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ORIGINAL ARTICLE

Characterization of *Tolypocladium cylindrosporum* (Hypocreales, Ophiocordycipitaceae) isolates from Brazil and their efficacy against *Aedes aegypti* (Diptera, Culicidae)

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Abstract

Aims: To survey and to characterize entomopathogenic fungi as natural enemies of mosquitoes in Central Brazil.
Methods and Results: *Tolypocladium cylindrosporum* (Hypocreales, Ophiocordycipitaceae) was isolated for the first time in South America by using *Aedes aegypti* (Diptera, Culicidae) as sentinel larvae in a stagnant mosquito breeding site in a secondary tropical forest. Two isolates were morphologically and molecularly identified, and their activity tested against *A. aegypti* eggs, larvae and adults.
Conclusions: Findings emphasize the importance of *T. cylindrosporum* as a natural fungal antagonist of mosquitoes.
Significance and Impact of the Study: Both isolates affected larvae and adults but were less effective against eggs; both have potential for development as a mycoinsecticide especially against larvae of *A. aegypti* the main vector of dengue, chikungunya, Zika and urban yellow fever.

Introduction

Mosquitoes (Diptera, Culicidae), especially *Aedes aegypti*, are vectors of such important human viral diseases as dengue, chikungunya, Zika and urban yellow fever in the tropics and subtropics (Weaver *et al.* 2018). Vector control is notably hampered by resistance to synthetic insecticides (Moyes *et al.* 2017), and there is an urgent need for innovative control methods for these pests. Natural antagonists, particularly and specifically those fungal entomopathogens that attack culicids, are of high interest for biological mosquito control (Scholte *et al.* 2004; Lacey 2017). Efforts to develop specific mycoinsecticide for mosquito control are still in early stages (Faria and Wraight 2007; Mascarin *et al.* 2018). There is now a first commercialized product based on *Beauveria bassiana* (Hypocreales, Cordycipitaceae) intended for use against *A. aegypti* in over 30 countries worldwide (http://www.in2care.org/mosquito-trap/registrations/ accessed 30 May 2018). High specificity, virulence and persistence together with simple large-scale production will determine the potential of a fungus for further development as a successful mycoinsecticide against mosquitoes. Another important criterion is the adaptation of the entomopathogen to regional climatic conditions such as challenging high temperatures, extreme low or high relative humidities, or the intense ultraviolet radiation. These limiting conditions are common in tropical sites where *A. aegypti* is frequent. The increased demands on potent fungal candidates for mosquito control render regional and specific studies on the natural occurrence of entomopathogenic fungi affecting these vectors indispensable.

Until now, the biodiversity of entomopathogenic fungi in Brazil has been known primarily from agricultural sites

(Sosa-Gómez *et al.* 2010). The scarcity of information about fungi from nonagricultural sites is primarily a result of the lack of efforts to collect and to culture such fungi. During an extended survey for fungal pathogens of mosquitoes and other dipteran vectors of serious human and animal diseases in Central Brazil between 2014 and 2017, *Leptolegnia chapmanii* (Peronosporomycetes, Saprolegniales) and *Coelomomyces santabrancae* (Blastocladiomycetes, Blastocladiales) were found in mosquito larvae. *Conidiobolus macrosporus* (Entomophthoromycetes, Entomophthorales) was detected from mosquito adults (Montalva *et al.* 2016a,b; Rueda-Páramo *et al.* 2017). Among these entomopathogens that were reported for the first time from Brazil, the oomycete *L. chapmanii* seems to be the most promising candidate. This pathogen is currently being intensely studied for the control of *A. aegypti* larvae (Gutierrez *et al.* 2017).

The hypocrealean fungus *Tolypocladium cylindrosporum* (Hypocreales, Ophiocordycipitaceae) has not been reported from mosquitoes in Brazil or any other countries in South America. This fungus, which is frequently found in soils, is known as a natural antagonist of mosquito larvae in Northern America, New Zealand and Europe and also from other dipterans, other insect orders, and from spiders in Northern America, Europe and Nepal (Wright *et al.* 2009; Humber *et al.* 2014). *Tolypocladium cylindrosporum* has also been reported as an endophyte from plants (Hanada *et al.* 2010; Sánchez Márquez *et al.* 2010; Gazis *et al.* 2014; Zabalgogeazcoa *et al.* 2018). Whereas the larvicidal activity of *T. cylindrosporum* against species of genera such as *Aedes*, *Culex*, *Culiseta* and *Ochlerotatus* is well documented (Scholte *et al.* 2004; Rocha *et al.* 2015), there is still little information on the activity of this fungus in other mosquito developmental stages such as adults and eggs (Luz *et al.* 2007; Rocha *et al.* 2015; Flor-Weiler *et al.* 2017). We report here the first record of *T. cylindrosporum* from Brazil, and document its activity against eggs, larvae and adults of *A. aegypti* in laboratory conditions.

Materials and methods

Sampling, isolating and preserving mosquito pathogens

Between May 2014 and March 2016, aquatic entomopathogenic fungi were surveyed with sentinel mosquito larvae in a secondary tropical forest in the Santa Branca farm close to the city of Terezópolis de Goiás in Central Brazil. Batches of 20–25 *A. aegypti* second or third instar larvae (L2 or L3) reared in the laboratory as explained below were set into a transparent floatable plastic containers (85 × 60 mm) described by Montalva *et al.* (2016a). Containers were generally placed in the morning (8–10 AM) in selected locations protected by overhanging foliage from continuous sunlight in stagnant, shallow (10–25 cm depth) transitory bodies of water with high organic contents. The ambient air temperature and relative humidity (RH) were routinely measured at the time of placing these sentinel larvae. Containers were retrieved after a 36–48 h exposure. Each batch of larvae, whether alive or dead, was transferred with a previously disinfected (ethanol 70%) pipette to a sterile plastic tube (50 ml; Falcon Plastics, Brookings, SD) and then brought immediately in a cooler to the laboratory.

