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EFFECT OF TEMPERATURE AND ULTRAVIOLET RADIATION ON GROWTH AND PATHOGENICITY OF *Metarhizium anisopliae*

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ABSTRACT

The efficacy of entomopathogenic fungi in the field depends on the influence of various environmental factors, including temperature and level of exposure to UV light. It is hypothesized that temperature and level of UV light exposure of *Metarhizium anisopliae* (Hypocreales, Clavicipiceae) may affect its efficiency in controlling the sweetpotato weevil (*Cylas formicarius*, Fabricius; Coleoptera, Brentidae). The objective of this study was to evaluate the influence of temperature and ultraviolet light radiation on the growth and pathogenicity of different native strains of Cuban origin of the *M. anisopliae* Sorokin complex with biological activity against *C. formicarius.* Colony diameter was measured at 28, 30, 32 and 34 °C temperature and exposed to ultraviolet light (254 nm) for 10, 15, 20, 30, 30, 60 and 120 minutes. Under laboratory conditions, the percentage germination of conidia of strains LBMa-11, LBM-30, LBM-41 and LBM-146 was determined, as well as their pathogenic capacity by means of bioassays with adults of *C. formicarius* in terms of the percentage of cumulative mortality and the mean lethal time (TL50). Results proved that temperature and UV light affect the ability of *M. anisopliae* to control *C. formicarius*. A delay in cumulative mortality was detected for all strains exposed to UV light, with an increase in TL50. The optimum temperature range was between 28 and 30 °C. The strain LBM-127 showed the highest sensitivity to temperature, LBMa-11 the highest tolerance to UV light, and LBM-146 was the most virulent according to Probit regression analysis.

Keywords: *Cylas formicarius*, temperature, ultraviolet radiation.

INTRODUCTION

Entomopathogenic fungi belonging to the genus *Metarhizium* Sorokin (Hypocreales, Clavicipitaceae) are considered effective biological control agents against a broad spectrum of insect orders (Rezende *et al.*, 2015). Globally, they are used in the

prevention and management of various insect species of agricultural interest (López-Lastra *et al*., 2019).

In Cuba, the use of *Metarhizium* spp. began in 1980 with studies of the physiology and pathogenicity of isolates *in vitro* and in the field against pests such as *Cosmopolites sordidus* Germar, *Lissorhoptrus brevirostris* Kuschel, *Lissorhoptrus oryzophilus* Kuschel and *Monecphora bicincta fraterna* Uhler. These results allowed the selection of *Metarhizium anisopliae* strain LBMa-11 for the control of *L. oryzophilus* and *Monecphora fraterna* in rice crops and pastures. The implementation of mass production technologies was implemented in the Entomophagous and Entomopathogen Reproduction Centers (CREE), as a strategy for insect control in Integrated Pest Management Programs in crops (Márquez-Gutiérrez *et al*., 2020).

Different environmental factors such as temperature, solar radiation, pH and humidity can limit the development of *M. anisopliae*, which is one of the disadvantages for its application in the field. Temperature has an influence on conidial germination, germ tube development and penetration, as well as on their ability to colonize and reproduce (Pereira-Junior *et al*., 2018). The evaluation of growth, sporulation and germination rates in relation to temperature is important to operate an efficient selection of *Metarhizium* spp. isolates with biocontrol potential (Torres *et al*., 2013).

Solar radiation has a direct impact on the survival and efficiency as a biocontrol agent of this fungus, in addition to play a fundamental role in its permanence on treated crops. Ultraviolet light affects the growth of *Metarhizium* spp. as a function of the intensity of the energy received and the time of exposure, which can limit or retard conidial germination by direct (mutations) or indirect genetic damage (López-Lastra *et al*., 2019).

Recent research on *Cylas formicarius*, a coleopteran that affects the productivity of several economically important crops, particularly sweet potato (*Ipomoea batatas*), focuses on the study of abiotic factors that influence the growth and development of the fungus. The goal is to select strains with greater potential for the biological control of pests and that can adapt to different environmental conditions to ensure their persistence and efficacy in field conditions.

