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Research Article

Quantitative expression analyses of *Dipeptidyl peptidase and Cytochrome P450 Monooxygenase* genes of *Beauveria bassiana* during growth on insect cuticles

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ABSTRACT- *Beauveria bassiana* is an entomopathogenic fungus that acts as a biological control agent and attacks varieties of insects by degrading and penetrating their cuticles. The relevant molecular basis of this action was investigated by comparing the expression analyses of *dipeptidyl peptidase* and *cytochrome P450 monooxygenase*, candidate genes involved in fungal pathogenesis, via Real-Time PCR. *B. bassiana* was grown in an artificial medium and in the cuticle extract media isolated from four species belonging to different insect orders. The expression of the two genes in the fungus was examined in different culture media. The results showed that *dipeptidyl peptidase* was upregulated in media containing insect cuticular extracts compared to the control synthetic medium, while *Cytochrome P450 monooxygenase* showed reduced expression in abundance. The effect of cuticle types in inducing gene expression was compared and discussed. It seems likely; information from different reactions of this fungus against different insect cuticles can be helpful in producing specific pesticides.

INTRODUCTION

Fungal biocontrol agents (FBCAs) are promising alternatives to chemical pesticides (Rios-Moreno et al., 2016). FBCAs are used to control pest populations, and unlike chemical pesticides, they pose no risk to human, animal or environmental health (Erler and Ates, 2015; Rios-Moreno et al., 2016).

Beauveria is a well-known genus of entomopathogenic fungi with numerous registered formulations around the globe such as mainly *B. bassiana* (Balsamo) Vuillemin and *B. brongniartii* (Saccardo) Petch (Thomas and Read, 2007). With over 700 host species, this genus has been used for biological control of vectors of human diseases as well as a wide range of insect pests (Galidevara et al., 2016; Ortiz-Urquiza and Keyhani, 2016). *Beauveria* can also endophytically colonize within a variety of plant species and may be useful to plants by aiding the efficient absorption of minerals (Vega, 2008). Fungal spore attachment to the target host cuticle triggers the initiation of infection that continues with spore germination and germ tube protrusion that accompanies the synthesis and secretion of different enzyme types (Ortiz-Urquiza and Keyhani, 2013; Vikhe et al., 2016). Upregulation of hydrolytic enzymes during insect host encounter has been reported for entomopathogenic fungi (Khan et al., 2012; Ortiz-Urquiza and Keyhani, 2016). Entomopathogenic fungi roles are reported to be in infection of insects and penetration within their cuticles (Xiao et al., 2012, Ortiz-Urquiza and Keyhani, 2013, 2016). Insect cuticles are mainly made up of proteins that are cross-linked to chitin as the major carbohydrate structural biomolecule. This dynamic mechanism helps insects prevent water loss and protects them against microorganisms, predators, and chemical and biological pesticides (Vincent and Wegst, 2004; Pedrini et al., 2013). A study to identify and analyse the expression of 26 *cytochrome p* (CYPs) genes in response to benzo[α]pyrene in *Brachionus rotundiformis* (small rotifers) showed that these CYPs including *cytochrome p450* may play a role in the detoxification mechanism and could be used as B [α] P molecular biomarkers in *B. rotundiformis* (Han et al., 2019). Knock out of *CYP52X1* in *B. bassiana* led to reduction in virulence capability once encountered with *Galleria mellonella* L. and complementation reverted the phenotype (Zhang et al. 2012). In another study, examination of the expression of this gene in *Plecoptera oculata* has shown that *PoCYP* genes can be used as targets for the production of new pesticides (Lyu et al., 2020).

Comparative analysis of fungal transcripts is a suitable mechanism to predict the behavior of any pathogen (Xiao et al., 2012). Here, expression patterns of two genes involved in virulence and cuticle degradation of *B. bassiana*, namely *dipeptidyl peptidase* and *cytochrome P450* (CYP) *monooxygenase* were analysed via RT-qPCR and the data were normalized against *γ-actin*, as a reference gene. The comparative expression analysis was carried out on fungal RNA once encountered with the cuticles of four insect pests including *G. mellonella* (Lepidoptera: Pyralidae), *Caliptamus italicus* L. (Orthoptera: Acrididae),

Eurygaster integriceps Puton (Hemiptera: Scutelleridae), *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), and a synthetic culture medium lacking cuticular extract. Information obtained from the differential expressions of regulatory genes helps to understand the molecular mechanism associated with *B. bassiana* metabolites.

