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Manuscript title: Increased chitin biosynthesis contributes to the resistance of *Penicillium polonicum* against the antifungal protein PgAFP.

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ABSTRACT

Antifungal proteins from molds have been proposed as a valuable tool against unwanted molds, but the resistance of some fungi limits their use. Resistance to antimicrobial peptides has been suggested to be due to lack of interaction with the mold or to a successful response. The antifungal protein PgAFP produced by *Penicillium chrysogenum* inhibits the growth of various ascomycetes, but not *Penicillium polonicum*. To study the basis for resistance to this antifungal protein, localization of PgAFP and metabolic, structural, and morphological changes were investigated in *P. polonicum*. PgAFP bound the outer layer of *P. polonicum* but not regenerated chitin, suggesting an interaction with specific molecules. Comparative two-dimensional gel electrophoresis (2D-PAGE) and comparative quantitative proteomics revealed changes in the relative abundance of several proteins from ribosome, spliceosome, metabolic, and biosynthesis of secondary metabolite pathways. The proteome changes and an altered permeability reveal an active reaction of *P. polonicum* to PgAFP. The successful response of the resistant mold seems to be based on the higher abundance of protein Rho GTPase Rho1 that would lead to the increased chitin deposition via cell wall integrity (CWI) signaling pathway. Thus, combined treatment with chitinases could provide a complementary means to combat resistance to antifungal proteins.

KEYWORDS

Antifungal proteins, proteomics, resistance, *Penicillium polonicum*, chitin, cell-wall integrity pathway

INTRODUCTION

The antifungal protein PgAFP produced by the strain *Penicillium chrysogenum* CECT 20922 (formerly RP42C) is within a group of small, highly basic and low molecular mass proteins (Rodríguez-Martín et al. 2010). PgAFP inhibits various pathogenic and spoilage ascomycetes of interest in foods, including strains of various *Aspergillus* spp., such as *A. carbonarius, A. flavus, A. ochraceus, A. fumigatus,* and *A. tubingensis*, as well as *Penicillium* spp., such as *P. commune, P. restrictum, P. nalgiovense,* and *P. chrysogenum* (Delgado et al. 2015a). However, *Penicillium polonicum* and the PgAFP-producer strain of *P. chrysogenum* were not inhibited by PgAFP.

Other antifungal proteins produced by ascomycetes are PAF from *P. chrysogenum* Q176 (Marx et al. 1995), Pc-Arctin from *P. chrysogenum* A096 (Chen et al. 2013), BP from *Penicillium brevicompactum* (Seibold et al. 2011) AFP and AFP$_{NN5353}$ from *Aspergillus giganteus* (Nakaya et al. 1990; Binder et al. 2011), Anafp from *Aspergillus niger* (Gun Lee et al. 1999), AcAFP and AcAMP from *Aspergillus clavatus* (Skouri-Gargouri and Gargouri 2008; Hajji et al. 2010), FPAP from *Fusarium polyphialidicum* (Galgóczy et al. 2013b) and NFAP from *Neosartorya fischeri* (Kovács et al. 2011). Mechanisms of action of antifungal proteins from molds have been described as multifactorial, where membrane permeabilization, changes in actin distribution, chitin biosynthesis inhibition, destabilization of cell wall, and oxidative stress lead to apoptosis (Leiter et al. 2005; Moreno et al. 2006; Hagen et al. 2007; Binder et al. 2010; Virágh et al. 2015; Delgado et al. 2015b). AFP binds chitin, inhibits chitin biosynthesis, permeabilizes the cell membrane, and penetrates into the cell and binds nucleic acids (Liu et al. 2002;

Moreno et al. 2006; Hagen et al. 2007) and AcAFP also binds chitin altering cell wall (Skouri-Gargouri et al. 2009), whereas PAF, NFAP, and PgAFP lead to apoptosis mediated by G-protein signaling (Binder et al. 2010; Binder et al. 2015; Virágh et al. 2015; Delgado et al. 2015b). Both PAF and NFAP activate the cAMP/Protein kinase A pathway via G-protein signaling (Leiter et al. 2005; Virágh et al. 2015) and PgAFP provoked a lower amount G-protein subunit β CpcB (Delgado et al. 2015b).

However, the defensive strategies of resistant molds are poorly described. The lack of electrostatic affinity or receptors in cell surfaces has been suggested as the cause of the resistance to antimicrobial peptides (Yeaman and Yount 2003). PgAFP does not bind to the producer strain *P. chrysogenum* CECT 20922 that withstands at least 312 μg/ml (Delgado et al. 2015a, b). The lack of interaction between antifungal proteins and mold surface results in the absence of major metabolic responses in the resistant fungi. Another successful strategy of resistant fungi to counteract AFP is chitin synthesis stimulation (Ouedraogo et al. 2011). The latter strategy implies interaction with the resistant fungus and active metabolic response to the antifungal protein. Thus, studying the mechanisms involved in the resistance requires in depth investigation of the metabolic response of resistant fungi to antifungal proteins.

Comparative proteomic analysis is a powerful tool to study metabolic changes at the molecular level (Kim et al. 2007). 2D-PAGE has the ability to separate complete proteins including those with post-translational modifications, but only a small percentage of the whole proteome is revealed (Görg et al. 2009). On the other hand, comparative quantitative proteomics is able to identify proteins not detectable by 2D-PAGE. These two techniques have been used to complementarily evaluate the effect of PgAFP on the proteins involved in signaling pathways and selecting adequate tests to study the metabolic response in molds (Delgado et al. 2015b). In addition, localization of the antifungal protein in non-sensitive molds can give valuable information on the possible interaction at the surface or inside the cell. Given that antifungal proteins provoke oxidative stress leading to apoptosis in sensitive mold, knowing the extent of these two phenomena in resistant molds would contribute to clarify the defence mechanism.

To study the effect of PgAFP on resistant molds, *P. polonicum* was chosen because it was the only resistant ascomycete known, apart from the PgAFP-producer *P. crhysogenum* CECT 20922 (Delgado et al. 2015a). *A. niger* has been used as sensitive control with various antifungal proteins (Kaiserer et al. 2003; Hagen et al. 2007; Kovács et al. 2011). *A. tubingensis* CECT 20932, formerly *A. niger* An261, has been used in the present work as sensitive control because it is the closest species to *A. niger* known to be sensitive to PgAFP (Delgado et al. 2015a).

The aim of this work was to investigate the effect of PgAFP on the proteome profile and selected characteristics to disclose the resistance response of *P. polonicum*. Localization of PgAFP was studied for a better understanding of the interaction with *P. polonicum*. This knowledge would allow designing new strategies to maximize the inhibition effect and spectra of PgAFP in molds.

MATERIAL AND METHODS

Strains

In vitro tests were carried out with three molds isolated from dry-cured ham available from the Spanish Type Culture Collection (CECT, Valencia, Spain): *P. chrysogenum* CECT 20922, *P. polonicum* CECT 20933 and *A. tubingensis* CECT 20932.

Purification of PgAFP

PgAFP was obtained from *P. chrysogenum* CECT 20922 grown in potato dextrose broth (PDB, Scharlab, Barcelona, Spain) pH 4.5, at 25 °C for 21 days, as described previously (Acosta et al. 2009). To get cell-free medium, mycelium was removed by filtering through Miracloth (Calbiochem, Darmstadt, Germany) and the culture medium was filtered through a nitrocellulose 0.22 µm-pore-size (Sartorius, Goettingen, Germany). Cell-free media were applied to an ÄKTA FPLC with a cationic exchange column HiTrap SP HP (Amersham Biosciences, Uppsala, Sweden) with 20 mM sodium acetate, pH 4.5. Adsorbed proteins were eluted with 20 mM sodium acetate buffer (pH 4.5) containing 1 M NaCl and detected at 214 nm. The fraction containing PgAFP protein was then gel filtered on a HiLoad 26/60 Superdex 75 column for FPLC (Amersham Biosciences, Uppsala, Sweden) using 50 mM sodium phosphate buffer, pH 7 containing 0.15 M NaCl as elution buffer. PgAFP concentration in a pooled stock solution was measured by Lowry method (Lowry et al. 1951), sterilised through 0.22 µm acetate cellulose filters (Fisher Scientific) and stored at -20 °C until use.