The research hypothesis assumes that temperature and level of UV light exposure of *Metarhizium anisopliae* (Hypocreales, Clavicipiceae) may affect its efficiency in controlling the sweetpotato weevil (*Cylas formicarius*, Fabricius; Coleoptera, Brentidae). The objective of this study was to evaluate the influence of temperature and the level of exposure to ultraviolet light on the growth and pathogenicity of native strains of Cuban origin of the *Metarhizium anisopliae* Sorokin complex with biological activity against *Cylas formicarius*.

MATERIALS AND METHODS

Strains LBM-5, LBM-10, LBMa-11, LBM-12, LBM-30, LBM-41, LBM-42, LBM-46, LBM-146 and LBM-267 belonging to the *Metarhizium anisopliae* strain complex from the microorganism collection of the Instituto de Investigaciones de Sanidad Vegetal

(INISAV), Havana, Cuba, were used. These strains were isolated from insects of the orders Hemiptera, Lepidoptera and Coleoptera. In particular, LBMa-11 is the most widely used, as it has been already, for the control of numerous pests under a mass production program of biopesticides in Cuba (Márquez-Gutiérrez *et al*., 2016).

Reactivation of *Metarhizium anisopliae* strains

The strains were preserved in mineral oil and reactivated by reinoculation on insect hosts in order to enhance the entomopathogenic action of each strain. They were transferred to Petri dishes with complete medium (MC) (Torres *et al*., 2013) and incubated at 25 ± 2 °C temperature for 10 d until complete sporulation of the cultures. Then, decimal dilutions were made with sterile distilled water and 0.1 % Tween 80 to prepare conidial suspensions at a concentration of 10^7 conidia mL^{-1}; 10 second-generation young adults of *C. formicarius* from a laboratory rearing kept at INISAV at a temperature of 28-30 °C and relative humidity of 85 % were used for insect reinoculation.

Insects were disinfected with 0.1 % sodium hypochlorite for 1 min, rinsed twice in sterile distilled water and immersed for 30 to 50 s in the conidial suspension of each of the previously prepared strains. Treated insects were placed in Petri dishes with sweet potato fragments as natural diet. The dishes were incubated at 25 ± 2 °C in the dark and checked daily until fungal emersion and sporulation were observed. Mycosed insects were re-isolated on MC dishes supplemented with rose bengal (0.5 g L^{-1}) and chloramphenicol (0.05 g L^{-1}) and placed in humid chamber. Monoconidial cultures were obtained, transferred to test tubes with MC and stored at 4 °C (10 replicates per isolate) for assay performance.

Evaluation of temperature on *M. anisopliae* colonies

The growth rate of *M. anisopliae* strains, with the exception of strain LBM-42, was determined at different temperatures. Conidial suspensions were prepared for each monoconidial culture at a concentration of 10^7 conidia mL^{-1}. Taking 0.1 mL of each suspension, surface seeding was performed using a Drigalski spatula. They were then incubated at 26 °C and, at 72 h, 4-mm discs were removed and transferred to the center of Petri dishes (9 cm in diameter) with MC culture medium and incubated at temperatures of 28, 30, 32, and 34 °C. Colony diameter was measured up to 10 d of growth and five replicates were considered per each strain.

Evaluation of ultraviolet light on *M. anisopliae*

The effect of ultraviolet light on the growth and cultural characteristics of *M. anisopliae* strains was evaluated through circular portions of unsporulated cultures, which were obtained by means of a 5 mm diameter borer and placed in the center of Petri dishes. The inoculated plates were exposed to UV light of 254 nm wavelength at a distance of 15 cm height in the biological safety cabinet, for 10, 15, 30, 45, 60 and 120 minutes, four replicates for each variant and a control treatment without UV exposure were

considered. They were then incubated at 25 ± 2 °C temperature and viability test by germ tube emission was performed.

