Impact of the antifungal protein PgAFP on the proteome and patulin production of *Penicillium expansum* on apple-based medium.

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Abstract

Apples are prone to be contaminated with *Penicillium expansum*, which produces the mycotoxin patulin, posing a risk for human health. Antifungal treatments are required to control this fungal pathogen, although consumers demand products free of synthetic additives. Then, the use of antifungal proteins produced by moulds represents a novel and promising strategy. Although its inhibitory effect on *P. expansum* has been reported, the impact of these proteins on patulin production has been scarcely studied, pointing to a possible patulin overproduction. The aim of this work was to evaluate the effect of the antifungal protein PgAFP on the proteome and patulin biosynthesis of *P. expansum* grown in apple-based agar, intending to decipher these effects without the apple *in vivo* physiological response to the fungal infection. PgAFP increased the production of patulin on three of the five *P. expansum* strains evaluated. The proteome of the PgAFP-treated *P. expansum* showed five proteins involved in patulin biosynthesis in higher abundance (fold change 2.8-9.8), as well as proteins related to pathogenicity and virulence that suggest lower ability to infect fruits. Additionally, several proteins associated with oxidative stress, such as glutathione peroxidase, redoxin, or heat shock proteins were found in higher abundance, pointing to a response against oxidative stress elicited by PgAFP. These results provide evidence to be cautious in applying this antifungal protein in apples, being of utmost relevance to provide knowledge about the global response of *P. expansum* against an antifungal protein with many shared characteristics with others. These findings significantly contribute to future studies of assessment and suitability of not only these antifungal proteins but also new antifungal compounds.

Keywords: biocontrol; antifungal proteins; proteomics; oxidative stress

1. Introduction

Apples and their based products are produced and consumed worldwide. The total production of apples in 2019 in the world was over 85 million Tm (FAO, 2019), being an important economic source for many geographical areas. The period in which the apples can be consumed greatly depends on the presence of postharvest diseases. In fact, it has been estimated losses of about 25 % in developed countries and up to 50 % in developing countries when postharvest handling and storage conditions are not optimal (Nunes, 2012). One of the main biological hazards that contaminates and spoils these fruits is the mould *Penicillium expansum*, which is able not only to infect them but also to produce toxic secondary metabolites, mycotoxins, such as patulin.

Patulin is a polyketide lactone that provokes various health disorders, such as neurological, gastrointestinal, and immunological adverse effects (Pal et al., 2017), having been considered as possibly genotoxic by the World Health Organization (WHO, 2005). The level of this mycotoxin found in apple or apple-based products has reached up to 328.5 µg/kg in the European Union (Ostry et al., 2018), being much higher than those levels considered as safe by international regulators (European Commission, 2002; FDA, 2005; WHO, 2005). Strategies to counteract *P. expansum* infection include the use of synthetic chemicals (Amiri and Bompeix, 2011; da Rocha Neto et al., 2016). However, the growing trend regarding consumer´s demand for products free of artificial preservatives leads to the use of natural and low environmental impact treatments, such as those based on biocontrol agents, mainly yeasts and bacteria (Agirman and Erten, 2020; Ren et al., 2021). Additionally, the use of antifungal proteins against *P. expansum* in apples has recently been evaluated (Delgado et al., 2019a; Gandía et al., 2021). These are small, cationic and cysteine-rich proteins, with antifungal effects on a variety of mould species (Delgado et al., 2015a; 2016b). Their effects on *P. expansum* virulence in apples are moderately effective, although their impact on patulin production is less documented and

71 the available data point to patulin overproduction (Delgado et al., 2019a). This fact highlights
72 that the inhibitory effect of any antifungal treatment should be evaluated both on the fungal
73 biomass reduction as well as the impact on the secondary metabolite profiles, particularly
74 when this antifungal activity is assessed on very well-known mycotoxin producer moulds.

75 The mould´s response to any antifungal treatment is affected by the interaction of external
76 factors, such as the fruit-pathogen interaction, where the former attempts to impede fungal
77 colonisation (Buron-Moles et al., 2015). In this sense, it is well known that during the
78 interaction between the pathogenic mould and apple, the generation of ROS by infected
79 apples is one of the measures to hamper the fungal burden (Torres et al., 2006). These
80 external factors make it difficult to unveil the isolated effect of the antifungal compound on *P.*
81 *expansum.* Under these circumstances, it is required the use of highly controlled variables that
82 could only be reached in an *in vitro* study, in contrast to natural systems that could hide these
83 effects (Crowther et al., 2018).