The protocols for isolating and preserving *in vitro* cultures of these pathogens were those used by Montalva *et al.* (2016a). The isolates were preserved in the culture collection (IP) of the Laboratory of Invertebrate Pathology at IPTSP at 4 and −80°C. In addition, they were co-deposited in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, NY) where these fungi are preserved cryogenically at −196°C.

Morphological evaluation

Isolates were grown at 25 ± 1°C and in darkness on quarter-strength Sabouraud dextrose agar plus yeast extract (SDAY/4; peptone 2·5 g l^{-1}, dextrose 10 g l^{-1}, yeast extract 2·5 g l^{-1}, agar 10 g l^{-1}) for 15 days. The fungi were investigated based on morphological characteristics, and semi-permanent slide mounts were prepared in lactophenol-cotton blue according to Bissett (1983) and Humber (2012). Fungal microstructures were examined by brightfield or phase contrast microscopy (Nikon Eclipse E600, Nikon Corporation, Tokyo, Japan), documented with a Nikon DS-Fi1 digital camera, and measured with Motic Images Plus 2.0 software. Measurements were based on 50 objects per microstructure from which we calculated mean values, standard error of the mean (±SE), and a range with the maximum and minimum values.

Molecular evaluations

The two isolates (IP 419 and IP 425) collected during the survey and the ex-type isolate *of T. cylindrosporum*, ARSEF 2920, were grown in 150 ml SDY/4 broth (SDAY/4 without added agar) for 7 days in a shaker at 125 rev min^{-1} and 25 ± 1°C. DNA was extracted from mycelium using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The internal transcribed spacer (ITS) regions ITS1, 5·8S and ITS2 were amplified by PCR using the primers ITS1 and ITS4 (White *et al.* 1990). Partial sequences of three other genes were also amplified: for the RNA polymerase II largest subunit (RPB1a) with primers RPB1C and RPB1Af (Stiller and Hall 1997); RNA polymerase II second largest subunit (RPB2a) with primers fRPB2-5F and RPB2-7cR

(Liu *et al.* 1999); and translation elongation factor 1-alpha (TEF exon) with primers 983F and 2218R (Rehner and Buckley 2005). PCR products were sent for purification and sequencing to Helixxa Genomic Services (Paulínia, SP, Brazil). Both strands of the PCR products were sequenced using the Applied Biosystems Big Dye ver. 3.1 kit and the same primers described above with an ABI 3500 automatic sequencer. Contigs of sequence data were assembled using Chromas Pro (ver. 1.5; Technelysium Pty Ltd, South Brisbaine, Qld, Australia), and sequence alignments were made with MEGA 7.0.26 by CLUSTALW (Kumar *et al.* 2016). All sequences were deposited in the GenBank database (Table 1). ITS sequences of other *Tolypocladium* species obtained from the GenBank (Table 1) were used to produce a Neighbor-Joining Tree using MEGA based on the *p*-distance method with 1000 replicates for bootstrap test. The parameter used to analyse gap/missing data was pairwise deletion.

GenBank accession numbers of further sequenced genes are RPB1a (MG228384); RPB2a (MG228387); TEF exon (MG228390) for ARSEF 2920; RPB1a (MG228382); RPB2a (MG228385); TEF exon (MG228388) for IP 419 and RPB1a (MG228383); RPB2a (MG228386); TEF exon (MG228389) for IP 425.

In vivo characterization

Aedes aegypti used in the reported studies on virulence originated from larvae collected in 2012 in Goiânia, Brazil and they were reared at 27 ± 5°C, RH 75 ± 10% and natural photophase (Lima *et al.* 2009; Rocha *et al.* 2015). The feeding technique was approved previously by the Ethics Commission for the Use of Animals of the Federal University of Goiás, Goiânia (CEUA 079/13, UFG, 10 February 2014).

The production and preparation of conidia of both isolates and the confirmation of their viability at the beginning of each assay were done as reported by Rocha *et al.* (2015). Three- to five-day-old eggs were prepared as reported by Luz *et al.* (2007), and then 50 μl of aqueous suspensions of conidia for each isolate were inoculated topically with a semi-automatic pipette onto the eggs at $3{\cdot}3 \times 10^3$; 10^4; $3{\cdot}3 \times 10^4$; 10^5 and $3{\cdot}3 \times 10^5$ conidia per cm^2. For the controls, 50 μl of sterile water were applied. Tests were run with a final number of 20 eggs for each isolate and conidial concentration. Further processing of eggs, incubation at 25 ± 1°C and RH >98% in a humid chamber up to 10 days, daily examination of fungal development on eggs and, after submersion in water, eclosion of larvae in the following 10 days were performed as reported by Luz *et al.* (2007).

