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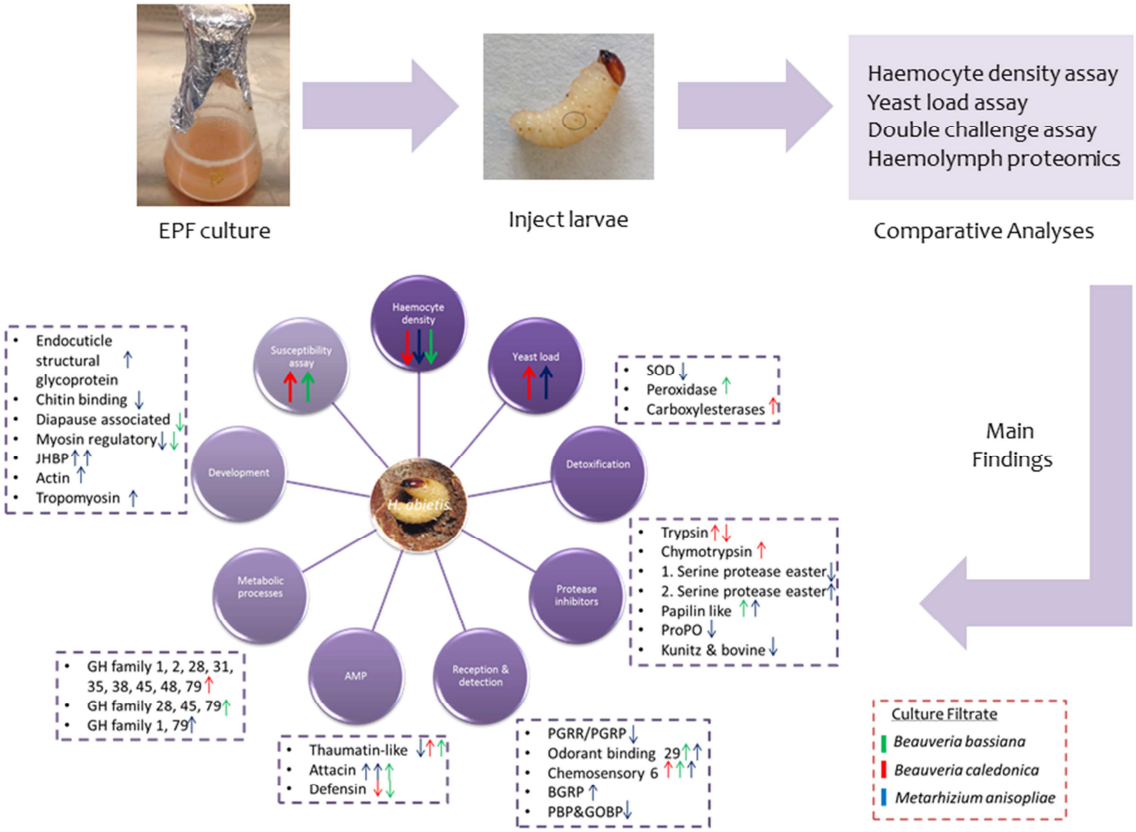

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The effect of entomopathogenic fungal culture filtrate on the immune response and haemolymph proteome of the large pine weevil, *Hylobius abietis*.

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Abstract

The large pine weevil *Hylobius abietis* L. is a major forestry pest in 15 European countries, where it is a threat to 3.4 million hectares of forest. A cellular and proteomic analysis of the effect of culture filtrate of three entomopathogenic fungi (EPF) species on the immune system of *H. abietis* was performed. Injection with *Metarhizium brunneum* or *Beauvaria bassiana* culture filtrate facilitated a significantly increased yeast cell proliferation in larvae. Larvae co-injected with either *Beauvaria caledonica* or *B. bassiana* culture filtrate and *Candida albicans* showed significantly increased mortality. Together these results suggest that EPF culture filtrate has the potential to modulate the insect immune system allowing a subsequent pathogen to proliferate. Injection with EPF culture filtrate was shown to alter the abundance of protease inhibitors, detoxifing enzymes, antimicrobial peptides and proteins involved in reception/detection and development in *H. abietis* larvae. Larvae injected with *B. caledonica* culture filtrate displayed significant alterations in abundance of proteins involved in cellulolytic and other metabolic processes in their haemolymph proteome. 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.

Keywords

Large pine weevil, entomopathogenic fungi, proteomic, transcriptome, immunomodulation, biocontrol

Abbreviations

PO, phenoloxidase; PPO, prophenoloxidase; EPF, entomopathogenic fungi; PCA, principal component analysis; AMP, antimicrobial peptide; LFQ, label free quantification; GH, glycoside/glycosyl hydrolase

1. Introduction

There is increasing interest in the exploitation of entomopathogenic fungi (EPF), particularly *Beauveria* spp and *Metarhizium* spp., for the biological control of insect pests. Biocontrol agents can be deployed where use of chemical pesticides is restricted or where resistance has developed. The large pine weevil *Hylobius abietis* L. is a major forestry pest in 15 European countries, where it is a threat to 3.4 million hectares of forest (Långström and Day 2004). Until recently, young trees were protected with cypermethrin or alpha cypermethrin as a control measure, but is no longer permitted in forests certified as sustainably managed. All stages of *H. abietis* are susceptible to strains of *Metarhizium* and *Beauveria* in laboratory assay (Ansari and Butt, 2012), but the performance of EPF in field trials has been disappointing (Williams et al., 2013). Failure of biocontrol agents to live up to expectations in the field is not uncommon. One approach to improving the success of biocontrol is to deploy a combination of agents against the pest. Synergistic interactions, where the success of the combination is greater than that of the individual agents, are frequently reported between EPF and other pathogens including nematodes (Ansari *et al*. 2006; 2008; Anbesse, 2008) and bacteria (Wraight and Ramos, 2005; Sayed and Behle, 2017). Synergy may result from the combined agents rendering the host more susceptible through modulating its immune system, prolonging developmental stages or by the two treatments acting on different components of the host population (Lacey *et al.* 2015).

The ability to modulate the immune response of an insect rendering it more susceptible to other pathogens would have great significance for integrated pest management. Both insects and their pathogens must constantly improve their defence and virulence, respectively, to survive (Wojda 2016; Joop and Vilcinskas, 2016). The insect immune system is composed of the cellular and humoral defences (Hoffmann 1995). Humoral defences include antimicrobial

peptides (AMPs), production of reactive forms of oxygen and nitrogen, soluble effector molecules and cascades that regulate clotting and melanisation of insect haemolymph (Strand 2008). Cellular defences encompass haemocyte mediated defences (Lavine and Strand 2002). There is an overlap between humoral and cellular defences in the recognition of pathogens; many humoral factors regulate the activity of haemocytes and haemocytes produce many humoral defence molecules such as defence peptides and stress proteins (Strand 2008, Grizanova et al. 2014, Wojda 2016).

Host colonization by EPF requires the ability to cope with host immune defences 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); 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). *Beauvaria bassiana* is known to produce cyclic peptides that are cytotoxic and immunosuppressive (Hung et al., 1993) and a diverse selection of secondary metabolites including nonpeptide 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).

Here we report a cellular and proteomic analysis of the effect of culture filtrate of three EPF species on the immune system of *H. abietis*. The primary aim of this work was to investigate the immunomodulatory potential of EPF on the insect immune response. This was achieved in part using label free quantitative (LFQ) mass spectrometry to investigate proteomic expression of pine weevils exposed to EPF extracts, a strategy that has been successfully applied to the lepidopteran *Galleria mellonella* (McNamara et al., 2017). To facilitate the proteomic analysis and to compensate the lack of genomic information for *H. abietis*; a *de novo* transcriptome for *H. abietis* was produced. The three species of EPF chosen for this work were *M. brunneum (Petch)(Met52), B. bassiana* and *B. caledonica*. *Beauveria bassiana*

and *Metarhizium* spp. are two of the most commonly employed EPF in biocontrol. Both have a wide host range and global distribution, and are used to control plant pests and vectors of human disease (Shah and Pell, 2003; Glare et al., 2008; Gao et al., 2011; Xiao et al., 2012; Lacey et al., 2015; Butt et al., 2016). *Beauveria caledonica* was found to be a naturally occurring pathogen of pine bark beetles in New Zealand (Glare et al., 2008; Reay et al., 2008) and *H. abietis* in Ireland (Glare et al., 2008; Williams et al., 2013). Morphologically, *B. caledonica* is similar to *B. brongniartii*, with cylindrical conidia, but molecular studies (Glare & Inwood 1998; Rehner and Buckley 2005), including the current research, have shown the species to be distinct. In contrast, *B. bassiana* has globulose conidida.

