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Research Article

Expression analyses of some *Beauveria bassiana* genes in response to cuticles of four different insects

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Abstract: *Beauveria* spp. as entomopathogenic fungi have extremely diverse insect host ranges. Here, a comparative transcript analysis of a *Bassiasin I* (*BSNI*), a *Dipeptidyl peptidase* (*DPP*), a *Cytochrom P450 monooxygenase* (*CYP52*), a *Mitogen activated protein kinase* (*MAPK*), and a *Neuronal calcium sensor 1* (*NCS*) in two isolates of *Beauveria bassiana*., with low and high virulence, were carried out. For this, induction media, containing isolated cuticles from four insects, and a check medium lacking any cuticular extract were prepared and isolates were allowed to grow on them. Interestingly, up-regulation of *BSNI* and *DPP* in induction media was noted in comparison to the check, following normalization with *γ-ACTIN*. *CYP52* was up-regulated in synthetic medium in comparison to cuticular extract. *MAPK* expression was greater in most virulent isolate once compared with the low virulent isolate. The expression of *MAPK* was greater in the presence of *Caliptamus italicus* cuticles and synthetic medium as opposed to others. *NCS* expression remained on changed in all media tested and can be suggested as a reference gene in transcriptomics of *Beauveria* spp. genes in future.

Keywords: Bassiasin I, Dipeptidyl peptidase, Cytochrome P450 monooxygenase, Mitogen activated protein kinase, Neuronal calcium sensor.

Introduction

Beauveria spp. are well-known entomopathogenic fungi that can be used as potential alternatives to chemical pesticides for the control of insect pests, especially where the humidity is high. *Beauveria* spp. have a broad-host-range, affecting both crop and invasive pests, and insects that act as vectors of human and animal diseases (Fan *et al*., 2012a; Faria and Wraight, 2007; Kirkland *et al*., 2004). Next to its capability as a biological control agent, *Beauveria* spp. has been considered as an emerging model organism. It has been used to study fungal growth and development in order to delineate host-pathogen interactions (Jin *et al*., 2010; Wanchoo *et al*., 2009). Considering the entomopathogenicity mechanism of the fungus, *Beauveria* spp. secretes suites of enzymes in order to penetrate through the cuticular barrier of the insect host (Freimoser *et al*., 2003; Goettel *et al*., 1989; Pendland *et al*., 1993). On the other hand, due to the great diversity amongst insects, their cuticles and epicuticles are quite different in terms of structure and constituents. Such differences are

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quite common even within species (Charnley, 1984; McCoy *et al*., 1988; St. Leger *et al*., 1994). In *Beauveria* spp., varieties of techniques such as EST (expressed sequence tag) and microarray have demonstrated the differences in the expression of pathogenic genes in response to different insect cuticles, plant extracts and nutrient depleted media (Freimoser *et al*., 2005; Wang *et al*., 2005). In such cases, global transcript analysis opens a window towards better insight into entomopathogenicity at a molecular level, leading to the discovery of enzymes and toxins that may have a role in efficient triumph over the insect host (Bagga *et al*., 2004; Dutra *et al*., 2004; Freimoser *et al*., 2003, 2005). This preliminary data can be followed up by further single/oligo gene comparative transcript analysis to further illustrate the actual role of such genes. This of course depends on the experimental set up and the treatments such as the presence of particular insect cuticle within the fungal culture medium and/or the type of used medium. Nevertheless, transcript analysis data needs to be complemented at the enzyme level via quantitative biochemical methods.

Here, the expression patterns of genes involved in encoding proteins responsible for proteolytic digestion of cuticles, namely cuticle degrading protease *Bassiasin I* (*BSNI*), *Dipeptidyl peptidase* (*DPP*), were analyzed. Since, the major component of cuticle is made up of proteins and if an entomopathogen is to break through this barrier, it should be able to penetrate through this barrier via the arsenal of proteases (Hepburn, 1985). The first coating that the fungal pathogen faces in an insect host would be the epicuticular layer, covered with lipids and carbohydrates (Pedrini *et al*., 2010). Accordingly, transcript analysis of *Cytochrom P450 monooxygenase* (*CYP52*) involved in degradation of such layers was analyzed by the two *Beauveria bassiana* isolates and cuticles of four insect hosts, namely *Galleria mellonella*, *Caliptamus italicus*, *Eurygaster integriceps*, *Tribolium castaneum*. The cytochrome P450 monooxygenases constitute a large superfamily of haem-thiolate proteins in almost all known organisms, playing pivotal roles in metabolism of both endogenous and xenobiotic compounds. They often are involved in conversion of a wide range of lipophilic compounds to more hydrophilic derivatives. The enzymes encoded by *CYP52* family of enzymes, specifically are involved in terminal hydroxylation of *n*-alkanes, named P450alk, (Pedrini *et al*., 2010). In *Beauveria*, *CYP52* family contributes to the penetration of the host cuticle via facilitating the assimilation of insect epicuticle lipids (Zhang *et al*., 2012).

Earlier studies have shown that a *Mitogen activated protein kinase* (*MAPK*) has a pivotal role in fungal virulence (Jin *et al*., 2012). Mitogen activated protein kinases are proteins involved in cell signaling and responses to environmental stimuli. Mutant analysis of *MAPK* in *Beauveria* spp. caused the fungus to lose its virulence (Zhang *et al*., 2009). Further analysis revealed that the MAPK is involved in conidial adhesion to insect cuticles, ability to construct appresorium, and to establish a molecular connection.

Neuronal calcium sensor 1 (*NCS*) has some roles pre-and during-penetration of fungus hyphae into its insect host (Fan *et al*., 2012b). *NCS* has been demonstrated to be involved in extracellular acidification and virulence of Beauveria spp. (Fan *et al*., 2012b). Therefore, analyses of their expressions were also considered in this study.

