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The effect of entomopathogenic fungal culture filtrate on the immune response of the greater wax moth, *Galleria mellonella*

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ABSTRACT

Galleria mellonella is a well-established model species regularly employed in the study of the insect immune response at cellular and humoral levels to investigate fungal pathogenesis and biocontrol agents. A cellular and proteomic analysis of the effect of culture filtrate of three entomopathogenic fungi (EPF) species on the immune system of *G. mellonella* was performed. Treatment with *Beauveria caledonica* and *Metarhizium anisopliae* 96 h culture filtrate facilitated a significantly increased yeast cell density in larvae (3-fold and 3.8-fold, respectively). Larvae co-injected with either *M. anisopliae* or *B. caledonica* culture filtrate and *Candida albicans* showed significantly increased mortality. The same was not seen for larvae injected with *Beauveria bassiana* filtrate. Together these results suggest that *B. caledonica* and *M. anisopliae* filtrate are modulating the insect immune system allowing a subsequent pathogen to proliferate. *B. caledonica* and *M. anisopliae* culture filtrates impact upon the larval prophenoloxidase (ProPO) cascade (e.g. ProPO activating factor 3 and proPO activating enzyme 3 were increased in abundance relative to controls), while *B. bassiana* treated larvae displayed higher abundances of alpha-esterase when compared to control larvae (2.4-fold greater) and larvae treated with *M. anisopliae* and *B. caledonica*. Treatment with EPF culture filtrate had a significant effect on antimicrobial peptide abundances particularly in *M. anisopliae* treated larvae where cecropin-D precursor, hemolin and gloverin were differentially abundant in comparison to controls. Differences in proteomic profiles for different treatments may reflect or even partially explain the differences in their immunomodulatory potential. Screening EPF for their ability to modulate the insect immune response represents a means of assessing EPF for use as biocontrol agents, particularly if the goal is to use them in combination with other control agents. Additionally EPF represent a valuable resource pool in our search for natural products with insect immunomodulatory and biocontrol properties.

1. Introduction

Beauveria bassiana and *Metarhizium anisopliae* are amongst the best characterised and widely used entomopathogenic fungi (EPF). These EPF have a global distribution, infect a wide range of insects and are used to control vectors of human disease and plant pests (Shah and Pell, 2003; Glare et al., 2008; Gao et al., 2011; Xiao et al., 2012; Lacey et al., 2015; Butt et al., 2016). EPF have great potential as biopesticides since, unlike bacteria and viruses, they infect hosts through direct cuticle penetration, allowing them to act as contact insecticides (Leger et al., 2011). In addition to their role as insect pathogens, EPF have a crucial role in natural ecosystems as endophytes, plant disease antagonists, rhizosphere colonizers, and plant growth promoters (Gao et al., 2011; Lacey et al., 2015). Despite being promising biocontrol agents EPF have not fulfilled their potential and often fail when tested in the field.

Biocontrol agents are generally slower acting than their chemical counterparts and their high specificity may necessitate the application of more than one agent to control a number of pests. To improve efficacy, biocontrol agents can be combined to increase the success of the application. Combining treatments may result in additive, antagonistic or synergistic interactions. If a combination resulted in a synergistic interaction, then the associated cost of EPF may be reduced. Combinations of EPF, especially *M. anisopliae,* with various species of entomopathogenic nematodes has resulted in synergy in lab and field trials against various insect pests (Ansari et al., 2006, 2008, 2010; Anbesse et al., 2008). Synergism can also occur when different species of EPF are applied together; *Metarhizium flavoviride* and *B. bassiana* can be combined to overcome the constraints of temperature in controlling thermoregulating grasshoppers (Inglis et al., 1997). In the case of synergism, combined applications may make the host more susceptible

Abbreviations: PO, phenoloxidase; PPO, prophenoloxidase; EPF, entomopathogenic fungi; PCA, principal component analysis; AMP, antimicrobial peptide

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through modulating their immune system, prolonging developmental stages or by the two treatments acting on different components of the host population (Lacey et al., 2015).

Knowledge of the insect immune response induced by fungal pathogens contributes to the understanding of both insect defenses and the fungal pathogenicity that defeats them. Ultimately uncovering fungal virulence determinants may give rise to opportunities to manipulate these virulence factors to improve the success of biocontrol agents (Gillespie et al., 2000). Despite the importance of EPFs as biocontrol agents and the cost benefits of achieving synergy through combinations of agents our understanding of the molecular basis by which EPF modulate the host immune response making it more susceptible to secondary pathogens is still limited.

Host colonization by EPF requires the ability to cope with host immune defenses and extract nutrients from the host (Gillespie et al., 2000) which is achieved through immune evasion by cryptic forms or immune system modulation through the action of secreted molecules (Schrank and Vainstein, 2010). *Metarhizium* spp. produce a diverse range of enzymes and secondary metabolites that are active against insects, fungi, bacteria, viruses and cancer cells (Roberts and St Leger, 2004; Gao et al., 2011). *Metarhizium* produce metabolites that are toxic to a broad range of targets, most notably the cyclic hexadepsipeptidic destruxins (Schrank and Vainstein, 2010) which display antiviral, antitumor, insecticidal, cytotoxic, immunosuppressive, phytotoxic and *anti*-proliferate effects (Kershaw et al., 1999; Sowjanya Sree et al., 2008; Liu and Tzeng, 2012). *Metarhizium* isolates that produce higher amounts of destruxin are generally more virulent (Amiri-Besheli et al., 2000; Sowjanya Sree et al., 2008). *B. bassiana* is known to produce cyclic peptides that are cytotoxic and immunosuppressive (Hung et al., 1993) and a diverse selection of secondary metabolites including non-peptide pigments and polyketides (e.g. oosporein), non-ribosomally synthesized peptides (e.g. beauvericin) and secreted metabolites that have roles in pathogenesis and virulence (Xiao et al., 2012). These metabolites have insecticidal properties and can also inhibit growth of other microorganisms (van der Weerden et al., 2013). *B. bassiana* products have both realized and proposed applications in industry, pharmaceuticals and agriculture.

A cellular and proteomic analysis of the effect of culture filtrate of three EPF species on the immune system of the lepidopteran greater wax moth *Galleria mellonella* was performed. *G. mellonella* is a well-established model species and is regularly employed in the study of the insect immune response at cellular (Vilcinskas et al., 1997a, 1997b; Vilcinskas and Götz, 1999) and humoral (Vilcinskas and Matha, 1997; Bergin et al., 2006; Wojda and Jakubowicz, 2007; Wojda et al., 2009) levels. Of greater relevance is the use of *G. mellonella* as a model to investigate fungal pathogenesis and biocontrol agents (Vilcinskas et al., 1997a; Cotter et al., 2000, Fröbius et al., 2000, Bergin et al., 2003; Scully and Bidochka, 2005) and although different insect species may vary greatly in their susceptibility to fungal toxins (Gillespie et al., 2000), *Galleria* is an excellent substitute for insects that are difficult to source or cultivate, particularly for preliminary investigations. The aim of this work was to investigate the immunomodulatory potential of EPF in order to assess their potential use and mode of action as synergists in combined applications with other control agents.

The three species of EPF chosen were *M. anisopliae*, *B. bassiana* and *B. caledonica*. *Beauveria caledonica* was found to be a naturally occurring pathogen of the pine bark beetles *Hylastes ater* and *Hylurgus ligniperda* in New Zealand (Glare et al., 2008; Reay et al., 2008) and the large pine weevil, *Hylobius abietis* in Ireland (Glare et al., 2008; Williams et al., 2013). Considering *B. caledonica* kills these pests in their natural environment, it makes this fungus an excellent candidate as a biocontrol agent of coleopteran forestry pests (Glare et al., 2008; Williams et al., 2013).

