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Differential expression of genes involved in entomopathogenicity of the fungi *Metarhizium anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum* (Clavicipitaceae)

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ABSTRACT. Expression analysis of the genes involved in germination, conidiogenisis and pathogenesis of *Metarhizium anisopliae* during its saprophytic and pathogenic life stages can help plan strategies to increase its efficacy as a biological control agent. We quantified relative expression levels of the nitrogen response regulator gene (*nrr*1) and a G-protein regulator of genes involved in conidiogenesis (*cag*8), using an RT-qPCR assay. Comparisons were made between *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum* during germination and conidiogenesis and at different stages of pathogenesis. The *cag*8 gene was repressed during germination and induced during conidial development and the pathogenic phase, and the *nrr1* gene was induced during germination, conidiogenesis and the pathogenic phase. Both genes were more expressed in *M. anisopliae* var. *anisopliae*, demonstrating

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that different varieties of *M. anisopliae* differ in activation of genes linked to virulence for certain environments and hosts. This suggests that differences among these varieties in the ability to adapt could be attributed not only to specific genomic regions and genes, but also to differential gene expression in this fungus, modulating its ability to respond to environmental stimuli.

Key words: Biocontrol; Entomopathogenic fungi; Quantitative RT-PCR; Pathogenicity gene

INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* can infect ca. 200 species from more than 50 insect families (Roberts and Humber, 1981), and is used globally as a biological control agent for many insect pests. Its advantages over chemical pesticides include high insect specificity, low toxicity to other organisms and low environmental impact (Miller et al., 1983). In addition, due to its multiple development processes of infection, insect resistance to *M. anisopliae* is less likely or develops more slowly than resistance to chemical pesticides (He and Xia, 2009). However, its use has been limited due to its relatively slow rate of killing when compared to chemical pesticides (St Leger et al., 1996).

Metarhizium anisopliae is a saprophytic organism with both soil-dwelling and pathogenic life stages (Roberts and Humber, 1981). The morphological, cytological and biochemical mechanisms involved in these two life stages are relatively well understood. However, attempts to improve the effectiveness of entomopathogenic fungi also require a clear understanding of molecular basis (Wang et al., 2005; Wang and St Leger, 2007). In particular, the molecular steps involved in attachment to the host and the penetration of the cuticle are crucial in the establishment of infection and are very attractive in scientific terms (Roberts and Humber, 1981; Wang et al., 2008). Several genes of *M. anisopliae* implicated in pathogenesis and other developmental stages have been cloned and characterized (St Leger et al., 1992a,b; Bogo et al., 1998; Joshi and St Leger, 1999; Screen et al., 2001; Baratto et al., 2006; He and Xia, 2009). Hence, a detailed study on the timing of the expression of these genes is crucial for elucidating their contribution in the adaptation of the fungus as saprophyte and pathogen.

Here, we show that the genes *cag*8 (regulator of G-protein signaling) and *nrr*1 (regulator of nitrogen) are differentially expressed in *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum* during germination, conidiogenesis and pathogenesis. It is likely that this difference in expression allows *M. anisopliae* to respond flexibly to adverse conditions and adapt to different environments and hosts.

MATERIAL AND METHODS

Biological material

Metarhizium anisopliae var. *anisopliae* URM 4921, isolated from *Mahanarva posticata*, and *M. anisopliae* var. *acridum* URM 4412, isolated from *Austracnis guttulosa*, were obtained from the mycological collection of the Department of Mycology, Federal University

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of Pernambuco (URM-UFPE). Cultures were grown on potato dextrose agar at 28°C for 12 days in order to obtain conidia.

Preparation of biological material for the study of gene expression during germination

Conidia were harvested in 0.01% Tween 80 aqueous solution, and the conidial suspension was filtered through glass wool to remove mycelia. Conidia were then added to YPD broth (0.2% yeast extract, 1% peptone, 2% dextrose) at a concentration of 2 x 10^7 conidia/mL. The conidia were collected by centrifugation at 8 h (ungerminated conidia-before germination), 10, 12 and 14 h (germinating conidia) after inoculation, immediately frozen in liquid nitrogen and maintained for 24 h at -80°C for subsequent extraction of RNA. Two biological replicates were performed for each time analysis. Conidial germination was monitored microscopically.

