Selection of Entomopathogenic Fungi for the Biological Control of *Demotispa neivai* (Coleoptera: Chrysomelidae) in Oil Palm Plantations in Colombia[1]

Luis Guillermo Montes-Bazurto[2,3], Yimer Peteche-Yonda[4], Hector Camilo Medina-Cardenas[3], and Alex Enrique Bustillo-Pardey[3]

Colombian Oil Palm Research Center (Cenipalma), Calle 98 # 70 91, Bogotá 111121, Colombia

J. Entomol. Sci. 55(3): 388–404 (July 2020)

Abstract *Demotispa neivai* (Bondar) is an economically important pest of oil palm, *Elaeis guineensis* Jacq., plantations in Colombia. During our search for alternatives to conventional chemical insecticides for controlling *D. neivai*, we initially screened 50 isolates of various entomopathogenic fungi against adults and larvae. At a concentration of 1×10^7 conidia/ml, 28 of the isolates caused fungal-induced mortality. Isolates of *Metarhizium anisopliae* (Metchnikoff) Sorokin, designated CPMa1502 and CeMa9236, caused the greatest mortality in adults and larvae. These isolates were further evaluated for efficacy against natural infestations of *D. neivai* in oil palm fruit bunches by applying each isolate at a rate of 1×10^{13} conidia/ha. The CPMa1502 isolate caused a significantly ($F = 39.22$; df = 6, 8; $P < 0.0001$) higher larval mortality (87.7%) than that of the CeMa9236 isolate. Three concentrations (5×10^{12}, 7.5×10^{12}, and 1×10^{13} conidia/ha) of the CPMa1502 isolate were then compared in a field efficacy test, and no significant differences were observed among the treatments. CPMa1502 also was applied to commercial oil palm plantations in two tests at a rate of 1×10^{13} on a large plot with 23 palms and a rate of 5×10^{12} conidia/ha on a 511-palm plot. Larval mortality in these field tests was greater than 62%.

Key Words palm oil, fruits scraper, *Metarhizium anisopliae*, biological control, *Elaeis guineensis*

Demotispa neivai (Bondar) (Coleoptera: Chrysomelidae) is one of the most economically important pests of oil palm, *Elaeis guineensis* Jacq., plantations in Colombia (Genty and Mariau 1973, Zenner and Posada 1992, Aldana et al. 2003, 2004, Corley and Tinker 2003, Calvache 2004, Valencia et al. 2007, Aldana-De La Torre et al. 2010, Bustillo-Pardey 2014). Damage is caused by larvae and adults that live in the palm bunches and feed on the fruits (Genty and Mariau 1973, Genty et al. 1978, Aldana et al. 2004, Aldana-De La Torre et al. 2010). Young oil palm spears also are affected when adults feed on the parenchyma, causing loss of leaf area (Urueta 1975, Aldana-De La Torre et al. 2010). *Demotispa neivai* damage to

[1]Received 10 June 2019; accepted for publication 5 August 2019.
[2]Corresponding author (email: lmontes@cenipalma.org).
[3]Pest and Diseases Program, Colombian Oil Palm Research Center (Cenipalma), Palmar La Vizcaína Experimental Field, Km 132 Vía Puerto Araujo-La Lízama, Barrancabermeja, Santander 111611, Colombia.
[4]Agronomy Engineer Student, Universidad Nacional de Colombia, Carrera 32 # 12 - 00 Palmira Valle del Cauca, Colombia.

the fruit epidermis results in a cork-like appearance, which makes it difficult to determine the degree of maturity, thus, interfering with proper identification of the optimum time to harvest (Genty et al. 1978, Valencia et al. 2007, Aldana-De La Torre et al. 2010). In addition, the damage to fruit causes a reduction in oil yield (Aldana et al. 2004, Valencia et al. 2007).