Materials and Methods

Materials

Peptone, Yeast extract, Dextrose and Agar were purchased from Lifechem (Milano, Italia). Taq DNA polymerase, 10× PCR buffer, $MgCl_2$, dNTPS, Safe-stain were obtained from Biosience (Cambridge, England). PCR primers were ordered from Bioneer (Dajon, Korea south,). 2-step RT-PCR kit containing 10 mM dNTP MIX, 50 mM $MgCl_2$, Taq DNA Polymerase, DNase Free Deionized water, 10 × Buffer M-MulV, M-MulV Reverse Transcriptase were purchased from Vivantis (Shahe Alam, Malaysia). Trizol (TRI Reagent) and agarose were obtained from Invitrogen (Carlsbad, California, USA). Loading dye, DNase and DNA ladder were from

Fermentase (Maryland, USA). Cyclohexamide, dodine, chloramphenicole, chloroform, isopropanol and ethanol were obtained from Sigma (St. Louis, Mo. USA). DNA Gel Purification Kit and DNA Gel Extraction Kit were from Norgen Biotek (Ontario, Canada). The other materials were purchased from Merck (Darmstadt, Germany).

Fungal isolation from soil

Galleria melonella bait method (Zimmerman, 1986) was used for fungal isolation from soil and using ITS as molecular marker, the species were determined to be *B. bassiana* (data not shown). The collected soil samples (20 g each) were strained through a 1 mm sieve and layered in 90 mm petri dishes (Meyling, 2007). Third or 4th instar *Galleria* larvae (10 larvae) were left over moist soil at 27 ± 1 ºC for 10 days in dark. The grown fungi on the dead larvae were surface sterilized with 1% sodium hypochlorite and transferred into SDAY, containing 0.5 g.l^{-1} cyclohexamide, 0.5 g.l^{-1} 65% dodine, 0.2 g.l^{-1} chloramphenicole, 0.3 g.l^{-1} $CuCl_2$ and 0.02 g.l^{-1} crystal violet (Sun and Liu, 2008). Dilution series were made from the obtained fungal isolates on the above selective medium to obtain single spores that were considered as pure isolates (El Damir, 2006). For this, a loopful of spores was picked and suspended in 0.2% (v/v) tween 80. The suspensions were then transferred into 1.2% agar and incubated for 72 h at 27 ± 1 ºC and 85 ± 3% humidity. Star-shaped mycelium was observed and considered as mycelium growing from a single spore. The mycelia were sub-cultured in fresh media and allowed to grow for 2 weeks.

DNA extraction

Modified Plaza *et al*. (2004) method was used for DNA extraction from single spore clones. Each clone was cultured on a rotary shaker (160 rpm) in liquid medium containing 0.2% (w/v) dextrose, 1% (w/v) pepton and 0.02% (w/v) yeast extract and incubated at 28 ºC for 72 h. Grown mycelia were passed through a filter paper and left on a fresh filter paper to dry for 1 h. Relatively dry mycelia (0.02 g) was ground to powder in the presence of liquid nitrogen using a dentistry condenser in an Eppendorf tube. CTAB buffer (700 µl of 2% buffer, containing 2 g CTAB, 1 g PVP, 100 mM Tris, 1.4 mM NaCl and 20 mM EDTA) was added to the ground cells in the presence of 100 µl 1% SDS. Tubes were vortexed for 5 min and followed as reported by Plaza *et al* (2004).

Molecular identification of fungal isolates

Polymerase Chain Reaction (PCR) was carried out on isolated DNA samples using ITS_1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS_4: 5'-TCCTCCGCTTATTGATATGC-3' primers (White *et al*., 1990) in a 25 µl volume. The in house PCR reaction contained 2.5 µl 10 × PCR buffer, 2.5 µl 25 mM $MgCl_2$, 1 µl of 5 mM dNTPS, 0.5 µl of 1 U Taq DNA polymerase, 1 µl of each of 10 pmol ITS_1 and ITS_4 primers and 1 µl of 50 nmoles of isolated fungal DNA. PCR started with 5 min at 94 ºC followed by 35 cycles of [94 ºC for 1 min: 55 ºC for 1 min: 72 ºC for 2 min] and a final incubation at 72 ºC for 10 min. The PCR amplified internal transcribed spacer sequence (ITS) was separated on 1% (w/v) agarose gel electrophoresis and stained with ethidum bromide. The band of about 540 bp in size was isolated from gel and purified using DNA Gel Extraction Kit. The isolated DNA fragment was sent for DNA sequencing to Bioscience (England), using ITS_1 and ITS_4 primers. ITS sequence was checked via BLAST at NCBI to determine the fungal genus (Tamura *et al*., 2011).

Virulence of fungal isolates to insects

Beauveria spp. (160 isolates) were isolated from different geographical provinces of Iran, mainly from citrus orchards in northern and southern parts of the country. After single clones were prepared for each isolate, in 0.2% (v/v) tween 80, spore suspensions were made in 0.2% (v/v) tween 80 and were adjusted to have concentrations of 1 × 10^4 conidia/ml. The suspensions were used in virulence evaluation on *Sitophilus oryzae* in three replicates. For this, 30 insects in triplicates were submerged for 30 s in the suspension and laid over a wet filter paper in a Petri dish and incubated for 24 h at 27 ± 1 ºC and 85 ± 3% humidity. The fourth sets was sunmerged in distilled water instead of fungal suspension and used as control. Wherever control insects died, the

values obtained from treated insects with *B. bassiana* isolates were adjusted with Abbott's formula (data not shown; Abbott, 1925).

The plates were checked on a daily basis and dead insects were removed and placed on a clean wet filter paper to evaluate if the cause of death was due to microorganism other than *Beauveria*. Out of these isolates, 65 demonstrated more than 22% mortality rate during a 12 day period. Therefore, the isolates were classified into three groups; namely low-(0-22%), average-(22-60) and high-virulent (60-80%). The isolates with the highest-(80.23% from *Minoodasht*) and the lowest-mortality (22.05% from *Hormozgan*) rates were chosen for further analysis.