84 As far as we are aware, there is only one available study regarding the effect of the antifungal
85 protein PgAFP on patulin production by *P. expansum*, linked to a high ROS generation (Delgado
86 et al., 2019a), although the whole impact of this protein on the mould is not known yet.
87 Proteomic analyses have been proved to be efficient tools for understanding the impact of
88 antifungal compounds on moulds (Delgado et al., 2019b; M. Li et al., 2020; Y. Wang et al.,
89 2021). The proteome changes elicited by PgAFP on *P. expansum*, in parallel to patulin
90 overproduction under highly controlled conditions, would serve to understand the global
91 impact of this antifungal protein on this fruit pathogenic mould.

92 Thus, the aim of this work was to evaluate the impact of PgAFP on the patulin production of
93 five *P. expansum* strains as well as to unveil the proteome changes in one of these strains
94 which overproduced patulin in response to PgAFP.

95 **2. Material and methods**

2.1 Microorganisms and culture conditions

Penicillium allii-sativi CECT 20922 (formerly *Penicillium chrysogenum* RP42C) isolated from dry-cured ham (Acosta et al., 2009) was used as a source of the antifungal protein PgAFP. This protein was assayed upon five *Penicillium expansum* strains (Table 1), which previously showed susceptibility against PgAFP (Delgado et al., 2019a). Each strain was 3-point inoculated on potato dextrose agar (PDA) plates and incubated for 10 days at 25 °C. Then, spores were harvested by addition of 4 mL of phosphate buffer saline (PBS) [0.32 g of NaH_2PO_4 (Scharlab, S. L.), 1.09 g of Na_2HPO_4 (Scharlab, S.L.), 9 g of NaCl (Scharlab, S.L.), 1 L of distilled water], scraping the plate surface with a glass spatula. Conidia concentrations were determined using a Thoma counting chamber Blaubrand® (Brand, Germany), visualising them in a microscope (NIKON, Japan) and adjusted to 10^5 spores/mL.

2.2 PgAFP purification

P. alli-sativi CECT 20922, PgAFP producer, was inoculated 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 statically incubated for 21 days at 25 °C. PgAFP was isolated and concentrated from the cell-free medium by fast protein liquid chromatography (FPLC), 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).

A pooled stock solution of PgAFP was measured by the 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.

2.3 Culture medium, inoculum, and experimental settings

An apple-based culture medium prepared as previously described (Delgado et al., 2019a) was used as a substrate to test the production of patulin by *P. expansum* and to collect mycelia for proteome analyses. Briefly, lyophilized apple powder (30 g/L) and bacteriological agar (20 g/L) were dissolved by stirring and sterilised at 121 °C for 16 min. Once the medium was cooled at 50 °C, three batches were prepared: a) PgAFP was added to reach a final concentration of 40 µg/g, b) the same molar concentration (60 µM) of H_2O_2 (30 % wt, Sigma Aldrich, Madrid, Spain), as a positive pro-oxidant control, and c) an untreated control. Every batch was poured into 55 mm diameter Petri dishes and kept at room temperature for two hours. Once the agar was solidified, a sterile sheet of cellophane was placed on every plate intended to be sampled for proteomic analysis to prevent cross-contamination between the apple matrix and the fungal biomass, as previously described (Delgado et al., 2019b). To assess patulin production, one hundred µL of the conidia stock solution (section 2.1) of the five *P. expansum* strains was spread onto the apple-based agar medium with a glass rod, and plates were incubated for 15 days at 25 °C. Three plates per strain were analysed (n=3) . The strain CMP-1 was selected for proteomic analysis given that it was the strain whose patulin overproduction was higher in response to PgAFP (section 3.1). For that purpose, five independent apple-based medium plates treated with PgAFP and another set of five plates used as non-treated control were overlaid with cellophane membranes and inoculated with the CMP-1 strain. After an incubation period of 10 days at 25 °C, mycelium from each individual plate was collected and stored for proteomic analyses. This sampling date was chosen to be performed before that to patulin quantification since the PgAFP mechanism of action is deployed before mycotoxin overproduction.

