UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

Metarhizium anisopliae E6 secretome reveals new insights in cattle tick infection and the identification of potential molecular players implicated in host specificity

LAURA RASCOVETZKI SACILOTO DE OLIVEIRA

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Trabalho de Conclusão de Curso apresentado ao Curso de Farmácia da Universidade Federal do Rio grande do Sul como requisito à obtenção do título de grau de Farmacêutico.

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Porto Alegre, 2022

AGRADECIMENTOS

Agradeço ao meu professor orientador e meu coorientador por toda paciência, todo apoio e todo suporte que me deram. Agradeço aos mesmo por sempre se dedicarem e estarem dispostos a ajudar e esclarecer as dúvidas que vieram no caminho. Agradeço meus colegas de laboratório que me ajudaram com os experimentos para o desenvolvimento dos resultados do artigo. Agradeço meus pais pelo suporte financeiro que possibilitou eu realizar a faculdade na UFRGS, uma vez que ela é situada em uma cidade diferente da minha cidade natal. Agradeço toda minha família pelo carinho e apoio que sempre me deram. Agradeço também o meu namorado por me apoiar e me ajudar sempre que necessário. Por fim, agradeço minha avó, que faleceu em janeiro/2022, mas que mesmo em seus últimos dias de vida estava lá torcendo por mim e dizendo o quanto acreditava no meu potencial e no sucesso que eu irei ter em minha carreira. Agradeço também a todos os professores e profissionais da UFRGS que tive o prazer de obter grande aprendizado. Concluir esse trabalho de conclusão de curso é o primeiro passo para minha futura carreira, dessa forma, agradeço a todos que me ajudaram de alguma forma nesse caminho.

APRESENTAÇÃO

Esse Trabalho de Conclusão de Curso foi redigido sob a forma de artigo ao qual foi elaborado segundo as normas da revista Microbiological Research, apresentadas em anexo.

Metarhizium anisopliae E6 secretome reveals new insights in cattle tick infection and the identification of potential molecular players implicated in host specificity

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Abstract
Although *Metarhizium anisopliae* being one of the most studied fungal biocontrol agents the molecular mechanisms of its infection on different hosts is far to be completely understood. Here we analyzed secreted proteins related to the infection of the cattle tick, *Rhipicephalus microplus*. The results showed relative abundance changes in the expression of 194 proteins after exposure to host cuticle. Several proteins related with adhesion, penetration, stress and fungal defense were identified. We further performed a comparative genomic distribution of the differentially expressed proteins of the *M. anisopliae* secretome with *Beauveria bassiana*. Among the analyzed families, almost all of them have had a superior amount of genes identified in *M. anisopliae* genome. An *in vivo* toxicity assay using *Galleria mellonella* model was also performed showing that the molecular results found at genomic and proteomic level confirmed the expected higher toxic effect of *M. anisopliae* E6 secretome related with the cattle tick infection, over the other secretomes tested, *B. bassiana* related with cattle tick and *M. anisopliae* E6 related with the cotton stainer bug infection, *Dysdercus peruvianus*. This new set of results may help to explain molecular aspects associated with host infection specificity due to evolutive/gene set and gene expression control at protein level differences in arthropod-pathogenic fungi.

Keywords
Metarhizium anisopliae; Biological Control; Biocontrol; Cattle tick; *Riphicephalus microplus*; Proteomics

1. Introduction

The use of chemical pesticides for the control of pests in agriculture and livestock has caused several damages to the environment and to human health. Besides, the number of resistant pests against pesticides and other chemicals is increasing annually. (Safiou et al., 2016; Klafke et al., 2017) Biological control has been an advantageous alternative higher specificity, neither affecting other ecologically important arthropods nor causing environmental contamination (Samish et al., 2004; Beys-da-Silva et al., 2020; Sullivan et al., 2022). The entomopathogenic fungus *Metarhizium anisopliae* has been widely studied as a biocontrol agent, due its variety of hosts, safety and easily to produce conidial mass. This fungus is considered a generalist, being able to infect different hosts (Lovett and St. Leger 2018; Beys-da-Silva et al. 2014; 2020; Sarven et al. 2020). In general, the infection process of *M. anisopliae* consists of six stages, which are adhesion, germination, appressorium formation, penetration, colonization, extrusion and sporulation. (Aw and Hue, 2017).

The cattle tick, *Rhipicephalus microplus* (Acari: Ixodidae), is the main ectoparasite that infests livestock, causing commercial losses around US$ 3 billion/year (Grisi et al., 2014) in Brazil, which has the largest commercial cattle herd in the world. Each tick sucks large amounts of blood daily during parasitism, which can cause anemia, anorexia, hemostatic changes, slimming and apathy, leading to significant losses in milk, meat and leather production, even death (Reck et al., 2009; Webster et al., 2015). The potential of entomopathogenic fungi as *M. anisopliae* to control ticks is due to many factors, including their ability to target different developmental stages of the host, penetrate through the cuticle, genetic variability and ability to penetrate through the cuticle. (Quinelato et al., 2012).

It is already known that this fungus has been proven to control the tick (Beys-da-Silva et al. 2020), including field trials (Webster et al. 2015). However, to be adopted as a commercial practice, is necessary to make the biocontrol process more effective and compatible with the reality of the producers (Lovett and St. Leger 2018; Sullivan et al., 2022) Therefore, the purpose of this study is to identify and characterize the proteins secreted by *M. anisopliae* during tick infection, thus revealing potential virulence factors and pathogenicity determinants, triggered by *R. microplus* compared to other related secretomes.

2. Materials and methods

2.1. Conidia production of *Metarhizium anisopliae*

The fungus *M. anisopliae* var. *anisoplia*, strain E6 (previously selected for control of the bovine tick) (Frazzon et al. 2000), was kept according to previously described (Beys-da-Silva et al. 2009). For the spore production, the methodology described by Beys-da-Silva et al (2009) was used. Briefly, the fungus was grown in polypropylene bags containing 100 g of rice with 30 mL of 0.5% peptone. A suspension of 10^6 conidia/mL was added and the bags were incubated at 28 ºC for 14 days. The spores were mechanically removed from the rice grains using a sieve and gentle shaking. The spore suspension was made with sterile distilled water, adjusted to the concentration of 10^8 conidia/mL.

2.2. Culture condition

M. anisopliae (10^7 conidia/mL) was cultured in 70 mL of basal medium (0.6% $NaNO_3$, 0.2% glucose, 0.2% peptone, 0.05% yeast extract) containing 0.7% *R. microplus* cuticles and 0.05% cholesteryl stearate, as induced infection condition (RM) (Beys-da-Silva, et al. 2014). This strategy is widely used for studies of expression of *M. anisopliae* during infection (Freimoser et al. 2005; Beys-da-Silva et al. 2010; Manalil et al. 2010; Beys-da-Silva, et al. 2014). A culture medium containing 1% glucose instead cuticle and cholesteryl stearate was used as control (C). Culture was performed at 28 ºC for 48h and 150 rpm agitation on an orbital shaker. After this time, 0.25% (v/v) Triton X-100 was added to cultures to remove proteins attached from external micelia (Beys-da-Silva et al. 2010; Beys-da-Silva, et al. 2014). Supernatants were recovered by filtration through filter paper Whatman no. 1. All experiments were performed in technical and biological triplicate.

2.3. Inactivation of endogenous proteolytic activity and protein quantification

Supernatants containing secreted proteins were boiled for 5 minutes for inactivation of endogenous proteases, as described previously (Beys-da-Silva et al. 2014). Posteriorly, the samples were lyophilized and kept at -80 ºC until use. Protein quantification was determined by the bicinchoninic acid method (Pierce, Rockford, IL) (Smith et al. 1985).

2.4. Sample preparation for mass spectrometry

Samples (100 µg of proteins/treatment) were re-suspended in digestion buffer (8M urea, 100mM tris-HCl pH 8.5), as previously described (Beys-da-Silva et al. 2014). Proteins were digested with trypsin (2 µg) (Promega, Madison, WI) for 16h at 37 °C. The reaction was stopped by the addition of 5% formic acid and the samples were stored at -80 ºC.

2.5. Mass spectrometry analysis

The digested proteins were packaged in a biphasic column containing 2.5 cm ion exchange resin (Partisphere SCX) and 2 cm reverse phase resin (Acqua C18) (Beys-da-Silva et al. 2014). Twelve steps of MudPIT salt separation were used, with a gradient ranging from 0 to 100% of buffer B (80% acetonitrile/0.1% formic acid), as described (Washburn et al. 2001). The peptides were loaded on a LTQ-XL system (Thermo Fisher, USA) according to the manufacturer's protocol. A cycle of one full-scan mass spectrum (300–2000 m/z) followed by five data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. To prevent repetitive analysis, dynamic exclusion was enabled with a repeat count of 1, a repeat duration of 30 s, and an exclusion list size of 200. Application of mass spectrometer scan functions and HPLC solvent gradients was controlled by the Xcalibur data system (Thermo, San Jose, CA).

Protein identification and quantification analysis were done with the IP2 analysis package (www.integratedproteomics.com/). The search for protein identification was made using *M. anisopliae* strain E6 genome, deposited in the NCBI (Staats et al. 2014). The peptide mass search tolerance was set to 3 Da, and carboxymethylation (+57.02146 Da) of cysteine was considered to be a static modification. The following parameters were used: the cross-correlation score (XCorr) and normalized difference in cross-correlation scores (DeltaCN) to achieve a false discovery rate of 1%. For each sample, six techniques replicates were performed.