Fungal culture media

The conidia from culture on SDAY medium were inoculated on (solid) minimal medium with nitrate (Pontecorvo, 1953) and 0.5% (w/v) insect cuticle. Conidia from 15-day-old culture on this minimal medium were harvested. An aqueous suspension of the conidia was made with 0.01% Tween-80. The conidial concentration in the suspension was adjusted to 10^7 ml^{-1} through hemocytometer (Neubauer Improved Bright Line, Hamburg, Germany). The suspension was used to inoculate the medium with insect cuticular extract (see following section). Thus, a serial transfer from a nutrient rich medium to insect cuticle was done.

Preparation of the cuticular extract

Cuticles were obtained from four insect species *C. italicus* (Orthoptera: Acrididae), *E. integriceps* (Hemiptera: Scutelleridae), *G. mellonella* (Lepidoptera: Pyralidae) and *T. castaneum* (Coleoptera: Tenebrionidae). In each case, sufficient numbers of insects were used to obtain approximately 2 g of dried cuticle. For *C. italicus*, the cuticle was stripped off the nymph instar, while for *E. integriceps*, the cuticle was dissected from the abdomen and scutellum of adults. For *G. mellonella*, the cuticle was dissected from the abdomen of adults. For *T. castaneum*, cuticle was obtained from adults. And since *T. castaneum* is very small, it was not possible to dissect out the inner organ and remove hemolymph. Instead, they each were individually pressed with pestle to squeeze out the hemolymph. The inner sides of the other insect cuticles were scraped free of any adhering material. The separated cuticles of the insects were washed with an aqueous solution of 1% (w/v) potassium tetraborate (Andersen, 1980). The cuticles were blotted on a blotting paper, dried to a constant weight in an oven at 32 ºC, ground to a fine powder and were stored in screw-capped bottles. Cuticle (0.5% w/v) medium was prepared in water. The cuticle powder of the respective insect species was wrapped in a sterile muslin cloth, dipped in sterile double distilled water and autoclaved for 5 min at 115 ºC to obtain a medium with cuticular extract (Pathan *et al*., 2007). The cuticle components rather than the topography of the cuticle were found to influence the gene expression pattern in the insect pathogenic fungus *Metarhizium anisopliae* (Wang *et al*., 2005). SDY medium was used as the synthetic control medium. For *B. bassiana* culture, 500 µl of a conidial suspension (10^7 ml^{-1} conidia) was inoculated in 50 ml of cuticular extract or artificial medium in 250 ml conical flasks and incubated at 27 ºC and 160 rpm, for 96 h (Florez *et al*., 2003). The mycelium was harvested by filtration, washed with sterile distilled water and used for RNA extraction immediately.

RNA extraction and cDNA synthesis

Total RNA was isolated from fungal mycelia grown on different insect cuticlar extracts and SDY medium using TRIzol reagent as instructed by manufacturer (Invitrogen, Carlsbad, California). Residual DNA was removed by DNase treatment. The integrity and quantity of RNA was determined by agarose gel electrophoresis and UV spectrophotometric measurement (JENWAY 6300 Spectrophotometer, Badfordshier, England). cDNA was synthesized from approximately 5 µg of total RNA with the Vivantis cDNA Synthesis Kit following the instructions of the manufacturer. Total RNA (5 µg of), 1 µl of 40 µM Oligo $d(T)_{18}$ primer and 1 µl 10 mM dNTPs mix incubated at 65 ºC for 5 min, chilled on ice for 2 min, 10 µl of the cDNA Synthesis Mix containing 2 µl 10× buffer M-MuLV, 100 unit M-MuLV Reverse Transcriptase and Nuclease-free Water was added

to the mixture. The mixture was incubated at 42 ºC for 60 min and at 85 ºC for 5 min to stop the reaction. The cDNA mixture was stored at-20 ºC for subsequent quantitative PCR analysis.

RT-PCR

Gene-specific primers (Table 1) for two *B. bassiana* proteases and *γ-ACTIN* gene, as a reference gene, were designed using IDT (2015). The specificity of primers was checked via BLASTX and TBLASTN at NCBI.

PCR amplification was carried out in a final volume of 25 µl containing 2.5 µl 10 × PCR buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP, 1 µl of each of reverse and forward primers, 1 µl of template cDNA (0.1 ng), 0.5 µl Taq DNA Polymeras (0.2 units). PCR was started with 94 ºC for 10 min followed by 20-35 cycles of [94 ºC for 30 s; annealing temperature based on Table 1 for 30 s; 72 ºC for 30 s] that finished by a cycle of 72 ºC for 7 min. PCR reactions were allowed to proceed for a different number of cycles to determine the exponential phase of amplification. Each sample was evaluated in 3 biological replicates. In order to show the cDNA bands of genes, the PCR products were run on 1.5% (w/v) agarose gel. A 1 kb DNA ladder was used as marker at this stage. Quantification of the bands was performed by Image J software (National Institute of Mental Health, Bethesda, Maryland, USA). In all experiments, collected data were normalized against the expression pattern of a reference gene that was against *γ-ACTIN* and compared not only among media containing insect cuticles, but also with a minimal medium lacking any cuticular extract.

Statistical analysis

The analyses of data obtained from gel band densitometry in a completely randomized block design were carried out using SAS, version 9.1 (SAS Institute Inc, Cary, NC 27513, USA). Differences were considered significant if p values were smaller than 0.05. The least significant difference (LSD) was performed to determine the mean difference in genes expression.

Results

Mycelium mass growth

The fungal growth for two isolates was monitored on SDY and in the presence of the insect cuticles (Pathan *et al*., 2007). As expected, the growth on SDY (3.7 g fresh weight) was profuse when compared with the other media (~0.6 g fresh weight). Moreover, the mycelial mass was greater in the presence of *G. mellonella* and *C. italicus* cuticles compared to the media containing cuticles of either *E. integriceps* or *T. castaneum* (Fig. 1).