MATERIALS AND METHODS

Fungus Isolation and Molecular Identification

The *G. mellonella* bait method was used to separate the fungus from the agricultural soils of different regions of Iran (Fialho et al., 2018). DNA was isolated from single clones of the fungus according to Plaza et al. (2004). PCR was carried out on isolated DNA samples using ITS1: 5'TCCGTAGGTGAACCTGCGG3' and ITS4: 5'TCCTCCGCTTATTGATATGC3' primers in a 25 µl volume (2.5 µl 10× PCR buffer, 2.5 µl 25 mM $MgCl_2$, 1 µl 5mM dNTPs, 0.5 µl 10.0 U *Taq* DNA polymerase (Biosience, Cambridge, England), 1 µl of 10 pM of each of primers (Bioneer, Korea south, Dajon), 1 µl 50 nM isolated fungal DNA) to amplify Internal Transcribed Spacer (ITS) of the fungus genome. PCR initiated at 94 °C for 5 min that followed by 35 cycles of (94 °C for 1min: 55 °C for 1 min: 72 °C for 2 min) and final incubation at 72 °C for 10 min. The amplified DNAs were separated by 1% (w/v) agarose gel electrophoresis and stained using ethidium bromide. The ITS amplified DNA (540 bp) was isolated from gel using DNA Gel Extraction Kit (Norgen Biotek, Ontario, Canada). The ITS1 and ITS4 primers were used for DNA sequencing at Bioscience (England) and the sequence was analysed according to Vega (2008).

Fungal Culture Media

The conidia grown on SDAY (Sabouraud Dextrose Agar Yeast) medium were used to inoculate both minimal medium with nitrate and minimal medium containing 0.5% (w/v) insect cuticle (see the following section). Conidia were isolated from the culture minimal medium (15 days) and their aqueous suspension was prepared with 0.01% Tween 80 (Merck, Darmstadt, Germany). The concentration of conidia in the suspension was adjusted to 10^7 conidia ml^{-1} using a hemocytometer. The suspension was inoculated with the culture medium containing insect cuticle extract and was transferred sequentially from the nutrient-rich culture medium to the cuticle-containing medium.

Preparation of Cuticles from Insects

Insect cuticles (2 g) of *G. mellonella*, *C. italicus*, *E. integriceps*, and *T. castaneum* were prepared according to the method of Andersen (1980) reported at Pathan et al. (2007). The cuticles were ground into a fine powder and stored in sealed containers. The insect cuticle powder was wrapped in a sterile cloth, immersed in sterile distilled water and autoclaved for 5 min at 115 °C. SDY medium was used as synthetic control medium. 500 µl of conidial suspension (10^7 conidia ml^{-1}) was inoculated into 50 ml of cuticular extract or artificial medium in 250 ml conical flasks and incubated at 27 ºC and 160 rpm for 96 h (Zhang et al., 2011). After the fungus has grown, mycelium was isolated using a filter, sterilized with distilled water and finally, RNA was extracted from them.

RNA Extraction and cDNA Preparation

Total RNA was isolated from 100 mg of fungal mycelium using TRIzol reagent (Invitrogen, Carlsbad, California) according to the company protocol. The quality of the isolated RNA was determined by 1.5% (w/v) agarose gel electrophoresis. Isolated nucleic acid (5 µg) was incubated with 2 µL of 1 U/µL DNaseI and 1 µL of 10× reaction buffer containing $MgCl_2$ at 37 °C for 30 min to remove genomic DNA. The reaction mixture was incubated with 1 µL of 50 mM EDTA at 65 °C for 10 min to inactivate the enzyme. The quantity of RNA was determined by UV spectrophotometer, and first-strand cDNA was prepared using Vivantis cDNA Synthesis Kit (Shah Alam, Malaysia). The cDNA was kept at -20 °C for qPCR analysis.