2.6. Molecular characterization of the secretome

Several bioinformatics programs were used to characterize molecular and functionally the secretome. The software PatternLab (Carvalho et al. 2016) was used for comparative analyses, identifying differentially expressed proteins (module TFold) and unique proteins (module AAPV). The following parameters were used: proteins that were not detected in at least four out of six runs per condition were not considered; a t-test (p value of 0.005) was applied and BH q-value of 0.05 (5% FDR) was set. Also, an absolute fold change greater than two was used to select differentially expressed proteins (Beys-da-Silva, et al. 2014).

The Blast2Go tool (http://www.blast2go.org) was used to categorize proteins by Gene Ontology annotation according to biological process and molecular function. The software BlastP (https://blast.ncbi.nlm.nih.gov/) was used to analyze hypothetical proteins identified in the secretome.

Other bioinformatics tools were used to investigate the characteristics of proteins identified, as TargetP 1.0 (cutoff>0.9), TMHMM 2.0, SignalP 5.0 (http://www.cbs.dtu.Dk/services/) and Wolf PSORT (http://www.genscript.com/wolfpsort.html) to predict signals of subcellular localization and secretion.

2.7. Sequence data and identification of protein families

M. anisopliae E6 and *Beauveria bassiana* ARSEF2860 predicted genomes were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/genome/browse/). The overall protein sequences of these genomes were extracted for investigations. Proteins identification was performed using HMMER 3.2.1 (http://hmmer.org/) with hmmsearch of profile hidden Markov models derived from the Pfam alignment flat files as follow: PF00082, PF00089, PF00135, PF00188, PF00199, PF00704, PF00734, PF01565, PF01822 and PF03583 (downloaded from the Pfam protein families database, http://pfam.xfam.org/) against the selected predicted genomes. A per-domain output option, with one data line per homologous domain detected in a query sequence for each homologous model was applied, and the cutoff of positive hits was set at E value of 10^{-3}.

2.8. Phylogenetic reconciliation analysis

Alignment of obtained protein sequences was performed by HMMER package with hmmalign of the corresponding profile hidden Markov models. Then, the phylogenetic trees from alignments were constructed by FastTree version 2.1.11 with maximum-likelihood method (http://www.microbesonline.org/fasttree/). Gene duplications and losses were inferred from reconciliation of the species and gene trees using Notung v2.9 (Vernot et al., 2008). The species tree was generated using the NCBI Taxonomy Browser (Sayers et al., 2009).

2.9. Toxicity assay using *Galleria mellonella in vivo* model

The proposed methodology by Inés-Molina et al. (2020) was performed with some changes. Groups of ten larvae in the final stage weighing 220-280 mg were used. Different groups of larvae were exposed to three different secretomes: *M. anisopliae* secretome cultured in medium containing *R. microplus* cuticle (MaR), *M. anisopliae* secretome in medium containing *Dysdercus peruvianus* cuticle (MaD) and *B. bassiana* secretome in medium containing *R. microplus* cuticle (BbR). 30µL per larvae was used. A culture medium containing 1% glucose instead cuticle was used as a positive control and DMSO was used as death control. Larvae were observed daily up to 6 days and were evaluated according to survival. The larvae were considered dead when they did not show any movement in response to touch. Experiments were performed by triplicate.

2.11. Statistical Analysis

Enzymatic assays were analyzed statistically using the Student's t-test and SPSS 21.0 for Windows program (SPSS, Inc., USA).

3. Results

3.1. Secretome overview

Comparing the medium containing cattle tick cuticle (RM), as inductor of the infection system, against the control (C), a total of 404 proteins were identified, being 133 uniquely identified in tick cuticle (Table S1) and 5 in the control condition (Fig. 1). Among 266 proteins identified in both conditions, 56 were considered differentially regulated proteins: 52 up-regulated and 4 down-regulated in RM. (Table S2). In addition, it is important to note that in the set of proteins exclusively and up-regulated in RM, proteins related with adhesion, as cell wall proteins, penetration and cuticle degradation, as subtilisin protein Pr1K and Chitooligosaccharide oxidase (ChitO), stress and fungal defense, as WSC domain containing

protein, CFEM domain containing protein and putative acid phosphatase, were identified. (Table S1 and S2).

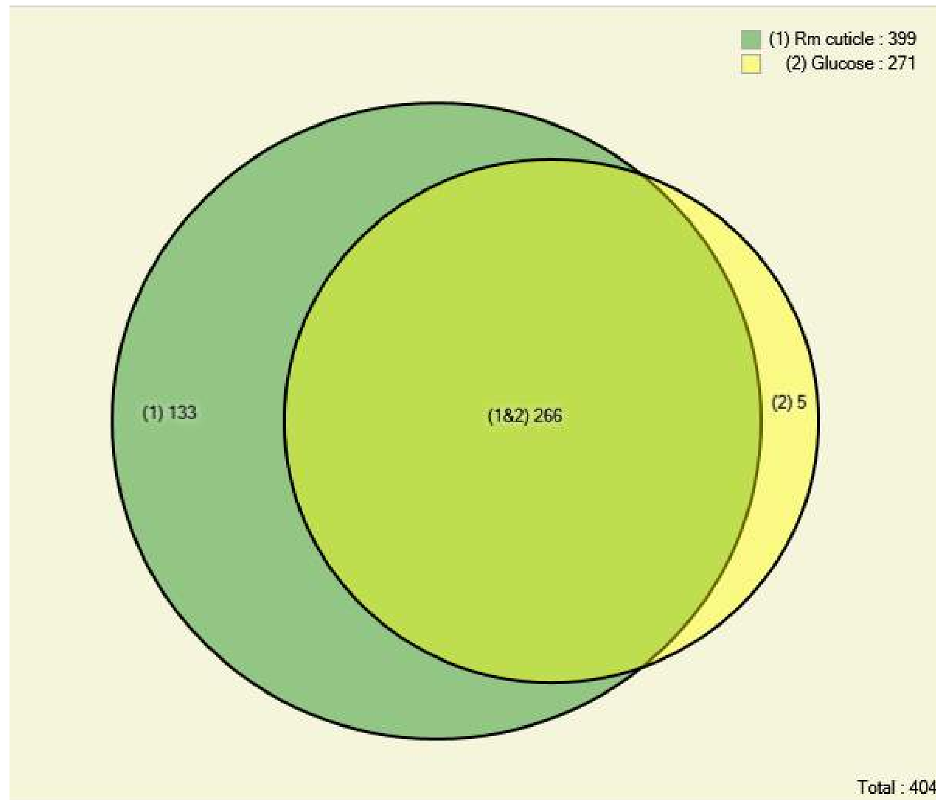

Fig. 1. Distribution and overlap of proteins from *M. anisopliae* supernatant when grown in *R. microplus* cuticle medium (RM) compared with glucose (C). Data were generated in PatternLab's AAPV module. Green circle: *R. microplus* cuticle; yellow circle: glucose.

3.2. Functional analysis

The set of proteins identified as exclusive and differentially expressed were submitted to Blast2Go analysis, in order to categorize in the level of biological process and molecular function. The top-ranked biological processes (BP) of upregulated proteins were carbohydrates metabolical process, proteolysis process and oxidation-reduction process and organic substance catabolic process (Fig. 2A). The top-ranked molecular function (MF) upregulated were hydrolase activity, hydrolyzing O-glycosyl compounds, oxidoreductase activity, nucleotide binding, tranferas activity and peptidase activity (Fig. 2B).

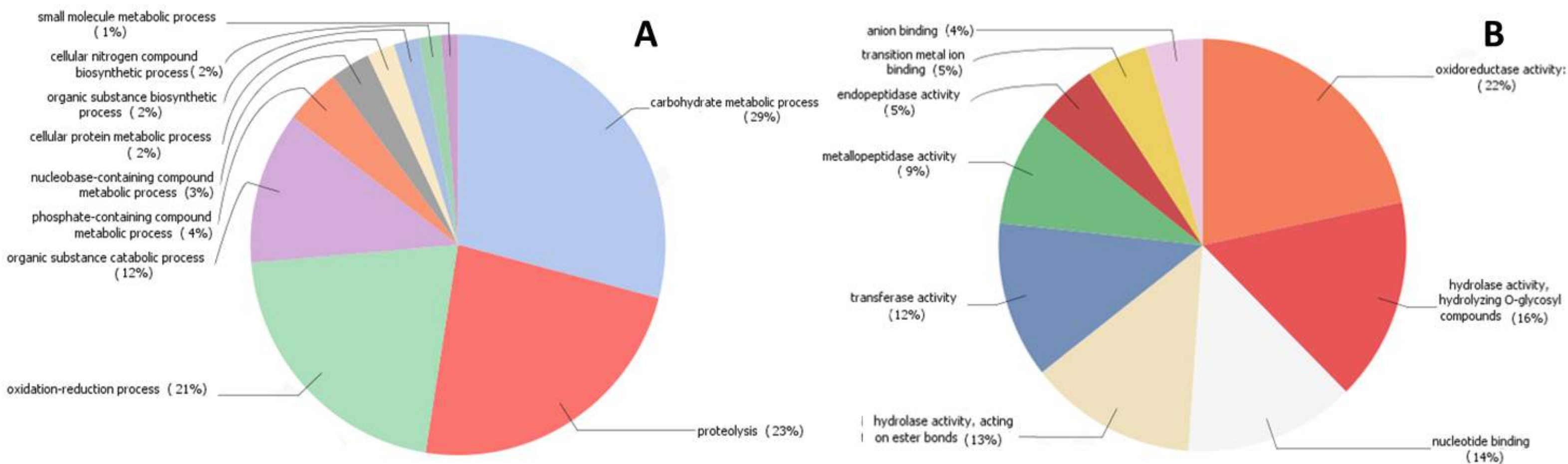

Fig. 2. Gene Ontology annotation. Categorization of differentially expressed proteins identified in RM medium versus C. Classification according to (A) biological processes or (B) molecular function.

