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The entomopathogenic fungus *Beauveria bassiana* activate Toll and JAK-STAT pathway controlled effector genes and anti-dengue activity in *Aedes aegypti*

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Abstract

Here we investigated the effect of *Beauveria bassiana* infection on the survival of *A. aegypti* mosquitoes and the modulation of their susceptibility to dengue virus infection. Application of *B. bassiana* caused a reduction in the life span of *A. aegypti* and hindered dengue virus replication in the mosquito midgut. Fungus infection induced the expression a variety anti-microbial and dengue virus restriction factor genes. Transient reverse genetic analyses showed that the JAK-STAT pathway is implicated anti-fungal defense of *Aedes* mosquitoes. Our data suggest that this *B. bassiana*-mediated anti-dengue activity is likely to be at least partly indirectly mediated through the activation of the mosquito's anti-dengue Toll and JAK-STAT pathways.

Keywords

Aedes aegypti; dengue virus; *Beauveria bassiana*; innate immunity

1. Introduction

Dengue fever has become the most important arboviral disease worldwide, with a dramatic increase in its incidence and negative impact on public health in recent decades (Gibbons and Vaughn, 2002; WHO, 2011). The World Health Organization has estimated that about half of the world's population is at daily risk of contracting dengue and that some 50 million cases occur every year worldwide (Mackenzie *et al.*, 2004; WHO, 2011).

Dengue is a positive single-stranded RNA virus that is primarily transmitted through the *A. aegypti* blood feeding, but *A. albopictus* also plays a relevant role in dengue transmission, especially in areas where *A. aegypti* is not present (Teyssou, 2009). After the mosquito has ingested an infected blood, the dengue virus invades the mosquito's midgut epithelium, where it undergoes replication and is then disseminated to other tissues such as the trachea, fat body, and finally the salivary glands. This whole process usually takes approximately 7-10 days, although in a minority of cases dengue virus can be detected in the mosquito's

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salivary glands as early as 4 days post-infection. Once in the salivary glands, the virus can be transmitted during probing and blood feeding for the rest of the mosquito's adult life (Salazar *et al.*, 2007).

The lack of an effective vaccine and the mosquito's resistance to insecticides has accentuated the need for the development of novel control strategies that can prevent dengue virus transmission by the mosquito vectors (Gubler, 2005; Guzman, 2005; Kay and Vu, 2005; Mackenzie *et al.*, 2004; Ritchie *et al.*, 2004; WHO, 2011). The entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, have gained interest as candidate bio-control agents of mosquitoes vectors mainly because of their ability to shorten the mosquitoes life span or reduce instantaneous blood feeding of mosquitoes (Blanford *et al.*, 2005; Howard *et al.*, 2010b; Knols *et al.*, 2010; Knols and Thomas, 2006; Michalakis and Renaud, 2005; Scholte *et al.*, 2004; Scholte *et al.*, 2006; Scholte *et al.*, 2005; Scholte *et al.*, 2003; Thomas and Read, 2007), and more importantly that pyrethroid resistance *A. gambiae* mosquitoes have increased susceptibility to these entomopathogenic fungi (Howard *et al.*, 2010a). *B. bassiana* is a soil-borne fungus that infects soil-dwelling insects, including both *Anopheles* and *Aedes* mosquitoes (Boucias DG, 1998). While *B. bassiana* is not as virulent to *Aedes* larvae as to anopheline larvae, the dried conidia are highly virulent to *A. aegypti* adult mosquitoes, which kill mosquitoes in only 5 days which suggests that application of conidia to adult *Aedes* mosquitoes is more effective (Clark *et al.*, 1968;). Laboratory bioassays of two development stages of *B. bassiana* have shown that blastospores (BS) have significant higher larvicidal potency than conidiospores (CS) (Miranpuri and Khachatourians, 1990). A recent publication has shown that exposure of *A. aegypti* to a filter paper impregnated with conidia is highly effective in infecting and killing mosquitoes, and the fungus can be transmitted between mosquitoes through mating (Garcia-Munguia *et al.*, 2011).

