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CHANGES IN THE EXPRESSION OF IMMUNE PROTEINS OF *DIATRAEA SACCHARALIS* (LEPIDOPTERA: CRAMBIDAE) CHALLENGED BY BACTERIALAGENTS AND ENTOMAP ATHOGENIC FUNGUS *BEAUVERIA BASSIANA*

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ARTICLE INFO	ABSTRACT
	In this study, the expression of proteins from hemolymph of Diatraea saccharalis (Lepidoptera: Crambidae) larvae was analyzed after septic challenge in order to verify the humoral response to Bacillus subtilis, Escherichia coli and entomopathogenic fungus Beauveria bassiana. Hemolymph samples were submitted to two-dimensional electrophoresis and mass spectrometry for identification of proteins and peptides. Orthologous immune proteins were identified using Mascot database and TagIdent software. After six hours of microbial septic challenge was observed that E. coli increased the expression of a putative defense protein and B. subtilis modulated negatively the expression of peptidoglycan recognition protein (PGRP). In this condition, B. subtilis also increased the expression of the same putative defense protein and others proteins like to attacin-A and inhibitor of serine-protease were induced. Septic challenge by six hours with B. bassiana induced the expression of Cecropin-A2 related protein and a possible Drosomycin-like protein. In addition twelve hours of septic challenge with B. subtilis and E. coli increased the expression of a probable lysozyme. A multifunctional protein Apolipophorin-3 was induced twelve hours after of fungal challenge. These results indicate that septic challenge of D. saccharalis larvae was able to induce or regulate the expression of putative proteins involved in immunological processes.

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INTRODUCTION

The cultivation of sugar cane is widely distributed in countries like India, Mexico, China, the United States and Brazil; the latter is considered the major global producer. Due to the high demand for renewable fuels in the last decades, the prevalent challenge is increased productivity, which sometimes inhibited by pest damage (Sanches *et al*., 2017). *Diatraea saccharalis* (Lepidoptera: Crambidae) is the major pest that attacks sugar cane crops, causing direct and indirect damage to the plant and leading to considerable impacts on productivity (Pauli, 2009). One of main factors that favors the attack and infestation of crops is the capacity of *D. saccharalis* larvae to respond to the

stress caused by pathogens in the environment, such as entomopathogenic microorganisms. Insects have an innate immune response divided into cellular and humoral responses. The cellular immune response is performed by hemocytes and includes processes like phagocytosis and encapsulation. The humoral immune response is characterized by processes in the hemolymph, such as coagulation and melanization, the production of enzymes by hemocytes and their release into the circulation (Lavine & Strand, 2002). Additionally, antimicrobial peptides (AMPs) are produced by insect fat body (Zhang *et al.,* 2017). The production of AMPs in insects has been described in model organisms such as *Bombyx mori, Drosophila melanogaster, Apismellifera* and others insects (Tian*et al*., 2017). AMPs are divided into a number of classes:

defensins, cecropins, atacins, lebocins, moricins and gloverins (Yi *et al.,* 2014). AMPs are low molecular weight, cationic and amphiphilic proteins and are released into the hemolymph within a few hours of pathogen invasion (Chung & Kumar, 2016; Vilcinskas, 2015). The mechanism of action of AMPs consists of inducing disturbances in the bacterial cell membrane; the effects of these molecules have become the object of study for possible treatments for antibiotic-resistant microorganisms (Lavicoli *et al.,* 2016; Meloni *et al.*, 2015). The immune response of *D. saccharalis,* as well as other insects, starts when the proteins responsible for patterns recognition (PRPs) recognize the pathogen (Kingsolver *et al.*, 2013); that signaling induces the activation of different cascades responsible for increased expression of AMPs, which then participate in the immune response (Gilbert , 2012). The evolution of genomic techniques has led to the genome sequencing of some species insects. This has improved the available databases, increased the characterization of new genes participating in the innate immune response in model and non-model insects and demonstrated the diversity of these biomolecules (Viljakainen, 2015). In addition to genomics, proteomic methods are one of the main tools applied to the characterization of AMPs in insects (Makarova*et al.*, 2016). Induction of the humoral immune response in *D. saccharalis* larvae have been demonstrated following challenge by microorganisms (Silva *et al.*,2010; Rocha *et al*., 2016). In this study, the objective was to analyze the expression of low molecular weight proteins in the hemolymph of *D. saccharalis* after septic challenge with *Bacillus subtilis*, *Escherichia coli*, as well as the entomopathogenic fungus *Beauveria bassiana,* which is widely used in the biological control of sugar cane borers.Applying proteomic techniques such as two-dimensional electrophoresis (2-DE) and mass spectrometry, we found putative orthologous of defense proteins from the immune response this important agricultural pest, thus generating new perspectives on the study of the immune response in *D. saccharalis*. This is the first report on the induction of protein expression in larvae challenged by the entomopathogenic fungus *B. bassiana*.

