

## Brief Report

# Germination and Culturability after UV Irradiation of *Metarhizium anisopliae* Native from Soils of Tropical Cattle Farms

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**Abstract:** The use of entomopathogenic fungi (EF) is a promising alternative for the control of *Rhipicephalus microplus*, an important tick affecting cattle globally. This study aimed to evaluate the effect of ultraviolet irradiation (UV) exposure on the percentage of conidia germination and number of colony-forming units of eight strains of *Metarhizium anisopliae* (MaV55, MaV35, MaV31, MaV25, MaV13, Ma08, MaV05, and MaV02). The UV (UV-A and UV-A+B) irradiation was carried out with an ultraviolet radiation emission lamp. The conidia of each strain were exposed to the UV irradiation treatments for 3 h. MaV25, MaV08, MaV05, MaV13, and MaV31 showed higher tolerance to UV-A radiation exposure, as assessed by conidia germination. UV-A+B radiation decreased the germination percentage of all the *M. anisopliae* strains. The eight evaluated strains showed good tolerance to UV-A radiation, as assessed by the development of colony-forming units (CFU). UV-A+B radiation did not significantly affect ( $p > 0.05$ ) the count of the CFU of six of the *M. anisopliae* strains evaluated (MaV35, MaV13, MaV08, MaV05, MaV31, and MaV02). The novel findings of the UV-tolerant *M. anisopliae* strains may potentially improve the effectiveness of EF under environmental conditions. Integral research under real tropical conditions is advised to evaluate the effectiveness of the EF strains.

**Keywords:** entomopathogenic; fungi; UV radiation resistance; conidia; germination



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## 1. Introduction

Ticks, notably *Rhipicephalus microplus*, are the primary ectoparasites of cattle in tropical and subtropical regions, leading to substantial economic losses and serious disease transmission. Although chemical acaricides have been the main line of defense against these ticks, the development of resistance necessitates the exploration of alternative control measures [1–3].

The utilization of entomopathogenic fungi (EF), particularly *Metarhizium anisopliae*, shows promise against different stages of *R. microplus* (eggs, larvae, nymphs, and adults) [4–6]. However, inconsistent field effectiveness has been found in some studies that have been attributed to environmental factors such as ultraviolet (UV) radiation, high temperatures, and humidity variations [3,5,7]. In this regard, it has been mentioned that UV radiation is one of the environmental characteristics that most affect the development of EF. The relationship between the ability of EF to tolerate UV exposure and the ability to affect ticks is high and must be considered from different approaches. The most important factor is that UV radiation has a detrimental effect on propagules and the entomocidal activity of fungal cells [8]. Due to that, the exposure of EF to UV radiation in practical applications such as directly on the cattle or sprayed on pastures to control ticks may result in inefficiency. When conidia are exposed to UV-A (320–400 nm) and UV-B (280–320 nm) sunlight, their viability decreases, reducing their pathogenic effect [9]. For this reason, strains with the ability to tolerate UV exposure

could exhibit better mortality effects against ticks in environmental conditions. Efforts to address these challenges include the addition of photoprotectors, the nighttime application of treatments, and the selection of UV-resistant strains [5,10].

The geographical origin of EF strains may influence their UV resistance, with strains potentially exhibiting higher tolerance by adaptation/evolution mechanisms [10,11]. Therefore, evaluating the UV resistance of native *M. anisopliae* strains under livestock conditions is crucial for selecting effective strains for field application [6]. In the north-central area of Veracruz, Fernandez-Salas [12] isolated 59 strains of *Metarhizium anisopliae* from soils in bovine production units. At least nine of these strains demonstrated high myco-acaricidal efficacy (>90%) against different stages of *R. microplus* [13]. However, their tolerance to sunlight, specifically UV-A and UV-B radiation, remains unknown.

Conducting in vitro studies to assess the resistance of *M. anisopliae* strains to UV radiation in livestock conditions is imperative since this information will facilitate the selection of EF strains with prolonged viability under field conditions. This study aimed to evaluate the conidial germination and culturability (number of colony-forming units) of eight strains of *M. anisopliae* isolated from tropical cattle farms under UV-A and UV-A+B radiation.

## 2. Materials and Methods

### 2.1. Study Area

This study was developed in the Animal Health Laboratory of the Center for Teaching, Research, and Extension in Tropical Livestock (CEIEGT) of the Faculty of Veterinary Medicine and Zootechnics (FMVZ) of the National Autonomous University of Mexico (UNAM). The CEIEGT is located in the Municipality of Tlapacoyan, Veracruz, Mexico (20°02'10.74" N, 97°06'18.17" W). The CEIEGT has an altitude of 110.9 masl with a climate classified as warm humid Af (m) W'' (e) with an annual temperature of 24.4 °C, an RH of 85%, and an average rainfall of 1990 mm [14].

