranges did not reduce the incidence nor the

severity of the green mould infection (Fig. 2).

The possible inhibitory activity of PgAFP during blue mould development in apple fruit was analysed using the strain CMP-1, which is moderately resistant to PgAFP. Golden Delicious apples were inoculated with 10⁴ conidia/mL of CMP-1 either alone or in the presence of PgAFP at 50 µg/mL or *P. chrysogenum* CECT 20922 at 10⁶ conidia/mL. By 5 dpi, there were no significant differences among treatments in disease incidence (Fig. 3A) or severity (Fig. 3B).

On the contrary, both purified PgAFP and *P. chrysogenum* CECT 20922 were able to delay blue mould development in Royal Gala apples (Fig. 3). As this variety is more resistant than Golden Delicious, a higher

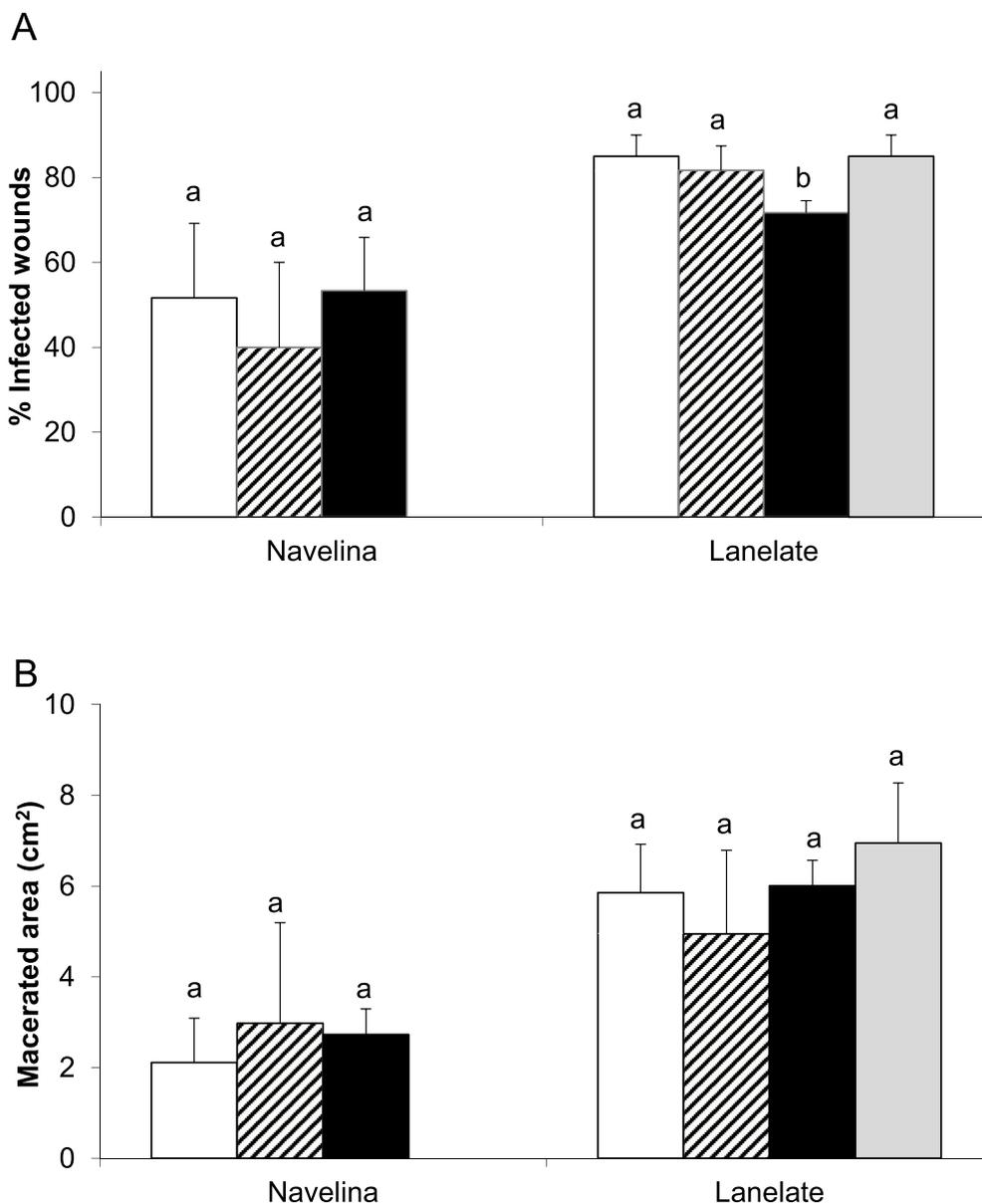


Fig. 2. Effect of PgAFP and *P. chrysogenum* CECT 20922 on the infection of citrus fruits by *P. digitatum* Pd1. Assays were conducted with Navelina and Lanelate oranges. A) Incidence of infection B) Macerated area. Wounds were inoculated with 10 µL of a spore suspension of *P. digitatum* Pd1 at 10⁴ conidia/mL, either alone (white bars) or in the presence of PgAFP (striped bars) at either 50 µg/mL (Navelina) or 100 µg/mL (Lanelate) or *P. chrysogenum* CECT 20922 at 10⁶ conidia/mL (black bars) or PgAFP at 100 µg/mL and pre-incubated for 5 h (grey bars). Bars represent the mean value and SD of the percentage of infected wounds (A) or the area of macerated tissue (B) at 5 five days post-inoculation (dpi) of three replicates of five fruits with four inoculated wounds per fruit. Values with the same letter are not statistically different according to Fisher's least significant difference test ($p < 0.05$).