Subsequently, four bioinformatic programs were used to predict subcellular localization and secretion signals. Interestingly, 72.16% of the proteins presented a positive prediction signal for secretion, in at least three of the four programs, attesting the enrichment of secreted proteins in our sample, as expected (Table S3).

As the secretome presented a high number of hypothetical proteins (n=103), these were separately analyzed in order to identify any characteristic (Table S4). Their sequences were searched using the BLAST tool against the NCBI non redundant database, and all proteins had a high similarity with other hypothetical proteins. The presence of hypothetical proteins in the secretome reached 25,5%. A further check of those sequences was made using BlastP in order to identify a corresponding homologous sequence or conserved domains for protein annotation. 57,3% of these hypothetical proteins were able to be re-annotated since an homologous protein was found and 5,8% of these sequences presented conserved domains. Most of the homologous proteins found had similarity with proteins of other *Metarhizium* species, related to processes of cuticle's adhesion and degradation, for example Cell wall beta-glucan synthesis [*Metarhizium brunneum* ARSEF 3297], glycoside hydrolase family 12 [*Metarhizium robertsii* ARSEF 23] and peptidase S1 domain protein [*Metarhizium robertsii*], oxidation-reduction, for example FAD-binding, type 2 [*Metarhizium robertsii* ARSEF 23] and L-amino acid oxidase [*Metarhizium robertsii* ARSEF 23], and fungal defense, for example WSC domain-containing protein [*Metarhizium robertsii* ARSEF 23] thus contributing to the link with infection described here. In addition, among the hypothetical proteins, some presented domains that suggest implications for the infection process, such as GPI-anchored superfamily, *Alternaria alternata* allergen 1 (AltA1), Dynein light intermediate chain (DLIC), G2F domain (Nidogen), Laminin_G_3 (LamG superfamily) and LIP (Secretory lipase).

3.3. Comparative genomic distribution of secretome

We performed a comparative genomic distribution of the differentially expressed proteins of the *M. anisopliae* secretome in order to compare *B. bassiana*, another well known entomopathogen (Table S5). Among the analyzed families, almost all of them have had a superior number of genes identified in *M. anisopliae* genome, as subtilase and trypsin,

carboxylesterase, glycosyl hydrolases family 18, catalase, FAD binding domain and WSC domain containing proteins, suggesting these proteins are important for fungal pathogenesis for both fungi.

3.4. Reconstruction of duplication history

Based on the reconciled phylogeny, the amount of duplication and gene loss events between *M. anisopliae* and *B. bassiana* were estimated during protein families diversification. Reconciliation is based on the observation that discordance between species and gene trees is evidence that genes diverged through other processes than speciation, including gene duplication, horizontal gene transfer and gene loss. A correspondence between genes and species evolutionary history was established with the same protein families analyzed in genomic distribution analysis (Table S6).

3.5. Toxicity assay using *Galleria mellonella in vivo* model

The group of larvae that was administered with *M. anisopliae* E6 secretome cultured in medium containing *R. microplus* cuticle (MaR) resulted in a higher mortality rate over the course of the experiment, compared to other groups (*B. bassiana* related with cattle tick (BbR) and *M. anisopliae* E6 related with the cotton stainer bug infection, *Dysdercus peruvianus* (MaD)). oreover, after 6 days this group presented the highest mortality. (Fig. 3) This result reinforces the hypothesis that the greater arsenal of proteins found in the secretome of *M. anisopliae*, when the infection system was artificially activated and induced by the cattle tick cuticle, results in a more toxic secretome for the arthropod model tested.

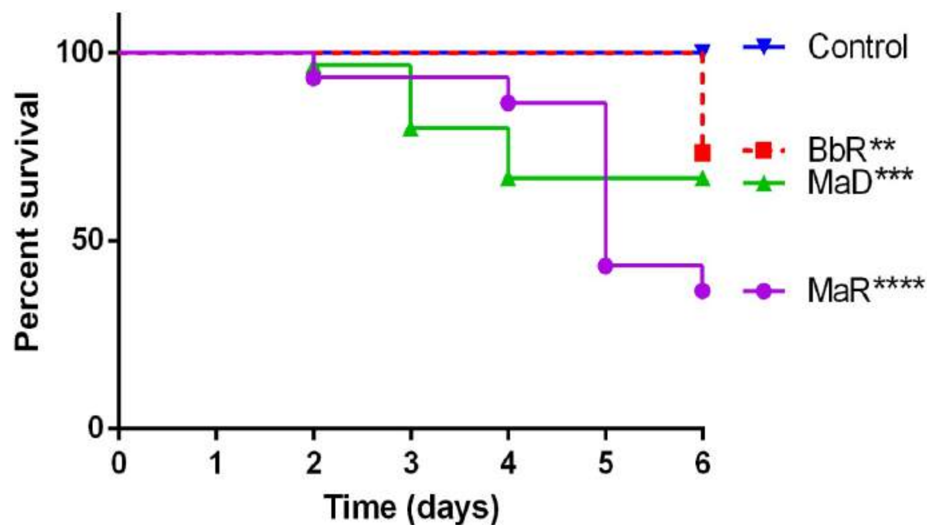

Fig. 3. *Galleria mellonella* toxicity assay of different fungal secretomes induced by host cuticles. The survival rates of the *G. mellonella* after inoculation with secretome of *M. anisopliae* cultured in medium containing cuticle of *R. microplus* (MaR), cuticle of *D. peruvianus* (MaD) and secretome of *B. bassiana* cultured in medium containing cuticle of *R. microplus* (BbR), were expressed by the Kaplan-Meier survival plot. Controls were performed using culture medium instead of cultured supernatant. *$p<0.05$; ns $p\geq0.05$

4. Discussion

M. anisopliae, known to be an efficient biological control agent and widely used worldwide, secrets a set of proteins and enzymes which are induced by host cuticle, as already described (Santi et al. 2010a, 2010b, 2018; Beys-da-Silva et al. 2013, 2014; Perinotto et al. 2014; Kordi et al 2015; Coutinho-Rodrigues et al. 2015). However, most of these studies were made using insect models, lacking an overview and in-deep analysis of molecular mechanisms involved in tick control. The tick *R. microplus* have an important economic impact, like other pests, and therefore have a potential hosted for study (Webster et al., 2015).

The infection of *M. anisopliae* involves several steps (Aw and Hue, 2017), and the success of the infection depends on the expression of specific proteins and other molecules. It was proposed that *M. anisopliae* acts by secreting proteins to degrade host components and to manage host physiology (Beys-da-Silva et al, 2014). Here, we identified 404 proteins in the secretome, with 194 proteins considered differentially expressed or unique. Recently, our group identified 71 proteins with differential expression, *M. anisopliae* E6 related to biocontrol of the cotton pest, *D. peruvianus* (Beys-da-Silva et al. 2014) and 82 proteins with differential expression in *B. bassiana* secretome related to biocontrol *R. microplus* (Santi et al. 2018). These studies had the same analysis strategy as our work and used the same experimental conditions and times. The higher number of proteins identified in this work suggest the differential specificity of the strain E6 for ticks, indicating greater potential for the development of better formulations to control this plague (Table 1).

Table 1. Comparison of differential fungal secretomes related to arthropod infection.

	Metarhizium anisopliae secretome related to *Riphicephalus microplus* infection (**MaR**)	*Metarhizium anisopliae* secretome related to *Dysdercus peruvianus* infection (**MaD**) *	*Beauveria bassiana* secretome related to *R. microplus* infection (**BbR**) **
Number of Differentially Expressed Proteins	194 proteins	71 proteins	82 proteins
Up-regulated Proteins	53 proteins	8 proteins	16 proteins
Uniquely Identified Proteins in the Infection-Related Condition	133 proteins	31 proteins	50 proteins
Serine proteases (Pr1)	Pr1K, Pr1G, Pr1C, Pr1H, Pr1A, putative serine peptidase, subtilisin-like serine protease	Pr1I, Pr1B, Pr1A, Pr1C, Pr1J, serine peptidase putative	bassiasin I, serine peptidase putative
Trypsin-like protease (Pr2)	trypsin-related protease	-	-
Carboxypeptidases	carboxypeptidase, carboxypeptidase	glutamate carboxypeptidase 2	Carboxypeptidase like protein
Other proteases	aminopeptidase 2, putative aminopeptidase, metallo-endopeptidase, metalloprotease-like protein, metalloprotease MEP1, putative Xaa-Pro aminopeptidase pepP, putative	aspartic protease precursor, leucine aminopeptidase	Peptidase family M28, Metalloprotease-like protein, peptidase family protein, peptidade S33, tripeptidyl-peptidase, peptidase S8, peptidase family S58, extracellular dipeptidyl-

	leucine aminopeptidase, tripeptidyl-peptidase 1 precursor, peptidase S1 domain protein***		peptidase DPP4, family 53 protease
Chitinases	endo-N-acetyl-beta-D-glucosaminidase D1, subgroup A chitinase A1, subgroup B chitinase B4, Subgroup A chitinase A6, subgroup B chitinase B7	-	chitinase-like protein
Lipases	Secretory lipase family protein, lipase superfamily***, LIP***, secretory lipase***	-	Secreted lipase 1 precursor

* Beys-da-Silva et al. 2014
** Santi et al. 2018
***hypothetical proteins with homologous match identification

A common concern in secretome analysis is cell lysis contamination. As previously mentioned, we used bioinformatic programs to identify the cellular localization of proteins, achieving more than 70% of predicted secreted proteins, which is higher than the average of 50% and other works previously published (Beys-da-Silva et al. 2014; Santi et al. 2018). In addition, some proteins could be secreted by non-classical secretion pathways, such as vesicles, which could explain the 27% remaining. Moreover, the presence of intracellular proteins in the secretome can be explained by autolysis or mechanical damage caused by the agitation of the liquid culture (Girard et al. 2013; McCotter et al. 2016). Therefore, the present study probably presents lower contamination of internal proteins, attesting the efficiency of culture condition mimicking infection as well.