Preparation of biological material for the study of gene expression during conidiogenesis

Conidia were harvested in 0.01% Tween 80 aqueous solution, and the conidial suspension was filtered through glass wool to remove mycelia. Conidia (2 x 10^7 conidia/mL) were spread onto cellophane (0.5 µM, pore) on YPD agar (0.2% yeast extract, 1% peptone, 2% dextrose). Scraped from Petri dishes, the mycelia were collected at 24 h (mycelial growth), 48 h (conidiophore development), 72 h (conidiophore with few conidia), and 96 h (conidiophores with a large number of conidia) after inoculation, immediately frozen in liquid nitrogen and maintained for 24 h at -80°C for subsequent extraction of RNA. Two biological replicates were performed for each time analyzed. Fungal conidiogenesis was monitored microscopically.

Preparation of biological material for the study of gene expression during pathogenesis

Third-instar larvae of *Diatraea saccharalis* (the sugar cane borer) were infected by immersion for 1 min in a conidial suspension of 2 x 10^8 conidia/mL. The insects collected at 20 h after infection, dead infected insects, insects with few emergent mycelia, insect cadavers mummified with conidia, and uninfected insect were immediately frozen in liquid nitrogen and maintained for 24 h at -80°C for subsequent extraction of RNA. Two biological replicates were performed for each phase analyzed.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from conidia, mycelia and insects described above. The frozen samples were ground with liquid nitrogen. For each sample, 100-150 mg powdered sample was placed in a cooled 2-mL tube. RNA samples were extracted with Trizol reagent (Invitrogen) according to manufacturer instructions. RNA was suspended in 50 µL DEPC-treated water. Purity of the total RNA extracted was determined based on the 260/280 nM ratio and integrity was checked by electrophoresis on a 1% agarose gel. Residual DNA was removed by treating RNA with RNase-free DNase I according to manufacturer instructions (Deoxyribonuclease I, Invitrogen). RNA was stored at -80°C until further use. An aliquot of

2 µg DNAse-treated RNA was transcribed into cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo-dT primers (Invitrogen).

Real-time quantitative polymerase chain reaction (qPCR) analyses

To obtain qPCR products, the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen) was used. Each qPCR mixture of 25 µL contained 12.5 µL qPCR SuperMix-UDG Kit (Invitrogen), 0.5 µL $MgCl_2$ (50 mM), 0.1 µL each of the forward and reverse primer (at 100 µM) (Beoneer), 10.8 µL nuclease-free water and 1 µL cDNA (20 ng/mL cDNA for all genes). The negative controls (with no DNA template) for each primer set were included in each run to ensure that there was no contamination. For each biological repetition, two reaction tubes were used. The reaction was performed with the iCycler system (Bio-rad). qPCR was carried out after a 1.5-min activation/denaturation step at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. After the PCR amplification, the specificity of primers was checked by melting curve analyses. In all experiments, a melting curve was performed from 72° to 96°C to verify the specificity of amplification, detected by the presence of a unique amplification product. All PCR amplifications were performed in duplicate, and the mean of all these values was used for final analysis.

The reference gene used was the gene *try* (involved in the biosynthesis of tryptophan) (Fang and Bidochka, 2006). Its choice was based on the constitutive expression of the gene, with highly similar values of quantification cycle (Cq) in all cDNA samples analyzed. The tested genes in this study were *cag*8 and *nrr*1 genes, which were investigated during germination, conidiogenesis and insect pathogenesis, in *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum*. The methods used for qPCR quantification were based on previously reported studies; gene *cag*8 (a regulator of G-protein signaling pathway involved in conidiogenesis) (Fang et al., 2007), and gene *nrr*1 (a nitrogen regulator response gene) (Screen et al., 1998). The primers for these genes and the lengths of amplified PCR products are shown in Table 1.