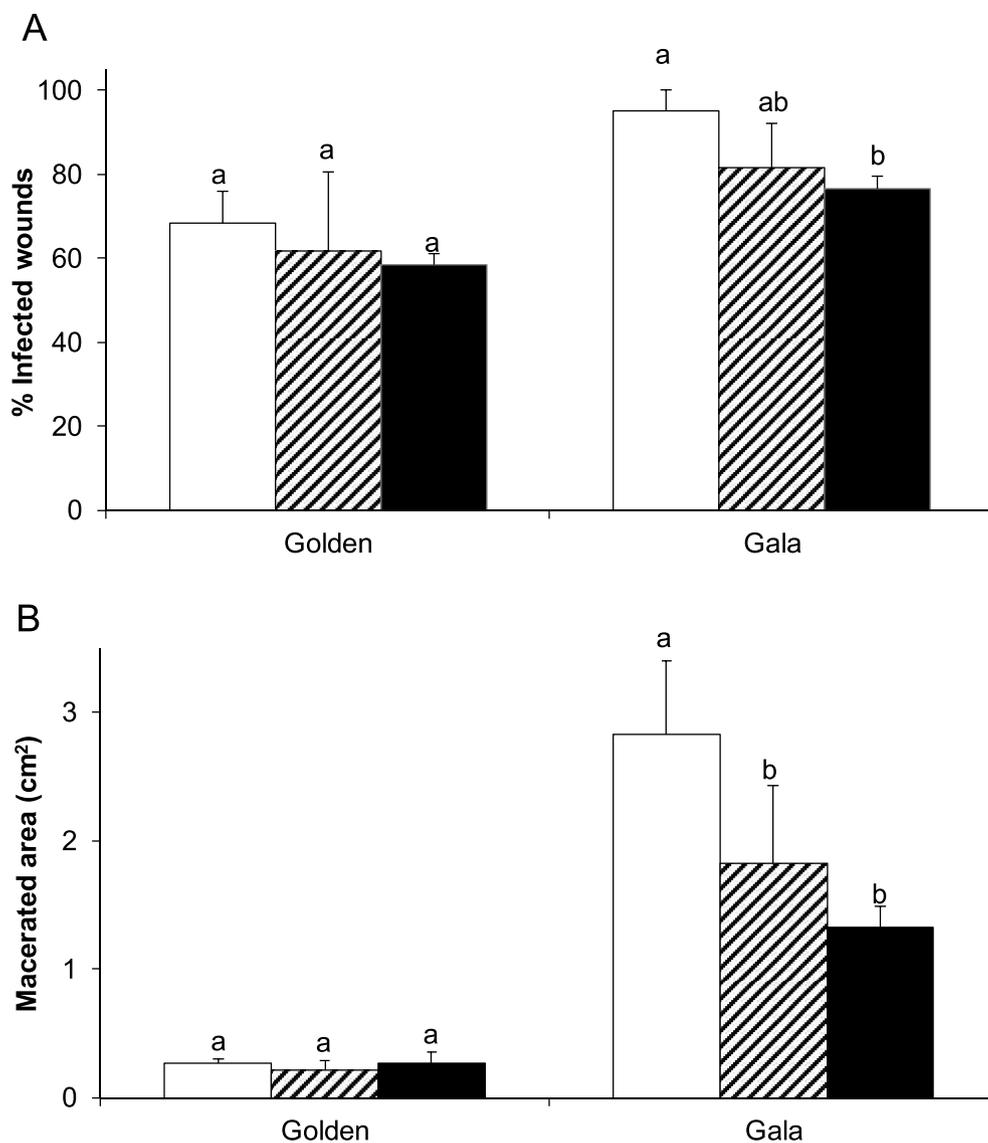


Fig. 3. Effect of PgAFP and *P. chrysogenum* CECT 20922 on the infection of apple fruits by *P. expansum* CMP-1. Assays were conducted with Golden Delicious and Royal Gala apples. A) Incidence of infection B) Macerated area. Wounds were inoculated with 10 μ L of a spore suspension of *P. expansum* CMP-1 at 10^4 or 10^5 conidia/mL in Golden Delicious or Royal Gala, respectively, either alone (white bars) or in the presence of PgAFP at 50 μ g/mL (striped bars) or *P. chrysogenum* CECT 20922 at 10^6 or 10^7 conidia/mL in Golden Delicious or Royal Gala, respectively (black bars). Bars represent the mean value and SD of the percentage of infected wounds (A) or the area of macerated tissue (B) at 5 five days post-inoculation (dpi) of three replicates of five fruits with four inoculated wounds per fruit. Values with the same letter are not statistically different according to Fisher's least significant difference test ($p < 0.05$).

inoculum of *P. expansum* was used (10^5 conidia/mL). The highest effect was observed with *P. chrysogenum* CECT 20922, with a reduction by 5 dpi of 20% in disease incidence and up to 50% in the area of the macerated tissue. It should be noted that in this particular experiment the dose of *P. chrysogenum* was 10^7 conidia/mL, 10 fold higher than in the other experiments, in order to keep a 100-fold excess over the pathogen. PgAFP at 50 μ g/mL also showed some efficacy by 5 dpi. It reduced disease incidence by 14% (Fig. 3A) and disease severity by 35% (Fig. 3B).

