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Using Transgenic Entomopathogenic Fungi to Prevent Dengue Fever: Current Status, Challenges and Perspectives

Etienne M. Bilgo[1,2*], Houeffa Adeline Tatiana Dokpomiwa[1,2,3], Abdoulaye Diabate[1,2]

[1]Biomedical and Public Health Department, Institute of Research in Health Sciences (IRSS), Western Regional Directorate, Bobo-Dioulasso, Burkina Faso
[2]Muraz Center, National Institute of Public Health (INSP), Bobo Dioulasso, Burkina Faso
[3]Rural Development Institute (IDR), Nazi Boni University (UNB), Bobo Dioulasso, Burkina Faso
Email: *bilgo02@yahoo.fr

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Abstract

Over the last few decades, dengue fever epidemics have increased in frequency and intensity worldwide, making it a major global concern for public health. Its prevention, which is essentially vector-based control, is already being compromised by reports of resistance of the main vector *Aedes aegypti* to insecticides. To tackle the rapid increase in insecticide resistance and outbreaks, the biological vector control is a promising approach. One of the strategies of this approach is the use of entomopathogenic fungi because of their great efficacy and their eco-friendly aspects. However, some aspects of their use, such as the low efficiency, the high cost of production and the sensitivity to various adverse conditions, need to be addressed for their successful large-scale application. Therefore, innovative technologies based on strains of transgenic fungi with improved biocontrol potentials by genetic engineering are actively pursued. Although these modified mycoinsecticides are acclaimed for their better effectiveness against target insects, the main concern remains their potential adverse effects on the environment and human health. The present review is dedicated to giving an update on recent developments in transgenic entomopathogenic fungi (TEF) for *Aedes* mosquito control. Future perspectives are also proposed to address the safety concerns related to the release of transgenic entomopathogenic fungi into the environment.

Keywords

Transgenic, Entomopathogenic, Fungi, Dengue, Control

1. Introduction

The global incidence of dengue has been steadily increasing over the past decades, resulting in public health problems and a heavy economic burden. The world's population is therefore witnessing periodic epidemics in endemic areas and an emergence in previously unexposed areas [1]. The distribution of dengue fever across the globe is largely dependent on the distribution of susceptible vector species, which varies with climate change [2]. In fact, relative humidity and temperature are important factors that can influence the transmission of dengue. Global warming provides optimal conditions for the multiplication of dengue vectors, thus increasing the vector density [1] [3] [4]. Other important factors in the resurgence of dengue cases are social order, characterized by rapid urbanization, human travel and commercial exchanges across the world. These international movements would favour the importation of dengue vectors, notably *Aedes aegypti* and *Aedes albopictus*, to areas where the latter were previously absent raising the need for vector control. Among the preventive measures for dengue vector control, environmental, mechanical, biological, chemical and genetic interventions are available to eradicate *Aedes* mosquitoes or limit their reproductive capacity [5]. Depending on the effectiveness, ecological impact and sustainability some of these control methods seem to be promising. Whereas chemical interventions are limited by the increasing development of resistance to insecticides, genetic modifications of mosquitoes raise ecological concerns about the potential long-term health and environmental risks. As no single approach is likely to be sufficient and/or always affordable, there is an urgent need to develop new vector control strategies. This has led to great interest in the use of wild or genetically modified entomopathogenic microorganisms to control dengue transmission. Biological vector control for *Aedes* mosquitoes based on microorganisms offers targeted, eco-friendly solutions to reduce mosquito populations. Techniques like the use of Wolbachia bacteria [6] and entomopathogenic fungi [7]-[9] limit mosquito reproduction, and disease transmission. This approach minimizes pesticide reliance, reducing environmental impact and resistance development, while promoting sustainable public health interventions.

Fungi are probably the most common pathogens of mosquitoes in nature, and Metarhizium spp. has a variety of properties that are useful in vector control. They have a wide host spectrum, infecting various insect species, while developing enzymatic systems to invade them and escape the host's immune response [7] [10]. Depending on their genomic composition and their ability to infect different hosts efficiently or not, a distinction is made between specialists and generalists. *Metarhizium* strains called specialists (very narrow host range) have lower physiological adaptability than generalists (wide host range) and need specific physical and chemical characteristics of the cuticle of their host to efficiently induce the fungal infection [11]. Natural strains of *Metarhizium* cannot be used as standalone mosquito control agents due to their slow kill rate (typically one to two weeks to achieve more than 90% of mortality) and unreliability, the latter largely due to their sensitivity to abiotic stress [10]. Consequently, unmodified *Metarhizium spp.* do not meet WHO Pesticide Evaluation Scheme (WHOPES) requirements for a vector control product [12]. This is reflected in the low presence of *Metarhizium* products for vector control on the market. It seems then judicious to find a way to circumvent the limits of natural strains of *Metarhizium* in order to exploit their great potential in vector control. Fortunately, rapid advances in genomics have revolutionized our understanding of entomopathogenic fungi with the sequencing of many species of *Metarhizium* [10] [11]. Moreover, these recent advances have also facilitated the identification of genes involved in fungal pathogenicity and physiology as well as genes encoding biologically useful molecules with implications for the development of genetically modified fungi and the use of these fungi as novel mycoinsecticides for disease vector control [10] [13].