Phylogenetic Analysis of Dipeptidyl Peptidase and Cytochrome P450 Monooxygenase

Dipeptidyl peptidase amino acid homologous sequences and Cytochrome P450 monooxygenase homologous gene sequences were obtained for entomopathogenic fungi. Two non-entomopathogenic fungi including *Endocarpon pusillum* and *Morchella brunnea* were used as outgroups. The obtained sequences (https://www.ncbi.nlm.nih.gov/) were aligned with Mega6 software (Tamura et al., 2013). The phylogenetic tree was generated with Mega6 software using the Maximum likelihood method with bootstrap replications of 1000.

RT-qPCR

Specific primers were designed for γ-actin internal control and two *B. bassiana* genes dipeptidyl peptidase and cytochrome P450 monooxygenase (Table 1) using IDT Oligo Analyzer (http://eu.idtdna.com/analyzer/Applications/ Oligo Analyzer/). The specificity of these primers was examined with BLASTX and TBLASTN at NCBI.

The relative quantification of mRNA of studied genes was investigated using a SYBR Green SYBRBIOPARSTM Kit (Gorgan University, Iran) on the iQ5 system (BioRad, USA). Each sample was examined in three replications. After the initial activation phase of the DNA polymerase at 95 °C for 3 min, samples were subjected to 35 cycles of amplification (95 °C: 10 sec, 59 °C: 10 sec, 72 °C: 10 sec).

Relative gene expression was calculated using a mathematical model described by Pfaffl (2001) and the expressions were examined by REST (http://www.WZW.tum.de/gene-quantification) software (Pfaffl et al., 2002). The melt curve was used to determine the specificity of the primers.

Ratio = (E_{target}) ΔCP target (unknown target sample)/ ($E_{ref.}$) ΔCP reference (control-sample) Eq. (1)

On the basis of Equation (Eq.) 1, the relative expression ratio of the target gene (ΔCP target) was computed, based on its real-time PCR efficiencies (E) and differential crossing point (ΔCP) of an unknown sample vs. a control sample (ΔCP control-sample). Therefore, the target gene expression was normalized by a non-regulated reference (ref.) gene expression.

Table 1. Primers used in Real time PCR

Gene name	Accession no.	Primer size (bp)	Annealing temperature (°C)	Primer sequence (5′→3′)	Amplic-on size
DPP	AY380550.1	26	57.5	TACTCCTTTGAGACTCACTCCAAGTC	332
		18	57.8	TTCTCTTCGTCAGCGGCG	
CYP52	ADK36660.1	27	60.3	TCTCTTTGACCGTTGCCTTTATCCTCC	305
		25	59.6	CGCCAGAATCGTCTTGATGTTCTCG	
γ - ACTIN	HQ232398.1	21	59.5	GTATCCACGCCACCACCTTCA	412
		25	59.0	GCCTCCTTGATTCTATCACACGCAT	

RESULTS AND DISCUSSION

The outermost cuticular layer of insects (wax layer) is composed of an inhomogeneous mixture of lipids including fatty acids, wax esters, and long-chain alkanes (Lin et al., 2011; Xiao et al., 2012; Pedrini et al., 2013, Perez et al., 2014). Hydrocarbon digestion plays a vital role in the ability of *B. bassiana* to infect certain insects, and alkane growth has been linked to increased virulence (Zhang et al., 2012).

Comparison of fungal cells grown in alkanes and glucose media showed that the mortality of fungal cells against the bean weevil *Acanthoscelides obtectus*, in alkanes was two to four times that of glucose (Thomas and Read 2007; Zhang et al., 2012). *B. bassiana* can grow in a wide range of long-chain alkanes and fatty acids (Pedrini et al., 2010). Cytochrome P450 monooxygenase family of enzymes is involved in the oxidation of organic compounds such as lipids that can be found in insect cuticles (Pedrini et al., 2010). For example, CYP52 family P450 monooxygenase helps in penetration to cuticle via epicuticular lipid digestion (Zhang et al., 2012), which justified this high level of expression with more access to organic compounds in SDY medium. Identification and study of *P450* gene expression in *Bradysia odoriphaga* associated with imidacloprid insecticide detoxification showed that four *P450* genes increased expression and were involved in imidacloprid detoxification (Chen et al., 2018).