2. Materials and methods

2.1. Fungal origin and entomopathogenic fungal culture filtrate production

A commercial strain of *M. anisopliae* (Met52) produced by Novozymes (Denmark) was used. Met 52 was purchased on rice grains from National Agrochemical Distributors, Lusk, Dublin. *B. bassiana* experimental strain 1694, was supplied by Becker Underwood (Littlehampton, UK). *B. caledonica* (2c7b) is a native strain isolated from a soil sample from soil close to a pine stump in a felled forest in Hortland, Co. Kildare (Ireland). The soil sample was baited with *G. mellonella* larvae and fungus from the infected cadaver was identified through DNA sequencing of an ITS PCR product (a region of the internal transcribed spacer unit of the ribosomal DNA, IST4, was amplified by PCR). EPF were cultured in Sabouraud Dextrose liquid medium (Oxoid) for 48 h, 72 h and 96 h in a shaking incubator at 25 °C and 250 rpm. After each time point the culture was filtered through 0.45 μm syringe filters and then through 0.2 μm syringe filters (Sartstedt). The filtrate was collected and stored at -80 °C.

2.2. Inoculation of Galleria mellonella larvae

Sixth instar larvae of G. *mellonella* (Mealworm Company, Sheffield, England) were stored in the dark at 15 °C in wood shavings prior to use. Larvae were stored immediately upon receipt from the supplier. Larvae that had been stored for 1–3 weeks and weighing 0.24–0.28 g were used. Larvae were inoculated with 20 μl of culture filtrate through the last pro-leg using a Myjector U100 insulin syringe (Terumo Europe, Leuven, Belgium). Control larvae were inoculated with 20 μl of Sabouraud dextrose liquid medium. Larvae were incubated at 20 °C.

2.3. Enumeration of haemocyte and yeast cell densities, and infection susceptibility assays

The density of circulating haemocytes in larvae was assessed as described previously (Bergin et al., 2003). All experiments were performed on three independent occasions.

To test the effect of EPF on the immune response to a subsequent infection, larvae were inoculated with EPF culture filtrate or Sabouraud dextrose (control) and incubated for 24 h at 20 °C, after which they received a second inoculation through the second proleg with *Candida albicans* (10^5 cells in 20 μl). *Candida albicans* MEN (serotype B, wild-type originally isolated from an eye infection (a gift from Dr. D. Kerridge, Cambridge, UK) was cultured to the stationary phase overnight in yeast extract peptone dextrose (YEPD) at 30 °C and 200 rpm on an orbital shaker (Browne et al., 2015). Following the second inoculation, larvae were incubated for a further 24 h or 48 h at 20 °C and were homogenized in 3 ml of sterile PBS. After serial dilution in PBS, 100 μl of each sample was spread on YEPD plates containing erythromycin (1 mg/ml). The plates were incubated for 48 h at 30 °C. Yeast cell density was subsequently calculated per larva.

To test whether EPF would make larvae more susceptible to a second pathogen, larvae were inoculated through the last proleg with 20 μl of culture filtrate or Sabouraud dextrose and incubated at 20 °C. After 24 h larvae were given a second injection with *C. albicans* (1×10^5/20 μl, culture as above), or PBS. There were ten larvae per treatment. Larvae were incubated at 20 °C and mortality was recorded for up to 14 days.

2.4. Protein sample preparation and mass spectrometry

Larvae were injected with 20 μl of culture filtrate or Sabouraud dextrose (controls) and incubated for 48 h at 20 °C. Five larvae per treatment were bled into a pre-chilled 1.5 ml centrifuge tube and spun at 1500 *g* for 5 min at 4 °C. Samples were diluted in PBS and a Bradford assay was carried out. Protein (100 μ*g*) was removed to a pre-chilled