2. Materials and Methods

2.1 Origin of EPF strains and preparation of culture filtrate

A commercial strain of *M. brunneum* (Met52; previously *M. anisopliae*) produced by Novozymes (Denmark) was used and 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, ITS4, 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 Hylobius abietis *larvae*

Late instar *H. abietis* larvae were collected from pine stumps and stored at 4 °C for a maximum of 3 weeks until used in experiments. For each of the laboratory bioassays larvae were injected with fungal culture filtrate through an abdominal spiracle using a Myjector

U100 insulin syringe (Terumo Europe, Leuven, Belgium). Larvae were placed in 24 well culture plates (Costar) with filter paper and stored 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 by Bergin *et al*., (2003). All experiments were performed with three biological replicates.

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 ^{o}C, after which they received a second inoculation through an abdominal spiracle with *Candida albicans* (10^4 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). In each of the three replications, five larvae were injected per treatment and time. 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 phosphate buffered saline (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 calculated per larva.

To test whether EPF would make larvae more susceptible to a second pathogen, larvae were inoculated through an abdominal spiracle 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* ($1x10^4$/20 µl, culture as above), or PBS. In each of the three replications, ten larvae were injected per treatment and time. Larvae were incubated at 20 °C and mortality was recorded for up to 14 days.

2.4 RNA extraction of H. abietis larvae

One larva was crushed to a fine powder in liquid nitrogen using a sterilized pestle and mortar. Trizol was added and the sample was homogenized with a power pestle. The sample was spun at 13,000 x g for 10 minutes at 4 °C. Then, 200 µl of chloroform was added to the

sample, vortexed and left at room temperature for 10 min. The sample was spun at 12,000 x g for 10 min 4 °C and the top clear layer was removed to a fresh centrifuge tube. Isopropanol was added to the clear layer and inverted several times. The sample was left for 10 min at room temperature. The sample was spun again at 12,000 x g for 10 min, and the resulting pellet was washed in 70 % ethanol. The sample was spun to remove ethanol, the pellet was allowed to air-dry. The pellet was resuspended in 100 µl of elution buffer (Sigma GenElute Mammalian Total RNA Miniprep Kit). A Sigma GenElute Mammalian Total RNA Miniprep Kit and protocol was used to do complete extraction of the sample. This method was carried out twice: 1. Untreated *H. abietis* larva, and 2. *H. abietis* larva injected with *M. brunneum* culture filtrate.

2.5 H. abietis transcriptome

The *H. abietis* transcriptome *de novo* study was completed by Beijing Genomics Institute (BGI, Hong Kong) using Illumina HiSeq 4000. After extraction of total RNA and treatment with DNase I, Oligo (dT) adapters were used to isolate mRNA. The mRNA was fragmented by mixing with the fragmentation buffer and cDNA was synthesized using the mRNA fragments as templates. Short fragments were purified and resolved with elution buffer for end reparation and single nucleotide A (adenine) addition. The short fragments were connected to adapters and suitable fragments were selected for the PCR amplification. During the QC steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used in quantification and qualification of the sample library. Then the library was sequenced using an Illumina HiSeq 4000. After sequencing, the raw reads were filtered for low-quality, adaptor-polluted and high content of unknown base (N) reads to get clean reads. *De novo* assembly was performed using Trinity with clean reads to obtain the Unigene set. After that, simple sequence repeats (SSR) detection, Unigene expression analysis, heterozygous single nucleotide polymorphisms (SNP) detection, and Unigene functional annotation were performed. Unigenes were divided into two classes; clusters with the prefix “CL” (comprising several Unigenes with sequence similarity of 70% and above) and singletons with the prefix “Unigene”. The predicted protein sequences for the *H. abietes* transcriptome was analysed using InterProScan (version 5.18-57.0) to provide functional annotations based on protein family (Pfam) domains (Jones et al., 2014). To assess the completeness of *H.*

abietis assembled transcriptome a BUSCO (Benchmarking Universal Single-Copy Orthologs) (Simão *et al.* 2015) assessment was carried out on the predicted nuceotide and protein fasta files. Raw sequence reads were deposited in the Sequence Read Archive (SRA) hosted by the National Center for Biotechnology Information under BioProject PRJNA419715 (https://www.ncbi.nlm.nih.gov/bioproject/419715) and BioSample SAMN08095620 https://www.ncbi.nlm.nih.gov/biosample/SAMN08095620/.

2.6 Protein sample preparation and mass spectrometry

Larvae were either injected with 20 µl of fungal culture filtrate or Sabouraud dextrose (procedural control) 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 1,500 x g for 5 min at 4 °C. Samples were diluted in PBS and a Bradford assay was carried out to determine protein quantity. Protein (100 µg) was removed to a pre-chilled 1.5 ml centrifuge tube and ice cold 100 % acetone was added at ratio of 1:3 (sample: acetone) and precipitated at -20 °C. The sample was centrifuged at 13,000 x 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 dithiotreitol (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. Three 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 QExactive (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.

2.7 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 predicted protein set derived from the *H. abietis de novo* transcriptome generated in this study. To insure that all identified proteins were derived from the insect and not the fungal supernatant an additional search of all MS data was performed against the predicted protein set for *Beauveria bassiana* (Joint Genome Institute, downloaded April 2018) derived from the *B. bassiana* genome initiative (Xiao et al., 2012). 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 PXD008232. 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 3 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 1.7 times the mean standard deviation (SD) of all measured values and a width of 0.33 times this SD. Volcano plots were generated in Perseus to visualize differentially abundant proteins between control and treatment groups by plotting negative log p-values from pairwise Student's t-tests on the y-axis and $\log_2$ fold-change values on the x-axis for each pair-wise comparison. Proteins with a p-value <0.05 were considered statistically differentially abundant. To reduce the numbers of proteins with minor fold changes proteins with a relative fold change < 1.5 were removed from the analysis. To obtain an overall proteomic profile of abundance for all significantly

expressed and exclusive proteins, hierarchical clustering (displayed as a heat map) on Z-score normalised intensity values was performed to resolve clusters of proteins with similar abundance and expression profiles. The Blast2Go suite (Conesa *et al.*, 2005; www.blast2go.com) of software tools was utilized to BlastP search all identified proteins against the NCBI non redundant database with the following search settings: number of blast hits: 5, high-scoring segment pair length cutoff: 33, Blast expect value: 1.0e-5. Blast2Go was used to assign gene ontology (GO) terms for biological processes, molecular function and cellular components in addition to enzyme codes and InterPro identifiers for all proteins. Annotations derived from the InterProScan-based Pfam annotation of the *H. abietis* transcriptome were used to provide annotations in cases where Blast2Go failed to provide one.

2.8 Statistical analysis

Statistical analysis was carried out using Minitab version16 statistical software and GraphPad Prism version 5. All data were first tested for normality, where data were found not to be normal, the data were either transformed before further analysis was carried out or a suitable non-parametric test was used. For alterations to haemocyte densities and yeast densities, data were analysed 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 *H. abietis* 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 both 24 h and 48 h (*B. caledonica*; $F_{3,16}=8.21$, $p<0.01$, *B. bassiana*; $F_{3,16}=49.36$, $p<0.001$ and *M. brunneum*; $F_{3,16}=8.89$, $p<0.001$) (Figure 1A). All significant differences in haemocyte densities between treatments and their appropriate controls were in

the direction of reduction. All three EPF caused a decrease in the haemocyte densities of larvae following inoculation with 96 h fungal culture filtrate relative to their appropriate controls.

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

Injection of larvae with *B. caledonica* culture filtrate did not have a significant effect on yeast cell density in larvae (Figure 1Bi). In larvae injected with *M. brunneum* and *B. bassiana* culture filtrate, both treatment (*M. brunneum*; $F_{3,16}$=36.85, p<0.001, *B. bassiana;* $F_{3,16}$=20.93, p<0.001) and time (*M. brunneum*; ($F_{3,16}$=36.43, p<0.001), p<0.001, *B. bassiana* $F_{3,16}$=77.85, p<0.001) had a significant effect, there was also a significant interaction between treatment and time (*M. brunneum*; ($F_{3,16}$=12.96, p<0.001), p<0.001, *B. bassiana* $F_{3,16}$=6.62, p<0.01). After a 24 h incubation, injection with *M. brunneum* 48 h, 72 h, and 96 h culture filtrate resulted in a significant alteration in yeast density, with a fold increase of 6.2, 2.1 and 16.8 respectively, relative to controls. After 48 h incubation, injection with 72 h and 96 h *M. brunneum* culture filtrate resulted in a significant alteration in yeast density, with a fold increase of 12.8 and 20.8 respectively, relative to controls (Figure 1Biii). After a 48 h incubation, injection with *B. bassiana* 72 h culture filtrate resulted in a significant alteration in yeast density (p<0.001), with a fold increase of 44.4 (Figure 1Bii).

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

Larvae injected with a combination of fungal culture filtrate and *C. albicans* showed higher mortality than those that received fungal culture filtrate only, and the difference was significant for both *B. bassiana* (T=-17, p<0.01) and *B. caledonica* (T=-4.91, p<0.05) (Figure 1C). Larvae that were injected with EPF culture filtrate before treatment with *C. albicans* also had higher mortality than larvae treated with *C. albicans* alone, where no death occurred.