Similar to other insects, mosquitoes use their innate immune system to fight pathogenic organisms and the three immune signaling cascades, Toll, Immune Deficiency (IMD), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways play key roles in these defenses (Cherry and Silverman, 2006; Dimopoulos, 2003; Dostert *et al.*, 2005; Garver *et al.*, 2009; Hoffmann and Reichhart, 2002; Pinheiro and Ellar, 2006; Souza-Neto *et al.*, 2009; Xi *et al.*, 2008). The Toll pathway is more specific for Gram-positive bacteria, fungi, and viruses, whereas the Imd pathway is mainly active against Gram-negative bacteria and *Plasmodium falciparum* (Cirimotich *et al.*, 2010; Shin *et al.*, 2006; Shin *et al.*, 2005). The lesser studied JAK-STAT pathway has been implicated in anti-viral defenses (Souza-Neto *et al.*, 2009). The Toll pathway has been shown to be activated in response to fungal infection, resulting in the transcriptional activation of several anti-microbial peptides and other immune genes (i.e. Cecropin A, Defensin A, Serpin 27A) (Shin *et al.*, 2005). We have previously shown that the Toll pathway is also activated in response to dengue virus infection in *A. aegypti* and that such activation, in turn, controls viral proliferation in the mosquito midgut (Xi *et al.*, 2008). Another key player in the *A. aegypti* anti-dengue defense is the JAK-STAT pathway, presumably through one or several STAT-regulated dengue virus restriction factors (DVRFs) (Souza-Neto *et al.*, 2009). Here we show that *B. bassiana* infection shortens the mosquito's life span and induces the Toll and JAK-STAT pathways which are known to exert anti-dengue defense. We establish the optimal dosage and route of infection of *B. bassiana* spores for reducing the life span of *Aedes* mosquitoes and the vector competence for dengue virus.

2. Material and methods

2.1. Mosquito rearing

Aedes aegypti mosquitoes of the Rockefeller strain were maintained in an insectary at 27°C and 80% humidity on a 12-h light/dark cycle according to standard rearing procedures. Larval stages were fed on pulverized fish food, and adults were provided with a 10% sucrose solution.

2.2. Culture of *B. bassiana*

Beauveria bassiana strain 80.2 (kindly provided by Dr. Neal Silverman, University of Massachusetts Medical School) was used for all assays (Lemaitre *et al.*, 1997). Prior to use, a glycerol stock of conidia stored at −80°C was inoculated on malt-agar medium and allowed to grow at room temperature for 2 weeks. Spores and hyphae were harvested according to standard procedures (Broekaert *et al.*, 1990) by using a sterile spatula and resuspended in PBS containing 0.05% Tween 20. The concentration of conidia was measured using a hemocytometer and adjusted to the following concentrations at 2.3×10^8 , 10^8 , 10^7 , 10^5 , and 10^3 conidia/ml.

2.3. Infection of mosquitoes with *B. bassiana* and survival assays

Different concentrations of conidia were applied to *A. aegypti* mosquitoes either through filter papers or by dipping mosquitoes in the conidia suspensions. For each trial of a bioassay with filter paper, 4 ml of diluted conidia was pipetted evenly onto a piece of filter paper (173 cm²) attached to the side of an autoclaved 8-oz cardboard cup. This fungus-containing cup was allowed to dry at room temperature for 10 min while the filter paper is still damp, after which the mosquitoes were placed inside the cups. For the dipping method, mosquitoes were cold-anesthetized and briefly dipped into the appropriate conidia suspension for 1 min, and then transferred to 8-oz cardboard paper cups. For both methods, at least three biological replicates (of 40 2-day-old females each) were used; control mosquitoes were treated with a PBS/0.05% Tween 20 solution (referred as PBS thereafter). The survival of *B. bassiana*-infected and control mosquitoes was monitored daily for up to 30 days. After this period, the LT₅₀ for each group was determined (Clements and Paterson, 1981). The mosquito survival rate was analyzed by Kaplan–Meier pairwise comparison (Everitt, 1994).