In a 2021 study by Zhang et al., the expression of two genes of *Cytochrome P450 monooxygenases* (*CYP52X1* and *CYP5293A*) involved in hydrocarbon digestion of the cuticle in *B. bassiana* exposed to the cuticle *Frankliniella occidentalis* were analyzed (Zhang et al., 2021). *CYP52X1* had the highest expression level in the early stages of infection. *CYP52X* showed an increase in expression about 20-25-fold after 18 h of fungal spray on the insect surface (Zhang et al., 2021). It was consistent with its predicted role in the digestion of host cuticle hydrocarbons and participation in the

insect cuticle penetration event (Zhang et al., 2012; Pedrini et al., 2013). The expression of *CYP5293A* was highest at 12 to 24 hours after inoculation and had a low expression level before that. Although the function and substrates of this enzyme are unknown, it can be said that it may be sequentially involved in distinct hydrocarbon assimilation pathways (Zhang et al., 2021).

In a study examining the expression of *P450 monooxygenase* gene in the minimal medium, it was shown that the expression of this gene is highly related to the length of alkanes (Pedrini et al., 2010; Huang et al., 2014). According to their study, the expression of certain *CYP52 P450 monooxygenase* was increased as the alkane length grew to C28 (long chain alkanes up to 28 carbon; Pedrini et al., 2010). The length of the fatty acyl chain can cause differences in gene expression between different cuticles, because their study showed that the longer the fatty acyl chain, the more it is expressed. The *CYP52* gene encodes for a strong Cytochrome p450 monooxygenase with 528 amino acids (GenBank accession No: ADK36660.1).

Based on the results of the current study, a low expression pattern of *cytochrome P450 monooxygenase* was observed for *T. castaneum*, *G. mellonella*, *E. integriceps* and *C. italicus* (Fig. 1). The highest expression of *CYP52* was in SDY artificial culture medium. This was probably due to the high level of access to organic compounds in this culture medium. The expression of gene in the presence of different insect cuticles showed a decrease in expression compared to the control culture medium. As it can be seen in Fig. 1, the highest expression of *CYP52* in the face of cuticle was related to *C. italicus*, *E. integriceps*, *G. mellonella* and T. castaneum, respectively. One of the possibilities for the change in expression of this gene in the presence of different cuticles can be related to the difference in the length of alkanes in different cuticles. However, since the exact composition of the cuticle of these insects has not been identified, no definite conclusion can be made.

In addition to carbohydrates, the cuticle is composed of proteins (Moussian, 2010). So, the expression pattern of a proteolytic enzyme, *dipeptidyl peptidase*, was investigated in this study. This gene encodes an enzyme with 687 amino acids. *Dipeptidyl peptidase* showed increase in expression, when the fungus was grown on a variety of insect cuticular extracts compared to synthetic media. The up-regulation was significant ($P < 0.01$) when *C. italicus* and *T. castaneum* cuticular extracts were used (Fig. 2), possibly due to the higher protein contents in these cuticular extracts compared to those in other cuticular extracts and the synthetic medium.

The expression of *dipeptidyl peptidase* is dependent on the availability of polypeptide chains. Although insect cuticles are poor in proteins, they still can induce *dipeptidyl peptidase* expression. In the present study, *dipeptidyl peptidase* expression was significantly high in the presence of cuticular extracts of tested insects (Fig. 2). In a study conducted by Dionisio et al. (2016), proteomics was used to examine the detection of exoenzymes of the fungus *B. bassiana* in an artificial culture medium containing carbon, nitrogen, and phosphate and another culture medium containing shrimp chitin and beetle *Periplaneta americana* cuticle. The enzyme dipeptidyl peptidase was one of the enzymes detected in the supernatant of culture medium containing beetle cuticle (Dionisio et al., 2016).