2.4 Patulin and mycelia sampling

Samples from the five *P. expansum* strains assayed, subjected to the three treatments: Control, PgAFP and H_2O_2, were taken. Around 1 g of sample (mycelium+culture medium from

145 the edge to the centre of the plate, across one of its diameters) was collected from every plate
146 (n=3 per strain and treatment) with a sterile scalpel and transferred to a 45 mL plastic tube.
147 The samples were kept at - 20 °C until their extraction.

148 Those plates inoculated with *P. expansum* CMP-1 for proteomic analyses, control and PgAFP
149 treatments were sampled at day 10. Around 300 mg of mycelium from every plate surface (n =
150 5 per treatment) were flash-frozen in liquid nitrogen and stored at - 80 °C until proteome
151 extraction.

152 **2.5 Patulin extraction**

153 Ethyl acetate (2 mL) was added to *c.a.* 1 g of mycelium+apple agar where *P. expansum* CMP-1
154 grew (Delgado et al., 2019a). The 45 mL plastic tube was vortexed for 2 min and centrifuged at
155 4192 *g* for 5 min. One mL from the supernatant was transferred to a new tube and evaporated
156 to dryness under a gentle stream of nitrogen. Samples were kept at −20 °C until resuspension
157 to be analysed.

158 **2.6 Patulin quantification**

159 Patulin was analysed as previously described (Delgado et al., 2019a), with some modifications.
160 Briefly, a uHPLC system, Thermo Scientific (United Kingdom) Dionex UltiMate 3000 coupled to
161 a Q exactive Plus (Thermo Scientific, Germany) was used. The column was a Thermo Fisher
162 Accucore Aq C18 reversed-phase column (150 × 2.1 mm) 2.6 µm particle size. The mobile
163 phase consisted of 0.1% formic acid (solvent A) and 0.1% formic acid-acetonitrile (solvent B).
164 The injection volume was 5 µL and the flow rate was set at 0.3 mL/min. The analysis was done
165 in an isocratic gradient of 10% B. MS detection of patulin was performed using the ion
166 155.0266 *m/z*, with a mass tolerance of 5 ppm, in ESI+ ionization mode. The total run time was
167 7 min, being the patulin detected at 1.6 ± 0.2 min. Signals were processed by FreeStyle
168 (Thermo Scientific, Germany).

Standard solutions of patulin (from 0.1 to 100 µg/mL) were used to build a calibration curve by uHPLC-MS/MS. This curve revealed a linear relationship ($R^2 \geq 0.995$) between the detector response and the amount of patulin standard (Sigma Aldrich, Spain).

2.7 Proteomic analysis

The mycelium from *P. expansum* CMP-1 (control and PgAFP treatments, n=5 per treatment) was lysed and partially run in SDS-PAGE and in-gel digested, as previously described (Álvarez et al., 2021). A total of 2 µg from every sample were analysed using a Q-Exactive Plus (Thermo Scientific, Germany) coupled to a Dionex Ultimate 3000 RSLCnano (Thermo Scientific). The gradient used ranged from 8 to 30% B (A: 0.1% formic acid (FA), B: acetonitrile, 0.1% FA) for 4 h on an Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. × 50 cm, nanoViper column (Thermo Scientific), thermostated at 45 °C in the oven compartment. Data were collected using a Top15 method for MS/MS scans (Delgado et al., 2017, 2016b; Dolan et al., 2014). Label-free comparative proteome abundance and data analysis were performed using MaxQuant software (v. 1.6.15.0; https://www.maxquant.org/) (Cox and Mann, 2008), with Perseus (v. 1.6.14.0) applied to organise the data and conduct statistical analysis. Carbamidomethylation of cysteines was set as a fixed modification, while oxidation of methionines and acetylation of *N*-terminals were set as variable modifications. Database searching was performed against a *P. expansum* protein database downloaded from Uniprot (https://www.uniprot.org/). The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The label-free quantitative algorithm (LFQ) was used to generate normalised spectral intensities and infer relative protein abundances (Luber et al., 2010). Proteins that matched with a contaminant database or the reverse database were removed from the list of proteins, as were those proteins only detected in one treatment with only two or fewer replicates. Quantitative analysis was performed using a *t*-test to compare the PgAFP treatment to the control ($p < 0.05$). Qualitative analysis was also

194 performed to detect proteins found in at least three replicates of a particular treatment but
195 undetectable in the compared treatment. For enrichment analysis, the proteins were
196 evaluated through ClueGO v. 2.5.7 (Bindea et al., 2009). To define term-term interrelations and
197 functional groups based on shared genes between the terms, the Kappa score was established
198 at 0.4. Three GO terms and 4% of genes covered were set as the minimum required to be
199 selected. The *p*-value was corrected by Bonferroni step down and set as $p \leq 0.05$.