MATERIAL AND METHODS

Culture of larvae of *D. saccharalis:* Larvaewere treated with a specific diet under controlled conditions (temperature of $26 \pm 1^0 C$ and humidity of 70%) until the V instar of development (Hensley&Hammond, 1968). After this, the larvae were removed from the diet tubes in a laminar flow hood and cleaned with 70% alcohol.

Preparation of bacterial inoculum and entomopathogenic fungal suspension: *Bacillus subtilis* (ATCC 6623) and *Escherichia coli* (ATCC 11229) were cultivated in Luria-Bertani medium (LB) overnight. After microbial growth, 1 mL of bacterial culture from the LB medium culture was transferred to a microtube and submitted to centrifugation (5 minutes, 12°C, 1.520 x g). The pellet was washed with PBS buffer (Na_2HPO_4 4.3 mM; NaH_2PO_4 14 mM; NaCl 137 mM). The optical density of the inoculum was determined to 600 nm on a spectrophotometer (Genesys 10S UV-Vis), standardizing the dilution 10^6 cells/µL microorganisms. The *B. bassiana* fungal suspension (Strain 88) was prepared by adding 10 mL of water and Tween 80 0.05% to a tube containing the fungus. After that, dilutions were prepared and the conidia were counted in a Neubauer chamber. The fungal suspension was standardized to 2×10^8 conidia/µL.

Immunological challenge of V instar larvae with microorganisms: The larvae were divided into groups with ±50 specimens: the control group, where hemolymph was collected directly (group 1), larvae challenged with *B. subtilis* (group 2), larvae challenged with*E. coli* (group 3) and larvae challenged with*B. bassiana* (group 4). Two types of assays were performed, one where septic injury lasted for about 6 hours and the other where septic injury lasted for about 12 hours. With the aid of a precision microapplicator (Burkard-PAX 100-3), 3 µL of the bacterial inoculum or fungal suspension 2×10^8 conidia/µL were inoculated in each larva according to the group.

Collection of hemolymph samples, extraction and protein concentration: The hemolymph was collected through a small cut made with an ophthalmic scissors and the exuding drop of hemolymph was immediately transferred to a microtube immersed in ice that contained a few crystals of phenylthiourea (Sigma®) using an automatic micropipette. The hemolymph samples were centrifuged (5 minutes, $4^0 C$, 2,580 x g) to separate the cellular components. The cell-free hemolymph was stored at -20 $^0 C$ until use. Low molecular proteins were extracted from the hemolymph using an extraction solution composed of methanol, acetic acid and water (90: 1: 9) (Zdybicka-Barabas*et al.*, 2017). The protein extracts were concentrated on 3000 Da columns (Vivaspin 500, Sartorius), which were centrifuged (45 minutes, 20 °C, 14.000 x g). A protease inhibitor (2 µL; Sigma®) was added to the fraction of interest (>3000 Da) and samples were then dried at 42 °C. After drying, the samples were solubilized in 100 µL of ultrapure water. The hemolymph protein concentration was assessed at 280 nm using a UV-Vis spectrophotometer (Genesys).

Isoelectric focusing (IEF) and two-dimensional electrophoresis (2-DE) analysis: About of 50 µg of total extracted proteins were applied to pH gradient strips (Immobilinedrystrips, pH 3-10, GE Healthcare®) for posterior focusing on the IPGphor system (Amersham Biosciences®). Isoelectric focusing was conducted at 20 $^0 C$ with an electric current of 0.05 mA for the IPG strip with five steps: Step 1: 200 Vhr; Step 2: 500 Vhr; Step 3: 800 Vhr; Step 4: 11.300 Vhr and Step 5: 12.000 Vhr, totaling 24.800 Vhr. IPG strips were placed in equilibrium solution (Tris-HCl 7.5 mM pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS, and a trace amount of bromophenol blue) supplemented with 1% dithiothreitol (DDT) for 30 minutes. The strips were kept in equilibrium solution, then 2.5% iodoacetamide (IAA) was added for another 30 minutes. The equilibrated strips were transferred to 12.5% Tricine SDS-PAGE (Schägger, 2006). Additionally, 15 µL of a molecular weight ladder was run on the SDS-PAGE (Pattern LMW, GE Healthcare®). After electrophoresis, two-dimensional gels (2-DE) were fixed and stained with Coomassie Colloidal (G-250) overnight and the 2-DE gels were digitalized with an Image Scanner (Amersham). The spots were identified using ImageMaster 2-DE Platinum 6.0 software. Gels from challenged samples were compared against the control gels to check if the corresponding spots were differentially expressed after septic challenge considering a cut-off of 1.5 in accordance to Song *et al.*, 2008.