Previous studies have determined that the most susceptible phenological stage of the oil palm fruit to *D. neivai* damage is at the filling developmental phase 703, according to international scale Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (Montes-Bazurto et al. 2019). Stage 703 occurs 28 to 31 days postanthesis when the fruit has reached 50% of its final size, the mesocarp is green, and endosperm is in the aqueous phase (Hormaza et al. 2010, Forero et al. 2012, Forero and Romero 2012).

Demotispa neivai is affected by different natural enemies, especially entomopathogenic fungi, which might represent an alternative to conventional chemical insecticides in managing the pest in oil palm (Valencia et al. 2007, Aldana-De La Torre et al. 2010, Bustillo-Pardey 2014). This research reported herein was aimed at identifying fungal isolates for further development for the management of *D. neivai* in oil palm production.

Materials and Methods

Fungal and insect colonies. Cenipalma's Entomopathogenic Microorganism Laboratory in Bogotá, Colombia, maintains a collection of entomopathogenic fungi that were isolated from insects feeding on oil palm. Fifty of these fungal isolates, representing 5 genera, were selected for screening of pathogenicity against *D. neivai* (Table 1).

Demotispa neivai larvae used in the pathogenicity bioassays were obtained from a laboratory colony. Adults collected from the field were placed in 8-cm diameter $\times$ 5-cm high PVC tubes, with ends closed by covering with muslin and palm fruit as a feeding and ovipositional substrate. When eggs were found, they were separated and held until eclosion to yield larvae for bioassays (Fig. 1). Adult *D. neivai* used in the assays were obtained from a breeding site established in oil palm bunches covered with an entomological sleeve (Fig. 2).

Bioassays. The pathogenicity bioassays were conducted at Palmeras Yarima Plantation Laboratory and in Cenipalma's Vizcaína Research Station of Colombian Oil Palm Research Center (Cenipalma) in rooms maintained at 26°C and 83 $\pm$ 3.8% relative humidity (RH). A suspension of each of the 50 fungal isolates was prepared by harvesting the conidia of each fungus growing on Sabouraud's dextrose agar (SDA) and mixing in distilled water with Tween 80® (0.1%, w/w) (Biopack, Buenos Aires, Argentina). Suspensions were diluted to yield a concentration of 1×10^7 conidia/ml by using a hemacytometer to enumerate spores in each suspension. Insects were immersed in the appropriate suspension for 1 min and then placed individually in containers constructed of PVC (8-cm diameter $\times$ 5-cm height), with muslim covering each end and damp sterile toweling and an immature palm fruit in each container. Control treatments were left untreated. Insects were observed daily for 15 days to determine cumulative fungus-induced mortality. These assays were conducted in a completely random design with 10 insects per treatment. Bioassays

Fig. 1. *Demotispa neivai* breeding system in PVC tubing to obtain larvae (Photo: L. Montes).

with adults were made in 8 independent evaluations, and there was only 1 assay with larvae. Mortality data obtained from the treatments were subjected analysis of variance (ANOVA), with treatment means separated by Tukey's honestly significant differences (hsd) (SAS 9.4).

Isolate comparison. The two fungal isolates that caused the highest mortality in the bioassays were compared in a 5-yr-old oil palm plot naturally infested with *D. neivai* at Palmeras Yarima Plantation in San Vicente del Chucurí, Santander

Fig. 2. *Demotispa neivai* breeding system on oil palm bunches to obtain adults (Photo: L. Montes).

Department (187 m above sea level). Conditions during the test were 26.5 ± 3.4°C, 93.8 ± 10.4% RH, and 91 mm of rainfall. Oil palm bunches in phenological stage 703 (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale) (Hormaza et al. 2010, Forero and Romero 2012, Forero et al. 2012) and those with damage by *D. neivai* were labeled. Treatments were arranged in a randomized complete block design and were replicated five times, with experimental units consisting of three bunches each.