Effect of PgAFP on mold growth

As a preliminary test, to confirm the known effect of PgAFP on growth of both sensitive and resistant molds in malt extract broth (Delgado et al. 2015a), *P. polonicum* and *A. tubingensis* were grown in PDB treated with PgAFP in the whole range of concentrations used in this work (0 to 75 µg/ml) for 96 h.

Proteomics

To obtain the protein extracts, *P. polonicum* CECT 20933 was cultured in triplicate in 50 ml of PDB, at 25 °C with continuous shaking at 200 rpm in either presence (10µg/ml) or absence of PgAFP. Mycelia were harvested, filtered, washed, and lysed as previously described (Carberry et al. 2006). Mycelial lysates were centrifuged to remove cell debris and the subsequent supernatant precipitated with TCA/acetone (Carpentier et al. 2005). The following two proteomic analysis were carried out from these precipitated lysates, similar to the procedure described by Delgado et al. (2015b).

Two-dimensional electrophoresis. For protein separation by 2D-PAGE, resuspended extracts containing 250 µg of protein were loaded onto Immobiline Dry strips (IPG strip; Amersham Biosciences) in the pH range 4–7, followed by electrofocusing, and electrophoresis as described previously (Carberry et al. 2006). Gels obtained from 3 biological replicates and 2 technical replicates per treatment were stained and analyzed using Progenesis^{TM} SameSpot software (TotalLab, Newcastle, UK) as previously described (O'Keeffe et al. 2013; Collins et al. 2013; Owens et al. 2014). Spot intensities were normalised in Progenesis SameSpots software (Delgado et al. 2015b). Protein spots showing differences ($p < 0.05$, fold change ≥ 1.5) were excised, destained, in-gel trypsin-digested (Shevchenko et al. 2007). Then, samples were sonicated and the digested supernatant was dried, resuspended in 0.1% formic acid, and filtered through 0.22 µm cellulose spin-filters according to Delgado et al. (2015b).

The samples were loaded onto a Zorbax 300 SB C-18 Nano-HPLC Chip and analysed by a 6340 Model Ion Trap LC-Mass Spectrometer (Agilent Technologies, Dublin, Ireland) using electrospray ionisation. The eluted peptides were ionized and analysed by mass spectrometry. MS^n analysis was carried out on the three most abundant peptide precursor ions at each time point, as selected automatically by the mass spectrometer. MASCOT MS/MS Ion search, NCBI (National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov/guide/proteins/) database and KEGG (Kioto Encyclopedia of Genes and Genome, www.genome.jp/kegg/) were used for protein identification and function, also BLAST® protein was employed to find orthologous proteins.

Label-free proteomics. Proteins precipitated from three biological replicates were resuspended in 8 M urea, dithiothreitol reduced and iodoacetic acid alkylated (Collins et al. 2013), and trypsin digested. Digested samples were desalted using C18 ZipTips® (Millipore, Darmstadt, Germany). One microgram from each digest was analysed via a Thermo Scientific Q-Exactive mass spectrometer coupled to a Dionex RSLCnano (Thermo Scientific, Waltham, MA, USA). Data was collected using a Top15 method for MS/MS scans (Dolan et al. 2014; O'Keeffe et al. 2014). Comparative proteome abundance and data analysis was performed using MaxQuant software (Version 1.3.0.5; www.maxquant.org/downloads.htm) (Cox and Mann 2008), with Andromeda used for database searching and Perseus (Version 1.4.1.3) used to organise the data, as per Delgado et al. (2015b). Data were searched against a *P. chrysogenum* database from Uniprot (www.uniprot.org; March 2014). In the absence of sequenced *P. polonicum* or any other species from Section *Fasciculata* (Houbraken and Samson 2011), *P. chrysogenum* also from subgenus *Penicillium*, was chosen for comparison. Quantitative analysis was performed using a t-test. Due to the high sensitivity and larger dynamic range of the gel-free proteomics analyses only proteins with a *p* value < 0.05 and fold change ≥ 2 were included in the quantitative results (Dolan et al. 2014; O'Keeffe et al. 2014). Qualitative analysis was also performed, to detect proteins that were found in at least 2 replicates of a particular sample, but undetectable in the comparison sample. Blast2GO analysis was utilized to further elucidate putative functions of proteins identified with abundance changes (Conesa et al. 2005).

Hyphal morphology

P. polonicum and *A. tubingensis* were grown on tubes containing 300 µl of PDB at 25 °C for 24 h in either the presence (75 µg/ml) or absence of PgAFP. Mycelia were collected by centrifugation and observed on a microscope Eclipse E200 equipped with a digital camera DS-Fi2 (Nikon, Tokyo, Japan).

Metabolic tests

To study the response to PgAFP, various metabolic tests were performed as described previously (Delgado et al. 2015b). For this, the resistant *P. polonicum* (c.a. 5×10^5 conidia per ml) was cultured in PDB at 25 °C for 24 h in static conditions with and without PgAFP. To rule out even potential weak effects, the highest concentration of 75 µg/ml PgAFP was used. Additionally, to study the effect on membrane permeability throughout a wide concentration range of PgAFP (i.e. 75, 37.5, 18.75, 9.38, 4.69, 2.34, 1.17, and 0 µg/ml) were assayed.

To test membrane permeability, cultures in microtiter plates were supplemented with SYTOX Green (Molecular Probes, Eugene, OR, USA) at final concentration of 0.2 µM. The fluorescence emitted was measured at 10, 30, and 210 min.

Metabolic activity was assessed by FUN-1 staining. Grown mycelia was washed with 10 mM HEPES (pH 7.5) before staining with 100 µl FUN-1 (Molecular Probes, Eugene, OR, USA) for 30 min at 25 °C as described previously (Kaiserer et al. 2003). Stained hyphae were visualized and photographed by fluorescence microscopy. Induction of reactive oxygen species (ROS) production was evaluated using 20 µM 2', 7' dichlorofluorescein diacetate (Molecular Probes, Eugene, OR, USA) according to Kaiserer et al. (2003), and observed by fluorescence microscopy.

Membrane integrity was assessed by the acridine orange/ethidium bromide (AO/EB) double staining. Hyphae were stained with 4 µg/ml of AO/EB (Sigma-Aldrich, St. Louis, MO, USA), incubated for 30 min, washed, and observed by fluorescence microscopy.

To distinguish between necrotic, late apoptotic, and viable cells, the Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, MO, USA), composed by Annexin V-fluorescein isothiocyanate/propidium iodide (AnV-FITC/PI), was used according to manufacturer's instructions.

For each of these metabolic tests, the sensitive *A. tubingensis* was used as a positive control to confirm the effect of PgAFP in the various assays.

Chitin staining

Conidia of *P. polonicum* were inoculated on 10 ml PDB in Petri dish containing a coverglass and incubated in presence (75µg/ml) and absence of PgAFP at 25 °C for 24 h. Mycelium was fixed, stained for 5 min with fluorescent brightener 28 (Sigma-Aldrich, St. Louis, MO, USA), and then washed, to visualize chitin (Harris et al. 1994) in a fluorescence microscope with an excitation wavelength of 387/11 nm.

