1   **Novel insights in the production, activity and protective effect of *Penicillium***

2   ***expansum* antifungal proteins.**

3   Mónica Gandía, Ana Monge, Sandra Garrigues[1], Helena Orozco, Moisés Giner, Jose F.

4   Marcos and Paloma Manzanares*

5   Food Biotechnology Department, Instituto de Agroquímica y Tecnología de Alimentos

6   (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Catedrático Agustín

7   Escardino Benlloch 7, 46980 Paterna, Valencia, Spain.

8   Mónica Gandía and Ana Monge contributed equally to this work

9   [1]Present address: Fungal Physiology, Westerdijk Fungal Biodiversity Institute & Fungal

10   Molecular Physiology, Utrecht University, Uppsalalaan 8, 3584 CT Utrecht, The

11   Netherlands.

12   *Corresponding author: Dr. Paloma Manzanares. IATA-CSIC, Catedrático Agustín

13   Escardino Benlloch, Paterna 46980, Valencia, Spain. e-mail: pmanz@iata.csic.es.

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Abstract

Antifungal proteins (AFPs) offer a great potential as new biofungicides to control deleterious fungi. The phytopathogenic fungus *Penicillium expansum* encodes three phylogenetically distinct AFPs, PeAfpA, PeAfpB and PeAfpC. Here, PeAfpA, a potent *in vitro* self-inhibitory protein, was demonstrated to control the infection caused by *P. expansum* in Golden apple fruits. We determined the production of the three proteins in different growth media. PeAfpA and PeAfpC were simultaneously produced by *P. expansum* in three out of the eight media tested as detected by Western blot, whereas PeAfpB was not detected even in those described for class B AFP production. Regardless of the culture medium, the carbon source affected *Peafp* expression. Notably, the production of PeAfpA was strain-dependent, but analyses of *PeafpA* regulatory sequences in the three strains studied could not explain differences in protein production. None of the PeAFPs was produced during apple infection, suggesting no relevant role in pathogenesis. PeAfpA together with PeAfpB and also with *Penicillium digitatum* PdAfpB showed synergistic interaction. The highly active antifungal PeAfpA also showed moderate antibacterial activity. We conclude that there is not a general pattern for *Peafp* gene expression, protein production or antimicrobial activity and confirm PeAfpA as a promising compound for postharvest conservation.

Keywords: PeAfpA, gene expression, pathogenesis, synergy, antibacterial activity.

1. Introduction

Pathogenic fungi resistant to current antifungal agents and the cross-resistance between clinic and crop pathogens are becoming a major concern [1, 2]. In human health, global mortality from fungal diseases exceeds that of malaria or breast cancer, while in agriculture phytopathogens cause crop yield losses of 20 % with an additional 10 % loss after harvest worldwide [1, 3, 4]. Consequently, the development of new antifungals with different properties from existing ones is of utmost interest.

Antifungal proteins (AFPs) secreted by filamentous fungi have attracted much attention as biofungicides to control deleterious fungi [5, 6]. AFPs are small, cationic, cysteine-rich proteins that are highly stable to pH, high temperatures, and proteolysis, and exhibit broad antifungal spectra and different mechanisms of action against opportunistic human, animal, plant and foodborne pathogenic filamentous fungi [7-9]. Furthermore, fungi have a complex repertoire of AFP and AFP-like sequences, whose experimental characterization might provide new antifungal agents or lead compounds for the next generation of fungicides.

In previous studies, we proposed the classification of fungal AFPs into three phylogenetic classes: A, B and C [10]. Remarkably, some fungal genomes encode more than one AFP from different classes. For instance, the biotechnology-relevant fungus *Penicillium chrysogenum* and the phytopathogenic fungus of pome fruits *Penicillium expansum* encode three phylogenetically different AFPs [10]. The genome of *Neosartorya fischeri* encodes two AFPs from classes A and C, and a new AFP, NFAP2, which seems to be the first member of a fourth class [11]. The first identified AFPs belong to class A, and they were abundantly secreted proteins, such as *Aspergillus giganteus* AFP [12] or *P. chrysogenum* PAF [13]. The first experimentally characterized AFP from class B was Anafp, isolated from the culture supernatant of *Aspergillus niger* [14] while

class C representative is the BP protein, an abundant component in *Penicillium brevicompactum* exudates [15].

Interestingly, some of the *afp* genes in fungal genomes do not result in protein production such as the class B proteins PdAfpB from *Penicillium digitatum* [10] or PAFB from *P. chrysogenum* [16], which remained undetectable in the culture medium although their encoding genes were transcribed at high levels. Only recently, cultivation conditions triggering *pafB* expression and protein secretion into the culture broth of *P. chrysogenum* have been described [17]. For *A. giganteus* AFP, PAF and Anafp, nutrient limitation and non-favorable growth conditions were suggested to be major triggers for their gene expression [9, 18, 19]. By contrast, the expression of *pafB* is strongly induced under nutrient excess during the logarithmic growth phase [17]. Also dependent of culture conditions is the production of *N. fischeri* AFPs, since class A NFAP was isolated from a complex medium [20] while NFAP2 but no NFAP was isolated from 7-day old minimal medium supernatants [11]. These differences in AFP regulation and production have led to the hypothesis that there are diverse functions for these proteins that go beyond their antifungal activity [19, 21].

P. expansum provides an excellent opportunity to address the biological role of its three AFP-encoding genes in the producer fungus and also during pathogenesis. In a previous work, we showed that the three *P. expansum* AFPs, PeAfpA, PeAfpB and PeAfpC, have different patterns of production and antifungal profiles [22]. Similarly to *A. giganteus* AFP and *P. chrysogenum* PAF, PeAfpA production seemed to correlate with nutrient limitation in the parental strain, whereas in such conditions, PeAfpB and PeAfpC remained undetectable [22]. Moreover, the heterologous production of PeAfpB and PeAfpC in a *P. chrysogenum* expression system [23] allowed the side-by-side comparison of PeAFP antifungal activity, which pointed out the high antifungal efficacy against

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human and plant pathogens and mycotoxin-producer fungi of PeAfpA. In this study, we further detail the cultivation conditions under which PeAFPs are produced and secreted to the culture medium and study the expression of the corresponding *Peafp* genes. Also, the relevance of PeAFPs during apple infection is discussed, as well as the effectiveness of PeAFPs to control *P. expansum* infection in apple fruits. Taking advantage of the existence of three *P. expansum* strains whose genomes were sequenced [24], we have shown that AFP production is a strain-dependent trait. Finally, the potential synergism among PeAFPs and their antibacterial activity are presented.