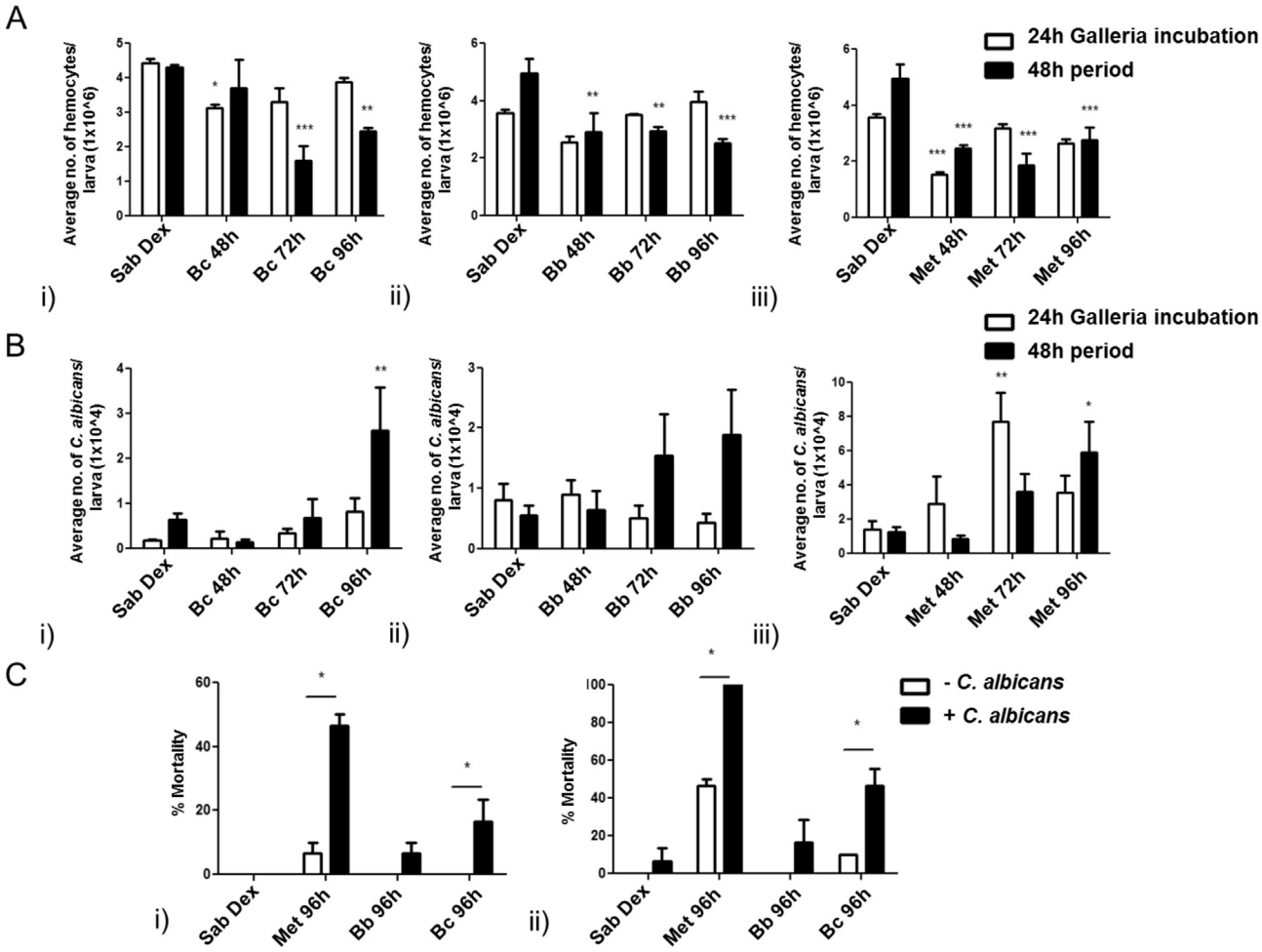

Fig. 1. Haemocyte and yeast cell densities and mortality in *G. mellonella* larvae pre-treated with EPF culture filtrate. (A) Haemocyte density (mean ± SE) in *G. mellonella* larvae treated with EPF culture filtrate. Following inoculation with fungal culture filtrate, larvae were incubated for 24 h or 48 h at 20 °C before bleeding and enumeration. Sab Dex: Control medium, (i) Bc: *B. caledonica*, (ii) Bb: *B. bassiana* and (iii) Met: *M. anisopliae*. X-axis represents length of time EPF was cultured for: 48 h, 72 h, 96 h. (B) Yeast cell density in *G. mellonella* larvae pre-treated with EPF culture filtrate. Number (mean ± SE) of *C. albicans* cells per larva after incubation for 24 h and 48 h at 20 °C. Larvae were treated with fungal culture filtrate 24 h prior to inoculation with *C. albicans*. Sab Dex: Control medium, (i) Bc: *B. caledonica*, (ii) Bb: *B. bassiana* and (iii) Met: *M. anisopliae*. X-axis represents length of time EPF was cultured for: 48 h, 72 h and 96 h. Asterisks indicate significant difference to relevant control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Mortality of *G. mellonella* larvae treated with EPF culture filtrate alone and in combination with *C. albicans*. + *C. albicans* indicates larvae that received a dose of *C. albicans* after 24 h, – *C. albicans* indicates larvae that did not. Sab Dex: Control media, Bc: *B. caledonica,* Bb: *B. bassiana* and Met: *M. anisopliae*. All EPF were cultured for 96 h. Mortality (i) one week and (ii) two weeks after infection with *C. albicans*. $^{*}p < 0.05$, $^{**}p < 0.01$.

1.5 ml centrifuge tube and ice cold 100% acetone was added at ratio of 1:3 (sample:acetone). Protein was precipitated at -20 °C. The sample was centrifuged at 13,000 *g* for 10 min and the protein pellet was resuspended in 100 µl of resuspension buffer (6 M urea, 2 M thiourea, 5 mM calcium chloride). Protein (75 µg) was reduced with dithiothreitol (200 mM) and alkylated with iodoacetamide (1 M). Samples were digested with sequence grade trypsin (Promega, Ireland) at a trypsin:-protein ratio of 1:40, overnight at 37 °C. Four replicate samples were prepared for each treatment.

Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply Company, Ireland) and 1 µg of peptide mix was eluted onto a Q Exactive (ThermoFisher Scientific, U.S.A) high resolution mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient (2–40%) on a Biobasic C18 PicofritTM column (100 mm length, 75 mm ID), using a 120 min reverse phase gradient at a flow rate of 250 nL /min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A full MS scan at 140,000 resolution and a scan range of 300–1700 *m/z* was followed by an MS/MS scan, at resolution 17,500, to select the 15 most intense ions prior to MS/MS.

2.5. Quantitative mass spectrometry data analysis

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; http://maxquant.org/) to correlate the data against a 6-frame translation of the EST contigs for *G. mellonella* (Vogel et al., 2011). The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium (Côté et al., 2012) via the PRIDE partner repository with the dataset identifier PXD005939. Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.0.31. Label free quantification (LFQ) intensities were $\log_2$-transformed and *t*-tests comparing EPF treated larvae with controls were performed using a p-value of 0.05. Proteins were kept in the analysis if they were found in all 4 replicates in at least one group. Principal component analysis (PCA) was used to emphasize variation and visualize strong patterns in the data. Proteins found to be absent (below the level of detection) in one or more treatments and present (above the level of detection) in

three or fewer treatments were termed 'uniquely detected proteins'. These proteins were also used in statistical analysis of the total differentially expressed group following imputation of the zero values with values that simulate low abundant proteins. These values were chosen randomly from a distribution specified by a downshift of 2.19 times the mean standard deviation (SD) of all measured values and a width of 0.36 times this SD. To obtain an overall proteomic profile of abundance for all significantly expressed and exclusive proteins, hierarchical clustering on Z-score normalised intensity values was performed and the relative protein expression values were displayed as a heat map. The Blast2Go suite (www.blast2go.com) of software tools was utilized to assign gene ontology terms (GO terms) relating to biological processes, molecular function and cellular components.

2.6. Statistical analysis

Statistical analysis was carried out using Minitab V.16 statistical software and GraphPad Prism V.5. All data were first tested for normality, where data were found not to be normal, the data were transformed before further analysis was carried out. For alterations to haemocyte densities and yeast loads, data were analysed separately for each EPF species using two-way ANOVA with EPF culture time and assessment time (24 or 48 h post injection) as the factors. Bonferroni post hoc tests were used to compare EPF treatments to relevant controls. To determine whether EPF culture filtrate increases susceptibility of *G. mellonella* to a subsequent infection, data for yeast-injected and PBS-injected larvae were compared using paired *T*-tests.

3. Results

3.1. Alterations in haemocyte densities following injection of larvae with EPF culture filtrate

Larvae injected with all three EPF culture filtrates showed a significant alteration in haemocyte densities at 24 h and 48 h (Fig. 1A). Administration of all three EPF induced a decline in the haemocyte densities of larvae following inoculation with 72 h or 96 h culture filtrate and incubation for 48 h, relative to their appropriate controls. There was also a significant interaction between treatment and time in all EPF tested; treatments perform differently after different incubation periods.

3.2. Alterations in yeast cell density following injection of larvae with EPF culture filtrate

In larvae injected with *B. caledonica* culture filtrate, treatment had a significant effect on yeast cell density ($F_{3,16} = 5.01$, $p < 0.05$). Inoculation with *B. caledonica* 96 h culture filtrate resulted in a significant difference ($p < 0.01$) in yeast cell density, with a 3-fold increase relative to controls (Fig. 1B i), when the larvae were incubated for 48 h following injection with *C. albicans*. In larvae injected with *M. anisopliae* culture filtrate, treatment had a significant effect on yeast load ($F_{3,16} = 0.16$, $p < 0.001$). Treatment with *M. anisopliae* 96 h culture filtrate induced a significant alteration in yeast load ($p < 0.05$) with a fold increase of 3.76 relative to controls, when the larvae were incubated for 48 h following injection with *C. albicans* (Fig. 1B iii). Additionally, treatment with *M. anisopliae* 72 h culture filtrate caused a 4.42-fold increase ($p < 0.01$) when the larvae were incubated for 24 h following injection with *C. albicans*. In contrast, treatment with *B. bassiana* (48 h, 72 h, 96 h culture filtrate) did not cause a significant alteration in yeast cell density in larvae relative to controls (Fig. 1B ii).

3.3. Effect of EPF culture filtrate on susceptibility of larvae to subsequent infection

More than 40% of larvae died within two weeks of injection with *M. anisopliae* culture filtrate alone, while all other single species infections (*B. bassiana*, *B. caledonica* or *C. albicans* only) resulted in negligible mortality (Fig. 1C ii). Larvae that were treated with *M. anisopliae* or *B. caledonica* culture filtrate before treatment with *C. albicans* had higher mortality than larvae treated with either culture filtrate or *C. albicans* alone. The difference between treatments with and without *C. albicans* was significant one week (*M. anisopliae*: $T = -6.93$, $p < 0.05$; *B. caledonica:* $T = -4.74$, $p < 0.05$; Fig. 1C i) and two weeks after *C. albicans* infection (*M. anisopliae*: $T = 7$, $p < 0.05$; *B. caledonica:* $T = -4.75$, $p < 0.05$; Fig. 1C ii) with *M. anisopliae* and *C. albicans* combined infection causing 100% mortality after two weeks. This pattern was not seen with *B. bassiana*.