### 2.2. Obtaining Entomopathogenic Fungi

The EF strains (MaV02, MaV05, MaV08, MaV13, MaV25, MaV31, MaV35, and MaV55) were selected from a mycological collection that is made up of at least 59 strains at the Animal Health Laboratory of CEIEGT, where the evaluations of their effect against *R. microplus* ticks in their different stages were carried out. All the strains were originally isolated from the soils of cattle farms in Veracruz, México, with a high prevalence of ticks and identified using molecular tools [12]. The EF were selected based on their high in vitro virulence against *R. microplus* [13], and they are part of an integrated project against ticks.

### 2.3. Production of Conidia and Preparation of Inoculum

The growth and reproduction of the selected strains were carried out in Petri dishes with 20 mL of Potato Dextrose Agar (PDA), 1% of yeast extract, and chloramphenicol (500 ppm) according to Cañedo and Ames [15]. The strains were incubated individually (27 °C, 90% of Relative Humidity, RH) for 15 d in a bacteriological culture oven. Subsequently, the conidia were collected by scraping and washing the surface of the culture medium with 20 mL of sterile distilled water. The collection was poured into 50 mL Falcon® tubes (Fisher Scientific®, Pittsburgh, PA, USA). The suspension was homogenized by vortex shaking (Thermo Scientific®, model No: M37615, Waltham, MA, USA) and later filtered through a cellulose nitrate membrane (25 mm diameter, 8 µm pores; Sartorius®, Göttingen, Germany) to obtain the suspension of conidia isolated without the surfactants [16]. A Neubauer chamber was used to determine the concentration of the conidia. The dilutions were performed with sterile distilled water and refrigerated at 4 °C for 24 h before exposure to UV radiation treatments.

For each *M. anisopliae* strain, 200 µL of conidia at a concentration of  $1.5 \times 10^5$  conidia/mL were seeded in a PDA culture medium enriched with yeast extract contained in sterile disposable Petri dishes (100 × 15 mm) with three divisions (3 mL per division).

Immediately afterwards, the strains were exposed to the different UV radiation treatments for 3 h.

#### 2.4. Experimental Design

Three treatments were used in the study. A negative control group of the eight strains was protected with an optical filter that did not allow the passage of waves shorter than 400 nm. A set of the eight strains was exposed to UV-A with the use of an optical filter. A set of the eight strains was exposed to UV-A+B with the use of an optical filter. Three replicates were used for strains in each treatment.

#### 2.5. Ultraviolet Irradiation

The UV irradiation (UV-A and UV-B) was carried out by exposition of conidia to a UV radiation emission in a laminar flow hood (Biosafe: BBS-H1300). The radiation spectrum of the UV lamp was determined before the start of the experiment utilizing a spectrophotometer (Ocean Optics Spectrometer serial number HR4B183).

The filters used to evaluate the effect of the UV waves (A or A+B) on the treatment of conidia were as follows: (1) Glass filter Thorlabs® (Newton, NJ, USA) model FGUV11s to allow a bandpass of 280 nm–370 nm, ensuring the passage of a UV-B wavelength (280–320 nm) and a fraction of UV-A (320–400 nm). This did not allow the passage of UV-C rays or visible light. (2) Glass filter Thorlabs® model FGB18 to avoid the bandpass of wavelengths lower than 400 nm (UV light). (3) Glass filter Thorlabs® model FGB75 to hinder the passage of UV-B and UV-C rays, while allowing the passage of UV-A and a portion of the visible light spectrum. The treatments were applied to the two groups of conidia separately, one to evaluate germination and the other to evaluate culturability. The EF conidia were covered with the filters and the variables of interest (germination and the development of colony-forming units) were monitored during the irradiation process. Additionally, temperature and RH inside the laminar flow hood were also monitored.

#### 2.6. Germination and Culturability Evaluation

The cultures were incubated at 28 °C and 90% of RH in complete darkness after the irradiation. The germination of the conidia was evaluated 12 h after being cultured and exposed to the irradiation treatment by observation with an optical microscope (40× objective). Conidia was considered germinated when the length of its germ tube was longer than the diameter of the conidia itself [16]. A total of 300 conidia were evaluated per treatment.