Table 1. Primer sequences for genes studied, the length of the polymerase chain reaction (PCR) products, GenBank accession No. and PCR efficiency.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank accession No.	Length of PCR products (bp)
try	TTGCAATGCATGTTTGATGTC	CAAAGAGTGGTATCGAGTTAC	AY245100	174
*cag*8	AAGCTGATGGCTAGCGATTC	TTGCGGTTGGAACGACTTTG	DQ826044	128
*nrr*1	ACTATTGATGAGCGTCGTAAC	TGCGTCGTTGTCCATGAAG	AJ006468	222

Relative quantification of gene expression

Generation of quantitative data by real-time PCR is based on the number of cycles required for optimal amplification fluorescence to reach a specific threshold of detection (Cq value) (Bustin et al., 2009). The relative expression ratios were calculated by a mathematical model, which included an efficiency correction for real-time PCR efficiency of the individual transcripts (Pfaffl, 2001). Real-time PCR amplification efficiencies (E) were determined for each set of primers with the slope of a linear regression model (Pfaffl, 2001). The cDNA samples were diluted at a range of 50, 25, 5, 1, and 0.25 ng/mL and were used as RT-qPCR

templates. The standard curves were generated by plotting the log cDNA values against Cq values obtained over the range of dilutions. The slope of the curves was used to determine the reaction efficiency (E) as $E = 10^{-1/[slope]}$.

During germination, the average Cq value of conidia collected after 8 h was set as the control treatment and the average Cq value of conidia collected at 10, 12 and 14 h was set as the sample treatment. During conidiogenesis, the average Cq value of mycelia collected over 24 h was set as the control treatment and the average Cq value of mycelia collected at 48, 72 and 96 h was set as the sample treatment. During pathogenesis, the average Cq value of insect 20 h after infection was set as the control treatment, and the average Cq value of insect with emergent mycelia from insect cadavers and insect cadavers mummified with conidia was set as the sample treatment.

After the calculation of the R values from each biological repetition, the relative gene expression levels for *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum* were presented as Log_2 R. In this way, values were directly and numerically correlated with the induction or repression of gene expression, and thus, from the zero level of expression, the genes analyzed could be relatively induced (positive values) or repressed (negative values). Afterwards, the data were subjected to analysis of variance (ANOVA) and means compared by the Tukey test at 5% probability using the Assistat 7.4 software (Silva and Azevedo, 2002).

RESULTS AND DISCUSSION

In this study, information was gathered supplying a better understanding of the role of the genes *cag*8 and *nrr*1, implied by differential expression during the adaptation of these fungi as saprophyte and pathogen.

According to the standard curve obtained from the slope of the dilutions of each gene evaluated, the PCR efficiency (E) varied from 98 to 108% and regression coefficient values (R^2) varied from 0.981 and 0.994. Analysis of the melting curves showed the specificity of the primers, with unique peaks for the amplifications with each primer set (Table 2). These results support the fidelity in representing the expression of target genes under distinct environmental conditions.

Table 2. E and R^2 values of linear regressions for dilutions of the reference and target gene transcripts.

Gene	E value (%)	R^2 value
*cag*8	98%	0.994
*nnr*1	105.4%	0.994
try	108%	0.981

E = efficiency value: R^2 = regression coefficient value.

The gene *cag*8 acts in regulating the G-protein signaling pathways on the conidial surface, and thus it is involved in the interaction with the lipid layer on the surface of the host, promoting the adhesion of conidia on the insect cuticle through hydrophobic mechanisms (Boucias et al., 1988; Hamm, 1998). In *M. anisopliae*, this gene also plays a role in the regulation of conidiation, virulence and hydrophobin synthesis (Fang et al., 2007). Corroborating these findings, we observed the differential expression of this gene in both *M. anisopliae* var.

anisopliae and *M. anisopliae* var. *acridum* during germination, conidiogenesis and pathogenesis. After 10, 12 and 14 h of germination, the expression of the *cag8* gene was differentially repressed when compared with 8 h (ungerminated conidia) in *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum*. In *M.* anisopliae var. *anisopliae* a significant difference was observed only after 14 h, whereas in *M. anisopliae* var. *acridum* the expression was differentially repressed at all times examined (Figure 1).