A few studies have shown that the expression of regulating proteases is complex and their induction occurs in nutrient-poor culture media (Dias et al., 2008; Dhar and Kaur, 2010). Insect cuticles and some protein molecules have been shown to induce the expression of protease genes. On the other site, nitrogen sources such as amino acids and ammonium have inhibitory effects on their expression (Dias et al., 2008; Gupta et al., 2002). For instance, two protease genes (*Pr1* and *Pr2*) showed to be expressed in the presence of insect cuticles and the availability of soluble/insoluble proteins were checked, respectively (Vincent & Wegst, 2004). On the other hand, in the study of Tartar and Boucias (2004), it was observed that *dipeptidyl peptidase* in *B. bassiana* is also expressed during growth in SDY and during proliferation in insect hemolymph (Tartar and Boucias, 2004).

To investigate the role of *Dipeptidyl peptidase* and *Cytochrome P450 Monooxygenase* genes of *B. bassiana* in the host infection process, Mega6 was used to align the homologous sequences and generate phylogenetic trees. For cytochrome P450 monooxygenase, comparison of the rate of evolution between genera (i.e., *Aspergillus*, *Beauveria*, and *Metarhizium*) seems to be similar (Fig. 3). This can be interpreted as a gene that is required for fungal survival through detoxification, degradation of xenobiotics, and the biosynthesis of secondary metabolites for pathogenesis (Cresnar and Petric, 2011; Forlani et al., 2014). In comparison, the evolution of dipeptidyl peptidase appeared to be different. *Metarhizium* was evolved earlier in time followed by *Beauveria* and *Aspergillus*, respectively (Fig. 4). Interestingly, within the *Metarhizium* genus the evolving pattern has not changed as much as *Aspergillus*. This indicates that what is formed as *dipeptidyl peptidase* for *Metarhizium* was functionally sufficient for its pathogenicity, while further changes were required for *Aspergillus* species. In the current study, the phylogenetic analyses based on both genes confirmed the findings of Xiao et al. (2012); reported that *B. bassiana* and *Cordyceps militaris* are closely related (Figs 3 and 4). In the study of Alkin et al. (2021), the same results were observed for *dipeptidyl peptidase* in a phylogenetic tree.

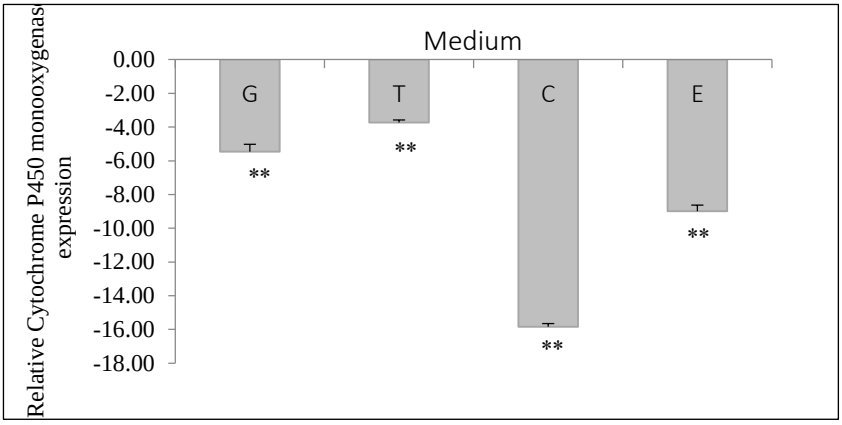

Fig. 1. Comparative expression values of *cytochrome P450 monooxygenase* Real-Time PCR was carried out using cDNA derived from mycelia of *B. bassiana* grown in media containing different cuticular extracts obtained from G, *G. mellonella*; T, *T. castaneum;* C, *C. italicus*; E, *E. integriceps*. **, significant (0.154 ≤ Error bar ≥ 0.447).