3.4 Transcriptome of H. abietis larvae

Approximately 17.7 Gb bases in total were generated after Illumina Hiseq sequencing. After assembly 49,960 unigenes were generated, with a total length of 59,001,875 bp, an average

length of 1,180 bp, N50 of 2,241 bp, and GC content 39.27 %. The unigenes were annotated with 7 functional databases; NR (Non Redundant Protein), NT (Non Redundant Nucelotide), GO (Gene Ontology), COG (Clusters of Orthologous Groups of proteins), KEGG ((Kyoto Encyclopedia of Genes and Genomes), Swissprot and Interpro. With functional annotation, 27,653 coding domains (CDS) were detected, and after ESTScan with the remaining unigenes, a further 2,936 CDS were found. 3,121 SSRs were detected on 2,788 unigenes. To determine the comprehensiveness of the assembly and annotation a BUSCO (Benchmarking Universal Single-Copy Orthologs) assessment was carried out on the nucleotide fasta file. The BUSCO analysis was conducted using the single-copy ortholog set of 1,658 conserved genes derived from the class Insecta. The *H. abietis* transcriptome used in this study contained ~97% (1,607/1,658) of the queried BUSCO orthologs (1214 Complete and single-copy, 340 Complete and duplicated, 53 Fragmented and 51 Missing). These conserved genes are expected to be present in the genomes of all Insecta species and given that a transcriptome captures what is constitutively expressed at the time of RNA extraction, the high number of BUSCO orthologs identified here indicates that our pine weevil transcriptome is particularly comprehensive.

3.5 LFQ analysis of H. abietis larval hemolymph following EPF culture filtrate treatment

Label free quantification (LFQ) was used to compare the haemolymph proteome of *H. abietis* larvae treated with EPF filtrate relative to control larvae. The groups analyzed were larvae treated with *M. brunneum, B. caledonica* and *B. bassiana* culture filtrate grown for 96 h and control larvae (treated with sabouraud dextrose media). In total, 157 proteins were identified, 155 proteins having two or more peptides (Table S1). Seventy seven of these proteins were either significantly changed in abundance (Table 1) or uniquely detected across the four treatments analyzed. Across the four sample groups, 43 proteins were deemed to display exclusive distribution either being present in at least one group but undetected in one or more groups. These proteins were termed ‘uniquely detected proteins’; notably, several of these were glycosyl hydrolase proteins uniquely detected in *B. caledonica* treated larvae only. These proteins were included in subsequent statistical analysis of the total differentially expressed group following imputation.

Proteomic analysis indicates that the larval response to the culture filtrate from *B. caledonica* was the most divergent relative to the control; this is evident in the PCA analysis (Figure 2 A). Hierarchical clustering resolved proteins that had similar expression profiles in response to treatment with different fungal culture filtrates (Figure 2B) and a number of protein clusters were identified (Table S2). Cluster A comprises proteins with higher levels of abundance in larvae treated with *M. brunneum* and *B. caledonica* relative to control larvae and includes several proteins involved in sensing and recognition such as odorant-binding 29, chemosensory 6 and β-1,3-glucan-binding. Cluster B comprises proteins with higher levels of abundance in larvae treated with *B. caledonica* relative to all other treatments and control larvae. It consists of a number of proteins involved in metabolic processes including numerous members of glycosyl hydrolase families 1, 2, 20, 28, 31 35, 45, 48 and 79 a member of the carboxylesterase family. Cluster C comprises proteins with lower levels of abundance in larvae treated with *M. brunneum* relative to all other treatments and control larvae. It includes proteins that may be involved in the proPO cascade such as serpin, serine protease easter and serine protease persephone isoform X2. Cluster D comprises proteins with higher levels of abundance in control larvae relative to all EPF treated larvae and includes the antimicrobial peptide (AMP) defensin. Two sample t-tests ($p<0.05$) identified 23, 37 and 33 statistically significant differentially abundant (SSDA) proteins in pine weevils treated with *B. bassiana, B. caledonica* and *M. brunneum* culture filtrates in comparison to control treatments, respectively (Table S3).

The Blast2GO annotation software was used to group proteins based on their conserved gene ontology (GO) terms in order to identify processes and pathways affected by the different EPF treatments (Figure 4; Table S4). In relation to cellular processes *M. brunneum* appears to affect the largest number of process in the insect haemolymph, with *M. brunneum* and *B. caledonica* affecting the most processes in common. In relation to molecular function *M. brunneum* appears to affect the largest number of functions in larval haemolymph including ion binding and hydrolase activity. *M. brunneum* and *B. bassiana* affect the most functions in common. While *B. caledonica* affects the least functions it has 25 proteins involved in hydrolase activity alone. In relation to biological processes *M. brunneum* appears to affect in the largest number of processes in the larval haemolymph. While *B. caledonica* affects the

359 least number of processes, it affects 23 proteins involved in organic substance metabolic
360 process and 22 proteins involved in primary metabolic process (Figure 4).

361 A number of the identified haemolymph proteins are typically associated with the insect
362 digestive system, highlighting the potential that the EPF filtrate damaged or altered midgut
363 cells which resulted in their release into the heamolymph. However, seven glycosyl
364 hydrolases were detected in the control samples (Table S5) which suggests that their presence
365 is not directly associated with exposure to EPF filtrate. To account for the possibility that
366 some of the identified proteins were components of the injected EPF filtrate MS data was
367 searched against the predicted proteome for *B. bassiana*. Only seven proteins were identified
368 with two or more peptides (Table S5) many of which are highly conserved eukaryotic
369 proteins. In addition a number of serine proteases annotated as trypsin or chymotrypsin were
370 identified in pine weevil haemolymph. These annotations were based on the presence of the
371 pfam motifs that matched to the trypsin reference (PF00089) or the top hit BLAST result
372 (Table S1). In total 17 proteins with the pfam code PF00089 were identified in pine weevil
373 haemolymph across all treatments and where no additional annotation was available they
374 were annotated as trypsin- or chymotrypsin-like.

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4. Discussion

The principal objective of most insect biocontrol studies is to identify efficient means of utilising entomopathogenic organisms. In many cases this is achieved through a better understanding of the molecular strategies adopted by the control agent and its interaction with its target and the latter's cellular and immunological systems. However the lack of available genomic resources for both target and biocontrol organism can dramatically inhibit our understanding of and insight into this interaction and therefore reduce the effectiveness of novel control strategies. This bottleneck can now be circumvented through advances in high throughput sequencing of genomes and transcriptomes which enable functional research on 'non-model' organisms, including those of great ecological or evolutionary importance (Haas *et al.* 2013). For the purposes of this study we produced an assembled transcriptome that was used to facilitate proteomic analysis of *H. abietis* exposed to culture filtrates of three EPF. A number of cellular immune assays were also conducted which highlighted differences in effects of EPF filtrate on *H. abietis*. Transcriptome characteristics, immune assays and significant altered protein pathways and processes are discussed in detail below.

4.1 H. abietis transcriptome

The comprehensivness of the *H. abietis* transcriptome was evaluated through a comparative analysis of highly conserved single-copy orthologs using 1,658 BUSCO for the class Insecta. BUSCO assessment allows for informative comparisons of, for instance, newly sequenced draft genome and transcriptome assemblies through comparisons to high quality model genomes (Simão *et al.* 2015). This *H. abietis* transcriptome produced in this study comprised 97% of the Insect BUSCO indicating a particularly high degree of comprehensiveness making it a suitable resource not only for the proteomic analysis conducted in this study but to the wider communities of plant-insect interactions, insect immunology, pest control and insect genetics and phylogenetics.

4.2 EPF culture filtrates induce variant immune responses in H. abietis.

Injection with filtrate led to reduction in circulating haemocyte number. One possibility for the reduction in haemocyte number is due to death and disintegration of haemocytes and/or reduced proliferation of haemocytes (Strand, 2008) or due to activated haemocytes becoming adhesive and attaching to inner organs such as the fat body (Browne *et al.*, 2013). Injection of

409 larvae with *M. brunneum* or *B. bassiana* filtrate facilitated a significantly increased yeast cell
410 density suggesting these filtrates are modulating the insect immune system allowing a
411 subsequent pathogen to proliferate. Larvae co-injected with *B. bassiana* or *B. caledonica*
412 filtrate and *C. albicans* showed significantly increased mortality. The proposed
413 immunomodulation of larvae by the EPF that is rendering the host more susceptible to a
414 subsequent pathogen is caused by spore-free culture filtrate which contains a diverse mixture
415 of enzymes, proteases and secondary metabolites (Vey *et al*. 2001; Sánchez-Pérez *et al*.
416 2014). Destruxin, the most abundantly produced secondary metabolite in *Metarhizium* spp.,
417 induced a similar response in *D. melanogaster* (Pal *et al*. 2007).

418 Proteomic analysis indicated that the response to *M. brunneum* culture filtrate was the most
419 divergent relative to the control (Figure 2). Injection with *M. brunneum* affects the largest
420 number of processes within the haemolymph. Injection with *B. caledonica* affects 25 proteins
421 involved in hydrolase activity alone and influences 23 proteins involved in organic substance
422 metabolic process and 22 proteins involved in primary metabolic process (Figure 4).