Conidial suspensions were formulated in an emulsifying oil (Carrier®; Agro SAS, Soacha, Cundinamarca, Colombia) and applied to the bunches by using a hand-operated 20-liter backpack sprayer fitted with a cone-shaped nozzle (RC 350B101X; Royal Condor, Bogotá, Colombia) and delivering 1 liter of suspension per palm at a rate of 1×10^{13} conidia/ha. The viability of conidias of isolates CPMa1502 and CeMa9236 were 94.5% and 97.4%, respectively, 24 h after inoculating in SDA at 25 ± 1°C the standard deviation of the incubation temperature. Controls received no spray. After 14 days, bunches were cut from the palms and transported to the laboratory where they were separated to recover *D. neivai* and record developmental stage, as well as record mortality and sporulation of fungi. Treatments were compared by ANOVA and Tukey's hsd (SAS 9.4).

Response to selected concentrations. This test was performed with the fungal strain that caused the highest mortality in the previous test in oil palm bunches. This test was conducted in a 4-yr-old oil palm plot naturally infested with *D. neivai* at Palmeras Yarima Plantation. Conditions during this test were 27.6 ± 4.0°C, 85.6 ± 18.2% RH, and 57-mm rainfall.

As in the previous test, bunches in phenological stage 703 and with *D. neivai* damage were selected and labeled. Treatments consisted of concentrations of conidia (5×10^{12}, 7.5×10^{12}, and 1×10^{13} conidia/ha) and an untreated control. Treatments were arranged in an randomized complete block design with two bunches per experimental unit and six replicates. The conidial suspensions were prepared in the Carrier emulsifying oil and applied with the backpack sprayer as previously described. The viability of conidias of isolate CPMa1502 was 97.3% 24 h after inoculating in SDA at 25 ± 1°C. Mortality was assessed at 14 days after application as described previously. Data were subjected to ANOVA and Tukey's hsd (SAS 9.4).

Efficacy trials. Two field-based efficacy trials were then conducted using the same fungal isolate. Conidial suspensions were prepared as described in the previous two tests. The first efficacy trial was conducted in a 0.16-ha area with 23 palms that were part of a 5-yr-old commercial plot on the Palmeras Yarima Plantation. The palms were naturally infested with *D. neivai*, and conditions during the trial were 27.6 ± 3.5°C and 92.0 ± 11.7% RH with no rainfall. A conidial suspension was sprayed on crowns of bunches in the 23 palms using a 20-liter backpack sprayer fitted with a gasoline-powered four-stroke Honda GX25 motor and cone-type nozzle (RC 350B101x) calibrated to deliver 1 liter of suspension per palm at a rate of 1×10^{13} conidia/ha. The viability of conidias of isolate CPMa1502 was 94.5% 24 h after inoculating in SDA at 25 ± 1°C. The control received no spray. Eleven days later, six bunches in phenological stage 703 that had been sprayed were randomly selected, removed from the plants, and transported to the laboratory where they were dissected and examined for fungus-induced insect

Table 1. Entomopathogenic fungal strains tested for pathogenicity bioassays against *Demotispa neivai* adults.

Fungus	Strain	Collection Location	Host	Host Development Stage
Beauveria bassiana	CPBb0403	Cenicafé	*Hypothenemus hampei*	Adult
Beauveria bassiana	CPBb0412	Puente Sogamoso (Santander)	*Leptopharsa gibbicarina*	Adult
Beauveria bassiana	CPBb0414	Puente Sogamoso (Santander)	*Dirphia gragatus*	Larva
Beauveria bassiana	CPBb0417	Puerto Wilches (Santander)	*Stenoma cecropia*	Larva
Beauveria bassiana	CPBb0419	Tucurinca (Magdalena)	*Brassolis sophorae*	Larva
Beauveria bassiana	CPBb0404	San Andrés de Tumaco (Nariño)	*Stenoma cecropia*	Larva
Beauveria bassiana	CPBb0420	San Carlos de Guaroa (Meta)	*Loxotoma elegans*	Larva
Beauveria bassiana	CPBb0502	Puerto Wilches (Santander)	*Euprosterna elaeasa*	Larva
Beauveria bassiana	CPBb1101	San Andrés de Tumaco (Nariño)	*Rhynchophorus palmarum*	Adult
Beauveria bassiana	CPBb1102	Barrancabermeja (Santander)	*Rhynchophorus palmarum*	Adult
*Cordyceps fumosorosea**	CPlf1001	No available	*Leptopharsa gibbicarina*	Adult
Cordyceps sp.**	CPlsp1201	San Alberto (Cesar)	*Stenoma cecropia*	Larva
Cordyceps sp.	CPlsp1203	San Carlos de Guaroa (Meta)	*Brassolis sophorae*	Larva
Cordyceps sp.	CPlsp1205	San Alberto (Cesar)	*Stenoma cecropia*	Larva
Cordyceps sp.	CPlsp1207	Cumaral (Meta)	*Loxotoma elegans*	Larva
Cordyceps sp.	CPlsp1301	Barrancabermeja (Santander)	*Anteotricha* sp.	Larva
Cordyceps sp.	CPlsp1303	San Carlos de Guaroa (Meta)	*Loxotoma elegans*	Larva
Metarhizium anisopliae	CeMa9236	Cenicafé	Not available	Not available

