Journal of Invertebrate Pathology 128 (2015) 14–21

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Assessing gene expression during pathogenesis: Use of qRT-PCR to follow toxin production in the entomopathogenic fungus *Beauveria bassiana* during infection and immune response of the insect host *Triatoma infestans*

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ARTICLE INFO

Article history:
Received 21 January 2015
Revised 10 April 2015
Accepted 13 April 2015
Available online 22 April 2015

Keyword:
Secondary metabolites
Nonribosomal peptides
Chagas disease vector
Humoral immunity
Fungus–insect interaction

ABSTRACT

Entomopathogenic fungi secrete toxic secondary metabolites during the invasion of the insect hemocoel as part of the infection process. Although these compounds have been frequently mentioned as virulence factors, the roles of many of them remain poorly understood, including the question of whether they are expressed during the infection process. A major hurdle to this issue remains the low sensitivity of biochemical detection techniques (e.g., HPLC) within the complex samples that may contain trace quantities of fungal molecules inside the insect. In this study, quantitative reverse transcription real-time PCR (qRT-PCR) was used to measure the transcript levels within the insect fungal pathogen *Beauveria bassiana*, that encode for the synthetase enzymes of the secondary metabolites tenellin (*BbtenS*), beauvericin (*BbbeaS*) and bassianolide (*BbbslS*) during the infection of *Triatoma infestans*, a Chagas disease insect vector. Absolute quantification was performed at different time periods after insect treatment with various concentrations of propagules, either by immersing the insects in conidial suspensions or by injecting them with blastospores. Both *BbtenS* and *BbbeaS* were highly expressed in conidia-treated insects at days 3 and 12 post-treatment. In blastospore-injected insects, *BbtenS* and *BbbeaS* expression peaked at 24 h post-injection and were also highly expressed in insect cadavers. The levels of *BbbslS* transcripts were much lower in all conditions tested. The expression patterns of insect genes encoding proteins that belong to the *T. infestans* humoral immune system were also evaluated with the same technique. This qPCR-based methodology can contribute to decifering the dynamics of entomopathogenic fungal infection at the molecular level.

1. Introduction

Beauveria bassiana s.l. is a generalist entomopathogenic fungus used in microbial control programs worldwide. It represents a promising tool to control blood-sucking arthropods (Fernandes et al., 2012; George et al., 2013; Juárez et al., 2000; Luz et al., 1998, 2004; Pedrini et al., 2009; Romaña and Fargues, 1992) because, similar to other hypocrealean fungi, it does not need to be ingested to initiate the disease, and can invade insect hosts by penetrating through their cuticle. There is abundant biochemical information about the ability of *B. bassiana* to degrade insect cuticular proteins, chitin, and lipids (mostly hydrocarbons) (Fang et al., 2005, 2009; Pedrini et al., 2007, 2010, 2013; St. Leger et al., 1986a,b, 1988; Zhang et al., 2012). Fungal hyphae penetrate into the hemocoel where the fungus colonizes and proliferates throughout the host by replicating as hyphal bodies (Lewis et al., 2009; Wanchoo et al., 2009).

During the invasive process, many strains secrete toxic or immunosuppressive compounds, often considered as secondary metabolites that facilitate and/or protect the fungal invasion process or confer resistance against host defense compounds, respectively (Ferron, 1985; Roberts et al., 1992; Trienens and Rohlfs, 2012). These compounds have been well characterized in a variety of filamentous fungi, and particularly include polyketide and nonribosomal peptides (Gupta et al., 1991; Molnar et al., 2010). *B. bassiana* s.l. produces the cyclooligomer nonribosomal peptides beauvericin and bassianolide, the diketomorpholine bassiatin, the cyclic peptides beauverolides, the dibenzoquinone oosporein, and the 2-pyridone tenellin (Gibson et al., 2014; Molnar et al., 2010).

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http://dx.doi.org/10.1016/j.jip.2015.04.004
0022-2011/

The biosynthesis of tenellin, beauvericin and bassianolide are mechanistically well known, and the genes encoding for tenellin synthetase (*BbtenS*), beauvericin synthetase (*BbbeaS*) and bassianolide synthetase (*BbbslS*) have been characterized (Eley et al., 2007; Xu et al., 2009, 2008). Although the importance of these toxins has been frequently mentioned as an important step for fungal infection, their roles remain poorly understood, and many details including whether they are even expressed during the infection process remain obscure. Although some of these compounds may not be required for infection, it is likely that non-lethal secondary metabolites contribute to the disease process in various subtle ways, e.g., by perturbing biochemical and physiological processes that have the potential to exert effects on the overall fungal infection; by weakening host defense systems; and/or by limiting competing microbes. Molecular approaches with high sensitivity in complex biological samples are need to help better understand where and how these compounds act during various phases of the pathogenic process (Rohlfs and Churchill, 2011).

