Journal of Oil Palm Research Vol. 36 (1) March 2024 p. 40-50
DOI: https://doi.org/10.21894/jopr.2022.0076

INITIAL CHARACTERISATION OF *Metarhizium anisopliae* CPMa1502 FOR THE DEVELOPMENT OF A BIOPESTICIDE AGAINST THE OIL PALM FRUIT SCRAPER *Demotispa neivai* (Coleoptera: Chrysomelidae)

GINNA QUIROGA-CUBIDES[1]*; FELIPE BORRERO-ECHEVERRY[2]; ANA MARÍA JIMÉNEZ[1]; LUIS GUILLERMO MONTES-BAZURTO[3,4]; ALEX BUSTILLO PARDEY[3]; MARTHA I GÓMEZ[1] and PAOLA E CUARTAS-OTÁLORA[1]

ABSTRACT

Entomopathogenic fungi are key components of biological pest control programs. Among these, Metarhizium anisopliae *is one of the best-studied and has been demonstrated to be particularly effective against coleopterans. The native* M. anisopliae *CPMa1502, previously selected for its insecticidal activity against* Demotispa neivai *(oil palm fruit scraper), was studied to establish its growth parameters under several culture conditions and its virulence on different stages of* D. neivai*. Considerable conidia production of CPMa1502 on MAYP (Maltose Agar Yeast with Potato Extract) and SMAYR (Sabouraud Maltose Agar with yeast extract and rice extract) agar was observed ($1x10^9$ conidia cm^{-2} at 14 days). The isolate was highly tolerant to a wide range of pH (5-9; germination >90%; radial growth rate: 0.95-1.02 mm day^{-1}) but moderately tolerant to high temperatures (>35°C). Additionally, the adhesion capacity to* D. neivai *cuticle and conidial hydrophobicity was strong (114 conidia mm^{-2}: 72%). Finally, the insecticidal activity of CPMa1502 was greater on larvae than on adults, with a mean lethal concentration (LC_{50}) of $1.8x10^4$ conidia mL^{-1} and a mean lethal time (LT_{50}) of 4.3 days, than on adults. These findings show that CPMa1502 is a promising candidate for further development as a biopesticide against* D. neivai*.*

Keywords: biocontrol activity, conidia production, *Elaeis guineensis*, fruit scraper, growth parameters.

Received: 11 May 2022; **Accepted:** 11 October 2022; **Published online:** 9 December 2022.

INTRODUCTION

Entomopathogenic fungi have been well studied and developed into biopesticides, due to their high virulence towards a wide range of insect pests, their ability to form resistant structures and persist in the environment, and their innocuity to the environment and human health (Zimmermann, 2007). The most studied entomopathogenic fungi belong to the genus *Metarhizium* (Meyling and Eilenberg, 2007; Zimmermann, 2007). *Metarhizium anisopliae* has been isolated from insect cadavers, soil, and roots. It synthesises various compounds (lipases, adhesives, proteases, and chitinases) to degrade insect cuticle (Schrank and Vainstein, 2010), allowing it to grow hyphae that colonise the hemolymph, leading to insect death and sporulation (Gul *et al*., 2014; Zimmermann, 2007). *Metarhizium anisopliae* has been shown to be particularly active against Coleoptera, making it

[1] Departamento de Bioproductos, Corporación Colombiana de Investigación Agropecuaria (Agrosavia), Km 14 vía Mosquera-Bogotá, Mosquera, Colombia.

[2] Centro de Investigación Tibaitatá, Corporación Colombiana de Investigación Agropecuaria (Agrosavia), Km 14 vía Mosquera-Bogotá, Mosquera, Colombia.

[3] Pest and Disease Program, Colombian Oil Palm Research Center (Cenipalma), Calle 98, No. 70-91 Piso 14, Centro Empresarial Pontevedra, Bogotá, Colombia.

[4] Sustainable Plant Protection Programme, Institute of Agrifood Research and Technology (IRTA), Carretera Cabrils, Km 2, Cabrils, Barcelona, Spain.

* *Corresponding author e-mail: gquiroga@agrosavia.co*

a prime candidate to control various pest beetles (Batta, 2004; Erler and Ates, 2015; Hajek *et al*., 2007).

Oil palm (*Elaeis guineensis* or the hybrid *E. oleifera* x *E. guineensis*) is one of the most important oleaginous agricultural crops in the world. In Colombia, there are currently more than 559 000 cultivated hectares of this crop, making it the fourth-largest producer in the world. Like other agricultural crops, oil palm is seriously affected by various insect pests and entomopathogenic fungi have been used effectively in their control. For instance, entomopathogenic fungi have been shown to have high efficacy for the control of palm pests such as *Metisa plana* (Walker) (mortality of more than 95.0% with *Beauveria bassiana*), *Oryctes rhinoceros* (Scarabaeidae) (mortality of 100% with *M. anisopliae*), *Pteroma pendula* (mortality of 93.8% with *Paecilomyces carneus*), *Stenoma impressella* (mortality from 61.0% to 96.0% with *Cordyceps cateniannulata*), *Coptotermes curvignathus* (mortality from 71.0% to 84.0% with *M. anisopliae*), and *Demotispa neivai* (mortality from 68.0% to 95.0% with *M. anisopliae*) (Bakeri *et al.*, 2009; Kin *et al.*, 2017; Montes-Bazurto *et al.*, 2020a; 2020b; Ramle *et al.*, 2006; Ramle and Basri, 2004).

The oil palm fruit scraper, *D. neivai* Bondar (Coleoptera: Chrysomelidae), is an economically impactful emerging pest in oil palm plantations in Colombia. Both larvae and adults feed on the epidermis of fruits causing superficial damage and changing fruit colour. Insect feeding affects fruit development resulting in losses of approximately 7% in oil extraction. Furthermore, the superficial damage on the bunches makes it difficult to determine fruit maturity in the field, causing an additional 8% fruit loss (Aldana *et al*., 2004; Valencia *et al*., 2007). A recent study demonstrated that a native isolate of *M. anisopliae* (CPMa1502) caused up to 95% mortality in *D. neivai* larvae and adults under laboratory conditions (Montes-Bazurto *et al*., 2020b) and 62% larval mortality under field conditions (Montes-Bazurto *et al*., 2019). These findings demonstrated a marked biocontrol potential of CPMa1502 for further development as the first biological pesticide against *D. neivai* in Colombia. Fungal biopesticides require that their active ingredients fulfil minimal requirements for production and commercialisation from early research stages, such as conidia yield, growth parameters, and virulence. Therefore, the aim of this study was to assess desirable attributes such as growth conditions (production media, temperature, and pH), virulence factors (adherence and hydrophobicity), and insecticidal activity (lethal concentrations and mean lethal time) of *M. anisopliae* CPMa1502.

