Journal of Medical Entomology, 57(2), 2020, 454–462
doi: 10.1093/jme/tjz161
Advance Access Publication Date: 27 September 2019
Research

Action of *Metarhizium brunneum* (Hypocreales: Clavicipitaceae) Against Organophosphate- and Pyrethroid-Resistant *Aedes aegypti* (Diptera: Culicidae) and the Synergistic Effects of Phenylthiourea

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Subject Editor: Roberto Pereira

Received 3 May 2019; Editorial decision 27 August 2019

Abstract

Dengue, yellow fever, Zika, and chikungunya arboviruses are endemic in tropical countries and are transmitted by *Aedes aegypti*. Resistant populations of this mosquito against chemical insecticides are spreading worldwide. This study aimed to evaluate the biological effects of exposure of pesticide-sensitive *Ae. aegypti* larvae (Rockefeller) to conidia of the entomopathogen, *Metarhizium brunneum*, laboratory strains ARSEF 4556 and V275, and any synergistic activity of phenylthiourea (PTU). In addition, to investigate the nature of any cross-resistance mechanisms, these *M. brunneum* strains were tested against the Rockefeller larvae and two temephos- and deltamethrin-resistant wild mosquito populations from Rio de Janeiro. Treatment of Rockefeller larvae with 10^6 conidia/ml of ARSEF 4556 and V275 fungal strains resulted in significant decreased survival rates to 40 and 53.33%, respectively ($P < 0.0001$), compared with untreated controls. In contrast, exposure to 10^4 or 10^5 conidia/ml showed no such significant survival differences. However, the addition of PTU to the conidia in the bioassays significantly increased mortalities in all groups and induced a molt block. Experiments also showed no differences in *Ae. aegypti* mortalities between the fungal treated, wild pesticide-resistant populations and the Rockefeller sensitive strain. The results show the efficacy of *M. brunneum* in controlling *Ae. aegypti* larvae and the synergistic role of PTU in this process. Importantly, there was no indication of any cross-resistance mechanisms between *Ae. aegypti* sensitive or resistant to pesticides following treatment with the fungi. These results further support using *M. brunneum* as an alternative biological control agent against mosquito populations resistant to chemical insecticides.

Key words: mosquito vector, entomopathogenic fungi, phenylthiourea, larvicidal, insecticide resistance

Aedes aegypti (Linnaeus 1762) is an important vector of flaviviruses such as dengue fever, Zika, and yellow fever as well as chikungunya fever caused by a togavirus. *Aedes aegypti* originated from the arid regions of Africa but between the 17th and 20th centuries, via international trading, colonized other tropical regions (Brown et al. 2011, 2014; Kraemer et al. 2015).

Dengue fever results in a general systemic infection and symptoms include aches, rashes, fatigue, and, in the most severe hemorrhagic phase, death (Gubler 1998, Simmons et al. 2015). According to the Brazilian Ministry of Health, in 2017, the disease incidence in Brazil was equivalent to 121.7 cases per 100,000 people (Ministério da Saúde do Brasil 2018).

Yellow fever is an acute viral hemorrhagic disease with high morbidity and mortality. In nature, in South America and Africa, transmission occurs between non-human primates and hematophagous females of wild mosquitoes, mainly by *Haemagogus* spp. (Diptera:

Culicidae) and *Sabethes* spp. (Diptera: Culicidae) (Monath and Vasconcelos 2015). *Aedes aegypti* is a potential vector of this virus and, by its synanthropic and anthropophilic behavior, results in an urban cycle of transmission (Couto-Lima et al. 2017). In Brazil, from 1 July 2017 to 28 February 2018, 723 cases of yellow fever were reported of which 237 people died (Ministério da Saúde do Brasil 2018).

Aedes aegypti can also incubate and transmit Zika virus which may cause fever, headache, arthralgia, edema, and retro-orbital pain, and be associated with neuropathology and microcephaly in newborn babies (Ioos et al. 2014, Plourde and Bloch 2016, Colón-González et al. 2017). In February 2016, 25 countries from the continents of Asia, Africa, and the Americas reported the occurrence of Zika virus in their territories (World Health Organization [WHO] 2016).

The symptoms of Chikungunya fever are similar to those of dengue; however, it may also cause more severe and long-lasting neuropathological effects (WHO 2016, Burt et al. 2017). In Brazil, in 2016 and 2017, 277,882 and 180,000 probable cases of Chikungunya fever were registered, respectively (Ministério da Saúde do Brasil 2018).

Since there is no therapeutic cure for these viruses and an efficient and safe vaccine only exists against yellow fever, the management of these diseases consists of control of the mosquito populations (Shragai et al. 2017, Paixão et al. 2018). Chemical control for *Ae. aegypti* primarily uses synthetic insecticides, including carbamates, organophosphates, and pyrethroids, with temephos and deltamethrin among the most widely used chemical insecticides in Brazil (Moyes et al. 2017, Paiva et al. 2017). The indiscriminate use of these substances, however, increases the incidence of resistant individuals, hindering efforts to combat disease transmission (Montella et al. 2007, Liu 2015, Viana-Medeiros et al. 2018). It is thus necessary to find alternative control strategies that do not affect the same cross-resistance mechanisms to those against the synthetic insecticides (Poopathi and Tyagi 2006, Von Seidlein et al. 2017).