After microbial invasion, insects trigger two types of innate immune reactions: the cellular and the humoral responses. The former involves phagocytosis, hemocyte aggregation and pathogen encapsulation. The latter includes the induction of several antimicrobial peptides (AMPs), lectins, and the prophenoloxidase cascade. Transcription of AMPs is regulated mainly by the Toll signal transduction pathway; the resulting peptides are then secreted into the hemocoel to prevent microbial proliferation (Pal and Wu, 2009). *Rhodnius prolixus* (Hemiptera: Reduviidae), a Chagas disease vector, induces AMPs such as defensin in both the hemolymph and the midgut after bacterial infection (Lopez et al., 2003). However, infection with the unicellular parasitic protozoa *Trypanosoma rangeli* does not modify defensin expression but activates lectins, affecting parasite motility and survival, and the prophenoloxidase cascade (Garcia et al., 2009).

B. bassiana is effective against all stages of the Chagas disease vector *Triatoma infestans*, in laboratory as well as in the field (Luz et al., 1998; Pedrini et al., 2009). Using a powder formulation containing *B. bassiana* conidia and diatomaceous earth, nymphs' mortalities ranged from 80% to 100% with median lethal time between 5 and 9 days; whereas 90% of adults were killed in 10–11 days (Forlani et al., 2011). An attraction–infection trap based on this powder formulation combined with a chemical cue has already been tested for indoor *T. infestans* control in an endemic zone of the Argentina–Bolivia border, diminishing in 50% the insect population after a 30-d intervention (Pedrini et al., 2009). Also, the horizontal transmission of fungal conidia was dependant of bug density, showing to contribute significantly to the overall infection of the insect population (Forlani et al., 2011).

The aim of this study was to develop a methodology useful to measure the time course expression of *B. bassiana* genes toxins including tenellin, bassianolide, and beauvericin biosynthesis (i.e., *BbtenS*, *BbbeaS* and *BbbslS*) during fungal infection of a target host, namely nymphs of *T. infestans*. We also investigated the gene expression levels of insect proteins belonging to the humoral immune response, including prophenoloxidase, hemolectin and defensin, in order to help understand, at the molecular level, their roles in the host-pathogen interaction that are triggered by the invasion of the fungus into the insect hemocoel.

2. Materials and methods

2.1. Fungal cultures

B. bassiana strain GHA (Laverlam International, Butte, MT) was grown on complete medium agar (CMA) plates containing 0.4 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.6 g $MgSO_4{\cdot}7H_2O$, 1 g KCl, 0.7 g $NH_4NO_3{\cdot}7H_2O$, 10 g glucose, 5 g yeast extract, and 15 g agar in 1000 ml of distilled water. Plates were incubated at 26 °C for 12 days. Suspensions of conidia were prepared by rinsing fungal cultures with sterile distilled water and rubbing the sporulating surface with a bent needle. After filtering debris, the liquid was diluted in sterile distilled water containing 0.01% Tween 80. Blastospores were produced in Sabouraud dextrose +1% yeast extract liquid broth cultures (SDY), using conidia harvested from CMA plates to final concentration of 5×10^5 conidia ml^{-1} as the inoculum. Cultures were grown for 3 days at 26 °C under shaking (200 rpm), and filtered (twice) through glass wool to remove mycelia. Blastospores were obtained by centrifugation and the pellet resuspended in sterile saline solution (0.9% w/v of NaCl). Final conidial and blastospore concentrations were determined by direct counts using a hemocytometer.

2.2. Insects inoculation with conidia and blastospores

Fourth nymphal instar *T. infestans* came from a colony regularly maintained and reared at 30 °C, 50–60% relative humidity, under a 12 h photophase, and fed on chickens, at the INIBIOLP, Facultad de Ciencias Médicas, La Plata, Argentina. For all the bioassays, 2-week-old nymphs were used, one week after a blood meal. Individual insects were either immersed for 6 s in conidial suspensions (concentrations of 0, 10^4, or 10^6 conidia ml^{-1}) or injected with 3 µl of fungal blastospore suspensions (0, 4×10^4, or 4×10^6 blastospores ml^{-1}) to yield a final dose of 0, 120 or 12,000 blastospores/insect. After treatment, insects were returned and maintained at the rearing conditions described above. At different time periods (3, 6, 9, and 12 days for conidia-immersed insects and 12, 24, and 48 h for blastospore-injected insects), three live insects for each treatment were separated, and their total RNA was extracted as described in Section 2.4. Mortality bioassays were performed with other batches of insects treated with the same concentrations of conidia (five replicates, ten insects per replicate) and blastospores (ten replicates, ten insects per replicate). Mortality was checked either weekly during four weeks (conidia) or daily during one week (blastospores). Dead insects were placed in individual humid chambers (relative humidity (RH) $\geqslant$98%) to confirm fungal infection. Median lethal time (MLT) was estimated as Σ ($days_n \times$ dead $nymphs_n$)/total dead nymphs (Moore et al., 1995).

2.3. Quantification of hyphal bodies in insect hemolymph

Hemolymph samples from live individuals previously treated with conidia or blastospores (as detailed in Section 2.2) were obtained as follows: each insect was surface-sterilized by completely dipping in 70% ethanol during 3 s and subsequently twice in sterile water for 3 s. The nymph's head was cut at the base with fine scissors, 10 µl of the leaking hemolymph were collected with a micropipette, immediately diluted tenfold in sterile saline solution and spread (using a Drigalski loop) onto SDY agar medium (SDYA) added with chloramphenicol (0.5 g/l) in a Petri dish (60 mm diameter) and incubated at 26 ± 1 °C, 75% ± 10 RH and 12 h photophase. The development and number of colony-forming units (CFU) on the medium were assessed up to 72 h of incubation. At least three replicates were done for each treatment.