MATERIALS AND METHODS

Fungal Isolate and Reactivation

Metarhizium anisopliae isolate CPMa1502 (MH673410, MH698453 - GenBank), was obtained from the Entomopathogen Collection of Cenipalma (Montes-Bazurto *et al*., 2020b). The fungus was originally isolated from *D. neivai* larvae, collected from oil palm plantations in San Vicente de Chucurí, Santander, Colombia (Montes-Bazurto *et al*., 2019). It was reactivated on *D. neivai* adults and then grown on Maltose Agar Yeast with 0.5% v/v Potato Extract (MAYP) (Edelstein *et al*., 2004), supplemented with 0.1% w/v chloramphenicol (Colmed® International, Colombia), and incubated at 25°C ± 2°C for 14 days in total darkness. Conidia were harvested from Petri plates by carefully scraping the surface with a sterile scalpel.

Growth Parameters

Conidia production. CPMa1502 conidia obtained in MAYP agar were suspended in a 0.1% Tween® 80 solution (Merck, Germany), and the concentration was adjusted to $1x10^6$ conidia mL^{-1} using a hemocytometer. Subsequently, eight agar media reported for *M. anisopliae* conidia production were inoculated with 0.1 mL of the conidial suspension: 1. Potato Dextrose Agar (PDA) (Gandarilla-Pacheco *et al*., 2012); 2. PDA with 0.5% peptone (PDAP) (Gandarilla-Pacheco *et al*., 2012); 3. PDA with 0.5% yeast extract (PDAE) (Gandarilla-Pacheco *et al*., 2012); 4. Yeast Malt Agar (YM); 5. MAYP (Edelstein *et al*., 2004); 6. Sabouraud Dextrose Agar (SDA); 7. Sabouraud Maltose Agar with 1% yeast extract (SMAY) (Edelstein *et al*., 2004); and 8. SMAY supplemented with 13% rice extract (SMAYR) (Edelstein *et al*., 2004). The culture media were then incubated at 25°C ± 0.5°C (Memmert, Germany) for 10, 12 and 14 days. At each time, the growing biomass was harvested and suspended in 10 mL 0.1% Tween® 80 solution. The conidia concentration of each suspension was assessed to determine sporulation or conidia yield per unit area (conidia cm^{-2}). The conidia production test was carried out in triplicate for each treatment (growth media* incubation time), and the whole experiment was replicated three times.

Growth temperature. CPMa1502 conidia were suspended in a 0.10000% Tween® 80 solution, and the concentration was adjusted to $1x10^8$ conidia mL^{-1} using a hemocytometer. Germination was assessed by transferring 100 µL of dilution 10^{-2} of the conidial suspension to Petri plates with YM agar with 0.00004% benomyl (Benlate, DuPont, Spain). Plates were incubated at six temperatures for 16 and 24 hr (Faria *et al*., 2015): 20°C ± 0.5°C (Binder,

Germany); 25°C ± 0.5°C (Memmert, Germany); 30°C ± 1°C (Sheldon Manufacturing, Inc., USA); 35°C ± 1°C (Binder, Germany); 40°C ± 2°C (Memmert, Germany); and 45°C ± 2°C (Memmert, Germany). An agar square of 1 cm^2 was removed and then a drop of lactophenol blue solution was added (Merck, Germany). Germinated and non-germinated conidia were determined using an optical microscope (Olympus®, Japan), and 100 conidia were scored per replicate. A germinated conidium must have a germ tube length of at least twice the conidia diameter (Ekesi *et al*., 1999).

The radial growth rate was determined by measuring colony radius daily in a Petri plate with MAYP agar with 0.1% w/v chloramphenicol, inoculated with 10 µL of a conidial suspension in the middle of the plate at the six temperatures mentioned above for 14 days and reported as mm day^{-1}. The growth temperature test was carried out in triplicate with three replicates per repetition.

Effect of pH. A conidial suspension at 1x10^8 conidia mL^{-1} was prepared to inoculate 15 mL glass flasks with phosphate buffer adjusted to pH 5, 6, 7, 8 and 9 [1:9/conidia: buffer (Merck, Germany)], as liquid culture media. Sterile distilled water was used as a control (pH=5.88 ± 0.45). The flasks were incubated on a heated orbital shaker at 150 rpm, 25°C ± 1°C for 24 hr (Heidolph, Germany). Subsequently, germination and radial growth were measured, as described above, at a single incubation temperature of 25°C ± 0.5°C. The test was repeated three times and replicated three times.

Conidial Adherence and Relative Hydrophobicity

Conidial adherence was determined based on the methodology developed by Boucias *et al*. (1988). Cuticle ghosts were prepared from *D. neivai* adults by dipping them in 15% KOH (Merck, Germany), and boiling until the fragments became translucent. Cuticle ghosts were then mixed in a conidial suspension of *M. anisopliae* CPMa1502, adjusted to 1x10^7 conidia mL^{-1} and maintained at 200 rpm for 1 hr at room temperature (ThermoFisher Scientific, USA). Subsequently, the mixture was filtered with a non-woven fabric to recover the cuticles which were then cleaned with sterile distilled water. The conidia attached to the cuticle (1 mm^2) were revealed with lactophenol blue (Merck, Germany) and counted using a hemocytometer. The results were reported as conidia per unit area of the *D. neivai* cuticle.

Hydrophobicity assays were carried out according to the phase exclusion methodology described by Boucias *et al*. (1988) and Shah *et al*. (2007). Briefly, 10 mg of dry conidia were suspended in a 0.1M KNO_3 solution (aqueous phase; Merck, Germany) and absorbance was read at 600 nm (Mecasys, Korea). A volume of 3 mL of the aqueous phase was then mixed with 1 mL of toluene (organic phase; Merck, Germany), gently vortexed for 20 s, and left to rest for 30 min. The aqueous phase was extracted and absorbance was assessed at 600 nm. Relative hydrophobicity percentage (*H*) was calculated using the following Equation (1):

$$H\,(\%) = \frac{A_0 - A_f}{A_f} \times 100 \qquad (1)$$

where H is the relative hydrophobicity, A_0 is the initial absorbance of the aqueous phase, and A_f is the absorbance of the aqueous phase after mixing with toluene.

Conidial adherence and relative hydrophobicity were assessed three times, with nine measurements carried out for adherence and three for hydrophobicity.