The relative culturability was evaluated by comparing the colony-forming units (CFU) of the irradiated assays against the controls of the same strain [10].

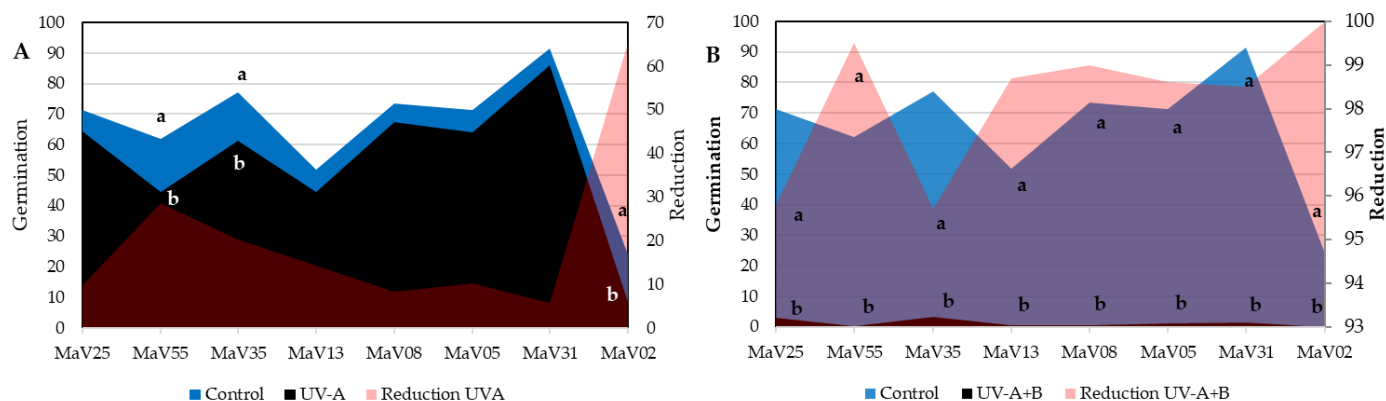
#### 2.7. Statistical Analysis

An ANOVA test was performed to evaluate the effect of UV radiation on the germination of EF conidia. The Kruskal–Wallis test was carried out to evaluate the effect of the UV light treatments on the culturability of the EF colonies. In both tests, a  $p < 0.05$  was used to determine statistically significant differences among the treatments. The Statgraphics™ software (version 17.1.02) was used to perform the analysis.

### 3. Results

#### 3.1. Germination of Conidia

The percentage of germination of the eight strains of *M. anisopliae* exposed to UV-A (A) and UV-A+B (B) is shown in Figure 1 and Table S1. UV-A radiation did not affect ( $p > 0.05$ ) the germination of MaV25, MaV13, MaV08, MaV05, and MaV31, exhibiting higher tolerance to exposure to UV-A radiation. Oppositely, MaV55, MaV35, and MaV02 showed a higher susceptibility to UV-A radiation on the germination of conidia ( $p < 0.05$ ).

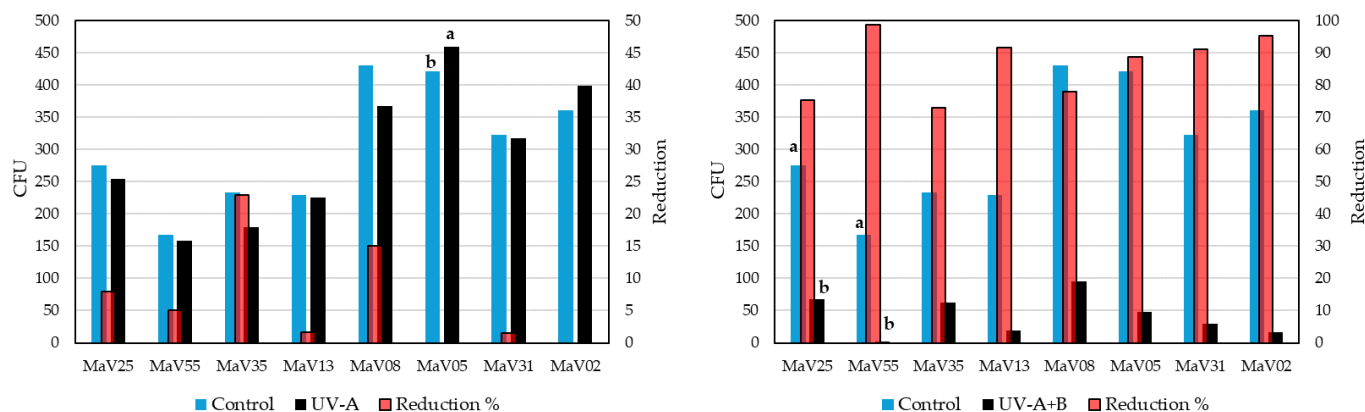


**Figure 1.** Conidia germination and reduction (%) of the eight strains of *Metarhizium anisopliae* irradiated with UV-A (A) or UV-A+B (B). <sup>a,b</sup> different literals in the same strain indicate significant differences ( $p < 0.05$ ) between the control and irradiation treatments.