Trypsin digestion, MALDI-ToF/ToF mass spectrometry and protein identification: The most evident and delimited spots from each gel were removed and submitted to trypsin digestion (Promega-USA) (Shevchenko*et al.*, 1996). The digested spots were submitted to MALDI-ToF/ToF mass spectrometry (Bruker Daltonics®, Autoflex II), in which the peptides were crystallized with a solid matrix (3,5-dimethoxy-4-hydroxycinnamic acid), then bombarded with a laser to induce sample ionization. The peptides were separated by a time of flight (ToF) analyzer according to mass and charge. Mass/charge spectra were analyzed using FlexAnalysis 2.0 software and compared against the NCBIprot and Swissprot databases with the MASCOT server specified for a search against *Drosophila melanogaster* and internal database of Lepidoptera proteins. The parameters of the search were adjusted to: carbamidomethyl (C), oxidation (M) and 200 ppm of tolerance. The TagIdent tool (https://web.expasy.org/tagident/) was used to search the UniProtKB/SwissProt database, considering the experimental molecular weight (Mw) and isoelectric point (pI) of the proteins.

RESULTS

Hemolymph proteins extraction and protein dosage: After the extraction protocol, an increase in the protein concentration of the hemolymph of *D. saccharalis* larvae (Figure 1) was observed after immunological challenge compared to control, with the exception of 6 hours of septic challenge with *B. bassiana*. The longer septic challenge, 12 hours, showed a significant increase in protein concentrations when compared with the shorter challenge; for example, septic challenge with *B. bassiana* resulted in a protein concentration of 11.19 mg/mL at 6 hours and 57.58 mg/mL at 12 hours.
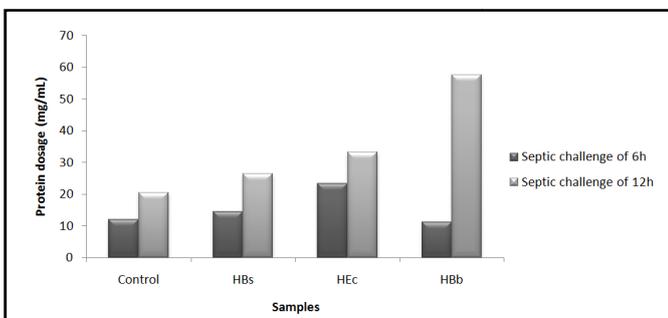

Figure 1. Protein extract of hemolymph samples from *D. saccharalis* collected after 6 and 12 hours of septic challenge. HBs: Hemolymph collected after septic challenge with *B. subtilis*. HEc: Hemolymph collected after septic challenge with *E. coli*. HBb: Hemolymph collected after septic challenge with *B. bassiana*. Ladder: LMW standards (GE Healthcare).