Effect of PgAFP combined with chitinase on *P. polonicum* growth

Four different batches were prepared by pouring the reagents onto 15 ml potato dextrose agar plates made with PDB (Scharlab, Barcelona, Spain) and 20 g/l bacteriological agar (Scharlab, Barcelona), as follows: a) 2.5 ml of 600 µg/ml PgAFP in phosphate elution buffer and 0.1 ml PBS, b) 2.5 ml of phosphate elution buffer and 0.1 ml of ≥ 60 units/ml chitinase from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, MO, USA) in PBS, c) and 2.5 ml of 600 µg/ml PgAFP in phosphate elution buffer and 0.1 ml of ≥ 60 units/ml chitinase from *S. griseus* (Sigma-Aldrich, St. Louis, MO, USA) in PBS, and d) 2.5 ml of phosphate elution buffer and 0.1 ml PBS as a control samples. Every plate was surface three-point inoculated with 10 µl of a suspension containing 10^4 conidia and incubated at 25 °C for 168 h. The diameter of the colonies were measured every 24 h. To elucidate whether the combined treatment of PgAFP and chitinase has an additive or synergistic effect, the expected efficacy of this combination was determined by the Abbott formula and the interaction ratio as described by Moreno et al. (2003). Interaction ratios between 0.5 and 1.5 are considered to be additive interactions and ratios over 1.5 are considered to be synergistic interactions.

Chitin binding ability of PgAFP

Regenerated chitin was prepared as previously described (Souza et al. 2009), using chitin powder from crab shells (Sigma-Aldrich, St. Louis, MO, USA) added to concentrated HCl with vigorous stirring, filtered, and precipitated with ethanol 95%. The precipitate was filtered and washed with water until neutral pH. Chitin-PgAFP binding assay was carried out as described Liu et al. (2002). Briefly, three different amounts of PgAFP were mixed with 4 mg of regenerated chitin in a 0.5 ml 0.1 M Tris-HCl pH 7.4, 0.15 M NaCl buffer, incubated in ice for 1 h with stirring every 15 min. After incubation, samples were centrifuged and the quantity of protein contained in the supernatant was measured by the method described by Lowry (Lowry et al. 1951).

PgAFP localization

PgAFP was labelled by DareBio S.L. (Elche, Spain) as described previously (Delgado et al. 2015b). For this, 100 µl of 20 mM fluorescein isothiocyanate (FITC; Anaspec, Fremont, CA, USA) in dimethylsulphoxide was added to 4 ml of PgAFP (369 µg/ml), and left for 8 h at room temperature in the dark. Then, 100 µl of 0.8 M Tris-HCl pH 8 were added and dialyzed against PBS.

P. polonicum and *A. tubingensis* were grown in PDB in presence of 20 µg/ml PgAFP-FITC for 24 h at 25 °C. Hyphae were washed twice with PBS and visualized by fluorescence microscopy with excitation wavelength of 482/35 nm.

Statistical analysis

Statistical analyses were performed with the IBM SPSS v.22 (www-03.ibm.com/software/products/es/spss-stats-standard). Growth inhibition and membrane permeability data were tested for normality (Kolmogorov-Smirnov with Lilliefors correction) and homoscedasticity (Levene's test). Given that these data were non-normally distributed, mean values were compared using nonparametric Kruskal–Wallis test. To compare treatments in pairs, Mann-Withney *U* test was applied ($p < 0.05$).

RESULTS

As expected, PgAFP showed no effect ($p > 0.05$) on *P. polonicum* grown in PDB in the whole range of concentrations tested. *A. tubingensis* growth was affected ($p < 0.05$) from 4.7 µg/ml PgAFP at 48 h (data not shown).

Effect on proteome

2D-PAGE comparative proteomic analysis, in presence or absence of 10 µg/ml of PgAFP, showed 37 spots with differences ($p < 0.05$) over 1.5 fold change in relative abundance between treated and untreated *P. polonicum*. The abundance in treated samples was higher (1.5-3 fold) in 9 spots and lower (1.5-4.1 fold) in the remaining 27 proteins, including 2 spots from separate isoforms (Table S1 in the Supplementary Material).

Comparative label-free quantitative proteomic analysis showed a total of 918 proteins from *P. polonicum*, 93 of them displayed altered relative abundance ($p < 0.05$) over two-fold change with PgAFP treatment (Table S2 in the Supplementary Material). Thirty eight proteins were found in higher amounts (2-12.4 fold) in treated samples, 19 were only detected in treated samples, 25 were obtained in lower amounts (2-663 fold) following treatment, and 11 were only detected in non-treated samples (Table S2 in the Supplementary Material).

Eight of the nine proteins found in higher amounts in treated samples by 2D-PAGE were also detected by label-free proteomic analyses, with six of them showing similar increases in both methods (Tables S1 and S2 in the Supplementary Material). Also 26 of the 27 proteins found in lower relative abundance in treated *P. polonicum* by 2D-PAGE were also detected by label-free proteomics. However, only 16 of them were also detected at a lower relative abundance in the latter.

According to KEGG pathway analysis, most of the 57 proteins from label-free proteomics with higher relative abundance or only detected in treated *P. polonicum* were ribosomal and spliceosomal proteins (39%), or involved in biosynthesis of secondary metabolites and metabolic pathways (14%), such as pyruvate decarboxylase, pyrimidine biosynthesis, glycerol kinase, and asparagine synthetase (Table 1). The remaining proteins with higher relative abundance or only detected in treated samples were distributed across various pathways, such as Rho GTPase Rho1 involved in MAPK signaling pathway and, interestingly, glucosamine-6-phosphate N-acetyltransferase involved in chitin biosynthesis. Additionally, the antifungal protein PgAFP was detected in each of the triplicate treated sample, but not in any non-treated sample. Most of the proteins found in lower quantity or only detected in non-treated samples were related to biosynthesis of secondary metabolites and metabolic pathways (33%), including phosphoglucomutase, and glucose 6-phosphate isomerase related to glycolysis and gluconeogenesis (Table 1). Only limited changes in stress-related proteins, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heat shock proteins, were found in treated *P. polonicum* (Table S1 and S2 in the Supplementary Material).

SYTOX Green uptake

Upon PgAFP exposure up to 4.7 µg/ml, a 7 (± 3.4) % increase (± standard error) in fluorescence was observed in *P. polonicum* ($p < 0.05$) at 210 min after SYTOX Green addition (Fig. 1). Fluorescence at intermediate PgAFP concentrations (9.37-18.75 µg/ml) did not differ from untreated control ($p > 0.05$), whilst concentrations higher than 18.75 µg/ml decreased ($p < 0.05$) permeability below the levels of untreated samples, being the fluorescence values up to 21 (± 7.1) % lower at 210 min after SYTOX Green addition. On the other hand, the sensitive *A. tubingensis* showed a high increase of permeability ($p < 0.05$) at all PgAFP concentrations assayed (Fig. 1), reaching with 9.37-18.75 µg/ml over 110 ± (9.8-15) % fluorescence higher than in the untreated control.

Hyphal morphology and FUN-1 staining

PgAFP exposure provoked no morphological change on either *P. polonicum* or the sensitive *A. tubingensis* (data not shown). To know whether PgAFP affects the metabolic activity, the viability was evaluated with FUN-1 using *A. tubingensis* as sensitive control. The FUN-1 metabolic staining showed red intravacuolar stains in both treated and untreated *P. polonicum* revealing no reduction in the metabolic activity (Fig. 2). Conversely, intravacuolar red stains were not observed in PgAFP-treated *A. tubingensis*, revealing a lower metabolic activity.

Chitin staining

To study the effect of PgAFP on chitin deposition on the resistant mold, the quantity of chitin was estimated by staining with fluorescent brightener 28. The observed fluorescence indicated a higher chitin deposition in the cell wall of treated than in non-treated *P. polonicum* (Fig. 3).

Effect of PgAFP-chitinase combined treatment on *P. polonicum* growth

For the whole incubation time, no statistically significant difference was found among growth of the untreated control and *P. polonicum* treated only with PgAFP (Fig 4). Chitinase treatment reduced growth compared to control batch. Growth of *P. polonicum* treated with combined PgAFP and chitinase was the lowest ($p < 0.05$). The interaction ratios between these antifungal compounds at 96 and 120 h incubation were 1.93 and 1.70, respectively. Thus, the slower growth in the combined treatment is attributed to a synergistic effect of chitinase and PgAFP.

PgAFP localization

PgAFP localization was investigated in *P. polonicum* by incubation with FITC-labelled PgAFP. *P. polonicum* showed green fluorescence only bound to the outer layer (Fig 5). However, the labelled protein was found both inside the hyphae and bound to the outer layer in *A. tubingensis*, revealing that PgAFP had entered *A. tubingensis*.