200 **2.8 Statistical analysis**

201 SPSS software v. 26 (IBM Corporation, USA) was used for patulin data analysis. After testing
202 their normality, these datasets showed a non-normal distribution. Thus, the non-parametric
203 tests Kruskal-Wallis and Mann-Whitney U were performed. The statistical significance was
204 established at $p \leq 0.05$.

205 **3. Results**

206 **3.1 Patulin production**

207 To determine patulin production, five different *P. expansum* strains were grown on intact
208 apple-based agar or the same medium supplemented with either H_2O_2 or PgAFP. The
209 production of this mycotoxin greatly depended on both the strain and the composition of the
210 medium, varying from 0.5 µg/g for MD-8 to 60 µg/g for T01 after 15 days of incubation at 25 °C
211 (Figure 1). Two strains, MD-8 and NRRL6231, produced low amounts of patulin and the
212 presence of H_2O_2 or PgAFP resulted in even lower production. On the contrary, strains d1 and
213 T01 produced above 20 µg/g of patulin on non-supplemented apple-agar and the presence of
214 H_2O_2 increased this production. The level of patulin produced by strains d1 and T01 was even
215 higher in the presence of PgAFP. Strain CMP-1 showed a somewhat intermediate behaviour; in
216 the absence of any stressor, the level of patulin was very low, similar to those produced by
217 strains MD-8 and NRRL6231. It slightly increased when H_2O_2 was added and increased

218 drastically in the presence of PgAFP to circa 3000% with respect to patulin production on
219 apple-agar medium, whereas the rise in strains d1 and T01 was about 200%.

220 **3.2 Proteomic results**

221 To better understand the impact of PgAFP on *P. expansum* biology, the proteome composition
222 of the mycelium of CMP-1 from both untreated and PgAFP-supplemented apple-based media
223 was analysed. This strain was used for proteomic analysis because it is the one that showed
224 the largest induction in patulin production in response to the application of the PgAFP protein.
225 After processing raw data, 1528 unique *P. expansum* proteins were identified (Supplementary
226 Table 1). Among them, 124 increased in abundance and 113 decreased (*t*-test, adjusted
227 $p<0.05$) in presence of PgAFP. The qualitative analysis showed 26 proteins only found in the
228 mycelium of *P. expansum* treated with PgAFP and 1 protein only found in the non-treated
229 control. In subsequent sections, we describe the identified proteins classified into three
230 different groups: patulin, virulence and stress-related proteins.

231 **3.2.1 Patulin related proteins**

232 Six proteins involved in the patulin biosynthesis pathway were detected (Table 2). Not
233 surprisingly, five of them were found significantly higher ($p<0.05$) in *P. expansum* treated with
234 PgAFP, whose fold change was between 2.88 and 9.80. They were PatE, PatF, PatN, PatD and
235 PatB. The remaining detected protein involved in this pathway, PatO, was found in the five
236 PgAFP-treated biological replicates and solely in one out of the five non-treated biological
237 control replicates (Supplementary Table 1). However, the statistical analysis failed to reveal
238 this protein as differentially expressed. Additionally, a patulin biosynthesis-related protein,
239 acetyl-coenzyme A synthetase, was found in higher abundance ($p<0.05$) in the PgAFP-treated
240 *P. expansum* with a fold change of 1.52 (Supplementary Table 1, data showed as Log_2). Finally,

241 a protein related to acetyl-CoA synthesis, acetyltransferase, a component of the pyruvate
242 dehydrogenase complex, was also found in higher relative quantity (p<0.05, fold change 1.67).