As expected, the secretome contains proteins related to adhesion, penetration, fungal defense, oxidative stress and signaling. An expressive number of proteases, chitinases and lipases was identified in the secretome. These findings may be linked to the composition of the tick cuticle, which needs to be degraded to allow fungal penetration in the infection. Tick cuticle, the first barrier to be transposed by fungus, is composed by two layers: the epicuticle, which is a thin external layer composed mainly by esterified lipids; and the procuticle, a thicker layer rich in proteins and chitin (Beys-Da-Silva et al. 2012).

M. anisopliae produces a diverse array of proteases, especially subtilisin-like Pr1 (Pr1A-K) (Bagga et al. 2004). The physiological integrity of the host is disrupted by these enzymes, thus pathogenic fungi have a strong selective advantage (Bagga et al., 2004, Bye and Charnley, 2008; Beys-da-Silva et al. 2012). Several serine proteases were differentially identified, including Pr1K (the most up-regulated protein), Pr1A, Pr1G, Pr1H and Pr1C. Other proteases such as trypsin (Pr2), metallo, aspartic-, carboxy- and aminopeptidases were also identified. Different Pr1 isoforms can act during pathogenesis, enabling the hydrolysis of different types of proteins present in the arthropod cuticles (Beys-da-Silva et al., 2012; 2014; Leão et al. 2015; Aw and Hue, 2017), being considered key for virulence and host specificity. For example, Pr1A was already described with differential expression for other hosts, including the insects *D. peruvianus* (Santi et al. 2011), *Callosobruchus maculatus* (Manalil et al. 2010), and *Diatraea saccharalis* (Leão et al. 2015). In the same way, the *Pr1C* RNA was related to *R. microplus* in a RDA (representational difference analysis) study (Dutra et al. 2004). Interestingly, this protease was a central node in interactomic analysis of secreted proteins during *D. peruvianus* infection (Beys-da-Silva et al. 2014). This particular Pr1 seems to be involved in infection and virulence, as part of a general response to nutrient deprivation, but not in host specificity.

As aforementioned, other enzymes are important to transpose the host cuticle, such as chitinases and lipases, releasing molecules for fungus nutrition (Butt et al., 2013). Chitinases are also involved in the modification of fungal cell wall, conidia release, hyphae differentiation (appressoria) and morphogenesis (Gooday et al., 1992). We identified several chitinases from class A and B (A1, A6, B4 and B7) and lipases, ceramidases and cutinases, which are probably involved in nutrition and morphogenesis. We also found others proteins involved in chitin degradation process, for example Chitooligosaccharide oxidase (ChitO), a enzyme known to catalyze the oxidation of chitooligosaccharides, oligomers of N-acetylated glucosamines derived from chitin degradation (Savino et al. 2020). Interestingly, this protein was exclusively identified in a culture medium containing the tick cuticle.

Furthermore, proteins related to adhesion, the first step of pathogenesis, were identified. Cell wall proteins (CWP) can act increasing the fungal hydrophobicity, supporting the adhesion of fungal spores to the tick cuticle (Li et al. 2010; Santi et al. 2018). Lipases also have an important role in the adhesion, by increasing the hydrophobic interactions between host and conidia, over the release of free fatty acids through its lipolytic activity (Santi et al. 2010a; Beys-da-Silva et al 2010).

It was noticed that the infection process is considered a stressful condition, not only for the host, but also for the pathogen (Lovett and St. Leger, 2015). In our analysis, we identified several proteins related to stress tolerance. The Gene Ontology analysis identified a higher percentage of proteins related to oxidoreductase, as previously found in other secretomes (Beys-da-Silva et al. 2014; Santi et al. 2018). Oxidoreductases are important in many aspects of fungal life, including infection, formation of specific structures, ecological processes, cellular communication, and signaling (Tudzynski et al. 2012). Proteins containing CFEM domain were also identified as up-regulated in the secretome. This domain is rich in cysteine and was described during pathogenesis in other pathogens, thus demonstrating its involvement in various processes including conidial production and stress tolerance (Kulkarni et al 2003; Liang et al. 2013; Vaknin et al. 2014; Zhu et al 2017; Santi et al. 2018). Other proteins involved in stress and adaptation of the fungus in different hosts were proteins containing the WSC domain. These proteins may also be associated with modulation of the host immune system (Liang et al. 2013; Sen-Miao et al. 2016; Tong et al 2016a, 2016b).

The secretome analyzed in this work presented a high number of hypothetical proteins. Our search for homologous proteins and conserved domains enabled the re-annotation of these proteins. Interestingly, most of the homologous proteins and conserved domains found were related to the infection system. The *A. alternata* allergen 1 (AltA1), for example, has been studied and some evidence has suggested that its role can be related to virulence and fungal infection pathogenicity (Gómez-Casado et al. 2014; Gabriel et al. 2017). Moreover, several hypothetical proteins were homologous to proteins group already described for being important to infection process as oxidoreductases, cell wall proteins, peptidases and lipases (Li et al. 2010; Tudzynski et al. 2012; Butt et al., 2013; Beys-da-Silva et al. 2014; Santi et al. 2018).

The use of Hidden Markov Models (HMM) profiles based pipelines for protein domains identification provides an easy approach to investigate the distribution and diversity of proteins/enzymes in predicted genomes, allowing the identification of unsought domains associated with the proteins of interest and the comparison of genome repertoires. Five families HMM profiles groups were used for genome identification in this study in order to compare *M. anisopliae* and *B. bassiana* protein/enzyme arsenal as follow: proteases, lipases, chitinases, ROS protection proteins and extracellular effectors (Table S5). As a greater number of pre-invasion phase enzymes families were observed in *M. anisopliae* genome (subtilase, trypsin,

carboxylesterase and glycosyl hydrolases family 18) we could suppose in a more virulent *M. anisopliae* infection elements dedicated to pre-invasion phase than in *B. bassiana* genome as it has already been observed (Rustiguel et al. 2018), pointing out to a better host range and versatility, once a more diversified enzyme arsenal make fungi more capable to infect a wider spectrum/range of invertebrates with a improved efficiency.

The evolution of *M. anisopliae* genes is characterized by expansion and reduction of these elements in genome, as inferred from a specie and gene tree reconciliation analysis using Notung software (Chen et al., 2000) Most of gene duplication events are result of whole genome duplication (WGD) or tandem duplication and provide conditions for neo-functionalization or sub-functionalization of paralogous genes, contributing to all kind of innovations, as gene regulatory network expansion and cellular and organismal diversification (Wolfe et al., 1997; Davis et al., 2005; Semon et al. 2007). Gene gain through duplication occurs more often in *M. anisopliae* than in *B. bassiana* in the hydrolytic enzymes analyzed for cuticle degradation (Table S6), although gene loss occurs more frequently than gain in all families analyzed, indicating a prevalence of gene loss on its evolutionary history. Together with data indicating a greater number of genes in *M. anisopliae* genome distribution, we are prone to believe in a divergent host specialization driven by distinct select pressures imposed by the divergent repertoire found in both genomes, notwithstanding the phylogenetic proximity of this two species. A strong evidence of this evolutionary process could be observed in the genetic divergence shown here, as mentioned elsewhere (Raffaele et al., 2010; Brunner et al., 2013; Poppe et al., 2015). Gene duplication is considered the main substrate for adaptive evolution, allowing adaptation in host-pathogen coevolution scenarios. Together with the secretomic analysis described here, we could infer in the sub-functionalization (function and expression sharing among paralogous after duplication) and neo-functionalization (acquisition of new functions after duplication) of the paralogous (Lynch et al., 2000; Krishnan et al., 2018).

The entomopathogenicity has evolved independently and repeatedly in all major fungi, and therefore interactions between fungi, hosts, and the environment are diverse and dynamic, moreover, the protein arsenal is due host specificity. (Wang et al. 2019) Thus, as our results show a greater arsenal of infection-related proteins, we suggest that *M. anisopliae* E6 has a greater potential specificity to *R. microplus*. Considering all genomic results comparing *M. anisopliae* and *B. bassiana* and related secroteme comparisons, we could expect that *M. anisopliae E6* secretome induced by tick cuticle would be potentially more lethal in *in vivo* assay due to its greater secreted protein arsenal. (Beys-da-Silva et al. 2014; Santi et al. 2018). To test the hypothesis that *M. anisopliae* E6 has a greater potential specialization to *R. microplus* the *in vivo* toxicity assay using *G. mellonella* larvae was performed comparing *M. anisopliae* E6 secretome induced by tick cuticle, induced by the cotton stainer bug cuticle and *B. bassiana* secretome induced by tick cuticle. The assay's result shows that the larvae group inoculated with *M. anisopliae* secretome, when cultivated with tick cuticle, kills larvae faster than the other groups (larvae group administered with *M. anisopliae* secretome when cultivated with *D. peruvianus* cuticle; and larvae group administered with *B. bassiana* secretome, when cultivated with tick cuticle). Thus, our results reinforced the formulated hypothesis based on molecular results found.