2. Materials and Methods

2.1. Strains, media and growth conditions

P. expansum strains CECT 20906 (CMP-1), CECT 20907 (d1) and CECT 20908 (MD-8) [24] and *P. digitatum* CECT 20796 (PHI26) [25] were used in this study. Fungi were cultured on Potato Dextrose Agar (PDA; Difco-BD Diagnostics, Sparks, MD, USA) plates for 7-10 days at 25 °C. For AFP production in liquid medium, *P. chrysogenum* Minimal Medium (PcMM) [23], Malt Extract Broth (MEB; 2 % malt extract, 0.1 % peptone) and Yeast Peptone Broth (YPB; 1 % yeast extract, 2 % peptone) with either 2 % D-sucrose (PcMMS, MEBS and YPBS) or 2 % D-glucose (PcMMG, MEBG [26] and YPBG) were evaluated. MEBS and MEBG media supplemented with 1 % yeast extract (MEBSY and MEBGY) were also tested. For PeAfpB and PdAfpB production 4 × PcMMS [17] and 4 × *P. digitatum* Minimal Medium (PdMMG) [23], respectively were evaluated. Media were inoculated with a final concentration of 10^6 conidia/mL of either *P. expansum* or *P. digitatum* and were incubated for 5-10 or 11 days, respectively at 25 °C with shaking. For antibacterial assays, bacteria were grown in Luria-Bertani (LB) medium with shaking. *Escherichia coli* JM109, and *Bacillus subtilis* CECT 498 were grown at 37 ºC. *Agrobacterium tumefaciens* AGL-1 was grown at 28 ºC.

2.2. Apple infection assays

Apple fruits (*Malus domestica* cv Golden Delicious and cv Royal Gala) were purchased in a local grocery. Inoculation of *P. expansum* CMP-1 on apple fruits and sample collection were performed as previously described [24]. For testing AFP production during apple infection, three replicates of five fruits were inoculated with 5 µL of conidial suspension (5×10^6 conidia/mL) at twelve wounds (four around the equator, four at the top and four at the bottom). Control mock-inoculations were carried out with 5 µL of sterile water. Additional controls consisted of non-wounded fruits. After inoculation, fruits were maintained at 20 °C and 90 % relative humidity, and tissue discs of 5 mm in diameter around the inoculation site were sampled after 1, 2, 3 and 9 days post inoculation (dpi). Tissue samples were crushed, frozen in liquid nitrogen, and stored at −80 °C for further protein extraction. Proteins were extracted in a ratio 1:2 (w/v) of ground apple tissue to the protein extraction buffer previously described [27], and were separated on 16 % denaturing polyacrylamide gel (SDS-PAGE) and Coomassie blue stained.

For apple protection assays, three replicates of five apple fruits were inoculated at four wounds around the equator with 5 µL of conidial suspensions (10^4 conidia/mL) that were pre-incubated for 24 h with 100 µg/mL concentration of PeAfpA, PeAfpB or PdAfpB. Apple fruits were stored at 20 °C and 90 % relative humidity. Each wound was scored daily for infection symptoms on consecutive dpi.

2.3. AFP purification and Western blot analyses

PdAfpB and the three PeAFPs were produced and purified as previously described [22, 28]. Total proteins from supernatants and apple tissues, and purified AFPs were separated by SDS-16 % polyacrylamide gels and transferred to Amersham Protran 0.20 µm NC nitrocellulose transfer membrane (GE Healthcare Life Sciences, Little Chalfont,

135 United Kingdom). Protein detection was accomplished using anti-PeAfpA, anti-PeAfpB

136 and anti-PeAfpC antibodies [22] diluted 1:2,500 for anti-PeAfpA and anti-PeAfpC and

137 1:1,500 for anti-PeAfpB. For PdAfpB detection anti-PAFB antibody diluted 1:1,000 was

138 used [29]. As secondary antibody, 1:20,000 dilution of ECL NA934 horseradish

139 peroxidase donkey anti-rabbit (GE Healthcare) was used and chemiluminescent detection

140 was performed with ECL^{TM} Select Western blotting detection reagent (GE Healthcare)

141 using a LAS-1000 instrument (Fujifilm, Tokyo, Japan). The experiments were repeated

142 at least twice.

143 *2.4. Total RNA extraction and quantitative RT-PCR*

144 Total RNA from mycelium of *P. expansum* CMP-1 was obtained using TRI

145 Reagent® (Sigma-Aldrich, St Louis, MO, USA) following manufacturer's instructions.

146 Total RNA was used to synthesize first strand cDNA, and quantitative RT-PCR was

147 performed and analysed as previously described [30].The gene-specific primers used for

148 qRT-PCR are shown in Supplementary Table 1. Three independent housekeeping genes

149 coding for *P. digitatum* β-tubulin, ribosomal protein L18a and 18S rRNA were used

150 simultaneously for normalization of the absolute gene expression analyses between

151 conditions.

152 *2.5. Antimicrobial assays*

153 Antibacterial assays were performed in 96-well, round-bottom microtiter plates

154 (Nunc, Roskilde, Denmark) as previously described [22]. Briefly, 50 µL of bacterial cells

155 (5×10^5 cfu/mL) in 1/5 diluted LB were mixed with 50 µL of twofold concentrated

156 proteins from serial twofold dilutions. Plates were statically incubated for 48 h at 28 °C.

157 Growth was determined every 24 h by measuring the optical density (OD) at 600 nm

158 using Spectrostar Nano microplate spectrophotometer (BMG labtech, Orlenberg,

159 Germany), and the OD$_{600}$ mean and standard deviation (SD) between three replicates were

160 calculated. These experiments were repeated at least twice. Minimum Inhibitory

161 Concentration (MIC) is defined as the protein concentration that completely inhibited

162 growth in all the experiments performed.

163   For synergy assays, different combinations and concentrations of AFPs were

164 tested in 96-well flat-bottom microtiter plates. Twenty-five µL of two different 4×

165 concentrated AFPs were mixed with 50 µL of *P. expansum* CMP-1 or *P. digitatum*

166 conidia (5×10^4 conidia/mL in 1/10 diluted potato dextrose broth (PDB) containing 0.02

167 % (w/v) chloramphenicol) (total volume 100 µL). Plates were statically incubated for 96

168 h at 25 °C. Data are expressed as OD_{600} mean ± SD of three replicates. Concentrations

169 for each AFP were: 0-8 µg/mL for PeAfpA, 0-16 µg/mL for PeAfpB, 0-32 µg/mL for

170 PeAfpC, and 0-16 µg/mL for PdAfpB.

171 *2.6. In silico analyses of PeafpA regulatory sequences.*

172   Promoter and terminator sequences from the three *PeafpA* genes from *P.*

173 *expansum* CMP-1 (gene ID: PEX1-077760), d1 (PEXP_059300(TO)) and MD-8

174 (PEX2_042150) were downloaded from the National Center for Biotechnology

175 Information server (http://www.ncbi.nlm. nih.gov). Multiple sequence alignments were

176 performed with Clustal Omega [31] (https://www.ebi.ac.uk/Tools/msa/clustalo/). The

177 presence of binding sites for the negative regulator mediating carbon catabolism

178 repression CREA ([G/C][C/T]GG[AG]G) [32], and for the pH-response transcription

179 factor PacC (GCCA[AG]G) [33] was searched using RSAT Fungi online tool [34]

180 (http://rsat-tagc.univ-mrs.fr/rsat/dna-pattern_form.cgi).

181 *2.7. Statistical analyses*

182   Statistical analyses were performed using IBM SPSS Statistics v.26 and Sigma

183 Plot v.14. One way ANOVA and Tukey's HSD test were carried out for infection assays

184 ($p < 0.05$). Student's t-test was achieved for antibacterial assays ($p<0.05$). Two-way

185 ANOVA analysis and Tukey's HSD test were performed to determine AFP interactions

186 ($p<0.05$).