Previously, the entomopathogenic fungi, *Beauveria bassiana* (Hypocreales: Cordycipitaceae) and *Metarhizium brunneum* (Petch, 1935), were shown to be viable alternatives for the biological control of insect populations resistant to chemical insecticides (Faria and Wraight 2007, Zimmermann 2007a,b). These fungi are also pathogenic for several medically important arthropods, including *Argaspersicus* sp. (Acari: Argasidae) (Pourseyed et al. 2010), *Triatoma infestans* (Hemiptera: Reduviidae) (Lazzarini et al. 2006, Luz et al. 2012), *Anopheles gambiae* (Diptera: Culicidae), *Anopheles stephensi* (Diptera: Culicidae) (Bukhari et al. 2011), and larvae of *Ae. aegypti* (Gomes et al. 2015).

One important infection mechanism *of M. brunneum* towards terrestrial arthropods consists of adhesion and mechanical penetration of the exoskeleton, aided by enzymatic activity that can determine the strain's virulence (Silva and Messias 1986, Dhar and Kaur 2010, Butt et al. 2016). In response to the early stages of fungal infection, the host mobilizes humoral and cellular immune systems (Butt et al. 2013) such as the activation of the prophenoloxidase (PPO) cascade (Yassine et al. 2012, Ramirez et al. 2018). PPO activation is triggered by surface molecules from invading microorganisms including lipopolysaccharides, peptidoglycans, and zymosan (Cerenius and Söderhäll 2004). This activation involves a cascade of serine proteases that converts PPO to phenoloxidase (PO) via toxic quinonic intermediaries and results in unpolymerized melanin capable of killing pathogens invading mosquitoes (Cerenius and Söderhäll 2004, Lu et al. 2014).

Recent studies on the effect of inhibition of PO by phenylthiourea (PTU) on the infectivity of entomopathogenic agents have been undertaken by Tokarev et al. (2018). They showed that *Nosema pyrausta*-resistant (Dissociodihaplophasida: Nosematidae) populations of *Galleria mellonella* (Lepidoptera: Pyraloidea) were more susceptible to conjugated treatments with PTU and *Bacillus thuringiensis* (Bacillales: Bacillaceae) when compared to the controls. The application of PTU as a synergistic agent in insect pest population control needs additional studies as well as elucidation of any possible impact on non-target species.

In the present study, the development and mortality of the *Ae. aegypti* Rockefeller population treated with fungal strains were standardized, since these mosquitoes are used as a sensitive reference population in studies of resistance to chemical insecticides (David et al. 2018, Garcia et al. 2018). Experiments were also performed to evaluate the synergistic effect of PTU, a strong competitive inhibitor of the enzymatic oxidation of DOPA by PO (Ryazanova et al. 2012). Subsequently, studies of fungal pathogenicity used sublethal doses of *M. brunneum* ARSEF 4556 and strain V275 against the larvae of two temephos- and deltamethrin-resistant wild *Ae. aegypti* populations.

Materials and Methods

Insects

The eggs of *Ae. aegypti* were supplied by the Laboratory of Physiology and Control of Vector Arthropods (LAFICAVE-IOC, Rio de Janeiro, Brazil). The insects were maintained in accordance with the Ethical Principles of Animal Experimentation approved by the Committee of Ethics in Animal Experimentation of Fiocruz (CEUA) and the Ministry of Science and Technology and Innovation (https://www.mctic.gov.br/mctic/opencms/institucional/concea), according to the protocols defined by the 'American Association for Animal Science', 'Federation of European Laboratory Animal Science Associations', and 'International Council for Animal Science and Association for Assessment and Accreditation of Laboratory Animal Care International'.

For the assays, Rockefeller strains (temephos/deltamethrin susceptible) and two populations resistant to temephos (an organophosphate insecticide) and deltamethrin (a pyrethroid insecticide) were used. These two latter strains are named, Nova Iguaçu (second generation) and Paquetá (third generation), whose 95% (RR_{95}) temephos-resistance ratios are 8.1 and 9.8, respectively (Dias 2015, Borges 2018), with both previously analyzed by LAFICAVE (http://www.ioc.fiocruz.br/laficave/Laficave/Laficave.html). Additionally, Nova Iguaçu and Paquetá populations are also resistant to deltamethrin (pyrethroid), RR_{95} = 44.4 and 40 (Dias 2015, Borges 2018), respectively, and both mosquito-resistant populations showed also high pyrethroid-resistant *kdr* allele frequencies (Borges 2018, Brito et al. 2018).

For egg hatching, a filter paper containing around one thousand adherent eggs was immersed in a beaker of dechlorinated water at 37°C and then placed in an incubator at 26°C for 50 min. The hatched larvae were then transferred to a tray containing 300 ml of water with 300 mg of shredded fish feed (Poytara Tropical Flakes®, Rio de Janeiro, Brazil). The larvae were maintained at 26°C until the third instar (L3) used in all bioassays.

Fungal Strains

The ARSEF 4556 and V275 strains of *M. brunneum* were maintained on Saburaud Dextrose Agar medium and conidia harvested from 14-d-old sporulating cultures (Greenfield et al. 2015). The conidia were suspended in a solution of 0.03% Tween 80 and filtered through a funnel with gauze. The suspension was then centrifuged at 2,000*g* for 10 min and the conidia resuspended in 0.03% Tween 80. The conidia were counted in a hemocytometer (Neubauer) for the preparation of the bioassays.

Bioassays

The experiments were carried out in disposable 50-ml plastic cups each containing 10 ml dechlorinated water with 10 third-instar *Ae. aegypti* larvae. Experimental groups of Rockefeller strain larvae were inoculated with 250 µl of the diluted conidia suspensions in 0.03% Tween 80, to obtain final concentrations of 10^4, 10^5, or 10^6 conidia/ml used in the bioassays. In the control larval groups, water alone was added (negative control, NC) or 0.03% Tween 80 (surfactant control, SC) instead of the conidial suspensions. The trays containing the cups were maintained at 26 ± 1°C and the groups were periodically monitored for viability and for counting larvae, pupae, and adults. The bioassays were made in triplicates, repeated at least twice and followed for 7 d, by which time all adults had emerged in the control groups.