Insecticidal Activity

Insects. *Demotispa neivai* adults and larvae used to establish the rearing or for bioassays were obtained from oil palm fruit bunches, collected on a naturally infested oil palm plot, located near Cenipalma's Palmar de la Vizcaína research station, Santander, Colombia. Collected individuals were subjected to a 10 day quarantine to avoid contamination by naturally occurring pathogens or parasitoids. Adults and larvae were placed in 8 × 5 cm PVC tubes, closed at both ends with muslin and allowed to feed and oviposit on ripe fruits. The eggs found on fruits were individualised and monitored until hatching. Ripe palm fruits were replaced as necessary. Insects were kept at 27°C ± 1°C, 90% ± 5% relative humidity (RH), and a photoperiod of 12:12 hr (light: dark), until they were needed for bioassays.

Concentration-mortality and time-mortality tests. The lethal concentrations for CPMa1502 conidia, grown on MAYP agar, were determined on adults and larvae of *D. neivai*, using laboratory bioassays (Montes-Bazurto *et al*., 2020b). For the adult bioassay, adults of *D. neivai* were immersed for 1 min in an aqueous suspension of *M. anisopliae* CPMa1502 conidia, adjusted to five different concentrations (1x10^4, 1x10^5, 1x10^6, 1x10^7, and 1x10^8 conidia mL^{-1}), in 0.1% Tween® 80 solution. For control treatments, a solution of 0.1% v/v Tween® 80 was used, whereas, for absolute controls, insects were untreated. Larvae were inoculated by placing a 15 µL drop of conidial suspension on the thorax, adjusted to the same concentrations as those used in the adult bioassay. After inoculation, insects were maintained individually in 8 cm diameter × 5 cm PVC tubes covered with muslin on both ends at 27°C ± 1°C and 90% ± 5% RH, under a natural photoperiod on oil

palm fruits. Insect mortality was recorded every day for 15 days. Mortality data for treatments was corrected with mortality data from the control, to determine the mean lethal concentrations LC_{50} and lethal concentration ninety LC_{90} were determined using Probit analysis (Finney, 1952). The lethal time (LT_{50}) was calculated using the mortality data of the pathogenicity bioassays for the treatment corresponding to a fungal concentration of $1x10^8$ conidia mL^{-1} for both adults and larvae.

A completely randomised design was used with five replicates per treatment (conidia concentration) for adult bioassay (n=5) and three per treatment for larvae bioassay (n=3). Each replicate consisted of 10 individual insects (experimental units) for a total of 50 adults, and 30 larvae used per treatment. Different methods were used for larval and adult bioassays since larvae drowned when the immersion method was used and drops rolled off adult elytra.

Statistical Analysis

For statistical purposes, germination was arcsine or square-root transformed. Statistical significance of treatments was determined using one-way analysis of variance (ANOVA), and mean separations were compared using Tukey HSD´s test with a confidence level of 95%. Analysis was carried out using Statistix® version 8.1 (Analytical Software 2003). Probit analysis (Finney, 1952) was used to determine the lethal concentrations using POLO-PLUS 1.0 (LeOra Software 2006) and lethal time data were subjected to Weibull survival analysis using the Generalised Linear Interactive Modeling Program (Crawley, 1993).

RESULTS AND DISCUSSION

Growth Parameters

Conidia production. All the media assessed allowed the growth and sporulation of CPMa1502. The fermentation started with the colonisation of white mycelium and the production of dark green aerial conidia after 6-7 days of incubation. The conidia yields at 10, 12 and 14 days are presented in *Figure 1a*. Sparse sporulation was observed in PDAP, PDAE, YM, and SDA agar, followed by PDA and SMAY media (*Figure 1b*). MAYP and SMAYR reached the highest conidia yields at all three times evaluated. Significant differences were found between the eight culture media on the 10th and 12th day of growth (10th day: $F_{7,16}$=4.93, p=0.0039; 12th day: $F_{7,16}$=4.32, p=0.0073); while no statistical differences were observed on the 14th day ($F_{7,16}$= 2.11; p= 0.1023). The largest yield was reached on the 14th day using MAYP and SMAYR (MAYP: 1.1 ± $0.4x10^9$ conidia cm^{-2}; SMAYR: 1.1 ± $0.3x10^9$ conidia cm^{-2}).

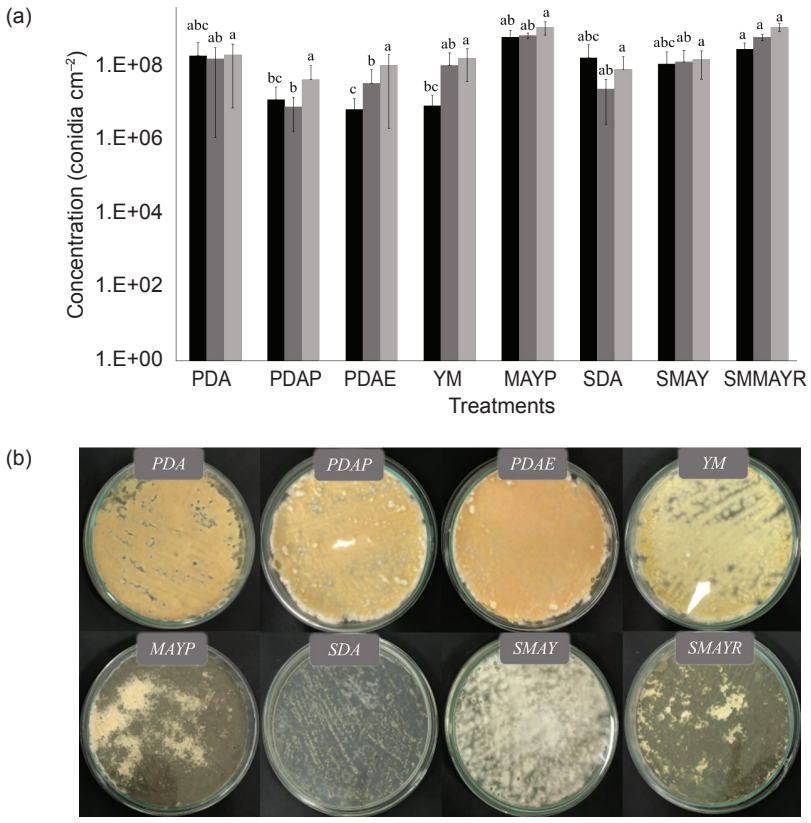

Figure 1. Conidia production of M. anisopliae *CPMa1502 on different agar media. (a) Conidia concentration (conidia cm^{-2}) at 10(■), 12(■), and 14(■) days incubation; (b)* In vitro *growth at 14 days. Values with different lowercase letters show statistical differences according to Tukey HDS Test* ($p<0.05$).