3.4. LFQ analysis of G. mellonella larval hemolymph following EPF culture filtrate treatment

Haemolymph of *G. mellonella* injected with EPF culture filtrate was subjected to LFQ proteomics to assess differences in protein abundance between treated and control larvae. In total, 100 proteins were identified with two or more peptides. Sixty-one of these proteins were either significantly altered in abundance or uniquely detected across the four treatments analysed (Supp. Table 1a and b). Ten proteins were found to be absent (below the level of detection) in one or more treatments and present (above the level of detection) in three or less sample groups. These proteins were termed 'uniquely detected proteins' (Table 1). These proteins were also used in statistical analysis of the total differentially expressed group following imputation (Fig. 3, Table 2) which resulted in the replacement of absent values (represented by NaNs or 0) with values that simulated low expression values. This permitted the treatment and visualisation of exclusively expressed and statistically significant proteins simultaneously. PCA was used to visualize the variation in protein abundances across all individual

Table 1
Proteins determined to be uniquely detected in one or more samples. Protein abundance is represented by the mean LFQ intensity values and the number of replicates (total n = 4) within which each protein was detected are given. n.d. = not detected.

Protein Annotation	Mean LFQ Intensities (log_2 transformed)				Number of replicates			
	Control	BB	BC	MET	Control	BB	BC	MET
Apyrase	4.5×10^7	8.3×10^7	0	7.3×10^7	2	3	0	4
Cecropin-D precursor	1.4×10^8	1.6×10^8	3.3×10^8	0	3	3	4	0
Peptidoglycan recognition-like	8.8×10^7	8.1×10^7	2.5×10^8	0	3	4	4	0
Aminoacylase-1-like	1.9×10^8	0	4.8×10^7	0	4	0	1	0
Prophenoloxidase activating factor 3	0	6.7×10^7	4.6×10^8	6.8×10^8	0	2	4	4
Gustatory receptor candidate 25	0	4.9×10^8	3.3×10^8	7.9×10^8	0	4	3	3
Contig22050_1	0	1.1×10^7	1×10^8	7.4×10^7	0	1	3	4
Beta actin	0	7.8×10^7	8.5×10^7	5.9×10^8	0	1	1	4
Peptidylprolyl isomerase B precursor	0	0	3.2×10^7	6.5×10^7	0	0	2	4
Contig20011_1.r1.	6.3×10^7	0	0	0	4	0	0	0

Table 2
Number of statistically significant differentially abundant (SSDA) proteins between EPF injected and control larvae. Annotations, mass spectrometry information and sequence characteristics for SSDA haemolymph proteins (two sample *t*-tests, $p < 0.05$) between treated and control larvae. Relative fold changes are given for EPF injected SSDA proteins with ↑ and ↓ representing higher or lower abundance relative to the controls.

Protein Annotation	Relative fold change			Intensity	MS/MS	Peptides	Mol. Weight (kDa)	Sequence length
	BB	BC	MET					
Gustatory receptor candidate 25	20.99↑	10.51↑	20.97↑	5.4×10^9	46	2	25.9	235
Scolexin	1.96↑	2.18↑	3.21↑	2.2×10^{10}	224	11	58.3	528
27 kDa hemolymph protein	1.68↑	1.98↑	3.06↑	8.1×10^9	125	7	44.7	405
Cationic peptide CP8 precursor	1.84↓	2.42↓	2↓	1.3×10^{11}	246	4	17.7	168
Contig04490_1	2.69↓	1.82↓	2.92↓	2.9×10^9	119	3	8.1	77
Heat shock protein 25.4 precursor	2.82↑	–	3.88↑	3.1×10^{10}	196	11	28.4	252
Contig_190.0	2.21↑	–	1.54↑	2.0×10^{10}	173	8	36.6	326
Bombyrin	1.72↓	–	1.84↓	3.3×10^{10}	166	7	37.3	322
Contig20220_1	1.97↓	–	3.5↓	2.8×10^{10}	162	10	14.8	133
Putative protease inhibitor 4	2.66↓	–	3.9↓	4.3×10^{10}	172	6	30.0	272
Aminoacylase	3.8↓	–	6.1↓	1.3×10^9	30	4	432	46.7
Contig19736_1	1.95↓	–	7.32↓	2.8×10^{10}	174	3	15.4	161
Serine proteinase	4.49↑	3.32↑	–	1.93×10^9	57	7	52.4	477
Prophenoloxidase activating factor 3	–	14.9↑	25.2↑	2.7×10^9	68	5	25.8	233
Contig_962.0	–	9.3↑	8.6↑	5.2×10^9	88	12	53.6	489
Contig_1159.0	–	4.5↑	7.3↑	1.1×10^9	41	2	35.3	330
Protease inhibitor 1 precursor	–	2.75↑	6.11↑	1.42×10^{10}	138	4	24.3	222
Prophenoloxidase activating enzyme 3	–	3.47↑	4.52↑	5.5×10^9	202	17	72.1	649
Peptidoglycan recognition protein S2 precursor	–	2.76↑	2.98↓	1.46×10^9	49	2	17.8	156
Gloverin-like protein	–	1.83↑	2.44↓	9.34×10^8	57	2	20.5	189
Alpha-esterase 45	–	2.06↓	2.03↓	3.77×10^{10}	338	13	33.6	291
Methionine-rich storage protein	–	2.25↓	2.39↓	6.47×10^{11}	823	20	15.2	129
Apolipophorin-2	–	1.76↓	2.88↓	1.25×10^{11}	288	8	8.7	78
Apolipoprotein D like	2.46↑	–	–	2.8×10^{10}	208	9	41.3	372
Alpha-esterase 45	2.35↑	–	–	6.6×10^9	104	9	71.2	670
Adhesion related protein, transmembrane	2.29↑	–	–	2.2×10^9	61	3	40.4	360
Integument esterase 2 precursor	1.97↑	–	–	5.6×10^{10}	425	11	56.5	503
Aldo-keto reductase AKR2E4-like	1.72↑	–	–	2.6×10^9	63	2	9.3	80
Chain A, Structure Of Active Serpin K	1.62↑	–	–	1.6×10^{11}	754	21	50.6	451
Contig_5682.0	1.75↓	–	–	1.3×10^{11}	333	11	28.0	245
Contig20011_1	2.04↓	–	–	4.4×10^8	24	2	57.6	539
Contig20268_1	2.21↓	–	–	5.7×10^9	61	9	25.2	222
Protease inhibitor-like protein	3.06↓	–	–	4.2×10^9	122	2	12.7	120
Glyceraldehyde-3-phosphate dehydrogenase	3.9↓	–	–	3.2×10^9	58	6	47.0	441
Serpin 3a	–	2.88↑	–	2.73×10^9	68	8	110.4	989
Beta-1,3-glucan-binding protein	–	2.4↑	–	$1.66x10^9$	44	2	23.4	214
Peptidoglycan recognition-like protein B	–	2.12↑	–	9.18×10^9	166	12	47.6	430
Insecticyanin	–	1.65↓	–	5.42×10^{10}	354	14	29.0	253
Beta Actin	–	–	14.8↑	1.9×10^9	39	6	30.1	279
Putative defense protein Hdd11	–	–	5.55↑	4.93×10^9	114	7	20.7	196
25 kDa silk glycoprotein	–	–	4.99↑	9.25×10^9	135	9	84.6	746
Contig22050_1	–	–	2.7↑	5.9×10^8	33	6	48.5	422
27 kDa hemolymph protein	–	–	2.31↑	6.88×10^{11}	1181	10	17.3	161
Peptidylprolyl isomerase B precursor	–	–	1.85↑	$3.1x10^8$	25	3	39.1	354
Twelve cysteine protein 1	–	–	1.63↑	1.8×10^{10}	185	7	35.6	320
Hemolin	–	–	1.63↑	3.96×10^{10}	161	6	15.3	140
Gelsolin	–	–	1.57↑	9.89×10^{10}	657	21	50.7	469
Methionine-rich storage protein 2	–	–	1.54↑	2.06×10^{12}	4415	68	56.7	489
Transferrin	–	–	1.57↓	3.81×10^{11}	1632	58	106.3	965
Hexamerin	–	–	1.68↓	2.5×10^{11}	1194	53	90.6	778
Aarylphorin	–	–	1.77↓	1.66×10^{11}	328	19	19.6	168
Sensory appendage protein 1	–	–	1.87↓	9.75×10^9	111	9	22.8	203
Imaginal disc growth factor-like protein	–	–	2.12↓	1.52×10^{11}	523	14	23.3	208
Contig19502_1	–	–	2.14↓	3.07×10^{10}	603	34	83.6	733
32 kDa ferritin subunit	–	–	2.2↓	3×10^{10}	204	9	43.0	381
Multicystatin and procathepsin F precursor	–	–	2.21↓	2.96×10^{10}	164	8	13.2	120
Nimrod B precursor	–	–	2.75↓	1.9×10^{10}	146	3	19.5	180
Contig22104	–	–	2.96↓	2.12×10^9	42	2	25.5	234
Carboxylesterase	–	–	4.71↓	5.83×10^9	52	6	30.2	272