For each replicate, circular portions were obtained and placed in 5 mL of water with Tween 80. Decimal dilutions up to 10^{-4} were then prepared for Neubauer chamber counting to determine the concentration of conidia at the UV exposure times considered; 0.01 mL of the selected dilution was seeded in MC culture medium in humid chamber and incubated at 25 ± 2 °C for 24 h. The viability of the strains was determined by optical microscopic observation of the germ tube emission; it was considered as germinated and viable when the conidium developed hyphal filaments around, and not germinated when visibly spherical conidial structures without lateral extensions were observed. Finally, records were taken (three for each strain) and the percentage of germinated conidia was calculated.

Evaluation of pathogenicity of *M. anisopliae* strains

Evaluation of the pathogenicity of *M. anisopliae* strains exposed to UV light was performed on 70 adults of *C. formicarius* from a laboratory rearing. The insects were disinfected using the same procedure developed for strain reactivation and separated into seven groups, each containing 10 insects. Using *M. anisopliae* strains irradiated with UV light and another group of non-irradiated strains (control +), suspensions were prepared and adjusted to the concentration of 10^7 conidia mL^{-1} for each treatment. The insects were immersed in the conidial suspensions for 50 s, except the insects in the control group which were not inoculated with the microorganism.

They were then placed on filter paper to remove excess moisture and placed in Petri dishes with the natural sweet potato-based diet, previously disinfected. The dishes were incubated at room temperature and live and dead insects were quantified daily to calculate the percentage of cumulative mortality, according to the formula of Abbott (1925). To evaluate the effect of the treatment on the biocontrol capacity of the strains, at the end of the bioassay, the dead insects that showed incipient mycelium formation and emission were placed in a humid chamber and incubated at 25 ± 2 °C temperature to recover and check the identity of the microorganism.

Statistical analysis

Statistica v. 8.0 was used for statistical analysis of the results. The Kolmogorov-Smirnov normality test was used for all variables and the homogeneity of variance test. The Newman-Keuls parametric test was used to compare means among treatments. By means of Probit regression equations, TL50 was determined to express the correlation between the percentage of cumulative mortality and the time of exposure to UV light.

RESULTS AND DISCUSSION

Effect of temperature on the growth of *Metarhizium anisopliae* colonies

Strains LBM-5, LBM-10, LBMa-11, LBM-12, LBM-30, LBM-41, LBM-127, LBM-146 and LBM-267 of M. anisopliae grown on MC culture medium at temperatures of 28, 30, 32

and 34 °C showed similar behaviour to that reported by Gato-Cárdenas *et al.* (2016) on Saboraud Dextrose Agar (SDA) medium. Rodrígues *et al.* (2016) indicated that temperature affects entomopathogenic fungi of the genus *Metarhizium* by affecting their metabolism, altering the processes of enzyme production, toxins, conidia germination, germ tube emission, mechanism of action, colonization and reproduction in the ecological niche. This effect was evident in the growth diameter quantified in the colonies of the different strains of *M. anisopliae* (Figure 1).

The optimum growth temperature was between 28 and 30 °C for these native strains that were isolated from insects collected in different agricultural areas of Cuba (Figure 1). Other authors mentioned a growth optimum between 25 and 30 °C for different strains of *M. anisopliae* isolated in tropical regions of the state of Veracruz, Mexico (Torres *et al*., 2013). In our study, 32 and 34 °C temperatures were unfavorable in the growth of all strains evaluated; in particular, LBM-5, LBM-12, LBM-41, LBM-127, LBM-146 and LBM-267 were especially sensitive to 34 °C ($p \leq 0.05$). It has been noted that most entomopathogenic fungi are mesophilic with growth between 10 and 40 °C and an optimum between 25 and 35 °C (Chandra and Rahman, 2016).

Strains treated at 34 °C had a marked decrease in growth. Likewise, this temperature limited strains LBM-41 and LBM-267, with growth only at the margins of the disc inoculated on agar. Chandra and Rahman (2017) evaluated four rhizospheric isolates of *M. anisopliae* in India and found increased growth of strains at 30 °C, with a lag from 35 °C, values that agree with the results achieved in this study. A great genetic variability in the thermotolerance of *Metarhizium* spp. was evidenced by Rangel *et al.* (2015), not only in mycelial growth capacity, but also in terms of their conidial germination potential, a characteristic directly linked to the virulence of the strains.