2.4. Gene expression analysis

Total RNA (insect + fungus) was extracted from both fungus-treated and control insects by employing the RNAeasy Mini kit (Qiagen, Hilden, Germany) with an on-column DNA digestion step (DNAse I, Qiagen, Hilden, Germany). RNA was quantified by a Nanodrop spectrophotometer (Thermo Scientific, Wilmington,

Table 1
Beauveria bassiana and *Triatoma infestans* real-time quantitative primer sequences.

Name	Forward (5′–3′)	Reverse (5′–3′)	Amplicon (bp)	Target
BbtenS	ACTGTCCGCATTGGCAGCTAAG	TGTCCTTTGGTGGTGGTGATGG	113	Fungus
BbbeaS	GTTCTTCCTCCGCATTCCGTTC	TAGAGCGCAACGTCTTTCGGTC	97	Fungus
BbbslS	CAATCGACTGAGACGCCATTCC	TTTGACCTGCGAATCCATACGG	156	Fungus
TiPPO	CACGCGGTATTGATATCTTGGG	GATCATGACAGAGCGCAATGG	117	Insect
TiHL	TCCCACCAAACTTCCACTCC	GGCAAAGATGAACCGCTACC	107	Insect
TiDEF	CGCCCTGGCTTACTCATATC	CACAGGTGGCTCTCTTCAGAC	122	Insect
Ti actin	CACCCCAGCAATGTATGTAG	ACCATCAGGAAGTTCGTAAG	352	Insect

DE), and its integrity was assessed on a 1% (w/v) agarose gel. Two-step real-time polymerase chain reaction (RT-PCR) was carried out with iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Amplification was performed in an Mx3000P qPCR System (Stratagene, La Jolla, CA) employing 40 ng reverse-transcribed total RNA for each sample. The primers used are listed in Table 1. The fungal sequences used for primer design were obtained from Genbank (*BbtenS*: AM409327, *BbbeaS*: EU886196, *BbbslS*: FJ439897), the insect sequences (*TiPPO*, *TiHL*, and *TiDEF*) were selected from an EST expression library obtained from the integument of *T. infestans* nymphs (G.M. Calderón-Fernández and M.P. Juárez, unpublished data). Additional information about insect sequences is shown in Fig. S1. The following amplification program was used: denaturation at 95 °C for 10 min, followed by 40 cycles with three-segment amplification (30 s at 95 °C for denaturation, 30 s at 55 °C for annealing, and 30 s at 72 °C for DNA chain elongation). In order to confirm that only single products were amplified, a temperature-melting step was then performed. Negative controls were performed by using 'cDNA' generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The assay was performed in duplicate, and three independent biological replicates were done. The ΔΔCt method was used to perform a relative quantification of the insect genes (Pfaffl et al., 2002). Because no fungal genes are present in control insects, the ΔΔCt method is not useful to evaluate them, thus, an absolute quantification was performed by using a calibration curve (see Section 2.5) for the genes of interest.

2.5. Standard curves for fungal gene quantification

The amplicons corresponding to *BbtenS*, *BbbeaS*, and *BbbslS* were ligated and cloned into a pGEM-T Easy vector (Promega, Madison, WI). The gene fragments sizes were 113, 97, and 156 bp for *BbtenS*, *BbbeaS*, and *BbbslS*, respectively. The constructs were transformed into *Escherichia coli* JM109, ampicillin-resistant colonies were isolated, and their plasmid purified (Qiagen, Hilden, Germany). The inserts were sequenced to confirm their identity. To calculate the number of construct gene copies (GC), the following formula was used: n° molecules pGEM T-Easy::insert/μl = [concentration of the pGEM-T-Easy::insert (ng/μl)/molecular weight (ng/mol)] × 6.022×10^{23} molecules/mol (Gómez-Doñate et al., 2012), employing serial dilutions of the stocks. Each point was amplified by quadruplicate, and the average of Ct results was used to plot the curves.

2.6. Statistical analysis

For mortality bioassays, differences between the mean values were determined by the Student's *t* test ($p < 0.05$). For gene expression experiments, differences among the mean values obtained for each dose and time period were determined by analysis of variance (ANOVA) and by using Student–Newman–Keuls test to separate treatment means ($p < 0.05$). Instat 3.05 (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses.