243 **3.2.2 Virulence/pathogenicity related proteins**

244 Proteins previously reported as involved in *P. expansum*'s virulence or pathogenicity, and
245 found altered in quantity (p<0.05) after the PgAFP treatment, have been collected in Table 3.
246 Cell wall degrading enzymes (CWDE) such as alpha-amylase and endo-polygalacturonase (Levin
247 et al., 2019) were found in higher relative abundance, whilst a pectin lyase fold/virulence
248 factor was found in reduced in quantity (Bradshaw et al., 2021; Jurick et al., 2020). An endo-
249 chitosanase and a chitinase were found lowered in quantity by the PgAFP treatment. The
250 protein t-SNARE (Bradshaw et al., 2021), was also found reduced by the effect of PgAFP. An
251 amidase protein (Jurick et al., 2020), was only found in the PgAFP treated samples, and the
252 glucose-methanol-choline oxidoreductase showed higher abundance in PgAFP-treated
253 samples. Interestingly, the peptidase S8/S53, subtilisin/kexin/sedolisin, also involved in *P.*
254 *expansum* virulence (Levin et al., 2019), was found among the total proteins identified but it
255 was on the verge of the set statistical threshold (p=0.0508, fold change 0.6) to be considered
256 as discriminant.

257 **3.2.3 Stress-related proteins**

258 Several stress-related proteins were found altered by the addition of PgAFP. The protein
259 thioredoxin was found reduced (p<0.05) by the effect of the antifungal protein, fold change
260 0.65, whilst glutathione peroxidase was increased (p<0.001) by 3.07-fold (Supplementary Table
261 1, data showed as Log_2). The relative quantity of the protein redoxin augmented (p<0.05) with
262 a 1.79-fold change. Other three proteins: lactoylglutathione lyase, glutathione S-
263 transferase/chloride channel, C-terminal and oxidoreductase, N-terminal were found in lower
264 quantity (p<0.05) after the treatment, with a common fold change of *c.a.* 0.77. Also, two heat

shock proteins were increased (p<0.05) by the presence of PgAFP: heat shock protein 70 family and heat shock chaperonin-binding, with a fold change of 1.33 and 1.2, respectively.

3.3 Gene ontology (GO) results

Results from a GO enrichment analysis (p<0.01) of proteins that increased in quantity by the effect of PgAFP, both quantitatively (p<0.05) and qualitatively, are depicted in Figure 2. Slightly more than half of the annotated proteins (52.17%) were included in the Golgi-associated vesicle group and 30.43% of the PgAFP-induced proteins belonged to nucleotide binding. Each of the organonitrogen compound biosynthetic process and carboxylic acid metabolic process groups represented 6.52%. Each of the remaining two groups represented 2.17% of the total enriched proteins of this analysis.

A similar analysis for those proteins found in lower quantity after PgAFP treatment revealed the different groups of proteins found through this GO enrichment analysis (p<0.01) as shown in Figure 3. The cell wall component represented 28.57% of the proteins, and each of the groups pyrimidine-containing compound metabolic process, organophosphate metabolic process, branched-chain amino acid metabolic process, generation of precursor metabolites and energy and magnesium ion binding, were assigned 14.29% of the total of the enriched proteins found in lower quantity.

4. Discussion

Fungal infection of apples by *P. expansum* is not only characterised by fungal burden. A further matter of concern about this pathogen when grown in apples is the production of toxic metabolites, such as patulin. To successfully evaluate the suitability of a given antifungal compound, both fungal infection and mycotoxin production should be addressed, since biocontrol strategies have been mainly focused on hindering mould growth, instead of reducing mycotoxin production (Medina et al., 2017). The overproduction of patulin by three

289 out of the five *P. expansum* strains by the PgAFP antifungal treatment (Figure 1) points to a
290 failure in one of the two aforementioned items to fulfil. The strain CMP-1, as previously
291 reported, was among those increasing the production of patulin in the presence of PgAFP
292 (Delgado et al., 2019a). This mycotoxin overproduction has been attributed to the PgAFP
293 ability to induce ROS in sensitive moulds (Delgado et al., 2019a; Delgado et al., 2015b).
294 However, the same concentration of a pro-oxidant compound, H_2O_2, usually found in apple
295 tissue in response to fungal infection (Torres et al., 2006), did not lead to a similar level of
296 patulin as that reached after PgAFP treatment, being strain CMP-1 the one that showed the
297 most remarkable differences between PgAFP and H_2O_2. Thus, in general, the mechanism that
298 PgAFP triggers on *P. expansum* leading to patulin overproduction seems to be different or
299 stronger than that triggered by H_2O_2. This fact leads to be pessimistic about the potential use
300 of this protein as antifungal treatment in apples, given that exerts an even more noxious effect
301 on the production of patulin than those provoked by the naturally produced levels of H_2O_2 by
302 apples in response to *P. expansum* infection (Buron-Moles et al., 2015).