2.4. Propagation of DENV-2 and infection assays

The *A. albopictus* cell line C6/36 was grown in minimal essential medium (MEM) with 10% heat-inactivated FBS, 1% L-glutamine, and 1% non-essential amino acids in a Thermo tissue culture incubator at 32°C supplied with 5% CO₂. The propagation of the New Guinea C strain of DENV-2 in C6/36 cells essentially followed the methods described in (Troyer *et al.*, 2001; Xi *et al.*, 2008). In brief, 80% confluent C6/36 cells were infected with the virus in a 75-cm² flask, at a multiplicity of infection (MOI) of 3.5 virus particles per cell. Infected cells were incubated for 5 to 7 days, harvested with a cell scraper, and lysed by repeated freezing and thawing in dry CO₂ and a 32°C water bath to release the viral particles. The virus suspension was then mixed with an equal amount of commercial human blood and 10% human serum and maintained at 37°C for 30 min prior to feeding. Two days before the blood feeding, 2-day-old female mosquitoes were treated with fungus at 2.3×10^8 conidia/ml as described above (both filter paper and dipping methods). All infections with DENV-2 and manipulation of DENV-2-infected mosquitoes were carried out in a Biological Safety Level-3 insectary.

2.5. DENV-2 virus titration

At 7 days after blood feeding, mosquitoes were briefly washed in 70% ethanol and then rinsed in sterile PBS five times. The midgut was dissected in sterile PBS, and a pool of five midguts was homogenized in 150 ml of MEM using a Kontes pellet pestle motor in a sterile environment, and five data points were collected for each replicate. Virus titers in the tissue homogenates were measured as previously described (<http://www.jove.com/index/Details.stp?ID=220>). The virus-containing homogenates were serially diluted, inoculated into 80% confluent C6/36 cells in 24-well plates and kept for 5-7 days at 32°C and 5% CO₂. Plaque-forming units (PFU) were visualized by peroxidase immunostaining, using mouse hyperimmune ascitic fluid (MHIAF) specific for DENV-2 and a goat anti-mouse HRP conjugate as the primary and secondary antibodies, respectively. All procedures involving DENV-2 infections were carried out in a Biological Safety Level 2 laboratory.