Chitin-PgAFP binding assay

Given that PgAFP was located at the outer layer of *P. polonicum*, a chitin-binding assay was performed. When PgAFP was added to a solution of regenerated chitin, over 91% of the antifungal protein was recovered from the supernatant after incubation, even at the lowest concentration tested (146 μg/ml). Thus, PgAFP does not specifically bind to regenerated chitin.

Effect on oxidative status and viability

To test the influence of PgAFP on ROS production, staining with 2’, 7’ dichlorofluorescein diacetate was used. Both treated and untreated *P. polonicum* showed similar levels of emitted fluorescence (data not shown), revealing that PgAFP does not increase ROS in the resistant *P. polonicum*. The effect of PgAFP on membrane integrity was evaluated by AO/EB double staining. EB was taken only by PgAFP-treated *A. tubingensis,* showing orange hyphae, whilst non-treated *A. tubingensis* and both treated and non-treated *P. polonicum* only showed green hyphae due to AO uptake (Fig. 6). These results reveal that *P. polonicum* membrane was not compromised by PgAFP, which is the opposite to *A. tubingensis*. The evaluation of apoptosis or necrosis confirmed the above reported effects on viability. *A. tubingensis* treated hyphae showed orange color as a consequence of AnV-FITC and PI staining, meaning a necrotic stage. Non-treated *A. tubingensis* and both treated and untreated *P. polonicum* were not dyed, showing no sign of apoptosis or necrosis (Fig 7).

DISCUSSION

Both proteomic methods used in this work revealed differences in the relative abundance of proteins after treatment of *P. polonicum* with PgAFP (Tables S1 and S2 in the Supplementary Material). Discrepancies were detected in the fold change estimated by each method. Such discrepancies can be explained by the fact that 2D-PAGE compares one isoform of a protein at a time, whereas label-free proteomics combines every isoform together and gives the final total abundance of that protein (Delgado et al. 2015b). Therefore, changes in the relative quantity of each isoform could be detected using 2D-PAGE, while in the label-free proteomics only a measure of total abundance of all isoforms of a given protein is carried out.

Label-free proteomics showed an increased abundance of 22 proteins related to ribosomes and spliceosomes in PgAFP-treated *P. polonicum*, according to KEGG. However, only two ribosomal proteins were found in higher amount by 2D-PAGE analysis. This fact can be explained by the narrow range of pH chosen for 2D-PAGE analysis (Görg et al. 2009). In particular, the analysis carried out is suitable for proteins with pI between 4-7, whilst proteins involved in ribosome structure or function are generally out of this range (Görg et al. 2004). Therefore, the combinatorial deployment of proteomic tools used in this study works complementarily to obtain further information about the effect of PgAFP on the proteome.

The higher relative abundance of proteins from ribosomal and spliceosomal pathways in PgAFP-treated *P. polonicum* could be regarded as a response of the mold to counteract the protein's antifungal activity. A higher relative abundance of a substantial number of ribosomal and spliceosomal proteins has also been described in a recent work on the effect of PgAFP on the sensitive *A. flavus* (Delgado et al. 2015b). Twelve of the 22 proteins from this group that increased with PgAFP in *P. polonicum* also increased in *A. flavus*. Thus, the increase in proteins from ribosomal and spliceosomal pathways solely would not explain the resistance mechanism in *P. polonicum*.

The changes observed in the proteins related to metabolic pathways and biosynthesis of secondary metabolites were heterogeneous, with 8 proteins increasing and 12 decreasing in PgAFP-treated *P. polonicum* (Table 1). From these proteins, only pyruvate decarboxylase, aldehyde dehydrogenase, and phosphatidylglycerol specific phospholipase showed similar changes in PgAFP-treated *A. flavus* (Delgado et al. 2015b). However, these enzymes are scattered among various metabolic routes, including glycolysis gluconeogenesis, purine metabolism, and aminoacyl-tRNA biosynthesis, making it unlikely that any of them explain the ability of *P. polonicum* to withstand PgAFP.

All the above changes in the abundance of the metabolic-related proteins did not entail dramatic changes in the metabolic activity, which in turn is consistent with the resistance of *P. polonicum* to PgAFP. The abundance of intracellular red spots in FUN-1 staining (Fig. 2) revealed that the metabolic activity in *P. polonicum* remained substantially unaffected by PgAFP, whereas it was greatly reduced in the sensitive *A. tubingensis* used as a control (Fig 2), as well as in PgAFP-treated *A. flavus* (Delgado et al. 2015b).

Other effects reported for antifungal proteins, including PAF, NFAP, and PgAFP, are increased ROS levels leading to programmed cell death in sensitive molds (Leiter et al. 2005; Galgóczy et al. 2013a; Delgado et al. 2015b). Increased ROS levels have been linked to higher relative abundance of proteins involved in the glutathione pathway and heat shock proteins in PgAFP-treated *A. flavus* (Delgado et al. 2015b). The limited changes in such stress-related proteins in treated *P. polonicum* do not reveal a strong response to oxidative stress. In addition, none of the negative effects related to oxidative stress was observed in PgAFP-treated *P. polonicum*, including increased ROS levels, loss of cell membrane integrity, and necrotic signs.

All the changes in the proteome discussed so far reveal that PgAFP interacts with the non-sensitive *P. polonicum*, but do not seem to explain the successful defence response. As discussed later, proteins from the cell wall integrity (CWI) pathway seem to be involved in the successful defence response.

Membrane permeabilization is a main effect described for other antifungal proteins (Thevissen et al. 1999; Hagen et al. 2007). Increased permeability also contributes to PgAFP inhibition on *A. flavus* (Delgado et al. 2015b). Similarly, the permeability of the sensitive *A. tubingensis* to SYTOX Green increased at all PgAFP concentrations tested (Fig. 1). However, membrane permeabilization in *P. polonicum* exhibited a two-step pattern: first increasing slowly at low PgAFP concentrations, then slowly declining even below the level of untreated controls with the two highest concentrations tested (Fig. 1). A similar two-step pattern in membrane permeabilization was also described for *Neurospora crassa* treated with plant defensins (Thevissen et al. 1999). The lower permeability at the highest concentrations of defensins has been explained by the apparent dependency of permeabilization on membrane polarization. The higher permeability of fungal membranes treated with defensins causes depolarization, which may ultimately decrease membrane permeability (Thevissen et al. 1996, 1999). The decline in *P. polonicum* membrane permeability at high PgAFP concentrations might be partially explained by membrane depolarization. As discussed later, other changes in membrane and cell wall can contribute to reach permeability levels well below that in untreated controls.

Growth inhibition by AFP, PAF, and PgAFP has been related to the ability to interact with specific molecules or anionic phospholipids in the cell wall and/or plasma membrane (Lacadena et al. 1998; Theis et al. 2003; Marx et al. 2008; Delgado et al. 2015b). Similarly NFAP might bind to a G-protein coupled receptor in a sensitive mold (Virágh et al. 2015) and $\mathrm{AFP_{NN5353}}$ does not bind to insensitive *Mucor circinelloides* (Binder et al. 2011). Interestingly, PgAFP was located at the outer layer in the resistant *P. polonicum* (Fig. 5). This binding may be due just to adherence to chitin or to specific receptors, but PgAFP did not bind to regenerated chitin in vitro. Given that PgAFP was not internalized by the resistant mold, no internal receptor can be detected. In addition, the proteome changes observed in PgAFP-treated *P. polonicum* can only be due to transduction signals derived from the interaction with outer layer receptors. Therefore, PgAFP may interact with specific molecules in the outer layer of *P. polonicum*, similarly to PAF (Marx et al. 2008; Batta et al. 2009). As a consequence, PgAFP-resistance could be related with the ability of *P. polonicum* to produce structural changes that prevent the interaction with the specific receptors or the negatively charged phospholipids.