replicates (Fig. 2A). Four distinct clusters were resolved representing each treatment, with each replicate resolving into their appropriate treatment. *M. anisopliae* was more divergent than the other EPF treatments relative to control larvae. Hierarchical clustering resolved four distinct clusters of proteins with similar expression profiles (Fig. 2B). Cluster A comprises proteins with higher levels of abundance in larvae treated with *M. anisopliae* and *B. caledonica* relative to control larvae and intermediate intensities in *B. bassiana* treated larvae. This cluster includes a number of proteins involved in the prophenoloxidase (ProPO) cascade; ProPO activating factor and enzyme 3. Cluster B comprises proteins with lower levels of abundance in larvae treated with *B. bassiana* relative to all other treatments and control larvae. Cluster C comprises proteins with lower levels of abundance in larvae treated with *M. anisopliae* relative to all other treatments and control

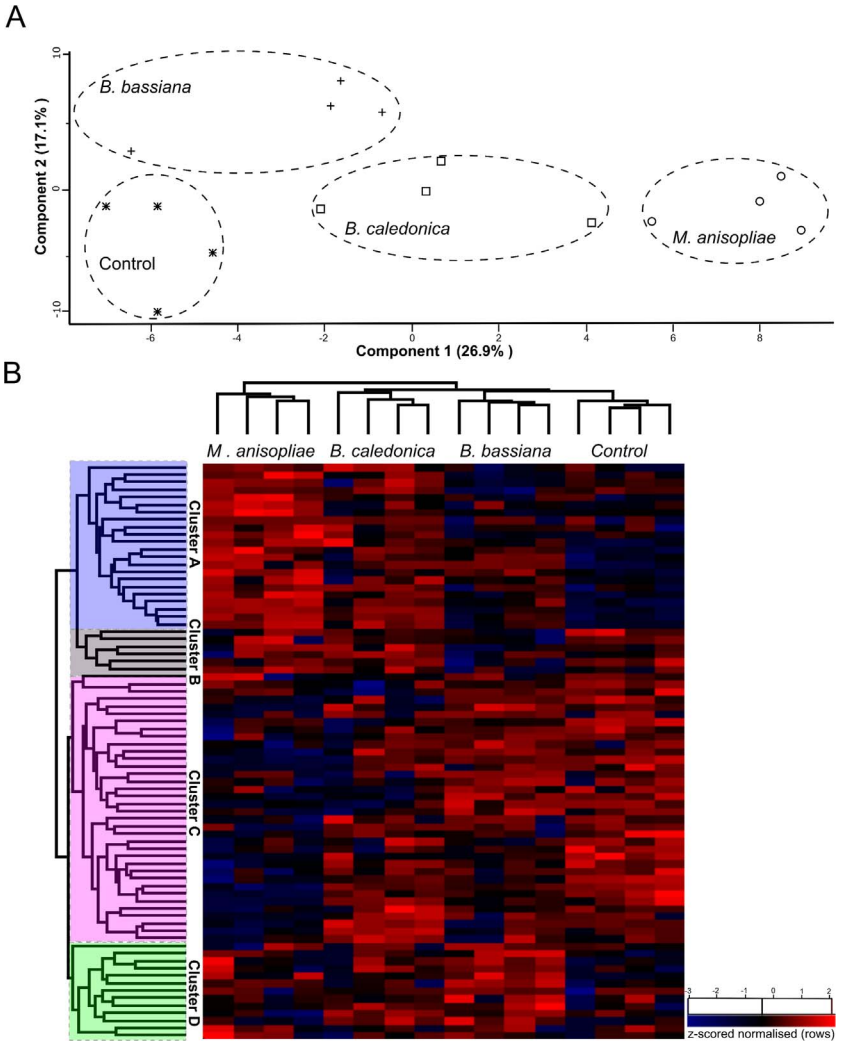

Fig. 2. Principal component analysis (PCA) and hierarchical clustering of hemolymph proteomic profiles of larvae treated with EPF culture filtrate versus control. (A) PCA of four replicates of each treatment included in LFQ analysis. Dashed circles denote sample groups. The two axes account for 44% of total variation within the dataset. (B) Heat map based on hierarchical clustering of the median protein expression values of all statistically significant differentially abundant and uniquely detected proteins. Hierarchical clustering (columns) resolved four distinct clusters comprising the replicates from their original sample groups and four protein clusters (rows) based on expression profile similarities.

larvae, including proteins involved in recognition; and peptidoglycan recognition-like protein. Cluster D comprises proteins with higher levels of abundance in larvae treated with *B. bassiana* relative to all other treatments and control larvae.

Blast2GO annotation software (www.blast2GO.com) was used to group proteins based on conserved gene ontology (GO) terms in order to identify processes and pathways altered in response to different EPF treatments. Changes in proteins involved in molecular and biological processes, as annotated by Blast2GO, is shown in Fig. 4.

4. Discussion

The aims of the experiments described here were to investigate immunomodulatory effects of EPF culture filtrate treatments on a model insect and specifically to determine whether EPF treatment rendered the insect more susceptible to subsequent pathogen attack. Understanding how insects resist and/or detoxify EPF secretory products and identifying the specific biological responses elicited in susceptible hosts is essential to the development of effective fungal biological control strategies (Rohlfs and Churchill, 2011). Our results give insight into the effect EPF culture filtrate has on the cellular and humoral immune response of *G. mellonella* larvae and provide information regarding the specific immunomodulation potential of EPF. *M. anisopliae* and *B. caledonica* 96 h culture filtrates exhibited clear immunomodulatory effects on *G. mellonella* larvae, increasing their susceptibility to pathogenic infection, whereas *B. bassiana* filtrate had no significant effect on susceptibility to pathogens.

4.1. *B. caledonica, B. bassiana and M. anisopliae culture filtrates induce varied immune responses in G. mellonella*

Culture filtrate of all three EPF caused a significant decline in the haemocyte density of larvae. Haemocyte death is important in innate immunity as haemocytes are associated with encapsulation and nodulation (Butt et al., 2016). Treatment with *B. caledonica* and *M. anisopliae* 96 h culture filtrate facilitated a significantly increased yeast cell density in larvae suggesting that filtrate components are modulating the immune system of the insect allowing a subsequent pathogen to proliferate. In contrast, treatment with *B. bassiana* culture filtrate did not allow a significant increase in yeast cell density in larvae relative to controls. Larvae co-injected with *M. anisopliae* or *B. caledonica* culture filtrate and *C. albicans* showed significantly increased mortality. The proposed immunomodulation that is rendering the host more susceptible to a subsequent pathogen is presumably caused by spore free culture filtrate, known to contain a diverse mixture of enzymes, proteases and secondary metabolites. Destruxin, the most abundantly