187 **3. Results**

188 *3.1. PeAfpA protection against P. expansum infection in apple fruits depends on apple*

189 *fruit variety.*

190 Previous *in vitro* antimicrobial results showed that PeAfpA and PeAfpB were self-

191 inhibitory proteins, whereas PeAfpC showed no effect [22]. We conducted laboratory

192 fruit inoculation experiments to assess the effectiveness of both PeAFPs to control the

193 postharvest decay caused by *P. expansum* CMP-1 on apple fruits. *P. digitatum* PdAfpB,

194 which has been previously described as a highly active AFP *in vitro* [29], was also

195 included. Fig. 1A-C shows the effects of 100 μg/mL AFPs in apple fruits from Golden

196 Delicious variety. At 4 dpi, infection was observed in non-treated apples (control) and in

197 those treated with PeAfpB and PdAfpB, whereas infection was not observed in PeAfpA-

198 treated apples (Fig. 1A). Moreover, PeAfpA showed control of infection throughout the

199 experiment ($p<0.01$). Although PdAfpB and PeAfpB did not control the infection from

200 the beginning of the experiment, both proteins showed protective effect from 5 and 6 dpi,

201 respectively. The average efficacy at 7 dpi was 87 % disease reduction for PeAfpA and

202 27 % for PeAfpB and PdAfpB. Fig. 1B shows representative images of AFP-treated

203 apples at 7 dpi. Finally, to confirm the control of *P. expansum* infection by PeAfpA, three

204 more independent assays were accomplished. In all of them, PeAfpA exerted significant

205 protective effect, although the efficacy after 7 dpi was lower than that observed in

206 experiment 1, and varied between 42 and 52 % disease reduction (Fig. 1C).

207 Next, the effect of apple fruit variety on PeAfpA protection was assessed (Fig.

208 1D-E). PeAfpA showed no significant control of *P. expansum* growth in Royal Gala

209 apples in any of the two independent infection experiments, suggesting that *in vivo*

210 antifungal effect of PeAfpA against *P. expansum* is dependent of apple fruit variety in the

211 conditions tested.

3.2. None of the PeAFPs is produced in apple infected tissues.

213 In order to determine if any of the PeAFPs are produced during fruit infection,

214 *P. expansum* CMP-1 was grown on apple fruits, and total proteins from fungus-infected

215 apple tissue were analysed by SDS-PAGE (Fig. 2A, top panel). To identify the putative

216 PeAFPs produced, protein detection was accomplished by Western blot (Fig. 2A, bottom

217 panels) using the polyclonal anti-PeAfpA, anti-PeAfpB and anti-PeAfpC [22]. No

218 immunoreaction signals were observed in any of the time-course protein extracts,

219 indicating that *P. expansum* does not produce any of the PeAFPs under the conditions

220 tested.

3.3. Medium composition affects PeAFP production.

222 To further study the effect of medium composition on PeAFP production, *P.*

223 *expansum* CMP-1 was grown in nutritionally rich media (MEB and YPB) and minimal

224 media (PcMM) with either glucose (MEBG, YPBG and PcMMG) or sucrose (MEBS,

225 YPBS and PcMMS) as carbon sources. Additionally, MEB supplemented with yeast

226 extract (MEBY) was also included to test if residual fungal components could induce

227 gene expression and accumulation of PeAFPs in cultures. Five-day culture supernatants

228 were analysed by SDS-PAGE (Fig. 2B, top panel). The largest amount of low molecular

229 mass proteins was detected in both YPB media and in PcMMS, from which protein bands

230 of apparent molecular mass similar to those of pure PeAFPs were observed. PeAFP

231 detection was accomplished by Western blot (Fig. 2B, bottom panels) using the

232 polyclonal anti-PeAFPs. PeAfpA- and PeAfpC-specific signals were immunodetected in

233 several of the supernatants evaluated. Three of the eight supernatants tested, PcMMS,

234 YPBS and YPBG, reacted with the anti-PeAfpA antibody. PeAfpC-specific signals were

also observed in these three supernatants in addition to PcMMG, MEBYG and MEBYS. None of the three PeAFPs was produced after 5 days of growth in MEBG or MEBS. Since no immunoreaction with the anti-PeAfpB antibody was observed in any of the supernatants tested, we evaluated the production of PeAfpB and PdAfpB under culture conditions that were recently reported to trigger PAFB production in *P. chrysogenum* [17]. For this, *P. digitatum* and *P. expansum* CMP-1 were grown in either 4 × PdMMG or 4 × PcMMS and culture supernatants were analysed by SDS-PAGE and Western blot. Neither PdAfpB nor PeAfpB specific signals could be observed indicating that PeAfpB and PdAfpB production is not triggered by nutrient excess in the conditions tested (data not shown).

Thereafter, expression analyses of *Peafp* genes in the different growth media were assessed at the same time point as protein production (Fig. 3). The highest level of expression corresponded to *PeafpA* in YPB, regardless of the carbon source, followed by expression in PcMMS, in accordance to PeAfpA production. Overall, *PeafpC* gene expression was approximately tenfold lower than that reached by *PeafpA* gene. Similarly to that described for *PeafpA*, the highest level of *PeafpC* expression was detected in YPBS, followed by PcMMS and YPBG. Subtle expression levels were recorded for *PeafpC* gene in MEB, slightly higher with yeast extract regardless of carbon source, as well as for *PeafpB* in all media tested. Our results show that *Peafp* gene expression is affected by the carbon source in the growth media, since gene expression in the presence of glucose was always lower than that found in the presence of sucrose.

3.4. PeAFP production is strain-dependent.

Similar to *P. expansum* CMP-1 genome, the genomes of *P. expansum* strains d1 and MD-8 harbour three genes that encode three AFPs which are orthologous to PeAfpA, PeAfpB and PeAfpC [24]. Growth of the different strains on PDA plates showed that

260 MD-8 strain exhibits differences in colony morphology and sporulation compared with

261 CMP-1 and d1, as previously described (Fig. 4A) [24]. Comparison of the PeAFPs

262 primary sequences revealed that PeAfpA and PeAfpC exhibited 100 % identity within the

263 three strains, whereas CMP-1 PeAfpB differed in one amino acid residue at position seven

264 in the pre-sequence (a threonine to alanine substitution) from d1 and MD-8 PeAfpBs

265 (Supplementary Fig. 1). To determine whether PeAFP production is a strain-dependent

266 trait, SDS-PAGE analysis (Fig. 4B, top panel) followed by Western blot of *P. expansum*

267 supernatants from PcMMS was accomplished (Fig. 4B, bottom panels). The Coomassie

268 blue staining of proteins in the corresponding siupernatants showed a distinctive pattern

269 in the case of MD-8. As expected, CMP-1 supernatants reacted with the anti-PeAfpA and

270 anti-PeAfpC antibodies, and the same was observed with supernatants from d1 strain.

271 Remarkably, in MD-8 supernatants only immunoreaction against PeAfpC was observed

272 although at much lower intensity than the other two strains, while no PeAfpA could be

273 detected indicating that PeAfpA production is strain-dependent under the conditions

274 tested. PeAfpB was not produced by any of the three strains.