423 *4.3 EPF culture filtrate alters abundance of serine proteases and their inhibitors*

424 Insect hemolymph contains numerous serine protease/proteinase inhibitors (serpins) that are
425 involved in diverse processes including development and defence (Kanost 1999; Broehan *et*
426 *al*. 2010; Vilcinskas 2010; Butt *et al.* 2016). Serine proteases are major components of the
427 insect immune proPO pathway, which is regulated numerous serpins (Butt *et al.* 2016). In
428 addition serpins directly inhibit fungal and bacterial proteinases, regulate coagulation and
429 activate cytokine signaling processes (Kanost 1999). A number of serine proteases and
430 serpins were differentially abundant in *H. abietis* larvae injected EPF filtrate in a species
431 specific manner. *M. brunneum* filtrate resulted in a significant alteration in abundance of the
432 serine proteases easter, papilin-like protein and stubble in addition to a serpin (annotated as
433 kunitz & bovine pancreatic trypsin inhibitor domain containing protein; Figure 3). Larvae
434 injected with *B. bassiana* filtrate had an alteration in abundance of papilin-like protein and a
435 melanin-inhibiting protein whereas larvae injected with *B. caledonica* filtrate had an
436 alteration in the abundance of serine proteases and their inhibitors including trypsin- and
437 chymotrypsin-like proteins (Figure 3). Although chymotrypsin and trypsin generally have
438 digestive functions in insects, the specific function of these particular proteins in pine weevil

haemolymph has yet to be determined. It is most likely that these proteins are poorly annotated and are members of the immune associated serine protease/serpin cascade pathways found in insect haemolymph. The fact that the EPF of the different fungal species alters different members of these cascades may explain the different levels of immunomodulation observed in the cellular bioassays. Interestingly similar observations were made by Mc Namara *et al*. (2017) who showed that the filtrate of *M. brunneum* and *B. caledonica* also altered the abundance of serine proteases and serpins in the haemolymph of *G. mellonella* larvae highlight potentially conserved immunomodulatory effects of the excretory/secretory products of entomopathogenic fungi.

4.4 EPF culture filtrate alters abundance of detoxification enzymes in haemolymph

Insects have developed mechanisms to deal with EPF and their secretory products; insects exposed to fungal toxins generally have higher antioxidant enzyme activity (Butt *et al.* 2016). Larvae of the Colorado potato beetle, *Leptinotarsa decemlineata*, demonstrated elevated activity of esterases and glutathione-S-transferase (GST) when infected with *M. brunneum* (Dubovskiy *et al.* 2010). *Hylobius abietis* injected with *B. caledonica* filtrate had increased abundance of carboxylesterases. In *G. mellonella* larvae injected with *B. bassiana* culture filtrate there was a higher level of expression of alpha-esterase and carboxylesterase when compared to *M. brunneum* and *B. caledonica* treated larvae (McNamara *et al.* 2017). Insects have been shown to produce an array of humoral defences to resist fungal infection including lectins, protease inhibitors, PO, AMPs and reactive oxygen and nitrogen radicals (Butt *et al.* 2016). However these reactive species can damage both the host and pathogen. Thus, both possess antioxidant systems and detoxifying enzymes, aimed at neutralizing these reactive species. In insects, these enzymes include superoxide dismutase (SOD), catalase, peroxidase and GST (Felton and Summers 1995, Butt *et al.* 2016). *Hylobius abietis* larvae treated with *M. brunneum* or *B. bassiana* filtrate demonstrated alterations in abundance of proteins involved in oxidative stress: copper and zinc SOD and peroxidase isoform X, respectively.

4.5 EPF culture filtrate alters the abundance of proteins involved in reception and detection

The ability to perceive, discriminate and respond to chemical cues by chemoreception strongly impacts on fitness and survival. This process is necessary for identification of food

resources, avoiding intoxication and to communicate with or detect other organisms including fungi (Boucias *et al.* 2012). *Hylobius abietis* larvae injected with *M. brunneum* filtrate had altered abundance of proteins involved in reception and detection: chemosensory 6, odorant-binding 29 and β-1,3-glucan-binding protein (GBP) were increased in abundance, while peptidoglycan-recognition SC2 and an odorant binding protein from the pheromone binding protein & general odorant binding protein family were decreased in abundance (Figure 3). In *G. mellonella* larvae injected with *M. brunneum* and *B. caledonica* there were alterations in abundance of peptidoglycan recognition-like (due to *M. brunneum*) and β-1,3-GBP and two peptidoglycan recognition proteins (due to *B. caledonica*) (Mc Namara *et al*. 2017). Insects can differentiate between major groups of microbes using pattern recognition receptor (PRRs) such as PGRPs, hemolin and β-1,3-GBP. PRRs function by binding to Pathogen-associated molecular patterns (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). These receptors are crucial to recognition of pathogens and activation of an appropriate immune response (e.g. proPO pathway). Two major gene families are involved in the perireceptor events of the chemosensory system: the odorant binding and chemosensory protein families (Vieira and Rozas 2011). Chemosensory 6 was increased in abundance in *H. abietis* larvae injected with *B. caledonica* filtrate. Chemosensory and odorant-binding 29 were increased in abundance following injection with *B. bassiana* filtrate (Figure 3).

4.6 EPF culture filtrate alters the abundance of AMP in the insect haemolymph

Biologically active peptides exhibiting antibacterial, antifungal and antiviral activity are found abundantly in insects. Most insects have high anti-microbial peptide (AMP) activity against Gram-positive bacteria but less against Gram-negative bacteria, fungi and yeasts (Faruck *et al.* 2016). Anti-microbial peptides (AMP) are expressed in the fat body and secreted into the haemolymph in response to infection. *Hylobius abietis* larvae treated with *M. brunneum* filtrate had an altered abundance of attacin and pathogenesis-related 5 (thaumatin). Thaumatin-like peptides were identified in *T. castaneum*, and were found to act as an AMP against filamentous fungi (Altincicek *et al.* 2008), potentially indicative of the insect mounting an immune response to EPF. Attacin is an antibacterial protein, originally isolated from haemolymph of *Hyalophora cecropia*, where it was produced in response to bacterial infection (Carlsson *et al.* 1998). The production of immune effectors is costly for

the insect, so production of several in lower concentrations that work together would be very advantageous (Butt *et al.* 2016). One implication of an upregulation in AMP active against bacteria, following treatment with EPF filtrate, is that antibacterial activity can be beneficial to EPF as it might help exclude opportunistic infections that would be disadvantageous to the fungus (Butt *et al.* 2016).

Hylobius abietis larvae treated with *B. caledonica* filtrate had increased abundance of pathogenesis-related 5 and decreased abundance of a defensin. Larvae treated with *B. bassiana* filtrate had increased abundance of attacin C and decreased abundance of a defensin. Defensins are anti-bacterial peptides highly active against Gram-positive bacteria (Faruck *et al.* 2016), they form voltage-dependent channels, leading to rapid leakage of K+ and other ions (Hoffmann 1995). A coleoptericin was increased in abundance in *M. brunneum* and *B. bassiana* treated larvae relative to *B. caledonica* treated larvae (Figure 3). Antibacterial coleoptericins have been identified in the yellow mealworm beetle, *Tenebrio molitor*, and were upregulated following bacterial challenge and paratisation (Zhu *et al.* 2014). *Galleria mellonella* larvae treated with EPF filtrate also displayed alterations in abundance of AMP (McNamara *et al.* 2017).

4.7 EPF culture filtrate affects the abundance of proteins involved in insect development

Susceptibility to infection can depend on insect developmental stage, recently moulted insects being particularly vulnerable as new cuticle is not fully sclerotized (Butt *et al.* 2016). *Hylobius abietis* larvae are more susceptible than adults to both EPF (Ansari and Butt 2012) and EPN (Williams *et al.* 2015), potentially in part due to differences in cuticle thickness. *Hylobius abietis* larvae injected with *M. brunneum* filtrate had an alteration in abundance of proteins involved in development, metamorphosis and structure: JHPB, endocuticle structural glyco ABD-4, tropomyosin 1 and actin 5C were increased in abundance, myosin regulatory light chain 2 and a chitin binding protein Peritrophic matrix 9 precursor were decreased (Figure 3). Although typically associated with muscle and the cytoskeleton many of these proteins are commonly reported as soluble components of insect haemolymph (Handke et al., 2013; Li et al., 2012; McNamara et al., 2017). Larvae injected with *B. bassiana* filtrate had a decrease in abundance in proteins involved in development: diapause-associated transcript-2 and myosin regulatory light chain 2 (Figure 3).

