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Beauveria bassiana and *Metarhizium anisopliae in vitro* germination in culture media supplemented with the phases of the development of *Aedes aegypti*

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Abstract
In this study was to evaluate the conidial germination of EF strains: *Metarhizium anisopliae* (1 strain) and *Beauveria bassiana* (2 strains) in two culture media (Agarose and Sabouraud Dextrose Agar [SDA]) supplemented with eggs, larvae, pupae and adults of *Aedes aegypti* vector. *M. anisopliae* 028 presented the highest germination on Agarose media supplemented with larvae or pupae (99% and 98% at 22 hours) and (97% and 98% at 12 hours) on SDA media supplemented with larvae or adults, *B. bassiana* 120 presented the highest germination percentages on the SDA media, as on Agarose (99% and 98% in 12 and 20 hours respectively) supplemented with larvae or pupae. Respect to *B. bassiana* 103 strain by the vector addition to the culture medium, no effect was observed. The results indicate that *M. anisopliae* 028 and *B. bassiana 120* strains are *Ae. aegypti* potentials biocontrollers agents.

Keywords: *Aedes*, Metarhizium, conidial germination, cuticle

Introduction

Diseases transmitted by mosquito vectors, involve asymptomatic cases such as fever Zika virus [1], incapacitants such as Chikungunya fever and more severe cases such as severe Dengue that can lead to death [2]. *Ae. aegypti* is the main vector of these diseases, distributed globally [3]. The most used strategy to reduce the vector density is with chemical insecticides: spatial spray and larval control; however, scientific studies have shown that chemical insecticides cause resistance in mosquitoes [4, 5], damage to human health [6] and negative effects on beneficial insects [7].

Facing to this problem, it is necessary to propose less aggressive alternatives that encourage the use reduction of insecticides; community participation is also very important [8]. In this sense, EFs against *Ae. aegypti* is a viable strategy to reduce chemical inputs for vector control; *B. bassiana* and *M. anisopliae* are the most used EFs [9-11]. EF strains differ in the range of hosts; while *B. bassiana* is more specific with its guests, *M. anisopliae* is a generalist and attacks a wide range of arthropods from crop pests to disease vectors in humans and animals [12].

The pathogenicity of EFs (*B. bassiana* and *M. anisopliae*) concerning *Ae. aegypti* depends mainly on conidial germination, which involves several factors such as relative humidity, temperature [13, 14] and availability of nutrients such as carbohydrates, among others [15]. The first step for EF colonization is the conidia adhesion to the insect cuticle [16], after adhesion, the conidia germinate, penetrate and invade their host [17], in addition, EFs can be disseminated secondarily to other host population members [18].

Research has shown that supplementation of culture media with insect cuticles induces virulence in EFs [19] and, that is related to enzymatic activity; mainly by the proteases and chitinases production [20-22]. The aim research was *in vitro* evaluate the *B. bassiana* and *M. anisopliae* conidial germination, in two culture media supplemented with the different development *Ae. aegypti* stages.

2. Materials and methods

2.1 *Ae. aegypti*

Ae. aegypti in different development stages (eggs, larvae, pupae and adults) were provided by Medical Entomology Lab., of Center for Research on Tropical Diseases of the Autonomous University of Guerrero, Mexico (CIET-UAGro); the breeding conditions were temperature to 30 ± 2 °C, 80 ± 5% HR, photoperiod 16/8 (L/D) [23]. Larval feeding was done with a commercial diet based on fishmeal. The adults were fed 10% glucose solution and were sacrificed 24 hours after hatching.

2.2 Entomopathogenic fungi

EFs used, two *B. bassiana's* (103 y 120) and one of *M. anisopliae* (028), were donated by Biotechnology Research Center, of the Autonomus University of Morelos, Mexico. The fungi propagation was carried out on SDA Petri dishes, incubated for 15 days at 30 ± 5 ºC, 75 ± 5% relative humidity and photoperiod of 12/12 (L/D).

2.3 Conidial suspension

Conidial suspension was prepared by pouring 10 ml of sterile distilled water plus 0.01% Tween 80 (v/v) into each plate with fungal growth, it was scraped with a Drigalski spatula to obtain the conidial biomass, which was collected in test tubes and stirred for two minutes with a Vortex®. Starting from the fact that each EF isolate has its own sporulation [24, 25], the conidia suspension was adjusted to 3.25 x 10^9, 3.5 x 10^8 y 2 x 10^9 conidia/ml (*M. anisopliae* 028, *B. bassiana* 103 y *B. bassiana* 120 strains, respectively).