5. Conclusion

In this work, the *M. anisopliae* strain E6 differential secretome showed a specific complexity related to tick infection, due to the high number of differentially expressed proteins compared to other studies. It was possible to confirm the enrichment of secreted proteins in the worked sample, since 72.16% showed a positive prediction signal for secretion, surpassing the average of 50% presented in other secretomic studies. Furthermore, the study allowed the potential identification of processes such as adhesion, degradation and penetration of the cuticle, as well as potential host immunomodulation through specific and differential proteins, compared to secretomes related to other hosts. Our molecular results shows, in genomic level (*B. bassiana* comparison) and in proteomics level (*B. bassiana* secretome comparison to *M. anisopliae* E6 secretome and the same strain with two differents hosts (*R. microplus* and *D. peruvianus*)), that *M. anisopliae* E6 has an more lethal secretome due the higher arsenal to proteins related to toxicity and infection, which the Galleria toxicity assay confirms. Thus, proteins potentially involved in tick-specific infection and pathogenicity determinants potentially found here should be analyzed individually in the future (Fig. 4). Therefore, to the best of our knowledge the results presented here comprise the largest differential secretome related with host infection identified up-to-date in *M. anisopliae*, and will greatly contribute to the molecular elucidation of the cattle tick, *R. microplus,* infection and biocontrol process.

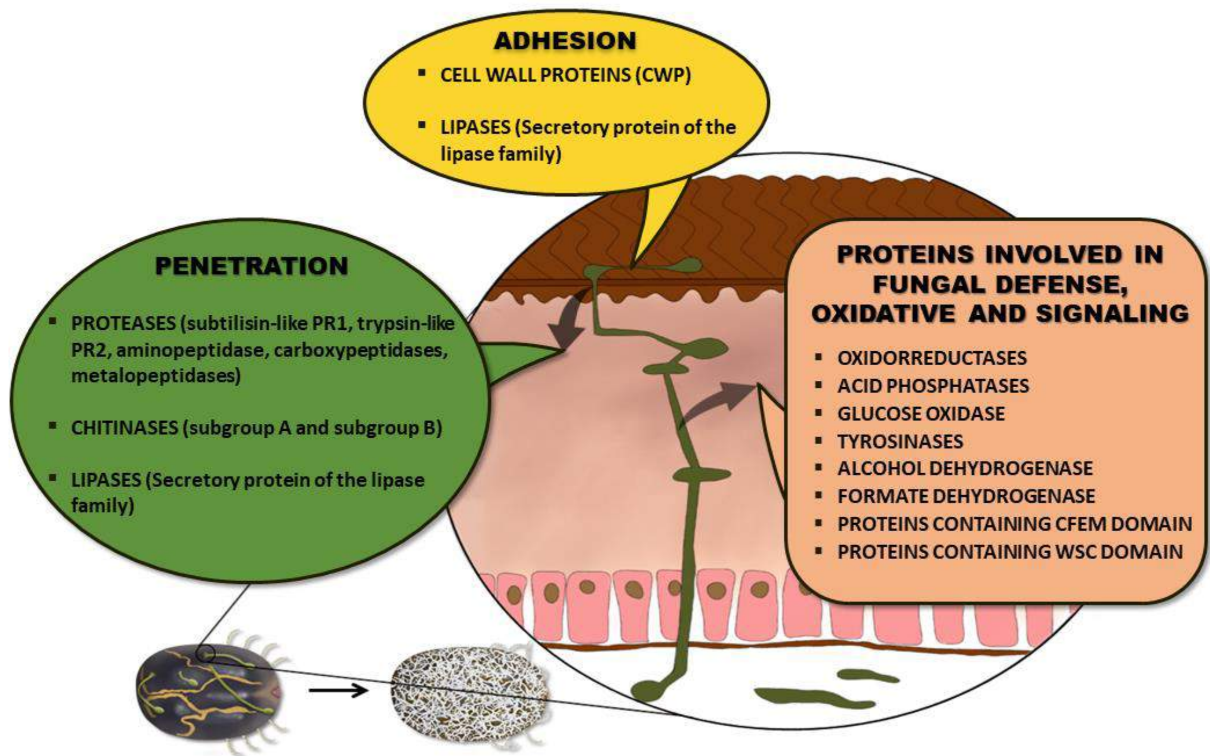

Fig. 4. Representation of the main protein groups found in this study.

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Supplementary Material

Table S1. Proteins exclusively identified in a culture medium containing the tick cuticle when compared to control

Accession number	Spec count	Protein name
gi|672383843|gb|KFG85949.1|	544	hypothetical protein MANI_011377
gi|672383272|gb|KFG85387.1|	381	ThiJ/PfpI family protein
gi|672378206|gb|KFG80457.1|	272	ER membrane protein Wsc4
gi|672380791|gb|KFG82986.1|	238	hypothetical protein MANI_021357
gi|672376434|gb|KFG78725.1|	210	carboxypeptidase
gi|672382737|gb|KFG84858.1|	201	hypothetical protein MANI_022411
gi|672377000|gb|KFG79277.1|	127	subtilisin-like protease PR1G
gi|672379708|gb|KFG81918.1|	115	chitooligosaccharide oxidase
gi|672384636|gb|KFG86731.1|	106	cell wall protein
gi|672385928|gb|KFG88014.1|	94	putative galactose oxidase, partial
gi|672382595|gb|KFG84717.1|	94	putative cutinase
gi|672383956|gb|KFG86059.1|	75	endo-N-acetyl-beta-D-glucosaminidase D1 (chitinase)
gi|672379284|gb|KFG81500.1|	70	carboxypeptidase
gi|672381594|gb|KFG83774.1|	64	aminopeptidase 2
gi|672378348|gb|KFG80590.1|	60	hypothetical protein MANI_119609
gi|672378126|gb|KFG80381.1|	60	hypothetical protein MANI_011023
gi|672377593|gb|KFG79861.1|	53	putative dioxygenase
gi|672378083|gb|KFG80338.1|	51	hypothetical protein MANI_010921
gi|672384941|gb|KFG87032.1|	48	putative cellulase
gi|672376628|gb|KFG78916.1|	48	subgroup A chitinase A1
gi|672375320|gb|KFG77657.1|	48	TRI14-like protein
gi|672380790|gb|KFG82985.1|	47	alpha/beta-hydrolase
gi|672381730|gb|KFG83907.1|	46	profilin
gi|672381209|gb|KFG83398.1|	46	hypothetical protein MANI_018182
gi|672383805|gb|KFG85912.1|	45	hypothetical protein MANI_011448
gi|672385181|gb|KFG87270.1|	44	Lcc2
gi|672383931|gb|KFG86035.1|	43	putative aminopeptidase
gi|672377748|gb|KFG80013.1|	42	hypothetical protein MANI_019648
gi|672380686|gb|KFG82883.1|	41	Citrate synthase
gi|672377598|gb|KFG79866.1|	40	acetylcholinesterase precursor
gi|672385773|gb|KFG87860.1|	38	6-phosphogluconate dehydrogenase
gi|672385160|gb|KFG87250.1|	36	hypothetical protein MANI_022892
gi|672376835|gb|KFG79118.1|	36	putative serine peptidase
gi|672375499|gb|KFG77823.1|	35	glucose-6-phosphate isomerase
gi|672382664|gb|KFG84786.1|	34	hypothetical protein MANI_010159
gi|672384382|gb|KFG86479.1|	33	transaldolase
gi|672377114|gb|KFG79389.1|	31	1,3-beta-glucanosyltransferase Gel2
gi|672383117|gb|KFG85233.1|	30	elongation factor 1-gamma
gi|672382899|gb|KFG85018.1|	29	tyrosinase 2