2.6. RNA sample preparation, semi quantitative RT-PCR and real-time RT-PCR (qRT-PCR)

At various time points (2, 5, 7, 9 days) after exposure to *B. bassiana* spores (via the filter paper and dipping method) at 10⁸/ml or PBS (control), six mosquitoes from each replicate were collected and homogenized in lysis buffer. RNA was extracted using the RNeasy kit (Qiagen). Reverse transcription was carried out at 42°C for 2 h using a Superscript II kit (Invitrogen) and 20-μL reaction mixtures containing oligo (dT) primers and 2 μg of total RNA. The PCR cycles were controlled to produce a non-saturated amount of PCR products, indicating linear amplification. For the semi-quantitative RT-PCR optimal cycle numbers were empirically determined from a test PCR run, and three replicates were obtained for each gene. The relative -fold induction or repression of gene expression in the experimental samples was determined by comparing these values to their respective controls after normalizing the transcript levels with the *A. aegypti* ribosomal S7 gene (Dong *et al.*, 2006). The qRT-PCR assays were performed according to standard protocol (Dong *et al.*, 2006) by using Sybr Green PCR Master mix (Applied Biosystems) and ABI StepOne real-time PCR system. The primers used for the RT-PCR were: attacin (AAEL003389) ATT-F: 5'-TTGGCAGGCACGGAATGCTTG-3', ATT-R: 5'-TGTTGTCGGGACCGGGAAGTG-3'; cecropin G (AAEL015515) CECG-F: 5'-TCACAAAGTTATTTCTCCTGATCG-3', CECG-R: 5'-GCTTTAGCCCCAGCTACAAC-3'; defensin A (AAEL003841) DEFA-F: 5'-CTGCCGAGGAAACCTATCAG-3', DEFA-R: 5'-GCAATGCAATGAGCAGCACAAG-3'; defensin C (AAEL003832), DEFC-F: 5'-TTGTTTGCTTCGTTGCTCTTT-3', DEFC-R: 5'-ATCTCCTACACCGAACCCACT 3'; dipterocin (AAEL004833) DIP-F: 5'-ATCCGATTCAGAATTCGCTTT-3', DIP-R: 5'-TTTACCGTCTCCCTGAAATCC-3'; ribosomal protein S7 (AAEL009496) S7-F: 5'-GGGACAAATCGGCCAGGCTATC-3', S7-R: 5'-TCGTGGACGCTTCTGCTTGTTG-3'. Primers for DOME, PIAS, DVRF1, and DVRF2 qRT-PCR were from (Souza-Neto *et al.*, 2009). All PCR reactions were performed in triplicate; to check for the specificity of the PCR reactions, melting curves were analyzed for each data point. The fold-change of the gene expression levels were determined by normalizing cDNAs using the AeS7 ribosomal gene and comparing to controls without *B. bassiana* challenge. P-values were calculated using either a one-way ANOVA as described in (Lajoie *et al.*, 2010) to determine the significance of the gene expression among different time points, or t-test to determine the significance of a single time point as described in (Garver *et al.*, 2009).

2.7 RNAi-mediated gene silencing and survival assays

RNAi assays were carried out according to previously established methodology with modifications (Blandin *et al.*, 2002; Dong *et al.*, 2006). Briefly, sense and antisense RNAs were synthesized from ~300 bp PCR-amplified fragments using the HiScribe T7 *In Vitro* Transcription kit (New England Biolabs) by using the same primers as listed in (Souza-Neto

et al., 2009). About 69 nl dsRNA in DEPC-water was introduced into the thorax of cold-anesthetized 3-5 days old female mosquitoes nano-microinjector (Drummond) with glass capillary needles. Both dsGFP- (control) and gene specific dsRNA-injected (experimental) groups were kept in the regular insectary conditions as described above. The dsRNA injection assay of different genes was repeated at least three times with at least 35 mosquitoes in each experiment, and the GFP dsRNA-injected mosquitoes served as controls. Verification of gene silencing was done through qRT-PCR by using the same verification primers as listed in (Souza-Neto *et al.*, 2009).

Two days post dsRNA injection, the conidia suspension (10^{10} conidia/ml) was applied to *A. aegypti* mosquitoes through a filter paper method or by rolling the mosquitoes in the petri dish with fully grown fungi as described earlier (Lemaitre *et al.*, 1997). The mortality of the mosquitoes was recorded up to 10 days, and at least 3 biological replicates were included in each assay. The significance of gene silencing on the mortality of mosquitoes to *B. bassiana* infection, compared with dsGFP-treated controls, was determined by Kaplan-Meier survival analysis by GraphPad Prism 5.0 and p-values were calculated through Wilcoxon test (Dong *et al.*, 2006).