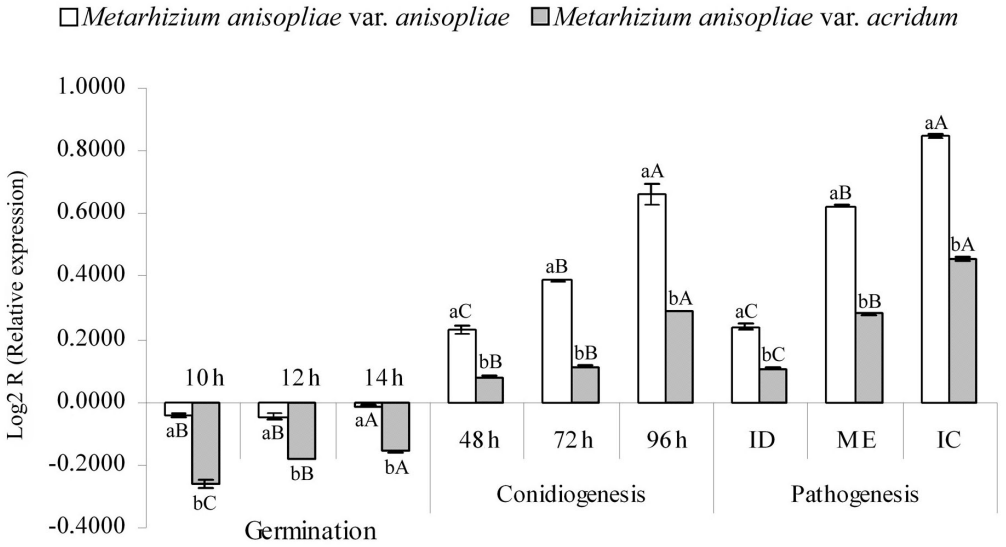


Figure 1. Relative expression levels of *cag*8 during germination, conidiogenesis and pathogenesis of *Metarhizium anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum*. ID = dead infected insect; ME = emergent mycelia from insect cadavers; IC = insect cadavers completely covered with conidia. Means followed by distinct letters differ by the Tukey test at 5% probability; lowercase letters represent differences between isolates at each time of culture, and uppercase letters represent the differences between times for each isolate.

Differences were also found in this gene expression during conidiogenesis. After 48, 72 and 96 h of conidiogenesis, *cag*8 was induced when compared to 24 h (mycelial growth) in both varieties. In *M. anisopliae* var. *anisopliae*, significant differences were observed at all times examined. However, in *M. anisopliae* var. *acridum* a statistical difference was observed only at 96 h. When comparing the two varieties, there were significant differences, with a greater induction in *M. anisopliae* var. *anisopliae* (Figure 1).

During the stages of pathogenesis, both *M. anisopliae* var. *anisopliae* and *M. anisopliae var. acridum* showed significantly different levels of induction when compared to those 20 h after infection, with a similar trend as observed during conidiogenesis. In both varieties, greater induction was observed in insects covered with conidia (conidiogenesis stage) and lower in the dead insect. When comparing the two varieties, *M. anisopliae* var. *anisopliae* showed higher induction (Figure 1).