Table 1 Primers used in RT-PCR**.**

Gene name	Accession number	Amplicon size (bp)	Primer size (bp)	Annealing temperature (°C)	Primer sequence (5'→3')
BSN1	AF104385.1	275	20	60.0	AACTTGACCGTCTCCGCCAC
			25	60.4	GTGACCATCTGTAGTGCCACTGACA
DPP	AY380550.1	332	26	57.5	TACTCCTTTGAGACTCACTCCAAGTC
			18	57.8	TTCTCTTCGTCAGCGGCG
CYP52	ADK36660.1	305	27	60.3	TCTCTTTGACCGTTGCCTTTATCCTCC
			25	59.6	CGCCAGAATCGTCTTGATGTTCTCG
MAPK	AY333430.1	312	25	59.0	CCGATGACCACTGCCAATACTTCAT
			20	58.4	GCTTCCCGCTTAGCATCTCG
NCS	JQ317159.1	300	20	60.4	TGTCACAGACGGCTGGTTGC
			27	59.4	CCTGATGGCGAAGCAGATTGTAATGTT
γ-ACTIN	HQ232398.1	412	21	59.5	GTATCCACGCCACCACCTTCA
			25	59.0	GCCTCCTTGATTCTATCACACGCAT

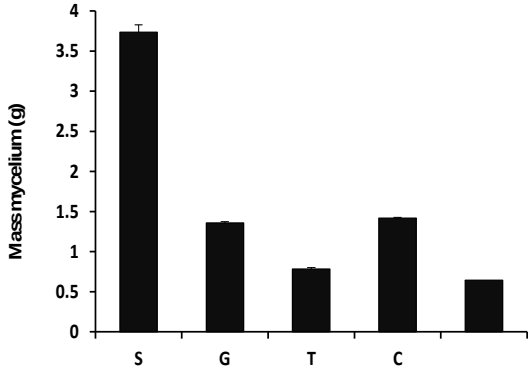

Figure 1 *B. bassiana* mycelium mass grown on different media. S, Synthetic. G, *Galleria mellonella* cuticular extract. T, *Tribolium castaneum* cuticular extract. C, *Caliptamus italicus* cuticular extract. E, *Erygaster integriceps* (LSD = 0.2153).

Transcript analysis via RT-PCR

γ-ACTIN was PCR amplified for 25 cycles and densitometry revealed its constant and stable expression in all tested experiments and therefore was used for normalization of the expression pattern of genes studied in this report (Fig. 2).

BSNI was amplified after 35 PCR cycles (Fig. 2). The gene encodes for a strong cuticle-degrading serine protease with 379 amino acids (GenBank accession No: AF104385.1). Analysis of variance of the normalized data was illustrative of all sources of variance including fungal isolate, media and isolate × media were statistically significant (Table 2). The expression pattern of *BSNI* was greater in the least virulent (LV; relative expression to control gene = 0.9122) *B. bassiana* (Fig. 3) compared to the most virulent isolate (MV; relative expression to control gene = 0.8990). The highest and the lowest expression of LV were noted in the presence of *C. italicus* cuticles (relative expression to control gene = 0.9923) and SDY (0.7885), respectively. Similar results were obtained for MV, *i.e. C. italicus* cuticles (0.9515) and SDY (0.8135). However no significant differences were seen among *G. mellonella* (0.9160), *T. castaneum* (0.9111) and *E. integriceps* (0.9033).

DPP gene (GenBank accession No: AY380550.1) encodes a protein with 687 amino acids in length that was amplified after 35 PCR cycles (Fig. 2). The data was normalized against *γ-ACTIN* expression pattern. Similar to *BSNI*, analysis of variance of *DPP* demonstrated to be significant for all sources of variance (Table 2). In contrast to *BSNI*, the expression of *DPP* in (MV; 0.9793) was significantly higher than (LV; 0.8539). No statistical differences were noted in expression pattern of *DPP* for MV in response to cuticles isolated from the four insects (Fig. 4). However, the expression was significantly different from SDY. For LV, the *DPP* expression was somewhat different and similar to what was noted for *BSNI*; the highest expression was for *C. italicus* cuticles (0.9239), no significant differences were observed among the other three insect cuticles, and SDY (0.7578) was the lowest.

CYP52 was amplified after 35 PCR cycle (Fig. 2). The gene encodes for a strong *Cytochrome P450 monooxygenase* with 528 amino acids (GenBank accession No: ADK36660.1). Analysis of variance of the normalized data was illustrative of all sources of variance including fungal isolate, media and isolate × media were statistically significant (Table 2). The expression pattern of *CYP52* was greater in least virulent (LV; 0.9122) *B. bassiana* compared to the most virulent isolate (MV; 0.8990). The highest expression was noted in SDY (0.9765; Fig. 5), which was significantly different from media containing cuticles of *G. mellonella* (0.91123), *T. castaneum* (0.86475), *C. italicus* cuticles (0.85529) and *E. integriceps* (0.78245).

MAPK was amplified after 25 PCR cycle (Fig. 2). The gene encodes for a strong *MAPK* with 356 amino acids (GenBank accession No: AY333430.1). Analysis of variance of the normalized data was illustrative of all sources of variance including fungal isolate, media and isolate × media, all of which were statistically significant (Table 2). The expression pattern of *MAPK* was greater in most virulent (MV; 0.8990) *B. bassiana* compared to the least virulent isolate (LV; 0.9122; Fig. 6). The highest and the lowest expression of MV were noted in SDY (1.072) in the presence of *C. italicus*

(1.063) and *G. mellonella* (1.057) cuticles. The difference was not significant between the two cuticles and SDY. Following these three, cuticles of *E. integriceps* (0.9595) and *T. castaneum* (0.9258) had the lowest value. Similar results were obtained for LV, *i.e.* in SDY (1.063), *C. italicus* cuticles (1.063) highest expression was noted. No significant statistical differences were seen among *E. integriceps* (1.031) *T. castaneum* (1.024) and *G. mellonella* (1.0).