Table 1. Continued.

Fungus	Strain	Collection Location	Host	Host Development Stage
Metarhizium anisopliae	CPMa0401	Puerto Wilches (Santander)	Not available	Not available
Metarhizium anisopliae	CPMa0602	Barrancabermeja (Santander)	*Demotispa neivai*	Adult
Metarhizium anisopliae	CPMa0603	Barrancabermeja (Santander)	*Demotispa neivai*	Adult
Metarhizium anisopliae	CPMa0604	Barrancabermeja (Santander)	*Demotispa neivai*	Adult
Metarhizium anisopliae	CPMa1107	Cumaral (Meta)	*Leucothyreus femoratus*	Larva
Metarhizium anisopliae	CPMa1205	San Andrés de Tumaco (Nariño)	*Rhynchophorus palmarum*	Adult
Metarhizium anisopliae	CPMa1206	San Andrés de Tumaco (Nariño)	*Rhynchophorus palmarum*	Adult
Metarhizium anisopliae	CPMa1208	Villanueva (Casanare)	*Haplaxius crudus*	Adult
Metarhizium anisopliae	CPMa1210	Cumaral (Meta)	*Strategus aloeus*	Adult
Metarhizium anisopliae	CPMa1211	Yarima (Santander)	*Demotispa neivai*	Larva
Metarhizium anisopliae	CPMa1301	Puerto Wilches (Santander)	*Rhynchophorus palmarum*	Adult
Metarhizium anisopliae	CPMa1302	Barrancabermeja (Santander)	*Rhynchophorus palmarum*	Adult
Metarhizium anisopliae	CPMa1303	No available	*Leucothyreus femoratus*	Larva
Metarhizium anisopliae	CPMa1304	Sabana de Torres (Santander)	*Demotispa neivai*	Adult
Metarhizium anisopliae	CPMa1305	Yarima (Santander)	*Demotispa neivai*	Larva
Metarhizium anisopliae	CPMa1306	Villanueva (Casanare)	*Strategus aloeus*	Larva
Metarhizium anisopliae	CPMa1308	Yarima (Santander)	*Demotispa neivai*	Larva
Metarhizium anisopliae	CPMa1310	Yarima (Santander)	*Demotispa neivai*	Larva

Table 1. Continued.