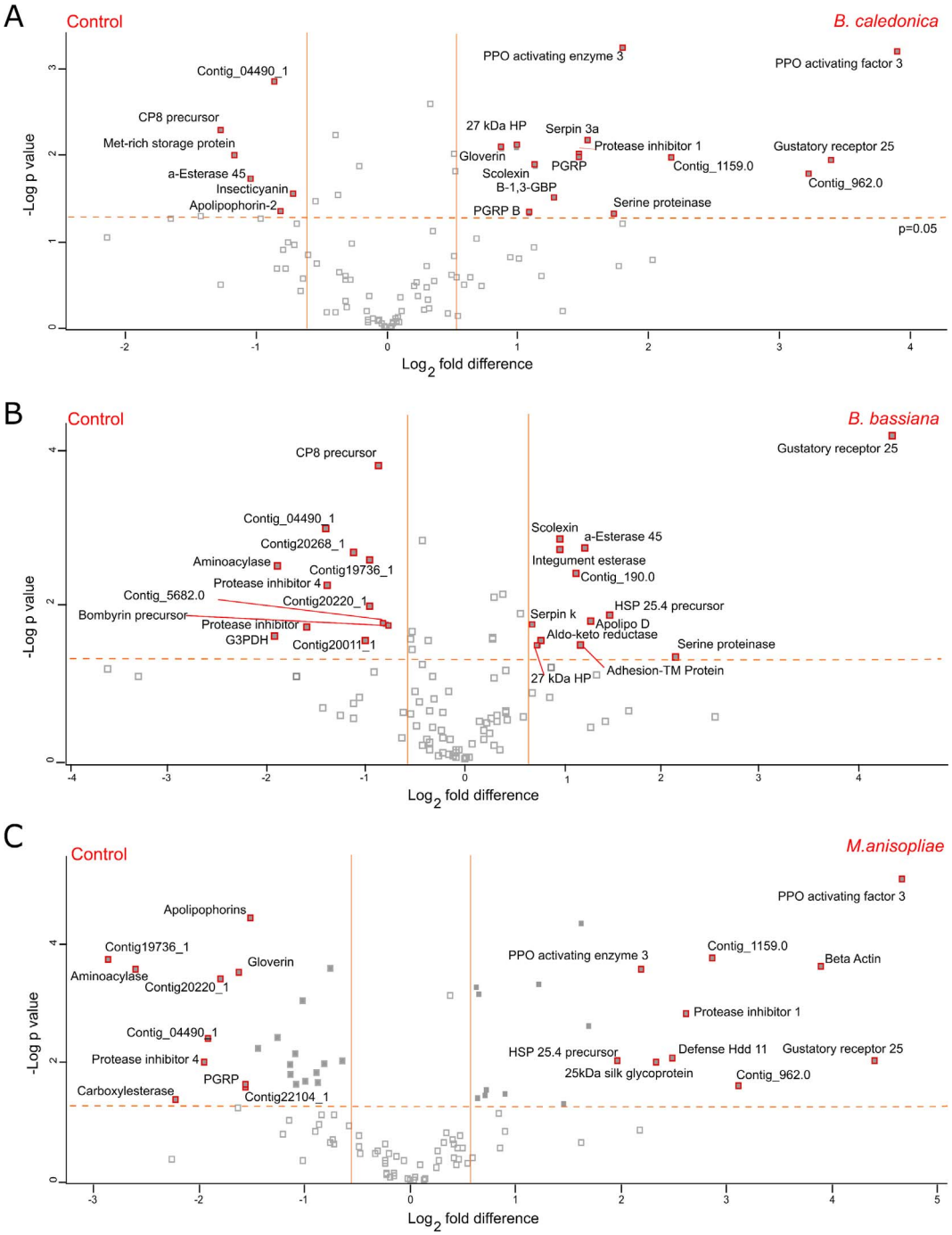

Fig. 3. Proteins altered in abundance in haemolymph of *G. mellonella* larvae following injection with EPF culture filtrate. Volcano plots represent protein intensity difference ($-\log_2$ mean intensity difference) and significance in differences ($-$ log P-value) based on a two-sided *t*-test. Proteins above the dashed line are considered statistically significant (p value < 0.05) and those to the right and left of the vertical lines indicate relative fold changes of ≥ 1.5. Annotations are given for the most differentially abundant proteins identified in larvae inoculated with (A) *B. caledonica*, (B) *B. bassiana and* (C) *M. anisopliae* culture filtrate versus control larvae (inoculated with Sabouraud Dextrose). These plots are based upon post imputed data.

produced secondary metabolite in *Metarhizium* spp., induced a similar response in *D. melanogaster* (Pal et al., 2007).

Proteomic analysis indicates that the response to the culture filtrate from *M. anisopliae* was the most divergent relative to the control, although some similarities in proteome alteration were observed between *B. caledonica* and *M. anisopliae*. This observation complements the bioassay results that show effects of *B. caledonica* are more similar to *M. anisopliae* than to *B. bassiana*. GO term annotation identified processes and pathways altered in response to the different EPF treatments with the *M. anisopliae* culture filtrate appearing to induce changes in the largest number of processes, including protein, lipid and ion binding.

B. bassiana treated larvae displayed higher abundances of alpha-esterase when compared to control larvae and larvae treated with *M. anisopliae* and *B. caledonica* culture filtrate. Insects infected with EPF often upregulate antioxidant genes, and fungal infection of insects is associated with increased total esterase and glutathione S-transferase activities in the hemolymph (Butt et al., 2016). Increased activity of detoxification enzymes represents the insect immune response to intoxication with metabolites produced by the pathogen or with