Exposure to UV-A+B radiation decreased ( $p < 0.05$ ) the germination of all the *M. anisopliae* strains by more than 95%, indicating that the germination process of these strains is highly affected by UV-A+B radiation.

### 3.2. Culturability

The effect of exposure to UV-A radiation and UV A+B on the culturability of the eight EF strains is shown in Figure 2 and Table S2. All the *M. anisopliae* strains showed good tolerance to exposure to UV-A radiation ( $p > 0.05$ ); the MaV02 strain even showed increased germination after the treatment. Exposure to UV-A+B radiation did not affect ( $p > 0.05$ ) the number of UFC of six strains (MaV35, MaV13, MaV08, MaV05, MaV31, and MaV02). Oppositely, the MaV25 and MaV55 strains showed low tolerance ( $p < 0.05$ ) to exposure to UV-A+B radiation (Figure 2).



**Figure 2.** Number of colony-forming units (CFU) and reduction (%) in the eight strains of *Metarhizium anisopliae* under UV-A and UV-A+B irradiation. Different literals in the same strain indicate significant differences ( $p < 0.05$ ) between the control and irradiation treatments.

## 4. Discussion

The exposure of the entomopathogenic fungi (EF) strains to ultraviolet (UV) radiation facilitated the differentiation and selection of strains with varying levels of tolerance to UV-A and UV-A+B irradiation. This study focused on eight particular strains of *M. anisopliae* selected from 59 strains isolated at the field level. Hence, fungal strains could decrease their acaricidal effectiveness due to abiotic factors, such as exposure to UV solar radiation. In this study, we evaluated the conidial germination and CFU count of eight strains of *M. anisopliae* exposed to UV irradiation. Five of them showed a higher ability for conidial

germination subjected to UV-A exposure. Also, the results indicate that all the strains did not show detrimental impacts on CFU count when exposed to UV-A irradiation. Despite the fact that UV-A radiation constitutes 95% of the solar spectrum reaching the earth in the UV range and that the conidia of hyphomycetes are very susceptible to UV-A [17], there are few studies on the effects of this radiation on the germination and CFU count of *M. anisopliae* and even more from the native strains of livestock ecosystems.

The findings in this study are promising, since the strains showed better germination percentages than some strains reported as ARSEF 23 [8], and similar to other strains which have also demonstrated high conidial tolerance to UV-A irradiation [18]. The negative impact caused by this fraction of UV radiation has been attributed to severe damage to conidia by generating harmful radicals and reactive oxygen species, for example, singlet oxygen, which interacts with intracellular chromophores [19] and causes cell death, delays their germination, and results in the inactivation of propagules [9,20]. The adherence and germination of the fungi on the host's cuticle are important events for the initiation of infection in their respective hosts [4,21,22]. Consequently, those strains of *M. anisopliae* that have exhibited notable resilience to UV-A radiation under controlled laboratory conditions could be deemed as potential candidates for field assessments; although it should be emphasized that this study was carried out in controlled laboratory conditions using UV radiation emission lamps.