Fungus	Strain	Collection Location	Host	Host Development Stage
Metarhizium anisopliae	CPMa1502	Yarima (Santander)	*Demotispa neivai*	Larva
Metarhizium anisopliae	CPMa0801	Yarima (Santander)	*Strategus aloeus*	Larva
Metarhizium anisopliae	CPMa1001	Puerto Wilches (Santander)	*Rhynchophorus palmarum*	Adult
Metarhizium anisopliae	CPMa1002	Puente Sogamoso (Santander)	*Strategus aloeus*	Pupa
Metarhizium anisopliae	CPMa1003	Cumaral (Meta)	*Strategus aloeus*	Larva
Metarhizium anisopliae	CPMa1004	San Andrés de Tumaco (Nariño)	*Strategus aloeus*	Larva
Metarhizium anisopliae	CPMa1101	Barrancabermeja (Santander)	*Strategus aloeus*	Pupa
Metarhizium anisopliae	CPMa1104	Puerto Wilches (Santander)	*Rhynchophorus palmarum*	Adult
Metarhizium anisopliae	CPMa1105	Puerto Wilches (Santander)	*Rhynchophorus palmarum*	Adult
Metarhizium anisopliae	CPMa1203	Puerto Wilches (Santander)	Unknown	Unknown
Metarhizium anisopliae	CPMa1207	Yarima rural area (Santander)	*Demotispa neivai*	Adult
Nomuraea rileyi	CPNr1201	Aracataca (Magdalena)	Noctuidae	Larva
Purpureocillium lilacinum	CPPl0601	Meseta San Rafael (Santander)	Not available	Pupa
Purpureocillium lilacinum	CPPl1201	Puerto Wilches (Santander)	Unknown	Not available

** Synonym *Isaria fumosorosea* (Kepler et al. 2017).
* Synonym *Isaria* sp. (Kepler et al. 2017)

mortality. Likewise, six bunches that had not been sprayed (controls) were selected and examined.

The second efficacy trial was conducted in a 4-yr-old, 3.6-ha commercial oil palm plantation (Zamarkanda Plantation in San Rafael de Lebrija in the Santander Department) naturally infested with *D. neivai* larvae. Conditions during this trial were 27.5 ± 3.2°C and 92.7 ± 11.6% RH, with 395-mm rainfall during the trial. The conidial suspension was prepared as in the previous efficacy trial and applied to a total of 511 palms by using a stationary gasoline-powered sprayer equipped with two cone nozzles (RC 350B101x) calibrated to deliver a 3.49×10^7 conidia/ml (equivalent to 5×10^{12} conidia/ha) in 1 liter of solution per palm at 30.6 kg/cm^2 of pressure. The viability of conidias of isolate CPMa1502 was 97.9% 24 h after inoculating in SDA at 25 ± 1°C. Controls ($n = 259$ palms) were not treated. An assessment of mortality and efficacy was conducted as previously described. In both trials, differences among the treatments were determined by calculation of mortality and 95% confidence intervals.

Molecular and phylogenetic analyses. The fungal isolate was grown on liquid media and harvested by filtration, and genomic DNA was extracted using the Qiagen DNeasy plant mini kit following the manufacturer's instructions. The DNA was frozen at −20°C until used. Polymerase chain reaction (PCR) was performed with the primers ITS4 and ITS5 (White et al. 1990), and partial β-tubulin 2 was amplified with the primers Bt2a and Bt2b (Glass and Donaldson 1995). Amplification was performed in a T3 thermocycler (Biometra, Gottingen, Germany), for each reaction, with a total volume of 25 μl containing 12.5 μl GoTaq® Green Master Mix kit (Promega), 0.3 μM of each primer, 50 ng of genomic DNA, and 9.5 μl of nuclease-free water. The conditions of each reaction in the thermocycler were the following: initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, annealing at 54°C for 30 s, and extension at 72°C for 1 min, ending with an extension at 72°C for 10 min. The PCR products were run on a 2% agarose gel. After electrophoresis, the gels were visualized under UV light. The bands of the expected size were cut directly from the gel, purified using the QIAquick Gel Extraction kit (Qiagen), and sent for sequencing to the Macrogen Company (South Korea) with the primers used in PCR.