Table 1 Reference species and strains used in *Tolypocladium* sp. phylogenetic analysis, with their respective country of origin and GenBank accession numbers for ITS sequences

Species	Strain	Country	ITS sequence
Tolypocladium album	MS490	Peru	JX155907
T. album	VPB140	Brazil	KF747263
T. album	VPB159	Brazil	KF747264
T. amazonense	LA100	Peru	HQ022485
T. amazonense	LA108	Peru	HQ022486
T. amazonense	MS308	Peru	JQ905653
T. capitatum	OSC 67004	–	EU834212
T. capitatum	FLAS-F-60359	USA	MF074845
*T. cylindrosporum**	ARSEF 2920	UK	MG228381
T. cylindrosporum**	**IP 419**	**Brazil**	**MG228379**
T. cylindrosporum**	**IP 425**	**Brazil**	**MG228380**
T. cylindrosporum	IHBF 2265	India	MF326612
T. cylindrosporum	NEFU45	China	KJ028796
T. endophyticum	MX575	Mexico	JX155949
T. endophyticum	MX486	Mexico	KF747245
T. endophyticum	NHB59	Brazil	KF747262
T. geodes	ARSEF 2684	–	FJ973059
T. geodes	–	–	Z54115
T. inflatum	NBRC 31668	Austria	AB103381
T. inflatum	NBRC 31669	USA	AB255606
T. inflatum	IFO 31669	–	AY245643
T. japonicum	BCRC FU30561	Taiwan	KT873533
T. longisegmentum	HMJAU6903	–	KJ866879
T. nubicola	ARSEF 3434	Canada	FJ973067
T. ophioglossoides	OSC 119566	–	EU834213
T. ophioglossoides	CBS_139.63	–	KU382154
T. ophioglossoides	CBS_100239	Denmark	KU382155
T. paradoxum	JFL14081002	–	KX017278
T. paradoxum	BA0332	–	KM357283
T. paradoxum	HKAS 87772	–	KX017279
T. parasiticum	ATCC 52203	–	U35305
T. parasiticum	–	–	U19039
T. parasiticum	–	–	Z54112
T. pustulatum	KaP8.2.2.1	–	KP698195
T. tropicale	MX337	Mexico	JQ905660
T. tropicale	IQ214	Peru	KF747254
T. tropicale	MX338	Mexico	KF747259
T. tundrense	ARSEF 3400	Canada	FJ973069
T. tundrense	–	–	U19041
T. tundrense	ATCC 56503	Canada	Z54108

*Denotes ex-type; **Denotes Brazilian isolates (in bold).

Ten third instar larvae (L3), 1–2 days after molting to this stage and without being fed for 24 h before treatment, were exposed in plastic cups (3·5 cm × 5 cm) to 25 ml of five conidial suspensions ($3{\cdot}3 \times 10^5$, 10^6, $3{\cdot}3 \times 10^6$, 10^7, or $3{\cdot}3 \times 10^7$ conidia per ml) of each isolate or to autoclaved distilled water for the control larvae. The larvae were then kept at 25 ± 1°C and a 12 h photophase for up to 10 days and fed on alternating days (Rocha *et al.* 2015). Water levels in cups were checked daily, and their original levels restored with sterile distilled water. Larval mortality in each cup was monitored daily, and any dead larvae were removed to an antibiotic water-agar medium (WA: 10 g l^{-1} agar, 1 g l^{-1} chloramphenicol, 10 mg l^{-1} crystal violet, 8 mg l^{-1} thiabendazol and pH 5·5; Leles *et al.* 2013) and incubated as mentioned. The development of fungi on dead larvae was checked daily for 10 days, and fungi were examined morphologically with a stereomicroscope (Leica EZ4, Leica Microsystems, Barra Funda, SP, Brazil) at 8–35× magnification.

Tests on adulticidal activity were run in transparent plastic cups (500 ml volume, 10 cm height, 7 cm diameter). The total inner surface (333 cm^2) was roughened with an abrasive paper (A-257, G220; Norton Saint-Gobain Abrasivos, Guarulhos, Brazil) in order to improve adhesion of conidia to the surface and provided with 50 randomly distributed circular openings of ≤1 mm diameter each to permit a permanent air exchange. Before treatment, cups were exposed for 15 min to ultraviolet light (UV-C Lamp Germicidal Ultraviolet G30T8; Royal Philips Electronics, Amsterdam, the Netherlands) to reduce the number of micro-organisms on their surfaces.

Conidia were suspended in water, and for each cup 100 ml of the suspension added to 200 mg of previously sterilized, finely powdered magnesium silicate as a carrier in a plastic dish in order to obtain a final concentration of $3{\cdot}3 \times 10^4$, 10^5, $3{\cdot}3 \times 10^5$, 10^6 or $3{\cdot}3 \times 10^6$ conidia per cm^2 treated surface. The carrier permitted both thee dilution and uniform delivery of conidia. Conidia and previously sterilized magnesium silicate were homogenized with a spatula, then dried in a desiccator at 4 ± 1°C for 3 days and then spread homogenously with a paintbrush on the whole inner surface of the cup. Subsequently, 10 adults, 1–3 days after emergence were set in each cup, and cups incubated at 25 ± 1°C, RH >98% and 12 h photophase up to 10 days. For the control groups, adults were exposed to cups treated with magnesium silicate only and maintained under the same conditions. During the test, adults were offered a 10% saccharose solution in a small glass container. Mortality was checked daily, dead individuals were transferred to WA, and the development of fungi on these cadavers was examined as mentioned.

Analysis of data

All tests against different stages of *A. aegypti* were carried out with four independent repetitions for all of the experiments. The relative values of mortality were arcsine-square root transformed and analysed with ANOVA and the Student-Newman-Keuls multiple range test to compare the means (STATISTICA 7.1; StatSoft, Tulsa, OK). Means were considered to be significantly different at $P < 0{\cdot}05$. Lethal times (LT_{50} and LT_{90}) and lethal concentrations (LC_{50} and LC_{90}) to kill 50 and 90% with their respective 95% confidence intervals were calculated by probit analysis for dependent and independent data, respectively (Preisler and Robertson 1989; Throne *et al.* 1995).