The differential thermal tolerance of this fungus lies in the composition of its cell wall. Souza *et al.* (2014) referred that the tolerance of conidial germination to temperatures

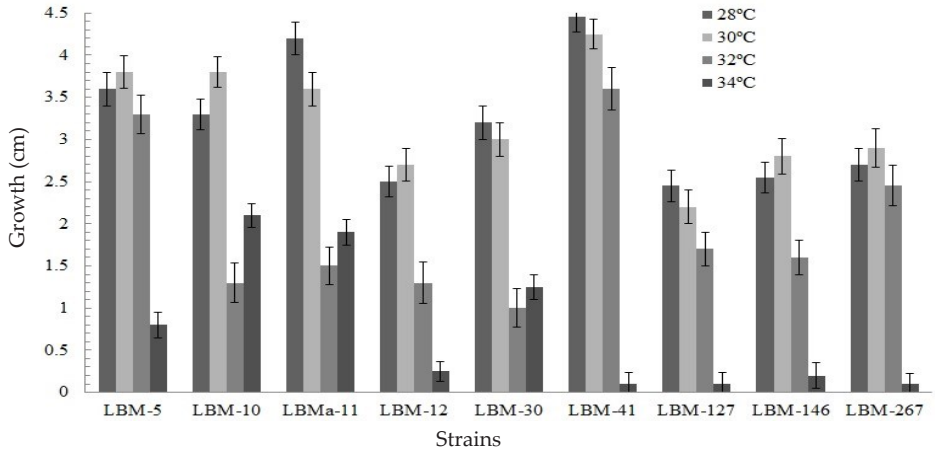

Figure 1. Growth of different strains of *Metarhizium anisopliae* at temperatures of 28, 30, 32 and 34 °C. Different letters indicate significant differences between each strain according to the Newman-Keuls test ($p \leq 0.05$).

above 30 °C is closely related to the presence of hydrophobic proteins in the cell wall of conidia that protect the fungus against heat stress. It is shown that temperature, in addition to affecting growth rate, influences cultural characteristics of *M. anisopliae*, such as sporulation pattern, pigmentation of the culture medium and colony morphology (Gato-Cárdenas *et al*., 2016).

The cultural characteristics of strain LBM-127 with 10 d of incubation at different temperatures showed very evident differences (Figure 2). At 28 °C it exhibited a cottony colony in the center, white in color, abundant aerial mycelium, incipient sporulation in the center of light olive green color, white mycelial margin and the reverse side of the colony was light yellow with regular edges; it also produced pigments that were exuded to the culture medium. At 30 °C the colony became cottony white with abundant aerial mycelium, diffuse sporulation and white mycelial margin; on the reverse side of the colony, its coloration and margins did not change. At 32 °C it maintained the same texture, but the diffuse sporulation was olive green with no other change of note.

Consistent with our results, Chandra and Rahman (2017) showed that temperature response is an important feature not only in selection, but in the reproduction of fungi and their growth capacity that can be related to their origin and gene pool.

Effect of ultraviolet light on the growth of *M. anisopliae*

The results of the statistical analysis showed significant differences between some of the UV exposure times for the strains evaluated, except the strain LBMa-11 which had no significant differences among treatments ($p > 0.05$). In strains LBM-30 and LBM-41 there were significant differences ($p \leq 0.05$) from 60 and 120 minutes of exposure, respectively, while strain LBM-146 only exhibited statistical differences ($p \leq 0.05$) between the extreme values of UV exposure (10 and 120 minutes) (Table 1). As the time of exposure to ultraviolet light increased, the number of germinated conidia per field decreased. This indicated a gradual decrease in the percentage of conidial germination in the strains evaluated.