3.4. Patulin production

The patulin production by *P. expansum* CMP-1 grown on apple agar with different concentrations of PgAFP was determined by u-HPLC-MS (Fig. 4). Both PgAFP treatments, 10 and 40 μ g/mL, induced a patulin overproduction by *P. expansum* CMP-1 ($p < 0.05$). In the non-treated samples, the patulin concentration was around 280 ppb, whilst in the batch treated with 10 μ g/mL of PgAFP was around 2500 ppb. The patulin concentration rose parallel to PgAFP concentration, reaching 8500 ppb in the samples treated with 40 μ g/mL of PgAFP.

3.5. ROS detection

PgAFP both in PDB (Fig. 5A) and apple broth (Fig. 5B), increased intracellular ROS on *P. expansum* CMP-1 at 48 h of incubation ($p < 0.05$). Both PgAFP concentrations tested equally increased the intracellular ROS in PDB ($p < 0.01$, Fig. 5A). However, in apple broth (Fig. 5B) 10 μ g/mL provoked a lower rise in ROS ($p < 0.05$) than 40 μ g/mL ($p < 0.01$). The parallel inhibition assay showed that at 48 h, both PgAFP concentrations tested (10 and 40 μ g/mL) provoked a remarkable inhibition of *P. expansum* CMP-1 in the two tested media ($p < 0.01$).

Additionally, a significant correlation was found between patulin production and intracellular ROS ($p < 0.05$), being the Spearman's ρ coefficient of 0.75.

4. Discussion

P. chrysogenum CECT 20922 isolated from dry-cured ham produces the antifungal protein PgAFP (Acosta et al., 2009; Rodríguez-Martín et al., 2010) active against toxigenic moulds, thus opening the

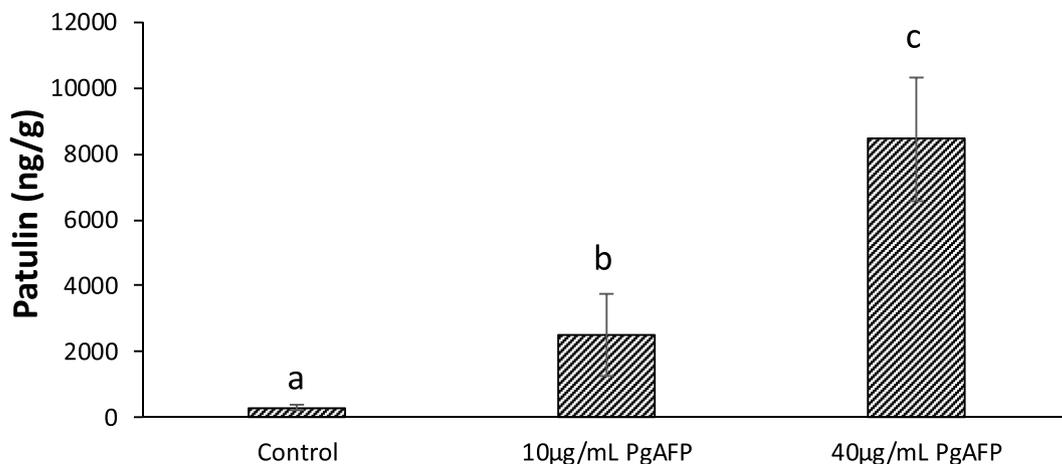


Fig. 4. Patulin production by *P. expansum* CMP-1 strain on apple agar supplemented with two PgAFP concentrations (10 and 40 µg/mL) and a control with no addition of PgAFP, at 25 °C for 15 days. Bars indicate standard deviation of the means. Significant differences between groups are noted with different letters ($p < 0.05$).