In the experiments using PTU (Sigma, São Paulo, Brazil), 10 mg PTU was solubilized by vigorous vortexing in 1 ml water, and then centrifuged at 5,000 × *g* for 30 s. Subsequently, the saturated supernatant was removed and 10 µl was added to those groups (final concentration < 1 mg/L) of experimental Rockefeller larvae also receiving 10^6 or 10^5 conidia/ml. The bioassays with PTU were extended from 7 to 8 d for the evaluation of any delays or interruption of molting. In these tests, there was an additional control group in which saturated solution of PTU was added without the conidia (PTU control [PTUC]).

In experiments to evaluate any *Ae. aegypti* cross-resistance against *M. brunneum* in the two temephos/deltamethrin-resistant populations of *Ae. aegypti*, Paquetá and Nova Iguaçu, larvae were tested with a 10^6/ml conidial concentration. The reactions of these resistant strains were compared with those of the temephos-sensitive Rockefeller larvae. The relative mortality was calculated by the mean of the difference in mortality of each replicate counted daily for groups of the resistant population in relation to the average mortality verified each day in the susceptible population.

Statistical Analysis

The graphs were created using Graphpad prism ver. 6.03 to analyze the survival (A) and the developmental course of larvae (B), pupae (C), and adults (D). The statistical significances from punctual analyses were performed using the Suissa and Shuster test with the R program and UNIX platform comparing the experimental groups with the surfactant (Figs. 1 and 2), PTU (Figs. 3 and 4), or Rockefeller (Fig. 5 and Table 1) controls, to evaluate survival differences and occurrence of remaining juvenile stages (unmolted) of *Ae. aegypti*. This test was also used to assess the daily survival difference between the sensitive (Rockefeller) and resistant (Nova Iguaçu and Paquetá) populations of *Ae. aegypti* (Fig. 5). The Gehan–Breslow–Wilcoxon statistical test, from programs Graphpad prism ver. 6.03 and PAST 3.20, was used to compare the whole curves that quantified insect survival (A) and the development of larvae (B) and adults (D) (Figs. 1–4 and Table 1). In all bioassays, the differences were only considered statistically significant for values of $P < 0.05$.

Results

Effects of *Metarhizium brunneum* ARSEF 4556 and V275 on *Aedes aegypti* Larvae (Rockefeller)

The bioassays with strain ARSEF 4556 of *M. brunneum* showed no significant effects on *Ae. aegypti* survival in concentrations of 10^4 or 10^5 conidia/ml (Fig. 1A and D). In contrast, treatment with 10^6 conidia/ml induced significant effects on survival from the third day after treatment, with 58.33 ± 4.01% survival ($P < 0.0001$),

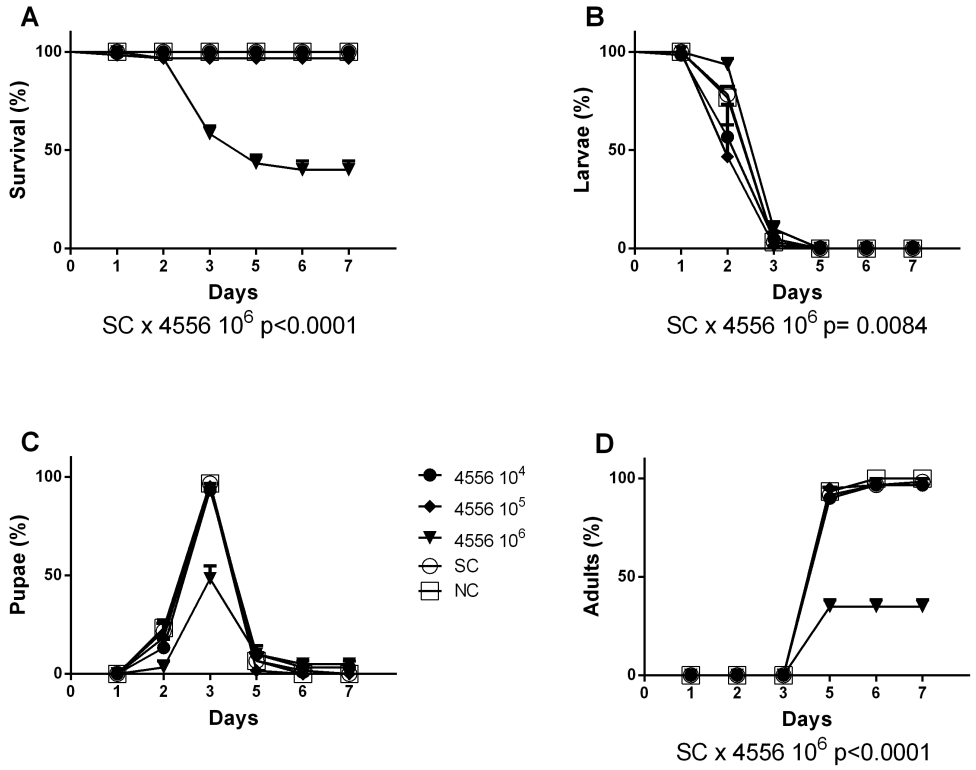

Fig. 1. Activity of *M. brunneum* strain ARSEF4556 (10^4, 10^5, or 10^6 conidia/ml) on *Ae. aegypti* (Rockefeller population) survival (A), and development of larvae from third instar (B), to pupae (C) and to adults (D) after the treatments; bars represent the SEM. NC, negative control; SC, surfactant control. The Gehan–Breslow–Wilcoxon statistical test was used to determine the whole survival and development curves in comparison with the SC (significance $P < 0.05$).