Results

During field collections in the Santa Branca farm during the dry season on 28 June and 12 July 2014, groups of sentinel larvae placed 20 m apart in the same location were recovered alive, brought to the laboratory and died in the following days. After transferring dead larvae to nutrient-free, antibiotic WA medium, mycelium and conidia that developed on the cadaver's surface were inoculated onto SDAY/4 medium supplemented with 1 g l^{-1} chloramphenicol (Montalva *et al.* 2016a). A total of two axenic cultures, designated as IP 419 (ARSEF 12855) collected on 28 June 2014, and IP 425 (ARSEF 12861), collected on 12 July 2014, in the two different locations was obtained and processed for use in the further studies.

Morphological findings

Both IP 419 and IP 425 formed wooly-floccose white colonies that subsequently turned to a slightly darker, off-white color after 15 days growth on SDAY/4. The cultures produced slender hyphae, short, cylindrical conidiophores with lateral or terminal whorls of phialides. The swollen phialides bore single-celled, oblong to cylindrical conidia; for IP 419, $4{\cdot}5 \pm 0{\cdot}1 \times 1{\cdot}4 \pm 0{\cdot}02$ μm ($3{\cdot}2$–$6{\cdot}1 \times 1$–$1{\cdot}8$ μm) and for IP 425, $4{\cdot}6 \pm 0{\cdot}1 \times 1{\cdot}3 \pm 0{\cdot}1$ μm ($3{\cdot}3$–$5{\cdot}6 \times 1$–$1{\cdot}7$ μm).

Molecular findings

A total of 3390 bp was obtained from the sequencing of four loci being 563 bp of the ITS region, 776 bp of RPB1, 1122 bp of RPB2 and 929 bp of TEF exon. Both Brazilian isolates IP 419 and IP 425 were 100% identical in all sequences of all genes being studied here. The sequences of the ITS, RPB1 and RPB2 regions of *T. cylindrosporum* ex-type isolate, ARSEF 2920 from the UK, were identical (100% similarity) to those found for the Brazilian isolates, and a single base pair of the TEF exon region differed between ARSEF 2920 and both IP 419 and IP 425 (99·89% similarity). The neighbor-joining phylogenetic analysis, based in ITS sequence obtained from GenBank, involved 16 species of *Tolypocladium* and 40 sequences of strains (Fig. 1). The Brazilian isolates clustered at a high bootstrap value (96%) with *T. cylindrosporum* ARSEF 2920 and NEFU45. The ITS sequence of these strains subsequently clustered with *T. cylindrosporum* IHBF 2265 which differed in only one base of the strains mentioned above (Fig. 1).