Two-dimensional electrophoresis (2-DE) and analysis of gels after 6 hours of septic challenge: Tricine SDS-PAGE 12.5% gels containing protein extracts from control and 6-hour challenged samples are shown in Figure 2. In the control protein extract, five spots were observed with range of molecular mass between 12 and 19 kDa and pI 5.21 to 8.44 (Figure 2a). After *B. subtilis* septic challenge, it was possible to identify nine spots with range of 12 to 22 kDa and pI 4.50 to 9.87 (Figure 2b). After the *E. coli* septic challenge, four spots were observed with range of 11 to 18 kDa and pI 4.96 to 8.48 (Fig. 2c). Lastly, following *B. bassiana* septic challenge, seven spots were observed with range of 12 to 24 kDa and pI 5.15 to 9.95 (Figure 2d).
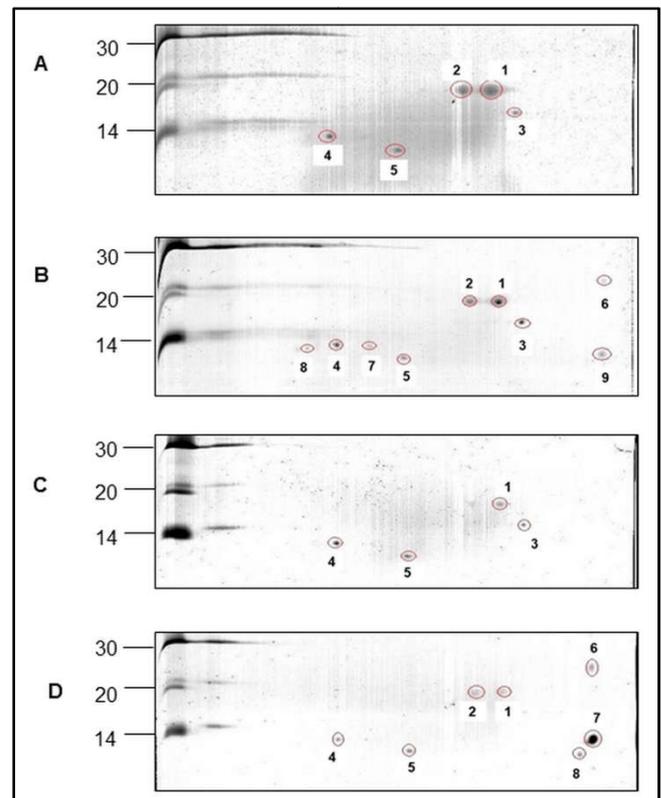

Figure 1. Two-dimensional electrophoresis (2-DE) by 12.5% Tricine SDS-PAGE of low molecular weight proteins extracted from the hemolymph of *D. saccharalis* larvae after 6 hours of septic challenge. (a) 2-DE of the control sample. (b) 2-DE of the protein extract obtained from larvae submitted to septic challenge with *B. subtilis*. (c) 2-DE of the protein extract obtained from larvae submitted to septic challenge with *E. coli*. (d) 2-DE of the protein extract obtained from larvae submitted to septic challenge with *B. bassiana*. Ladder: LMW standards (GE Healthcare).

A comparative analysis was performed between the septic challenge gels and the control gels. The corresponding spots were compared to each other and a cut-off value of 1.5 was provided by the software, showing which proteins were differentially expressed (Song *et al.*, 2008). A difference in expression can indicate an increase or decrease in the proteins concentration under septic challenge conditions.

Table 1. Spots differentially expressed in the protein extract from the hemolymph of *D. saccharalis* larvae after 6 hours of septic challenge with different microorganisms

Spot number	[a]Mw (kDa)	[b]pI	[c]Expression change
Challenge with *B. subtilis*			
Positive regulation			
3	16	8.39	2.21
Negative regulation			
2	18	7.45	1.54
Challenge with *E. coli*			
Positive regulation			
3	15	8.48	2.26
4	13	4.96	2.36
5	11	6.29	2.81
Challenge with *B. bassiana*			
Negative regulation			
1	19	8.22	4.92
2	19	7.72	3.17
4	13	5.15	3.63
5	12	6.49	2.14

[a] Molecular weight. [b] Isoelectric point. [c] Expression change at least 1.5 times.

The table 1 shows the results obtained after the comparative analysis between each sample from the 6-hour septic challenge and the control sample. We identified that septic challenge

with *B. subtilis* caused positive regulation by 2.21-fold for Spot 3 protein and negative regulation by 1.54-fold for Spot 2 protein. After septic challenge with *E. coli*, Spots 3, 4 and 5 showed increased expression by 2.26-, 2.36- and 2.81-fold, respectively, and no proteins were suppressed. After septic challenge with *B. bassiana*, it was observed that no proteins underwent positive regulation, and all the matches showed decreased expression after 6 hours of septic injury, where Spots 1, 2, 4 and 5 were suppressed by 4.92-, 3.17-, 3.63- and 2.14-fold, respectively. Septic challenge for 6 hours resulted in changes in the expression of some proteins and induced some spots in the hemolymph of *D. saccharalis* (Table 2). Septic injury with *B. subtilis* induced the expression of three proteins absent in the control sample (Spots 6, 7 and 9). Immunological challenge with *E. coli* did not induce any additional proteins compared to the control. Following septic challenge with *B. bassiana*, three spots were visualized under these conditions (Spots 6, 7 and 8).

Table 2. Spots expressed in the protein extract from the hemolymph of *D. saccharalis* larvae only after 6 hours of septic challenge

Spot number	[a] Mw (kDa)	[b] pI
Challenge with *B. subtilis*		
6	22	9.87
7	13	5.64
9	12	9.84
Challenge with *B. bassiana*		
6	24	9,9
7	13	9.94
8	12	9.68

[a] Molecular weight. [b] Isoelectric point.