NCS was amplified after 35 PCR cycles (Fig. 2). The gene encodes for a strong NCS with 205 amino acids (GenBank accession No: AF104385.1). Analysis of variance of the normalized data illustrated the effect of medium being significant on gene expression (Table 2). The highest expression of *NCS* was noted in the presence of *C. italicus* cuticle (0.9923; Fig. 7), while the expression remained the same for the other media.

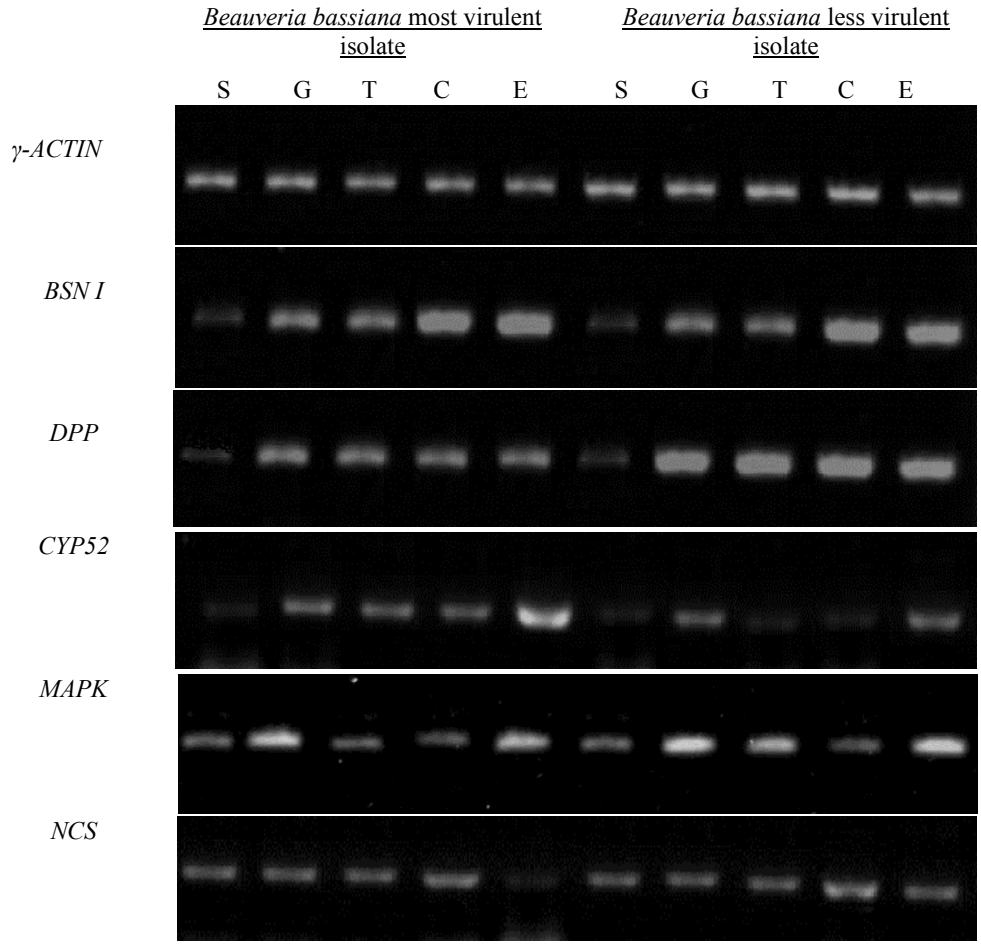

Figure 2 RT-PCR quantitative estimation of *γ-ACTIN, BSN I*, *DPP*, *CYP52*, *MAPK* and *NCS* transcripts in two *B. bassiana* isolates grown on different media. S, Synthetic medium. G, *Galleria mellonella* cuticular extract. T, *Tribolium castaneum* cuticular extract. C, *Caliptamus italicus* cuticular extract. E, *Erygaster integriceps*.

Table 2 Analysis of variance of relative expression of *cuticle-degrading protease*, *dipeptidyl peptidase*, *Cytochrome P450*, *mitogen activated protein Kinase*, *Neuronal calcium sensor 1* genes in two fungal isolates of *Beauveria* grown on different media.

Sources of variance		Mean squares (MS)				
	df	*Cuticle-degrading protease*	*Dipeptidyl peptidase*	*Cytochrome P450*	*Mitogen activated protein Kinase*	*Neuronal calcium sensor 1*
Replication	2	0.000040^{ns}	0.000043^{ns}	0.003020^{ns}	0.000038^{ns}	0.003846^{ns}
FI	1	0.001300**	0.117900**	0.006046**	0.003190**	0.003700^{ns}
M	4	0.024800**	0.037500**	0.030950**	0.009990**	0.006614**
M × FI	4	0.001900**	0.004200**	0.001990^{ns}	0.005940**	0.000665^{ns}
Error	18	0.000039	0.000035	0.002300	0.0000314	0.001540
Coefficient of variation (%)		1.340	1.223	10.790	3.530	8.850

ns: non significant. **: significant at $p < 0.01$, df: degrees of freedom. FI: Fungal isolate. M: Medium.