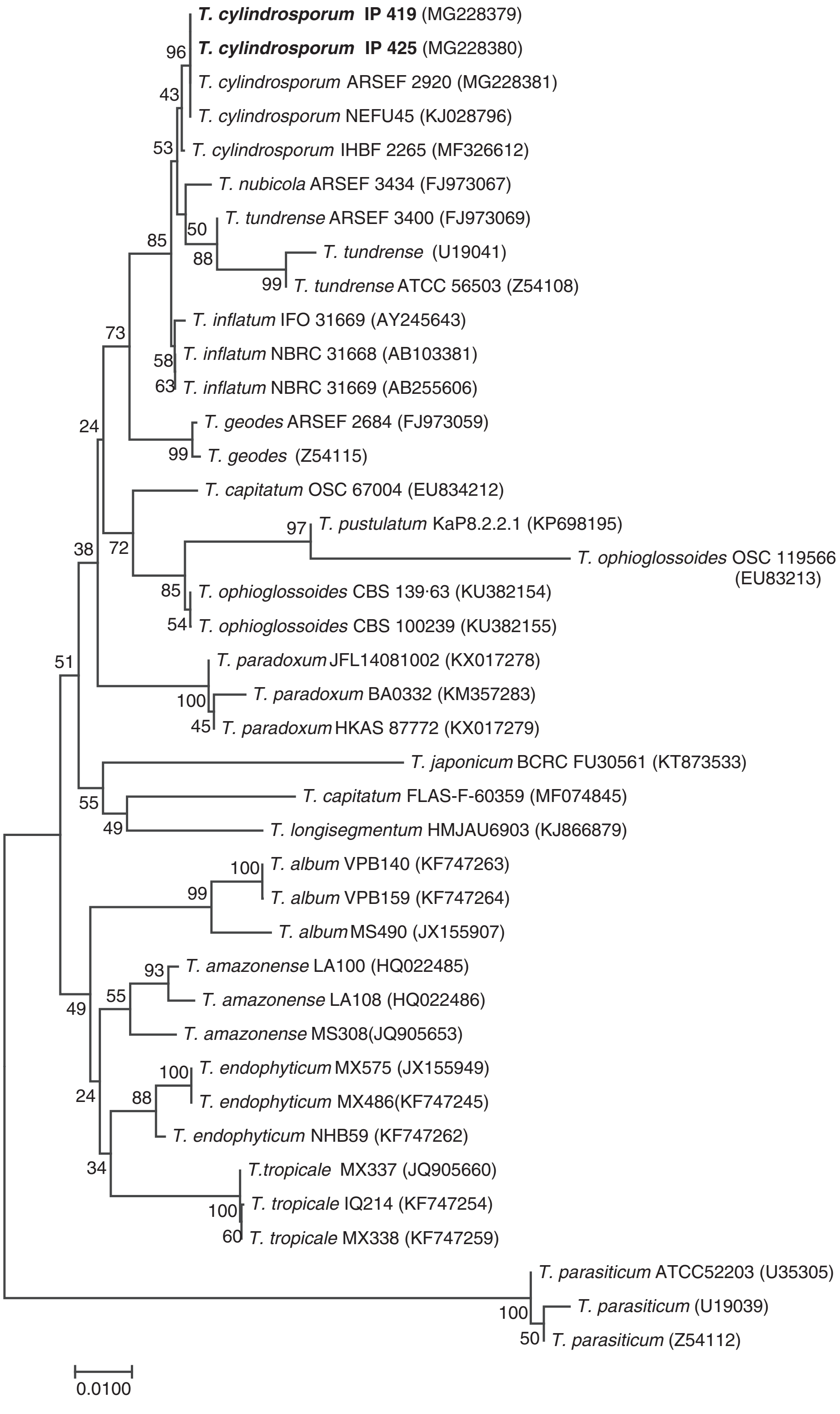

Figure 1 Phylogenetic tree based in Neighbor-Joining analysis of rRNA-ITS gene sequences. Molecular distances were calculated using the parameter of *p*-distance. Bootstrap percentages are shown for nodes (1000 replicates). GenBank codes of each sequence are included in parentheses next to the strain identifications.

Activity against *Aedes aegypti*

On eggs, the first visible hyphae and distinct mycelium were detected 2 and 3 days, respectively, after treatment with conidia regardless of the isolate tested. Conidiogenesis started 4 days later. After a 10-day incubation at 25 ± 5%, the eggs treated with IP 419 presented mycelium and conidia of *T. cylindrosporum* on their surface, whereas 8 ± 5% of eggs treated with IP 425 were found to bear mycelium but no conidia at the same time. In addition, a saprobic *Penicillium* sp. (Eurotiomycetes, Trichocomaceae) contaminant developed on ≤35% of the eggs regardless of the isolate and control; no attempt was made to identify this contaminant to the species level.

After submersion of eggs in water the first larvae enclosed immediately. The cumulative eclosion, 10 days after incubation of eggs in water ranged between 60·2 ± 2·3% (control, IP 425) and 85 ± 3·1% (IP 425 at 10^{5} conidia per cm^{2}) without significant effect of the conidial concentration (controls included) of either isolate on eclosion ($F_{5,15} \leq 1{\cdot}2$; $P \geq 0{\cdot}17$; Fig. 2).

Larvae reduced their movements and started dying within the first 24 h after application of conidia at highest $3{\cdot}3 \times 10^{7}$ conidia per ml. Cumulative mortality increased quickly at higher conidial concentrations in the next days and reached values up to 100% after a 5- and 10-day exposure without significant effect of the conidial concentration on mortality for IP 419 ($F_{4,15} \leq 2{\cdot}81$; $P \geq 0{\cdot}06$) but for IP 425 ($F_{4,15} \geq 5{\cdot}46$; $P \leq 0{\cdot}0064$; Table 2). Control mortality did not exceed 5% until the end of the tests. The values of LT_{50} and LT_{90} for both isolates decreased significantly with increasing conidial concentrations based on their confidence intervals; LT_{50} for IP 419 ranged from 3·7 days ($3{\cdot}3 \times 10^{5}$ conidia per ml) to 0·3 days ($3{\cdot}3 \times 10^{7}$ conidia per ml) and for IP 425 from 8·9 to 2·7 days at the same conidial concentrations (Table 2). Values of LT_{90} ranged from 6·5 to 2·8 days for IP 419 and from 15·8 to 6 days for IP 425 at the same minimal and maximal concentration of conidia tested (Table 2). While the values of LC_{50} for IP 419 ($3{\cdot}5 \times 10^{4}$ conidia per ml) and IP 425 ($5{\cdot}9 \times 10^{5}$ conidia per ml) differed significantly, those of LC_{90} ($\geq 6{\cdot}8 \times 10^{6}$ conidia per ml; Table 3) did not.

Dead larvae generally sank to the bottom of the cups, and after transfer onto WA medium, ≤51·5 ± 2·3% and ≤70·2 ± 3·6% for IP 419 and IP 425, respectively, at the different concentrations tested developed the mycelium and conidia typical for *T. cylindropsorum*.

The first dead adults were found 1 day after exposure to conidia at the highest concentrations for both IP 419 and IP 425. Mortality increased in the next days with a significant effect of the concentration on cumulative mortality, 10 and 15 days after incubation for IP 419 ($F_{4,15} \geq 3{\cdot}9$; $P \leq 0{\cdot}023$; Table 4) but not for IP 425 at the same periods ($F_{4,15} \leq 2{\cdot}22$; $P \geq 0{\cdot}12$; Table 4). Values of LT_{50} for adults treated with conidia varied between highest 16·8 days (IP 419 at 10^{5} conidia per cm^{2}) and lowest 7·1 days (IP 419 at $3{\cdot}3 \times 10^{6}$ conidia per cm^{2}) with significant difference for IP 419 but not for IP 425 (Table 4). Lowest LT_{90} of 12·9 days (IP 419 at $3{\cdot}3 \times 10^{6}$ conidia per cm^{2}) differed however without clear significance from most other values found for both IP 419 and IP 425, regardless of the concentration, and reached a highest LT_{90} value of 28·2 days (IP 419 at 10^{5} conidia per cm^{2}; Table 4). No differences were found for values of LC_{50} ($2{\cdot}1 \times 10^{4}$ conidia per cm^{2}; IP 419 and $2{\cdot}8 \times 10^{4}$ conidia per cm^{2}; IP 425) for both isolates and LC_{90} with $2{\cdot}3 \times 10^{6}$ conidia per cm^{2} (IP 419) and $1{\cdot}4 \times 10^{7}$ (IP 425) conidia per cm^{2} between both isolates