303 The amounts of patulin found in our study are much higher than the maximum level allowed in
304 drinks in the European Union, which is set to 0.05 µg/g (European Commission, 2006). We
305 found patulin concentrations between 0.5 to 25 µg/g in control samples, and up to 60 µg/g
306 when treated with PgAFP. These levels, although cautiously taken for being an *in vitro* assay,
307 raise awareness about the necessity of biocontrol strategies to counteract this contamination,
308 as well as the risk that the use of these antifungal proteins could entail despite the reported
309 reduction in the fungal burden (Delgado et al., 2019a; Gandía et al., 2021).

310 Proteomics studies on *P. expansum* have contributed to a better comprehension of different
311 biocontrol strategies on this fruit pathogen (Lai et al., 2014; Li et al., 2020). However, to the
312 best of our knowledge, this is the first proteomic study for unveiling the *P. expansum* response
313 upon treatment with an antifungal protein that triggers patulin overproduction.

As expected from the observed mycotoxin overproduction, several proteins involved in the biosynthesis of patulin were found in higher quantities when PgAFP was present in the medium. With regard to the involvement of these proteins in the *P. expansum* virulence, their implication has been contradictory.

The first studies conducted with gene knockout mutants lacking single genes that are required for the biosynthesis of patulin suggested that patulin production was not necessary for infection of apple fruits, as the mutants infected apple fruits as well as the parental strain (Ballester et al., 2015; Li et al., 2015). These results were obtained independently by two groups working with different *P. expansum* strains. However, other authors have shown that although patulin is not required to initiate the infection process, it may act as a cultivar-dependent aggressiveness factor for *P. expansum* (Snini et al., 2016). In this regard, it is worth mentioning that the CMP-1 strain showed reduced pathogenicity towards apple fruits in the presence of PgAFP, although the patulin levels were not assessed in those experiments (Delgado et al., 2019a). A similar result has recently been observed in a study comparing the effect of different antifungal proteins on the infection of apple fruits by the same *P. expansum* strain (Gandia et al., 2021). Our results suggest a lack of correlation between patulin production and *P. expansum* pathogenicity because the treatment with PgAFP triggered the production of patulin but at the same time reduced the infection capability of *P. expansum*. Nonetheless, the simple fact of patulin overproduction, being supported by a high relative quantity of proteins involved in its biosynthesis pathway, would hamper the use of this antifungal protein as a biocontrol agent.

Several CWDEs, considered as determinants of virulence and pathogenic success in the fungal colonisation of apples (Bradshaw et al., 2021), were found altered in quantity by PgAFP. Some of them were induced by PgAFP, whereas others were repressed. Thus, on one hand, alpha-amylase and endo-polygalacturonase were increased, as well as glucose-methanol-choline

oxidoreductase, C-terminal, being the latter described as relevant in the degradation of the apple cell wall during the early infection of apple tissues by this mould (Li et al., 2019). Although decreased, another protein pointing to this direction was an amidase, described in lower abundance in broth from a virulent wild type *P. expansum* (Jurick et al., 2020), which would lead to increased virulence.

On the other hand, the CWDE pectin lyase fold/virulence factor was reduced by the presence of PgAFP, as well as an endo-chitosanase and a chitinase. The protein t-SNARE, whose orthologs have been identified to affect pathogenesis and/or virulence (Dou et al., 2011; Li et al., 2017), were also found reduced by the action of PgAFP. These would be consistent with a lower virulence of *P. expansum* by the effect of PgAFP (Delgado et al., 2019a). Therefore, on average, the reaction of *P. expansum* to PgAFP did not show a clear relationship with either a virulent or an avirulent response, both lowering and increasing proteins linked to virulence or pathogenicity. However, it is worth to mention that this strain showed a lower infection capacity towards apple fruit when treated with PgAFP (Delgado et al., 2019a, Gandia et al., 2021), which suggests that the inhibition by PgAFP of CWDEs involved in pathogenicity has a deeper impact than the induction of other CWDEs.