gi|672378963|gb|KFG81188.1|	29	Subtilisin-like protease PR1H
gi|672378426|gb|KFG80666.1|	29	hypothetical protein MANI_017731
gi|672375020|gb|KFG77467.1|	29	subtilisin-like serine protease
gi|672380299|gb|KFG82505.1|	28	NADP-dependent glycerol dehydrogenase
gi|672376736|gb|KFG79021.1|	28	adhesin-like protein 1, partial
gi|672384663|gb|KFG86758.1|	26	spermidine synthase
gi|672379653|gb|KFG81867.1|	26	extracellular cell wall glucanase Crf1
gi|672385963|gb|KFG88048.1|	25	putative alpha/beta fold family hydrolase
gi|672382772|gb|KFG84893.1|	24	hypothetical protein MANI_115390
gi|672382606|gb|KFG84728.1|	24	hypothetical protein MANI_010251
gi|672382448|gb|KFG84574.1|	23	mitochondrial pyruvate dehydrogenase kinase
gi|672379036|gb|KFG81258.1|	23	ECM33-like protein
gi|672377796|gb|KFG80060.1|	23	metallo-endopeptidase
gi|672383659|gb|KFG85768.1|	22	Complex I intermediate-associated protein 30
gi|672375422|gb|KFG77749.1|	22	hypothetical protein MANI_005297
gi|672379453|gb|KFG81668.1|	21	hypothetical protein MANI_019335
gi|672378993|gb|KFG81215.1|	21	putative endoglucanase
gi|672382282|gb|KFG84413.1|	20	outer membrane protein porin
gi|672377130|gb|KFG79405.1|	19	alanine--glyoxylate aminotransferase
gi|672375552|gb|KFG77873.1|	19	hypothetical protein MANI_004329
gi|672385878|gb|KFG87964.1|	18	putative antigenic cell wall galactomannoprotein
gi|672382980|gb|KFG85097.1|	18	hypothetical protein MANI_002202
gi|672381720|gb|KFG83897.1|	18	mannan endo-1,6-alpha-mannosidase-like protein
gi|672383519|gb|KFG85631.1|	17	translation elongation factor 1 alpha
gi|672382815|gb|KFG84935.1|	17	putative serine-threonine rich protein
gi|672382148|gb|KFG84282.1|	17	Phosphodiesterase/alkaline phosphatase D
gi|672376333|gb|KFG78628.1|	17	Cel5b putative endoglucanase
gi|672385923|gb|KFG88009.1|	16	proteinase inhibitor I4
gi|672379235|gb|KFG81454.1|	16	subtilisin-like serine protease PR1C
gi|672378469|gb|KFG80706.1|	16	hypothetical protein MANI_111186
gi|672375493|gb|KFG77817.1|	15	hypothetical protein MANI_004456
gi|672381383|gb|KFG83567.1|	14	putative endoglucanase
gi|672375782|gb|KFG78097.1|	14	neutral ceramidase precursor
gi|672381865|gb|KFG84031.1|	13	glutathione-disulfide reductase
gi|672380515|gb|KFG82719.1|	13	Secretory lipase family protein
gi|672380467|gb|KFG82671.1|	13	malate dehydrogenase
gi|672377540|gb|KFG79808.1|	13	metalloprotease-like protein
gi|672375604|gb|KFG77924.1|	13	Subgroup B chitinase B4
gi|672382275|gb|KFG84406.1|	12	hypothetical protein MANI_010825
gi|672377590|gb|KFG79858.1|	12	proline rich protein 5MeD
gi|672384869|gb|KFG86962.1|	11	alcohol dehydrogenase
gi|672383917|gb|KFG86022.1|	11	putative phospholipase
gi|672383753|gb|KFG85861.1|	11	hypothetical protein MANI_027536
gi|672383580|gb|KFG85690.1|	11	arginine deiminase type-3
gi|672383290|gb|KFG85405.1|	11	hypothetical protein MANI_005023

gi|672378896|gb|KFG81125.1|	11	hypothetical protein MANI_014530
gi|672377571|gb|KFG79839.1|	11	hypothetical protein MANI_008919
gi|672384303|gb|KFG86401.1|	10	hypothetical protein MANI_002345
gi|672383541|gb|KFG85652.1|	10	hypothetical protein MANI_004698
gi|672383011|gb|KFG85128.1|	10	secreted aspartic proteinase
gi|672381667|gb|KFG83846.1|	10	hypothetical protein MANI_027043
gi|672380646|gb|KFG82843.1|	10	putative extracellular protein
gi|672383668|gb|KFG85777.1|	9	outer membrane autotransporter
gi|672383383|gb|KFG85496.1|	9	regulatory P domain-containing protein
gi|672383174|gb|KFG85290.1|	9	peptidyl-prolyl cis-trans isomerase B precursor
gi|672380281|gb|KFG82487.1|	9	putative glycosyl hydrolase
gi|672377234|gb|KFG79508.1|	9	metalloprotease MEP1
gi|672376413|gb|KFG78705.1|	9	candidapepsin-4 precursor
gi|672375620|gb|KFG77940.1|	9	hypothetical protein MANI_004425
gi|672375241|gb|KFG77583.1|	9	putative restculine oxidase precursor
gi|672383898|gb|KFG86003.1|	8	hypothetical protein MANI_010408
gi|672383827|gb|KFG85933.1|	8	hypothetical protein MANI_004014
gi|672383679|gb|KFG85788.1|	8	hypothetical protein MANI_007526
gi|672383358|gb|KFG85471.1|	8	alpha-galactosidase
gi|672379239|gb|KFG81458.1|	8	galactose oxidase precursor
gi|672378833|gb|KFG81062.1|	8	isoflavone reductase family protein
gi|672378802|gb|KFG81031.1|	8	formate dehydrogenase
gi|672377760|gb|KFG80025.1|	8	putative Xaa-Pro aminopeptidase pepP
gi|672377093|gb|KFG79368.1|	8	cell surface protein (Mas1)
gi|672376632|gb|KFG78920.1|	8	hypothetical protein MANI_007473
gi|672385701|gb|KFG87788.1|	7	alpha/beta hydrolase fold domain containing protein
gi|672385664|gb|KFG87751.1|	7	hypothetical protein MANI_001032
gi|672384157|gb|KFG86257.1|	7	major allergen Asp f 2-like protein
gi|672381305|gb|KFG83494.1|	7	adhesin-like protein 1, partial
gi|672377990|gb|KFG80248.1|	7	Alpha-N-arabinofuranosidase Precursor
gi|672377374|gb|KFG79646.1|	7	hypothetical protein MANI_019146
gi|672377235|gb|KFG79509.1|	7	putative cell surface spherulin 4-like protein
gi|672376253|gb|KFG78550.1|	7	hypothetical protein MANI_002983
gi|672381673|gb|KFG83852.1|	6	Leukotriene A-4 hydrolase
gi|672378081|gb|KFG80336.1|	6	hypothetical protein MANI_010995
gi|672377798|gb|KFG80062.1|	6	ThiJ/PfpI family protein
gi|672386154|gb|KFG88237.1|	5	isopentenyl-diphosphate delta-isomerase
gi|672384745|gb|KFG86839.1|	5	beta-glucosidase
gi|672381709|gb|KFG83886.1|	5	hypothetical protein MANI_116252
gi|672381296|gb|KFG83485.1|	5	extracellular serine-rich protein
gi|672380947|gb|KFG83141.1|	5	putative WSC domain protein
gi|672380127|gb|KFG82333.1|	5	putative nuclease PA3
gi|672378815|gb|KFG81044.1|	5	hypothetical protein MANI_014475
gi|672378560|gb|KFG80795.1|	5	collagen-like protein Mcl1

gi|672376341|gb|KFG78636.1|	5	hypothetical protein MANI_002826
gi|672375992|gb|KFG78299.1|	5	hypothetical protein MANI_020368
gi|672384220|gb|KFG86320.1|	4	hypothetical protein MANI_009105
gi|672382616|gb|KFG84738.1|	4	putative ferulic acid esterase (FaeA)
gi|672375342|gb|KFG77678.1|	4	hypothetical protein MANI_016460

Table S2. Differentially expressed proteins identified in *M. anisopliae* secretome comparing the tick cuticle–containing medium versus control medium

Accession number	Fold Change	pValue	Description
gi|672381981|gb|KFG84128.1|	75,71857923	1,00E-05	subtilisin-like protease PR1K
gi|672378997|gb|KFG81219.1|	74,8015873	0,000721005	riboflavin aldehyde-forming enzyme
gi|672378772|gb|KFG81001.1|	54,5625	0,000191149	putative effector 14
gi|672380759|gb|KFG82955.1|	31,66666667	0,000607485	Subgroup A chitinase A6
gi|672380312|gb|KFG82517.1|	27,31944444	0,003244297	trypsin-related protease
gi|672383693|gb|KFG85802.1|	24,90509259	0,031737742	1,2-a-D-mannosidase
gi|672375501|gb|KFG77825.1|	24,4	0,004165984	hypothetical protein MANI_004468
gi|672384717|gb|KFG86811.1|	22,88304094	0,000281884	WSC domain containing protein
gi|672376542|gb|KFG78830.1|	22,88095238	0,003841831	putative thioredoxin reductase
gi|672380302|gb|KFG82508.1|	20,91666667	1,00E-05	carboxy-cis,cis-muconate cyclase
gi|672376342|gb|KFG78637.1|	20,25333333	0,006996017	TRI14-like protein
gi|672381881|gb|KFG84047.1|	20,0625	0,000706334	CFEM domain containing protein
gi|672383750|gb|KFG85858.1|	19,57407407	0,000559651	putative acid phosphatase
gi|672378164|gb|KFG80416.1|	18,76190476	0,001502208	subgroup B chitinase B7
gi|672375398|gb|KFG77728.1|	16,55769231	0,000831715	hypothetical protein MANI_005253
gi|672383059|gb|KFG85176.1|	15,79816514	0,015286951	secreted protein
gi|672385215|gb|KFG87304.1|	14,73333333	1,00E-05	beta-1,3-glucanase precursor
gi|672375490|gb|KFG77814.1|	14,49122807	1,92E-05	putative penicillin-binding protein
gi|672384588|gb|KFG86683.1|	13,02424242	0,00023715	subtilisin-like protease Pr1A
gi|672385451|gb|KFG87539.1|	10,73170732	0,029076998	hypothetical protein MANI_001730
gi|672382702|gb|KFG84823.1|	10,63207547	1,00E-05	alpha-glucosidase, partial
gi|672375566|gb|KFG77887.1|	10,25294118	0,002005488	cell wall protein
gi|672382950|gb|KFG85069.1|	9,560185185	0,001469935	endonuclease/exonuclease/phosphatase family protein
gi|672382099|gb|KFG84234.1|	9,542735043	0,000333323	Cel3b putative secreted beta-glucosidase
gi|672378086|gb|KFG80341.1|	9,158333333	0,002092821	acid trehalase