The sequence obtained was edited through the BioEdit 7.0.5.3 program (Hall 1999), after which its identity was confirmed with the GenBank database by using BLASTn software (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, the sequences presented in Table 2 were aligned, analyzed, and edited manually with the MUSCLE algorithm (Edgar 2004) included in the MEGA6 program (Tamura et al. 2013). Based on the matrix obtained, the nucleotide substitution model was determined through Bayesian Information Criterion by using the ModelGenerator version 0.851 software (Keane et al. 2006). The phylogenetic relationship was determined by the maximum likelihood method, and the support of the nodes was determined by using the bootstrap method with 1,000 repetitions. The species *Metarhizium frigidum* (ARSEF 4124), *Metarhizium flavoviride* (ARSEF 2133), and *Nomuraea rileyi* (DT2011N7) were used as outgroups. The sequences of this work were deposited in GenBank under the accession numbers for internal transcribed spacer (ITS) region (MH673410) and β-tubulin (MH698453).

Table 2. Isolate codes, taxonomic identification, and accession numbers reported in the GenBank.

		GenBank Accession Numbers	
Strain	**Taxa**	**ITS**	**β-Tubulin**
ARSEF 7450	*M. anisopliae*	HQ331464.1	EU248823.1
ARSEF 7487	*M. anisopliae*	NR_132017.1	EU248822.1
CBS 257.90	*M. pingshaense*	HQ331450.1	EU248820.1
ARSEF 3210	*M. pingshaense*	HQ331449.1	EU248819.1
ARSEF 2107	*M. brunneum*	NR_132023.1	EU248826.1
CBS 258.90	*M. guizhouense*	HQ331448.1	EU248834.1
ARSEF 1914	*M. majus*	NR_152952.1	EU248840.1
ARSEF 7488	*M. lepidiotae*	HQ331456.1	EU248837.1
ARSEF 4587	*M. lepidiotae*	AY646386.1	EU248835.1
ARSEF 2596	*M. globosum*	NR_132020.1	EU248814.1
ARSEF 7486	*M. acridum*	NR_132019.1	EU248813.1
ARSEF 4124	*M. frigidum*	NR_132012.1	EU248828.1
ARSEF 2133	*M. flavoviride*	NR_131992.1	EU248827.1
DT2011N7	*M. rileyi*	KX641194.1	KX641195.1

Results

Screening bioassays. In our assays, 22 of the entomopathogenic fungal isolates caused no mortality of *D. neivai* adults. The isolates CPBb0419, CPBb0502, CPlf1001, CPlsp1201, CPlsp1203, CPlsp1205, CPlsp1207, CPlsp1301, CPlsp1303, CPMa0603, CPMa0604, CPMa1208, CPMa1210, CPMa1306, CPMa0801, CPMa1002, CPMa1003, CPMa1004, CPMa1101, CPNr1201, CPPl0601, and CPPl1201 were discarded during the reactivation process. A total of 28 isolates caused at least some mortality of *D. neivai* adults (Table 3). The *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota: Hypocreales) CPMa1502 and CeMa9236 isolates caused the highest mortality of *D. neivai* adults in bioassay 8 (Table 3; Fig. 3). In the evaluation against larvae, we observed no significant differences among them, except in comparison to the untreated control ($F = 188.53$; df = 2, 12; $P < 0.0001$) (Table 4; Fig. 4). These two isolates were, thus, selected for evaluations and testing in oil palm bunches.

Isolate and concentration comparisons. The application of conidial suspensions to oil palm bunches showed that the *M. anisopliae* CPMa1502 isolate caused 87.7% mortality of *D. neivai* larvae, which was significantly higher ($F = 39.22$; df = 6, 8; $P < 0.0001$) than that obtained after treatment with the CeMa9236

Fig. 3. Adult of *Demotispa neivai* infected by *Metarhizium anisopliae* CPMa1502 strain (Photo: L. Montes).

isolate (Table 5). The CPMa1502 strain was, thus, selected for comparing concentrations of conidial suspensions in plantation conditions. However, mortality resulting from the three concentrations tested yielded no significant differences among the concentrations. Mortality in all three concentrations was significantly higher ($F = 8.72$; df = 8, 15; $P = 0.0002$) than that observed in the untreated control (Table 6).