3. Results

3.1. Bioassays

In order to define the time frame for the gene expression experiments, cumulative mortality was previously evaluated in both conidia- and blastospore-treated insects. In conidia-treated insects (Table 2), cumulative mortalities obtained at the end of the assay (four weeks) were 57 ± 9% (10^4 conidia ml^{-1}) and 83 ± 6% (10^6 conidia ml^{-1}) ($p = 0.046$); the MLT values were 15.4 ± 1.0 days (10^4 conidia ml^{-1}) and 10.3 ± 1.4 days (10^6 conidia ml^{-1}) ($p = 0.017$). Mortality of the control insects at the same period was 7 ± 3%. In blastospore-injected nymphs (Table 3), mortalities at day 7 were 88 ± 6% (120 blastospores/nymph) and 97 ± 1% (12,000 blastospores/nymph) ($p = 0.171$), and MLTs were 4.3 ± 0.1 days and 2.4 ± 0.2 days ($p < 0.0001$) for 120 and 12,000 blastospores per nymph, respectively. No mortality was observed in control insects.

Hyphal bodies were quantified in hemolymph extracted at the same time periods than those assayed for gene expression. An increment in CFU/μl with time post fungal inoculation was observed either in insects immersed in conidia suspensions (Table 4) or injected with blastospore suspensions (Table 5).

3.2. Standard curves

The cloned gene fragments were serially diluted and used as templates for qRT-PCR. The Ct values obtained were plotted against the logarithm of gene copies. Fig. 1 shows the curves obtained for the three fungal genes, the coefficients determined (r^2) were 0.996 (*BbtenS*), 0.999 (*BbbeaS*), and 0.993 (*BbbslS*).

3.3. Expression of B. bassiana genes encoding non-ribosomal peptides

In nymphs immersed in conidial suspensions, the expression levels of fungal genes were dependent on both the original conidial concentration used and the time period assayed ($F = 8.4$;

Table 2
Mortality and median lethal time of 4th nymphal instar *Triatoma infestans* immersed in conidial suspensions of *Beauveria bassiana*.

Concentration (con ml^{-1})	Cumulative mortality (% ± SEM)				MLT (days ± SEM)
	Day 7	Day 14	Day 21	Day 28	
1×10^4	10 ± 5	30 ± 5*	40 ± 9*	57 ± 9*	15.4 ± 1.0*
1×10^6	29 ± 7	67 ± 8*	81 ± 6*	83 ± 6*	10.3 ± 1.4*
Control	3 ± 3	3 ± 3	7 ± 3	7 ± 3	n.d

con: conidia, MLT: median lethal time, n.d: non determined.
* Differences between treatments are significant ($p < 0.05$) after Student's *t* test.

Table 3
Mortality and median lethal time of 4th nymphal instar *Triatoma infestans* injected with blastospore suspensions of *Beauveria bassiana*.

Dose (bl/nymph)	Cumulative mortality (% ± SEM)							MLT (days ± SEM)
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
120	4 ± 2	10 ± 4*	16 ± 5*	36 ± 2*	80 ± 7*	88 ± 6	88 ± 6	4.3 ± 0.1*
12,000	13 ± 5	60 ± 10*	89 ± 5*	97 ± 1*	97 ± 1*	97 ± 1	97 ± 1	2.4 ± 0.2*
Control	0	0	0	0	0	0	0	n.d

bl: blastospores, MLT: median lethal time, n.d: non determined.
* Differences between treatments are significant ($p < 0.05$) after Student's *t* test.

Table 4
Quantification of *Beauveria bassiana* hyphal bodies in hemolymph extracted from 4th nymphal instar *Triatoma infestans* previously immersed in conidial suspensions.

Concentration (con ml^{-1})	Hyphal bodies/µl hemolymph (CFU ± SEM)			
	Day 3	Day 6	Day 9	Day 12
1×10^4	0.1 ± 0.1	20.4 ± 11.7	41.2 ± 22.9	19.0 ± 19.0*
1×10^6	0.6 ± 0.3	57.5 ± 17.8	>1000	≫1000

con: conidia, CFU: colony-forming units.
* Most of the surviving insects were not infected.

Table 5
Quantification of *Beauveria bassiana* hyphal bodies in hemolymph extracted from 4th nymphal instar *Triatoma infestans* previously injected with blastospore suspensions.

Dose (bl/nymph)	Hyphal bodies/µl hemolymph (CFU ± SEM)		
	12 h	24 h	48 h
120	0.1 ± 0.1	2.1 ± 1.6	27.2 ± 6.7
12,000	16.6 ± 6.0	89.2 ± 26.0	≫1000

bl: blastospores, CFU: colony-forming units.

df = 23,48; $p < 0.0001$). At 10^6 conidia ml^{-1}, both *BbtenS* and *BbbeaS* were expressed 3 days after inoculation reaching 1334 ± 440 and 1298 ± 475 GC/ng RNA, respectively (Fig. 2). Following this initial burst, transcript levels significantly dropped by day 6 (*BbtenS*: $p < 0.001$, *BbbeaS*: $p < 0.01$) and day 9 (*BbtenS*, $p < 0.01$; *BbbeaS*, $p < 0.001$). At day 12 post-treatment, a second burst of transcription, reaching expression levels similar to the values seen for day 3 (1432 ± 113 and 1313 ± 460 GC/ng, respectively) was noted. At low dose of the 10^4 conidia ml^{-1}, where it was anticipated that the infection process may take longer to start, *BbtenS* and *BbbeaS* transcript levels showed a peak at day 9 post-treatment, with values of 882 ± 104 ($p < 0.001$) and 261 ± 71 GC/ng RNA ($p < 0.05$), respectively. Unlike for infections with the higher dose, a second peak of expression was not seen within the 12 day time course.