Two-dimensional electrophoresis (2-DE) and analysis of gels after 12 hours of septic challenge: Tricine SDS-PAGE 12.5% gels containing protein extracts from control and 12-hour challenged samples are shown in Figure 3. In the gel obtained from the control protein extract, it was possible to observe seven spots with range of 11 to 25 kDa and pI 5.25 to 9.36 (Figure 3a). After *B. subtilis* immunological challenge, 12 spots were observed with range of 11 to 24 kDa and pI 5.07 to 9.65, however, the Spots 10, 11 and 12 were detected in low concentration and at the limit of detection by the software (Figure 3b). After *E. coli* septic challenge, 10 spots were seen with range of 11 to 23 kDa and pI 5.16 to 9.37 (Figure 3c). After *B. bassiana* septic challenge, six spots were visualized with range of 11 to 25 kDa and pI 5.02 to 8.53 (Figure 3d).

Table 3.

Spot number	[a] Mw (kDa)	[b] pI	[c] Expression change
Challenge with *B. subtilis*			
Positive regulation			
5	16	9.65	2.81
7	11	6.72	5.23
Negative regulation			
2	24	5.57	2.07
6	12	5.43	1.96
Challenge with *E. coli*			
Positive regulation			
5	14	9.37	2.31
7	11	6.56	1.74
Negative regulation			
4	15	8.87	4.39
6	12	5.22	2.34
3	17	8.32	1.63
Challenge with *B. bassiana*			
Negative regulation			
4	15	8.53	7.07
6	11	5.43	4.59

[a] Molecular weight. [b] Isoelectric point. [c] Expression change at least 1.5 times.
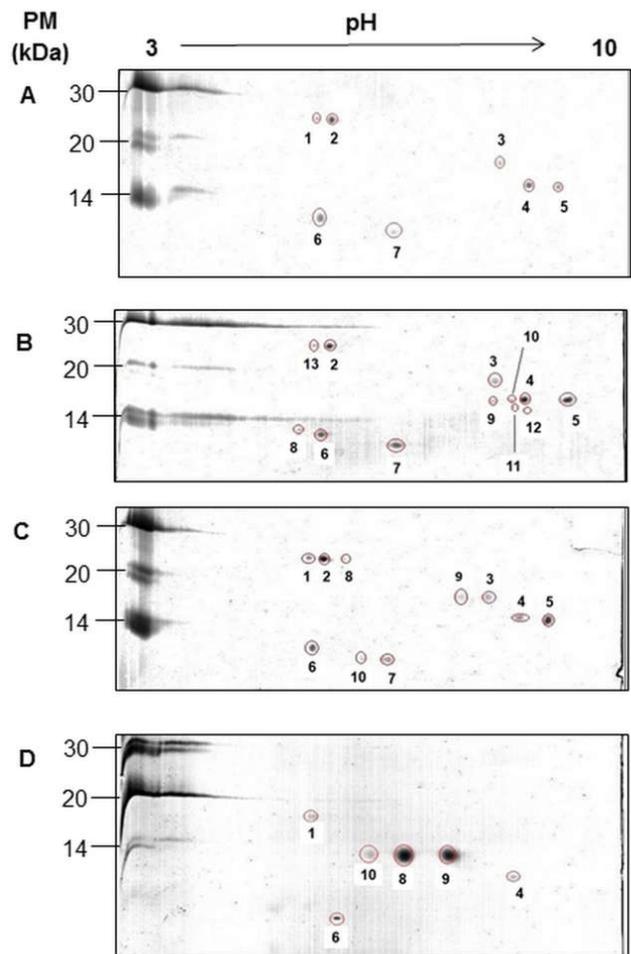

Figure 2. Two-dimensional electrophoresis (2-DE) by 12.5% Tricine SDS-PAGE of low molecular weight proteins extracted from the hemolymph of *D. saccharalis* larvae after 12 hours of septic challenge. (a) 2-DE of the control sample. (b) 2-DE of the protein extract obtained from larvae submitted to septic challenge with *B. subtilis*. (c) 2-DE of the protein extract obtained from larvae submitted to septic challenge with *E. coli*. (d) 2-DE of the protein extract obtained from larvae submitted to septic challenge with *B. bassiana*. Ladder: LMW standards (GE Healthcare).