2.4 Treatments

Eggs, larvae, pupae and adults of *Ae. aegypti* were disinfected with sodium hypochlorite 0.5% (v/v), they were macerated in porcelain crucible in sterile distilled water and sterilized to 120 ºC (15 lb/pul^2) for 20 minutes, each treatment consisted 50 ml of each medium (Agarose and SDA) at a final concentration of 1% (w/v) of each mash; ten treatments were obtained: Agarose (control), Agarose+eggs, Agarose+larvae, Agarose+pupae, Agarose+adults, SDA (control), SDA+eggs, SDA+larvae, SDA+pupae and SDA+adults. On slides (75 x 25 mm), 3.5 ml of each treatment was placed, forming thin layers of culture medium, after solidification, 100 µl of the inoculum was added, finally, they were incubated in humid chambers (90 mm Petri dishes with moistened filter paper) to 30 ± 5 °C, 75 ± 5% HR [26, 27].

2.5 Conidial germination

Conidial germination was measured in 10 time levels (Table 1). A conidia was defined as germinated when its germ tube was equal to or greater than its width. Germination was estimated using the compound microscope Primo Star Carl Zeiss ® (objective 40x), the number of germinated and ungerminated conidia in two fields was considered (50 per field), expressing the result as a germinated conidia percentage, each experimental unit was evaluated for triplicate.

2.6 Statistical analysis

The data were subjected to a two-factor variance analysis (ANOVA), where one of them has repeated measures. According to two culture media (Agarose and SDA), the analysis was carried out making segmentations and, five treatments per medium (control, eggs, larvae, pupae and adults) on the conidial germination of three EFs (*M. anisopliae* 028, *B. bassiana* 103 and 120). Intra-subject factor was defined as time with ten levels (0, 2, 4, 8, 12, 14, 16, 18, 20 y 22) and, as an inter-subject factor the treatment with five levels (control, eggs, larvae, pupae and adults). The significant differences between the mean conidial germination over time and five treatments, were applied the Lambda Wilks and Roy Root Multivariate tests, as well as the intra-subject effects tests, assumed Sphericity, Greenhouse-Geisser, Huynh-Feldt and lower limit. To evaluate the significant differences between treatments, were used the sums of squares and the F-Fisher statistic. To observe on conidial germination, the groups in the factor intra-subjects (time), multiple comparisons were made *a posteriori* with Bonferroni´s adjustment; the comparisons were made between time factor levels, with segmentation of data matrix from factors such as strain and media. All statistical tests were performed with statistical software (IBM SPSS Statistics 23, 2016).

3. Results

According to Lambda Wilks and Roy Root multivariate tests, in media (Agarose and SDA) with the three strains, significant differences were observed ($P \leq 0.05$) as with respect to conidial germination percentage, both in the intra-subject factor (time) and, in the corresponding interactions between time and treatment.

Observing the evidence of intra-subject effects such as assumed Sphericity, Greenhouse-Geisser, Huynh-Feldt as well as the lower limit, it is possible to corroborate the results of multivariate tests. In this way, in Agarose media with the three strains (*M. anisopliae* 028; *B. bassiana* 103 and *B. bassiana* 120) significant differences in conidial germination were observed over time, in addition, the interaction between time and treatment was statistically significant ($P \leq 0.05$) to *M. anisopliae* 028 and *B. bassiana* 120 strains. Regarding SDA media, it was observed significant differences ($P \leq 0.05$) for the three strains (*M. anisopliae* 028; *B. bassiana* 103 and *B. bassiana* 120) with respect to germination over time and effect on the interaction between time and treatment.

3.1 Time effect on conidial germination

Regarding conidial germination as a function of time expressed as a percentage, *M. anisopliae* 028 strain, showed on Agarose significant differences ($P > 0.05$) in all time levels (0 ± 0.00% 0 h to 98 ± 0.33% 22 h) meanwhile, there were only significant differences on SDA ($P > 0.05$) from 2 h (1 ± 0.24%) to 14 h (99 ± 0.34%). *B. bassiana* 103 strain showed significant differences ($P > 0.05$) between the two media (Agarose and SDA), 2 h (0 ± 0.00% for both) to 18 h (99 ± 0.18% and 99 ± 0.25% respectively). Germination of *B. bassiana* 120 strain on Agarose, presented significant differences ($P > 0.05$) in all the time levels (0 ± 0.00 0 h to 92 ± 0.50% 22 h); however, on SDA significant differences ($P > 0.05$) were observed from 0 h (0 ± 0.00%) to 8 h (99 ± 0.47%) (Table 1).