CPMa1502 achieved the highest yield on MAYP and SMAYR agar, and which could be attributed to the sources of carbon, nitrogen, proteins, vitamins, and amino acids favoring the conidiogenesis process. Yeast extract has been identified as a sporulation inductor, and enables increased biomass production due to its vitamin B content (Patil *et al*., 2014). Also, the starches used in these media can also induce the enzymatic capacity for the degradation of polysaccharides, similar to insect cuticles, and activate several virulence mechanisms (Clarkson and Charnley, 1996; Permadi *et al*., 2020). The yields achieved were 10 times higher than the values reported for *Metarhizium* sp., using YM agar ($7.7x10^6$ conidia cm^{-2}) (Aguirre *et al*., 2009). MAYP has also been found to favour biomass production and increase the growth rate for other species of *Metarhizium* (Edelstein *et al*., 2004).

Growth temperature. Germination after 16 and 24 hr was greater than 85% for temperatures between 20°C-35°C, and was no different (16 hr: $F_{5,48}$=149, p<0.0001; 24 hr: $F_{5,48}$=101, p<0.0001) (*Table 1*). There was a 3%-24% decrease in germination after 16 hr of incubation, and 9%-18% at 24 hr at 40°C ± 2°C of incubation, compared with the values found at 20°C-35°C (16 hr: $F_{3,32}$=11.6, p<0.0001; 24 hr: $F_{3,32}$=6.06, p=0.0007). Furthermore, at 45°C ± 2°C, the reduction at both times ranged between 85% and 98% (16 hr: $F_{3,32}$=165, p<0.0001; 24 hr: $F_{3,32}$=115, p<0.0001). Radial growth was not affected by the temperature range between 20°C to 35°C. The growth rates at 40°C ± 2°C and 45°C ± 2°C were significantly lower than the findings at 20°C-35°C, and no growth was observed at 45°C ($F_{5,48}$=352, p<0.0001).

The high-temperature tolerance of *M. anisopliae* CPMa1502 conidia will enable its persistence in the environment, despite daily thermal fluctuations in tropical regions for longer periods, ensuring its effectiveness in pest control. Thermotolerant conidia will also be able to resist formulation processes, such as drying and storage at high temperatures (30°C-40°C) (Alves *et al*., 2017; Athanassiou *et al*., 2017; Oliveira *et al*., 2016; Rangel *et al*., 2005). *Metarhizium anisopliae* CPMa1502 showed optimal growth at a temperature range between 20°C and 35°C, and no appreciable decrease in germination was observed up to 40°C after 24 hr (>80%). High viability at temperatures above 25°C could be due to the geographical region where this microorganism was isolated, which has a maximum temperature of 40°C (IDEAM, 2018; 2019). Other *Metarhizium* sp. isolated in tropical regions showed a similar response, suggesting a direct relationship between the isolates' geographical area and their tolerance to temperature (Fargues *et al*., 1997; Fernandes *et al*., 2010; Hallsworth and Magan, 1996; 1999; Rangel *et al*., 2005).

Effect of pH. CPMa1502 germination (16 and 24 hr) was above 95% for the range of pH evaluated and no differences were observed (16 hr: $F_{5,48}$=0.94; p=0.4660; 24 hr: $F_{5,48}$=0.68; p=0.6421) (*Table 2*). Similarly, pH did not affect the radial growth rate as shown in *Figure 2* ($F_{3,32}$=0.39, p=0.8451).

TABLE 1. EFFECT OF GROWTH TEMPERATURE ON CONIDIAL GERMINATION AT 16 hr AND 24 hr, AND RADIAL GROWTH RATE (mean ± SD)

Temperature (°C)	Germination at 16 hr (%)	Germination at 24 hr (%)	Radial growth rate (mm day^{-1})
20 ± 0.5	89.5 ± 1.5[a]	95.4 ± 1.1[a]	1.14 ± 0.08[a]
25 ± 0.5	89.5 ± 3.5[a]	93.1 ± 0.9[a]	1.07 ± 0.17[a]
30 ± 1.0	89.6 ± 2.5[a]	92.6 ± 1.8[a]	1.13 ± 0.15[a]
35 ± 1.0	89.3 ± 1.2[a]	93.5 ± 0.6[a]	1.16 ± 0.10[a]
40 ± 2.0	78.5 ± 1.8[b]	82.7 ± 1.4[b]	0.12 ± 0.01[b]
45 ± 2.0	3.9 ± 0.3[c]	5.9 ± 1.6[c]	0.00 ± 0.00[c]

Note: Values with different lowercase letters show statistical differences according to Tukey HDS Test (p<0.05).

TABLE 2. EFFECT OF pH ON CONIDIAL GERMINATION AT 16 hr AND 24 hr, AND RADIAL GROWTH RATE (mean ± SD)

pH	Germination at 16 hr (%)	Germination at 24 hr (%)	Radial growth rate (mm day^{-1})
Control	98.2 ± 1.4[a]	99.1 ± 1.3[a]	0.97 ± 0.04[a]
5	96.2 ± 4.3[a]	98.5 ± 2.2[a]	0.95 ± 0.07[a]
6	97.6 ± 1.7[a]	98.9 ± 1.6[a]	0.99 ± 0.06[a]
7	96.7 ± 2.7[a]	98.1 ± 1.8[a]	1.02 ± 0.11[a]
8	95.3 ± 4.4[a]	97.4 ± 3.5[a]	1.00 ± 0.09[a]
9	96.4 ± 3.8[a]	97.1 ± 3.2[a]	0.99 ± 0.07[a]

Note: Values with different lowercase letter show statistical differences according to Tukey HDS Test (p<0.05).

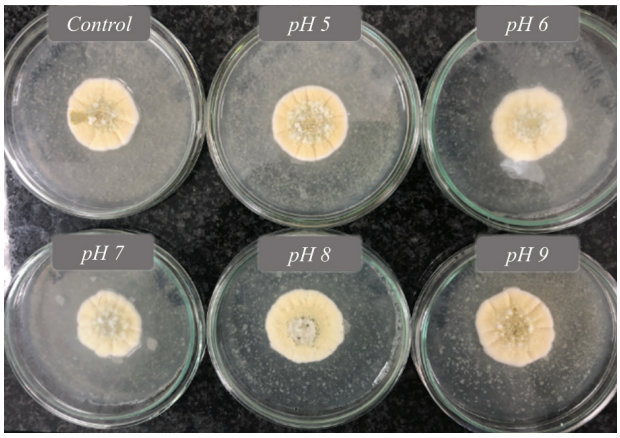

Figure 2. Effect of pH on the radial growth rate of M. anisopliae *CPMa1502.*

Tolerance to varying pH is also relevant since it facilitates the mass production of the fungi and subsequent formulation processes. Moreover, in-field applications are more reliable in pH-tolerant strains since the pH of water used in the field varies. This could reduce the efficacy of pH-sensitve isolates by altering cell structures and modifying micronutrient assimilation, resulting in changes to cell morphology and duplication (Glazebrook *et al*., 1992; Liu *et al*., 2007; Tamerler *et al*., 1998). Previous studies have shown that *M. anisopliae* can grow in pH range between 2.5 to 10.5 (Hallsworth and Magan, 1996; St Leger *et al*., 1999). Its ability to grow in acidic and basic environments is due to its ability to modulate the cytosolic production of organic acids to maintain pH homeostasis (St Leger *et al*., 1999).