Insect growth, development and reproduction are regulated by juvenile hormone (JH). Its presence during larval moulting prevents metamorphosis, and it reappears in the adult to regulate female reproductive maturation (Jindra *et al.* 2013). Insecticides have been developed that mimic the action of insect growth and developmental hormones; the steroidal 20-hydroxyecdysone and the sesquiterpenoid JH (Dhadialla *et al.* 1998). The cuticle is the first and most important barrier to EPF and chitin is a major component of the cuticle. EPF produce an extensive array of enzymes such as lipases, proteases and chitinases, with some of these cuticle-degrading enzymes being considered virulence determinants (Butt *et al.* 2016). Insect growth and morphogenesis are dependent on the capability to remodel chitin-containing structures. Thus, insects repeatedly produce chitin synthases and chitin-lytic enzymes. These alterations in abundance of developmental proteins following injection with EPF filtrate may be indicative of the insect trying to regenerate and protect itself from pathogens or it could be a reflection of EPF natural products (e.g. enzymes or secondary metabolites) within the culture filtrate having an impact on the insect.

4.8 EPF filtrate has a significant effect on proteins involved in cellulolytic and other metabolic processes in H. abietis

Larvae injected with *B. caledonica* filtrate had a higher abundance of proteins involved in metabolic processes (Figure 4) with GO term mapping indicating that a considerable portion were involved in cellulolytic processes. It was surprising to identify the large number of cellulases and carbohydrolases (annotated as glycoside or glycosyl hydrolases (GHs) in *H. abieties* haemolymph, although GHs have been identified previously in insect haemolymph (Zhang et al., 2014; Rocha et al., 2016). In total 24 GHs were identified across all treatments and a number of these were present in the haemolymph of non-exposed larvae indicating that they are endogenous to haemolymph and not artefacts of damage to the gut by the filtrate contents. We also explored the potential that these proteins were fungal in origin, with homology to insect GHs and were delivered into the insect via the filtrate. However no GH proteins were identified when the mass spectrometry data was searched against a reference proteome for *Beauvaria.*

The largest effect on GH abundance was observed in larvae injected with *B. caledonica* filtrate, which had higher abundance of proteins from GH families 1, 2, 31, 35, 38, 48 and 79

with proteins annotated as members of GH families 28 and 45 having relative fold changes of over 100 in comparison to the non EPF exposed controls. A smaller number of GHs were altered in abundance in *B. bassiana* (GH family 28 and 45) and *M. brunneum* (GH family 1) treated *H. abietis* larvae, but not to the same extent as in *B. caledonica* treated larvae (Figure 3). Although typically associated with cellulytic activity many of these protein families display considerably diverse functions in insects. GH family 1 consists of β-glucosidase, 6-phospho-β-glucosidase and β-galactosidase that are involved in carbohydrate transport and metabolism whereasGH family 2 contains β-galactosidase, β-mannosidase and β-glucuronidase activities involved in chlorophyll, carbohydrate and starch metabolic processes. GH family 28 includes polygalacturonase and rhamnogalacturonase, enzymes that are important in cell wall metabolism. GH family 31 is comprised of key enzymes of carbohydrate metabolism whereas members of GH family 45 are endoglucanases which function in the hydrolysis of soluble β-1, 4 glucans. GH48 are in most cases components of complex proteins that include additional functional domains. APAP I, from family GH48 from the leaf beetle *Gastrophysa atrocyanea* has chitinase activity but is also involved in diapause termination by JH (Fujita *et al*., 2006).

A number of these proteins belonging to GH families are potential cell wall degrading enzymes (PCWDEs). Xylophagous insects, such as *H. abietis*, are well adapted to feeding on wood and possess efficient systems to convert cellulosic biomass in their bodies (Watanabe and Tokuda 2010). PCWDEs degrade cellulose, hemicellulose, or pectin in plant cell walls, liberating sugars, minerals, and other nutrients from woody plant tissues. Although many of these GHs have been well characterized in other insects considerable analysis has now to be performed to determine the correct annotation, source and functional assignment of the GH proteins identified here. Although initially thought to be absent in insects (through the analysis of the genomes of model insect organisms including *D. melanogaster* and *B. mori*), recent work has shown that PCWDEs are in fact both present and diverse in insects (Pauchet *et al*., 2010; Watanabe and Tokuda, 2010), particularly in the Coleoptera. The PCWDEs present in the *H. abietis* transcriptomes were found in other beetle species previously; mountain pine beetle (Keeling *et al*., 2013), asian longhorned beetle (McKenna *et al*., 2013), coffee berry borer (Vega et al., 2015), Colorado potato beetle (Schoville *et al*., 2018). A

pectinesterase was also increased in abundance following injection with *B. caledonica* culture filtrate. Given the presence of PCWDEs including pectinesterases are carbohydrate esterases that belong to family 8 (CE8) (Markovic and Janecek, 2004; Kirsch *et al*., 2016) and demethylate galacturonic acid residues of homogalacturonan to facilitate the action of polygalacturonases (GH28). These enzymes are commonly found in species of Curculionidae and have important biotechnological applications for the processing of pectin (Habrylo *et al*., 2018). Thus the determination of a treatment of pine weevil larvae that results in the considerable over expression of potential PCWDEs and other enzymes of potential importance highlights the potential biotechnological significance of our work. However considerable analysis of the GH proteins is warranted to determine the specific function and origin of these diverse and abundant group of proteins not typically associated with insect haemolymph.

Conclusion

Elucidating how EPF modulate the immune response leaving insects more susceptible to subsequent pathogens may have application in improving biocontrol in the field in a number of ways: selecting superior strains with immune modulating characteristics to overcome problems with EPF killing target pests inefficiently compared to their chemical counterparts, selecting strains that could be used in combination with other plant protection products to enhance control (achieve synergy). 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 on the insect immune response using larvae of the economically important forestry pest *H. abietis* larvae.

The immune responses induced in *H. abietis* larvae 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 that can 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. Injection with *M. brunneum* or *B. bassiana* culture filtrate facilitated a significantly increased yeast cell density in larvae. Larvae co-injected with either *B. caledonica* or *B. bassiana* culture filtrate and *C. albicans* showed significantly increased mortality. Injection with EPF culture filtrate was shown to alter the abundance of protease inhibitors, detoxifing enzymes, antimicrobial peptides and proteins involved in reception/detection and development in *H. abietis* larvae. Larvae injected with *B. caledonica* culture filtrate displayed significant alterations in abundance of proteins involved in cellulolytic and other metabolic processes in their haemolymph proteome. Together these results suggest that EPF culture filtrate has the potential to modulate the insect immune system which may allow subsequent pathogens to proliferate.

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856 **Fig. 1.** Haemocyte and yeast cell densities and mortality in *H. abietis* larvae pre-treated with EPF
857 culture filtrate. (A) Haemocyte density (mean ± SE) in *H. abietis* larvae treated with EPF culture
858 filtrate. Following inoculation with fungal culture filtrate, larvae were incubated for 24 h or 48 h at 20
859 °C before bleeding and enumeration. Sab Dex: Control medium, (i) Bc: *B. caledonica*, (ii) Bb: *B.*
860 *bassiana* and (iii) Met: *M. brunneum*. X-axis represents length of time EPF was cultured for: 48 h, 72
861 h, 96 h. Asterisks indicate significant difference to relevant control * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
862 (B) Yeast cell density in *H. abietis* larvae pre-treated with EPF culture filtrate. Number (mean ± SE)
863 of *C. albicans* cells per larva after incubation for 24 h and 48 h at 20 °C. Larvae were treated with
864 fungal culture filtrate 24h prior to inoculation with C. *albicans*. Sab Dex: Control medium, (i) Bc: *B.*
865 *caledonica*, (ii) Bb: *B. bassiana* and (iii) Met: *M. brunneum*. X-axis represents length of time EPF
866 was cultured for: 48 h, 72 h and 96 h. Asterisks indicate significant difference to relevant control *
867 $p<0.05$, ** $p<0.01$, *** $p<0.001$. (C) Mortality of *H. abietis* larvae treated with EPF culture filtrate
868 alone and in combination with *C. albicans*. *+ C. albicans* indicates larvae that received a dose of *C.*
869 *albicans* after 24 h, *- C. albicans* indicates larvae that did not. Sab Dex: Control media, Bc: *B.*
870 *caledonica*, Bb: *B. bassiana* and Met: *M. brunneum*. All EPF were cultured for 96 h. Mortality one
871 week after infection with *C. albicans*. Data were tested for significance using paired T-tests. *
872 $p<0.05$, ** $p<0.01$.

873

874

875 **Fig. 2.** Principal component analysis (PCA) and hierarchical clustering of haemolymph proteomic
876 profiles of larvae treated with EPF culture filtrate versus control. (A) PCA of three replicates of each
877 treatment included in LFQ analysis. Dashed circles denote sample groups. The two axes account for
878 74.1 % of total variation within the dataset. (B) Heat map based on hierarchical clustering of the
879 median protein expression values of all statistically significant differentially abundant and uniquely
880 detected proteins. Hierarchical clustering (columns) resolved four distinct clusters comprising the
881 replicates from their original sample groups and four protein clusters (rows) based on expression
882 profile similarities.