Table 1: Conidia germination percentage of *M. anisopliae* 028, *B. bassiana* 103 and *B. bassiana* 120 strains on Agarose and SDA culture media.

Time (h)	Entomopathogenic fungi					
	***M. anisopliae* 028**		***B. bassiana* 103**		***B. bassiana* 120**	
	Agarose	**SDA**	**Agarose**	**SDA**	**Agarose**	**SDA**
0	0±0.00[j]	0±0.00[e]	0±0.00[g]	0±0.00[g]	0±0.00[j]	0±0.00[e]
2	1±0.12[i]	1±0.24[e]	0±0.00[g]	0±0.00[g]	5±0.28[i]	6±0.49[d]
4	4±0.18[h]	2±0.18[d]	3±0.45[f]	5±0.35[f]	9±0.21[h]	20±0.58[c]
8	10±0.70[g]	38±0.88[c]	28±0.85[e]	42±0.50[e]	23±0.60[g]	65±0.99[b]
12	18±0.46[f]	85±0.83[b]	63±0.59[d]	83±0.48[d]	38±0.84[f]	99±0.47[a]
14	25±0.63[e]	99±0.34[a]	82±0.62[c]	93±0.51[c]	65±0.98[e]	100±0.00[a]
16	38±0.61[d]	100±0.00[a]	95±0.47[b]	97±0.45[b]	70±1.06[d]	100±0.00[a]
18	55±0.63[c]	100±0.00[a]	99±0.18[a]	99±0.25[a]	82±0.55[c]	100±0.00[a]
20	83±0.95[b]	100±0.00[a]	100±0.00[a]	100±0.00[a]	86±0.57[b]	100±0.00[a]
22	98±0.33[a]	100±0.00[a]	100±0.00[a]	100±0.00[a]	92±0.50[a]	100±0.00[a]

Average ± EE (Standard error), values with different letter represent significant differences (adjusted by Bonferroni $p \leq 0.05$).
h= Time measured in hours.

3.2 Time-treatment interaction effect on conidial germination

About *M. anisopliae* 028 strain, all treatments on Agarose the maximum germination was reached in 22 h (>96 ± 0.74%), without observing significant differences. Regarding *M. anisopliae* 028 strain conidial germination and time-treatment interaction, all treatments showed significant differences ($P>0.05$), germination at 14, 16 and 18 h on Agarose media supplemented with pupae (37 ± 1.40%, 65 ± 1.37%, 72 ± 1.39%, respectively) it was greater in comparison with the other treatments in those same times; however, at 20 h, supplemented treatments with larvae and adults developed greater germination (88 ± 2.11%) than those supplemented by pupae (82 ± 2.11%). In contrast, for treatments on SDA media, maximum germination was reached at 14 h (>97 ± 0.76%), without significant differences (Fig 1 and 2).

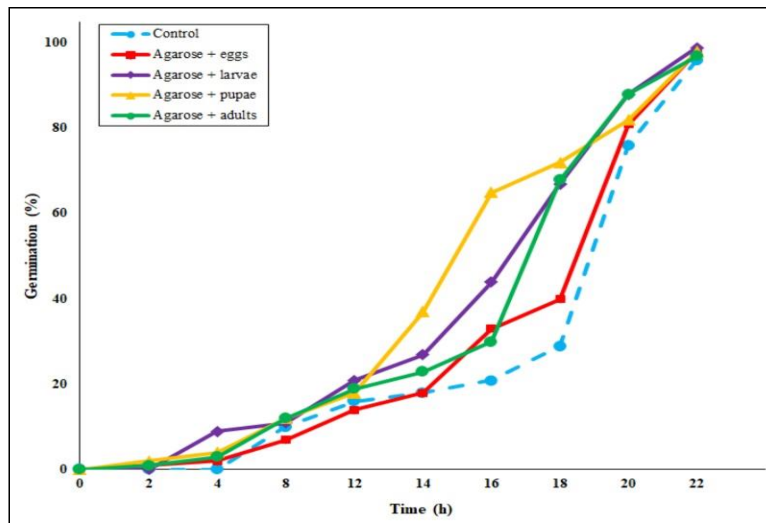

Fig 1: Conidia germination kinetics of *M. anisopliae* 028 in Agarose. The segmented line represents the Control = Agarose without supplementing; each continuous line with a different marker represents a different treatment; each treatment was supplemented with each of the stages of development of *Aedes aegypti*.