Conidial Adherence and Relative Hydrophobicity

Groups of more than 10 conidia were observed in some areas of the host cuticle without showing a specific distribution pattern, affecting the CV>20%. Despite this, the mean conidial adherence was 114 ± 36 conidia mm^{-2} without significant differences among repetitions ($F_{2,24}$=0.68, p=0.5172). Mean relative hydrophobicity was near 72%, with a CV<10% ($F_{2,6}$=2.38, p=0.1735).

Conidial adherence and hydrophobicity are critical factors that determine the interaction between fungal propagules and their host insects, and fungal dispersion (Jeffs *et al*., 1999; Sevim *et al*., 2012). Fungal propagules have different adhesion properties as a response to adaptation to environmental conditions. Aerial conidia tend to adhere strongly to hydrophobic surfaces, while submerged conidia can adhere to both hydrophilic and hydrophobic surfaces (Holder and Keyhani, 2005). Additionally, the high adherence to the fungal conidia allows for the induction of a continuous infection (Staples and Milner, 2000). The adherence of *M. anisopliae* CPMa1502 conidia to *D. neivai* cuticle represented 11% of the initial conidia concentration ($1x10^7$ conidia mL^{-1}). The conidia adhered irregularly to the insect, and preferred areas of the host cuticle with tiny spines or other irregularities, leading to high variability in adherence (Boucias *et al*., 1988). Fernandez *et al*. (2001) assessed the number of conidia adhered to different parts of the insect body with diverse inoculation methods, and found a higher conidia density on the dorsal surface compared to other parts of insects, regardless of the inoculation method. Villamizar and Cotes (2003) reported similar findings using *M. anisopliae* conidia obtained from submerged fermentation; when the authors assessed the adherence with conidia from solid-state fermentation, the values increased between three and six fold. Therefore, each type of propagule has different adhesion and hydrophobicity properties as a response to adaptation to environmental and nutritional conditions.

High hydrophobicity is also a helpful characteristic due to the interaction between amphipathic proteins (hydrophobins) involved in conidia host and fruits interactions since both the waxy surface of oil palm fruits and the lipid layer of the insect cuticle is hydrophobic (Dubey *et al*., 2014). Similar to adherence, conidia hydrophobicity can be influenced by the nutritional composition of the media (Holder and Keyhani, 2005; Rosenberg *et al*., 1980). A comparable hydrophobicity of *M. anisopliae* CPMa1502 was reported by Jeffs *et al*. (1999) using *M. anisopliae* isolates, showing hydrophobicities between 65%-69% using a salt-mediated aggregation and sedimentation assay (SAS). Luke *et al*. (2014) observed a relative hydrophobicity between 62%-71% with the SAS assay, and 95%-98% with the phase exclusion method for four *Metarhizum* species. Villamizar and Cotes (2003) achieved comparable values of relative hydrophobic with conidia recovered from solid-state fermentation. Boucias *et al*. (1988) found a relative hydrophobicity of 100% in *M. anisopliae* conidia grown on a synthetic medium.

Insecticidal Activity

Concentration-mortality test. Mortality of adults and larvae of *D. neivai* was demonstrated at different fungal concentrations and oscillated between 4.0% and 78.0% in *D. neivai* adults and between 51.9% and 100% in larvae (*Figure 3*). There were significant differences between the different concentrations evaluated in the mortality of both adults ($F_{5,24}$=13.90, p<0.0001) and larvae ($F_{7,74}$=5.12, p=0.0018). Mortality in the absolute controls was 2% for adults (Standard error of the mean, *SEM*=0.63), and 14.8% for larvae (*SEM*=2.47), values acceptable for this type of assay. The beetles, killed by the action of the fungus, showed progressive stiffness until complete mummification, with cadavers covered with white mycelia that later sporulated with powdery green conidia (*Figure 4*). Mortality data

was corrected, and the dose-mortality relationships were calculated (*Table 3*). A linear relationship between dose and mortality for both larvae and adults was found (p>0.05). Mortality increased comparably with increased concentrations for both larvae and adults (*Figure 3* and *Table 3*).

The LC_{50} was $9.4x10^6$ and $1.8x10^4$ conidia mL^{-1} for adults and larvae, respectively, and the LC_{90} was $1.6x10^9$ and $9.2x10^6$ conidia mL^{-1} for adults and larvae, respectively. Mortality increased in a comparable manner with increased concentrations for both larvae and adults (Adults: p= 0.5, df= 3, χ^2 =2.13 and Larvae: p= 0.5, df= 3, χ^2= 2.35).

Time-mortality test. The mean lethal time (LT_{50}) was estimated at a fungal concentration of $1x10^8$ conidia mL^{-1} for both adults and larvae. The LT_{50} for adults was 10.1 days [95%, Confidence intervals (C.I.) 8.1-16.3] and was 4.3 days (95 % C.I. 3.8-4.7) for larvae.

Data available on the biological activity of *M. anisopliae* show great variability in activity with LC_{50} values between $1.7x10^3$ conidia mL^{-1} and $1.4x10^7$ conidia mL^{-1} (Quesada-Moraga *et al*., 2004; Shi and Feng, 2009). Our findings are comparable with the values found for *Hoplia philanthus* control (LC_{50}: $2.5x10^4$-$4.0x10^6$ conidia mL^{-1}) (Ansari *et al*., 2004). *Eurygaster integriceps* (Hemiptera:

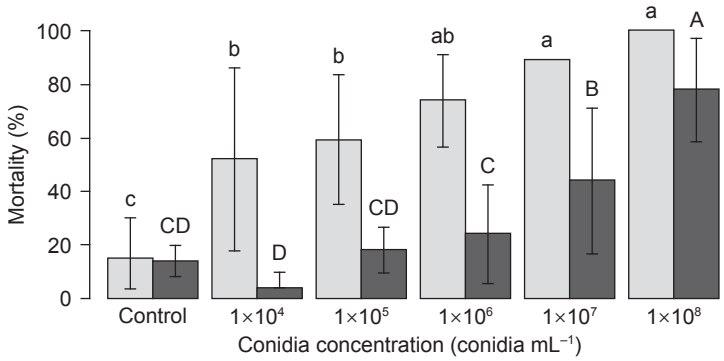

Figure 3. Mortality caused by M. anisopliae *CPMa1502 on* D. neivai *larvae(□) and adults(■) under laboratory conditions. Values with different uppercase (adults) or lowercase (larvae) letters did not show statistical differences according to the LDS Test* (p<*0.05).*