3. Results and discussion

3.1. Survival of *A. aegypti* mosquitoes after fungal infection

Previous studies have shown that the application of the entomopathogenic fungus *M. anisopliae* to female *A. aegypti* mosquitoes at 10^{10} conidia/m² results in the killing of half the treated mosquitoes by 4 days after fungal infection (Scholte *et al.*, 2007). The main objective of this study was to investigate the use of a fungus-infection as a way to induce mosquito resistance to the dengue virus in addition to shortening its life span. To avoid extensive mosquito mortality in our co-infection assays with *B. bassiana* and the dengue virus, we first performed experiments to determine the optimal dosage of the fungus by treating the mosquitoes with five different concentrations of *B. bassiana*. Mosquitoes were exposed to the fungus via two routes: (i) continuous exposure to immobilized fungus on a filter paper and (ii) one-time exposure by a single dip into a conidial fungus solution. After challenge, we monitored daily mosquito survival for up to 30 days (Fig. 1). For both filter paper and dipping methods, all the *B. bassiana*-treated mosquitoes died significantly earlier than the PBS-treated control mosquitoes (see statistical analysis in Table S1), except of those exposed to 10^3 conidia/ml *B. bassiana* through the dipping method, and the half-survival rate (LT₅₀) was significantly lower for the *B. bassiana*-treated mosquitoes compared to the controls (Table S1), regardless of fungal dosage. However, the mosquitoes exposed to fungal spores through the dipping method died at a slower rate compared to those exposed through the filter paper method when treated with a lower dosage of the *B. bassiana* conidia solution (from 10^3 to 10^5 conidia/ml) (Figs. 1A and 1B, Table S1), suggesting that a one-time exposure of fungi spores requires longer time for developing pathogenicity to mosquitoes. However, when mosquitoes were treated at higher concentrations of *B. bassiana* spores (from 10^7 to 2.3×10^8 conidia/ml), the mortality after fungus exposure through the two different methods were similar but the mortality of mosquitoes through dipping methods had a large variation between different biological replicates (Fig. 1B). This is most likely because the one-time treatment does not result in a homogenous infection with the fungal spores. In order to confirm that the mosquitoes were dying from fungal infection, dead mosquitoes were transferred to a Petri dish containing a distilled water-moisturized piece of filter paper, which revealed the growth of vigorous *B. bassiana* hyphae at 24 h and sporulation at 3 days after exposure (Fig S1).

Our data show that regardless of the application method of fungal spores, infection with *B. bassiana* results in a significant increase in mosquito mortality, suggesting that this fungus

could be a suitable candidate for use as a bio-control agent for dengue vectors. Our results are similar to those of previous laboratory and field studies that used *B. bassiana* and *M. anisopliae* to shorten the lifespan of the African malaria vector *A. gambiae* (Blanford *et al.*, 2005; Scholte *et al.*, 2005). In sum, we show that a prolonged exposure to immobilized fungus on a filter paper is more effective than a one-time exposure method in infecting and killing the *Aedes* mosquito.

3.2. *B. bassiana* infection renders mosquitoes more resistant to dengue virus infection

Blanford and colleagues (Blanford *et al.*, 2005) have shown that treatment of anopheline mosquitoes with *B. bassiana* not only reduces the lifespan of *Plasmodium*-infected mosquitoes but also dramatically decreases the prevalence of mosquitoes carrying sporozoites in the salivary glands, suggesting that the fungal infection is directly or indirectly (possibly through the activation of the mosquito's immune system) inhibit *Plasmodium* development in the mosquito. In order to investigate the hypothesis that *B. bassiana* can have a deleterious effect on dengue virus replication in *A. aegypti*, we treated mosquitoes with the fungus (1×10^8 conidia/ml) and fed them 2 days later on a DENV2-containing blood meal. Similarly to the survival assays, mosquitoes were exposed to *B. bassiana* conidial solution through the two routes. Seven days after the DENV-2 infected blood meal, the midguts were removed, and the viral load was measured by plaque assays and compared to that of non-fungus-exposed PBS-treated control mosquitoes (Fig. 2). Dengue infection was significantly reduced by a 2-fold ($p < 0.01$) in the midguts of mosquitoes continuously exposed to *B. bassiana* using the filter paper method (Fig. 2). The viral load was also approximately a 2-fold lower in the mosquitoes dipped once into the conidial solution, but this effect was variable between different individual mosquitoes and was therefore statistically insignificant ($p = 0.22$) (Fig. 2), indicating that a prolonged exposure to the fungus is necessary to achieve an efficient suppression of viral infection. In order to determine whether the fungus could directly inhibit dengue virus replication, we conducted an *in vitro* study in which the virus was incubated for 4 h with *B. bassiana* spores. No significant influence of the fungus on virus viability was observed (data not shown), suggesting that the effect of *B. bassiana* on dengue virus infection is indirect and likely to be at least partly mediated by the activation of the mosquito's immune response to fungus infection.