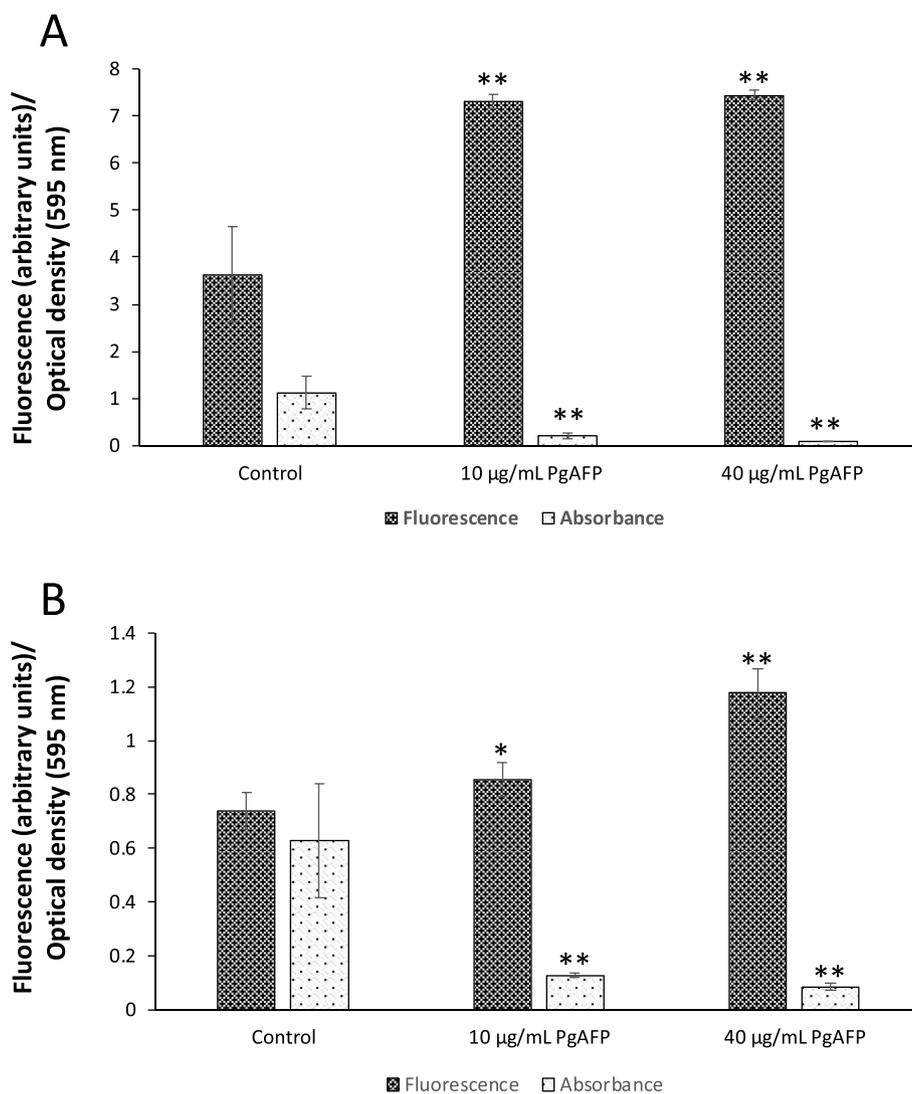


Fig. 5. ROS production measured through fluorescence (striped bars) and mould growth measured through absorbance (dotted bars) from *P. expansum* CMP-1 grown in PDB (A) or apple broth (B). Bars indicate standard deviation of the means. Asterisks mean significative differences with their control (* $p < 0.05$) and ** ($p < 0.01$).

possibility to be used as a biocontrol agent in dry-cured meat products. Several filamentous fungi showed different sensitivity profiles to PgAFP, from highly susceptible species with IC₅₀ values as low as 1.2 µg/mL to non-sensitive species (Delgado et al., 2015a).

In this work, we have extended this previous study to analyze the antifungal activity of PgAFP against eleven different isolates from three *Penicillium* species that are major postharvest pathogens of citrus (*P. digitatum* and *P. italicum*) and pome (*P. expansum*) fruits. Our results show the importance of using different isolates from the same species when analyzing the antifungal activities of different compounds, either of chemical or biological origin.

The five *P. expansum* strains were more resistant to PgAFP than the *P. digitatum* strains. This result agrees with the *P. expansum* strain analysed in a previous study and classified as a low sensitive mould (Delgado et al., 2015a). MIC values were also higher than in *P. digitatum* whilst the two *P. italicum* strains were even more resistant.

There are three major classes of fungal antifungal proteins (AFPs) (Garrigues et al., 2015), although a fourth one has been recently proposed (Tóth et al., 2016). PgAFP belongs to class B, which also contains other proteins from different *Penicillium* species, such as *P. digitatum* AfpB and *P. expansum* PeAfpB. The mature PgAFP protein shows an identity of 88% and 77% with AfpB and PeAfpB, respectively. Taking into account the differences in the protocols followed to conduct the antimicrobial assays, the inhibitory activity of PgAFP against *P. digitatum* PHI26 is similar to those described for AfpB from *P. digitatum* (Garrigues et al., 2017) and PeAfpB from *P. expansum* (Garrigues et al., 2018) against the same strain of *P. digitatum*. Thus, after 72 h of growth in 5% PDB, the MIC value of AfpB and PeAfpB against *P. digitatum* PHI26 were 3.2 µg/mL and 12 µg/mL, respectively, whilst PgAFP showed a MIC value of 4.7 µg/mL at 72 h of growth in full strength PDB medium. It should be noted that the antifungal activity of PgAFP could be underestimated with respect to AfpB and PeAfpB by the use of complete PDB medium rather than 5% PDB, as the antifungal effect is greatly influenced by the culture medium nutrient composition. Both *P. digitatum* AfpB and *P. expansum* PeAfpB showed the same MIC values against *P. digitatum* and *P. expansum* (3.2 µg/mL and 12 µg/mL, respectively). However, PgAFP showed lower activity against *P. expansum*, with MIC values \geq 18.8 µg/mL after 72 of growth.

On the other hand, the antifungal activity of PgAFP against the three *Penicillium* species is much higher than that of *P. chrysogenum* PAF (Garrigues et al., 2017), which showed a MIC value of 50 µg/mL against *P. digitatum* and \geq 200 µg/mL against *P. expansum* and *P. italicum*. The fact that *P. chrysogenum* AFP belongs to class A AFPs does not explain its much lower antifungal activity against these three species, since PeAfpA from *P. expansum* (Garrigues et al., 2018), which also belongs to class A, shows MIC values between 1 and 2 µg/mL against these Penicillia.