Here, we review the scientific evidence supporting the use of transgenic fungi to control the transmission of dengue fever and discuss the related risks, challenges and perspectives

2. Rising Cases of Dengue Fever, a Current Challenge of Vector Control Strategies

Dengue fever is a vector-borne viral disease and currently, one of the major arbovirosis in the world. It represents an emerging or re-emerging infectious disease depending on the region, as a consequence of demographic changes, rapid large-scale urbanization, exponential changes in transportation and environmental change [2]. It is expanding rapidly and its geographic distribution worldwide overlaps with that of its main vector, *Aedes aegypti*. The actual number of dengue cases is underreported because the majority of cases are asymptomatic or mild and self-managed. However, the number of dengue cases reported to WHO has increased more than eightfold over the past two decades, from 505,430 cases in 2000 to more than 2.4 million cases in 2010 and 4.2 million cases in 2019. The number of reported deaths from the disease between 2000 and 2015 increased from 960 to 4,032 [14]. More than 3.9 billion people, nearly half of the world's population, are now at risk of contracting the disease. The condition is now endemic in approximately 129 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the Western Pacific [14]. To date, there is no specific treatment for dengue, however one dengue vaccine Dengvaxia® developed by Sanofi Pasteur has been licensed, and additional dengue vaccine candidates are in clinical development [15]. The main concern with this vaccine is the requirement of a primary infection before it can be safely administered.

As the transmission of the dengue virus to humans is based on a bite of infected female mosquitoes, vector control has been the key strategy to limit or prevent transmission of dengue virus. Indeed, *Aedes aegypti* is the main vector of dengue, and occasionally *Aedes albopictus*, many vector control strategies have been developed based on the use of environmental management and/or chemical control. Environmental management consists of changing the environment to make it unfavourable for mosquito development, while chemical control relies on the use of chemical insecticides to control mosquito populations. Chemical control, although still used today, is increasingly threatened by the emergence of insecticide resistance [16]. Several studies conducted in West Africa have revealed the resistance of *Aedes aegypti* to some classes of insecticides. Thus, cases of resistance of *Aedes aegypti* to pyrethroids [17] [18] and to carbamates [18] were reported in Burkina Faso. To tackle the rapid increase in insecticide resistance and outbreaks, the biological vector control is a promising approach. This approach involves the use of mosquito predators or pathogens. The use of larvivores fish such as *Betta splendens*, *Trichogaster trichopterus* and *Poecilia reticulata* is often mentioned as a biological approach to control *Aedes aegypti* larvae [19] [20]. Another environmentally friendly alternative to chemical insecticides is the use of fungal entomopathogens in biocontrol programs [21]-[23]. However the limitations with the use of entomopathogenic fungi were their low virulence and longer lethal time [24]-[26] during which the infected mosquitoes can continue transmit parasites or virus to humans and their inconsistent efficacy on field applications [27]. Fortunately, the recent biotechnological advances allow to consider the improvement of the biocontrol potential of entomopathogenic fungi by genetic engineering.

3. Potential of TEF to Control *Aedes* Mosquitoes

Enhancing the efficacy of myco-insecticides by improving their virulence and tolerance to environmental stress is made possible with genetic engineering. The determining factors of the cost-effectiveness and efficacy of mycoinsecticides during field applications are high virulence and fungal stress tolerance.

3.1. Genetic Improvement of Fungal Virulence

In the context of the control of *Aedes* mosquitoes, the improvement of the virulence of entomopathogenic fungi by genetic engineering is based essentially on the expression of insect toxins, host enzymes or host-specific genes in the fungus [26] [28] (**Table 1**).

Table 1. Recent developments of improved virulence fungi against Aedes mosquitoes.