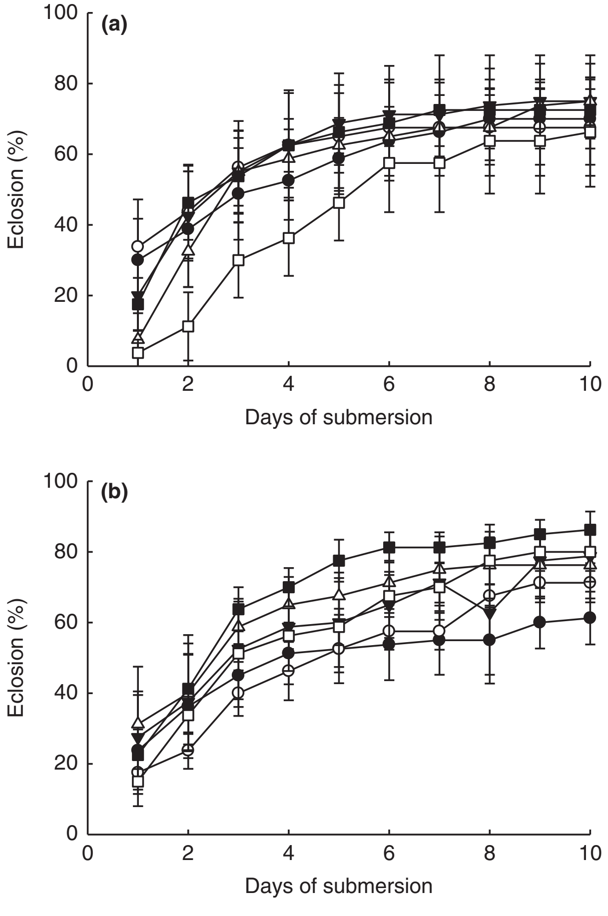

Figure 2 Relative cumulative mean eclosion (±standard error of the mean) of *Aedes aegypti* larvae after topical treatment of eggs with *Tolypocladium cylindrosporum* IP 419 (a) or IP 425 (b) conidia at $3{\cdot}3 \times 10^{3}$–$3{\cdot}3 \times 10^{5}$ conidia per cm^{2} or water only (control), incubation of eggs for 10 days at 25 ± 1°C and >98% humidity and subsequent submersion of eggs in water up to 10 days (–●– control; –○– $3{\cdot}3 \times 10^{3}$; –▼– 10^{4}; –△– $3{\cdot}3 \times 10^{4}$; –■– 10^{5}; –□– $3{\cdot}3 \times 10^{5}$).

Table 2 Relative cumulative mean mortality ± standard error (SE) and lethal time (days) to kill 50 or 90% (LT_{50} and LT_{90}) with their respective confidence interval (CI) and slope ± standard error of the mean (SE) of *Aedes aegypti* third instar larvae treated with *Tolypocladium cylindrosporum* IP 419 or IP 425 conidia (3·3 × 10^5–3·3 × 10^7 conidia per ml) and incubated at 25 ± 1°C and 12 h photophase for 10 days

		Mortality ± SE				
Isolate	Conidia per ml	5 days	10 days	LT_{50} (CI)	LT_{90} (CI)	Slope ± SE
IP 419	3·3 × 10^5	75 ± 8·7	90 ± 7·1	3·7 (2·9–4·5)^{b}	6·5 (5·6–7·8)^{b}	0·5 ± 0·07
	10^6	90 ± 7·1	92·5 ± 7·5	3 (2·4–3·5)^{b}	5·1 (4·4–6·1)^{b}	0·6 ± 0·09
	3·3 × 10^6	92·5 ± 7·5	97·5 ± 2·5	2·1 (1–3·1)^{ab}	5·7 (4·6–7·6)^{b}	0·4 ± 0·06
	10^7	100	100	2 (0·5–3·1)^{ab}	6 (4·5–9·6)^{b}	0·3 ± 0·07
	3·3 × 10^7	100	100	0·3 (0–1·1)^{a}	2·8 (2–4·2)^{a}	0·5 ± 0·12
	$F_{4,15}$	2·81	0·9	–	–	–
	P	0·06	0·49	–	–	–
IP 425	3·3 × 10^5	22·5 ± 8·5^{b}	52·5 ± 11·1^{c}	8·9 (7–11·6)^{b}	15·8 (12·8–22·1)^{b}	0·2 ± 0·04
	10^6	25 ± 12·6^{b}	60 ± 4·1b^{c}	8 (6–10·1)^{b}	14·3 (11·8–19·2)^{b}	0·2 ± 0·04
	3·3 × 10^6	40 ± 14·1^{b}	72·5 ± 7·5^{bc}	5·4 (3·7–7·2)^{ab}	11·7 (9·4–15·8)^{ab}	0·2 ± 0·04
	10^7	70 ± 20^{ab}	82·5 ± 11·1^{b}	3·4 (2·3–4·4)^{a}	7 (5·8–9)^{a}	0·4 ± 0·05
	3·3 × 10^7	95 ± 2·9^{a}	100^{a}	2·7 (0·1–4·7)^{a}	6 (4·1–11·4)^{a}	0·4 ± 0·06
	$F_{4,15}$	5·46	9·57	–	–	–
	P	0·0064	0·0005	–	–	–

Values, based on four repetitions at each concentration, in the same column for each isolate followed by different letters were significantly different based on ANOVA and SNK test (cumulated mortalities; a–c) or their CI (LT; a and b). Cumulative control mortality was ≤5% at a 10-day incubation.