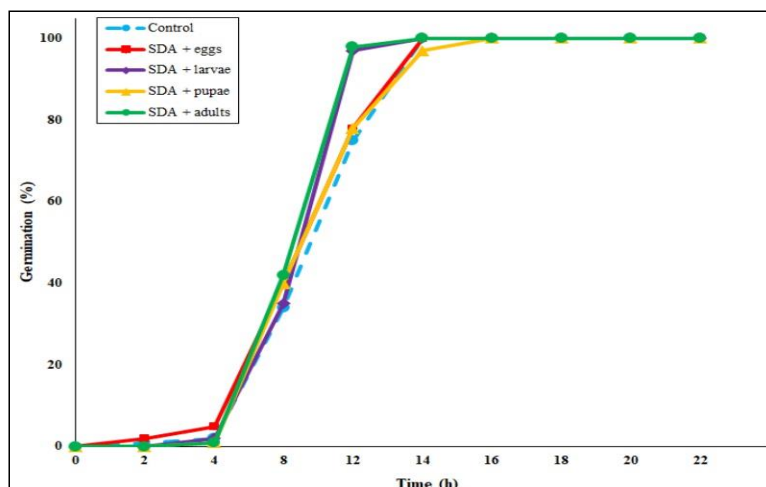

Fig 2: Conidia germination kinetics of *M. anisopliae* 028 in SDA. The segmented line represents the Control = SDA without supplementing; each continuous line with a different marker represents a different treatment; each treatment was supplemented with each of the stages of development of *Aedes aegypti*.

The treatments of SDA media supplemented with larvae and adults, presented the highest germination averages (97 ± 1.86% and 98 ± 1.86% respectively) at 12 h, showing significant differences (P>0.05) with respect to the rest of the treatments (Table 2).

Table 2: Time-treatment interaction effect of *M. anisopliae* 028 strain conidial germination on Agarose and SDA media.

Treatments	***M. anisopliae* 028 in Agarose**									
	Time (h)									
	0	**2**	**4**	**8**	**12**	**14**	**16**	**18h**	**20**	**22**
Control	0±0.00^{a}	0±0.26^{b}	0±0.39^{c}	10±1.56^{a}	16±1.03^{c}	18±1.40^{c}	21±1.37^{d}	29±1.39^{d}	76±2.11^{b}	96±0.74^{a}
Eggs	0±0.00^{a}	1±0.26^{b}	2±0.39^{c}	7±1.56^{a}	14±1.03^{c}	18±1.40^{c}	32±1.37^{c}	40±1.39^{c}	81±2.11^{b}	98±0.74^{a}
Larvae	0±0.00^{a}	0±0.26^{b}	9±0.39^{a}	11±1.56^{a}	21±1.03^{a}	27±1.40^{b}	44±1.37^{b}	68±1.39^{b}	88±2.11^{a}	99±0.74^{a}
Pupae	0±0.00^{a}	2±0.26^{a}	4±0.39^{b}	12±1.56^{a}	18±1.03^{b}	37±1.40^{a}	65±1.37^{a}	72±1.39^{a}	82±2.11^{b}	98±0.74^{a}
Adults	0±0.00^{a}	1±0.26^{b}	3±0.39^{b}	12±1.56^{a}	19±1.03^{b}	23±1.40^{b}	30±1.37^{c}	68±1.39^{b}	88±2.11^{a}	97±0.74^{a}
	M. anisopliae 028 in SDA									
Control	0±0.00^{a}	1±0.54^{a}	2±0.39^{b}	34±1.97^{b}	75±1.86^{b}	100±0.76^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
Eggs	0±0.00^{a}	2±0.54^{a}	5±0.39^{a}	40±1.97^{a}	78±1.86^{b}	100±0.76^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
Larvae	0±0.00^{a}	0±0.54^{a}	2±0.39^{b}	35±1.97^{b}	97±1.86^{a}	100±0.76^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
Pupae	0±0.00^{a}	0±0.54^{a}	1±0.39^{b}	40±1.97^{a}	78±1.86^{b}	97±0.76^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
Adults	0±0.00^{a}	0±0.54^{a}	1±0.39^{b}	42±1.97^{a}	98±1.86^{a}	100±0.76^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}

Average ± EE (Standard error), values with different letter represent significant differences (adjusted by Bonferroni p≤0.05).
h= Time measured in hours.

No significant differences were observed between *B. bassiana* 103 strain conidial germination (time-treatment interaction) on Agarose and SDA (Table 3). In both media the maximum germination was observed at 18 h (>98%) (Fig 3 and 4).

Table 3: Interaction time-treatment effect on *B. bassiana* 103 conidial germination on Agarose and SDA media.