The fungal cell wall acts as an initial barrier in contact with hostile environments (Latgé 2007). The main components of the cell wall that may act as a barrier against antifungal proteins are polysaccharides, including glucans, glucomannans and chitin. A lower chitin content in the fungal cell wall has been related to a higher permeability (Mellado et al. 2003; Rementeria et al. 2005), suggesting a barrier role of chitin. The higher amount of chitin observed in the cell wall of *P. polonicum* treated with the highest PgAFP concentration (Fig. 3) can be responsible for the lower permeability observed, being a key factor in the successful response to this antifungal protein. Chitin synthesis is also stimulated by AFP in resistant fungi (Ouedraogo et al. 2011), but not by AFP, PAF, and PgAFP in sensitive molds (Hagen et al.

2007; Binder et al. 2010; Delgado et al. 2015b). In addition, PAF and NFAP provoke delocalized chitin deposition at the hyphal tips (Binder et al. 2010; Virágh et al. 2015). Therefore, the altered chitin deposition can be related to sensitivity to antifungal proteins in contrast to our findings in *P. polonicum*.

To confirm if the increased chitin deposition itself is enough to explain the resistance to PgAFP, a joint treatment of PgAFP and chitinase was applied to *P. polonicum*. The slowest growth obtained with the combined treatment strongly infers that the increased chitin content plays a key role in the resistance of *P. polonicum* to PgAFP. Therefore, we propose that chitin cell wall reinforcement is responsible for the successful response of *P. polonicum*, due to a hampered interaction of PgAFP with specific receptors or the negatively charged phospholipids.

From the proteins involved in chitin biosynthesis, glucosamine-6-phosphate N-acetyl transferase was only found in treated *P. polonicum* (Table 1). The gene coding for this protein, as well as the gene encoding an α-1,3-glucan synthase, is upregulated in *A. niger* treated with sublethal doses of caspofungin (Meyer et al. 2007). Given that an increase of glucan but not chitin synthesis results in an ineffective survival response (Hagen et al. 2007), the increase in glucosamine-6-phosphate N-acetyl transferase could be important for *P. polonicum* to counteract PgAFP. The increased chitin content can also be related with CWI signaling activation. The stress signals sensed by the receptor protein Wsc are transmitted to Rho1, which has been considered the master regulator of cell wall integrity signaling pathway in yeasts (Levin 2005). Then, Rho1 binds and activates Pkc (Nonaka et al. 1995; Kamada et al. 1996; Lodder et al. 1999), and the signals channeled through the Mpk signaling lead to activation of genes involved in cell wall synthesis (Igual et al. 1996; Jung and Levin 1999), resulting in an elevated chitin content (Munro et al. 2007). Rho1 and Pkc1 have been suggested as the only proteins of CWI pathway that could be involved in the survival response of AFP-resistant *Saccharomyces cerevisiae*, but its relevance has not been established (Ouedraogo et al. 2011).

In sensitive molds, chitin synthesis is not increased by antifungal proteins, as for *A. nidulans* treated with PAF (Binder et al. 2010) or *A. niger* treated with AFP (Hagen et al. 2007). The resistant *P. polonicum* showed an increased abundance of Rho1 and in chitin synthesis when treated with PgAFP (Table 1 and Fig. 3). Conversely, the sensitive *A. flavus* showed a lower relative abundance of Rho1 and a lower chitin deposition when treated with PgAFP (Delgado et al. 2015b). Therefore, it seems that the efficient response of CWI pathway activation by Rho1 could be a key role in the resistance to PgAFP, in contrast to the basal ineffective compensatory response of this pathway in sensitive molds.

In conclusion, the proteome changes and the altered permeability imply an active reaction of *P. polonicum* to PgAFP, where the increased chitin content can be related with a higher abundance of glucosamine-6-phosphate N-acetyltransferase and Rho1. Moreover, the combined treatment with chitinase could provide a complementary means to combat resistance to antifungal proteins.

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ETHICAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE CAPTIONS

Fig. 1 SYTOX Green uptake with different concentrations of PgAFP on *P. polonicum* and *A. tubingensis* at 24 h (bars represent standard deviation of the mean).

Fig.2 Metabolic activity of *P. polonicum* (panel A) and *A. tubingensis* (panel B) tested with FUN-1 staining. Non-treated hyphae (left) showed intravacuolar activity as red spots. Hyphae treated with 75 µg/ml PgAFP for 24 h (right) showed intravacuolar activity in *P. polonicum*, but very low metabolic activity in *A. tubingensis*.

Fig. 3 Chitin distribution on *P. polonicum* stained with fluorescent brightener 28. Left: non-treated hyphae; right: hyphae treated with 75 µg/ml PgAFP for 24 h.

Fig. 4 Effect of PgAFP and chitinase combined treatment on *P. polonicum* growth. Untreated control: added with 2.5 ml of phosphate elution buffer and 100 µl PBS; PgAFP: added with 2.5 ml of 600 µg/ml PgAFP in phosphate elution buffer and 100 µl PBS. Chitinase: added with 2.5 ml of phosphate elution buffer and 100 µl of ≥ 60 units/ml chitinase from *Streptomyces griseus*; PgAFP + Chitinase: added with 2.5 ml of 600 µg/ml PgAFP in phosphate elution buffer and 100 µl PBS.

Fig. 5 PgAFP localization in *P. polonicum* (left) and *A. tubingensis* (right) treated with 20 µg/ml FITC-labelled PgAFP for 24 h. PgAFP was found solely bound to the outer layer in *P. polonicum* but mainly inside *A. tubingensis*.

Fig. 6 Effect of 75 µg/ml PgAFP on membrane integrity of *P. polonicum* (left) and *A. tubingensis* (right) evaluated with vital acridine orange (AO) / ethidium bromide (EB) staining. *P. polonicum* hyphae showed intense green color due to only AO penetration through non-compromised membrane. *A. tubingensis* hyphae showed intense orange color due to both AO and EB penetration through compromised cell membrane.

Fig. 7 Effect of 75 µg/ml PgAFP on *P. polonicum* and *A. tubingensis* hyphae viability evaluated with apoptosis detection kit at 24 h of incubation. A: Non-treated *P. polonicum.* B: PgAFP-treated *P. polonicum*. C: Non-treated *A. tubingensis*. D: PgAFP-treated *A. tubingensis*. Left: Annexin V/FITC-Propidium Iodide (An/FITC-PI) staining. Right: The corresponding bright field view. No intense green or orange color due to apoptosis or necrosis was observed in PgAFP-treated *P. polonicum*. Only PgAFP-treated *A. tubingensis* showed intense orange color due to necrosis.

Table 1. Selected proteins whose relative abundance was affected by PgAFP in *Penicillium polonicum* reaching over 2.0 fold change in Label-Free Proteomics (LFP) analysis or 1.5 fold change in 2D-PAGE. Data are given according to four groups of metabolic pathways.