In view of the high antifungal activity of PgAFP against *P. digitatum* and *P. expansum*, we conducted fruit inoculation assays to evaluate its potential as postharvest treatment and to compare its activity with that of the producer fungus, *P. chrysogenum* CECT 20922. As differences in antifungal susceptibility among isolates from the same species were observed, the less favorable scenario was chosen by including in the assays the relatively resistant isolates *P. digitatum* Pd1 and *P. expansum* CMP-1 for citrus and apple assays, respectively. For citrus fruit assays, two orange varieties that differ in maturity along the season were used. Navelina is an early harvest variety, while Lanelate is a late harvest variety. For both varieties, no significant reduction in disease incidence of *P. digitatum*, determined as the percentage of infected wounds, or disease severity, measured as the macerated area, was observed with PgAFP treatment at either 50 or 100 µg/mL. The co-inoculation of a 100-fold excess of *P. chrysogenum* CECT 20922 conidia only led to a slight reduction in disease incidence in Lanelate fruits. This lack of *in vivo* antifungal activity of PgAFP was also observed when the protein was co-incubated with conidia for up to 5 h before inoculation. A similar lack of *in vivo* control of *P. digitatum* has been described for *P.*

digitatum AfpB when applied up to 15 µM (Garrigues et al., 2018), which corresponds to 100 µg/mL. In the same study, PeAfpA was shown to have a potent *in vivo* activity against *P. digitatum*, being able to reach a high control of disease incidence even at low concentrations. It would be interesting to analyze the antifungal activity of this protein against *P. digitatum* under conditions more closely resembling those found in real practice, where the standard application of fungicides takes place once the fruits are in the packinghouse and infection may already be incipient. In this context, it is interesting to note the marked effect that the co-incubation of the antifungal protein with conidia before inoculation has on disease development. Thus, the synthetic peptide BP21 applied at 8 µM was able to reduce *P. digitatum* development from 100% to 3% when it was co-incubated with the conidia for 16 h prior to fruit inoculation (Wang et al., 2018). However, when fruit inoculation was done without any pre-incubation step, disease control was much lower, being disease incidence 87%. Similarly, the successful control of blue mold development in Golden apples by the antifungal peptide BP76 was highly dependent on the inoculation protocol (Badosa et al., 2009). A 10-h pre-incubation step of the peptide at 500 µM with *P. expansum* conidia, led to a drastic reduction in lesion diameter, whereas there was no effect on disease development when the mixture of spores and peptide was applied immediately onto the wounds.

The potential of PgAFP against blue mould development in apple fruits was also checked. For this, two different varieties that differ in susceptibility to *P. expansum* infection were used. In Golden Delicious apples, the most susceptible variety, no disease control by applying either PgAFP or *P. chrysogenum* was observed. However, with the less susceptible variety Royal Gala, the application of PgAFP at 50 µg/mL resulted in a significant reduction in disease severity, although the effect on disease incidence was not significant. On the other hand, the application of *P. chrysogenum* CECT 20922 at 10⁷ conidia/mL vs 10⁵ conidia/mL of the pathogen was more effective than the antifungal protein, with a 50% reduction in disease severity. As far as we know, there are no published data on the control of blue mold development on apples by fungal AFPs, so we cannot compare the performance of PgAFP with other AFPs. The antagonistic effect of *P. chrysogenum* CECT 20922 could be attributed to the production of PgAFP (Rodríguez et al., 2015), but the competition for nutrients and space may be considered, as it has been described for *Penicillium frequentans* in the biocontrol of brown rot in stone fruit (Gujjarro et al., 2017).

Although a relatively successful *in vitro* and *in vivo* inhibition by PgAFP was achieved on *P. expansum* CMP-1, an increase of patulin production was observed in parallel to PgAFP concentrations on apple agar. This stimulating effect on mycotoxin production has also been observed for *Aspergillus parasiticus* grown in calcium-free culture medium treated with 10 µg/mL PgAFP, although the addition of calcium caused the opposite effect on the mycotoxin production (Delgado et al., 2018). Additionally, a similar concentration of PgAFP was able to lower the ochratoxin A production by two *Aspergillus carbonarius* strains grown on simulating raisin media (Fodil et al., 2018), and the concentration of three mycotoxins produced by *Alternaria tenuissima* on the wheat matrix (da Cruz Cabral et al., 2019). The effect of antifungals on patulin production has not been investigated in previous works that have evaluated the antifungal effect of several compounds or microorganisms on the growth of *P. expansum* in apples (Calvo et al., 2017, 2019; Cerioni et al., 2013). According to our results, the possible increase in mycotoxin production needs to be taken into account in these biocontrol studies. In fact, it has been highlighted that the efforts in biocontrol have been focused on achieving mould growth inhibition instead of reducing mycotoxin production (Medina et al., 2017).

The very different behaviour found in different systems regarding the induction of mycotoxins should be a consequence both of the variety of species and substrates used in the different studies. This makes necessary the test of the PgAFP activity combining fungal strains of interest as well as substrates where the moulds grow in their usual environment. The use of model media is a first simplified step for this

kind of studies, allowing to discern the implied mechanisms under highly controlled conditions, which could be hidden in natural systems (Crowther et al., 2018). Then, the apple agar is likely the best possible approximation to apple, given that the substrate is very similar and no other microorganisms interfere in the results.