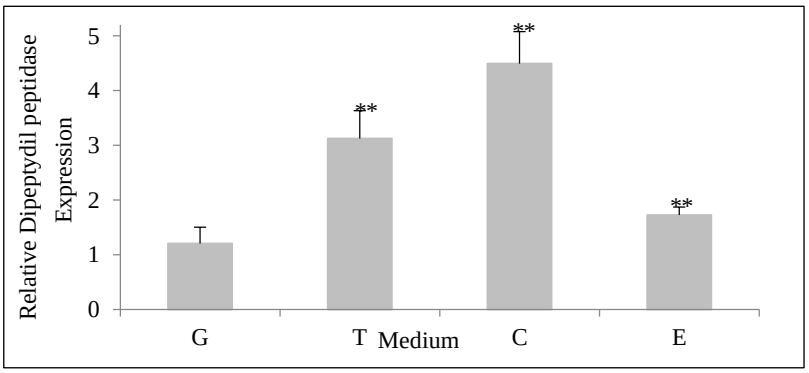

Fig. 2. Comparative expression value of *dipeptidyl peptidase*. Quantitative Real-Time PCR was carried out using cDNA derived from mycelia of *B. bassiana* grown in different media containing cuticular extracts of G, *G. mellonella;* T, *T. castaneum;* C, *C. italicus;* E, *E. integriceps*. **, significant (0.142 ≤ Error bar ≥ 0.587).

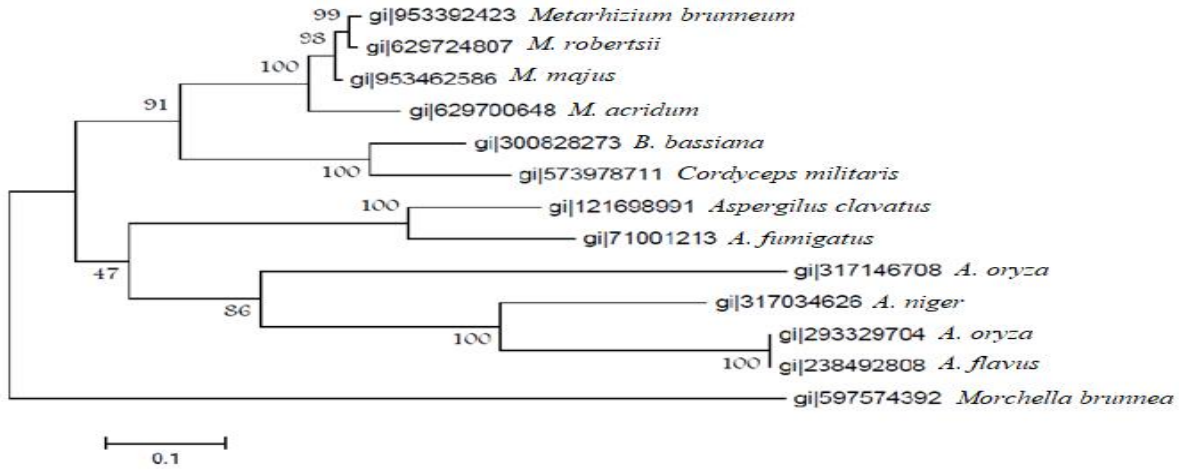

Fig. 3. Phylogenetic tree of *Beauveria bassiana* for *cytochrome P450 monooxygenase* sequences generated by maximum likelihood. Tree confidence was confirmed by 1000 bootstraps (presented numbers on nodes). Pathogenic fungi: gi: 953392423, *Metarhizium brunneum*; gi: 629724807, *M. robertsii*; gi: 953462586, *M. majus*; gi: 629700648, *M. acridum*; gi: 300828273, *B. bassiana*; gi: 573978711, *Cordyceps militaris*; gi: 121698991, *Aspergilus clavatus*; gi: 71001213, *A. fumigatus*; gi: 317146708, *A. oryza* RIB40; gi: 317034626, *A. nige*; gi: 293329704, *A. oryza* gi: 238492808, and *A. flavus*; and non-enthemopathogenic fungus: gi: 597574392, *Morchella brunnea*. gi (GenInfo Identifier).