275 *3.5. In silico analyses of PeafpA regulatory sequences.*

276   Since our results suggest that PeAfpA production is strain-dependent, the *PeAfpA*

277 promoters and terminators from CMP-1, d1 and MD-8 were analysed *in silico*. Our results

278 showed that *PeafpA* terminator sequences from the PeAfpA-producer strain CMP-1 and

279 the non-producer MD-8 shared 100 % identity, whereas terminator sequence from strain

280 d1, which also produces PeAfpA, was more divergent (Supplementary Fig. 2). These

281 results indicate that terminator sequences are not responsible for the disparities found in

282 protein production pattern among these strains. Promoter sequences also show huge

283 sequence conservation between the strains. However, several differences are noticeable

284 (Supplementary Fig. 3). *PeafpA* promoter from MD-8 strain shows a two nucleotides

285   deletion at positions 134 and 135, a nucleotide insertion at position 923, and two

286   nucleotide changes at positions 446 and 491 that are not present in the PeAfpA-producer

287   strains CMP-1 and d1. Remarkably, CMP-1 shows a big deletion between positions 440-

288   448, but this deletion does not correlate with PeAfpA production since it is absent in d1

289   and MD-8 strains. Further analyses of the promoter sequences were performed. We

290   searched for DNA binding sites for several regulatory proteins that have been reported to

291   play a role in the regulation of the expression of *afp* genes. Contrary to *paf* and *afp*

292   promoters [35], no PacC binding sites were found in any of the three *PeafpA* promoters

293   under study. However, several CREA binding sites were found, in correlation with what

294   was reported for *paf* promoter. *PeafpA* promoters from CMP-1 and d1 have five CREA

295   binding sites, while MD-8 has four (Supplementary Fig. 3). These results indicate that

296   carbon catabolite repression (CCR) may not be the main cause underlying the absence of

297   PeAfpA production in MD-8 strain.

298   *3.6. Synergistic interactions among PeAFPs*

299   The fact that *P. expansum* encodes one AFP of each phylogenetic class led us to

300   hypothesize that PeAFPs might act synergistically. To address this possibility, *P.*

301   *expansum* CMP-1 was assessed for susceptibility to the combination of PeAfpA with

302   either PeAfpB or PeAfpC. Subinhibitory concentrations of PeAfpA (0, 0.25, 0.5 and 1

303   µg/mL) were tested together with PeAfpB. Fig. 5A (top panel) shows that PeAfpB caused

304   a concentration-dependent inhibition of fungal growth in the presence of subinhibitory

305   concentrations of PeAfpA (notice the lack of effect of the PeAfpA subinhibitory

306   concentrations in the dose-response curve of PeAfpA in the middle panel). Moreover,

307   growth inhibition of *P. expansum* by PeAfpB occurred at lower concentration as the

308   concentration of PeAfpA increased. A clear PeAfpA concentration-dependent decrease

309   in the MIC value of PeAfpB was observed, and the two-way ANOVA analysis indicated

the existence of statistically significant interaction between both PeAFPs (Supplementary Table 2). With respect to the combination PeAfpA and PeAfpC, and since PeAfpC does not have any effect against *P. expansum*, the synergy experiment was designed with non-inhibitory concentrations of PeAfpC (0, 0.5, 4 and 32 μg/mL). As shown in Fig. 5A middle panel, the dose-response effect of PeAfpA was not modified by the presence of PeAfpC suggesting that both proteins do not act synergistically in the conditions tested (Supplementary Table 2). With the aim of evaluating the potential synergism of another class B AFP with PeAfpA, PdAfpB was included in the study. The interaction between both proteins towards *P. expansum* can be seen in Fig. 5A bottom panel. Likewise for PeAfpB, PdAfpB and PeAfpA showed statistically significant positive interaction towards *P. expansum* growth when combined, as suggested by the decrease in the MIC value of PdAfpB (Supplementary Table 2).

Given the synergy existing between PeAfpA and the two class B AFPs tested, further antifungal assays were conducted to determine whether the proteins could act synergistically towards *P. digitatum*, highly sensitive to PeAfpA [22]. For *P. digitatum*, the results indicated statistically significant synergistic interaction with both AfpBs, whereas no apparent synergy was observed between PeAfpA and PeAfpC (Fig. 5B and Supplementary Table 3).

3.7. PeAfpA showed moderate antibacterial activity

PeAFPs were tested for their antimicrobial activity towards the Gram negative bacteria *E. coli* and *A. tumefaciens*, and the Gram positive *B. subtilis*. Differences in antibacterial activity were observed among the three PeAFPs (Fig. 6). PeAfpA inhibited to some extent the growth of *E. coli* and *A. tumefaciens,* and it showed a MIC value of 64 μg/mL against *B. subtilis*. PeAfpB and PeAfpC were inactive against *E. coli* and

B. subtilis, and showed a very faint inhibition of *A. tumefaciens* growth at the highest concentrations evaluated.

4. Discussion

In the present study, we describe new features of the three AFPs from the phytopathogenic fungus of pome fruits *P. expansum,* including cultivation conditions triggering expression and secretion into the culture broth, the effect of the fungal strain in PeAFP production and their potential synergism.

We had showed previously that PeAfpA exerts protection against *P. digitatum* in oranges and *Botrytis cinerea* in tomato leaves at concentrations as low as 1-10 µg/mL [22]. Here we have evaluated the pathosystem *P. expansum*-apple fruit and found that PeAfpA was effective in controlling the blue mold rot caused by *P. expansum* infection to Golden Delicious but not to Royal Gala apples, pointing out to a variety-dependent *in vivo* efficacy. The effect of *P. chrysogenum* class B PgAFP on pome fruit infection by the same *P. expansum* strain as that used in this study was also described as variety-dependent [36]. However, and contrarily to our results, PgAFP showed a slight reduction of disease incidence in Royal Gala but not in Golden Delicious apples. In our infection experiments, PeAfpB and PdAfpB with 76 and 88 % amino acid identity with PgAFP, respectively, showed a modest disease reduction in Golden Delicious apples although their effectiveness in Royal Gala apples was not tested. The inhibition effect of *P. expansum* infection in Golden apple fruits by the rationally designed antifungal undecapeptides BP22 and BP76 was also reported and, as described here, variations in the percentage of disease reduction among experiments were observed [37]. It should be mentioned the difficulties to compare inter-laboratories *in vivo* experiments mainly due to different protocols of AFP application and fungal inoculation. Interestingly, when a commercial formulation of the fungicide imazalil is applied in similar *in vivo* experiments

359   to those described here, the average efficacy of disease reduction is not significantly

360   different to that provoked by PeAfpA and rationally-designed peptides [37]. Additional

361   studies are required to confirm the relevance of the AFP-fruit variety binomial.

362   Until recently, AFPs were assumed to be non-active towards their producer fungi.

363   However, the characterization of PdAfpB [29], PAFB [16] and PeAfpA [22] identified

364   those proteins as potent *in vitro* self-inhibitory AFPs. Here we have shown that PeAfpA

365   is also active against *P. expansum in vivo*, although to a lesser extent that *in vitro*.

366   Notably, the effectiveness of PeAfpA to control *P. expansum* infection in apples was

367   observed at a concentration of 100 μg/mL, much higher than that needed to control *P.*

368   *digitatum* in oranges (1-10 μg/mL) despite the similar *in vitro* MIC values [22, 29].