In blastospore-injected insects, significant differences were also found between the doses and time periods assayed ($F = 53.7$; df = 14,3; $p < 0.0001$). Twelve hours after the injection, both *BbtenS* and *BbbeaS* were significantly more expressed at 12,000 blastospores/nymph than at 120 blastospores/nymph ($p < 0.001$) (Fig. 3). Twenty-four hours after injection, *BbtenS* transcripts were more abundant regardless of the dose employed than for with other time periods ($p < 0.001$), and reached values of 3472 ± 230 GC/ng RNA (120 blastospores per insect) and 5028 ± 644 GC/ng RNA (12,000 blastospores per insect). The difference between these transcript levels was also significant ($p < 0.01$). In cadavers, significant differences ($F = 8.587$; df = 5,12; $p = 0.0012$) were found between the expression levels in dead insect either exposed or not to >98% RH. The values obtained for the former were 3745 ± 1000 (*BbtenS*) and 2603 ± 780 (*BbbeaS*) GC/ng RNA, with significant differences between cadavers exposed or not to

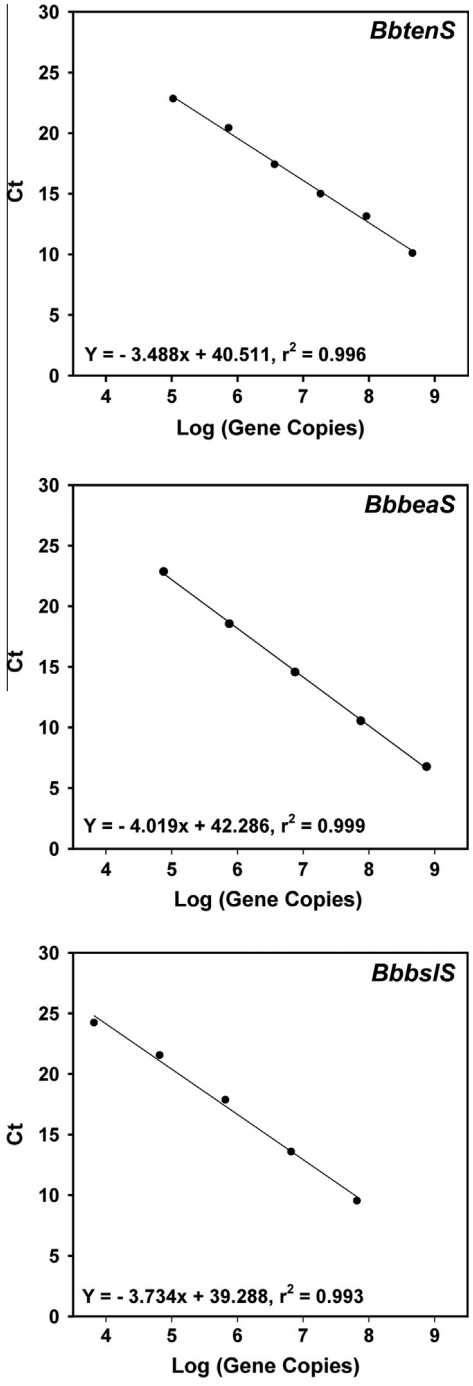

Fig. 1. *Beauveria bassiana* real-time quantitative PCR standard curve using serial dilutions of recombinant plasmids containing fragments of tenellin synthetase (*BbtenS*), beauvericin synthetase (*BbbeaS*) and bassianolide synthetase (*BbbsIS*) genes. Data presented are the average of four replicates.

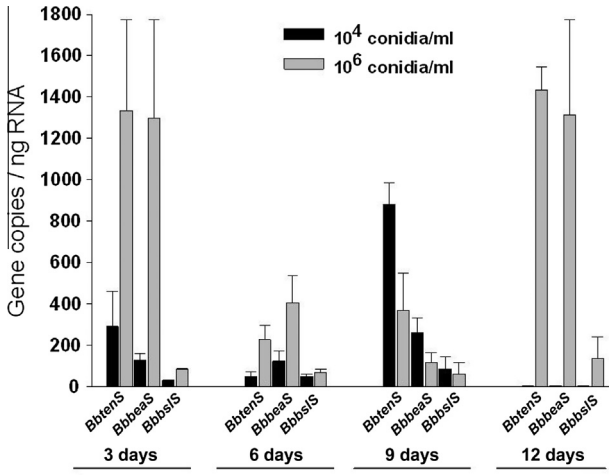

Fig. 2. Absolute quantification of *Beauveria bassiana* transcripts encoding tenellin (*BbtenS*), beauvericin (*BbbeaS*) and bassianolide (*BbbslS*) synthetases into 4th instar nymphs of *Triatoma infestans* at different time periods after insect immersion in conidial suspensions. Values are means of three replicates ± SEM.