Genes	Sources	Modified Fungi	Target Host	References
		Genes from insects predators		
AaIT	*A. australis*	*M. anisopliae*	*Aed. aegypti*	[25]
AaIT	*A. australis*	*B. bassiana*	*Aed. Albopictus*	[30]
		Genes from bacterium		
Cyt2Ba	*B. thuringiensis*	*B. bassiana*	*Aed. Aegypti*, *Aed. Albopictus*	[29]
		Genes from insects		
TMOF	*Aed. aegypti*	*B. bassiana*	*Aed. aegypti*	[31]
aaemiR-8	*Aed. aegypti*	*B. bassiana*	*Aed. aegypti*	[26]
aaemiR-375	*Aed. aegypti*	*B. bassiana*	*Aed. aegypti*	[26]

3.1.1. Transgenic Entomopathogenic Fungi Expressing Insecticidal Toxins

Anti-insect toxins are powerful compounds secreted by insect predators such as scorpions or arachnids with purpose to paralyze or kill their prey. In the context of insect pest control, several neurotoxins have been identified and studied such as the scorpion sodium channel blocker AaIT1, or the cytolytic toxin Cyt2Ba of *Bacillus thuringiensis* [28] [29]. In 2007, through a pioneer study, Wang and St Leger have increase by nine fold-change the toxicity of *Metarhizium anisopliae* in *Aedes aegypti* with a strain of *M. anisopliae* encoding the neurotoxin AaIT of *Androctonus australis [25]*. The expression of this scorpion toxin in *Metarhizium anisopliae* also reduced the survival time of infected mosquitoes by 38% compared to the wild-type strain of *M. anisopliae* associated with a reduction of mosquitoes mobility and feeding [25]. Similarly, a group of researchers improved the pathogenicity of *Beauveria bassiana* against *Aedes albopictus* by manipulating it to express the neurotoxin AaIT. They reported that the recombinant fungus had higher virulence and faster action than the wild-type strain against *Aedes albopictus* adults and larvae [30]. Few months later, following a study revealing that Cyt2Ba toxin of the bacterium *Bacillus thuringiensis* was toxic to mosquitoes of the genus *Culex*, *Anopheles* and *Aedes*, Deng and collaborators developed a transgenic strain of *Beauveria bassiana* against *Aedes aegypti* and *Aedes albopictus.* They indicated that the transgenic fungus displayed increased virulence against larval and adult *Aedes* mosquitoes compared with the WT. Indeed, at the concentration of 10^8 conidia/ml, the median lethal time (LT50) was decreased by 19% on *Aedes aegypti* adults and by 42% on larvae. For *Aedes albopictus* at the same concentration, the median lethal time was decreased by 23% on adults and by 33% on larvae. Likewise, the modified fungus infection has led fecundity reduction in the of *Aedes* mosquitoes [29]. Despite the proven potential of fungal strains expressing an insecticidal toxin, it raises questions about the occurrence of resistance to the toxin and thus a failure of the strategy as well as a host specificity compromission.

3.1.2. Transgenic Entomopathogenic Fungi Expressing Host Enzyme

It seems judicious to consider the development of a strain of entomopathogenic fungi with increased virulence in target mosquitoes associated with a low probability of resistance development. In this regard, Keyhani's team speculated that a host hormone exogenously produced (by the modified fungi) would disrupt the endocrine or neurological balance of the insect making it more susceptible to fungal infection [31]. For this purpose, they developed a strain of *Beauveria bassiana* expressing *Aedes aegypti* TMOF (Trypsin Modulating Oostatic Factor) [31]. They observed a reduction in the infecting dose of conidia (6 to 7 times less than the wild strain), a 25% reduction in the survival time of *Aedes aegypti* larvae and adults, as well as adverse consequences on mosquitoes fertility and fecundity [31].

3.1.3. Transgenic Entomopathogenic Fungi Exploiting Host Immune Mechanisms

Recently, significant insights into the molecular mechanisms controlling host selectivity by entomopathogenic fungi have been achieved and RNA interference (RNAi) has been considered for controlling insect pests. Cui *et al.* reported that host miRNAs with immunosuppressive activities can be exploited to increase fungal virulence. Indeed, they engineered *B. bassiana* to express *Aed. aegypti* miRNAs, aaemiR-8 and aae-miR-375, that are negative regulators of the Toll immune signalling pathway, which both upregulated Cactus (the inhibitor of Toll pathway) but silenced Toll5B and Rel1A, respectively. Then the expression of the host miRNAs in *B. bassiana* induce cross-kingdom RNAi to suppress mosquito immunity by targeting multiple host genes, thereby dramatically increasing fungal virulence against *Aedes aegypti*. In addition, they noticed a fungal lethality increase to laboratory-reared insecticide-susceptible and wild-caught insecticide-resistant *Aed. aegypti* mosquito suggesting that pathogen-mediated RNAi (pmRNAi) approach provides an innovative strategy to enhance the efficacy of fungal insecticides and eliminate the threat of resistance development.