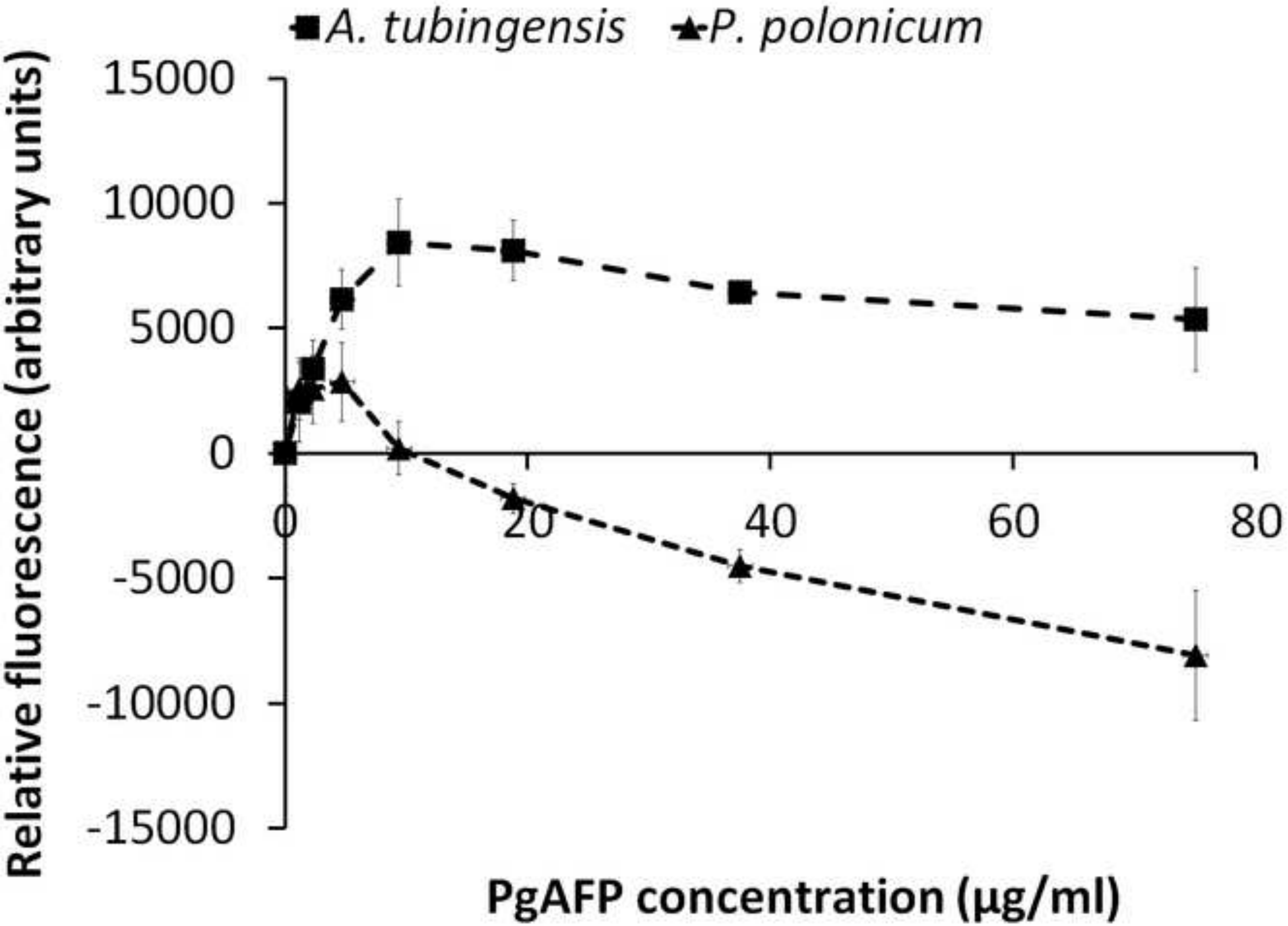
Proteins involved in pathways	**Fold change**	**Detection method**
Ribosomal and spliceosomal proteins		
Pc12g05940 40s ribosomal protein s13	T [a]	LFP
Pc13g01870 60s ribosomal protein l16	T	LFP
Pc16g04770 formin binding protein	T	LFP
Pc20g10480 small nuclear ribonucleoprotein	T	LFP
Pc22g08360 pre-mrna branch site protein p14	T	LFP
Pc20g00680 60s ribosomal protein l23	+11.16	LFP
Pc20g13260 ribosomal protein l14	+6.32	LFP
Pc13g02890 60s ribosomal protein l27	+5.32	LFP
Pc13g05540 60s ribosomal protein l18	+4.17	LFP
Pc18g04110 60s ribosomal protein l34	+4.05	LFP
Pc22g02060 60s ribosomal protein l8	+3.88	LFP
Pc21g16520 60s ribosomal protein l4	+3.83	LFP
Pc16g14740 40s ribosomal protein s22	+3.21	LFP
Pc16g09160 60s ribosomal protein l15	+3.14	LFP
Pc22g00880 40s ribosomal protein s18	+2.73	LFP
Pc21g18200 60s ribosomal protein	+2.51	LFP
Pc16g12990 60s ribosomal protein l17	+2.47	LFP
Pc13g07190 60s ribosomal protein l11	+2.32	LFP
Pc13g05920 60s ribosomal protein l7	+2.21	LFP
Pc20g03340 60s ribosomal protein l33	+2.19	LFP
Pc13g06740 60s ribosomal protein l13	+2.06	LFP
Pc20g02900 40s ribosomal protein s4	+2.02	LFP
Biosynthesis of secondary metabolites and metabolic pathway		
Pc21g21940 bifunctional pyrimidine biosynthesis protein	T	LFP
Pc22g06070 glycerol kinase	T	LFP
Pc22g17940 asparagine synthetase	T	LFP
Pc22g23800 glucosamine 6-phosphate N-acetyl transferase [b]	T	LFP
Pc21g15760 glutamyl-trna synthetase	+12.44	LFP
Pc20g07710 sulfate adenylyltransferase	+6.9	LFP
Pc22g07020 nitrilase	+3.49	LFP
Pc18g01490 pyruvate decarboxylase	+2.01	LFP
Phosphoglucomutase	-2	2D-PAGE
Pc16g05080 adenosylhomocysteinase	-2	LFP
Pc12g05750 d-xylulose kinase	-2.31	LFP
Pc21g04710 phospho-2-dehydro-3-deoxyheptonate aldolase	-2.52	LFP
Pc22g19440 aspartate aminotransferase	-2.58	LFP
Pc21g03190 glycerate dehydrogenase	-2.66	LFP
Pc22g02810 methylmalonate-semialdehyde dehydrogenase	-3.05	LFP
Pc22g19730 glucose-6-phosphate isomerase	-3.49	LFP
Pc15g01900 putative oligo-glucosidase	-6.9	LFP
Pc15g01880 phosphatidylglycerol specific phospholipase	-633	LFP
Pc13g03600 thiamine biosynthetic bifunctional	NT [c]	LFP
Pc14g00170 phosphatidylglycerol specific	NT	LFP
Pc22g24860 aldehyde dehydrogenase	NT	LFP
CWI pathway		
Pc22g23800 glucosamine-6-phosphate N-acetyl transferase [b]	NT	LFP
Pc14g01930 protein Rho gtpase rho1	+9.04	LFP
Pc21g11950 UDP-N-acetylglucosamine pyrophosphorylase	-2	2D-PAGE
UDP-glucose 4-epimerase	-1.5	2D-PAGE
Gamma-actin act	-1.6	2D-PAGE

[a] T: Protein only detected in treated samples.

[b] Protein involved in more than one pathway.

[c] NT: Protein only detected in non-treated samples.

Figure1



■ A. tubingensis
▲ P. polonicum
Relative fluorescence (arbitrary units)
15000
10000
5000
0
-5000
-10000
-15000
0
20
40
60
80
PgAFP concentration (µg/ml)