The repression of this gene during germination and induction in conidiogenesis, as well as in the conidiogenesis stages of pathogenesis, reinforce the association between *cag*8 and conidiogenesis. The conidium is the propagation structure that is involved in disease

transmission (Fang et al., 2007). Thus, an understanding in the level of gene expression involved in conidiogenesis is an important step to the commercial development and improvement of this biocontrol fungus. Here, we observed that *M. anisopliae* var. *anisopliae* showed higher expression of this gene during the process of pathogenesis. Lima (2005) observed that *M. anisopliae* var. *anisopliae* showed higher potential pathogenicity in *D. saccharalis* than *M. anisopliae* var. *acridum*. Fang et al. (2007) analyzed an *M. anisopliae cag8* loss-of function mutant strains and observed a decreased virulence in *Galleria mellonella*. Thus, we suggest that varieties of *M. anisopliae* that have higher expression of this gene may show a higher virulence in accordance with the host.

Another gene analyzed in this study is related to nitrogen processing by the fungi. The fungi have elaborate control mechanisms to provide a constant supply of nitrogen during distinct stages of differentiation, such as during the pathogenesis process. Recent studies have pointed to the fact that even genes encoding regulatory molecules or those that are involved in growth and secretion could also be considered to be pathogenicity related, since they may play important functions during the process of adaptation to the various insect cuticles (Casadevall and Pirofski, 2001; Freimoser et al., 2005; Wang et al., 2005). The nitrogen response regulator gene *nrr*1 has been shown to be important in the pathogenicity of *M. anisopliae* (Screen et al., 1998), since several enzymes involved in the penetration of the insect cuticle are also regulated in response to nitrogen availability (St Leger et al., 1997). We observed that *nrr*1 was constitutively induced at 10, 12 and 14 h of germination when compared to 8 h (ungerminated conidia), at 48, 72 and 96 h of conidiogenesis when compared to 24 h (mycelial growth) and during the stages of pathogenesis when compared to the insect at 20 h after infection. However, no interaction was observed between varieties and time for germination ($F = 4.3607$, $P = 0.0677$), conidiogenesis ($F = 3.3102$, $P > 0.10000$) and pathogenesis ($F = 1.166$, $P > 0.10000$) (Figure 2).

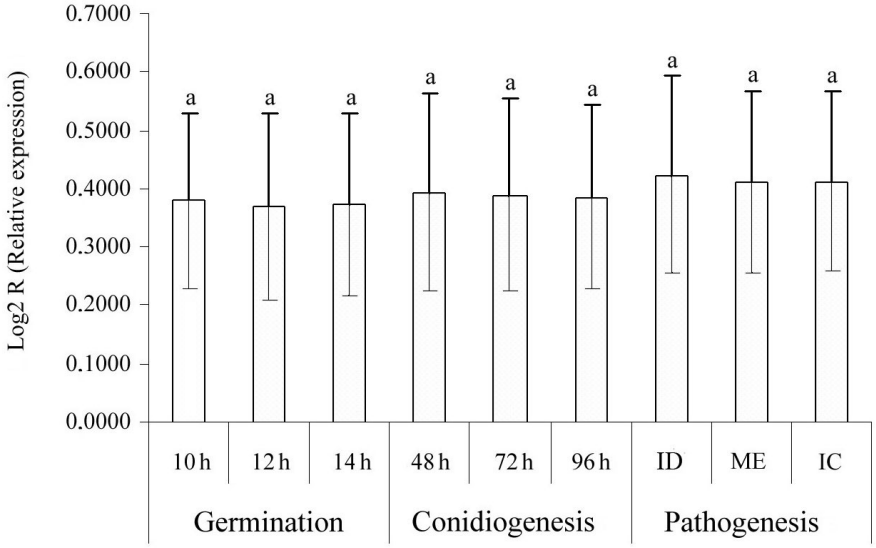
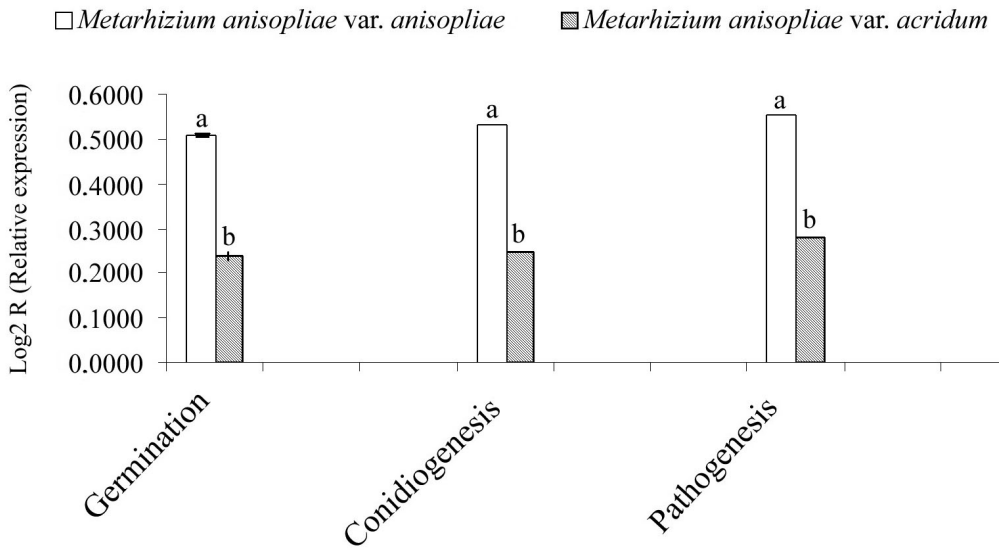