Comparing septic challenge with *B. subtilis* and *E. coli* after 6 and 12 hours, increased expression of proteins was observed on the 2-DE gels, indicating that a longer duration of immunological challenge can lead to the expression in a larger number of proteins involved in the immunological response in *D. saccharalis*. The comparative analysis between samples challenged for 12 hours and the respective control showed that 11 spots underwent a change in expression (Table 3). Septic challenge with *B. subtilis* showed two proteins that were positively regulated, i.e. Spot 5 (2.81-fold) and Spot 7 (5.23-fold), and two proteins that were negatively regulated, i.e. Spot 2 (2.07-fold) and Spot 6 (1.96-fold). After septic challenge with *E. coli*, Spots 5 and 7 showed increased expression, by 2.31- and 1.74-fold, respectively. Other proteins showed decreased expression after the challenge, i.e. Spot 4 (4.39-fold), Spot 6 (2.34-fold) and Spot 3 (1.63-fold). After *B. bassiana* septic challenge, Spots 4 and 6 showed decreased expression by 7.07 and 4.59-fold, respectively. After 12 hours of septic challenge, some spots showed induced expression (Table 4). After *B. subtilis* septic challenge, six proteins were visualized under challenge conditions (Spots 8, 9, 10, 11, 12

and 13). *E. coli* challenge was able to induce the expression of three proteins (Spots 8, 9 and 10), and *B. bassiana* fungal challenge induced the expression of three proteins as well (Spots 8, 9 and 10).

Peptidoglycan recognition protein (PGRP), identified by the TagIdent tool, corresponds to Spot 2 in the control sample and after 6 hours of immunological challenge with *B. subtilis*.

Table 4 Spots expressed in the protein extract from the hemolymph of *D. saccharalis* larvae only after 12 hours of septic challenge

Spot number	[a] Mw (kDa)	[b] pI
Challenge with *B. subtilis*		
8	13	5.07
9	15	8.36
10	16	8.69
11	15	8.72
12	15	8.92
13	24	5.33
Challenge with *E. coli*		
8	23	5.79
9	17	7.84
10	11	6.09
Challenge with *B. bassiana*		
8	18	6.63
9	18	7.40
10	18	6.04

[a] Molecular weight. [b] Isoelectric point.

Table 5. Putative proteins from *D. saccharalis* hemolymph identified by mass spectrometry (MASCOT) or PM and pI (TagIdent)

Spot number	Challenge	[a] Mw (kDa)	[b] pI	Protein (TagIdent/MASCOT)	[c] Mw (kDa)	[d] pI
Control						
1	6 hours	18	8.01	CU22_BOMMO (O02388) Larval cuticle protein LCP-22	17	8.07
2	6 hours	19	7.54	PGRP_BOMMO (Q9XTN0) Peptidoglycan recognition protein	19	6.71
3	6 hours	16	8.44	DFP_BOMMO (Q008X1) Putative defense protein	16	8.65
2	12 hours	24	5.50	HGLY_MANSE (Q25513) 27 kDa hemolymph glycoprotein	24	5.64
5	12 hours	15	9.36	LYS_GALME (P82174) Lysozyme	14	9.27
6	12 hours	12	5.31	CU66_HYACE (P45590) Larval/pupal rigid cuticle protein 66	12	4.80
Challenge with *B. subtilis*						
6	6 hours	22	9.87	ATTA_TRINI (P50725) Attacin-A	20	9.16
2	6 hours	18	7.45	PGRP_TRINI (O76537) Peptidoglycan recognition protein	18	6.82
3	6 hours	16	8.39	Chitin-binding protein	64	8.41
4	6 hours	13	5.04	ABP2_MANSE (P31417) Fatty acid-binding protein 2	14	5.09
8	6 hours	13	4.50	PBURS_(Q566B2) BOMMO Partner of bursicon	13	4.68
9	6 hours	12	9.83	Serine-protease inhibitor-like	9.8	8.61
5	12 hours	15	9.36	LYS_GALME (P82174) Lysozyme	14	9.27
Challenge with *E. coli*						
3	6 hours	16	8.44	DFP_BOMMO (Q008X1) Putative defense protein	16	8.65
5	12 hours	14	9.36	LYS_GALME (P82174) Lysozyme	14	9.27
Challenge with *B. bassiana*						
7	6 hours	13	9.94	Cecropin A2	5.8	10.43
8	6 hours	12	9.68	Drosomycin-like	8.8	8.11
10	12 hours	18	6.04	APL3_SPOLT (O77248) Apolipophorin-3	18	6.00

[a] Experimental molecular weight. [b] Experimental isoelectric point. [c] Protein molecular weight. [d] Protein isoelectric point.