Figure2

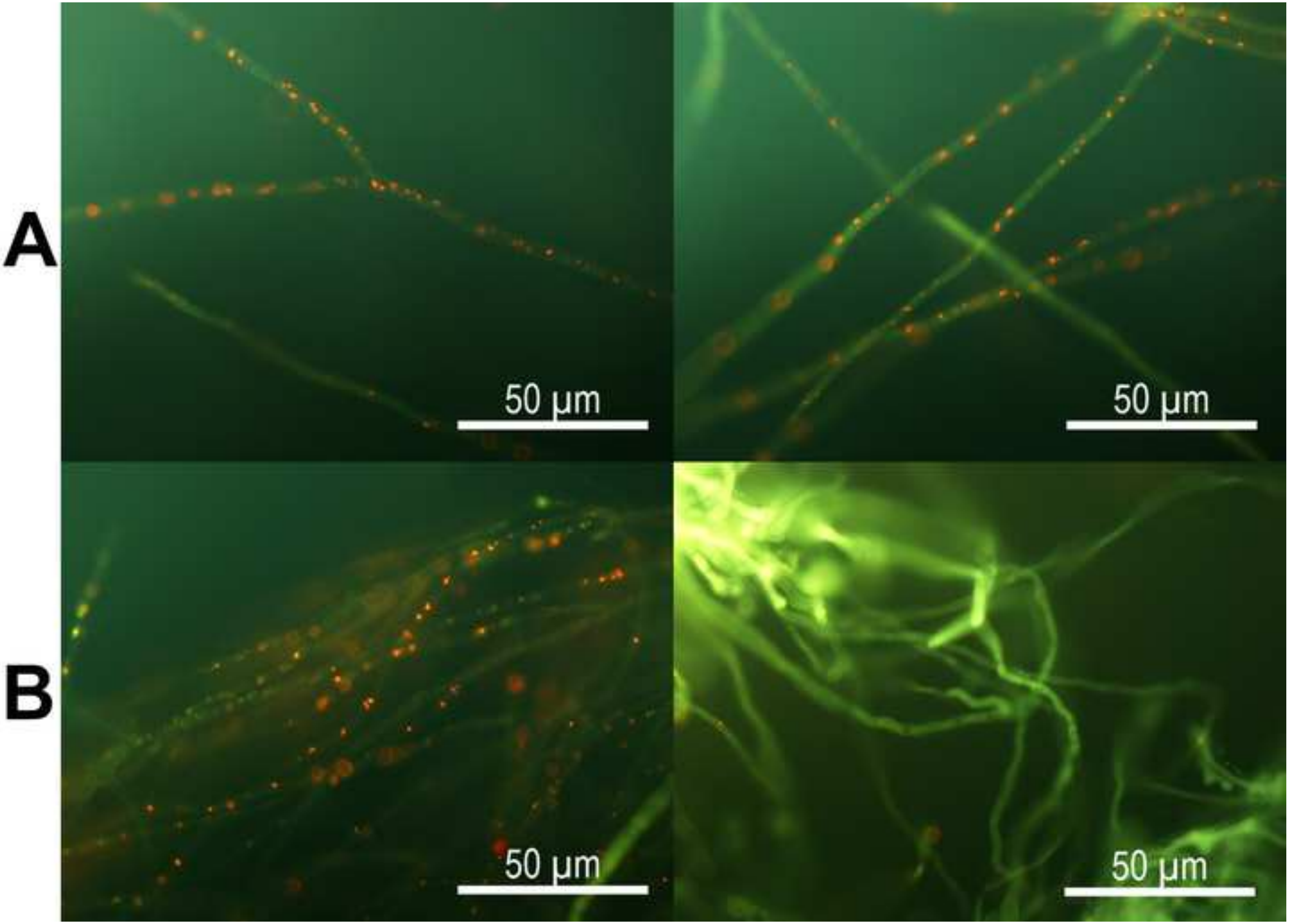


Figure 3

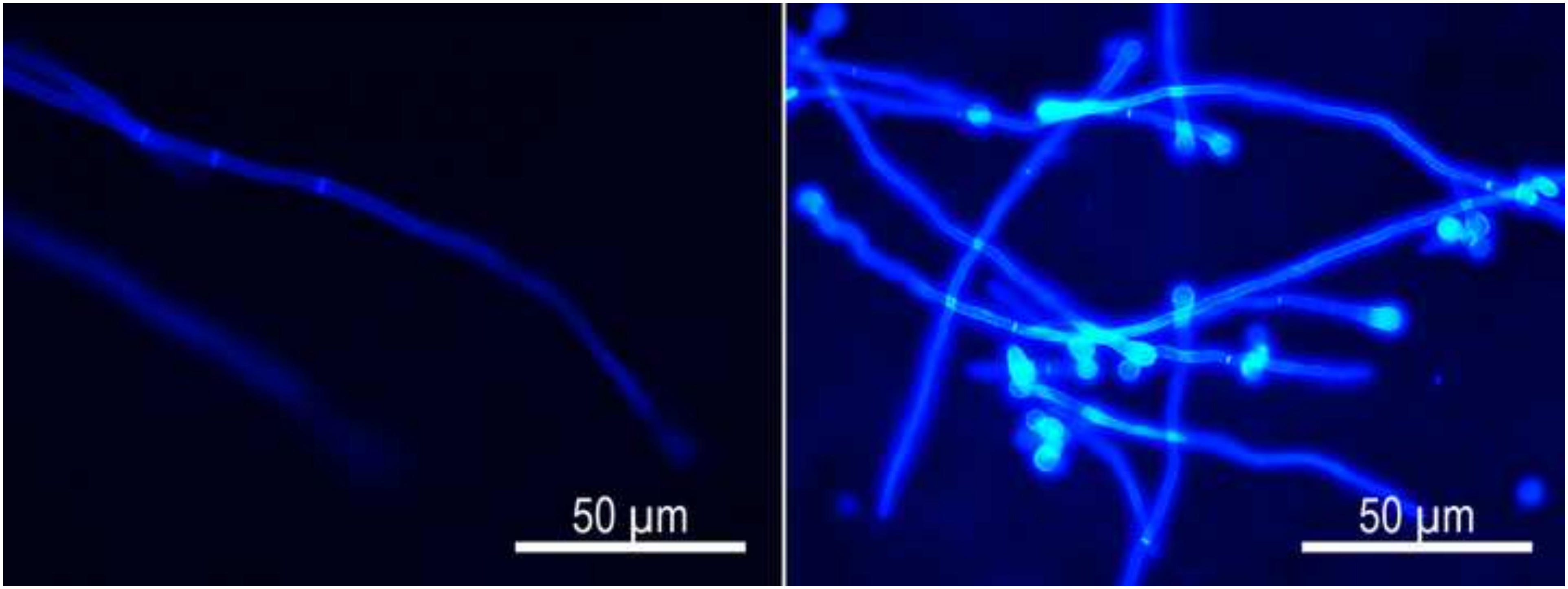
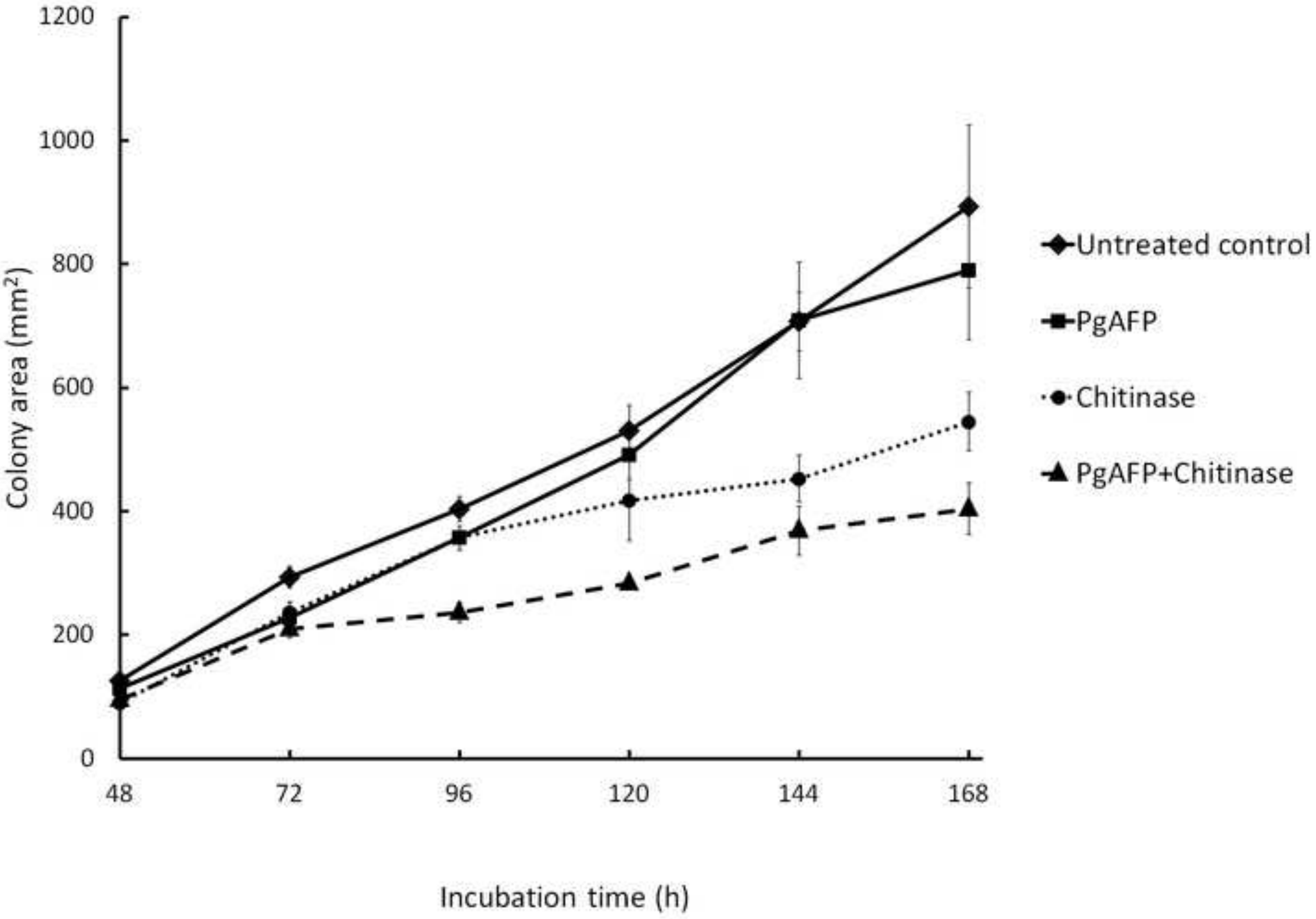