The mechanism of action of antifungal proteins in general and PgAFP, in particular, is multifactorial, involving cell wall integrity disturbance, membrane permeabilization, inhibition of metabolic activity, ROS and necrosis and apoptosis phenomena (Delgado et al., 2016). The ability of PgAFP for provoking intracellular ROS has been previously observed (Delgado et al., 2015b), whilst the ROS and the higher mycotoxin production mediated by β -oxidation has also been reported (Reverberi et al., 2012). This study displays a clear correlation between ROS levels and patulin production, then a relationship between PgAFP concentration, ROS quantity and patulin production can be set. It is interesting to highlight that the same factor, which seems to contribute to the fungal inhibition in the short term, provokes a mycotoxin overproduction if the mould survives this antifungal under the tested conditions, as occurs with the fungistatic effect of PgAFP.

Interestingly, PgAFP caused a dramatic ROS increase in *P. expansum* grown in PDB, whilst this ROS induction was much lower when the fruit pathogen grew in apple broth. This difference might be due to the higher antioxidant content in apple broth (Lu and Yeap Foo, 2000). The antioxidant effect of several compounds diminishes mycotoxin production (Kim et al., 2008; Reverberi et al., 2006, 2005). However, the high antioxidant content seems not to be enough to completely counteract the stimulation effect of PgAFP on mycotoxin production. Therefore, taking together the results from the fruit assays and the mycotoxin production, PgAFP does not seem to be adequate as a bio-protective agent on apple.

5. Conclusions

The analysis of the antifungal activity of *P. chrysogenum* PgAFP against a group of isolates from the three major *Penicillium* species that cause postharvest diseases in citrus and pome fruits revealed that *P. digitatum* is the most susceptible species, followed by *P. expansum* and *P. italicum*. Interestingly, we have found intraspecific differences in susceptibility toward PgAFP, a fact that deserves to be taken into account in future studies aimed at the development of AFPs as alternative control treatments. Besides the good *in vitro* antifungal activity, the *in vivo* fruit assays with oranges showed poor antifungal activity when PgAFP, or the producing fungus. In apple fruits, we observed some control on Royal Gala, but not in Golden Delicious, being better the application of the fungus than the antifungal protein. Nevertheless, the stimulation of patulin production by *P. expansum* in the presence of PgAFPs, probably as a consequence of a ROS stress, points to a new overlooked risk.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design, collection of data, and its interpretation; in the writing of the manuscript; and in the decision to publish the manuscript.