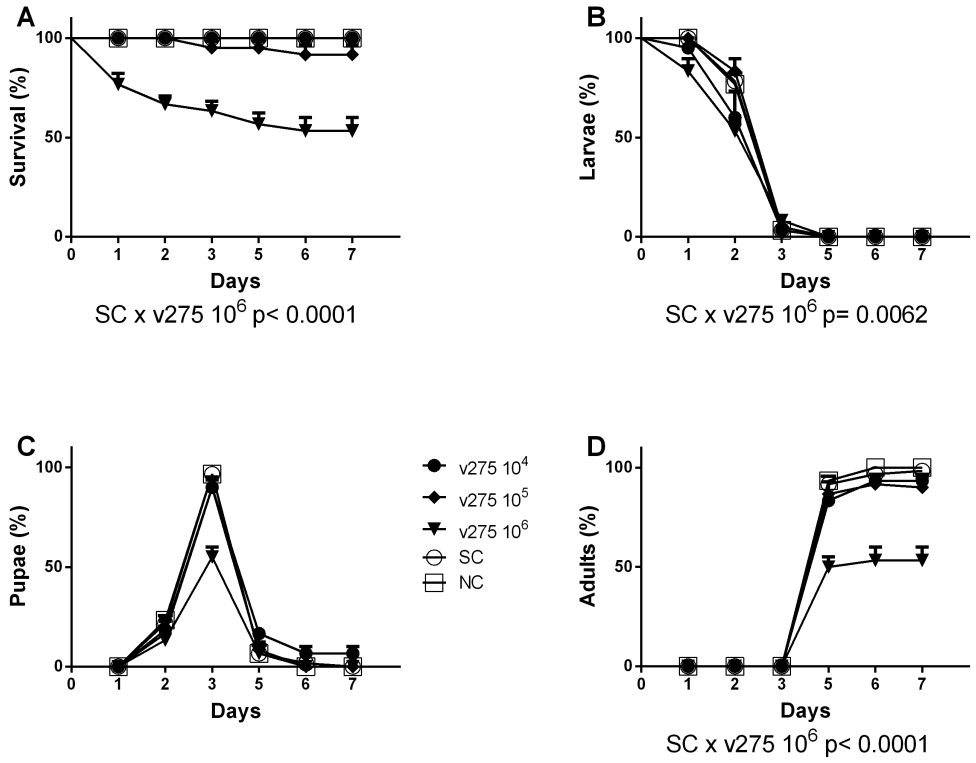

Fig. 2. Activity of *M. brunneum* strain V275 (10^4, 10^5, or 10^6 conidia/ml) on *Ae. aegypti* (Rockefeller population) survival (A), and development of larvae from third instar (B), to pupae (C) and to adults (D) after the treatment; bars represent the SEM. NC, negative control; SC, surfactant control. The Gehan–Breslow–Wilcoxon statistical test was used to determine the whole survival and development curves in comparison with the SC (significance $P < 0.05$).

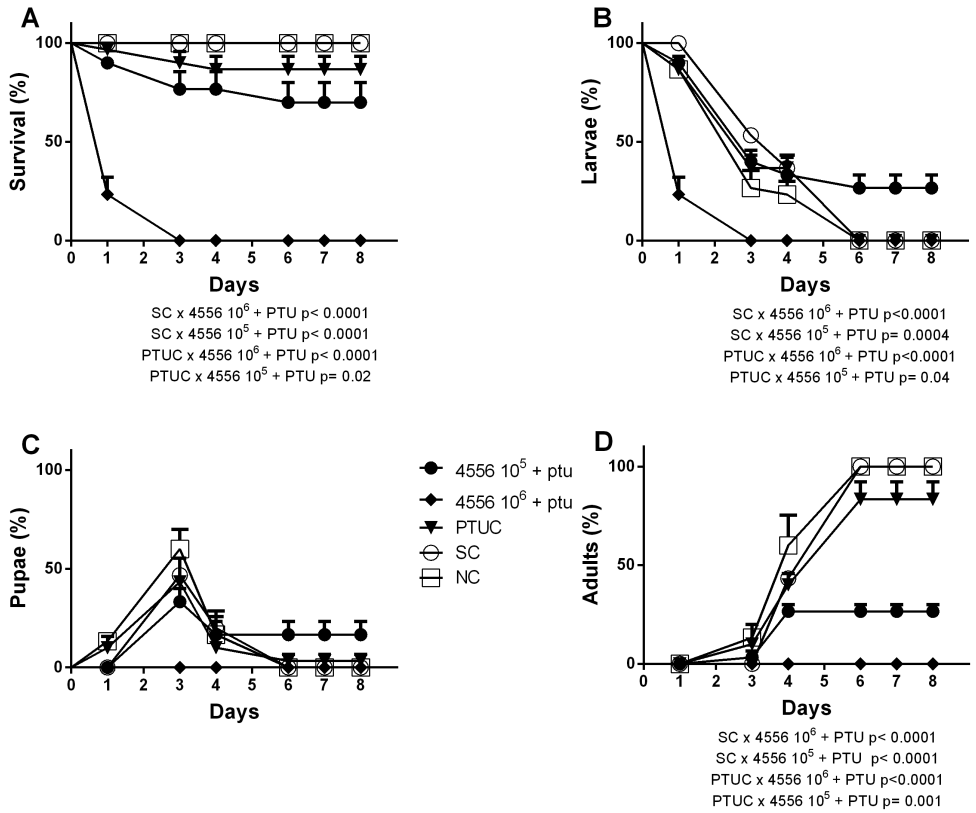

Fig. 3. Activity of *M. brunneum* strain ARSEF4556 (10^5 and10^6 conidia/ml) combined with PTU on *Ae. aegypti* (Rockefeller population) survival (A) and development of larvae from third instar (B), to pupae (C) and to adults (D) after the treatment; bars represent the SEM. NC, negative control; SC, surfactant control; PTUC, PTU control. The Gehan–Breslow–Wilcoxon statistical test was used to determine assessed the whole survival and development curves in comparison with SC and PTUC (significance $P < 0.05$).