Table 3 Lethal concentration (conidia per ml) to kill 50 or 90% (LC_{50} and LC_{90}) with their respective confidence interval (CI 95%) and slope ± standard error (SE) of *Aedes aegypti* third instar larvae treated with *Tolypocladium cylindrosporum* isolates and incubated 25 ± 1°C and 12 h photophase for 10 days

Isolate	LC_{50} (CI)	LC_{90} (CI)	Slope ± SE
IP 419	3·5 × 10^4 (3·3 × 10^2–1·7 × 10^5)^{a}	6·8 × 10^6 (2·7 × 10^6–4·1 × 10^7)^{a}	0·2 ± 0·04
IP 425	5·9 × 10^5 (2·5 × 10^5–1·1 × 10^6)^{b}	1·3 × 10^7 (7·5 × 10^6–2·6 × 10^7)^{a}	0·3 ± 0·04

Values, based on four repetitions at each concentration (3·3 × 10^5–3·3 × 10^7 conidia per ml), in the same column followed by different letters (a and b) were significantly different based on their CI. Cumulative control mortality was ≤5% at a 10-day incubation.

tested at a 15-day incubation (Table 5). Regardless of the conidial concentration applied, a total of 85·5 ± 2·2% and 65·7 ± 2·5% of the dead adults treated with IP 419 or IP 425, respectively, was found with mycelium and conidia of *T. cylindrosporum* on their surface after exposure on WA medium for 15 days. No mycelium was found on dead control adults incubated as mentioned.

Discussion

Both fungal isolates were readily identifiable as *T. cylindrosporum* of the Ophiocordycipitaceae family based on their morphological characteristics and genomic sequences (Weiser and Pillai 1981; Humber 2008, 2012; Quandt *et al.* 2014). The genus *Tolypocladium* was described by Gams (1971), and *T. cylindrosporum* was recognized as a species and insect pathogen by Weiser and Pillai (1981).

Further records in insects indicated that the natural host range of *T. cylindrosporum* includes insects from the orders Diptera, Coleoptera, Hymenoptera and Lepidoptera (Samson and Soares 1984; Scholte *et al.* 2004; Wright *et al.* 2009; Humber *et al.* 2014).

In South America, *T. cylindrosporum* had been reported previously only from soils in southern Argentina (Sosa-Gómez *et al.* 2010; Scorsetti *et al.* 2012). An unidentified *Tolypocladium* species was reported as an entomopathogen of *Leptopharsa heveae* (Hemiptera, Tingidae) in the Brazilian Mato Grosso (Humber *et al.* 2014). Other species of this genus have been found as endophytes of various plants in the Amazon region (Hanada *et al.* 2010; Gazis *et al.* 2014). To the best of our knowledge, this is the first report on the isolation of *T. cylindrosporum* in Brazil, and the first record of this species as a possible antagonist of mosquitoes or as a fungal pathogen present in any mosquito breeding sites in South America. Elsewhere, however, *T. cylindrosporum* has been repeatedly isolated from mosquito larvae in North America, Europe, Nepal and New Zealand (Weiser and Pillai 1981; Scholte *et al.* 2004; Humber *et al.* 2014). In

Table 4 Relative cumulative mean mortality ± standard error (SE), and lethal time (days) to kill 50 or 90% (LT_{50} and LT_{90}) with their respective confidence interval (CI) and slope ± standard error of the mean (SE) of *Aedes aegypti* adults treated with *Tolypocladium cylindrosporum* IP 419 or IP 425 conidia ($3{\cdot}3 \times 10^4$–$3{\cdot}3 \times 10^6$ conidia per cm^2) and incubated at 25 ± 1°C, >98% relative humidity and 12 h photophase for 15 days

Isolate	Conidia per cm^2	Mortality ± SE		LT_{50} (CI)	LT_{90} (CI)	Slope ± SE
		10 days	15 days			
IP 419	$3{\cdot}3 \times 10^4$	50 ± 12·2^{ab}	70 ± 12·2^{ab}	11·2 (6·6–17·2)^{ab}	21 (15·6–36·1)^{ab}	0·2 ± 0·02
	10^5	27·5 ± 4·8^{b}	47·5 ± 8·5^{b}	16·8 (12·8–22)^{b}	28·2 (22·4–41·7)^{b}	0·2 ± 0·04
	$3{\cdot}3 \times 10^5$	55 ± 6·5^{ab}	85 ± 2·9^{ab}	10·7 (5·2–17·3)^{ab}	20·1 (14·6–39·3)^{ab}	0·2 ± 0·02
	10^6	37·5 ± 16^{b}	70 ± 12·3^{ab}	12·1 (9·7–15·1)^{ab}	21·5 (18·1–27·8)^{ab}	0·2 ± 0·03
	$3{\cdot}3 \times 10^6$	80 ± 0^{a}	95 ± 2·9^{a}	7·1 (4·1–10)^{a}	12·9 (10·1–18·9)^{a}	0·2 ± 0·03
	$F_{4,15}$	4·18	3·9	–	–	–
	P	0·018	0·023	–	–	–
IP 425	$3{\cdot}3 \times 10^4$	27·5 ± 7·5	55 ± 11·9	14·4 (12·3–17)^{a}	22·2 (18·9–28·9)^{a}	0·2 ± 0·03
	10^5	17·5 ± 11·1	60 ± 12·9	14·6 (7·5–34·5)^{a}	26·4 (17·8–87·8)^{a}	0·2 ± 0·03
	$3{\cdot}3 \times 10^5$	40 ± 11·5	62·5 ± 12·5	13·8 (8·8–22·2)^{a}	26·2 (19·2–48·5)^{a}	0·2 ± 0·03
	10^6	42·5 ± 6·3	80 ± 7·1	11·6 (9·9–13·4)^{a}	18·1 (15·8–21·9)^{a}	0·2 ± 0·02
	$3{\cdot}3 \times 10^6$	32·5 ± 18·9	87·5 ± 7·5	11·6 (7·3–16·6)^{a}	18·3 (14·3–32·9)^{a}	0·2 ± 0·02
	$F_{4,15}$	0·84	2·22	–	–	–
	P	0·52	0·12	–	–	–

Values, based on four repetitions for each concentration in the same column followed by different letters were significantly different based on ANOVA and SNK test (cumulated mortalities; a–b) or their CI (LT; a–b). Cumulative control mortality was ≤10% at a 15-day incubation.