***B. bassiana* 103 in Agarose**										
Treatments	**Time (h)**									
	0	**2**	**4**	**8**	**12**	**14**	**16**	**18**	**20**	**22**
Control	0±0.00^{a}	0±0.00^{a}	0±1.00^{a}	30±1.91^{a}	63±1.33^{a}	81±1.38^{a}	92±1.05^{b}	99±0.39^{a}	100±0.00^{a}	100±0.00^{a}
Eggs	0±0.00^{a}	0±0.00^{a}	3±1.00^{a}	28±1.91^{a}	63±1.33^{a}	82±1.38^{a}	98±1.05^{a}	100±0.39^{a}	100±0.00^{a}	100±0.00^{a}
Larvae	0±0.00^{a}	0±0.00^{a}	3±1.00^{a}	28±1.91^{a}	65±1.33^{a}	82±1.38^{a}	96±1.05^{a}	100±0.39^{a}	100±0.00^{a}	100±0.00^{a}
Pupae	0±0.00^{a}	0±0.00^{a}	3±1.00^{a}	28±1.91^{a}	63±1.33^{a}	81±1.38^{a}	96±1.05^{a}	100±0.39^{a}	100±0.00^{a}	100±0.00^{a}
Adults	0±0.00^{a}	0±0.00^{a}	3±1.00^{a}	27±1.91^{a}	63±1.33^{a}	83±1.38^{a}	95±1.05^{a}	98±0.39^{a}	100±0.00^{a}	100±0.00^{a}
***B. bassiana* 103 in SDA**										
Control	0±0.00^{a}	0±0.00^{a}	2±0.79^{b}	42±1.13^{a}	82±1.07^{a}	93±1.14^{a}	99±1.00 ^{a}	100±0.56^{a}	100±0.00^{a}	100±0.00^{a}
Eggs	0±0.00^{a}	0±0.00^{a}	4±0.79^{b}	42±1.13^{a}	83±1.07^{a}	93±1.14^{a}	96±1.00 ^{a}	100±0.56^{a}	100±0.00^{a}	100±0.00^{a}
Larvae	0±0.00^{a}	0±0.00^{a}	8±0.79^{a}	45±1.13^{a}	85±1.07^{a}	96±1.14^{a}	99±1.00 ^{a}	100±0.56^{a}	100±0.00^{a}	100±0.00^{a}
Pupae	0±0.00^{a}	0±0.00^{a}	4±0.79^{b}	41±1.13^{a}	82±1.07^{a}	92±1.14^{a}	95±1.00 ^{a}	98±0.56^{a}	100±0.00^{a}	100±0.00^{a}
Adults	0±0.00^{a}	0±0.00^{a}	5±0.79^{b}	42±1.13^{a}	82±1.07^{a}	92±1.14^{a}	95±1.00 ^{a}	98±0.56^{a}	100±0.00^{a}	100±0.00^{a}

Average ± EE (Standard error), values with different letter represent significant differences (adjusted by Bonferroni p≤0.05).
h= Time measured in hours.

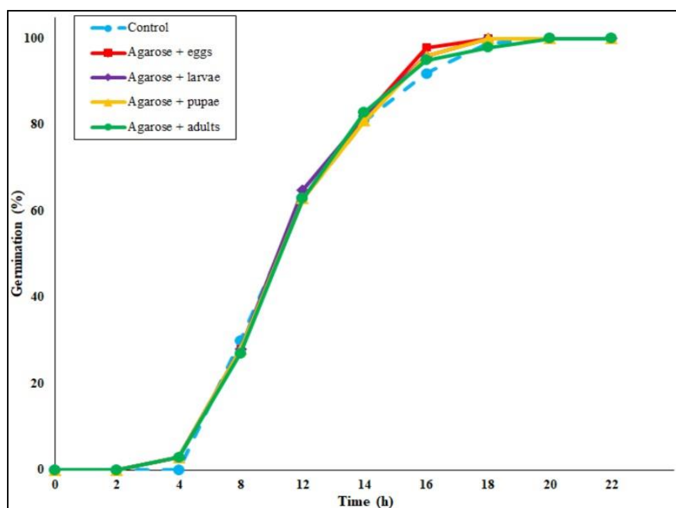

Fig 3: Conidia germination kinetics of *B. bassiana* 103 in Agarose. The segmented line represents the Control = Agarose without supplementing; each continuous line with a different marker represents a different treatment; each treatment was supplemented with each of the stages of development of *Aedes aegypti*.