Figure 2. Relative expression levels of *nrr*1 during germination, conidiogenesis and pathogenesis. ID = dead infected insect; ME = emergent mycelia from insect cadavers; IC = insect cadavers completely covered with conidia. Means followed by distinct letters differ by the Tukey test at 5% probability.

Significant differences were observed for the three developmental stages only when analyzing the two varieties, with a higher expression in *M. anisopliae* var. *anisopliae* (Figure 3), which may be related to its high virulence against *D. sacchacalis*, since the synthesis of enzymes involved in the penetration of *M. anisopliae* in arthropods is regulated in response to nitrogen availability. The consistent expression of these genes on YPD broth or agar and in insects suggests that this gene may mediate nitrogen catabolite depression. Fang and Bidocchka (2006) also observed constitutive expression of this gene during germination, spore production and pathogenesis of *M. anisopliae*.



Figue 3. Relative expression levels of *nrr*1 during germination, conidiogenesis and pathogenesis of *Metarhizium anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum* independent of time. Means followed by distinct letters differ by the Tukey test at 5% probability.

M. anisopliae var. *anisopliae* and *M. anisopliae* var. *acridum* are facultative saprophytes with both free-living (saprophytic) and pathogenic life stages. However, *M. anisopliae* var. *anisopliae* is a cosmopolitan pathogen and has been reported from over 200 insect species (Samuels et al., 1989). In contrast, *M. anisopliae var. acridum* is less plastic in its physiological responses and has a very limited host range (Inglis et al., 2001). Thus, a better estimate of the range of *M. anisopliae* pathogenicity determinants may be obtained by studying both subspecies (St Leger et al., 1988, 1992b, 1996; Freimoser et al., 2003). This exhibited plasticity could be attributed not only to the presence of specific genomic regions and genes, but also to the differential gene expression in this fungus, modulating its ability to respond to environmental stimuli. Identifying genes that are up- or down-regulated in the fungus in response to a given host insect or growth condition certainly contributes to the knowledge of the genetic mechanisms involved in host specificity and adaptation (Pathan et al., 2007). Corroborating these findings, our results showed clear differences in the expression of the genes analyzed during germination, conidiogenesis and pathogenesis between *M. anisopliae* var. *anisopliae*

and *M. anisopliae* var. *acridum*, i.e., higher expression of the genes *cag*8 and *nrr*1 in *M. anisopliae* var. *anisopliae*. Additionally, it was also remarkable that these varieties use different strategies to infect diverse groups of insects and adapt to different environments. This knowledge of differential gene expression helps to elucidate the molecular mechanisms associated with the metabolic versatility of *M. anisopliae*, representing an important tool for programs of genetic improvement of strains used in the biological control of pests.

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