The findings of the present research also revealed a reduction in the germination of conidia in all the strains of *M. anisopliae* when exposed to UV-A+B. Braga et al. [10] reported high variability in susceptibility to UV-B radiation among the different strains of *M. anisopliae* isolated from various latitudes. Previous research has documented that UV-B exposure leads to a delay in the germination of the surviving conidia of *Metarhizium* fungi, affects replication, and causes mutations and the death of cells by altering the DNA, predominantly forming cyclobutane pyrimidine dimers [20,23–25]. Furthermore, the extent of delay in conidial germination is directly correlated with the dosage of irradiation subjected to [20] and the time of irradiation exposition [9,26,27]. Braga et al. [16] identified that UV radiation affects the differently distinct phases of the EF cell cycle. For *M. anisopliae*, the final phase of germination, particularly during or after the emergence of the germ tube, is the most susceptible to UV radiation due to DNA replication occurring during the concluding stages of germination in various filamentous fungi species, which makes this phase particularly vulnerable to radiation-induced damage [20]. The variation in the tolerance or susceptibility of *M. anisopliae* to UV radiation can be considered multifactorial. Braga et al. [25] suggested that colony pigmentation may stem from genetic expression acquired during the evolutionary process or strain selection, serving as an indicator of environmental adaptation. Geographical origin is another factor contributing to the variability in UV tolerance [21]. *M. anisopliae* has been isolated across a wide geographic range, from latitudes higher than 60° N to latitudes near 55° S. This generates an exposition of the strains to diverse environmental factors and different levels of irradiation. Higher latitudes and lower altitudes result in decreased irradiance [10]. It is assumed that strains from locations subject to higher radiation levels will exhibit higher tolerance and vice versa. This study focused on *M. anisopliae* strains isolated from the soils of bovine production units in the tropics of Veracruz, Mexico. The high susceptibility of entomopathogenic fungi to UV irradiation, particularly UV-B, is corroborated, despite the fact that the fungal isolation area of the present study is characterized by high temperatures and long periods of sunlight per year. This susceptibility coincides with the reports of other authors [21,26,28]. While factors such as genetic expression, geographical origin, environmental exposure, and isolation habitats [29] may contribute to the observed tolerance or susceptibility of the evaluated strains, further evaluation of these strains' characteristics is necessary, including genetic diversity, the degree of pigmentation, metabolite production, and field behavior. Furthermore, considering the high effectiveness demonstrated by the fungal strains of this study against ticks [13], the use of UV protectant formulation could be a suitable option to maintain their virulence at the field level.



Our study also revealed that exposure to UV-A+B radiation had an impact on the culturability of the 25 MaV and 55 MaV strains. These findings align with previous reports by Braga et al. [20] indicating a delay in conidial germination, consequently affecting the CFU count. However, some studies have found strains that have the ability to maintain the number of CFU even when exposed to UV-B radiation [30]. The different response of the conidial culturability of the EF is due to the delay in conidial germination that is linked to the cellular capacity of different strains to repair radiation-induced damage before progressing in the cell cycle. This temporary delay aims to prevent the replication of damaged DNA, which could heighten susceptibility in subsequent generations and safeguard the integrity of genetic material [11,20,24]. However, this temporary interruption in germination could compromise the efficacy of the fungus as a bioinsecticide, as the virulence of *M. anisopliae* is closely tied to germination speed [4]. Strains that germinate rapidly and penetrate the host cuticle have a higher likelihood of establishing a successful infection [20]. Rangel et al. [21] found that the UV radiation tolerance and germination speed of *M. anisopliae* conidia may vary depending on the substrate or culture medium (nutritional environment) where it is cultivated. The quantity of endogenous nutritional reserves in each conidium appears to influence the germination initiation time. Moreover, the culture medium can influence colony morphology and pigmentation [11]. Manipulating these variables could yield conidia with enhanced UV radiation tolerance and reduced germination time.

## 5. Conclusions

This original study of *M. anisopliae* strains demonstrated a range of UV-A and UV-A+B tolerance or susceptibility, indicating that UV exposure can indeed differentiate strains based on their UV tolerance levels. These strains demonstrated good tolerance to UV radiation (A or A+B) in terms of conidia germination and CFU count. These results may be of high novelty and interest to the scientific community that studies biological pest control and are original advances in the knowledge and understanding of the use of EF in tropical problems. It is recommended to continue carrying out in vitro and in vivo studies to assess other factors or characteristics (natural abiotic factors) influencing the effectiveness of *M. anisopliae* and the determination of lethal concentrations to apply the best effective doses at the field level as an alternative tick control method under real tropical conditions.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microbiolres15030089/s1>, Table S1. Conidia germination and reduction (%  $\pm$  SD) of eight strains of *Metarhizium anisopliae* irradiated with UV-A or UV-A+B. Table S2. Number of CFU of eight strains of *Metarhizium anisopliae* under irradiation UV-A and UV-A+B.

**Author Contributions:** M.Á.A.-D.: conceptualization, methodology, and writing the manuscript. M.d.L.L.-V.: methodology, sampling, and writing the manuscript. I.A.G.-G.: methodology, analyzed the data statistically, and the revision of the final manuscript. A.F.-S.: methodology, investigation, and the revision of the final manuscript. All authors have read and agreed to the published version of the manuscript.

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