369   Previously we described that PdAfpB, with a MIC of 4 μg/mL against its producer fungus,

370   did not show *in vivo* effect against *P. digitatum* in oranges [22, 29]. Recently, *P.*

371   *chrysogenum* PAF, but not the rationally designed variant PAF[opt], was proven to inhibit

372   *B. cinerea* infection in tomato plant leaves, despite the fact that both proteins inhibited

373   *B. cinerea* growth *in vitro* [38]. Taken together, these results suggest that antifungal

374   activity observed in *in vitro* assays does not always correlate with the *in vivo* efficacy and

375   emphasize the need for *in vivo* protection assays as those described here.

376   The biological role of *afp* genes in filamentous fungi is not completely understood,

377   but still more intriguing is the function of those genes in phytopathogenic fungi. Previous

378   results indicated that *PdafpB* gene is dispensable for the pathogenicity and virulence of

379   *P. digitatum* [10]. Regarding *P. expansum*, putative virulence factors identified by means

380   of a transcriptomic analysis of apple fruits during the course of fungal infection did not

381   include any antifungal protein [24]. Here our results also suggest that none of the three

382   PeAFPs would have a key function during fruit infection. Other phytopathogenic fungi

383   that encode in their genomes putative AFPs are *Penicillium italicum*, *B. cinerea*; and

several *Fusarium* species [10, 39]. However, most of these putative AFPs as well as their function in the producer fungus have not been in-depth characterized. One exception is the *F. graminearum* FgAFP, which seems to be specific of fungi that compete for maize colonization and infection *in vivo* [40]. Fungal competition has also been suggested as the key function for the recently described AFP from the entomopathogenic fungus *Beuveria bassiana* (BbAFP1) [41]. Remarkably, and in accordance with our results, BbAFP1 was not produced during infection of target insect hosts, confirming than AFPs seem not involved in pathogenesis.

The knowledge of culture conditions that trigger *afp* gene expression and protein production is essential to unravel the biological role(s) of these proteins and improve protein yields which will allow further AFP applications. Our previous results suggested that PeAfpA production might be linked to nutrient limitation as described for PAF and *A. giganteus* AFP, and that glucose might suppress production [22]. In this study we examined the effect of the carbon source on PeAFP production when the fungus grows in PcMM but also in two nutritious complex media containing peptone and either malt (MEBG and MEBS) or yeast extract (YPBG and YPBS) in their composition, which have been previously used for AFP production [26, 42]. Our results show that *Peafp* expression is affected by the carbon source in the growth media, since gene expression in the presence of glucose was always lower than that found in the presence of sucrose, independently of the media used (see Fig. 3). However, glucose did not suppress PeAFP production since PeAfpA and PeAfpC were detected in supernatants of several growth media regardless the carbon source used. The exception is PcMMG where PeAfpA was not detected in the culture broth. The highest expression levels of the *PeafpA* gene corresponded to the three media where the protein was immunodetected (MMS, YPBS and YPBG). In the other media evaluated where PeAfpA remained under the limit of

409   detection, *PeafpA* gene expression dropped drastically. Although *PeafpC* expression was

410   always lower than that detected for *PeafpA*, expression levels were enough to make the

411   protein detectable in six out of the eight media evaluated. Contrarily to results described

412   here, in our previous study PeAfpC was neither immunodetected in supernatants of

413   PcMMS nor identified by peptide mass fingerprinting from an in-gel digestion of the

414   putative PeAFP bands [22]. This discrepancy might be explained by the fact that *PeafpC*

415   expression levels might be close to the threshold value necessary to detect the protein in

416   the supernatant, and that it might be necessary a sustained transcription over time to reach

417   detectable PeAfpC yields in the culture broth, as described for PAFB [17]. Additionally,

418   intrinsic variability found among fungal growth morphologies in submerged cultures, as

419   those observed in *P. expansum* growths, could also explain the discrepancies in PeAfpC

420   detection. Fungal growth can result in dispersed hyphae, compact pellets or intermediates

421   of these growth types, which strongly affects gene expression and resulting product titers

422   [43]. Thus, growth morphology variability might cause *PeafpC* gene expression to fall

423   below its detection threshold. Remarkably, the addition of yeast extract to MEB triggered

424   PeAfpC production. Yeast extract is a complex hydrolysate produced by yeast autolysis

425   with high content of amino acids, peptides, vitamins, growth factors, trace elements and

426   energy sources such as carbohydrates, which makes it difficult to know which

427   compound(s) could be responsible for the observed effect. In any case, yeast extract is

428   not essential for PeAfpC production, since the protein was also immunodetected in

429   PcMM. None of the three PeAFPs was detected in MEB media, despite the fact that

430   MEBG was successfully used to produce class B PgAFP in large quantities (up to 600

431   μg/mL) [26, 44]. Furthermore, a very similar medium to MEBG was employed for the

432   purification of the *A. giganteus* AFP [42]. In contrast to our results, glucose enhanced the

433   production of AcAFP secreted by *Aspergillus clavatus*, improving the AcAFP production

434   yields reached with sucrose [45].

435   Production of both PeAfpA and PeAfpC was observed in three out of the eight

436   cultures broth tested. By contrast, PeAfpB and PdAfpB remained undetectable despite

437   the use of growth media with nutrient excess as described for PAFB production [17].

438   PAFB amounts in the supernatants correlated with increasing nutrient availability

439   reaching the highest amount in $4 \times$ PcMMS, where also PAF production was detected

440   [17]. Thus, our results suggest that PeAfpB and PdAfpB would not have the same

441   biological role in *P. expansum* and *P. digitatum* as that of PAFB in *P. chrysogenum*.

442   Additional studies are required to elucidate the conditions triggering the production of the

443   two class B proteins PeAfpB and PdAfpB and the co-expression of the three PeAFPs.

444   Notably, our results show that the production of PeAfpA but not of PeAfpC is

445   strain-dependent. PeAfpA was not immunodetected in the PcMMS supernatants of MD-

446   8 strain. This strain exhibits differences in sporulation compared with CMP-1 and d1

447   when grown on PDA (see Fig. 4A) [24]. Remarkably, deficiencies in sporulation were

448   reported for the *P. chrysogenum* null mutant Δ*paf* [46], a strain that does not produce the

449   PAF protein, which belongs to the same phylogenetic class as PeAfpA. However, no

450   differences in sporulation were observed for the *A. niger* Δ*Anafp* [19] and *P. digitatum*

451   Δ*afpB* mutants [10], which do not produce the class B proteins Anafp and PdAfpB. To

452   the best of our knowledge this is the first time that AFP production is shown to be strain-

453   dependent in a side-by-side study of different fungal strains. Whether this dependency is

454   also influenced by the growth medium requires further research.