An exacerbation of ROS as a primary reaction after *P. expansum* treatment with PgAFP (Delgado et al., 2019a) as well as in another sensitive mould, *Aspergillus flavus* (Delgado et al., 2015b) has been reported. Thus, proteins altered in quantity in PgAFP-treated *P. expansum* with biological significance in redox reactions were trawled. Glutathione peroxidase, outstandingly increased in abundance, plays a pivotal role on scavenging peroxides. In fact, it has been reported to be essential for ROS and antifungal tolerance in *Alternaria alternata* (Yang et al., 2016). Based on this reported tolerance function, *P. expansum* would be able to survive to PgAFP and its ROS production by this key protein. A glutathione S-transferase, found in lower quantity in the treated *P. expansum,* has been also reported to be decreased by

PgAFP on another sensitive mould, *A. flavus* (Delgado et al., 2015b). Additionally, two heat shock proteins were found to increase in quantity in response to PgAFP and its ROS induction. These proteins have been linked to a mechanism to neutralize the deleterious effects of ROS, induced by PgAFP, on moulds (Delgado et al., 2015b). Other proteins, such as catalase and superoxide dismutase, have been reported as an antioxidant response to ROS promoted by cinnamaldehyde in *A. flavus* and *P. expansum*, respectively (Sun et al., 2015; Wang et al., 2018). These authors described higher ROS rates in parallel to lower mycotoxin biosynthesis, after the cinnamaldehyde treatment. In our study, both proteins were identified (Supplementary Table 1) but without significant differences. This differential antioxidant response against two antifungal compounds could explain the discrepancies about the mycotoxin production from our results and those previously described.

In addition to the aforementioned changes in the proteome, the global impact of PgAFP on the proteome of this sensitive *P. expansum* was assessed, and these variations were grouped by a GO enrichment analysis (Figures 2, 3). To the best of our knowledge, this is the first evaluation reported so far about the global changes produced by an antifungal protein on *P. expansum*, in parallel to its patulin overproduction. The most prominent group evidenced by ClueGO analysis among the proteins found in higher quantities was the Golgi-associated vesicle (Figure 3). According to their cellular location, PatB, PatD, PatF and PatN are localized in the cytosol and PatE is an extracellular protein located in the cell wall (Li et al., 2019). Thus they do not have an apparent relationship with the Golgi apparatus. However, PatB, PatE and PatO have putative signal peptides in their N-terminal extremities and several putative N-glycosylation sites, and they could enter the endoplasmic reticulum/Golgi secretory pathway (Barad et al., 2016). This relationship would explain the Golgi-associated vesicle proteins findings with some proteins involved in patulin biosynthesis.

Finally, the lower quantity of generation of precursor metabolites and energy proteins, grouped by ClueGO, is likely related to the inhibitory mechanism of PgAFP in the energy metabolism as previously reported in *A. flavus* (Delgado et al., 2015b).

It should be highlighted that all the results have been obtained from a highly controlled *in vitro* assay, without the involvement of a fruit physiological response. Thus, further analyses on diverse apple varieties, as well as different fruit maturation stages, deserve to be carried out to gather the information on the effect of PgAFP on *P. expansum* about ROS production and patulin production.

5. Conclusions

This work has revealed that the antifungal treatment PgAFP, under controlled conditions excluding the fruit physiology, generally induced the overproduction of patulin in *P. expansum*. The proteomic changes on the strain that showed the highest patulin overproduction were related to patulin biosynthesis and glutathione, whilst the impact on CWDEs that may act as virulence factors was variable. Thus, this fact should lead to being cautious to apply this antifungal protein in apples, although further *in vivo* assays should reveal the suitability of this tool. These proteomic analyses also pave the way to better comprehend how this mould is impacted by the isolated effect of PgAFP. Altogether, this information will serve to provide knowledge about the global response of *P. expansum* against an antifungal protein with many shared characteristics with other antifungal proteins, to be used in future studies of assessment and suitability of new antifungal compounds.

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582

583 **Table 1**. Fungal strains used in this study.