Field efficacy trials. Further evaluation of the CPMa1502 isolate in field testing yielded increased larval mortality in treated bunches. In the first trial, the treatment using a concentration of 1×10^{13} conidia/ha resulted in larval mortality of 68.2% in 11 days, whereas only 0.8% mortality was recorded in the untreated control (Table 7). In the second trial, the treatment of 5×10^{12} conidia/ha yielded a larval mortality of 62.0% at 15 days and 80.8% at 30 days, whereas mortality in the untreated control was 0% at 15 days and only 12.2% at 30 days (Table 8). However, there was no sporulation observed on larval cadavers (Table 8).

Molecular identification of the CPMa1502 isolate. The partial sequencing of rDNA and β-tubulin produced amplicons of 530 and 330 bp, respectively. The comparison of both regions against GenBank using BLAST allowed identification, with 100% identity, of *M. anisopliae*. Alignment based on the regions ITS1, ITS2, 5.8S rDNA, and β-tubulin of 15 taxa comprised 978 characters, including the gaps. Of those characters, 681 were conserved sites, 257 were variable, and 129 were informative parsimonious sites. The phylogenetic analysis allowed the grouping of the CPMa1502 isolate into a subclade, where the *M. anisopliae* isolates ARSEF 7450 and ARSEF 7487 were grouped, with a relative support on the branches of 98%. In the same way, Inside the clade with CPMa1502, the species Metarhizium pingshaense, Metarhizium brunneum, Metarhizium guizhouense, and Metarhizium majus grouped with a support of 100% (Fig. 5, indicated in blue).

Table 3. Average *Demotispa neivai* adult mortality caused by entomopathogenic fungal strains in different bioassays under laboratory conditions (26°C; 83 ± 3.8% RH).

Entomopathogenic Fungus	Strain	Adults Inoculated (*n*)	Mortality (%)*
Bioassay 1 ($F = 5.81$; df = 4, 20; $P = 0.0028$)			
Metarhizium anisopliae	CPMa1105	50	30.0 a
Metarhizium anisopliae	CPMa1207	50	16.0 ab
Metarhizium anisopliae	CPMa1203	50	14.0 ab
Beauveria bassiana	CPBb0404	50	4.0 b
None	Control	50	0.0 b
Bioassay 2 ($F = 12.63$; df = 4, 20; $P < 0.0001$)			
Metarhizium anisopliae	CPMa1205	50	26.0 a
Metarhizium anisopliae	CPMa1211	50	18.0 ab
Beauveria bassiana	CPBb1101	50	8.0 bc
Metarhizium anisopliae	CPMa1001	50	2.0 c
None	Control	50	0.0 c
Bioassay 3 ($F = 1.51$; df = 5, 24; $P < 0.2231$)			
Beauveria bassiana	CPBb0420	50	23.9 a
Metarhizium anisopliae	CPMa1104	50	17.4 a
Metarhizium anisopliae	CPMa1207	50	15.2 a
Beauveria bassiana	CPBb1102	50	13.0 a
None	Control	50	8.0 a
Bioassay 4 ($F = 3.93$; df = 6, 28; $P < 0.0056$)			
Metarhizium anisopliae	CPMa0602	50	26.0 a
Beauveria bassiana	CPBb0403	50	18.0 ab
Beauveria bassiana	CPBb0414	50	12.0 ab
Beauveria bassiana	CPBb0417	50	10.0 ab
Beauveria bassiana	CPBb0412	50	8.0 ab
None	Control	50	0.0 b

Table 3. Continued.