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References

- Acosta, R., Rodríguez-Martín, A., Martín, A., Núñez, F., Asensio, M.A., 2009. Selection of antifungal protein-producing molds from dry-cured meat products. *Int. J. Food Microbiol.* 135, 39–46. <https://doi.org/10.1016/j.ijfoodmicro.2009.07.020>.
- Badosa, E., Ferré, R., Francés, J., Bardají, E., Feliu, L., Planas, M., Montesinos, E., 2009. Sporidial activity of synthetic antifungal undecapeptides and control of *Penicillium* rot of apples. *Appl. Environ. Microbiol.* 75, 5563–5569. <https://doi.org/10.1128/AEM.00711-09>.
- Ballester, A.-R., Marcet-Houben, M., Levin, E., Sela, N., Selma-lázaro, C., Carmona, L., Wisniewski, M., Droby, S., González-candelas, L., Gabaldón, T., 2015. Genome, transcriptome, and functional analyses of *Penicillium expansum* provide new insights into secondary metabolism and pathogenicity. *Mol. Plant Microbe Interact.* 28, 232–248. <https://doi.org/10.1094/MPMI-09-14-0261-FI>.
- Bernaldez, V., Rodríguez, A., Martín, A., Lozano, D., Córdoba, J.J., 2014. Development of a multiplex qPCR method for simultaneous quantification in dry-cured ham of an antifungal-peptide *Penicillium chrysogenum* strain used as protective culture and aflatoxin-producing moulds. *Food Control* 36, 257–265. <https://doi.org/10.1016/j.foodcont.2013.08.020>.
- Calvo, H., Marco, P., Blanco, D., Oria, R., Venturini, E. M., 2017. Potential of a new strain of *Bacillus amyloliquefaciens* BUZ-14 as a biocontrol agent of postharvest fruit diseases. *Food Microbiol.* 63, 101–110. <https://doi.org/10.1016/j.fm.2016.11.004>.
- Calvo, H., Mendiara, I., Arias, E., Blanco, D., Venturini, E. M., 2019. The role of iturin A from *B. amyloliquefaciens* BUZ-14 in the inhibition of the most common postharvest fruit rots. *Food Microbiol.* 82, 62–69. <https://doi.org/10.1016/j.fm.2019.01.010>.
- Cerioni, L., Lazarte A., M., Villegas M., J., Rodríguez-Montelongo, L., Volentini I., S., 2013. Inhibition of *Penicillium expansum* by an oxidative treatment. *Food Microbiol.* 33, 298–301. <https://doi.org/10.1016/j.fm.2012.09.011>.
- Chen, C., Dickman, M.B., 2004. Dominant active Rac and dominant negative Rac revert the dominant active Ras phenotype in *Colletotrichum trifolii* by distinct signalling pathways. *Mol. Microbiol.* 51, 1493–1507. <https://doi.org/10.1111/j.1365-2958.2003.03932.x>.
- Crowther, T.W., Boddy, L., Maynard, D.S., 2018. The use of artificial media in fungal ecology. *Fungal Ecol.* 32, 87–91. <https://doi.org/10.1016/j.funeco.2017.10.007>.
- Da Cruz Cabral, L., Delgado, J., Patriarca, A., Rodríguez, A., 2019. 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. *J. Food Microbiol.* 292, 48–55. <https://doi.org/10.1016/j.ijfoodmicro.2018.12.005>.
- Delgado, J., Acosta, R., Rodríguez-Martín, A., Bermúdez, E., Núñez, F., Asensio, M.A., 2015a. Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products. *Int. J. Food Microbiol.* 205, 23–29. <https://doi.org/10.1016/j.ijfoodmicro.2015.03.029>.
- Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A., Núñez, F., 2016. Manuscript title: antifungal proteins from moulds: analytical tools and potential application to dry-ripened foods. *Appl. Microbiol. Biotechnol.* 100, 6991–7000. <https://doi.org/10.1007/s00253-016-7706-2>.
- Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A., Núñez, F., 2015b. Impact of the antifungal protein PgAFP from *Penicillium chrysogenum* on the protein profile in *Aspergillus flavus*. *Appl. Microbiol. Biotechnol.* 99, 8701–8715. <https://doi.org/10.1007/s00253-015-6731-x>.
- Delgado, J., Owens, R.A., Doyle, S., Núñez, F., Asensio, M.A., 2017. Quantitative proteomics reveals new insights into calcium-mediated resistance mechanisms in *Aspergillus flavus* against the antifungal protein PgAFP in cheese. *Food Microbiol.* 1–10. <https://doi.org/10.1016/j.fm.2017.03.015>.
- Delgado, J., Rodríguez, A., García, A., Núñez, F., Asensio, M., 2018. Inhibitory effect of PgAFP and protective cultures on *Aspergillus parasiticus* growth and aflatoxins production on dry-fermented sausage and cheese. *Microorganisms* 6 <https://doi.org/10.3390/microorganisms6030069>. 69.
- FAO, 2011. *Global Food Losses and Food Waste – Extent, Causes and Prevention*. Rome.
- Fodil, S., Delgado, J., Varvaro, L., Yaseen, T., Rodríguez, A., 2018. Effect of potassium sorbate (E-202) and the antifungal PgAFP protein on *Aspergillus carbonarius* growth and ochratoxin A production in raisin simulating media. *J. Sci. Food Agric.* <https://doi.org/10.1002/jsfa.9128>.
- Garrigues, S., Gandía, M., Castillo, L., Coca, M., Marx, F., Marcos, J.F., Manzanares, P., 2018. Three antifungal proteins from *Penicillium expansum*: different patterns of production and antifungal activity. *Front. Microbiol.* 9, 1–15. <https://doi.org/10.3389/fmicb.2018.02370>.
- Garrigues, S., Gandía, M., Marcos, J.F., 2015. Occurrence and function of fungal antifungal proteins: a case study of the citrus postharvest pathogen *Penicillium digitatum*. *Appl. Microbiol. Biotechnol.* 100, 2243–2256. <https://doi.org/10.1007/s00253-015-7110-3>.
- Garrigues, S., Gandía, M., Popa, C., Borics, A., Marx, F., Coca, M., Marcos, J.F., Manzanares, P., 2017. Efficient production and characterization of the novel and highly active antifungal protein AfpB from *Penicillium digitatum*. *Sci. Rep.* 7, 1–13. <https://doi.org/10.1038/s41598-017-15277-w>.
- Guijarro, B., Hernández-Escribano, L., Larena, I., Melgarejo, P., De Cal, A., 2017. Competition is the mechanism of biocontrol of brown rot in stone fruit by *Penicillium frequentans*. *Biocontrol* 62, 557–566. <https://doi.org/10.1007/s10526-017-9808-x>.
- Jayashree, T., Subramanyan, C., 2000. Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. *Free Radic. Biol. Med.* 29, 981–985. [https://doi.org/10.1016/S0891-5849\(00\)0398-1](https://doi.org/10.1016/S0891-5849(00)0398-1).
- Julca, I., Droby, S., Sela, N., Marcet-Houben, M., Gabaldón, T., 2016. Contrasting genomic diversity in two closely related postharvest pathogens: *Penicillium digitatum* and *Penicillium expansum*. *Genome Biol. Evol.* 8, 218–227. <https://doi.org/10.1093/gbe/evv252>.