The literature reports great variation of entomopathogenic fungi to UV light, although there are isolates very sensitive to high doses of radiation, which cause total mortality of conidia; the response depends on the isolate. Rangel *et al.* (2015) demonstrated that

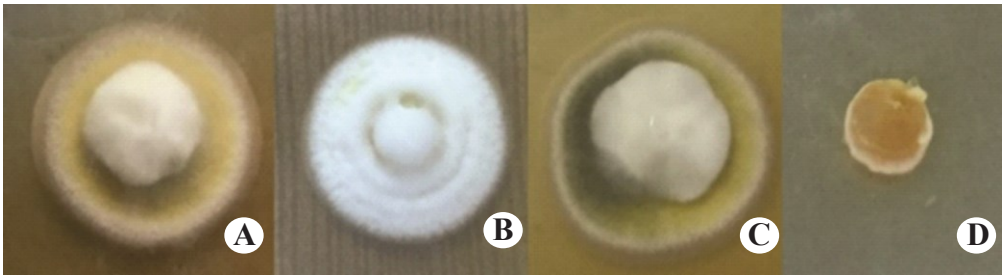

Figure 2. Cultural characteristics of *Metarhizium anisopliae* strain LBM-127 on MC medium at the temperatures evaluated after 10 d of incubation. A: 28 °C, B: 30 °C, C: 32 °C, and D: 34 °C.

Table 1. Diameter of *Metarhizium anisopliae* strains (DC) and percentage germination of conidia (G) at different UV light exposure times.

Strains	Exposure time (minutes) 0		10		15		30		45		60		120	
	DC	G	DC	G	DC	G	DC	G	DC	G	DC	G	DC	G
LBMa-11	7.77a[†]	96	7.72a	90	7.12a	88	7.52a	77	7.02a	72	7.52a	70	7.97a	65
LBM-30	5.50a	100	5.27a	98	5.40a	98	5.30a	95	5.40a	95	4.55b	90	5.87c	87
LBM-41	6.65a	99	6.52a	97	6.52a	91	6.65a	91	6.40a	90	6.35a	87	7.10b	80
LBM-146	4.22ab	98	4.02b	88	4.77a	86	4.35ab	80	4.20ab	73	4.17ab	70	4.98a	69

[†] Different letters indicate significant differences between rows for the DC values of each strain, according to the Newman-Keuls test ($p \leq 0.05$).

ultraviolet light impacts the growth and manifestation of morphological characters of *Metarhizium;* a wavelength of 254 nm inhibited conidial germination with direct and indirect DNA damage.

The germination test of the strains showed the presence of conidia with elongation of the germ tube and formation of lateral filaments, which indicated their viability. However, it was noted that the longer the exposure time to ultraviolet light, the lower the number of conidia per field. This represented a gradual decrease in the germination percentage of the strains. In strains LBMa-11 and LBM-146, a conidia germination percentage of 65 and 69 % was obtained at the longest exposure time; the lowest affectation was found in strain LBM-30 with 87 % conidia germination. Strain LBMa-11 (with 10 minutes of exposure), LBM-41 (from 10 to 45 minutes of exposure), and strain LBM-30 in all treatments (except 120 minutes), showed a percentage of conidia germination higher than 90 %.

This characteristic is highly desirable in the agronomic field, since the rapid development of the germinative tube of conidia in entomopathogenic fungi favours the infection process and, consequently, affects the time of exposure to adverse environmental factors when applied under field conditions.

Both damages interfere with DNA replication by causing mutations and even cell death, depending on the energy intensity and time of exposure. It is noted that the olive green coloration of strain LBM-146 changed to yellow in contrast to the control strain, which maintained the intense olive green coloration after 10 d of incubation, typical of some strains grown under appropriate growth conditions.

In general terms, these results agree with those obtained by Rodrígues *et al.* (2016), who obtained a reduction in the growth of *M. anisopliae* and *Beauveria bassiana* strains when exposed to ultraviolet light. Resistance to ultraviolet light may be due to a complex of genes influenced by environmental factors. Among them are those related to the synthesis of pigments such as melanin and carotenoids located on the cell surface that can block radiation. Also included are detoxifying enzymes that inactivate toxic and mutagenic substances such as free radicals, and other enzymes and proteins involved in the repair of radiation damage. Fernandes *et al*. (2015) found that geographic origin

and host are important, as at low latitudes entomopathogenic fungi are more resistant to radiation.