gi|672375616|gb|KFG77936.1|	9,097378277	1,24E-05	putative non-hemolytic phospholipase C precursor
gi|672382585|gb|KFG84709.1|	8,434782609	0,00500367	putative GPI anchored protein
gi|672385232|gb|KFG87321.1|	7,590196078	0,001280665	beta-1,3-glucanosyltransferase
gi|672382220|gb|KFG84351.1|	6,967228464	0,015959113	GPI-anchored cell wall beta-1,3-endoglucanase EglC
gi|672379028|gb|KFG81250.1|	6,948148148	0,003425832	hypothetical protein MANI_024182
gi|672376523|gb|KFG78811.1|	6,422330097	0,002582521	hypothetical protein MANI_007455
gi|672378681|gb|KFG80912.1|	6,319444444	0,008319856	glutamyl-tRNA(Gln) amidotransferase subunit A
gi|672375358|gb|KFG77692.1|	6,248858447	1,89E-05	protein tyrosine phosphatase
gi|672379163|gb|KFG81383.1|	5,933333333	0,000239942	malate dehydrogenase
gi|672380754|gb|KFG82950.1|	5,6875	0,002216533	hypothetical protein MANI_022604
gi|672379705|gb|KFG81915.1|	5,484893512	3,99E-05	hypothetical protein MANI_000861
gi|672385369|gb|KFG87457.1|	5,115740741	1,00E-05	putative glyoxal oxidase precursor
gi|672385526|gb|KFG87613.1|	5,090909091	1,00E-05	hypothetical protein MANI_001481
gi|672376244|gb|KFG78542.1|	4,931034483	0,000854636	putative cell wall glycosyl hydrolase YteR, partial
gi|672378859|gb|KFG81088.1|	4,877873563	0,000869238	DNase1 protein
gi|672376005|gb|KFG78310.1|	4,567264574	0,010476653	GPI-anchored cell wall beta-1,3-endoglucanase EglC
gi|672383910|gb|KFG86015.1|	3,837037037	2,00E-05	phosphorylcholine phosphatase
gi|672383228|gb|KFG85344.1|	3,823002755	0,036081882	glycerophosphoryl diester phosphodiesterase family protein
gi|672381896|gb|KFG84062.1|	3,809895833	1,00E-05	5'-nucleotidase precursor
gi|672384190|gb|KFG86290.1|	3,761904762	4,38E-05	nucleoside diphosphate kinase 1
gi|672375617|gb|KFG77937.1|	3,74906367	0,001218451	putative leucine aminopeptidase
gi|672378520|gb|KFG80755.1|	3,362421384	0,023823123	putative glucose oxidase
gi|672384737|gb|KFG86831.1|	2,683333333	0,000595794	tripeptidyl-peptidase 1 precursor
gi|672384654|gb|KFG86749.1|	2,468599034	0,025026251	hypothetical protein MANI_113561
gi|672385419|gb|KFG87507.1|	2,389397407	0,02273437	acid phosphatase
gi|672375619|gb|KFG77939.1|	2,06698821	0,001490895	beta-1,6-glucanase
gi|672382503|gb|KFG84628.1|	2,035037879	0,030875283	hypothetical protein MANI_019971
gi|672377834|gb|KFG80097.1|	-2,327819549	0,0008707	Ribonuclease Trv
gi|672376381|gb|KFG78674.1|	-3,736757624	0,000234792	Guanyl-specific ribonuclease F1

gi|672385099|gb|KFG87189.1|	-5,013824885	7,10E-05	hypothetical protein MANI_000444
gi|672384426|gb|KFG86522.1|	-20,47619048	0,000575519	hypothetical protein MANI_013783

Table S3. Predicted localization and possible secretion of all proteins identified in *M. anisopliae* as differentially expressed under infection condition

Protein	TargetP 1.0 (cuttof >0,9)	WoLF PSORT	TMHMM 2.0	SignalP 5.0
gi|672375020|gb|KFG77467.1|	S	S	S	S
gi|672375241|gb|KFG77583.1|	S	S	S	S
gi|672375320|gb|KFG77657.1|	S	S	S	S
gi|672375342|gb|KFG77678.1|	_	C	S	_
gi|672375358|gb|KFG77692.1|	S	S	S	S
gi|672375398|gb|KFG77728.1|	S	S	S	S
gi|672375422|gb|KFG77749.1|	S	S	S	S
gi|672375490|gb|KFG77814.1|	S	S	S	S
gi|672375493|gb|KFG77817.1|	S	S	S	S
gi|672375499|gb|KFG77823.1|	_	PX	S	_
gi|672375501|gb|KFG77825.1|	S	S	S	S
gi|672375552|gb|KFG77873.1|	S	S	T	S
gi|672375566|gb|KFG77887.1|	S	S	S	S
gi|672375604|gb|KFG77924.1|	S	S	S	S
gi|672375616|gb|KFG77936.1|	M	S	S	S
gi|672375617|gb|KFG77937.1|	S	S	S	S
gi|672375619|gb|KFG77939.1|	S	S	S	S
gi|672375620|gb|KFG77940.1|	S	S	T	S
gi|672375782|gb|KFG78097.1|	S	S	T	S
gi|672375992|gb|KFG78299.1|	_	C	S	_
gi|672376005|gb|KFG78310.1|	*	S	S	S
gi|672376244|gb|KFG78542.1|	_	S	S	_
gi|672376253|gb|KFG78550.1|	S	S	S	S
gi|672376333|gb|KFG78628.1|	_	S	S	_
gi|672376341|gb|KFG78636.1|	_	S	T	_

gi|672376342|gb|KFG78637.1|	S	PX	S	S
gi|672376381|gb|KFG78674.1|	S	S	S	S
gi|672376413|gb|KFG78705.1|	S	S	S	_
gi|672376434|gb|KFG78725.1|	S	S	S	S
gi|672376523|gb|KFG78811.1|	S	S	S	S
gi|672376525|gb|KFG78813.1|	S	S	S	S
gi|672376542|gb|KFG78830.1|	S	S	S	S
gi|672376628|gb|KFG78916.1|	S	S	S	S
gi|672376632|gb|KFG78920.1|	S	S	S	S
gi|672376736|gb|KFG79021.1|	_	C	S	_
gi|672376835|gb|KFG79118.1|	S	S	S	S
gi|672377000|gb|KFG79277.1|	S	S	S	S
gi|672377093|gb|KFG79368.1|	S	S	S	S
gi|672377114|gb|KFG79389.1|	S	S	S	S
gi|672377130|gb|KFG79405.1|	_	C	S	_
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gi|672377540|gb|KFG79808.1|	S	S	S	S
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gi|672377590|gb|KFG79858.1|	S	S	S	S
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gi|672377598|gb|KFG79866.1|	S	S	S	S
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gi|672378833|gb|KFG81062.1|	*	M	S	_
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gi|672379028|gb|KFG81250.1|	S	M	S	S
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gi|672379239|gb|KFG81458.1|	S	S	S	S
gi|672379284|gb|KFG81500.1|	S	S	S	S
gi|672379453|gb|KFG81668.1|	_	PX	S	_

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gi|672380302|gb|KFG82508.1|	S	S	S	S
gi|672380312|gb|KFG82517.1|	S	S	S	S
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gi|672380759|gb|KFG82955.1|	S	S	S	S
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gi|672381209|gb|KFG83398.1|	S	S	S	S
gi|672381296|gb|KFG83485.1|	S	S	S	S
gi|672381305|gb|KFG83494.1|	S	S	S	S
gi|672381383|gb|KFG83567.1|	S	S	S	S
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gi|672381881|gb|KFG84047.1|	_	S	S	_
gi|672381896|gb|KFG84062.1|	S	S	S	S
gi|672381981|gb|KFG84128.1|	S	S	S	S
gi|672382099|gb|KFG84234.1|	S	S	S	S
gi|672382148|gb|KFG84282.1|	*	S	S	_
gi|672382220|gb|KFG84351.1|	S	S	S	S
gi|672382275|gb|KFG84406.1|	S	S	S	S
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gi|672382585|gb|KFG84709.1|	S	P	S	_
gi|672382595|gb|KFG84717.1|	S	PX	S	S
gi|672382606|gb|KFG84728.1|	S	PX	S	S
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gi|672384717|gb|KFG86811.1|	S	S	S	S
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gi|672384745|gb|KFG86839.1|	S	S	S	S
gi|672384869|gb|KFG86962.1|	_	S	S	_
gi|672384941|gb|KFG87032.1|	S	S	T	S
gi|672384989|gb|KFG87080.1|	S	S	S	S
gi|672385099|gb|KFG87189.1|	S	S	S	S
gi|672385160|gb|KFG87250.1|	*	N	S	_
gi|672385181|gb|KFG87270.1|	S	S	S	_
gi|672385215|gb|KFG87304.1|	S	S	S	S
gi|672385232|gb|KFG87321.1|	S	S	T	S
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gi|672385664|gb|KFG87751.1|	_	S	T	_
gi|672385701|gb|KFG87788.1|	S	S	S	S
gi|672385773|gb|KFG87860.1|	*	M	S	_
gi|672385878|gb|KFG87964.1|	S	C	S	S
gi|672385923|gb|KFG88009.1|	_	M	S	_
gi|672385928|gb|KFG88014.1|	_	C	S	_
gi|672385963|gb|KFG88048.1|	_	C	S	_
gi|672386154|gb|KFG88237.1|	_	C	S	_
gi|672383843|gb|KFG85949.1|	S	S	S	S

(S: secreted; M: mitochondria; T: transmembrane; C: cytosol; PX: peroxisomes; P: plasma membrane; N: core; *: below the cutoff; _: other localization)