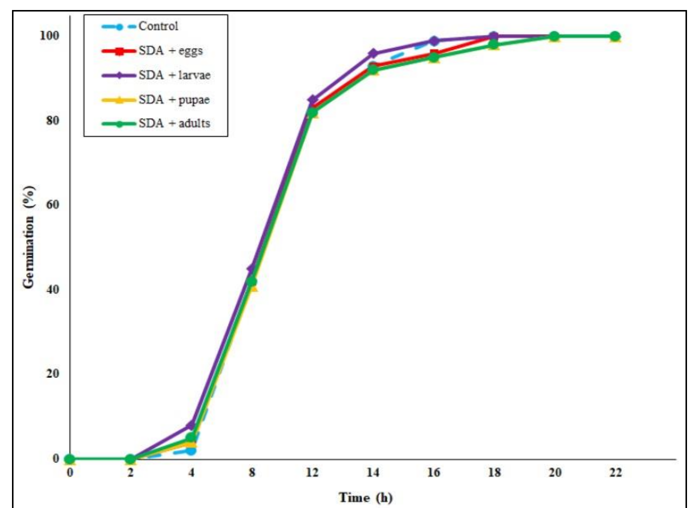

Fig 4: Conidia germination kinetics of *B. bassiana* 103 in SDA. The segmented line represents the Control = SDA without supplementing; each continuous line with a different marker represents a different treatment; each treatment was supplemented with each of the stages of development of *Aedes aegypti*.

The greater conidial germination of *B. bassiana* 120 strain (time-treatment interaction) was presented at 12 h on Agarose media; supplemented with pupae (54 ± 1.87%), followed by the one supplemented with adults (51 ± 1.87%); both showed significant differences (P>0.05) with respect to the rest of treatments in that same time (14, 16, 18, 20 and 22 h); on the other hand, the treatments with greater germination were the media supplemented with larvae (88 ± 2.20%, 92 ± 2.37%, 96 ± 1.22%, 99 ± 1.27% and 99 ± 1.13%) and pupae (88 ± 2.20%, 92 ± 2.37%, 94 ± 1.22%, 98 ± 1.27% and 100 ± 1.13%) with significant differences (P>0.05) with respect to the rest of treatments. On SDA media, the supplement treatment with larvae reached 50% (± 1.29) germination at 4 h with significant differences (P>0.05) with respect to the rest of the treatments. At 8 h, the treatments supplemented with larvae and pupae reached the highest values of conidial germination (72 ± 2.21% and 74 ± 2.21% respectively), with significant differences (P>0.05), compared to the other treatments (Table 4); on Agarose, the maximum germination it was observed in 20 h (>99%) and at 12 h on SDA (>98%), see Fig 5 and 6.

Table 4: Interaction time-treatment effect on *B. bassiana* 120 conidial germination on Agarose and SDA media.

***B. bassiana* 120 in Agarose**										
Treatments	**Time (h)**									
	0	**2**	**4**	**8**	**12**	**14**	**16**	**18**	**20**	**22**
Control	0 ± 0.00^{a}	0 ± 0.63^{c}	0±0.47d	18 ± 1.34^{b}	21 ± 1.87^{c}	37 ± 2.20^{b}	43 ± 2.37^{c}	62 ± 1.22^{d}	71 ± 1.27^{c}	83 ± 1.13^{c}
Eggs	0 ± 0.00^{a}	3 ± 0.63^{c}	10±0.47c	20 ± 1.34^{b}	25 ± 1.87^{c}	29 ± 2.20^{b}	39 ± 2.37^{c}	69 ± 1.22^{c}	72 ± 1.27^{c}	81 ± 1.13^{c}
Larvae	0 ± 0.00^{a}	5 ± 0.63^{b}	12±0.47b	20 ± 1.34^{b}	40 ± 1.87^{b}	88 ± 2.20^{a}	92 ± 2.37^{a}	96 ± 1.22^{a}	99 ± 1.27^{a}	99 ± 1.13^{a}
Pupae	0 ± 0.00^{a}	5 ± 0.63^{b}	10±0.47c	28 ± 1.34^{a}	54 ± 1.87^{a}	88 ± 2.20^{a}	92 ± 2.37^{a}	94 ± 1.22^{a}	98 ± 1.27^{a}	100 ± 1.13^{a}
Adults	0 ± 0.00^{a}	13 ± 0.63^{a}	15±0.47a	28 ± 1.34^{a}	51 ± 1.87^{a}	82 ± 2.20^{a}	84 ± 2.37^{b}	90 ± 1.22^{b}	92 ± 1.27^{b}	95 ± 1.13^{b}
***B. bassiana* 120 in SDA**										
Control	0 ± 0.00^{a}	6 ± 1.10^{b}	12 ± 1.29^{b}	54 ± 2.21^{c}	98 ± 1.05^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Eggs	0 ± 0.00^{a}	6 ± 1.10^{b}	15 ± 1.29^{b}	61 ± 2.21^{b}	98 ± 1.05^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Larvae	0 ± 0.00^{a}	8±1.10a	50 ± 1.29^{a}	72 ± 2.21^{a}	99 ± 1.05^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Pupae	0 ± 0.00^{a}	4 ± 1.10^{b}	11 ± 1.29^{b}	74 ± 2.21^{a}	98 ± 1.05^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}
Adults	0 ± 0.00^{a}	5 ± 1.10^{b}	13 ± 1.29^{b}	63 ± 2.21^{b}	100 ± 1.05^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Average ± EE (Standard error), values with different letter represent significant differences (adjusted by Bonferroni $p\leq0.05$).
h= Time measured in hours.