The manifestation of tolerance, according to Fernandes *et al.* (2015), may reflect an adaptive change to environmental conditions. In our study, strain LBMa-11 proved to be the most thermotolerant, a factor that under field conditions influences the mechanism of action of the fungi and affects their persistence. This characteristic may explain, among other aspects, the efficacy achieved in the field by this strain and its extensive application for the reduction of insect pests in Cuba (Márquez *et al.*, 2016).

Evaluation of the pathogenicity of *M. anisopliae* strains exposed to UV radiation against *Cylas formicarius*

The evaluation of *M. anisopliae* strains exposed to UV light treatments in the bioassay to determine the affectation of their pathogenic capacity on *C. formicarius* indicated that irradiated strains of the fungus showed a highly variable cumulative mortality percentage among them and among treatments (Figure 3).

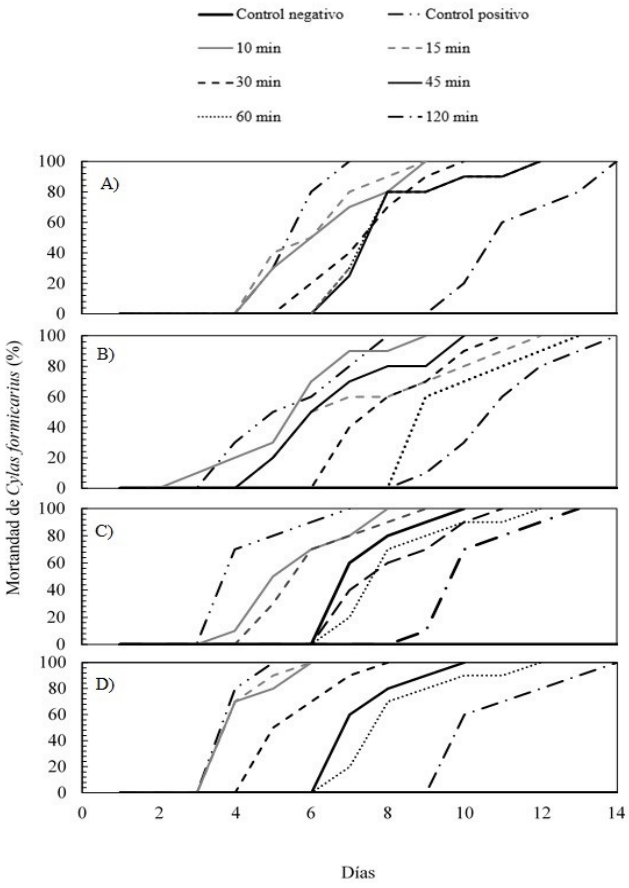

Figure 3. Percentage of cumulative mortality during the bioassay with *Metarhizium anisopliae* strains exposed to 254 nm UV light. A: LBMa-11, B: LBM-30, C: LBM-41, D: LBM-146.

Irradiated as well as non-irradiated (control +) strains reduced the number of live insects from the third day after inoculation and between 4 and 14 d, in contrast to insects not treated with *M. anisopliae* (control -) which remained alive.

Mortality percentages of 100 were achieved in insects treated with strains exposed and not exposed to UV light. However, this action was observed faster in insects infested with strains not irradiated with UV light, which was particularly evident for LBM-146 and LBM-41. No exposure time to UV light at the wavelength equal to 254 nm considered in this study nullified the pathogenic capacity of *M. anisopliae* strains, indicating that there could be other factors related to virulence, such as enzymatic activity involved in the degradation process of the insect cuticle. However, the efficiency of the biological action was affected in relation to the times when the highest percentages of mortality were obtained. The delay in biological action may be due to the fact that the amount of viable conidial inoculum decreased with time of exposure to UV light, as may have occurred in the 45, 60 and 120 min treatments.