Table S4. Putative Classification of *M. anisopliae* Hypothetical Proteins Identified under Infection Condition

	Accession number	BlastP homologous protein / conserved domains
DEP	gi|672385451|gb|KFG87539.1|	Cell wall beta-glucan synthesis [*Metarhizium brunneum* ARSEF 3297] / GPI-anchored superfamily
	gi|672379028|gb|KFG81250.1|	tape measure protein [*Metarhizium brunneum* ARSEF 3297] / -
	gi|672376523|gb|KFG78811.1|	- / *Alternaria alternata* allergen 1 (AltA1)
	gi|672379705|gb|KFG81915.1|	quinoprotein amine dehydrogenase beta chain-like protein [*Metarhizium robertsii* ARSEF 23] / -
	gi|672385526|gb|KFG87613.1|	- / Dynein light intermediate chain (DLIC)
	gi|672382503|gb|KFG84628.1|	catalytic protein [*Metarhizium majus* ARSEF 297] / MhpC
	gi|672384426|gb|KFG86522.1|	deoxyribonuclease nucA/NucB domain-containing protein [*Pochonia chlamydosporia* 170] / DNase_NucA_NucB
Exc RM	gi|672383843|gb|KFG85949.1|	- / *Alternaria alternata* allergen 1 (AltA1)
	gi|672382737|gb|KFG84858.1|	Allergen V5/Tpx-1-related protein [*Metarhizium robertsii* ARSEF 23] / SCP_PRY1_like
	gi|672378126|gb|KFG80381.1|	quercetin 2,3-dioxygenase [*Metarhizium robertsii* ARSEF 23] / QdoI - Cupin domain protein related to quercetin dioxygenase
	gi|672378083|gb|KFG80338.1|	FAD-binding, type 2 [*Metarhizium robertsii* ARSEF 23] / FAD_binding_4; FAD_lactone_ox;
	gi|672381209|gb|KFG83398.1|	malate dehydrogenase [*Metarhizium robertsii* ARSEF 23] / -
	gi|672383805|gb|KFG85912.1|	Barwin-related endoglucanase [*Metarhizium brunneum* ARSEF 3297] / -
	gi|672377748|gb|KFG80013.1|	- / PRK14718 (ribonuclease III)
	gi|672378426|gb|KFG80666.1|	Glycoside hydrolase, superfamily [*Metarhizium robertsii* ARSEF 23] / Glycosyl hydrolase catalytic core
	gi|672382772|gb|KFG84893.1|	catalytic protein [*Metarhizium anisopliae* ARSEF 549] / Hydrolase_4
	gi|672382606|gb|KFG84728.1|	Lysozyme-like domain protein [*Metarhizium majus* ARSEF 297] / rad23
	gi|672379453|gb|KFG81668.1|	Ubiquitin-activating enzyme [*Metarhizium anisopliae* BRIP 53293] / ECM4; GST_C_Omega_like; GST_C_2
	gi|672375552|gb|KFG77873.1|	glycosyl hydrolase family 16 [*Metarhizium brunneum* ARSEF 3297] / Glyco_hydro_16
	gi|672382980|gb|KFG85097.1|	endoglucanase [*Metarhizium majus* ARSEF 297] / Glyco_hydro_61
	gi|672378469|gb|KFG80706.1|	tyrosinase [*Metarhizium anisopliae* ARSEF 549] / Tyrosinase
	gi|672375493|gb|KFG77817.1|	peptidase S1 domain protein [*Metarhizium robertsii*] / Trypsin Superfamily
	gi|672382275|gb|KFG84406.1|	- / G2F domain (Nidogen)
	gi|672378896|gb|KFG81125.1|	Alpha/beta hydrolase fold-3 domain protein *[Metarhizium anisopliae* ARSEF 549] / Acetyl esterase/lipase Superfamily
	gi|672377571|gb|KFG79839.1|	Cerato-platanin [*Metarhizium majus* ARSEF 297] / Cerato-platanin
	gi|672384303|gb|KFG86401.1|	prolyl aminopeptidase (secreted protein) [*Metarhizium robertsii* ARSEF 23] / LIP (Secretory lipase)
	gi|672383541|gb|KFG85652.1|	cell wall protein [*Metarhizium brunneum* ARSEF 3297] / HsbA
	gi|672375620|gb|KFG77940.1|	glycoside hydrolase family 12 [*Metarhizium robertsii* ARSEF 23] / Glycosyl hydrolase family 12
	gi|672383898|gb|KFG86003.1|	polysaccharide lyase [*Metarhizium robertsii*] / Polysaccharide lyase
	gi|672383827|gb|KFG85933.1|	Beta-N-acetylglucosaminidase [*Metarhizium robertsii* ARSEF 23] / beta-N-acetylglucosaminidase
	gi|672383679|gb|KFG85788.1|	protein related to glucan 1, 4-alpha-glucosidase [*Metarhizium robertsii* ARSEF 23] / -
	gi|672376632|gb|KFG78920.1|	Proteinase inhibitor, propeptide [*Metarhizium robertsii* ARSEF 23] / -
	gi|672377374|gb|KFG79646.1|	lipase/esterase family protein [*Pochonia chlamydosporia* 170] / -
	gi|672376253|gb|KFG78550.1|	Cupin, RmlC-type [*Metarhizium robertsii* ARSEF 23] / -
	gi|672378081|gb|KFG80336.1|	Extracellular membrane protein, CFEM domain protein [*Metarhizium robertsii* ARSEF 23] / CFEM Superfamily

	gi|672381709|gb|KFG83886.1|	ATPase, AFG1-like protein [*Metarhizium anisopliae* ARSEF 549] / AFG1_ATPase
	gi|672378815|gb|KFG81044.1|	Lipase, secreted [*Metarhizium robertsii* ARSEF 23] / LIP
	gi|672384220|gb|KFG86320.1|	WSC domain-containing protein [*Metarhizium robertsii* ARSEF 23] / -
Exc C	gi|672381490|gb|KFG83674.1|	glutamyl-tRNA(Gln) amidotransferase [*Metarhizium majus* ARSEF 297] / Amidase Superfamily
	gi|672381634|gb|KFG83813.1|	collagen-like protein Mcl1 [*Metarhizium robertsii* ARSEF 23] / -

DEP = differentially expressed proteins; Exc RM= exclusively identified proteins in RM; Exc C = exclusive identified in control condition

Table S5. Comparative genomic distribution of the differentially expressed proteins of the *M. anisopliae* secretome compared to *B. bassiana*

	Pfam entry accession	ID	Description	MaE6	Bb2860
Proteases	PF00082	Peptidase_S8	Subtilase family	53	40
	PF00089	Trypsin	Trypsin	23	20
Lipases	PF03583	LIP	Secretory lipase	15	17
	PF00135	COesterase	Carboxylesterase family	27	20
Chitinases	PF00704	Glyco_hydro_18	Glycosyl hydrolases family 18	26	23
	PF00734	CBM_1	Fungal cellulose binding domain	3	4
ROS protection	PF00199	Catalase	Catalase	7	5
	PF01565	FAD_binding_4	FAD binding domain	32	29
Extracellular effectors	PF01822	WSC	WSC domain	20	15
	PF00188	CAP	Cysteine-rich secretory protein family	6	6
	PF00235	Profilin	Profilin	3	2
	PF00246	Peptidase_M14	Zinc carboxypeptidase	5	4
	PF00285	Citrate_synt	Citrate synthase, C-terminal domain	7	4
	PF00342	PGI	Phosphoglucose isomerase	1	1
	PF00393	6PGD	6-phosphogluconate dehydrogenase, C-terminal domain	3	2
	PF00775	Dioxygenase_C	Dioxygenase	8	5
	PF00923	TAL_FSA	Transaldolase/Fructose-6-phosphate aldolase	2	1
	PF01433	Peptidase_M1	Peptidase family M1 domain	3	4
	PF02866	Ldh_1_C	lactate/malate dehydrogenase, alpha/beta C-terminal domain	3	3
	PF03198	Glyco_hydro_72	Glucanosyltransferase	6	8
	PF07992	Pyr_redox_2	Pyridine nucleotide-disulphide oxidoreductase	149	135

Table S6. Amount of duplication and genes loss events between M. anisopliae and B. bassiana

	Pfam entry accession	Family	Specie	Duplications	Losses
Proteases	PF00082	Subtilase	*M.anisopliae*	14	16
			B.bassiana	7	22
	PF00089	Trypsin	*M.anisopliae*	5	10
			B.bassiana	2	10
Lipases	PF03583	Secretory lipase	*M.anisopliae*	3	6
			B.bassiana	4	5
	PF00135	Carboxylesterase family	*M.anisopliae*	3	3
			B.bassiana	1	8
Chitinases	PF00704	Glycosyl hydrolases family 18	*M.anisopliae*	5	5
			B.bassiana	3	6
	PF00734	Fungal cellulose binding domain	*M.anisopliae*	0	2
			B.bassiana	0	1
ROS protection	PF00199	Catalase	*M.anisopliae*	0	0
			B.bassiana	0	3
	PF01565	FAD binding domain	*M.anisopliae*	5	5
			B.bassiana	4	7
Extracellular effectors	PF01822	WSC domain	*M.anisopliae*	3	4
			B.bassiana	0	6
	PF00188	Cysteine-rich secretory protein family	*M.anisopliae*	1	2
			B.bassiana	1	2

MICROBIOLOGICAL RESEARCH

AUTHOR INFORMATION PACK

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