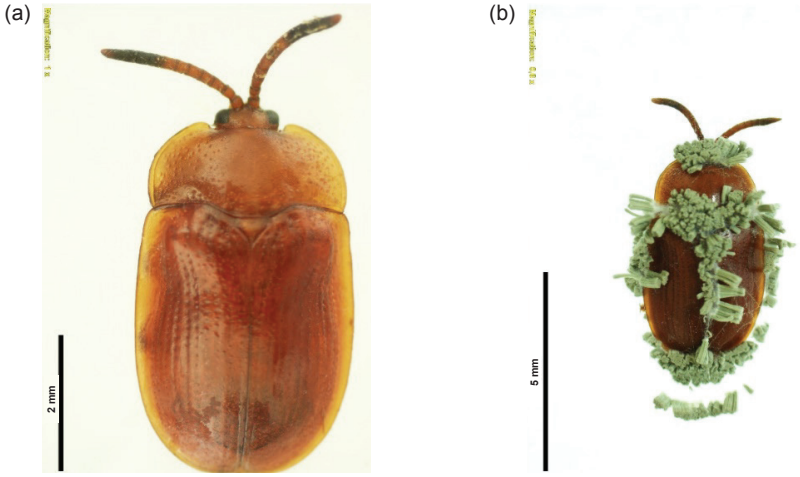

Figure 4. Typical symptoms of infected beetles by M. anisopliae *CPMa1502: (a) healthy beetle from a control treatment; (b) fungi-infected beetle with mycelia formation and sporulation.*

TABLE 3. ESTIMATION OF LC_{50} AND LC_{90} OF *M. anisopliae* CPMa1502 ON *D. neivai* ADULTS AND LARVAE UNDER LABORATORY CONDITIONS

Life stage	LC_{50} (conidia mL^{-1})	Confidence intervals (C.I.) (conidia mL^{-1})		LC_{90} (conidia mL^{-1})	Probit analysis			
		Lower	Upper		df	X^2	p	Slope
Adults	$9.4x10^6$	$4.5x10^6$	$2.3x10^7$	$1.6x10^9$	3	2.1322	0.5454	0.576
Larvae	$1.8x10^4$	$2.5x10^3$	$5.9x10^4$	$9.2x10^6$	3	2.3540	0.4740	0.474

Note: df - degree of freedom; χ^2 - chi-square value; p - probability value.

Scutelleridae) nymphs were shown to be more susceptible than adults to different isolates and concentrations of *M. anisopliae,* with LT_{50} values between five and nine days for nymphs and 12 to 30 days for adults (Sedighi *et al*., 2013). The diverse susceptibility of different life stages of insects to entomopathogenic fungi when they are used as pest control agents suggests that they should be applied when the insect is most susceptible to achieve the best efficacy, to curtail the spread of pests.

CONCLUSION

The growth parameters and biological characteristics of the CPMa1502 assessed in this study are fundamental to model its performance during further development as well as its fitness under real application conditions to control *D. neivai*. Results showed its high potential as an alternative for chemical pest control: Considerable conidia productivity on MAYP agar ($1.1x10^9$ conidia cm^{-2}), high tolerance to different growth conditions [pH (5-9) and temperature (below 30°C)], which will enable it to survive common conditions of oil palm plantations in Colombia. Conidia interaction with cuticle also suggests that CPMa1512 is a good candidate for further development (adherence: 112 conidia mm^{-2}; hydrophobicity 72%). Lastly, the high susceptibility of *D. neivai* to CPMa1502 (Adults LC_{50} $9.4x10^6$ conidia mL^{-1}; Larvae LC_{50} $1.8x10^4$ conidia mL^{-1}) show its insecticidal capacity and the potential for the development of the first biopesticide for this insect pest. This research provides the background needed to continue with the next steps of biopesticide development, standardising the fungal production, and formulation, and determining the biological activity of the formulated product under field conditions.

ACKNOWLEDGEMENT

The authors would like to thank the Oil Palm Development Fund administered by Fedepalma and the Ministerio de Agricultura y Desarrollo Rural (MADR) of Colombia (through public funds assigned to Agrosavia), for funding this study under the agreement AV17-03 between Cenipalma and Agrosavia. We would also like to thank Agrosavia's technicians Oscar Monroy, Stella Rincon, and Javier Pulido; Universidad de La Paz's student Luis Fernando Buitrago, Cenipalma´s technicians Harold Giordanelly Cortes, and the staff at Palmeras Yarima, Costa de Oro, and Zamarkanda plantations, for their support in all of the activities related to this research.

REFERENCES

Aguirre, N; Villamizar, L; Espinel, C and Cotes, P (2009). Efecto del pH y de la actividad de agua sobre el desarrollo de *Nomuraea rileyi* (Hyphomycetes). *Revista Colomb. Entomol., 35*: 138-144.

Aldana, J; Calvache, H; Cataño, J; Valencia, C and Hernández, J (2004). Aspectos biológicos y alternativas de control de *Imatidium neivai* Bondar raspador del fruto de la palma de aceite. *Revista Palmas, 25:* 240-248.

Alves, F; Bernardo, C; Paixão, F; Barreto, L; Luz, C; Humber, R and Fernandes, É K (2017). Heat-stressed *Metarhizium anisopliae*: Viability (*in vitro*) and virulence (*in vivo*) assessments against the tick *Rhipicephalus sanguineus*. *Parasitol. Res., 116(1)*: 111-121. DOI: 10.1007/s00436-016-5267-z.

Analytical Software (2003). *Statistix 8.1 User Manual*. Analytical Software. Tallahassee. 463 pp.

Ansari, M; Vestergaard, S; Tirry, L and Moens, M (2004). Selection of a highly virulent fungal isolate, *Metarhizium anisopliae* CLO 53, for controlling *Hoplia philanthus*. *J. Invertebr. Pathol., 85(2)*: 89-96. DOI: 10.1016/j.jip.2004.01.003.

Athanassiou, C; Kavallieratos, N; Rumbos, C and Kontodimas, D (2017). Influence of temperature and relative humidity on the insecticidal efficacy of *Metarhizium anisopliae* against larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae) on wheat. *J. Insect Sci., 17(1)*: 22. DOI: 10.1093/jisesa/iew107.