3.3. *B. bassiana* infection induces key factors and downstream effectors of the toll and JAK-STAT pathways

In response to fungal infection, the Toll pathway is activated in both *Aedes* and *Drosophila*, and this immune pathway regulates the transcription of a number of antimicrobial peptide and other immune genes (Lemaitre *et al.*, 1996; Xi *et al.*, 2008). In *A. aegypti*, the Toll pathway is also stimulated in response to dengue virus infection, and the transient activation of this pathway prior to virus infection increases the mosquito's resistance to the virus (Visintin *et al.*, 2010; Xi *et al.*, 2008). The prominent activation of the *A. aegypti* Toll pathway in response to fungal, bacterial, and dengue virus infections suggests that this pathway controls multiple immune-related functions and is therefore able to defend against diverse pathogens. We therefore hypothesized that the activation of the Toll pathway during mosquito infection with *B. bassiana* could be at least partly responsible for the inhibition of dengue virus proliferation in the mosquito midgut.

To assess the Toll pathway activation after the exposure of mosquitoes to *B. bassiana*, we performed preliminary experiments by using semi-quantitative PCR to monitor the transcript abundance of the Toll pathway marker genes *attacin*, *defensin*, and *cecropin*, and other antimicrobial peptide genes (*dipteracin* and *lysozyme C*) after fungal infection. At 48 h after exposure to *B. bassiana* through the filter paper method, the expression of these genes

(*attacin*, *cecropin G*, *defensin A* and *C*, *dipterecin*) was significantly up-regulated except *lysozyme C*. *Cecropin* and *Defensin* displayed the strongest induction (Fig. S2) which agrees with previous published data where *cecA* and *defA* were strongly induced upon *B. bassiana* challenge (Shin *et al.*, 2005).

Given that a one-time exposure of mosquitoes to the fungus yielded a lesser suppression of dengue viral loads compared to the prolonged exposure to the fungus, suggests that the latter type of exposure might induce a stronger anti-dengue immune response. In order to compare the changes of immune gene transcript abundance after two different modes of exposure to the fungus temporally and quantitatively, we also conducted real-time qRT-PCR-based analyses. As shown in Fig. 3, all assayed genes showed a stronger induction when mosquitoes were exposed to fungus through the filter paper method, especially *Rel1*, the transcription factor of the Toll pathway. *Rel1* was induced as soon as 2 days after fungal exposure through the filter paper method, while the one-time exposure method resulted in *Rel1* induction at 7 days post fungal challenge. Marked increases in transcript abundance of immune genes were observed at 5 to 9 days post exposure to fungal challenge, which is also the time when the dengue virus reaches the highest titres (from 3 to 7 days post dengue infected blood feeding). This result provides confirmatory evidence that *B. bassiana* infection activates the Toll pathway in mosquitoes, corroborating the hypothesis that fungus infection mediates resistance to the virus, at least partly, by the activation of an immune response. The prolonged exposure to fungus induced the Toll pathway -mediated immune responses stronger and earlier, which might have contributed to the observed stronger anti-dengue effect. These results show a synergy between anti-fungal and anti-dengue defenses, as previously suggested (Xi *et al.*, 2008). We can however not exclude the possibility that fungal infection also induces some other physiological changes that restricts dengue virus infection.