Protein identification by spectral analysis and biochemistry data: Protein identification was performed using databases (MASCOT and TagIdent tools) and the results of these analyses are shown in Table 5. Thirteen proteins were identified using the MASCOT or TagIdent tools. Five of them were not directly associated with the immunological response in *D. saccharalis*; however, these proteins are usually found in the hemolymph, i.e. larval cuticle protein (LCP-22), 27 kDa hemolymph glycoprotein, larval/pupal rigid cuticle protein 66, fatty acid-binding protein 2 and partner of bursicon. In the group of immune related proteins found in our experiments, we highlight the ortolougues proteins Attacin-A (Spot 6), Peptidoglycan recognition protein (Spot 2), Putative defense-like protein (Spot 3), Protein-like Serine protease inhibitor (Spot 9), Lysozyme (Spot 5), Cecropin A2 (Spot 7) and Drosomycin-like protein (Spot 8). Attacin-A corresponds to Spot 6 and was present after 6 hours of septic challenge with *B. subtilis*. It was identified by the TagIdent tool as a defense protein of the immune system with a molecular mass of 20.6 kDa and pI 9.16, close to the values found in our experiment (22 kDa and pI 9.87).

This protein underwent mild negative regulation after injury (1.54-fold) and showed 18 kDa and pI 6.82, similar to the experimental values with 18 kDa and pI 7.45. Spot 3 was present in the control sample and after 6 hours of septic challenge with *B. subtilis* and *E. coli*. Injury by *B. subtilis* positively changed the expression of this protein by 2.21-fold. Using the TagIdent tool to analyze the biochemical data for Spot 3, this protein showed pI and Mw similar to that of a putative defense-like protein from *Bombyx mori*. Protein-like Serine protease inhibitor was induced by 6 hours of septic challengewith *B. subtilis* (Spot 9) and identified by the MASCOT tool, showing 12% homology with this protein in *Drosophila serrata*. Spot 5 was present in the control sample and after 12 hours of septic challenge with *B. subtilis* and *E. coli*. This protein was identified as a lysozyme with 14 kDa and pI 9.27, close to the experimental values. *B. subtilis* challenge resulted in 2.81-fold increased expression of lysozyme, while *E. coli* challenge increased the expression of this protein by 2.31-fold, indicating that it is involved in the immune response. Six hours of septic challengewith the fungus *B. bassiana* induced the production of two proteins identified

by MASCOT. Cecropin A2 (Spot 7) showed identity and 37% homology in SwissProt and was considered an ortholog in spite of the difference in the real molecular weight of 5.8 kDa compared to the experimental value of 13 kDa. Drosomycin-like protein (Spot 8) showed 15% identity with the *D. melanogaster* peptide, suggesting involvement in the antifungal response. Another interesting protein was induced after 12 hours of *B. bassiana* septic challenge, i.e. Apolipophorin-3 (Spot 10), identified by the TagIdent tool. The experimental values for Mw and pI (18 kDa and 6.04) were very close to the characteristic values of this protein (Mw 18 kDa and pI 6.00).

DISCUSSION

The ortholog protein to peptidoglycan recognition protein (PGRP) was identified in both control and injured samples after challenge with the Gram-positive bacterium *B. subtilis* (Spot 2), with a slight decrease in expression under challenge conditions. PGRPs that recognize Gram-positive bacteria were isolated for the first time from *B. mori*(Li,*et al*., 2015). PGRPs are intimately associated with several functions of the innate immune response, like Toll and IMD receptor activation and the PPO cascade; they can induce hydrolysis of the peptidoglycan of bacterial wall and thus antimicrobial activity, and can moreover induce phagocytosis Li*et al*., 2014). Induction of PGRP following septic challenge of *B. mori* with *Pseudomonas aeruginosa* and *Staphylococcus aureus*was confirmed with increased expression of PGRP-S5 (Chen*et al*., 2014). The same study also confirmed the participation of PGRP in PPO activation and inhibition of bacterial growth. In this study, septic challengeof 6 hours with the Gram-positive bacterium *B. subtilis* induced the expression of an ortholog protein of Attacin-A (Spot 6). The atacins have molecular weights between 20 and 23 kDa and can be divided into basic (A to D) and acidic (E to F) molecules (Li,*et al*., 2015). Others studies have confirmed the spectrum of atacin activity, mainly against Gram-negative bacteria (Wang*et al*., 2009; Hu &Askoy, 2005). In addition to these reports also was observed increased early expression of Attacin-A in *D. melanogaster* associated with *Wolbachia*and challenged with *P. aeruginosa,* another Gram-negative bacterium (Gupta*et al*., 2017). Spot 3 protein identified in this study was positively regulated by 6 hours of septic challengewith *B. subtilis.* The molecular weight and isoelectric point are compatible with a putative defense protein DFP_BOMMO (Q008X1), indicating the probable involvement of this protein in the immunological response, which was shown through changes with the microbial challenge.