883

884 **Fig. 3.** Volcano plots of post imputed data highlighting proteins altered in abundance in haemolymph
885 of *H. abietis* larvae following injection with EPF culture filtrate. Proteins above the dashed line are
886 considered statistically significant (p-value < 0.05) and those to the right and left of the vertical lines
887 indicate relative fold changes of ≥1.5. Volcano plots are annotated with the most differentially

888 abundant proteins identified in larvae inoculated with (A) *B. caledonica*, (B) *B. bassiana* and (C) *M.*
889 *brunneum* culture filtrate versus control larvae (inoculated with sabouraud dextrose).
890
891
892 **Fig. 4.** Alterations in biological processes at level 3 gene ontology following injection of *H. abietis*
893 larvae with fungal culture filtrate in comparison to control larvae. Only processes identified in all
894 three EPF treatments are given.

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Table 1 Number of statistically significant differentially abundant (SSDA) proteins between EPF filtrate 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 ID	**Protein Annotation**	**Relative fold change**			**Intensity**	**MS/MS**	**Peptides**	**Mol. Weight**	**Sequence length**
		BB	**BC**	**MET**					
Unigene8018	Chemosensory 6	10.71↑	7.41↑	130.5↑	$1.0x10^{10}$	98	13	14.9	129
Unigene10957	Glycosyl hydrolase family 79	2.347↑	17.19↑	3.46↑	$4.8x10^{8}$	35	9	52.46	468
Unigene3665	FKBP-type peptidyl-prolyl cis-trans isomerase	2.56↑	2.28↑	3.09↑	$1.7X10^{9}$	81	12	24.13	220
CL416.Contig1	Heat shock 70 kDa cognate 4	1.5↑	2.1↑	2.7↑	$2.8x10^{8}$	53	11	71.8	655
CL2534.Contig1	Lectin C-type domain	2.14↓	2.74↓	2.17↓	$2.7x10^{8}$	33	6	16.02	143
CL1928.Contig2	Odorant-binding 29	1.6↑	-	3.6↑	$1.1x10^{11}$	313	17	14.8	136
CL943.Contig7	Papilin-like Protein	2.01↑	-	1.73↑	$4.8x10^{8}$	89	19	278.9	2546
Unigene7330	Attacin C	1.66↑	-	1.51↑	$4.1x10^{10}$	128	11	13.8	133
Unigene5426	Myosin regulatory light chain 2	6.35↓	-	18.56↓	$7.0x10^{8}$	30	6	22.1	206

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CL4537.Contig3	No annotation	9.4↑	144.2↑	-	$1.4x10^{9}$	20	2	12.7	120
Unigene3946	Glycosyl hydrolase family 28	2.52↑	130.12↑	-	$2.5X10^{9}$	53	8	38	364
Unigene9634	Glycosyl hydrolase family 45	3.88↑	105.8↑	-	$2.4x10^{9}$	18	2	23.8	225
CL2640.Contig1	Pathogenesis-related 5	1.52↑	48.4↑	-	$3.2x10^{9}$	46	10	27.0	253
Unigene2311	Alpha-L-fucosidase	2.45↑	25.2↑	-	$3.9x10^{8}$	28	8	49.2	429
Unigene3970	Glycosyl hydrolase family 28	1.76↑	22.81↑	-	$7.0x10^{8}$	39	11	35.8	335
Unigene5303	Defensin	1.54↓	1.76↓	-	$1.2x10^{10}$	72	3	9.1	85
Unigene28106	Glycosyl hydrolase family 1	-	26.88↑	2.6↑	$1.3X10^{9}$	18	7	25.8	227
Unigene11176	Peritrophic matrix 9 precursor	-	1.67↓	2.17↓	$1.2x10^{9}$	60	7	29.7	268
CL1224.Contig1	Lectin C-type domain	-	4.16↓	2.12↓	$1.9x10^{9}$	73	6	13.588	123
CL515.Contig1	Diapause-associated transcript-2	6.18↑	-	-	$3.2x10^{8}$	22	5	18.8	162
Unigene9585	No annotation	1.57↑	-	-	$3.3x10^{10}$	183	10	17.4	160
Unigene2445	Regulatory CLIP domain of proteinases	1.55↑	-	-	$1.8x10^{9}$	43	3	8.1	71
CL2420.Contig2	Melanin-inhibiting protein	1.53↑	-	-	$1.1x10^{10}$	182	14	32.4	286
CL2563.Contig1	Peroxidase isoform X1	1.51↑	-	-	$2.9x10^{9}$	301	35	80.4	716

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CL5881.Contig1	Actin	6.7↓	-	-	$1.4x10^{9}$	27	12	39.8	360
CL61.Contig2	Arylphorin	26.22↓	-	-	$1.0x10^{10}$	33	73	85.7	717
Unigene11986	Glycoside hydrolase family 48	-	887.1↑	-	$2.7x10^{10}$	230	26	70.52	633
CL5500.Contig2	Glycoside hydrolase family 48	-	884.3↑	-	$4.5x10^{10}$	264	29	70.9	638
Unigene3825	Glycosyl hydrolase family 45	-	624.9↑	-	$1.5X10^{10}$	52	3	23.9	227
Unigene7925	Neutral alpha-glucosidase C	-	266.3↑	-	$5.5x10^{9}$	175	31	96.6	844
Unigene9562	No annotation	-	162.2↑	-	$5.6x10^{9}$	98	15	40.6	366
Unigene12087	Glycosyl hydrolase family 2	-	143.1↑	-	$3.5x10^{9}$	133	32	101.79	894
Unigene13818	Glycosyl hydrolase family 28	-	96.9↑	-	$4.7x10^{9}$	67	10	36.4	350
Unigene19514	Pectinesterase	-	74.1↑	-	$1.9x10^{9}$	63	12	39.8	380
Unigene6962	Glycosyl hydrolase family 1	-	73.9↑	-	$2.6x10^{9}$	40	11	56.1	498
CL921.Contig2	Glycoside hydrolase family 31	-	41.65↑	-	$1.4x10^{9}$	69	16	70.7	626
Unigene12565	Glycosyl hydrolase family 35	-	35.95↑	-	$1.7x10^{9}$	70	17	71.9	640
Unigene8511	Glycosyl hydrolase family 38	-	30.85↑	-	$6.8x10^{8}$	55	18	11.9	988
Unigene13343	Carboxylesterase family	-	29.3↑	-	$1.5x10^{9}$	32	10	60.4	545
Unigene3841	Glycosyl hydrolase family 45	-	28.67↑	-	$4.4X10^{8}$	12	3	25.8	236

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CL2700.Contig4	Carboxylesterase family	-	14.1↑	-	$5.6x10^{8}$	32	12	58.5	527
Unigene3953	Prostatic acid phosphatase	-	9.89↑	-	$3.0X10^{8}$	25	7	28.9	381
CL1864.Contig4	Glycosyl hydrolase family 2	-	7.81↑	-	$4.2x10^{8}$	31	8	71.7	631
Unigene3489	Serpin	-	2↑	-	$1.6X10^{9}$	52	3	6.99	62
Unigene1589	Trypsin-like protein	-	1.74↑	-	$9.4x10^{8}$	64	11	42.1	379
CL797.Contig4	Chymotrypsin--like protein	-	1.67↑	-	$2.6x10^{8}$	45	8	49.7	448
CL5549.Contig2	Sodium channel 60E	-	1.54↓	-	$6.7x10^{8}$	50	9	43.8	380
Unigene13338	Trypsin-like protein	-	2.1↓	-	$4.6x10^{9}$	200	22	55.7	488
Unigene12317	JHBP	-	-	23.78↑	$8.5x10^{8}$	36	7	36.7	248
Unigene17410	JHBP		-	22.17↑	$1.3x10^{9}$	48	15	26.9	242
Unigene8077	Endocuticle structural glycoprotein ABD-4	-	-	11.51↑	$2.1x10^{8}$	15	3	11.04	102
Unigene10626	No annotation	-	-	6.67↑	$5.7x10^{8}$	33	9	41.4	384
CL3921.Contig1	Tropomyosin 1	-	-	5.2↑	$6.9x10^{8}$	38	17	32.7	283
CL466.Contig4	Aerine protease easter	-	-	3.24↑	$6.1x10^{8}$	57	20	41.2	374
Unigene2302	Major royal jelly protein	-	-	2.35↑	$1.4x10^{10}$	323	26	46.1	411