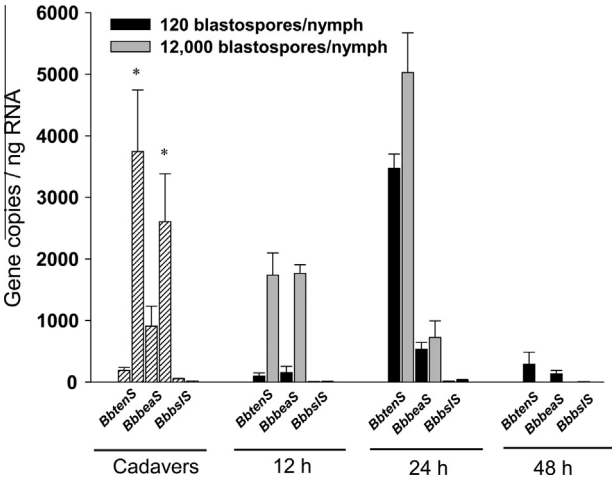

Fig. 3. Absolute quantification of *Beauveria bassiana* transcripts encoding tenellin (*BbtenS*), beauvericin (*BbbeaS*) and bassianolide (*BbbslS*) synthetases into both 4th instar nymphs of *Triatoma infestans* at different time periods after insect injection with blastospore suspensions and insect cadavers. Values are means of three replicates ± SEM. Asterisks indicate cadavers exposed to humid chambers (relative humidity ⩾98%) before RNA extraction.

humid chambers ($p < 0.01$ and $p < 0.05$ for *BbtenS* and *BbbeaS*, respectively). The levels of *BbbslS* transcripts detected in both conidia- and blastospore-treated insects were much lower than those obtained for *BbtenS* and *BbbeaS* transcripts.

3.4. Expression of T. infestans genes encoding humoral immune proteins

To evaluate the insect response to the fungal challenge at the molecular level, the expression of some genes belonging to the insect humoral immune system was studied in the same samples used for fungal gene quantification. For this purpose, we used an *T. infestans* EST expression library to select one gene involved in each mechanism described as part of this process: the prophenoloxidase cascade (*TiPPO*), a lectins (*TiHL*), and an AMP defensin (*TiDEF*). Insects immersed in 10^4 conidia ml^{-1} exhibited a peak in

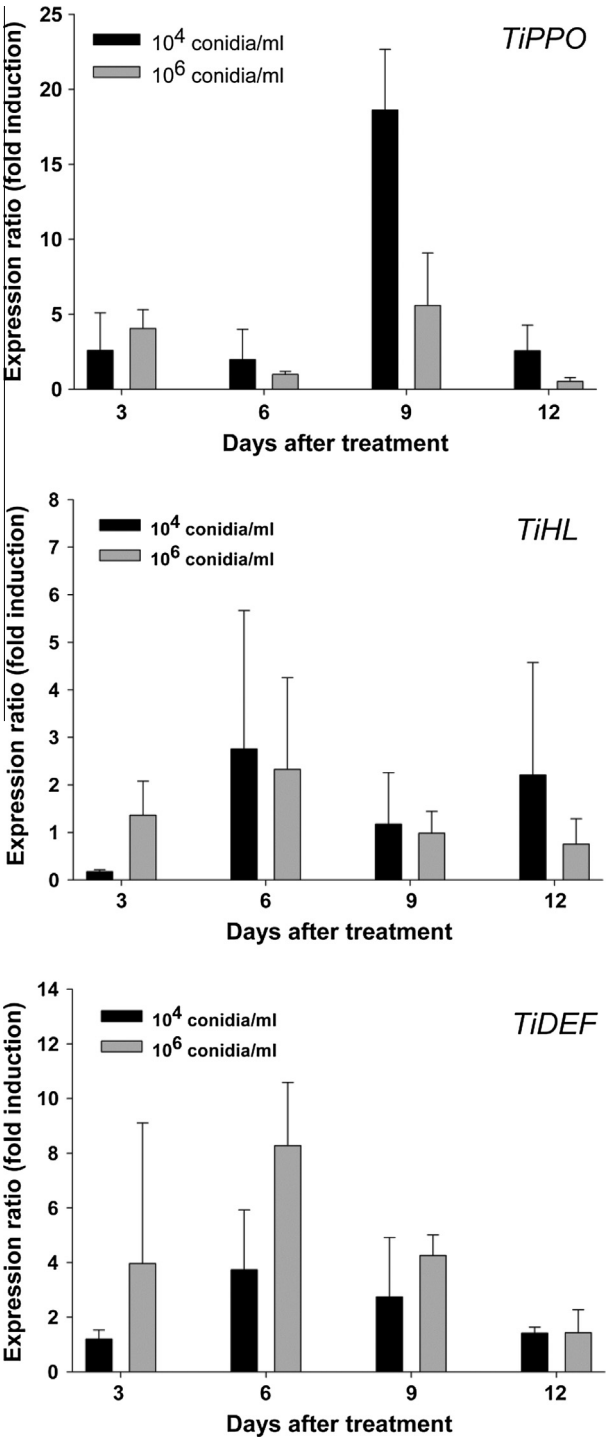

Fig. 4. Expression analysis of genes belonging to the *Triatoma infestans* humoral immune system at different time periods after immersion of 4th instar nymph in conidial suspensions of *Beauveria bassiana*. The genes analyzed were a prophenol oxidase (*TiPPO*), a lectin (*TiHL*) and a defensin (*TiDEF*). Values are means of three replicates ± SEM.