3.2. Genetic Improvement of Fungal Tolerance to Environmental Stress

Extremes temperature is an important adverse factor limiting the effectiveness of entomopathogenic fungi by reducing virulence and persistence in field applications. Tolerance of entomopathogenic fungi to heat stress can be enhance by the expressions of some genes. To our knowledge, there is no documented research on the improvement of stress tolerance of entomopathogenic fungi tried under field conditions in the context of *Aedes* mosquito control. However, against the silkworm *Bombyx mori* and the mealworm *Tenebrio molitor* a significant increase in UV resistance and virulence was observed with *B. bassiana* genetically modified to overexpress a tyrosinase gene [32]. Heat shock proteins (HSPs) are involved in multiple stress responses in different organisms. Liao and collaborators revealed that overexpressing of hsp25 in *Metarhizium robertsii* increased fungal growth under heat stress and enhanced the tolerance of heat shock-treated conidia to osmotic stress [33]. More recently, overexpression of transcription factor MaSom1 gene, involved in cAMP/PKA pathway, improves some biocontrol traits of *Metarhizium acridum* such as tolerance to UV and heat shock as well as conidial yield [34].

In view of all above, genetic modification is an effective way to improve the efficacy of entomopathogenic fungi. Depending on the regulations in each country, the development of these genetically modified strains will enhance the cost-effective application of mycoinsecticides for the control of disease vectors.

4. Large-Scale Application of Transgenic Entomopathogenic Fungi

The potential of using recombinant entomopathogenic fungi for insect control has been widely recognized. Numerous studies have been conducted at the laboratory to confirm the efficacy of modified entomopathogenic fungi against insect pests. Thus, entomopathogenic fungi have been improved to control plant pests [25] [35]-[37], and against vectors with medical interest [25] [26] [38] [39]. However, to our knowledge, there are no commercial products based on TEFs to control insect pests or vectors on the market yet. The greatest progress has been made with the transgenic *Metarhizium pingshaense* expressing spider neurotoxin Hybrid which kill mosquitoes faster and at lower spore doses than wild-type strains [38]. Indeed, already successfully tested in semifield environment, this alternative in phase 2 of development begins its deployment phase according to the WHO framework for GM mosquitoes [40]. It is therefore possible to envisage the marketing of products resulting from this alternative for an effective and safe fight against malaria vectors. However, to our knowledge, such progress has not yet been made in the fight against the main vector of arbovirosis of medical interest, *Aedes aegypti*. The development and deployment of transgenic fungi for dengue fever control will certainly follow the pathway for malaria control proposed par Lovett *et al*. [39] [40] (**Figure 1**). The experiments proposed in the **Figure 1** are part of the larger goal to bring this fungal biotechnology out into the field. For the dengue control, experiments are in phase one, and each phase requires to plan ahead and carefully check if scientists are ready to move onto the next with go/no-go criteria for efficacy, safety, regulators and stakeholders. The next step for using transgenic fungi for dengue control would be both the laboratory cages studies and physical confined field studies.

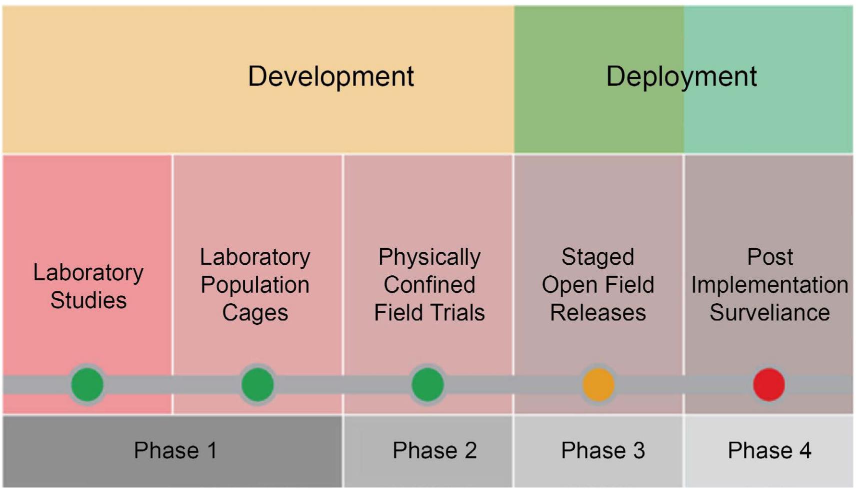

Figure 1. Phases of development and deployment of new vector control strategies [40].