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Unigene27113	28 kDa desiccation stress	-	-	2.22↑	$9.8X10^{9}$	265	14	26.2	229
CL1928.Contig3	Odorant-binding 29	-	-	1.88↑	$2.0x10^{11}$	387	15	14.96	136
CL3504.Contig2	Beta-1,3-glucan-binding protein	-	-	1.69↑	$1.7x10^{9}$	132	15	53.6	479
CL492.Contig1	Attacin	-	-	1.69↑	$2.4x10^{10}$	169	10	15.1	140
CL5881.Contig6	Actin-5C	-	-	1.58↑	$9.3x10^{8}$	43	21	41.8	376
Unigene8093	Cu^{2+},Zn^{2+} superoxide dismutase	-	-	1.67↓	$6.6x10^{9}$	52	2	16.8	168
CL3832.Contig2	Peptidoglycan-recognition SC2	-	-	1.7↓	$3.9x10^{9}$	75	7	20.3	186
Unigene3995	Serine protease easter-like	-	-	1.96↓	$8.9x10^{9}$	208	16	40.3	365
CL1617.Contig2	Serine proteinase stubble-like	-	-	2.13↓	$5.7x10^{9}$	47	21	48.52	447
CL2247.Contig3	PBP&GOBP family	-	-	2.19↓	$9.8x10^{11}$	1217	13	13.56	124
CL3607.Contig1	Kunitz trypsin inhibitor			2.32↓	$1.8x10^{11}$	261	6	11.5	102
Unigene4030	Thaumatin	-	-	2.63↓	$5.6x10^{10}$	421	14	26	236
Unigene6368	No annotation	-	-	2.86↓	$2.9x10^{8}$	55	17	71.8	615
Unigene417	Aspartyl protease	-	-	6.84↓	$2.3x10^{8}$	25	5	47.7	417

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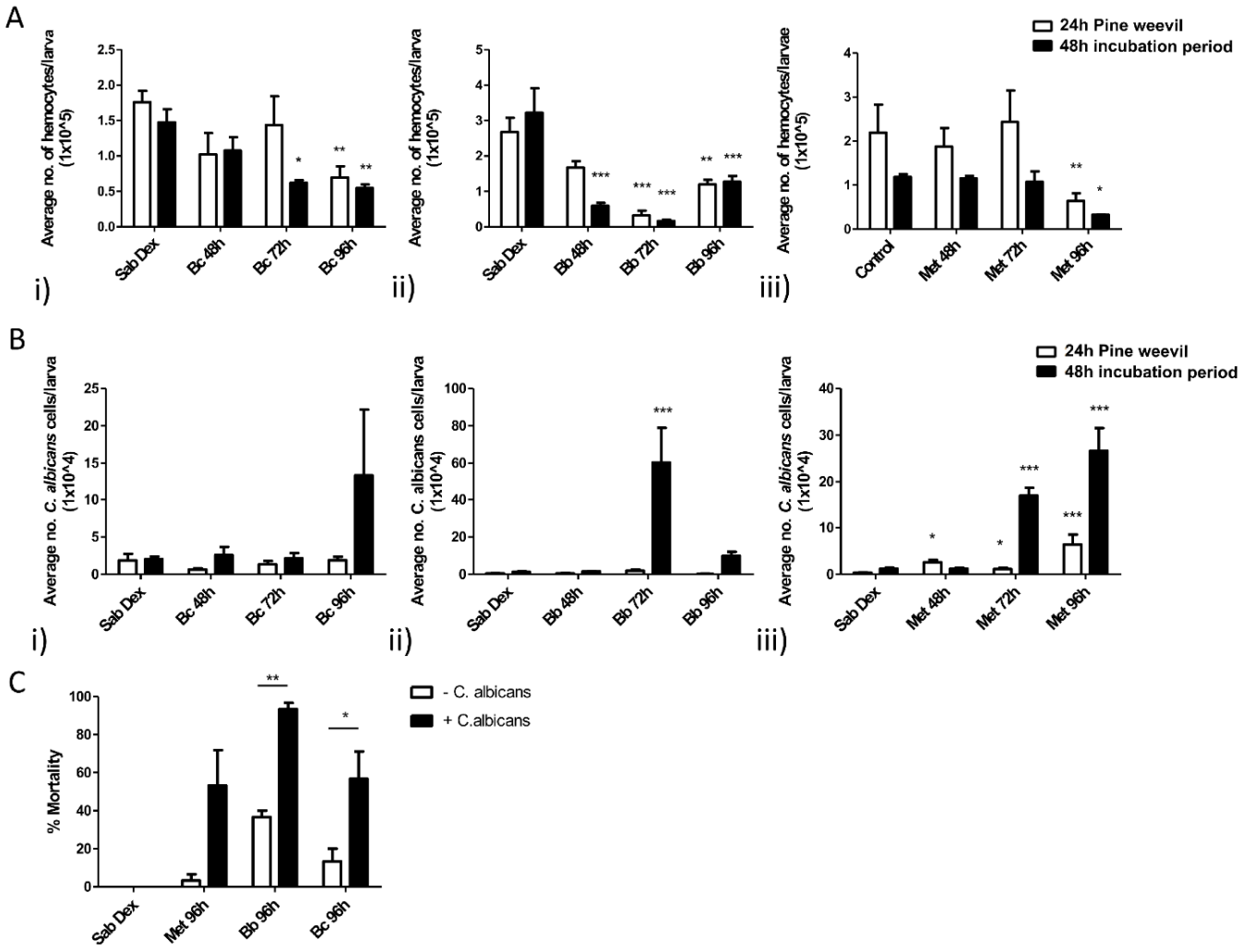
A
Average no. of hemocytes/larva (1x10^5)
Sab Dex
Bc 48h
Bc 72h
Bc 96h
i)
Bb 48h
Bb 72h
Bb 96h
ii)
Average no. of hemocytes/larvae (1x10^5)
24h Pine weevil
48h incubation period
Control
Met 48h
Met 72h
Met 96h
iii)
B
Average no. C. albicans cells/larva (1x10^4)
C
% Mortality
- C. albicans
+ C.albicans