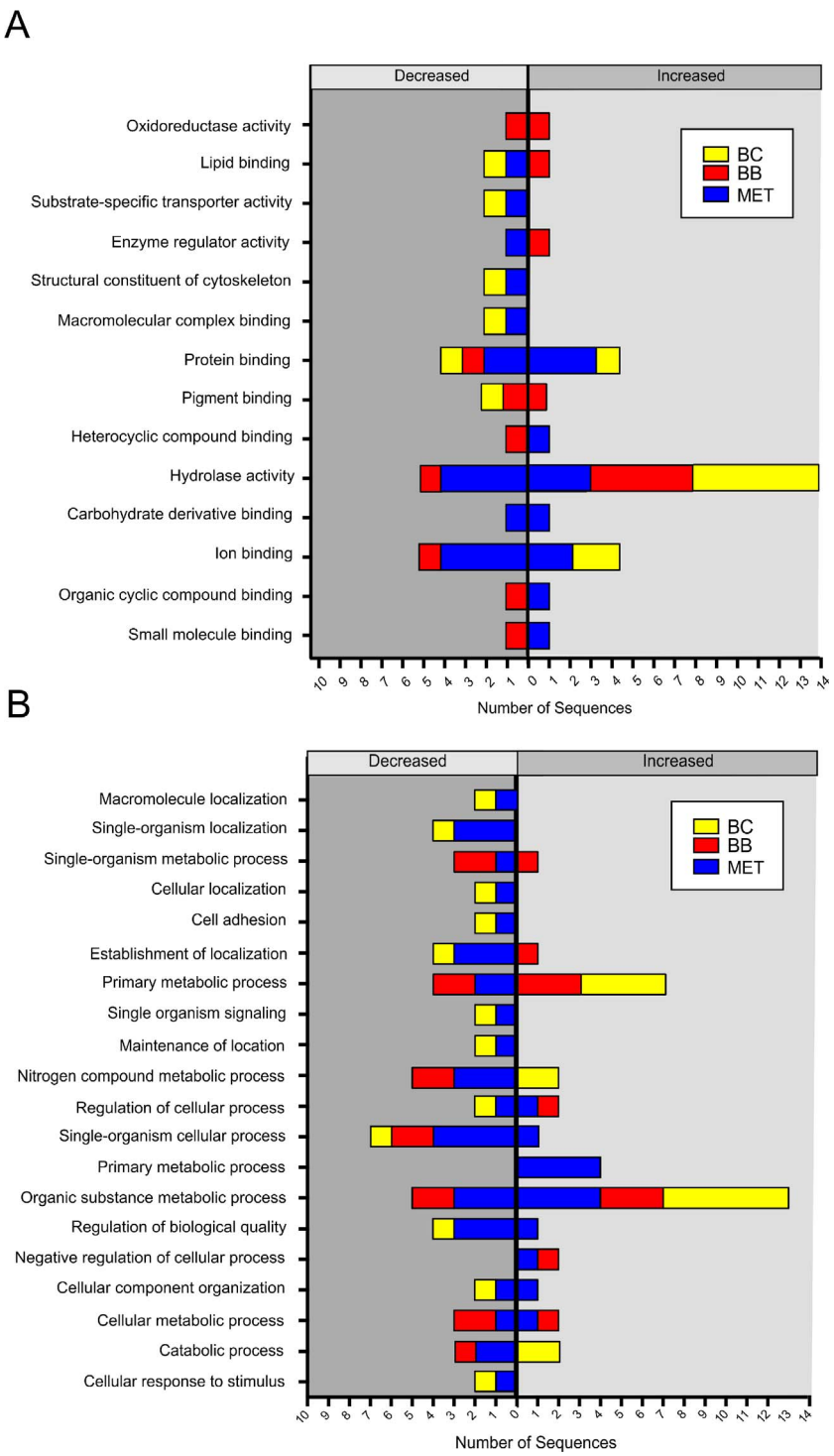

Fig. 4. Level 3 gene ontology terms for (A) molecular function and (B) biological process for proteins of altered abundance in *G. mellonella* larvae following injection with fungal culture filtrate.

products of host tissue degradation. Conversely, inhibition of detoxification enzymes increases insect death rate from fungal infection (Serebrov et al., 2006). Induction of additional esterase isoforms and increased glutathione S-transferase activity in *G. mellonella* larvae with mycoses can decrease their sensitivity to chemical insecticides (Hemingway and Ranson, 2000; Serebrov et al., 2006). A lethal dose of *M. anisopliae* was found to increase activity of detoxifying enzymes in the locust nymphs on the third day after infection, and a reduction in esterase and GST activity during acute mycosis was linked with effective inhibition of the host defense systems (Dubovskiy et al., 2012). Alpha esterase is decreased in abundance in *B. caledonica* treated larvae relative to control larvae, while alpha esterase and carboxylesterase is decreased in expression in *M. anisopliae* treated larvae. Thus it could be postulated that detoxifying enzymes induced by treatment with *B. bassiana* culture filtrate may have had some role in the reduction of susceptibility of those larvae to subsequent pathogen attack.

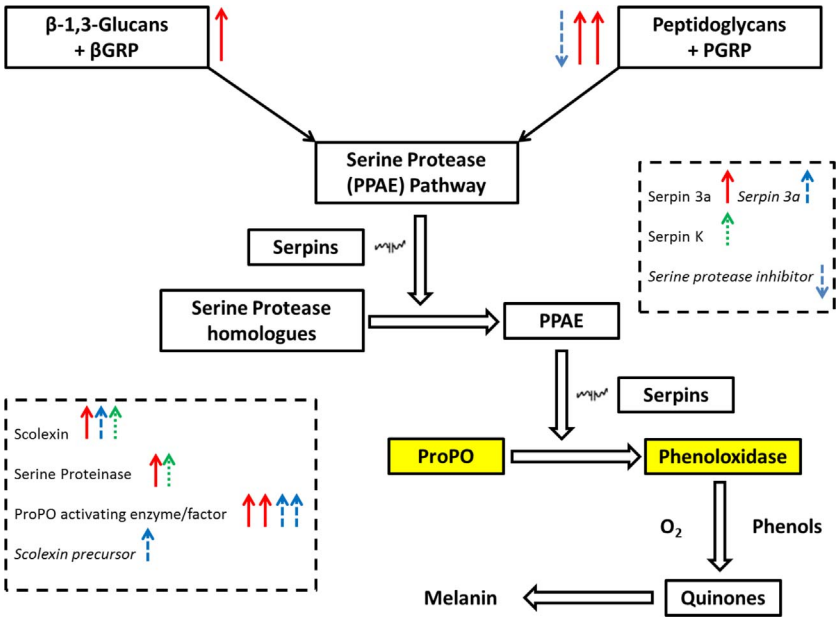

Fig. 5. Schematic overview of a general model of the proPO activation system in insects. Arrows denote changes in abundance of proteins associated with the proPO activation system following treatment with EPF culture filtrate. ↑ indicates increased abundance ↓ indicates decreased abundance. Solid arrows signify alterations following treatment with *B. caledonica*, dashed arrows *M. anisopliae* and dotted arrows represents *B. bassiana*. Italicized protein names signify changes observed also in 2D PAGE analysis (not presented here). Image adapted from González-Santoyo and Córdoba-Aguilar (2012).

4.2. B. caledonica and M. anisopliae culture filtrates impact upon the ProPO cascade in G. mellonella

LFQ analysis of the effect of *M. anisopliae* and *B. caledonica* culture filtrate on the proteome of *G. mellonella* hemolymph demonstrated an alteration in abundance of proteins implicated in the proPO (prophenoloxidase) cascade (summarised in Fig. 5), in both regulation and maintenance of PO (phenoloxidase). ProPO activating factor 3 (PPAF 3), proPO activating enzyme 3 (PPAE 3), serpin 3a, serine proteinase, protease inhibitor 1 precursor and scolexin were all increased in expression in larvae injected with *B. caledonica* filtrate. PO activities are vital for early host responses to EPF on the cuticle, during encapsulation responses to EPF in the haemocoel and for production of melanin and reactive oxygen and nitrogen species. Serine proteinases and the molecules they produce can be damaging to both the insect host and the fungal pathogen, affecting nucleic acids, peptides and oxidation of lipids (Jiang et al., 2010; Butt et al., 2016). Serpin 3 is increased in abundance following treatment with *B. caledonica*. Many serpins are irreversible inhibitors, key modulators of the proPO cascade and deactivators of microbe-derived proteases (Jiang et al., 2010; Butt et al., 2016). Serpin 3 was found to be constitutively expressed in *M. sexta* at low levels and increased in expression after microbial challenge. Recombinant serpin 3 blocks proPO activation in hemolymph and forms complexes with proPO activating proteinases (Zhu et al., 2003). Together these alterations in proPO pathway proteins (Fig. 5), indicate both a response in the insect to the treatment and an attempt to prevent the deleterious effects of uncontrolled PO activity (Sadd and Siva-Jothy, 2006).

A number of similarities were observed in the proteome response to *B. caledonica* and *M. anisopliae* culture filtrates which may explain similar susceptibilities to subsequent pathogens. ProPO activating factor 3, protease inhibitor 1 and proPO activating enzyme 3, for example, had increased abundance in *B. caledonica* and *M. anisopliae* treated larvae. Protease inhibitor 4 was downregulated in larvae treated with *M. anisopliae* and *B. bassiana*. Scolexin was observed in higher abundance in larvae following administration of all three EPF filtrates, but at a higher fold change in *M. anisopliae* and *B. caledonica*. Thus it seems that there is a clear similarity in proPO cascade protein profiles in larvae treated with the EPF filtrates, which may explain the similarity in pathogen susceptibility. This immunomodulation potential of EPF on the insect immune response may have implications for the utilisation of EPF as a biocontrol agent. The ability of EPF to suppress the insect immune system making it more susceptible to subsequent pathogens may potentially enhance EPF ability to act synergistically with other control agents in the field.