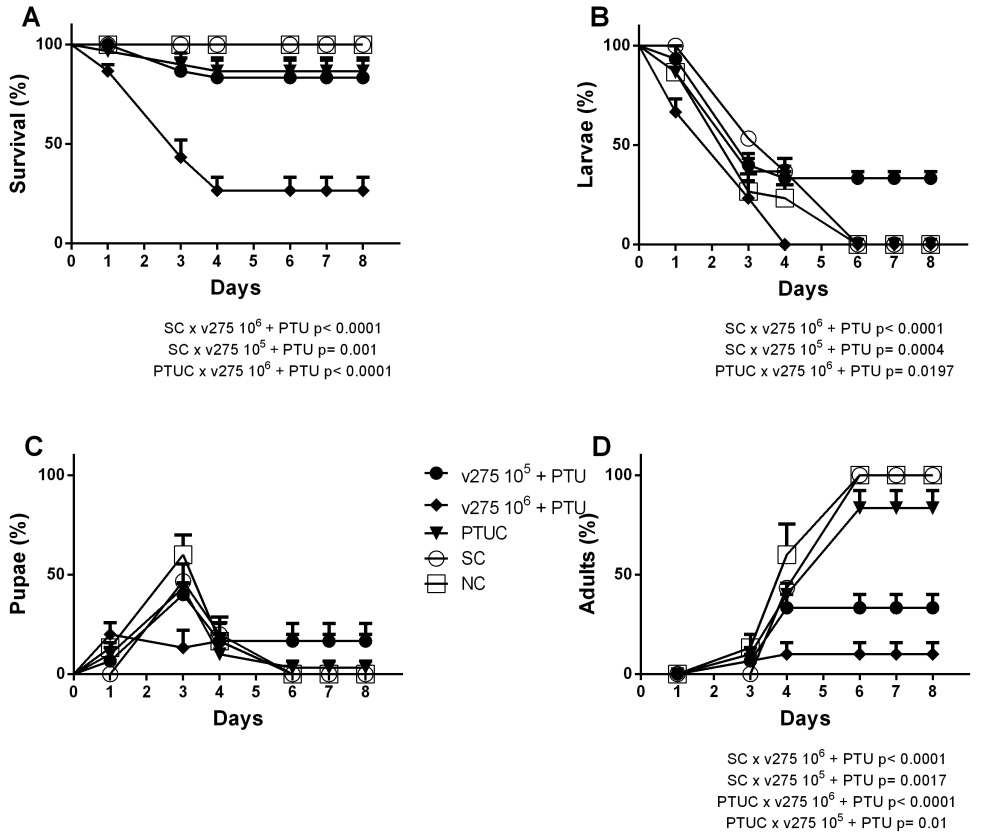

Fig. 4. Activity of *M. brunneum* strain V275 (10^5 and10^6 conidia/ml) combined with PTU on *Ae. aegypti* (Rockefeller population) survival (A) and development of larvae from third instar (B), to pupae (C) and to adults (D) after the treatment; bars represent the SEM. NC, negative control; SC, surfactant control; PTUC, PTU control. The Gehan–Breslow–Wilcoxon statistical test was used to determine assessed the whole survival and development curves in comparison with SC and PTUC (significance $P < 0.05$).