Species	Strain	Origin	GenBank acc. No.	Reference
Penicillium allii-sativi	CECT 20922*	Spain	Not sequenced	(Rodríguez-Martín et al., 2010)
Penicillium expansum	CMP-1 (CECT 20906)	Spain	JQFX00000000.1	(Ballester et al., 2015)
Penicillium expansum	MD-8 (CECT 20908)	USA	JQFZ00000000.1	(Ballester et al., 2015)
Penicillium expansum	T01	China	AYHP00000000.1	(Li et al., 2015)
Penicillium expansum	NRRL 62431**	USA	ALJY00000000.1	(Yang et al., 2014)
Penicillium expansum	d1 (CECT 20907)	Israel	JQFY00000000.1	(Ballester et al., 2015)

584 *This strain was previously classified as *Penicillium chrysogenum.*
585 **This strain was previously classified as *Penicillium aurantiogriseum*.
586

587 **Table 2**. Proteins involved in the patulin biosynthesis pathway identified in the *P. expansum*
588 proteome. Fold change referred to changes in protein abundance provoked by 40 µg/g of
589 PgAFP on *P. expansum* grown on apple-based medium with respect to the untreated control.

Protein name	**Protein ID**	***p* value**	**Fold change.**
Patulin synthase (patE)	A0A075TRK9	0.00527017	9.80
Patulin biosynthesis cluster protein (patF)	A0A075TR27	0.0149198	6.57
Isoepoxydon dehydrogenase (patN)	A0A075TRB3	0.0359649	4.00
Alcohol dehydrogenase (patD)	A0A075TMP0	0.0258044	2.88
Carboxylesterase (patB)	A0A075TXZ3	0.0311149	6.84
FAD-linked oxidoreductase (patO)	A0A075TR33	1*	5.53

590 * detected in the five PgAFP-treated biological replicates and solely in one out of the five non-
591 treated biological control replicates

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596 **Table 3**. Proteins involved in virulence/pathogenicity identified in the *P. expansum* proteome.
597 Fold change referred to changes in protein abundance provoked by 40 µg/g of PgAFP on *P.*
598 *expansum* grown on apple-based medium with respect to the untreated control.

Protein group Protein name	**Protein ID**	***p* value**	**Fold change.**
Cell wall degrading enzymes			
Alpha-amylase	A0A0A2KWC5	0.0190424	2.23
Pectin lyase fold/virulence factor	A0A0A2JWF1	0.00813428	0.39
Endo-polygalacturonase	A0A0A2J3W	0.0102587	3.4
Endo-chitosanase	A0A0A2IVN2	0.0266003	0.29
Chitinase	A0A0A2J7H8	0.00173259	0.43
Other virulence/pathogenicity related proteins			
t-SNARE	A0A0A2IAL4	0.0357122	0.54
Amidase	A0A0A2I946	-	Only PgAFP*
Glucose-methanol-choline oxidoreductase	A0A0A2KX84	0.0120758	2.97

599 * only detected in the PgAFP-treated biological replicates and non-detected in any of the non-
600 treated biological control replicates

601

602 **Figure captions**

603 Figure 1. Patulin production by *Penicillium expansum* strains tested on apple-based agar and
604 treated with 60 µM H_2O_2 or 40 µg/g of PgAFP. Asterisks denote significant differences ($p \leq 0.05$)
605 of any treatment with the untreated control.

606 Figure 2. Gene Ontology enrichment analysis of increased proteins in relative abundance in
607 *Penicillium expansum* CMP-1 after being grown for 10 days on apple-based agar and treated
608 with 40 µg/g of PgAFP. Percentage calculated from the number of terms per group. **
609 Statistical differences ($p < 0.01$).

610 Figure 3. Gene Ontology enrichment analysis of decreased proteins in relative abundance in
611 *Penicillium expansum* CMP-1 after being grown for 10 days on apple-based agar and treated
612 with 40 µg/g of PgAFP. Percentage calculated from the number of terms per group. **
613 Statistical differences ($p < 0.01$).

614 Supplementary Table 1. *Penicillium expansum* CMP-1 proteins identified in this study along
615 with fold change (Log_2 Student's T-test Difference, column AE) in red, and significance values
616 (*p*) of each treatment in comparison to the non-treated control in violet. LFQ intensities
617 (columns A-J and X-Z) are expressed as Log_2.

618