4.3. EPF culture filtrate alters abundance of pathogen recognition proteins in G. mellonella

Administration of three EPF culture filtrates resulted in increased abundance in a gustatory protein in the insect haemolymph. This may be linked to the fact that the production and maintenance of the proPO activating pathway is diet-dependent (Siva-Jothy and Thompson, 2002; González-Santoyo and Córdoba-Aguilar, 2012) and as there are changes to proteins related to the proPO pathway induced by all three EPF it may be necessary for the larvae to increase their dietary intake to sustain the cascade. The cost of activating and using immune defenses can itself reduce host fitness. Hosts may compensate for increased demands by increased resource uptake (Moret and Schmid-Hempel, 2000). A different explanation may be found in the work of Lee et al. (2010). They demonstrated that *Drosophila* can detect minute (0.05%) concentrations of DEET, the most widely used insect repellent worldwide, through the gustatory response and that it suppressed feeding. This behavior was mediated by direct activation of avoidance gustatory receptor neurons. Compounds that inhibit feeding have been shown to stimulate gustatory receptor cells in the mouthpart sensilla of certain orthopteran and coleopteran insects and a number of larval Lepidoptera (Chapman, 2003), highlighting the importance of gustatory receptors in toxin avoidance.

Administration of EPF culture filtrate to *G. mellonella* larvae impacted upon proteins instrumental in pathogen recognition that ultimately lead to activation of an appropriate immune response. Insects differentiate between major groups of microbes using PRRs such as peptidoglycan recognition proteins (PGRPs), hemolin and β-1,3-glucan binding protein (βGBPs). PRRs function by binding to PAMPs on microbial cells such as β-1,3-glucan from fungi that acts as a signal to activate the antifungal functions of Toll (Stokes et al., 2015). Three proteins involved in recognition were increased in abundance following

treatment with *B. caledonica*: βGBP and two PGRPs. These receptors are crucial to recognition of pathogens and activation of an appropriate immune response (e.g. proPO pathway). Both *M. anisopliae* and *B. caledonica* treated larvae display a lower abundance of apolipophorins which in addition to lipid transport can function as pattern recognition receptors by binding to bacterial and fungal cell walls (Zdybicka-Barabas and Cytryńska, 2013). *M. anisopliae* and *B. caledonica* treated larvae have a lower abundance of apolipophorins than larvae treated with *B. bassiana* or controls. In addition a PRR peptidoglycan recognition-like protein was observed in all samples except *M. anisopliae* treated larvae where it was undetected (essentially absent). These receptors are crucial to recognition of pathogens and the activation of an appropriate immune response such as the proPO pathway.

4.4. EPF culture filtrate alters antimicrobial peptide production in G. mellonella

Treatment with EPF culture filtrate had a significant effect on AMP abundances particularly in *M. anisopliae* treated larvae where cecropin-D precursor, hemolin and gloverin were differentially abundant in comparison to controls (Table 1 and 2). Cecropin-D precursor, a member of the 4 kDa cecropins found in Lepidoptera and Diptera, was detected in the haemolymph of all treatments except in those larvae treated with *M. anisopliae* culture filtrate. These proteins are membrane active antibiotics that act on Gram-positive and -negative bacteria by forming channels that permeabilise the lipid bilayer (Hoffmann, 1995; Jiang et al., 2010). Some cecropins also demonstrate antifungal activity (Faruck et al., 2015). Hemolin was only significantly increased in expression in *M. anisopliae* treated larvae. Hemolin is a 47 kDa hemolymph protein present at a low constitutive level that is increased upon bacterial challenge. It is thought to have a role in immune recognition and in modulation of defensive responses in *H. cecropia* and *M. sexta*. Hemolin has a role in antibacterial defense indicated by its structural similarity to cell adhesion molecules and its increased expression induced by bacteria. Hemolin can bind to surfaces of bacteria and haemocytes and can stimulate the phagocytic activity of haemocytes (Gillespie et al., 1997, 2000). A glycine rich AMP peptide gloverin was significantly decreased in expression in *M. anisopliae* treated larvae only. Glycine rich peptides have antifungal activity and are active against yeasts (Faruck et al., 2015) so their downregulation may contribute in part to the increased proliferation of *C. albicans* in larvae pretreated with *M. anisopliae* culture filtrate. Gloverin was increased in abundance in larvae treated with *B. caledonica* culture filtrate. This AMP is effective against Gram-negative bacteria. Gloverin acts by inhibiting the synthesis of vital outer membrane proteins resulting in an increase in permeability of the outer membrane of the bacteria (Bulet et al., 1999). One implication of an upregulation in AMP active against bacteria, is that antibacterial activities can be highly beneficial to EPF as it might help exclude opportunistic infections that would be disadvantageous to the fungus (Butt et al., 2016).

The lower abundance of proteins, such as cecropin and PRR, that are instrumental in the insect's ability to recognise and respond to pathogens are indicative of *M. anisopliae* immunomodulation potential observed in bioassays. The immune responses in this work are induced by cell- and spore-free culture filtrate which contains many secreted products. A direct relationship has previously been established between destruxin production and the virulence of *M. anisopliae*. Pal et al. (2007) injected *Drosophila* with destruxin that lead to a reduction in expression of AMP genes including *Cecropin.* Co-injection of destruxin with *E. coli* caused a significant decrease in survival of *Drosophila*. Thus it was suggested that a lowering in AMP production induced by destruxin allows *E. coli* to proliferate and colonize the fly to accelerate its demise (Pal et al., 2007). Through selectively reducing bacterial AMPs with the secretion of destruxin *M. anisopliae* may create an advantageous environment where bacteria can proliferate and, thereby, contribute to accelerating the demise of its insect host (Rohlfs and Churchill, 2011). Pal et al. (2007) complements the observation in this work that treatment with *M. anisopliae* culture filtrate causes a reduction in cecropin abundance among other immune relevant proteins and in parallel there is increased yeast proliferation and mortality when *G. mellonella* are injected with fungal culture filtrate before injection with *C. albicans*. Thus suggesting that EPF culture filtrate and the secreted products contained within can suppress the immune response of insects.

5. Conclusion

Understanding how EPF modulate the immune response leaving insects more susceptible to subsequent pathogens may have application in selecting superior strains with these characteristics to overcome problems with EPF killing target pests too slowly or inefficiently compared to their chemical counterparts. Additionally, EPF isolates could be screened for their ability to produce particular secreted products that induce immunomodulation in target insects. This aim of this work was to investigate the effect of culture filtrates from three EPF species (two widely used EPF and one less well studied species) on the insect immune response using *G. mellonella* larvae as a model system. The immune responses induced in *G. mellonella* were in response to injection with spore free culture filtrate, so it is a reflection of the immune response induced by EPF secreted products. A number of fungal secreted products are known to be important virulence determinants. It has been previously demonstrated that abundant EPF secreted products such as destruxin and oosporein induce changes to immune response of insects affecting AMP and the proPO cascade as well as the cellular immune response. These findings aid in understanding how the desired synergism between biocontrol agents could mechanistically occur e.g. interfering with the proPO cascade and the production of AMP.

Bioassays allowed assessment of the immunomodulation of different treatments and proteomic analysis aided in understanding mechanistically how these variations may have occurred e.g. alterations to proteins/pathways that may render the insect more susceptible to subsequent pathogens. The results indicate that *M. anisopliae* and *B. caledonica* culture filtrate exhibit an immunomodulation effect on *G. mellonella* larvae. Together the bioassay results and proteomic profiling indicate that *M. anisopliae* culture filtrate has the greatest impact on the insect immune system. Differences in proteomic profiles for different treatments may reflect or even partial explain the differences observed in bioassays. For example, higher levels of detoxifying enzymes in *B. bassiana* treated larvae than in those treated with *M. anisopliae* and *B. caledonica* may be one alteration reflective of their differences in immunomodulation. This proteomic work gives an insight into the mechanisms behind the observed differences in immunomodulation of the tested EPF and is intended to fit into the wider investigation of EPF immunomodulation in insects.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

All proteins identified from the haemolymph of EPF supernatant treated and control *Galleria mellonella* larvae. Statistically significantly differentially abundant proteins (2 sample *t*-tests; $p < 0.05$) and relative fold change differences for all comparisons for the post imputed data set and the exclusively expressed proteins (pre imputation) are also given.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2017.05.009.

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