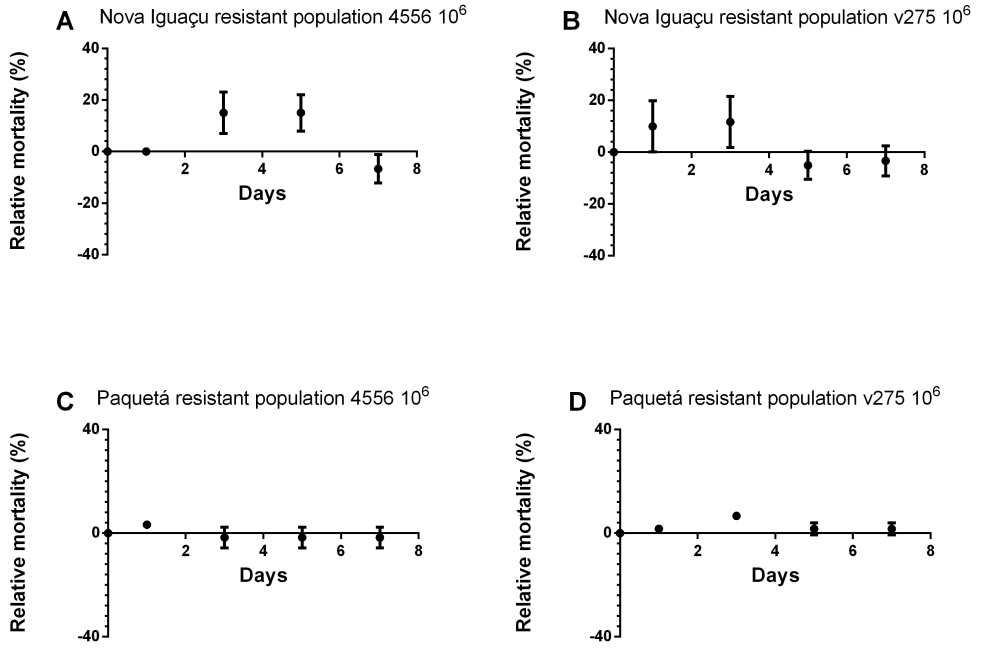

Fig. 5. Relative mortality of insects of the temephos/deltamethrin resistant populations from Nova Iguaçu (A, B) and Paquetá (C, D) in comparison with a susceptible population (Rockefeller) of *Ae. aegypti*. The insects were treated from third-instar larvae with the concentration of 10^6 conidia/ml of strain ARSEF4556 (A, C) and V275 (B, D) of *M. brunneum*. The bars represent the SEM. The Suissa and Shuster statistical test was used to determine the significance of treatment mortality in relation to SC and no significant differences were found. The x-axis corresponds to the average of the mortality verified each day for the Rockefeller population, while each point of the graph corresponds to the positive or negative difference between the averages of the mortality of the resistant populations in relation to the sensitive population (Rockefeller).

Table 1. Comparative activity of strains ARSEF4556 and V275 of *M. brunneum* (10^6 conidia/ml) on *Ae. aegypti* (Rockefeller population) survival in the absence (–) or presence (+) of PTU

Days[a]	4556 Survival (±SE%)		V275 Survival (±SE%)		4556 × V275, *P* value	
	–PTU	+PTU	–PTU	+PTU	–PTU	+PTU
1	100.00 ± 0	23.33 ± 8.82	76.67 ± 5.58	86.67 ± 3.33	**<0.0001**	**<0.0001**
3	58.33 ± 4.01	0 ± 0	63.33 ± 4.94	43.33 ± 8.82	0.6816	**<0.0001**
5	43.33 ± 4.22	0 ± 0	56.67 ± 5.58	26.67 ± 6.67	0.1706	**0.0027**
6	40.00 ± 4.47	0 ± 0	53.33 ± 6.67	26.67 ± 6.67	0.1647	**0.0027**
7	40.00 ± 4.47	0 ± 0	53.33 ± 6.67	26.67 ± 6.67	0.1647	**0.0027**
Curve[b]	–	–	–	–	**<0.0001**	**<0.0001**

Significant differences in bold (n = 12).

[a]Suissa and Shuister – punctual survival analysis.

[b]Gehan–Breslow–Wilcoxon – whole survival curve analysis.

decreasing from the fifth to seventh day, to 43.33 ± 4.22 and 40.00 ± 4.47% (P < 0.0001) (Table 1 and Fig. 1A), respectively. Similarly, with *M. brunneum* strain V275, there was no significant effects on survival of *Ae. aegypti* treated with 10^4 or 10^5 conidia/ml, with only a weak effect on survival, 10.00 ± 4.47% (P = 0.1182),with the latter concentration (Fig. 2A and D). The group treated with V275 10^6 conidia/ml, on the first day, showed a survival rate of 76.67 ± 5.58% (P < 0.0001), decreasing to 63.33 ± 4.94% and 53.33 ± 6.67% on the third and seventh days, respectively (P < 0.0001) (Table 1 and Fig. 2A).

Despite the early mortality of strain V275-treated larvae (10^6 conidia/ml), detected on the first day of testing (Fig. 2A and Table 1), on subsequent days, mortality was higher, although not significantly (P > 0.05), in insects treated with ARSEF 4556 (Table 1). The statistical analyses of the whole survival curve of *Ae. aegypti* indicated a significant difference (P < 0.0001) between strains ARSEF 4556 and V275 *M. brunneum* activity (Table 1). However, a significant difference (P < 0.0001) between both strains was only detected after the first day of treatment when the punctual analysis of daily survival was used (Table 1).

Furthermore, in treatments with ARSEF 4556 10^4 or 10^6 conidia/ml, 3.33 ± 3.33% and 5.00 ± 2.24% of insects, respectively, remained as pupae at least up to the last day (seventh) of treatment (P > 0.05) (Fig. 1C). However, with V275 only 10^6 conidia/ml induced insects to remain as pupae (6.67 ± 3.33%) (Fig. 2C), but no insects remained as larvae (Figs. 1B and 2B). In all experiments, no statistical differences in insect development or survival between negative and surfactant controls were detected (P > 0.05).

Effects of *Metarhizium brunneum ARSEF* 4556 and V275 + PTU on *Aedes aegypti* Larvae (Rockefeller)

There was no significant activity on *Ae. aegypti* Rockefeller larvae with 10^4 or 10^5 conidia/ml of fungi and only partial mortality with 10^6 conidia/ml, so that additional experiments were conducted using only 10^5 or 10^6 conidia/ml with the inclusion of PTU. The addition of PTU increased the activity of the entomopathogenic fungus in all groups.

In experiments with ARSEF 4556, 10^6 conidia/ml + PTU, on the first day, the survival was only 23.33 ± 8.82% (P < 0.0001), with all insect killed after 3 d (P < 0.0001, Table 1, Fig. 3A). With 10^5 conidia/ml + PTU, killing was significant from the first day with a survival rate of 90.00 ± 0% (P = 0.0134) which on the third day decreased to 76.67 ± 5.58% (P = 0.0001), reaching 70.00 ± 10.00% (P < 0.0001) on the sixth to final day (Fig. 3A). In addition, in this group treated with 10^5 conidia/ml + PTU, 26.67 ± 4.22% of insects remained in the larval stage (P = 0.0004), while 16.67 (P = 0.001) ± 6.67% remained as pupae until the last day of the test (Fig. 3B and C). Thus, only 26.66% of larvae became adults by the end of the assay (Fig. 3D).