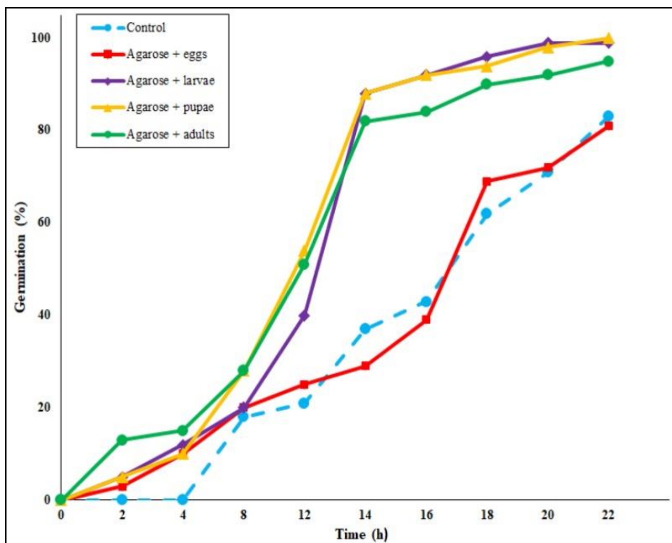

Fig 5: Conidia germination kinetics of *B. bassiana* 120 in Agarose. The segmented line represents the Control = Agarose without supplementing; each continuous line with a different marker represents a different treatment; each treatment was supplemented with each of the stages of development of *Aedes aegypti*.

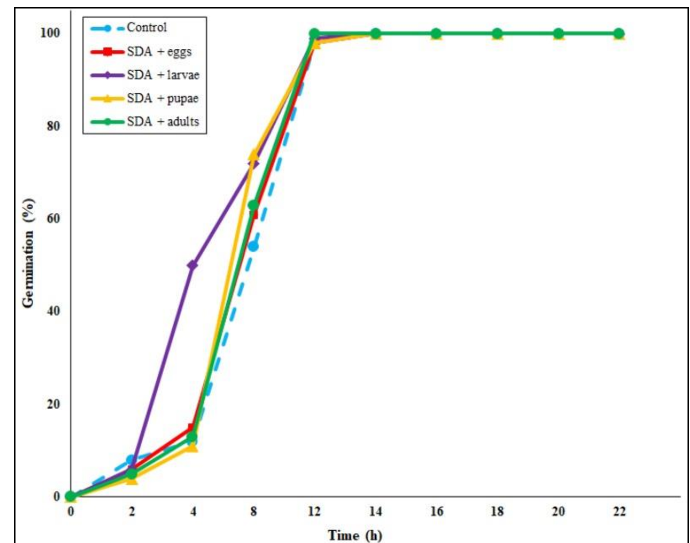

Fig 6: Conidia germination kinetics of *B. bassiana* 120 in SDA. The segmented line represents the Control = SDA without supplementing; each continuous line with a different marker represents a different treatment; each treatment was supplemented with each of the stages of development of *Aedes aegypti*.

4. Discussion

Considering that 80% of EF commercial products are made with *Metarhizium* and *Beauveria* [28]. In the present study, the conidial germination of three entomopathogenic fungi strains (*M. anisopliae* 028, *B. bassiana* 103 and 120) was compared on Agarose and SDA media supplemented individually with the different stages of *Aedes aegypti* (eggs, larvae, pupae and adults).

Regarding Agarose and SDA media, the conidial germination speed was increased as a time function. On Agarose the maximum germination for the three studied strains was at 22 h, however, on SDA media, the maximum percentage of conidial germination was obtained in less time: 16, 20 and 14 h for *M. anisopliae* 028, *B. bassiana* 103 and 120 strains, respectively, which coincides with other studies that highlight the culture media influence on germination [29, 30], for example, it has been shown that use of nutrient-rich media as SDA and YEA (Yeast Extract Agar) produce greater conidial germination on *M. anisopliae* and *B. bassiana* strains compared to unenriched media [30-32]. In addition, they have been used to increase virulence, by passing through the host to reactivate the strains [33].