the expression level of *TiPPO* at day 9 ($F = 30$, df = 3,9; $p < 0.01$), with significant differences with insect treated with 10^6 conidia ml^{-1} ($p < 0.05$). *TiHL* and *TiDEF* were more expressed at day 6 after inoculation with the two concentrations tested, although no significant differences were found at different time periods (*TiHL*: $F \leqslant 1.4$, df = 3,9, $p \geqslant 0.3$; *TiDEF*: $F \leqslant 3.6$, df = 3,8;3,9, $p \geqslant 0.05$) (Fig. 4). In insects injected with blastospores (Fig. 5), a significant

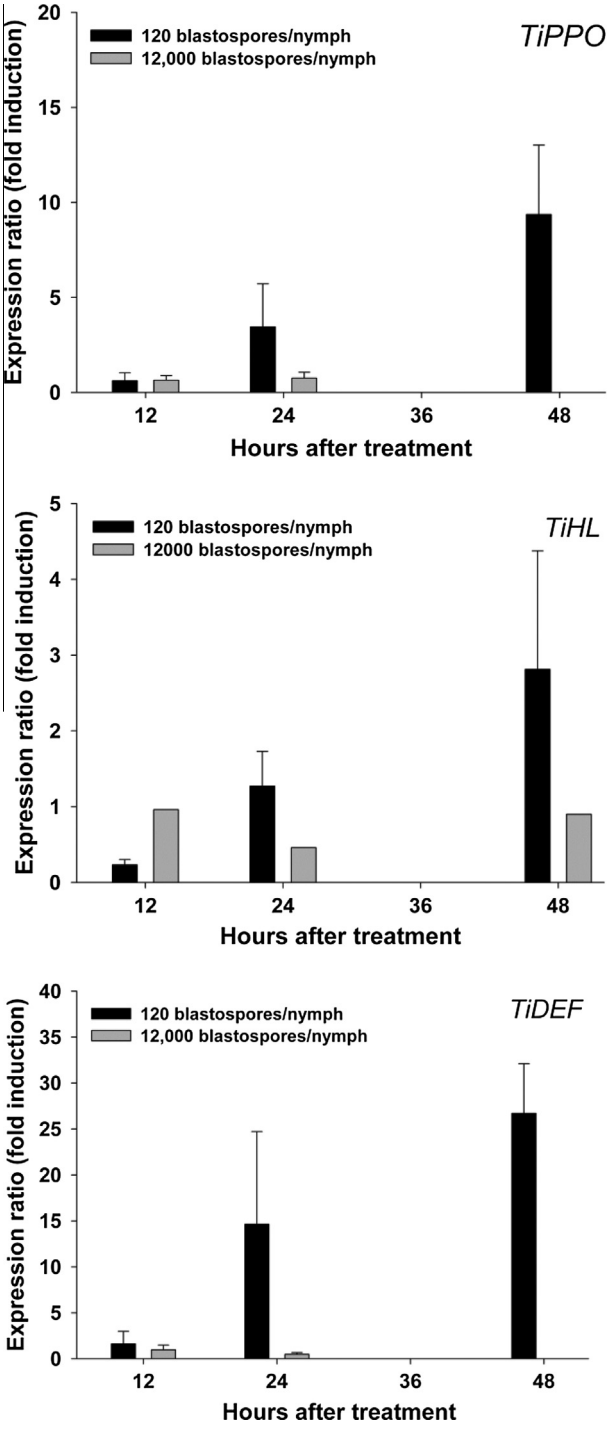

Fig. 5. Expression analysis of genes belonging to the *Triatoma infestans* humoral immune system at different time periods after injection of 4th instar nymph with blastospore suspensions of *Beauveria bassiana*. The genes analyzed were a prophenol oxidase (*TiPPO*), a lectin (*TiHL*) and a defensin (*TiDEF*). Values are means of three replicates ± SEM.

increase in the expression pattern of all genes was found at the lower dose tested (120 blastospores/nymph) ($F \leqslant 45.3$ df = 2, 6, $p \leqslant 0.01$). In contrast, no induction was observed in either controls or nymphs injected with 12,000 blastospores with expression values for 12 h and 24 h after treatment, respectively, as low as 0.4 ± 0.2 and 1.1 ± 0.2 (*TiPPO*), 0.8 ± 0.4 and 0.9 ± 0.1 (*TiHL*) and 1.0 ± 0.3 and 0.5 ± 0.1 (*TiDEF*).