To investigate whether the JAK-STAT pathway-controlled anti-dengue immune genes are responsive to fungal infection, we used qRT-PCR to assess changes in gene transcript abundance of *Dome* and *DVRFs* at different time points (day 2, 5, 7, 9) post exposure to a *B. bassiana* conidial solution. Our previous study showed that the transcription factor of JAK-STAT (*Dome*) and STAT-regulated anti-dengue restriction factors (*DVRF-1* and *-2*) were strongly induced upon dengue infection (Souza-Neto *et al.*, 2009). As shown in Fig. 3, both *Dome* and *DVRF1* transcript abundance significantly increased at 5 and 7 days post exposure to *B. bassiana* through the filter paper method. *DVRF1* was only induced at 9 days post exposure to fungus, which might explain part of the fungal-mediated anti-dengue effect when application of fungus occurred through the dipping method. *DVRF2* also showed a trend to be induced after fungal exposure through the filter paper method (data not shown, due to the lesser number of replicates). Future experiments will be necessary to elucidate the molecular mechanisms involved in the interplay of fungus induced infection responses and antiviral activity in a greater detail.

3.4. The JAK-STAT pathway exerts an anti-fungal defense

Our gene expression analyses showed that *Dome* and *DVRF1* are induced upon *B. bassiana* challenge at 5 and 7 days post infection, suggesting that the JAK-STAT pathway may play a role in the mosquito's anti-fungal defense. To address this hypothesis we monitored the susceptibility of *Dome*, *PIAS* and *DVRF1* gene silenced mosquitoes to fungal challenge. After 2 to 3 days recovery following dsRNA-injection, the treated mosquitoes were exposed to a *B. bassiana* conidial solution of 10^{10} conidia/ml to assure that the mortality of mosquitoes would take place within the time-window when the dsRNA-mediated gene silencing is still efficient, since the *B. bassiana* strain used here is the least virulent strain to mosquitoes (half of the mosquitoes were killed at around 16 days after exposure to a 10^8 conidia/ml dosage). The *Dome* dsRNA-treated mosquitoes (65% silencing efficiency) were

significantly more sensitive to the fungal infection compared to the *GFP* dsRNA injected mosquitoes, and the median LT_{50} was around 6 days, which is significantly lower than that of the control groups. At the end of the survival monitoring time (10 days) at least 70% of ds*GFP*-injected control mosquitoes had survived ($p < 0.001$) and the LT_{50} could therefore not be determined (Fig. 4, Table S1). DVRF1 depletion upon fungus challenge resulted in a trend of higher mortality compared to the control group, but this effect was only significant in 2 replicates, (Table S2). In accordance to our previous study, Dome and DVRF1 gene silencing resulted in a 3-fold increase of virus loads at 7 days post dengue virus infected blood meal, suggesting a relationship between anti-fungal and anti-dengue immune defences and pathways. Interestingly, gene silencing of *PIAS*, the negative regulator of JAK-STAT pathway, did not influence survival of mosquitoes upon fungus challenge, while we have previously shown that it renders mosquitoes more resistant to dengue infection. This may indicate some as yet unknown complexity of this pathway or that the pathway can't be further boosted to cope with a fungal infection at these conditions. The timing of infection assays and gene silencing are also different between the two experiments with fungus and dengue infection.

The increased susceptibility to fungal infection of Dome and DVRF1 depleted mosquitoes indicate a role of the JAK-STAT pathway anti-fungal immune defense, and that fungus infected mosquitoes may exert their anti-dengue activity partly through STAT-regulated effector molecules including the DVRF1. Our study points at a synergy between anti-fungal and anti-dengue defenses through the Toll and JAK-STAT pathways.