It has been demonstrated that conidial germination speed variability is related to both species and strain [34, 35], in these studies, most *M. anisopliae* strains have a higher germination rate than *B. bassiana* strains (10-14 h y 14-25 h, >95% germination, respectively), however, the results of the present study show the opposite with *M. anisopliae* 028 and *B. bassiana* 120 strains (*M. anisopliae* 028 85% and *B. bassiana* 120 99% at 12 h).

The results of the present study showed differences between the germination of *B. bassiana* and *M. anisopliae* depending on the culture media and the addition of cuticles type of *Ae. aegypti* (Fig 1-6). The treatments supplemented with larvae or pupae were associated with greater conidial germination, which is attributed to their cuticle composition and is consistent with studies involving the cuticle in adherence and germination [36, 37]. It has been reported that the addition of insect cuticles to culture media increases conidial germination and induces greater virulence [38, 19].

Rodríguez-Gómez *et al.* [39] evaluated two *B. bassiana* strains as a germination function and virulence rate on SDA exuvia supplemented of grasshopper and unsupplemented SDA, no significant differences were found in germination rate with one or the other media, later the strains were submitted in bioassays against larvae and adults of *Tenebrio molitor* (flour worm), in which better results were obtained in adults.

The conidial germination of two *M. anisopliae* strains was evaluated at 48 h and obtained 89-92% with the supplemented media with wings and legs (lobster) compared with that supplemented with wheat bran 61-66% [40], when evaluating the virulence on *Rhammatocerus schistocercoides* adult locusts, 100% mortality was obtained at 15 days (wheat bran or SDA+wings or lobster legs) against rice or unsupplemented liquid media. On the other hand, although the main component of insect cuticle is chitin, the epicuticle comprises a complex mixture of non-polar lipids that play an important role in the prevention of desiccation, penetration of insecticides [41] and chemical communication events [42]. The surface of the conidia consists of a hydrophobic layer that interacts with the hydrocarbon epicuticle (EHC) of the insect and is related to virulence [43].

Extensive studies on insect cuticular lipids have shown that hydrocarbons (HC) are widespread in most insect's orders and in many cases, are the predominant component [44]. In addition, they favor *B. bassiana* and *M. anisopliae* germination because by epicuticle degrading the energy is produce that is incorporate into its cellular components [38].

It has been reported that in culture media for *B. bassiana* supplemented with *Triatoma infestans* EHC extract (n-C29, 31 and 33) as the sole carbon source, the conidia production was twice as high as that obtained with epicuticular extract without hydrocarbons (mostly fatty alcohols and free or esterified fatty acids); in addition, these extracts work better than those that include synthetic alkanes [38]. In Diptera case, it has been reported that saturated HC predominantly in the house fly (*Musca domestica*) (C23-C29) [45]. Differently, in the tsetse fly (*Glossina morsitans*) the HC are saturated (C29-C37) and mostly with methyl branches [46]. Consistently the *Anopheles gambiae* cuticle is constituted by saturated straight HC (C27-C29) and monomethyl alkanes (C29 and C31) [47, 48]. Similarly, the alkanes of *Ae. aegypti* are mostly C25-C29 and monomethylalkanes (C29-C33) [49].

The virulence increase is often related to rapid germination and high growth rates; a high germination percentage can help increase the likelihood of infection before the spores are removed from the cuticle [50]. Germination acceleration improves pathogenicity [51] and therefore the addition of insect cuticles works as virulence inducers [29, 30].

Studies have shown that the germination increase is related to the increase virulence levels [29, 30, 52]. Therefore, if germination accelerates, virulence is likely to increase in the same direction. For all the above, it is important to develop new culture media that accelerate the conidial germination of potential biocontrol agents.

5. Conclusions

Conidial germination kinetics of three EF strains evaluated show that *M. anisopliae* 028 and *B. bassiana* 120 strains are potential candidates for the vector control of Dengue, Chikungunya and Zika. In addition, it was shown that supplementation with *Ae. aegypti* larvae function as virulence inducers, by accelerating germination consistently, in times of shorter and higher percentages. For the results obtained in the evaluation of supplements of the different phases of *Ae. aegypti* in culture media, it is recommended to continue pathogenicity bioassays and virulence in supplemented media mainly with larvae or pupae in the search to enhance their effectiveness as biocontrol agents of *Ae. aegypti.*

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