4. Discussion

The biosynthesis of secondary metabolites by *B. bassiana* is known to proceed by iterative reactions catalyzed by enzymes encoded by gene clusters, including non-ribosomal peptides synthetases (NRPS), polyketides synthetases (PKS), and hybrid NRPS–PKS genes (Süssmuth et al., 2011). Comparisons among the fungal genomes sequenced to date indicate that entomopathogens are particularly abundant in these particular core genes, although the products of most of these pathways remain unknown (Gibson et al., 2014). A handful of these pathways have, however, been chemically and genetically characterized in *B. bassiana*, including those for tenellin, beauvericine, and bassianolide synthesis (Eley et al., 2007; Xu et al., 2009, 2008). For other chemically known secondary metabolites including beauverolides, oosporein and bassiatin the genes involved in their biosynthesis remain unknown. The precise roles of these compounds in the entomopathogenic activities of this fungus remain uncertain. By synthetase gene disruption approaches, bassianolide was proposed to be a highly significant virulence factor against *Galleria mellonella*, *Spodoptera exigua*, and *Helicoverpa zea* (Xu et al., 2009). In contrast, beauvericin plays an important but dispensable role in virulence against the same insect hosts (Xu et al., 2008); tenellin, however, does not appear to contribute to *B. bassiana* virulence against *G. mellonella* (Eley et al., 2007). Despite these data, there has not yet been any conclusive evidence that these secondary metabolites are produced by the fungus inside the insect during infection. A major hurdle to this issue has been that the usual biochemical techniques used for their detection fail when attempting to trace these fungal molecules inside an host, although liquid-chromatography (LC) or high-performance liquid chromatography (HPLC) were useful for detecting secondary metabolites from mycelial cakes and free-cell cultures (Gupta et al., 1995; Xu et al., 2007), and also from pooled insects infected with either *Metarhizium anisopliae* (Amiri-Besheli et al., 2000; Skrobek et al., 2008) or *Beauveria brongniartii* (Strasser et al., 2000). The analysis of individual mycosed insects rather than from pooled samples is essential in order to provide an indication of the expression pattern of secondary metabolites during the time course of fungal infection (Molnar et al., 2010). In addition, the sensitiveness of these techniques does not permit the detection of the few molecules that were expected to be produced by the fungus when it is growing inside the insect hemocoel. In this regard, the expression of genes involved in the biosynthesis of these metabolites when the fungus grows within its insect host might help to better understand their role in pathogenesis.

Most of the secondary metabolite core genes are silent during laboratory cultivation in artificial media, and require specific situations that induce their expression such as are provided by the natural environment during the infection (Gibson et al., 2014). In this study, we demonstrated that *BbtenS*, *BbbeaS*, and to a lesser extent *BbbslS* are expressed within the infected insects (*T. infestans*) regardless of the methodology used for fungal inoculation. The three genes were, however, differentially expressed at different stages of infection thus suggesting that each has a discrete role in various phases of the pathogenic process. Both *BbtenS* and *BbbeaS* were found to be highly expressed in conidia-treated insects at day 3 and day 12 post-treatment, which co-incided with the mortality peaks previously observed in *T. infestans* infected with *B. bassiana* (Luz et al., 1998). The high expression levels of *BbtenS* and *BbbeaS* detected at day 12 post-treatment agrees with the high levels of secondary metabolites found in moribund insects; Skrobek et al. (2008) reported that destruxin levels peaked at the time of insect death. In blastospore-injected insects, *BbtenS* expression peaked at 24 h after treatment and was also highly expressed in insect cadavers, as was the *BbbeaS* gene. These results

agree with the well-known role of those secondary metabolites that display antibiotic activity; mycosed cadavers apparently produce them to prevent development of other microorganisms that might eventually compete for the same substrata. *M. anisopliae*-mycosed silkworm larval cadavers have been reported to contain levels up to 30-fold higher than those detected at day 5 post-infection, when the insects are still alive (Suzuki et al., 1971).

The expression of insect immune genes showed a differential behavior during the fungal infection. In conidia-treated insects, the corresponding transcripts were the most abundant at days 6 and 9 after fungal treatment, coincident with the lowest levels of expression of the fungal synthetase genes. This expression pattern suggests that host immune insect responses are critical in attempts to limit or to stop at limiting the fungal infections. In blastospore-injected insects, a correlation between the expression of insect humoral genes and the time course of infection with the lower fungal dose used was noted. These data suggest that immune responses are modulated by the dose, and this finding is an important conclusion from this work because host responses may not be uniform, even to a particular pathogen, and are likely to depend upon the exposure level (inoculum) to a potential pathogen.

The results showed here demonstrate that qRT-PCR is a useful technique to follow entomopathogenic fungal gene expression inside an infected insect host. The methodology is sufficiently sensitive to detect a wide range of fungal transcripts in a mixed (insect and fungus) cDNA sample. Moreover, the possibility to measure the expression levels of insect genes from the same sample expands the ability to monitor the time course evolution of the infection process, and could potentially be applied to other fungal-host systems.

Acknowledgments

We wish to thank Nemat O. Keyhani and Richard A. Humber for revising the manuscript, Carla Huarte-Bonnet for technical assistance in cloning of the gene fragments, and to the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil) for fellowship to L.S.L. This work was partially supported by grants of the National Agency for Science and Technology Promotion in Argentina (PICT 2012 1964) to N.P, and CAPES PCPP 016/2011 to C.L and L.S.L. Both M.P.J. and N.P. are members of the CONICET Researcher's Career, Argentina.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2015.04.004.

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