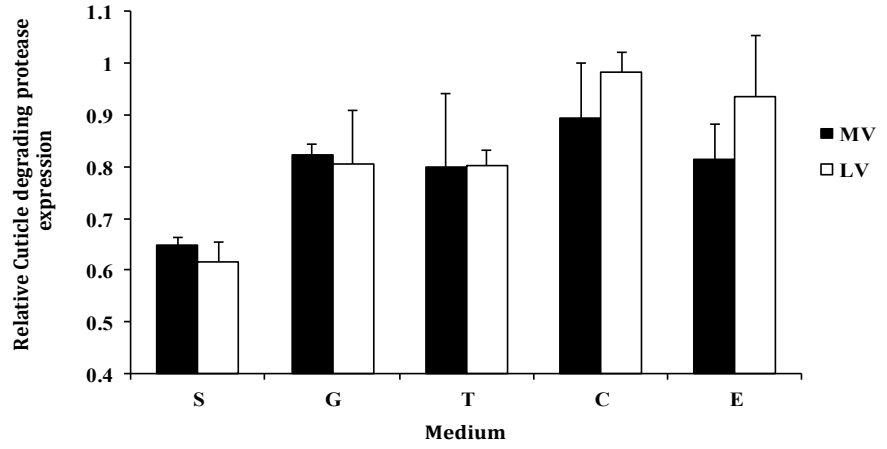

Figure 3 Relative expression of *cuticle degrading protease* (*BSNI*). Semi quantitative RT-PCR was performed using cDNA derived from mycelia of two *B. bassiana* isolates grown in different media. S, Synthetic medium. G, *Galleria mellonella* cuticular extract. T, *Tribolium castaneum* cuticular extract. C, *Caliptamus italicus* cuticular extract. E*, Erygaster integriceps*, MV = most virulent, LV = least virulent (LSD = 0.01071).

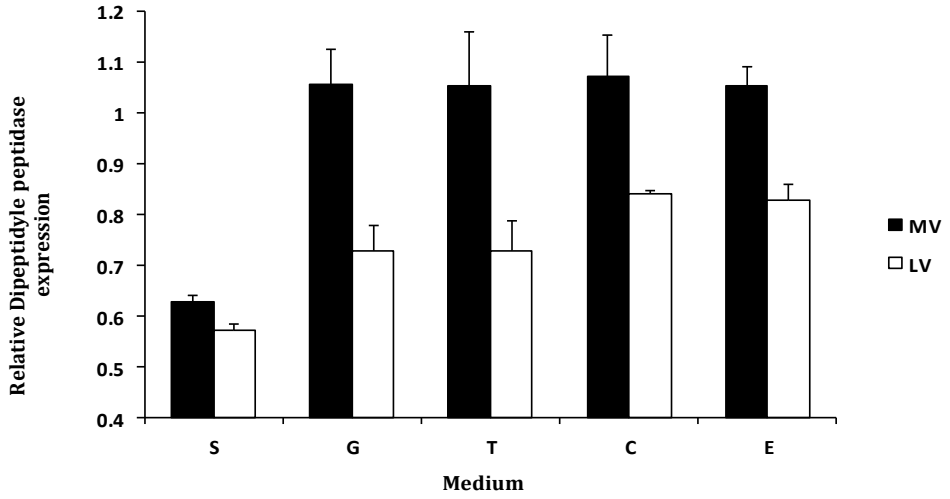

Figure 4 Relative expression of *dipeptidyle peptidase*. Semi quantitative RT-PCR was performed using cDNA derived from mycelia of two *B. bassiana* isolates grown in different media. S, Synthetic medium. G, *Galleria mellonella* cuticular extract. T, *Tribolium castaneum* cuticular extract. C, *Caliptamus italicus* cuticular extract. E, *Erygaster integriceps*, MV = most virulent, LV = least virulent (LSD = 0.01015).

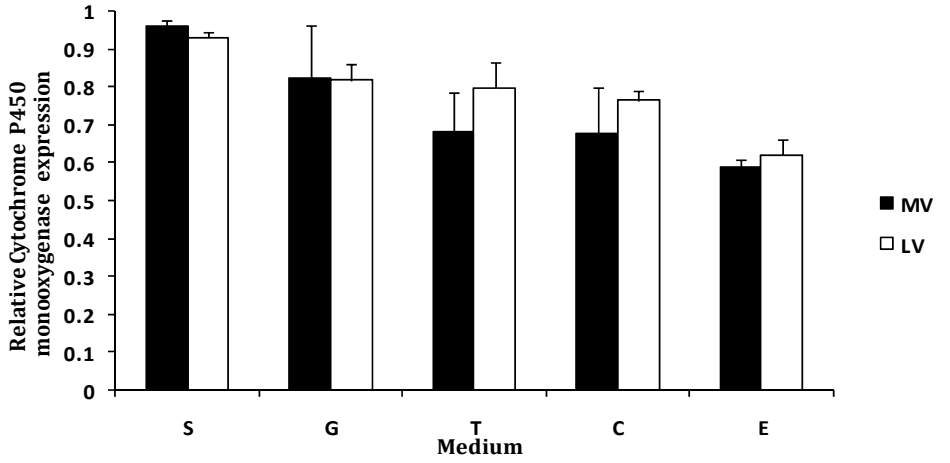

Figure 5 Relative expression of *Cytochrome P450 monooxygenase*. Semi quantitative RT-PCR was performed using cDNA derived from mycelia of two *B. bassiana* isolates grown in different media. S, Synthetic medium. G, *Galleria mellonella* cuticular extract. T, *Tribolium castaneum* cuticular extract. C, *Caliptamus italicus* cuticular extract. E*, Erygaster integriceps*, MV = most virulent, LV = least virulent (LSD = 0.059).

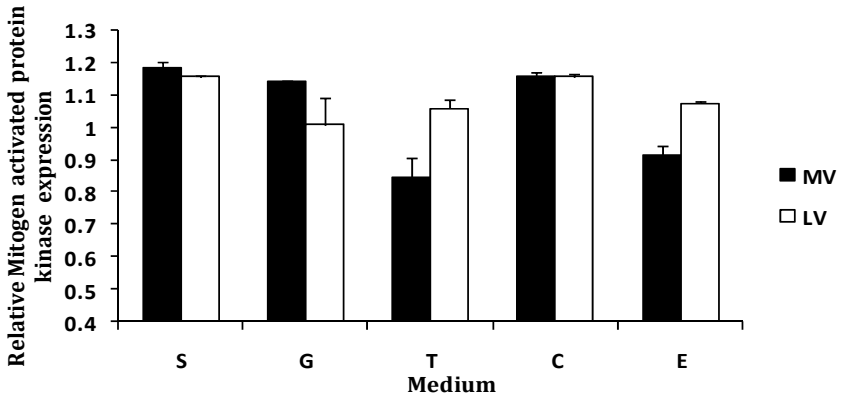

Figure 6 Relative expression of *Mitogen activated protein kinase*. Semi quantitative RT-PCR was performed using cDNA derived from mycelia of two *B. bassiana* isolates grown in different media. S, Synthetic medium. G, *Galleria mellonella* cuticular extract. T, *Tribolium castaneum* cuticular extract. C, *Caliptamus italicus* cuticular extract. E, *Erygaster integriceps*, MV = most virulent, LV = least virulent (LSD = 0.03040).