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899 **Fig.** **1**

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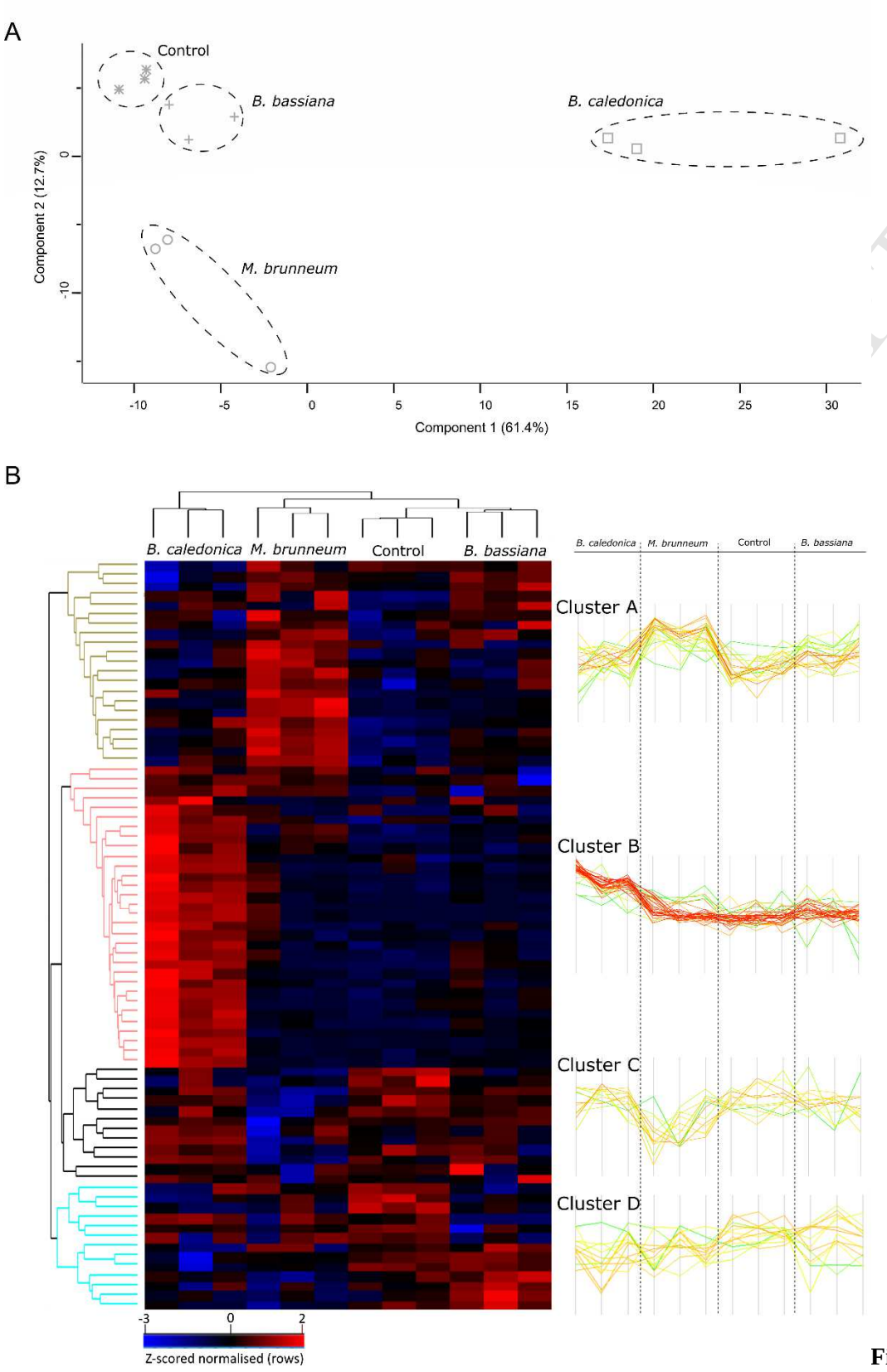
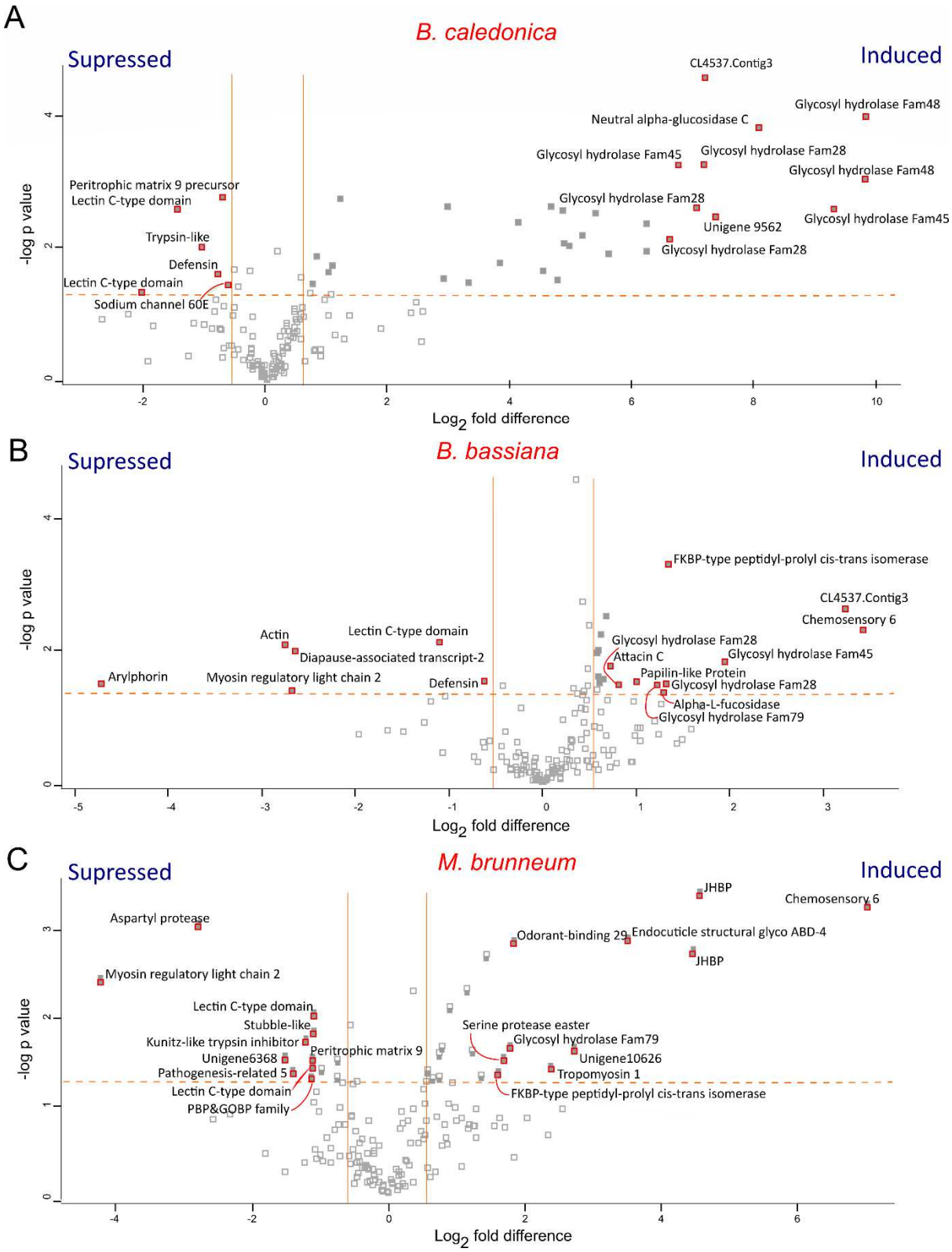

A
Control
B. bassiana
B. caledonica
M. brunneum
Component 2 (12.7%)
0
-10
-10
-5
0
5
10
15
20
25
30
Component 1 (61.4%)
B
B. caledonica
M. brunneum
Control
B. bassiana
Cluster A
Cluster B
Cluster C
Cluster D
-3
0
2
Z-scored normalised (rows)

900 **Fig. 2**

901



902

903 **Fig. 3**

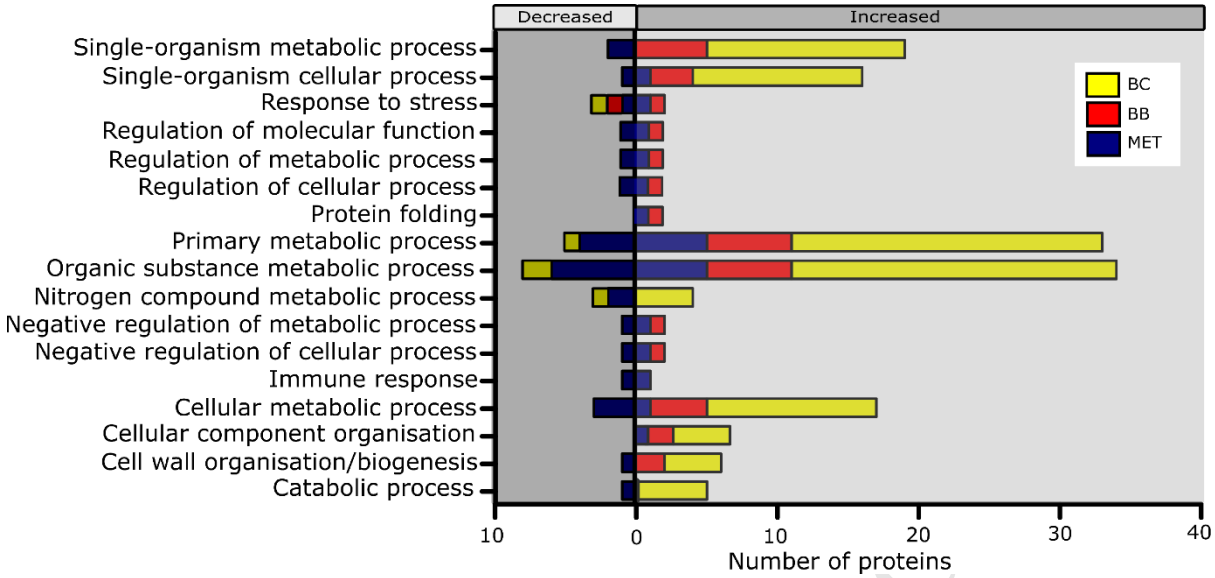


Fig. 4. Bar chart showing number of proteins changed in biological processes at level 3 ontology following injection of *H. abietis* larvae with fungal culture filtrate. Number of proteins changed in biological processes common to larvae treated with all three EPF.

SUPPORTING INFORMATION

Table S1 MS identified proteins from the haemolymph of the large pine weevil, *Hylobius abietis* after treatment with the culture filtrate of *B. bassiana*, *B. caledonica* and *M. brunneum* and Sabouraud Dextrose liquid medium. (XSLX)

Table S2 Proteins groups identified after hierchical clustering of SSDA and exclusive proteins. Four clusters of proteins with similar expression and abundance profiles were identified.

922 **Table S3** Statistically significantly differentially abundant *Hylobius abietis* haemolymph
923 proteins (2 sample t-tests ; $p<0.05$) and relative fold change differences for comparisons of *B.*
924 *bassiana* culture filtrate to control; *B. caledonica* culture filtrate to control and *M. brunneum*
925 to control. (XSLX)

926 **Table S4** Blast2Go results for all identified proteins with assigned InterPro ids, enzyme code
927 and gene ontology (GO) terms for biological processes, molecular function and cellular
928 components.

929 **Table S5** The glucosyl hydrolases identified from pine weevil haemolymph. Seven of the 20
930 proteins annotated as glycosyl hydrolases were present in control (not exposed to fungal
931 supernatant filtrate). The results for all MS/MS data searched against the predicted protein set
932 for *Beauveria bassiana* are provided. Of the 15 proteins seven were supported by more than
933 one peptide and only a single protein was annotated as a glucosidase, highlighting that the 20
934 glycosyl hydrolases identified here are more likely insect in origin. (XSLX)

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Highlights

- Injection with EPF filtrate facilitated an increased yeast cell density in larvae
- Co-injection with EPF and *Candida albicans* caused significantly increased mortality
- EPF can modulate insect immune system allowing proliferation of subsequent pathogens
- Injection with EPF culture filtrate significantly altered the haemolymph proteome
- *Beauveria caledonica* altered abundance of proteins involved in cellulolytic/metabolic processes

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