- Kaiserer, L., Oberparleiter, C., Weiler-Görz, R., Burgstaller, W., Leiter, E., Marx, F., 2003. Characterization of the *Penicillium chrysogenum* antifungal protein PAF. *Arch. Microbiol.* 180, 204–210. <https://doi.org/10.1007/s00203-003-0578-8>.
- Kim, J.H., Yu, J., Mahoney, N., Chan, K.L., Molyneux, R.J., Varga, J., Bhatnagar, D., Cleveland, T.E., Nierman, W.C., Campbell, B.C., 2008. Elucidation of the functional genomics of antioxidant-based inhibition of aflatoxin biosynthesis. *Int. J. Food Microbiol.* 122, 49–60. <https://doi.org/10.1016/j.ijfoodmicro.2007.11.058>.
- Li, B., Zong, Y., Du, Z., Chen, Y., Zhang, Z., Qin, G., Zhao, W., Tian, S., 2015. Genomic characterization reveals insights into patulin biosynthesis and pathogenicity in *Penicillium* species. *Mol. Plant Microbe Interact.* 28, 635–647. <https://doi.org/10.1094/MPMI-12-14-0398-FI>.
- Lowry, O.H., Rosebrough, N.J., Farr, L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Lu, Y., Yeap Foo, L., 2000. Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food Chem.* 68, 81–85. [https://doi.org/10.1016/S0308-8146\(99\)00167-3](https://doi.org/10.1016/S0308-8146(99)00167-3).
- Marcet-Houben, M., Ballester, A.R., de la Fuente, B., Harries, E., Marcos, J.F., González-Candelas, L., Gabaldón, T., 2012. Genome sequence of the necrotrophic fungus *Penicillium digitatum*, the main postharvest pathogen of citrus. *BMC Genomics* 13, 1–17. <https://doi.org/10.1186/1471-2164-13-646>.
- Marx, F., 2004. Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application. *Appl. Microbiol. Biotechnol.* 65, 133–142. <https://doi.org/10.1007/s00253-004-1600-z>.
- Medina, A., Mohale, S., Samsudin I.P., N., Rodriguez-Sixtos, A., Magan, N., 2017. Biocontrol of mycotoxins: dynamics and mechanisms of action. *Curr. Opin. Food Sci.* 17, 41–48. <https://doi.org/10.1016/j.cofs.2017.09.008>.
- Rana, S., Bhushan, S., 2016. Apple phenolics as nutraceuticals: assessment, analysis and application. *J. Food Sci. Technol.* 53, 1727–1738. <https://doi.org/10.1007/s13197-015-2093-8>.
- Reverberi, M., Fabbri, A.A., Zjalic, S., Ricelli, A., Punelli, F., Fanelli, C., 2005. Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production. *Appl. Microbiol. Biotechnol.* 69, 207–215. <https://doi.org/10.1007/s00253-005-1979-1>.
- Reverberi, M., Punelli, M., Smith, C.A., Zjalic, S., Scarpari, M., Scala, V., Cardinali, G., Aspite, N., Pinzari, F., Payne, G.A., Fabbri, A.A., Fanelli, C., 2012. How peroxisomes affect aflatoxin biosynthesis in *Aspergillus flavus*. *PLoS One* 7, e48097. <https://doi.org/10.1371/journal.pone.0048097>.
- Reverberi, M., Zjalic, S., Ricelli, A., Fabbri, A., Fanelli, C., 2006. Oxidant/antioxidant balance in *Aspergillus parasiticus* affects aflatoxin biosynthesis. *Mycotoxin Res.* 22, 39–47. <https://doi.org/10.1007/BF02954556>.
- Rodríguez-Martín, A., Acosta, R., Liddell, S., Núñez, F., Benito, M.J., Asensio, M.A., 2010. Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. *Peptides* 31, 541–547. <https://doi.org/10.1016/j.peptides.2009.11.002>.
- Rodríguez, A., Bernáldez, V., Rodríguez, M., Andrade, M.J., Núñez, F., Córdoba, J.J., 2015. Effect of selected protective cultures on ochratoxin A accumulation in dry-cured Iberian ham during its ripening process. *LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.)* 60, 923–928. <https://doi.org/10.1016/j.lwt.2014.09.059>.
- Schmidt-Heydt, M., Stoll, D., Schütz, P., Geisen, R., 2014. Oxidative stress induces the biosynthesis of citrinin by *Penicillium verrucosum* at the expense of ochratoxin. *Int. J. Food Microbiol.* 192, 1–6. <https://doi.org/10.1016/j.ijfoodmicro.2014.09.008>.
- Skouri-Gargouri, H., Ali, M. Ben, Gargouri, A., 2009. Molecular cloning, structural analysis and modelling of the AcAFP antifungal peptide from *Aspergillus clavatus*. *Peptides* 30, 1798–1804. <https://doi.org/10.1016/j.peptides.2009.06.034>.
- Sun, X., Ruan, R., Lin, L., Zhu, C., Zhang, T., Wang, M., Li, H., Yu, D., 2013. Genomewide investigation into DNA elements and ABC transporters involved in imazalil resistance in *Penicillium digitatum*. *FEMS Microbiol. Lett.* 348, 11–18. <https://doi.org/10.1111/1574-6968.12235>.
- Tóth, L., Kele, Z., Borics, A., Nagy, L.G., Váradi, G., Virágh, M., Takó, M., Vágvölgyi, C., Galgóczy, L., 2016. NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization. *Amb. Express* 6 <https://doi.org/10.1186/s13568-016-0250-8>. 75.
- Wang, W., Liu, S., Deng, L., Ming, J., Yao, S., Zeng, K., 2018. Control of citrus post-harvest green molds, blue molds, and sour rot by the cecropin a-melittin hybrid peptide BP21. *Front. Microbiol.* 9, 1–9. <https://doi.org/10.3389/fmicb.2018.02455>.
- Yang, Y., Zhao, H., Barrero, R.A., Zhang, B., Sun, G., Wilson, I.W., Xie, F., Walker, K.D., Parks, J.W., Bruce, R., Guo, G., Chen, L., Zhang, Y., Huan, X., Tang, Q., Liu, H., Bellgard, M.I., Qiu, D., Lai, J., Hoffman, A., 2014. Genome sequencing and analysis of the paclitaxel-producing endophytic fungus *Penicillium aurantiogriseum* NRRL 62431. *BMC Genomics* 15, 1–14. <https://doi.org/10.1186/1471-2164-15-69>.