455   Contrarily to *afp* [18] and *paf* [35] promoter sequences, *PeafpA* promoters do not

456   contain any PacC binding sites, indicating that alkaline pH conditions would not affect

457   *PeafpA* gene induction. However, similar to *paf* promoter [13], *PeafpA* promoter from all

458   three strains do contain putative CREA binding sites suggesting glucose repression, as

459   observed in our study. Due to a single nucleotide change, *PeafpA* promoter in MD-8

460   strain, which does not produce PeAfpA, contains four putative CREA binding sites,

461   whereas CMP-1 and d1 strains contain five. However, the presence of many putative

462   CREA binding sites in promoter sequences does not guarantee CCR, since two different

463   and adjacent binding sites are required for CCR *in vivo* [32]. This would explain why *afp*

464   gene from *A. giganteus* was not subject to CCR despite the presence of one putative

465   CREA binding site in its promoter [18], and in this context, we could assume that only

466   the two first CREA binding sites which are present in the *PeafpA* promoters of the three

467   *P. expansum* strains seem to be functional *in vivo* (Supplementary Fig. 3).

468   Many species produce mixtures of antimicrobial peptides with known synergistic

469   interactions [47-50], and thus the existence of three different PeAFPs allowed us to

470   speculate that they might act synergistically. Here we have shown that PeAfpA acts

471   synergistically with PeAfpB against *P. expansum* and *P. digitatum*. Interestingly, synergy

472   was also detected between PeAfpA and the other class B representative tested, PdAfpB

473   from *P. digitatum.* These results point to a potential synergy not only between AFPs from

474   the same fungus but also between AFPs from different fungal species. To the best of our

475   knowledge, this is the first report about synergism between AFPs. Whether the synergistic

476   activity of PeAfpA combined either with PeAfpB or PdAfpB might reflect synergy

477   between AFPs from different phylogenetic classes requires further research. In a previous

478   study we showed that two PdAfpB-derived antifungal peptides called PAF112 and

479   PAF118 showed positive synergistic interaction when combined against *P. digitatum*

480   [51]. Moreover, the synthetic antifungal hexapeptide PAF26 [52] also displayed

481   synergistic interaction with both PdAfpB-derived peptides, as well as with the *P.*

482   *chrysogenum* PAF [51]. Now our results show the feasibility of combining two AFPs to

improve their efficacy. The close-related antifungal plant defensins act synergistically with established antifungal drugs against *Candida albicans* [53, 54]. Also, synergy between the plant defensin NaD1 and the antifungal serine protease inhibitor BPT1 against the phytopathogens *F. graminearum* and *Colletotrichum graminicola* was reported [55]. The study also identified synergy between NaD1 and a group of peptides that do not individually affect fungal growth *in vitro*. This does not seem to be the case of the so far inactive PeAfpC, for which combination with PeAfpA against *P. expansum* and *P. digitatum* did not exhibit any positive interaction, suggesting that the role of PeAfpC might not be the enhancement of PeAfpA activity in the conditions tested.

Finally, we have described the antibacterial activity of PeAfpA, adding a new function to this highly active protein against economically important filamentous fungi and clinically relevant yeasts [22]. To date, only one AFP, AcAMP from *A. clavatus*, has been reported to show antimicrobial activity against Gram positive and Gram negative bacteria, including the pathogenic *Staphylococcus aureus* [56]. PeAfpA antibacterial activity against non-laboratory strains and/or pathogenic isolates deserves future research.

5. Conclusion.

In summary, this study provides additional knowledge about the three PeAFPs for which a common pattern of production, gene expression and antimicrobial activity cannot be concluded. The protection observed here upon application of PeAfpA on apple fruits reinforces the potential use of PeAfpA for postharvest protection. Future efforts are currently directed to clarify the biological role(s) of the three PeAFPs.

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686

687   **Captions to illustrations**

688   **Fig. 1. Effect of different antifungal proteins on the infection of apple fruits by**

689   ***Penicillium expansum.*** (A-C) Effect of PeAfpA, PeAfpB and PdAfpB on the infection

690   of apple fruits cv Golden Delicious. (A) Incidence of infection of inoculated wounds.

691   Apple fruits were inoculated with 10^4 conida/mL of *P. expansum* either alone (Control)

692   or in the presence of 100 µg/mL of AFPs. Bars show the mean values of the percentage

693   of infected wounds and SD of three replicates of five apples at 4, 5, 6, and 7 days post-

694   inoculation (dpi). Asterisks show statistical significance of the infection incidence

695   compared to the control samples at each independent day (One way ANOVA and Tukey's

696   HSD test, $*p<0.05$, $**p<0.01$). (B) Representative images of treated apples with AFPs at

697   7 dpi. (C) Mean values of the percentage of infected wounds at 7 dpi from four

698   independent infection experiments (Student´s t-test, $*p<0.05$, $**p<0.01$). Experiment 1

699   corresponds to that showed in (A). (D-E) Effect of PeAfpA on the infection of apple fruits

700   cv Royal Gala. (D) Incidence of infection of inoculated wounds in two independent

701   experiments. Apples were inoculated with 10^4 conida/mL of *P. expansum* either alone

702   (Control) or in the presence of 100 µg/mL of PeAfpA. Bars show the mean values of the

703   percentage of infected wounds and SD of three replicates of five apples at 4, 5, 6, and 7

704   dpi. (E) Representative images of PeAfpA-treated apples at 7 dpi.

Fig. 2. Western blot analyses of apple infected tissue and growth supernatants. (A) SDS-PAGE (top) and Western blot analyses (bottom) of proteins extracted from non-infected (control), wounded apples (wound) and *P. expansum* infected apples at 1, 2, 3 and 9 dpi. (B) SDS-PAGE (top) and Western blot analyses (bottom) of fungal supernatants after 5 days of growth in different media: PcMM with sucrose (S) or glucose (G); MEB medium with S, with S and yeast extract (SY), with G or with G and yeast extract (GY) and yeast peptone medium (YPB) with S or with G. Two µg of pure PeAFPs were added as controls. M: SeeBlue R® Pre-stained protein standard. Western blot analyses were performed using the three specific PeAFPs antibodies.

Fig. 3. Absolute expression of *P. expansum afp* genes in different growth media. Graph shows absolute expression at 5 days in growth media detailed in Fig. 2. Bars show the mean ± standard error (SE) of three replicates.

Fig. 4. Colony morphology and Western blot analyses of growth supernatants of *P. expansum* strains. (A) Growth on PDA plates of *P. expansum* CMP-1, d1 and MD-8 strains. (B) SDS-PAGE (top) and Western blot analyses (bottom) of CMP-1, d1 and MD-8 strains supernatants after 9 days of growth in PcMMS. Two µg of pure PeAfpA, PeAfpB and PeAfpC were added as controls. M: SeeBlue R® Pre-stained protein standard. Western blot analyses were performed using the three specific PeAFPs antibodies.

Fig. 5. Interactions between AFPs against *P. expansum* (A) and *P. digitatum* (B) *in vitro* growth. Synergy between PeAfpA and PeAfpB (top panel), PeAfpA and PeAfpC (middle panel) and PeAfpA and PdAfpB (bottom panel). Data show the mean ± SD of three replicates.

Fig. 6. Inhibition of bacterial growth by PeAFPs. Growth inhibition of *E. coli* (A), *B. subtilis* (B) and *A. tumefaciens* (C) in the presence of increasing concentrations of PeAFPs (0, 8, 32 and 64 µg/mL). Data are shown after 24 h of incubation. Bars show the mean

value ± SD of the percentage of growth as compared to the 100 % control defined as growth in the absence of PeAFP (0) at 24 h. Asterisks denote statistically significant differences in comparison to the 100 % control (Student´s t-test; *$p<0.05$; **$p<0.01$).