Table 5 Lethal concentration (conidia per cm^2) to kill 50 or 90% (LC_{50} and LC_{90}) with their respective confidence interval (CI 95%) and slope ± standard error (SE) of *Aedes aegypti* adults treated with *Tolypocladium cylindrosporum* IP 419 ou IP 425 and incubated 25 ± 1°C, ≥98% relative humidity and 12 h photophase for 15 days

Isolate	LC_{50} (CI)	LC_{90} (CI)	Slope ± SE
IP 419	$2{\cdot}1 \times 10^4$ ($2{\cdot}9 \times 10^3$–$5{\cdot}5 \times 10^4$)	$2{\cdot}3 \times 10^6$ ($9{\cdot}3 \times 10^5$–$1{\cdot}5 \times 10^7$)	0·4 ± 0·06
IP 425	$2{\cdot}8 \times 10^4$ ($1{\cdot}2 \times 10^3$–$8{\cdot}5 \times 10^4$)	$1{\cdot}4 \times 10^7$ ($2{\cdot}9 \times 10^6$–$2{\cdot}3 \times 10^9$)	0·4 ± 0·05

Values, based on four repetitions at each concentration ($3{\cdot}3 \times 10^4$–$3{\cdot}3 \times 10^6$ conidia per cm^2). Cumulative control mortality was ≤10% at a 15-day incubation.

addition, it was reported to cause epizootics in *Aedes sierrensis* (Diptera, Culicidae) populations in tree holes in California, USA (Sanders 1972).

Both Brazilian isolates were detected using *A. aegypti* sentinel larvae in the same location at sites separated by a short distance in 2014 during the dry season in stagnant, shallow transitory bodies of water with high organic contents in a secondary tropical forest. The natural hosts of those isolates are not known but dipterans–especially culicid aquatic larval stages occurring throughout the year in these breeding sites–are the most likely hosts. *Aedes aegypti* larvae do not commonly occur in these locations and were, therefore, useful as sentinels to detect specific entomopathogenic fungi since they were known to be susceptible to infection with this particular fungus. Previous studies have shown that both virulence and the susceptibility of various mosquito host larvae varies according to the fungal isolate used (Soares *et al.* 1986; Rocha *et al.* 2015) and raised the interest to investigate the activities of such fungi against the egg and adult stages of this important vector.

Neither of the Brazilian *T. cylindrosporum* isolates had any clear effect against *A. aegypti* eggs in the period of 10 days or within the range of conidial concentrations tested ($3{\cdot}3 \times 10^3$–$3{\cdot}3 \times 10^5$ conidia per cm^2). The fungus was obviously able to develop mycelium and new conidia on the chorion of a small fraction of eggs but did not affect unenclosed larvae inside those eggs nor induce premature eclosion as reported in other studies with the same fungus (Flor-Weiler *et al.* 2017). The little information available on *T. cylindrosporum* suggests that the ovicidal activity of this entomopathogen against aedine eggs depends on the isolate, a high conidial concentration (5×10^6 conidia per cm^2, not tested in the present study), and a more extended incubation time (>10 days) at high moisture (Luz *et al.* 2007; Rocha *et al.* 2015; Flor-Weiler *et al.* 2017). In fact, other fungi, especially those from the genus *Metarhizium* (e.g., *M. anisopliae* s.l. IP 46), seem to be much more effective as egg antagonists for biological control of *A. aegypti*. These fungi act against eggs much more rapidly and at lower conidial concentrations than does *T. cylindrosporum* (Luz *et al.*

2008; Albernaz *et al.* 2009; Santos *et al.* 2009; Sousa *et al.* 2013).

Both isolates were highly active against larvae but less so against adults. IP 419 was able to kill larvae more rapidly and at lower conidial concentrations than IP 425. While the adults of *A. aegypti* were susceptible to both of these isolates, there were no clearly significant differences in either their lethal times or effective conidial concentrations. Both isolates required 10–15 days to kill most of the adults and were able to develop on larvae and adults set onto WA medium.

Tolypocladium cylindrosporum is best known for its activity against aquatic mosquito larvae (Scholte *et al.* 2004; Rocha *et al.* 2015) but, depending on the isolate, this species can also affect aedine eggs and adults (Luz *et al.* 2007; Rocha *et al.* 2015; Flor-Weiler *et al.* 2017). *Aedes aegypti* larvae seem to be the key stage to target with *T. cylindrosporum*, because the recycling of the pathogen on mycotized larvae in transient breeding sites should eventually result in infections of other larvae and gravid females coming to these sites to oviposit. It is remarkable that two isolates with such high genetic similarity collected in the same location and at a short time difference showed such divergent virulence characteristics against *A. aegypti* larvae. These findings emphasize the importance of collecting natural antagonists of mosquito vectors. In the present study, IP 419 proved itself to be a promising native candidate for fungus-based biological control of *A. aegypti* in Brazil with *T. cylindrosporum* due to its high virulence, especially against larvae.

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Conflict of Interest

There are no conflicts of interest to declare.

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