Bakeri, S A; Ali, S R A; Tajuddin, N S and Kamaruzzaman, N E (2009). Efficacy of entomopathogenic fungi, *Paecilomyces* spp., in controlling the oil palm bagworm, *Pteroma pendula* (Joannis). *J. Oil Palm Res., 21(2)*: 693-699.

Batta, Y A (2004). Control of rice weevil (*Sitophilus oryzae* L., Coleoptera: Curculionidae) with various formulations of *Metarhizium anisopliae*. *Crop Protect., 23(2)*: 103-108. DOI: 10.1016/j.cropro.2003.07.001.

Boucias, D G; Pendland, J C and Latge, J P (1988). Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Appl. Environ. Microbiol., 54(7)*: 1795-1805. DOI: 10.1128/aem.54.7.1795-1805.1988.

Clarkson, J and Charnley, A K (1996). New insights into the mechanisms of fungal pathogenesis in insects. *Trends Microbiol., 4(5)*: 197-203. DOI: 10.1016/0966-842x(96)10022-6.

Crawley, M (1993). *GLIM for Ecologists*. Blackwell Scientific. Cambridge and Boston. 392 pp.

Dubey, M; Jensen, D and Karlsson, M (2014). Hydrophobins are required for conidial hydrophobicity and plant root colonization in the fungal biocontrol agent *Clonostachys rosea*. *BMC Microbiol., 14(18)*. DOI: 10.1186/1471-2180-14-18.

Edelstein, J; Lecuona, R and Trumper, E (2004). Selection of culture media and *in vitro* assessment of temperature-dependent development of *Nomuraea rileyi*. *Neotrop. Entomol., 33(6)*: 737-742. DOI: 10.1590/S1519-566X2004000600011.

Ekesi, S; Maniania, N K and Ampong-Nyarko, K (1999). Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Sci. Technol., 9(2):* 177-185. DOI: 10.1080/09583159929767.

Erler, F and Ates, A (2015). Potential of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* (Coleoptera: Scarabaeidae), as biological control agents against the June beetle. *J. Insect Sci., 15(1)*: 44. DOI: 10.1093/jisesa/iev029.

Fargues, J; Ouedraogo, A; Goettel, M and Lomer, C (1997). Effects of temperature, humidity and inoculation method on susceptibility of *Schistocerca gregaria* to *Metarhizium flavoviride*. *Biocontrol Sci. Technol., 7(3)*: 345-356. DOI: 10.1080/09583159730758.

Faria, M; Lopes, R; Souza, D and Wraight, S (2015). Conidial vigor *vs*. viability as predictors of virulence of entomopathogenic fungi. *J. Invertebr. Pathol., 125*: 68-72. DOI: 10.1016/j.jip.2014.12.012.

Fernandes, É K; Keyser, C; Chong, J; Rangel, D; Miller, M and Roberts, D (2010). Characterization of *Metarhizium* species and varieties based on molecular analysis, heat tolerance and cold activity. *J. Appl. Microbiol., 108(1)*: 115-128. DOI: 10.1111/j.1365-2672.2009.04422.x.

Fernandez, S; Groden, E; Vandenberg, J and Furlong, M (2001). The effect of mode of exposure to *Beauveria bassiana* on conidia acquisition and host mortality of Colorado potato beetle, *Leptinotarsa decemLineata*. *J. Invertebr. Pathol., 77(3)*: 217-226. DOI: 10.1006/jipa.2001.5022.

Finney, D (1952). *Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve.* Cambridge University Press. Cambridge. 318 pp.

Gandarilla-Pacheco, F; Arévalo-Niño, K; Galán-Wong, L; Coronado, C and Quintero-Zapata, I (2012). Evaluation of conidia production and mycelial growth in solid culture media from native strains of entomopathogenic fungi isolated from citrus-growing areas of México. *Afr. J. Biotechn., 11(79)*: 14453-14460. DOI: 10.5897/AJB12.1658.

Glazebrook, M; Vining, L and White, R (1992). Growth morphology of *Streptomyces akiyoshiensis* in submerged culture: Influence of pH, inoculum, and nutrients. *Can. J. Microbiol., 38(2)*: 98-103. DOI: 10.1139/m92-016.

Gul, H; Saeed, S and Khan, F (2014). Entomopathogenic fungi as effective insect pest management tactic: A review. *Appl. Sci. Bus. Econ., 1(1)*: 10-18.

Hajek, A; Huang, B; Dubois, T; Smith, M and Li, Z (2007). Field studies of control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) using fibre bands containing the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria brongniartii*. *Biocontrol Sci. Technol., 16(4)*: 329-343. DOI: 10.1080/09583150500335962.

Hallsworth, J and Magan, N (1996). Culture age, temperature, and pH affect the polyol and trehalose contents of fungal propagules. *Appl. Environ. Microbiol., 62(7)*: 2435-2442. DOI: 10.1128/AEM.62.7.2435-2442.1996.

Hallsworth, J and Magan, N (1999). Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. *J. Invertebr. Pathol., 74(3)*: 261-266. DOI: 10.1006/jipa.1999.4883.

Holder, D and Keyhani, N (2005). Adhesion of the entomopathogenic fungus *Beauveria (Cordyceps) bassiana* to substrata. *Appl. Environ. Microbiol., 71(9)*: 5260. DOI: 10.1128/AEM.71.9.5260-5266.2005.

IDEAM (2018). Mapa solar Colombiano. http://atlas.ideam.gov.co/visorAtlasRadiacion.htmL, accessed on February 2018.

IDEAM (2019). *Información aeronáutica* climatologica. http://bart.ideam.gov.co/paginas/index.htmL, accessed on June 2019.

Jeffs, L; Xavier, I; Matai, R and Khachatourians, G (1999). Relationships between fungal spore morphologies and surface properties for entomopathogenic members of the general *Beauveria*, *Metarhizium*, *Paecilomyces*, *Tolypocladium*, and *Verticillium*. *Can. J. Microbiol., 45(11)*: 936-948. DOI: 10.1139/w99-097.

Kin, P K; Moslim, R; Azmi, W A; Kamarudin, N and Ali, S R A (2017). Genetic variation of entomopathogenic fungi, *Metarhizium anisopliae* and *Isaria amoenerosea* and their pathogenicity against subterranean termite, *Coptotermes curvignathus*. *J. Oil Palm Res., 29(1)*: 35-46.

LeOra Software (2006). *POLO-Plus 1.0 Probit and Logit Analysis*. LeOra Software. Petaluma. 19 pp.

Liu, B; Rou, T; Rao, Y and Tzeng, Y (2007). Effect of pH and aeration rate on the production of destruxins A and B from *Metarhizium anisopliae*. *Int. J. Appl. Sci. Eng., 5*: 17-26.