Highlights

- The three *P. expansum* antifungal proteins (PeAFPs) have been studied more in depth
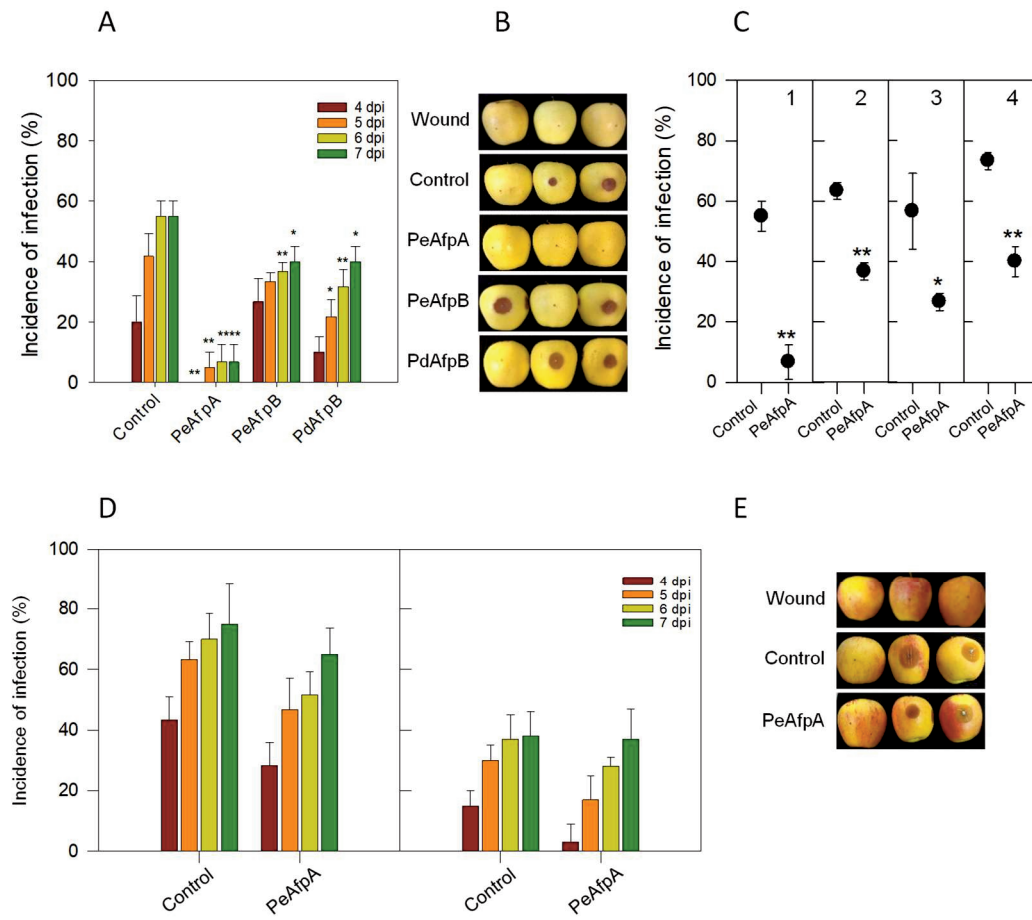- PeAFPs have no common patterns of gene expression, protein production or activity
- Synergistic interaction between AFPs has been demonstrated for the first time
- PeAfpA controls the infection caused by *P. expansum* in apple fruits
- PeAfpA is a promising alternative to control postharvest diseases