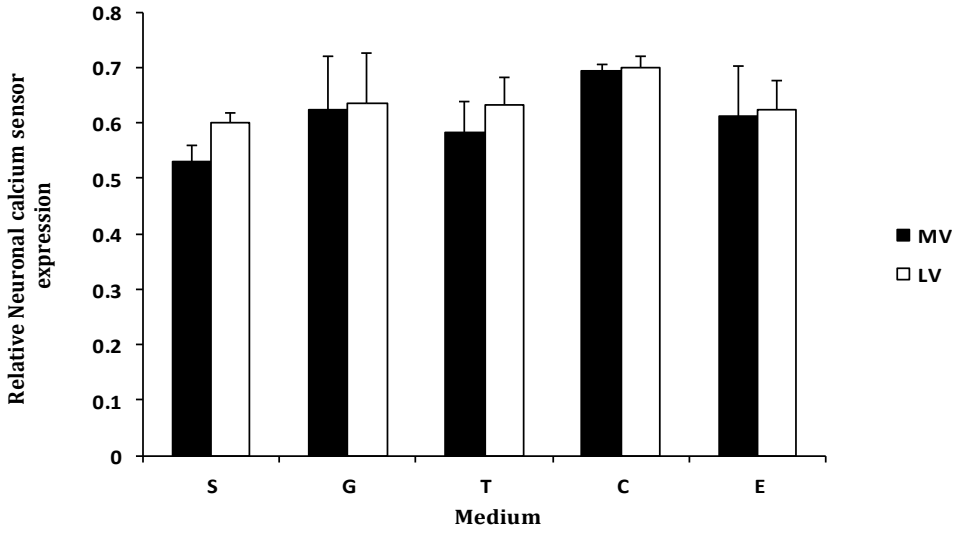

Figure 7 Relative expression of *Neuronal calcium sensor 1*. Semi quantitative RT-PCR was performed using cDNA derived from mycelia of two *B. bassiana* isolates grown in different media. S, Synthetic medium. G, *Galleria mellonella* cuticular extract. T, *Tribolium castaneum* cuticular extract. C, *Caliptamus italicus* cuticular extract. E, *Erygaster integriceps*, MV = most virulent, LV = least virulent (LSD = 0.067).

Discussion

Entomopathogenic fungi invade their hosts by direct penetration of the host cuticle. The cuticle has two layers, the outer epicuticle and the procuticle. The epicuticle is a very complex thin structure that lacks chitin but contains phenol-stabilized proteins and is covered by a waxy layer containing fatty acids, lipids and sterols (Hackman, 1984). The procuticle forms the majority of the cuticle and contains chitin fibrils embedded into a protein matrix together with lipids and quinones (Neville, 1984). Protein may account for up to 70% of the cuticle. In many areas of the cuticle the chitin is organized helically giving rise to a laminate structure. This rather complex structure helps insects to keep their bodily water and acts as a shield against microorganisms, parasites and predators (Vincent and Wegst, 2004). Therefore, fungal entomopathogens need to somehow break through this barrier. It seems that hydrolytic enzyme digestion allows the fungal species to make openings into the cuticle and find their way through. Initially, proteolytic enzyme digestions help to remove the protein coat. This follows with chitinase(s) activities that generate holes with proper sizes allowing fungal penetration via hyphae (Hegedus and Khachatourians, 1995).

Here, representative members of four insect orders, Lepidoptera, Coleoptra, Hemiptera and Orthoptera, due to their probable differences in terms of cuticular content and structure were selected. The complex structure is different in insect species and therefore it is expected that fungal entomopathogens behave differently (Charnley, 1984; McCoy *et al*., 1988; Pathan *et al*., 2007). These differences can be traced back to the expression pattern of genes involved in encoding proteins that are capable of cuticle degradation. Such studies may reveal the effective genes and proteins against insect hosts that later can be used in engineering highly pathogenic fungal entomopathogens towards pest biocontrol. A candidate gene approach was taken into consideration, looking into the expression patterns of *BSNI*, *DPP*, *CYP52*, *MAPK*, *NCS* in two *B. bassiana* isolates, most and least aggressive. For this purpose, five different media each containing cuticlar suspension from one of the four different insect species or lacking any cuticular extract as check were used.

As stated above, since cuticles are highly abundant in proteins, expression patterns of two proteolytic enzymes, *BSNI* and *DPP*, were studied. Recent studies have shown that regulation of proteases expressions is somewhat complex and their production becomes induced in poor media. Complex substrates such as insect cuticles and protein molecules induce protease expression, while simple sources of nitrogen such as amino acids and ammonium have inhibitory effect. Nevertheless, different responses can be expected from varieties of proteases. For instance, it has been shown that Pr1 becomes induced only in the presence of an insect cuticle, while Pr2 acts less sensitively and expresses in the presence of both soluble and insoluble proteins (Hegdus and Khachatourians, 1995). *BSNI* is a strong serine protease and our results were indicative of its higher expression in media containing insect cuticular extracts, suggesting a probable role in cuticle degradation (Fig. 3). *BSNI* expression was the highest in *C. italicus*. This may indicate that the cuticle has particular types of proteins that trigger *B. bassiana* membrane receptors more efficiently. Accordingly, during cuticle isolation more proteins have peeled off to the solution and caused such response in *B. bassiana* Nevertheless, to better comprehend such responses, a thorough structural analyses and comparisons of insect cuticles need to be performed and further experiments need to be done with intact structure. Comparative analyses between the two *B. bassiana* isolates in all media showed that the *BSNI* expression was greater in least virulent isolate. This might be due to expression of other proteolytic enzymes that makes one isolate more virulent. Furthermore, often there is no linear correlation between mRNA and protein contents, and therefore mRNA stability might be different between the two isolates.