Assays of larvae treated with V275 at 10^6 conidia/ml + PTU, after the first day, had a survival rate of 86.67 ± 2.12% (P = 0.0038) (Fig. 4A) which on the third day decreased to 43.33 ± 8.82% (P < 0.0001) and from the sixth to the final day was reduced to 26.67 ± 4.22% (P < 0.0001) (Fig. 4A). The 10^5 level was less pathogenic than the 10^6 with 86.67 ± 6.67% surviving, after the third day, decreasing to 83.33 ± 8.82% (P = 0.01) from fourth to the final day (Fig. 4A). There was no significant difference between this group (ARSEF V275 at 10^5 conidia/ml + PTU) and the control treated only with PTU. This group with a concentration of 10^5 + PTU had 33.33 ± 2.12% (P = 0.0004) of larvae remaining at the end of the experiment (day 8) (Fig. 4B). With both 10^6 and 10^5 conidia/ml of ARSEF V275 + PTU, 16.67% (±2.12% and 5.58%, respectively) insects remained as pupae (P = 0.001) (Fig. 4C), reducing the number of mosquitoes that became adults suitable for reproduction by the end of the experiment (Fig. 4D).

The group treated with only PTU solution had, at the end of the test, a survival rate of 86.67 ± 6.67% (P = 0.0134) and a total of 3.33 ± 2.12% remaining as pupae (P > 0.05) (Fig. 4A and C). In the bioassays containing PTU, the strain ARSEF 4556 was significantly (P ≤ 0.0027) more lethal against *Ae. aegypti* than V275 on all days analyzed (Table 1).

Effects of *Metarhizium brunneum* ARSEF 4556 and V275 on Temephos/Deltamethrin-Resistant Populations of *Aedes aegypti*

Since 10^4 and 10^5 conidia/ml failed to kill the Rockefeller temephos/deltamethrin-sensitive larvae, assays with temephos/deltamethrin-resistant populations of *Ae. aegypti* from Nova Iguaçu and Paquetá were carried out with 10^6 conidia of ARSEF 4556 and ARSEF V275/ml. The relative mortality was calculated by the difference between mortality found in each replicate of the resistant populations in relation to the average of mortality verified in Rockefeller, for each day analyzed. No significance statistical differences were found between the temephos/deltamethrin-resistant Nova Iguaçu and Paquetá mosquitoes compared with the sensitive Rockefeller population (Fig. 5) treated with the two strains of the entomopathogenic fungi.

In comparison with the bioassays with Rockefeller, the tests with ARSEF 4556 and the Nova Iguaçu mosquitoes, on the third and fifth days, mortalities were nonsignificantly higher (15.00 ± 8.03%, P = 0.095 and 15.00 ± 7.03%, P = 0.1214, respectively) and on the

seventh day the result was lower (−6.67 ± 5.58%, $P = 0.7917$), but also nonsignificantly (Fig. 5A). With V275, on the first and third days, resulted in a higher mortality rate (9.98 ± 9.88%, $P = 0.2093$ and 11.67 ± 9.92, $P = 0.2093$, respectively) and on fifth and seventh days a lower activity (−5 ± 5.42%, $P = 0.5313$ and −3.33 ± 5.78, $P = 0.7917$, respectively). As observed with the *Ae. aegypti* population of Nova Iguaçu, the population of Paquetá, also resistant to temephos and deltamethrin, did not differ significantly from the Rockefeller (sensitive) population in relation to mortality when treated with the 10^6 conidia of ARSEF 4556 or V275 strains of *M. brunneum* (Fig. 5C and D), indicating the absence of any cross-resistance mechanisms between *Ae. aegypti* sensitive to resistant to pesticides following treatment with the fungal strains.

Discussion

In the present study, although strain 4556 killed more mosquitoes than v275 strain (10^6 conidia/ml), from the third to the last day of the test, these punctual differences were not significant (Table 1). However, on the first day of testing, the V275 showed an early and statistically significant virulent effect compared to ARSEF 4556 (Table 1). The statistical analysis of the whole survival curve also indicated a significant difference in the temporal course of insecticide activity of ARSEF 4556 in comparison with V275 (Table 1). Thereby, the current study supports the findings of earlier studies that *M. brunneum* strain ARSEF4556 is more aggressive than V275 against *Ae. aegypti* (Greenfield et al. 2015, Alkhaibari et al. 2016). It appears that conidia of this strain are more virulent independent of the country of origin or resistance to chemical pesticides.

Some heterogeneity found among results from different experiments to evaluate the activity of *M. brunneum* may be related to the variation in stability, virulence, and/or conidia production which depend on the fungal strain, number of subcultures and the nutrients in the culture medium (Frigo and Azevedo 1986, Shah et al. 2007, Hutwimmer et al. 2008, Ansari and Butt 2011, Petlamul and Prasertsan 2012, Riaz et al. 2013).

The main forms of infection from entomopathogenic fungi in terrestrial insects consists of the attachment of conidia on to the cuticle, involving a diversity of adhesin-like proteins, followed by germination and penetration, promoted by subtilisins, trypsins and cysteine proteases (Yassine et al. 2012, Ramirez et al. 2018). In contrast, the main route of infection by fungi in aquatic larvae involves the ingestion of conidia and the subsequent synthesis of proteases Pr1 and Pr2, which with simultaneous action of high levels of hydrogen and oxygen reactive species, triggers apoptosis in the midgut epithelial cells and entry of fungi (Butt et al. 2013). Generally, in response against invading organisms, insects and other arthropods have efficient defense mechanisms, involving physical structures such as the cuticle itself, antibacterial molecules and defensive cells (Hultmark 1993, Pedrini 2018). Molecules on the surface of fungi can also activate the PPO cascade and the products generated with oxidation of phenolic groups show potent antifungal activity (Hajek and St. Leger 1994, Alkhaibari et al. 2016).