Figure 1 (Gandía et al., 2020)
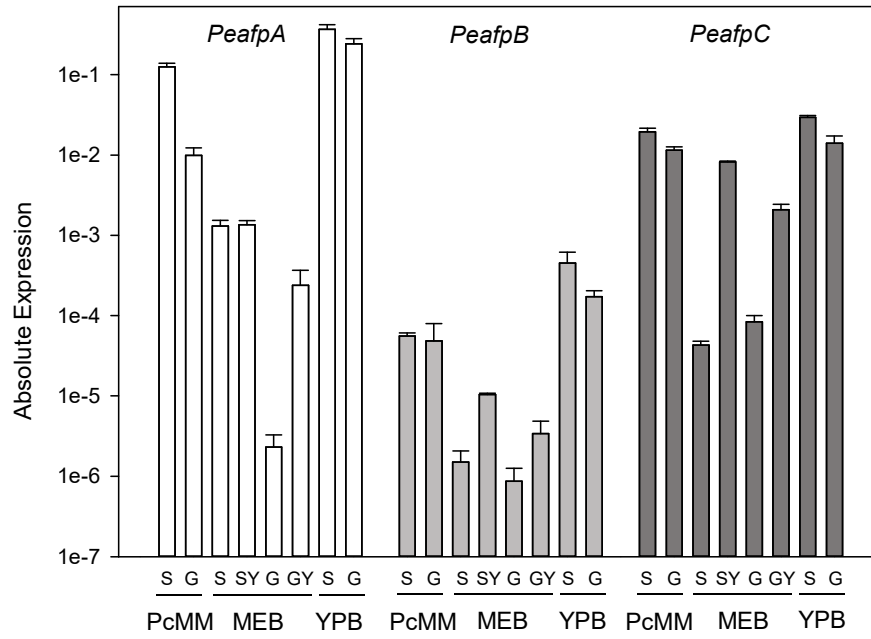
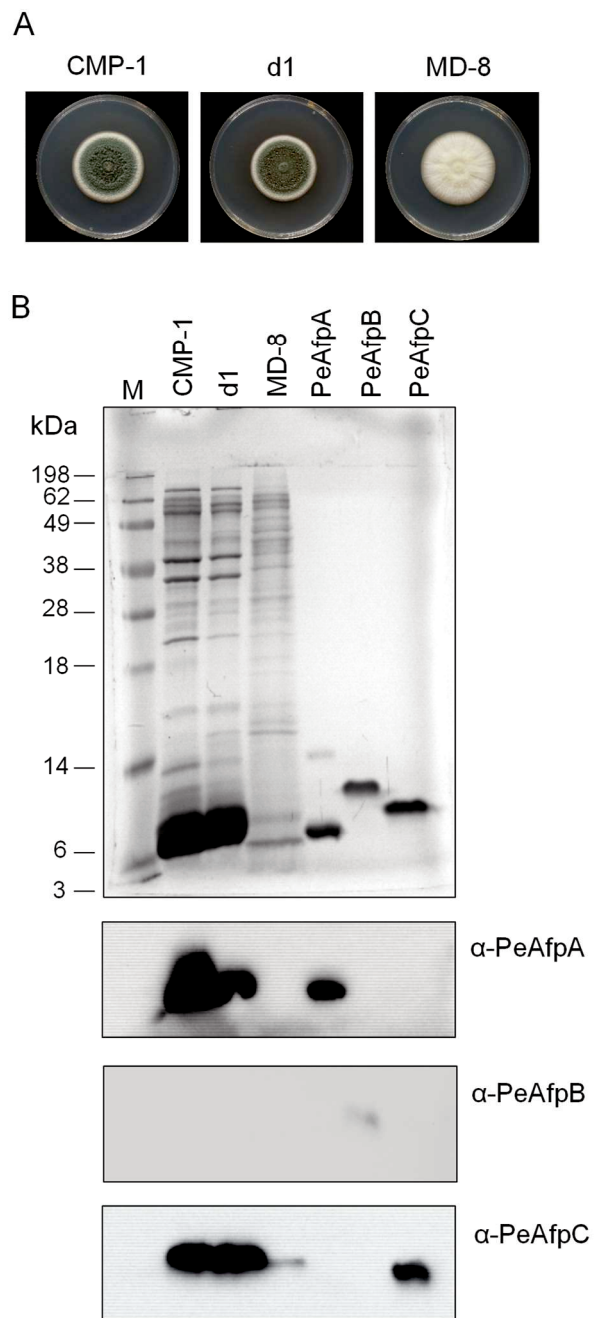Figure 2 (Gandía et al., 2020)
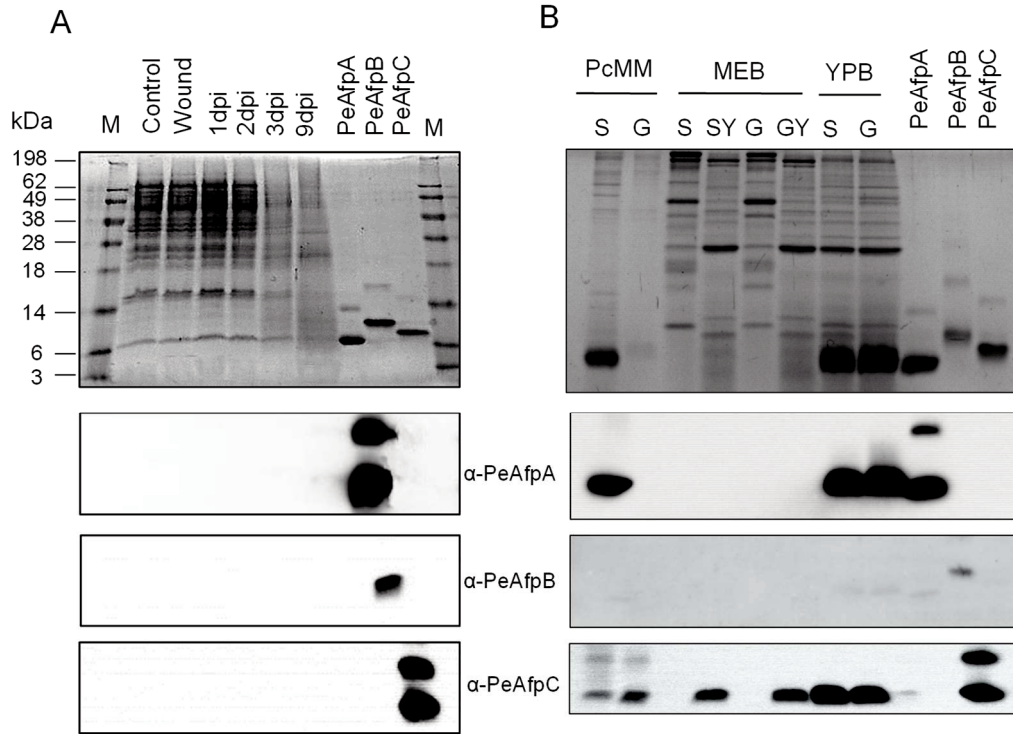
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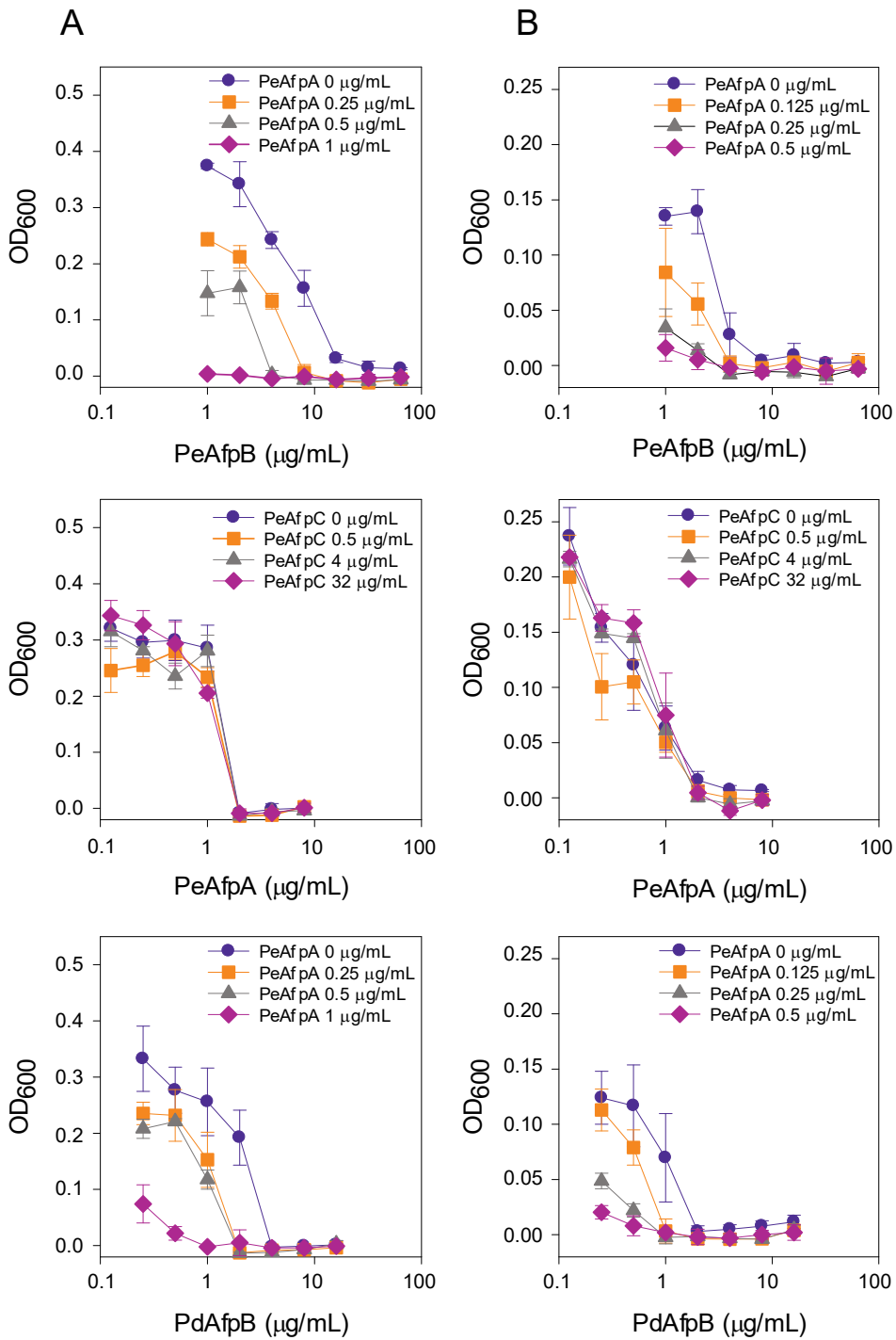
Figure 3 (Gandía et al., 2020)

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Figure 4 (Gandía et al., 2020)

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Figure 5 (Gandia et al., 2020)

A

B

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Figure 6 (Gandía et al., 2020)

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