The TL50 values obtained by evaluating the time-mortality correlation of each of the UV light treatments (Table 2) indicated that the lowest TL50 corresponded to the non-

Table 2. Relationship between UV radiation exposure time and mortality of *Metarhizium anisopliae* strains.

Strain	Treatment	Equation	R^2	TL50
LBMa-11	Control (+)	Y=17.25X-7.58	0.99	5.4
	10	Y=6.82X-0.29	0.99	6
	15	Y=7.93X-0.93	0.95	5.6
	30	Y=12.10X-5.36	0.99	7.2
	45	Y=4.88X+1.5	0.96	5.2
	60	Y=8.52X-2.34	0.79	7.3
	120	Y=13.38X-8.98	0.95	11.1
LBM-30	Control (+)	Y=5,34X+1,24	0.96	5.1
	10	Y=6,74X+0,24	0.92	5.1
	15	Y=5,27X+0,64	0.92	6.7
	30	Y=9,37X-3,22	0.94	7.5
	45	Y=7,78X-1,70	0.92	7.3
	60	Y=11,82X-6,01	0.98	8.5
	120	Y=16,34X-11,84	0.98	10.7
LBM-41	Control (+)	Y=4.26X+2.93	0.97	3.1
	10	Y=8.69X-1.33	0.9	5.3
	15	Y=6.64X-0.08	0.98	5.8
	30	Y=9.37X-3.22	0.95	7.5
	45	Y=9.44X-2.71	0.99	6.6
	60	Y=10.48X-4.33	0.86	7.8
	120	Y=19.53X-14.55	0.87	10
LBM-146	Control (+)	Y=4.54X+3.11	0.99	2.6
	10	Y=3.27X+3.55	0.99	2.8
	15	Y=7.82X+0.82	0.99	3.4
	30	Y=8.71X-1.14	0.98	5.1
	45	Y=9.44X-2.71	0.98	6.6
	60	Y=10.48X-4.33	0.86	7.8
	120	Y=9.81X-3.71	0.98	9.5

irradiated *M. anisopliae* strains (control +). The Probit analysis had a good goodness of fit (R^2) as all values were greater than 0.70.

As the time of exposure to UV light increased, the TL50 of each of the tested strains increased. The intervals were between 2.6 - 11.1 d in all treatments, due to the affectation of the *Metarhizium* strains caused by the time of exposure to ultraviolet light. For reference, Gato-Cárdenas *et al.* (2017) calculated TL50 and TL90, where they obtained values between 1.5 and 7.95 d, and 4.01 and 16 d under optimal conditions.

Strain LBM-146 presented the lowest TL50 at 120 minutes, the exposure time of highest incidence, in contrast to strains LBMa-11, LBM-30 and LBM-41. Thus, it proved to be the most virulent and could be a candidate for the development of a new insecticide product. Rojas-Gutiérrez *et al.* (2017) mentioned that differences in pathogenicity among *M. anisopliae* isolates may be related to their origin. Abiotic factors can limit the process and capacity of infection against pest insects. However, the expression of virulence-related factors, particularly through cuticle-degrading enzymes that destroy or modify the structural integrity of the host, inhibit its selective processes and interfere with its regulatory system. Furthermore, intermediate UV light resistance further benefits the effect of this fungus on *C. formicarius* and persistence in the field.

CONCLUSIONS

The results of this research showed that temperature and ultraviolet radiation affect the biocontrol efficiency of *Metarhizium anisopliae* against *Cylas formicarius*. In most cases at 32 and 34 °C radial growth was inhibited by 50 %. Exposure to UV light for 45 to 120 min negatively influenced strain growth and conidial germination. The percentage of cumulative mortality was variable among strains and treatments under the *in vitro* conditions tested. Strain LBMa-11 demonstrated to be the most tolerant to ultraviolet light and temperature. The irradiated strains did not decrease their lethal effect, although the increase in TL50 was notorious, which represented a delay in insecticidal activity.

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