4. Conclusions

We demonstrate that *B. bassiana* can significantly shorten the life span of *A. aegypti* mosquitoes at infection with as low as 10^3 conidia/ml. Furthermore, we have for the first time we have shown that fungal treatment can significantly suppress the dengue virus in the mosquitoes' midgut, at least partly through the immune activation of the Toll and JAK-STAT pathway-controlled effector genes. We also show for the first time that the JAK-STAT pathway plays a role in the *Aedes* mosquito's anti-fungal defense. The fact that both these events can influence the dynamics of dengue transmission renders *B. bassiana* a potentially promising agent for the development of dengue control strategies. *B. bassiana* has previously been used for the bio-control of agricultural pests, is commercially available, and is harmless to humans (reviewed in Kanzok and Jacobs-Lorena, 2006). A field study carried out in Tanzania suggests that application of *M. anisopliae*-impregnated black cotton sheets to mosquito's resting places could lead to a reduction of 75% in malaria transmission (Scholte *et al.*, 2005). Considering that *A. aegypti*, like *A. gambiae*, is highly anthropophilic and endophilic (Harrington *et al.*, 2001), the use of entomopathogenic fungus to infect field populations of *A. aegypti* appears to be a promising approach for reducing dengue transmission and therefore deserves further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Survival curves of 2-day-old female mosquitoes treated with different concentrations of *B. bassiana* conidia through the filter paper method (A) or dipping method (B). Three independent experiments were performed and all cohorts were monitored daily for survival until day 30th. The survival percentage that represents the mean survival percentage for all three biological replicates of 40 mosquitoes each, along with the standard errors of means shown. Statistical significance was determined by Kaplan-Meier survival analysis with GraphPad Prism5 software, and *p*-values were determined by Wilcoxon test (the detailed information is listed in Table 1).

Fig. 2.

Effect of *B. bassiana* infection on DENV-2 replication in *A. aegypti* midguts. Dengue titers in the midguts of *B. bassiana*-treated mosquitoes were determined by plaque assays in C6/36 cells at 7 days post-feeding on DENV-2 infected blood. Mosquitoes were treated with *B. bassiana* 2 days before dengue infection by the filter paper method (left panel) or dipping method (right panel). Data for control mosquitoes (PBS) and those treated with *B. bassiana* at 10^8 conidia/ml (B.b.) are shown. At least three biological replicates are included, and each point indicate the absolute number of DENV-2 PFU in an individual midgut, and the horizontal lines indicate the median values. The statistical significance of the effects of fungal treatment on dengue replication was determined by Mann-Whitney test.

Fig. 3.

Temporal expression analysis of Toll and JAK-STAT pathway marker genes after exposure of *A. aegypti* to *B. bassiana* through the filter paper method (A) or dipping method (B). Bars indicate fold change (Log₂ transformed) in expression of the assayed genes at 2, 5, 7, 9 days after *B. bassiana* infection, compared to PBS-treated control mosquitoes. Each bar indicates the mean value from at least 3 replicates, and the error bar indicates standard error among three biological replicates. A ribosomal RNA gene S7 was used as internal control. P-values (<0.05, <0.01, <0.001, <0.0001) calculated from one-way ANOVA analysis are indicated at the bottom of the bars. The p-values from pair wise comparisons (compared to the PBS-treated controls) were calculated through a student's *t*-test and indicated on each bar (a: *p*<0.05; b: *p*<0.01; c: *p*<0.001; d: *p*<0.0001). Dome: *Domeless*; DVRF1: dengue virus restriction factor #1 (AAEL008492); CecG: *Cecropin G*; DefA: *Defensin A*; DefC: *Defensin C*; Dipt: *Diptericin*.

Fig. 4. The JAK-STAT pathway modulates *Aedes* mosquitoes' susceptibility to *B. bassiana* infection. Survival rates of *PIAS* dsRNA-, *DOME* dsRNA-, *DVRF1* (AAEL008492) dsRNA-, and *GFP* dsRNA-injected mosquitoes upon *B. bassiana* infection. The significance of gene silencing on the mortality of mosquitoes upon *B. bassiana* infection, compared to *GFP* dsRNA-treated controls, was determined by Kaplan-Meier survival analysis and p-values are listed in Table S2. The mean survival percentage for all three biological replicates along with the standard errors are shown.