Earlier study by Tartar *et al*. (2004), demonstrated that *DPP* is being expressed equally in SDY and insect hemolymph. *DPP* is an exopeptidase, cleaving two peptides from one end of a protein molecule (Tartar and Boucias, 2004). Whenever the media is rich with amino acids such as having either pepton or hemolymph, there is no need for the expression of *DPP* since there is no protein to digest. However in poor complex media containing for example insect cuticle, it is expected that *DPP* and similar enzymes would be induced. Here, *DPP* expression was significantly higher in MV, following the virulence and aggressiveness pattern. However, no significant changes for any of the 4 cuticles for each isolate were evident (Fig. 4). *DPP* seems to have no gene family and a mutant analysis can provide information about its function in *B*. *bassiana*.

Here, the cytochrome P450 monooxygenase is constitutively being expressed (Fig. 5) with greater amounts in SDY and more specifically in LV isolate the expression in SDY was significantly greater than the media containing insect cuticles. This higher expression can be explained by the greater availability of organic compounds within SDY. Apart from the level of expression in control medium, no difference between other media containing insect cuticles was evident except for *E. integriceps* that appeared to have the minimum expression. Earlier study by Pedrini *et al*. (2010) in relation to P450 monooxygenase was indicative that the expression is greatly dependent on the alkane length (Pedrini *et al*., 2010). They demonstrated that the expression of *CYP52* declined as the alkane length grew to C28. Here, we have speculated that the differences in genes expressions are due to the length of the fatty acyl chain; whenever the fatty acyl chain was longer the expression was higher. The least expression pattern was evident for *E. integriceps* and again it is expected that it has the smallest alkane chain amongst the insects studied. Nevertheless, a detailed cuticular structure analysis seems to be inevitable to comprehensively correlate the changes in expression patterns with the coticule structure and content.

The expression of *MAPK* was significantly weaker in induced media in contrast to SDY with no cuticle for both isolates (Fig. 6). The results are inconsistent with what was reported by Zhang *et al*. (2009). These differences in results may be due to the cuticle extraction technique; in our experiment the isolated insect cuticles were finely ground to powder, while in Zhang *et al*. (2009) intact larvae of *Pieris brassicae* was used for bioassay. In the latter procedure the proteins may have kept their 3D structure in a way that makes the phosphorylation sites better exposed to MAPKs and therefore these genes become activated. Whereas, in our experiment such intact structures might have been disrupted and finding phosphorylation sites that may trigger this feedback loop would be hampered. In most virulent (MS) isolate of *B*. *bassiana*, the *MAPK* induction was significantly higher in farm pests, *G. mellonella*, *C. italicus*, than stored products pests, *E. integriceps*, *T. castaneum* (Fig. 6). In least virulent species, the highest *MAPK* expression was noted for *C*. *italicus* at similar expression level with MS isolate and was significantly higher than that of the other three insect cuticles.

Here, no differences were evident between media and the fungal isolates for *NCS* (Fig. 7), similar to what Pathan *et al*. (2007) reported on cuticle of four other insects (Pathan *et al*., 2007). Accordingly, we would like to suggest *NCS* as a reference gene for transcript analysis of *Beauveria* spp.. In our experiment, the expression pattern of *NCS* remained relatively low during 5 days of culturing, similar to that reported by fan *et al*. (2012b).

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آنالیز بیان برخی ژن‌های قارچ *Beauveria bassiana* در مواجهه با کوتیکول چهار حشره متفاوت

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چکیده: قارچ بیمارگر حشرات .*Beauveria* spp دامنه میزبانی وسیعی دارد. در این تحقیق، به آنالیز ترانسکریپتوم مقایسه‌ای ژن‌های *باسیاسین I*، *دای پپتیدیل پپتیداز*، *سیتوکروم P450 مونواکسیژناز*، *میتوژن پروتئین کیناز فعال* و *سنسور کلسیم* در دو ایزوله از *Beauveria bassiana* با بیماریزایی قوی و ضعیف پرداخته شد. در اینجا محیط‌کشت‌های القایی حاوی کوتیکول‌های جدا شده از چهار حشره متفاوت و یک محیط کشت کنترل فاقد هیچ نوع عصاره کوتیکولی تهیه شد و به ایزوله‌ها اجازه داده شد تا در آنها رشد کنند. به‌صورت جالب‌توجهی افزایش بیان دو ژن *باسیاسین* و *دای پپتیدیل پپتیداز* در محیط کشت القایی در مقایسه با محیط کشت کنترل مشاهده شد، که در ادامه با میزان بیان ژن *گاما اکتین* نرمال گردید، *سیتوکروم P450 مونواکسیژناز* در محیط کشت سنتتیک در مقایسه با عصاره کوتیکولی افزایش بیان نشان داد. بیان *میتوژن پروتئین کیناز فعال* در ایزوله با بیماریزایی بالا در مقایسه با ایزوله با بیماریزایی پایین بیش‌تر بود. بیان این ژن در حضور کوتیکول‌های ملخ ایتالیایی و محیط کشت سنتتیک برخلاف سایر محیط کشت‌ها بیش‌تر بود. بیان *سنسور کلسیم* در محیط کشت‌های مختلف مورد استفاده، تغییر نکرد. لذا به‌عنوان ژن منبع در مطالعات ترانسکریپتوم آینده ژن‌ها در .*Beauveria* spp پیشنهاد می‌گردد.

واژگان کلیدی: باسیاسین I، دای پپتیدیل پپتیداز، سیتوکروم P450 مونواکسیژناز، پروتئین کیناز، سنسور کلسیم