5. Other Biotechnological Advancements on EPFs Development for *Aedes* Control

Recent biotechnological advances have expanded the field of research into alternatives for public health problems, particularly in vector control. In addition to the development of transgenic entomopathogenic fungi against vectors of the genus *Aedes*, many studies have reported the potential of applying genetic engineering to entomopathogenic fungi (EPF). Although still under development, these alternatives also seem promising. We find in the first line of these alternatives, the synthesis of nanoparticles based on entomopathogenic fungi. Thus, from *Cochliobolus lunatus*, hydrophilic silver nanoparticles were formed and showed significant larvicidal activity against *Aed. aegypti* and *Ano. stephensi* [41]. Similarly, effective larvicidal and pupicidal effect of mycosynthesized silver nanoparticles (AgNPs) using an entomopathogenic fungi *Trichoderma harzianum* against developmental stages of the dengue vector *Aedes aegypti* was assessed [42]. In addition, Banu and Balasubramanian demonstrated the effectiveness of silver nanoparticles synthesized from *Isaria fumosorosea* and *Beauveria bassiana* against the larvae of *Aedes aegypti* [43] [44].

6. Challenges and Perspectives to Application of TEFs for *Aedes* Control

As with other genetically modified organisms, safety concerns related to the release of recombinant organisms into the environment have been controversial. Although the scientific community agrees that transgenic entomopathogenic fungi have advantages such as improved virulence and stability under field conditions, concerns related to this technology include the pathogenicity of the modified fungi to non-target organisms, gene flow to wild strains, the development of resistance in target insect pests, and unintended effects on the environment [28] [45] [46].

Overcoming these concerns would begin with identifying and exploiting insect-specific strains of entomopathogenic fungi to reduce the possibility that the recombinant fungus could infect non-target or beneficial organisms such as bees. Another strategy to greatly reduce the likelihood of compromising fungal strain specificity is to develop fungal strains that express physiological regulators of the target insect instead of toxin [31] [47]. Similarly, expression of host miRNAs in fungal strain engineering would minimize the likelihood of resistance development in the target insects [26]. In spite of the progress made in the development of transgenic entomopathogenic fungi, their marketing still requires extensive studies. As a first step, studies to assess the impact of recombinant mycoinsecticides on non-target hosts and the risk of these biopesticides to environmental safety would be considered. It would also be wise to determine the effectiveness of recombinant transgenic fungi on insecticide-resistant mosquitoes in order to assess their potential in the fight against insecticide resistance. Further studies are also needed to examine the impact of the modified fungi on the transmission of pathogens; it would be modified fungi in order to improve their virulence as well as to block/prevent the development of pathogens in mosquitoes in order to develop safer and more effective biopesticides. Finally, scientists should engage in education and training programs to provide better information about the benefits and risks of recombinant technology.

7. Conclusion

The advent of transgenic entomopathogenic fungi is a boon for vector control.

Through genetic engineering, these new control technologies reinforce the arsenal of biological control strategies with the improvement of virulence and or tolerance to abiotic stresses of entomopathogenic fungi. They will help to overcome the limitations of the large-scale use of entomopathogenic fungi in the control of disease-carrying mosquitoes. In the context of the control of dengue vectors, several studies have been carried out to develop strains of entomopathogenic fungi with high control potential. These include fungi with improved virulence through the expression of transgenes from insect predators or bacteria with insecticidal properties. In addition, other approaches to increase the virulence of the fungi and prevent the occurrence of resistance to the different transgenes were tested. These included the exogenous expression of host physiological regulators or the expression of RNAi peptides in the fungus in order to disrupt the endocrine system or suppress the immune defenses of the mosquito respectively, thus making it vulnerable to fungal infection. Furthermore, as with all genetically modified organisms, the use of transgenic entomopathogenic fungi raises many concerns, including environmental safety. To address this major concern and facilitate the adoption of these new pest management tools, many investigations still need to be conducted. Despite the controversy, the benefits of this new strategy are enormous, and as long as the benefit-risk ratio remains positive, biotechnological advances in the control of disease vectors have a bright future ahead of them. Meanwhile, promising techniques, such as those discussed in this manuscript, have already proven their effectiveness but remain laboratory studies and require more studies both in different settings and conditions closer to the field to be deployed in the vector-control agenda.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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