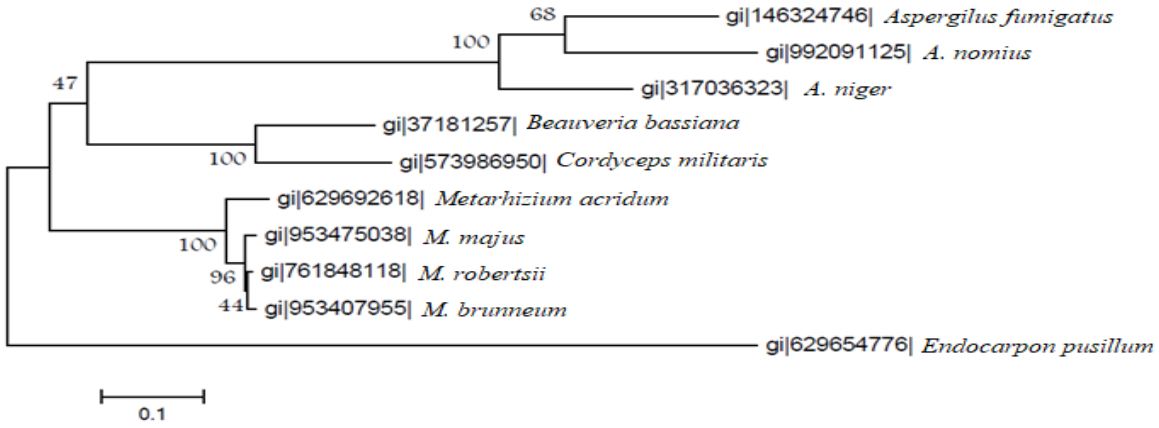

Fig. 4. Phylogenetic tree of *dipeptidyl peptidase* sequences of *B. bassiana* using Maximum likelihood. Tree confidence was confirmed by 1000 bootstraps (presented values on the tree). Pathogenic fungi: gi: 146324746, *A. fumigatus*; gi: 992091125, *A. nomius*; gi: 317036323, *A. niger*; gi: 371811257, *B. bassiana*; gi: 573986950, *C. militaris*; gi: 629692618, *M. acridum*, gi: 953475038, *M. majus*; gi: 761848118, *M. robertsii*; gi: 953407955; *M. brunneum*; and non-enthemopathogenic fungus: gi: 629654776, *E. pusillum*. gi (GenInfo Identifier).

CONCLUSIONS

A limited number of transcript analyses have been reported for filamentous fungi, especially for plant and arthropod pathogens. Transcript analyses performed in the current studty on the fungus *B. bassiana* growing on different media, indicated genes involved in pathogenicity and cuticle digestion. In the case of the two genes studied, the *DPP* gene showed increased expression in the presence of all tested insect cuticles in comparison with the control culture medium. However, *CYP52* gene showed decreased expression in all culture media containing insect cuticles compared to the control culture medium. This information, along with expression information about other genes involved in cuticle digestion and pathogenesis, helps us to produce specific pesticides.

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آنالیز بیان کمی ژنهای دای‌پپتیدیل پپتیداز و سیتوکروم p450 مونواکسیژناز در *Beauveria bassiana* طی رشد روی کوتیکول حشرات

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رونوشت

چکیده - *Beauveria bassiana* یک قارچ بیمارگر حشرات است، که به عنوان یک عامل کنترل بیولوژیک می باشد. *Beauveria bassiana* با تخریب و نفوذ در کوتیکول به انواع حشرات حمله می‌کند. با آنالیز بیان ژن های دای‌پپتیدیل‌پپتیداز و سیتوکروم p450 مونواکسیژناز با اسفاده از روش پی سی آر در زمان واقعی (Real-Time PCR) اساس مولکولی عمل این قارچ مورد بررسی قرار گرفت. بوریا باسیانا در یک محیط کشت مصنوعی و محیط های عصاره کوتیکولی چهار گونه از راسته های متفاوت حشرات رشد داده شد، و بیان دو ژن معرفی شده در قارچ در محیط کشت‌های مختلف (حاوی کوتیکول گونه های مختلف حشرات) مورد بررسی قرار گرفت. نتایج نشان داد بیان ژن‌دای‌پپتیدیل-پپتیداز در محیط‌های حاوی عصاره های کوتیکول حشرات در مقایسه با محیط کشت مصنوعی افزایش بیان نشان داد، در حالیکه سیتوکروم p450 مونواکسیژناز کاهش بیان نشان داد. در نهایت تاثیر انواع کوتیکول در القای بیان ژن مقایسه و مورد بحث قرار گرفت. به نظر می‌رسد اطلاعات حاصل از پاسخ متفاوت این قارچ در برابر کوتیکول حشرات مختلف می تواند در تولید آفت‌کش‌های اختصاصی کمک کننده باشد.