PTU is an inhibitory agent of the PPO cascade, although its mechanism of action in insects is not yet completely understood (Hajek and St. Leger 1994, Cerenius and Söderhäll 2004, Lu et al. 2014). The toxicity of PTU to humans is controversial but considered potential, so it must be handled with care (Wheatcroft et al. 1972).

In the current work, experiments added PTU in the test water together with ARSEF 4556 or V275 *M. brunneum* conidia and larvae of *Ae. aegypti*. A significant increase in mortality was observed in all the experimental groups treated with fungi and PTU, and there was also an effect on the molting process (Figs. 3 and 4). Furthermore, the results illustrated again the greater effectiveness of *M. brunneum* strain ARSEF 4556 against the survival of *Ae. aegypti*, in comparison with V275 (Table 1).

Previously, Beresky and Hall (1977) applied PTU to *Ae. aegypti* parasitized by *Neoaplectana carpocapsae* (Rhabditida: Steinernematidae) and verified its interference with the immune system, inhibiting melanin synthesis in vitro and delaying melanization and encapsulation against the nematode.

Studies with *Galleria mellonella* melanocytic populations, obtained by laboratory selection, have also shown that these insects, with higher PO activity, are more resistant to the fungus, *B. bassiana* (Dubovskiy et al. 2013). In contrast, however, the same effect was not observed in *M. brunneum*-treated melanocytic larvae (Dubovskiy et al. 2013). Recent results also show that *B. bassiana* spores previously treated with PPO become less virulent to insects, due to increased spore hydrophobicity and reduced adhesion to the cuticle (Zhang et al. 2017).

In the present study, strain ARSEF 4556 remained the most effective, killing all insects while still in the larval phase, at the highest conidia concentration (10^6/ml). Also, with 10^5 conidia/ml, less than 30% of the insects emerged to adults at the end of the experiment, taking into account both dead insects and surviving unmolted juveniles (Figs. 3 and 4). In mosquitoes treated with V275, although mortality was lower, the effect of molting inhibition was evident at both, 10^5 and 10^6 conidia/ml (Table 1 and Fig. 4). These results suggest that inhibition of the prophenoloxidase cascade in insects by PTU may cause immunosuppression and even affect the development of *Ae. aegypti*.

Cell line 4a-3B, from *A. gambiae*, can express protein from the PPO cascade under stimulus by 20-hydroxyecysone, suggesting a probable immune regulation mediated by the ecdysteroid hormone in insects (Müller et al. 1999). The synergistic effect of PTU applied with fungi, not only increases microbial pathogenicity and has insect control potential, but can also interrupt the molting process, inhibiting metamorphosis and, consequently, the reproductive activity of mosquitoes.

Results of experiments in the present paper, using temephos- and deltamethrin-resistant Nova Iguaçu and Paquetá *Ae. aegypti*, failed to show cross-resistance or an increase in susceptibility to *M. brunneum* treatment. In contrast, studies with *Anopheles gambiae* resistant to pyrethroid insecticide showed that these were more susceptible to both, *M. brunneum* and *B. bassiana*, in comparison with a non-resistant population. (Howard et al. 2010). In our results, both resistant populations, Nova Iguaçu and Paquetá, showed no statistically significant differences in mortalities relative to the susceptible population (Rockefeller), for both strains of *M. brunneum*, ARSEF 4556 and V275 (Fig. 5). Similar results were obtained with temephos-resistant *Ae aegypti* populations that were sensitive to methoprene, an analogue of juvenile hormone (Braga et al. 2005). Moreover, a recent paper showed that the resistance to pyrethroids may persist in populations of *Ae. aegypti* for long periods after the interruption of the use of these insecticides (Macoris et al. 2018).

In conclusion, *M. brunneum* ARSEF 4556 and strain V275 are virulent for *Ae aegypti* irrespective of whether the larvae are resistant or sensitive to insecticides and do not discriminate between which insecticides the mosquitoes are resistant to. Furthermore, PTU works synergistically with the more virulent strain of *M. brunneum* (ARSEF 4556) resulting in 0% mosquito survival and can also delay larval development—reducing number of generations of mosquitoes which could be quite important in wet seasons when mosquitoes breed rapidly and transmit diseases. These results confirm that the

M. brunneum, ARSEF 4556 and V275 entomopathogenic fungal strains, could be used as viable alternatives in the control of *Ae. aegypti* in integrated management programs and may even provide a substitute for chemical insecticides in endemic arboviruses areas where the mosquito resistance to organophosphates and pyrethroids may persist.

Acknowledgments

The authors thank Luciana dos Santos Dias (IBEX-RJ-Brazil) for technical assistance and to Dr. Fernando A. Genta (IOC-FIOCRUZ) for the suggestions that improved the paper. T.B. was supported by grants funded jointly by the Biotechnology and Biological Sciences Research Council, the Department for Environment, Food and Rural Affairs, the Economic and Social Research Council, the Forestry Commission, the Natural Environment Research Council and the Scottish Government, under the Tree Health and Plant Biosecurity Initiative. N.A.R. was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Brazil (E-26/201.787/2017) and R.P. (Fellowship) by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

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