Figure 4



1200
1000
800
600
400
200
0
Colony area (mm²)
48
72
96
120
144
168
Incubation time (h)
Untreated control
PgAFP
Chitinase
PgAFP+Chitinase

Figure 5

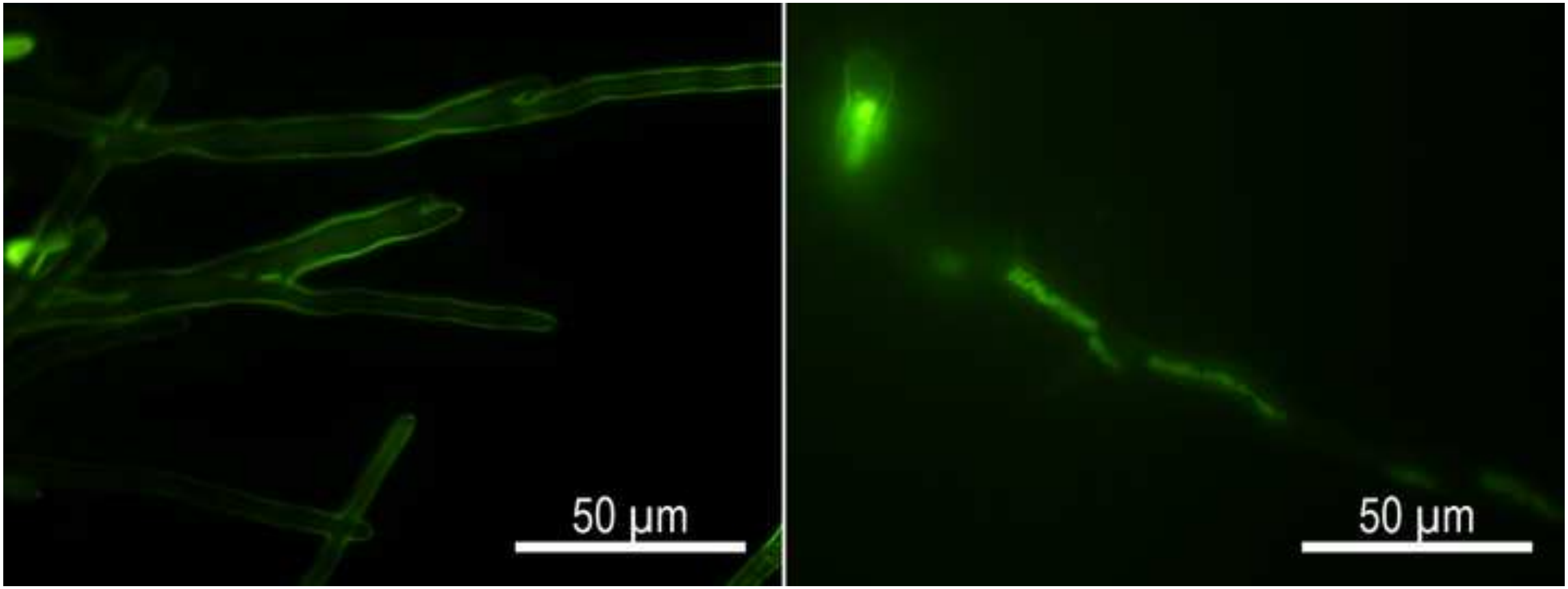


Figure 6

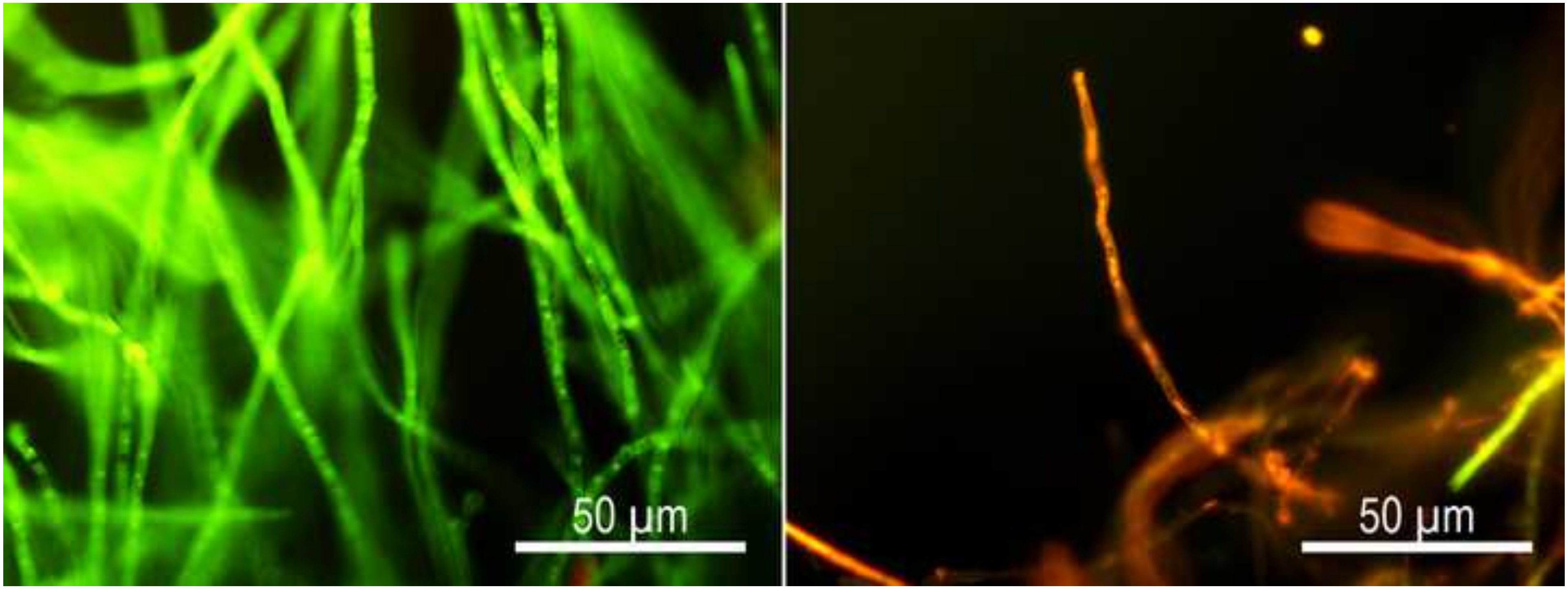


Figure7