Luke, B; Faull, J and Bateman, R (2014). Using particle size analysis to determine the hydrophobicity and suspension of fungal conidia with particular relevance to formulation of biopesticide. *Biocontrol Sci. Technol., 25(4)*: 383-398. DOI: 10.1080/09583157.2014.979396.

Meyling, N and Eilenberg, J (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biol. Control, 43(2)*: 145-155. DOI: 10.1016/j.biocontrol.2007.07.007.

Montes-Bazurto, L; Peteche-Yonda, Y and Bustillo, A (2019). Life cycle and natural enemies of *Demotispa neivai* (Coleoptera: Chrysomelidae). *J. Entomol. Sci., 54(3)*: 213-222. DOI: 10.18474/jes18-61.

Montes-Bazurto, L G; Bustillo-Pardey, A E and Medina-Cárdenas, H C (2020a). *Cordyceps cateniannulata*, a novel entomopathogenic fungus to control *Stenoma impressella* Busck (Lepidoptera: Elachistidae) in Colombia. *J. Appl. Entom., 144*: 788-796. DOI: 10.1111/jen.12818.

Montes-Bazurto, L; Peteche-Yonda, Y; Medina-Cardenas, H and Bustillo-Pardey, A (2020b). Selection of entomopathogenic fungi for the biological control of *Demotispa neivai* (Coleoptera: Chrysomelidae) in oil palm plantations in Colombia. *J. Entomol. Sci., 55(3)*: 388-404. DOI: 10.18474/0749-8004-55.3.388.

Oliveira, M; Monteiro, A; Scala Júnior, N; Barbosa, J and Mochi, D (2016). Sensibilidade de isolados de fungos entomopatogênicos às radiações solar, ultravioleta e à temperatura. *Arq. Inst. Biol., 83*: 1-7. DOI: 10.1590/1808-1657000042014.

Patil, S; Kadam, J; Chandele, A; Wagh, S and Jadhav, R (2014). Growth, development and viability of *Metarhizium anisopliae* on media with various nutrient sources. *Inter. J. Plant Prot., 7(2)*: 420-423. DOI: 10.15740/HAS/IJPP/7.2/420-423.

Permadi, M; Mukhlis, B; Siregar, D and Wayni, M (2020). Physiology characterization of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* on different carbohydrate sources. *J. Phys.: Conference Series, 1477*: 072007. DOI: 10.1088/1742-6596/1477/7/072007.

Quesada-Moraga, E; Santos-Quirós, R; Valverde-García, P and Santiago-Álvarez, C (2004). Virulence, horizontal transmission, and sublethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). *J. Invertebr. Pathol., 87(1)*: 51-58. DOI: 10.1016/j.jip.2004.07.002.

Rangel, D; Braga, G; Anderson, A and Roberts, D (2005). Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *J. Invertebr. Pathol., 88(2)*: 116-125. DOI: 10.1016/j.jip.2004.11.007.

Ramle, M and Basri, M W (2004). The effects of oils on germination of *Beauveria bassiana* (Balsamo) Vuillemin and its infection against the oil palm bagworm, *Metisa plana* (Walker). *J. Oil Palm Res., 16(2)*: 78-87.

Ramle, M; Mohd, B W; Norman, K; Hisham, H and Siti Ramlah, A A (2006). Research in the commercialization of *Metarhizium anisopliae* (Hyphomycetes) for biocontrol of the rhinoceros beetle, *Oryctes rhinoceros* (Scarabaeidae), in oil palm. *J. Oil Palm Res., Special Issue - April*: 37-49.

Rosenberg, M; Gutnick, D and Rosenberg, E (1980). Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett., 9(1)*: 29-33. DOI: 10.1111/j.1574-6968.1980.tb05599.x.

Schrank, A and Vainstein, M H (2010). *Metarhizium anisopliae* enzymes and toxins. *Toxicon, 56(7)*: 1267-1274. DOI: 10.1016/j.toxicon.2010.03.008.

Sedighi, N; Abbasipour, H; Askary, H; Gorjan, A S and Karimi, J (2013). Pathogenicity of the entomopathogenic fungus *Metarhizium anisopliae* Var. major on different stages of the sunn pest, *Eurygaster integriceps*. *J. Insect. Sci., 13(1)*: 150. DOI: 10.1673/031.013.15001.

Sevim, A; Donzelli, B; Wu, D; Demirbag, Z; Gibson, D M and Turgeon, B G (2012). Hydrophobin genes of the entomopathogenic fungus, *Metarhizium brunneum*, are differentially expressed and corresponding mutants are decreased in virulence. *Curr. Genet., 58(2)*: 79-92. DOI: 10.1007/s00294-012-0366-6.

Shah, F A; Allen, N; Wright, C J and Butt, T M (2007). Repeated *in vitro* subculturing alters spore surface properties and virulence of *Metarhizium anisopliae*. *FEMS Microbiol. Lett., 276(1)*: 60-66. DOI: 10.1111/j.1574-6968.2007.00927.x.

Shi, W and Feng, M (2009). Effect of fungal infection on reproductive potential and survival time of *Tetranychus urticae* (Acari: Tetranychidae). *Exp. Appl. Acarol., 48(3)*: 229-237. DOI: 10.1007/s10493-009-9238-2.

St Leger, R; Nelson, J and Screen, S (1999). The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. *Microbiol., 145(10)*: 2691-2699. DOI: 10.1099/00221287-145-10-2691.

Staples, J and Milner, R (2000). A laboratory evaluation of the repellency of *Metarhizium anisopliae* conidia to *Coptotermes lacteus* (Isoptera: Rhinotermitidae). *Sociobiol., 36(1)*: 113-148.

Tamerler, C; Ullah, M; Adlard, M and Keshavarz, T (1998). Effect of pH on physiology of *Metarhizium anisopliae* for production of swainsonine. *FEMS Microbiol. Lett., 168(1)*: 17-23. DOI: 10.1111/j.1574-6968.1998.tb13249.x.

Valencia, C; Ayala, I; Benítez, E; Torres, N; Barrera, E and Herrera, A (2007). Evaluación de estrategias de control y cuantificación de las pérdidas causadas por *Demotispa neivai* Bondar, raspador del fruto de la palma de aceite. *Revista Palmas, 28(1)*: 41-51.

Villamizar, L and Cotes, A (2003). Efecto de las condiciones de cultivo sobre parámetros del modo de acción de *Metarhizium anisopliae*. *Revista Col. Entomol., 29(2)*: 121-126.

Zimmermann, G (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci. Technol., 17(9)*: 879-920. DOI: 10.1080/09583150701593963.