Serine-protease inhibitor-like protein (Spot 9) ortholog showed induced expression after 6 hours of septic challengewith *B. subtilis*. These proteins are also known as Serpins and are involved in physiological processes and defense reactions in mammals and invertebrates, playing a role in the control of serine-protease activity and preventing inappropriate immune responses (Gilbert, 2012; Yang*et al*., 2017). The expression of a Serine-protéase inhibitor-like protein after septic challenge in this study is corroborated by reports in dipterans and Lepidoptera. In *Drosophila,*serpins are related to immune response regulation mediated by the Toll receptor. In *B. mori* serpin-15 was found in high quantities in the hemolymph and was capable of inhibiting PPO activation and AMP production (Liu*et al*., 2015). Lysozyme (Spot 5) ortholog protein was observed in both control and challenged samples by 12 hours;

as shown in the 2-DE gel it was possible to observe that after 12 hours of bacterial challenge or septic injury was confirmed the increase in lysozyme expression. Lysozymes play an important role in the immune response and are able to act synergistically with AMPs to hydrolyze the bonds between N-acetylmuramic acid and N-acetylglycosamine in the wall of Gram-positive bacteria (Sowa-Jasiłek*et* al., 2014; Hillyer, 2016), this could be causing the expression in the challenge with *B. sublitlis*. Lysozyme also acts against Gram-negative bacteria such as *E. coli*, verified using isolated lysozymes from members of the order Lepidoptera (Elmogy*et al*., 2015).

Septic injury with *B. bassiana* induced the expression of a protein homology to Cecropin A2 (Spot 7) after 6 hours of septic challenge. The cecropins have low molecular weights (3-5 kDa) and were the first reported AMPs in insects, isolated from *Hyalophora cecropia* hemolymph. These AMPs show a broad spectrum of action and can be active against Gram-positive bacteria, Gram-negative bacteria, fungi, parasites and HIV-1 (Yi*et al*., 2014; Wei*et al*., 2017). According to these reports, it was recently reported that Cecropin A2 from *Aedesaegypti* also showed activity against clinical isolates of *P. aeruginosa*, a Gram-negative bacterium (Zheng*et al*., 2017). Despite the high molecular weight of spot 7, this homologous protein reported here could be related to the immune system, conserving at least part of domains with antimicrobial activity like to cecropins, however, this hypothesis remains uncertain and still needs further studies. Peptide like to Drosomycin (Spot 8) was induced by 6 hours of septic challengewith *B. bassiana*. Drosomycin is an AMP of defensins class, isolated for the first time in *D. melanogaster* hemolymph after bacterial septic challenge. However, it has a narrow spectrum of activity, and is active only against some filamentous fungi, indicating that the expression induced by *B. bassiana* is reasonable (Zhang & Zhu, 2009; Rao *et al*., 2011). In *Drosophila melanogaster* was reported that Drosomycin and Drosomycin-2 were able to inhibit the growth of the fungi *Neurosporacrassa* and *Geotrichumcandidum* and the yeast *Saccharomyces cerevisiae* (Tian*et al*., 2008). In this present study, an ortholog protein of Apolipophorin-3 (Spot 10) was found to be induced 12 hours of septic challengewith *B. bassiana*. This protein was characterized in other species as a multifunctional protein with a role in the immune response, performing functions like pathogen pattern recognition, activation of the PPO cascade, stimulation of AMP release, synergistic effects with lysozyme and lipid transport (Butt*et al*., 2016; Wen *et al*., 2016). In accordance with our study also was observed that immunological challenge with fungus was able to induce the expression of Apolipophorin-3 in *Galleria mellonella* larvae (Zdybicka-Barabas*et al*., 2015).

Conclusion

Lastly, the results of this study indicate that the septic challenge of larvae with different microorganisms was able to induce or regulate the expression of proteins involved in immunological processes in *D. saccharalis*. All proteins reported in this study have been previously described in the immunological response in Lepidoptera. In the future the characterization of these proteins will allow a better understanding of the immune system of *D. saccharalis*, which can assist in the control of this important agricultural pest.

Authors Contribution: The authors CORSATO, A.C.M and SEUCHUCO, C. performed the experiments. MALLER A.

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