Entomopathogenic Fungus	Strain	Adults Inoculated (*n*)	Mortality (%)*
Bioassay 5 ($F = 2.92$; df = 4, 20; $P < 0.0469$)			
Metarhizium anisopliae	CPMa1301	50	22.0 a
Metarhizium anisopliae	CPMa1107	50	14.0 ab
Metarhizium anisopliae	CPMa1211	50	14.0 ab
Metarhizium anisopliae	CPMa1206	50	4.0 ab
None	Control	50	0.0 b
Bioassay 6 ($F = 2.57$; df = 4, 20; $P < 0.0696$)			
Metarhizium anisopliae	CPMa1305	50	16.0 a
Metarhizium anisopliae	CPMa1303	50	16.0 a
Metarhizium anisopliae	CPMa1304	50	14.0 a
Metarhizium anisopliae	CPMa1302	50	10.0 a
None	Control	50	0.0 a
Bioassay 7 ($F = 4.03$; df = 4, 20; $P < 0.0148$)			
Metarhizium anisopliae	CeMa9236	50	30.0 a
Metarhizium anisopliae	CPMa0401	50	18.0 ab
Metarhizium anisopliae	CPMa1310	50	18.0 ab
Metarhizium anisopliae	CPMa1308	50	14.0 ab
None	Control	50	0.0 b
Bioassay 8 ($F = 280.25$; df = 2, 11; $P < 0.0001$)			
Metarhizium anisopliae	CPMa1502	50	95.0 a
Metarhizium anisopliae	CeMa9236	50	50.0 b
None	Control	50	2.0 c

* Data followed by different lowercase letters in the same column and the same bioassay indicate that significant differences were found according to Tukey's test ($P = 0.05$).

Discussion

This study is the first screening of strains to select an entomopathogenic fungus to control *D. neivai* and is the first time that *M. anisopliae* has been evaluated in pathogenicity bioassays against *D. neivai* in Colombia. Valencia et al. (2007) recorded the fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota:

Fig. 4. Larva of *Demotispa neivai* infected by *Metarhizium anisopliae* CPMa1502 strain on a bunch of oil palm (Photo: L. Montes).

Hypocreales) as being pathogenic to *D. neivai* adults; however, our results showed that *D. neivai* adult mortality caused by *B. bassiana* isolates was very low (<30%).

The evaluation of the two strains of *M. anisopliae* against *D. neivai* was the first conducted in Colombia in oil palm bunches. The results we obtained, however, were similar to those of Teixeira and Franco (2007) in Brazil with *Cerotoma arcuata* Olivier, a defoliating insect pest within the same taxonomic family. Furthermore, *D. neivai* larval mortality was higher in oil palm bunches treated with *M. anisopliae* CPMa1502 than natural levels of mortality observed in our study and in previous observations of natural mortality in oil palm grown in Colombia (58.3%) (Montes-Bazurto et al. 2019).

Table 4. *Metarhizium anisopliae* pathogenicity test on *Demotispa neivai* larvae under laboratory conditions (26°C and 83 ± 3.8% RH).

Fungus	Strain	No. of Larvae Inoculated	Mortality (%)*	Corrected Mortality (%)**
Metarhizium anisopliae	CPMa1502	50	96.0 a	95.7
Metarhizium anisopliae	CeMa9236	50	84.0 a	83.0
None	Control	50	6.0 b	

* Data followed by the same lowercase letter in the same column were not significantly different according to Tukey's test ($P = 0.05$).

** According to the Schneider–Orelli formula (Püntener 1981).

Table 5. *Demotispa neivai* larval mortality caused by the *Metarhizium anisopliae* CPMa1502 fungal strain, 14 days after spraying at a dosage of 1×10^{13} conidia/ha in oil palm plantation (26.5 ± 3.4°C, 93.8 ± 10.4% RH, and 91-mm rainfall during the experiment).

Entomopathogenic Fungus	Strain	No. of Bunches Evaluated	Mortality (%)*	Corrected Mortality (%)**
Metarhizium anisopliae	CPMa1502	15	87.7 a	87.3
Metarhizium anisopliae	CeMa9236	15	68.4 b	67.5
None	Control	15	2.9 c	

* Data followed by the same lowercase letter in the same column were not significantly different according to Tukey's test ($P = 0.05$).
** According to Schneider–Orelli formula (Püntener 1981).

Our results indicate that the *M. anisopliae* CPMa1502 isolate may have potential for development as a biological control agent for *D. neivai* management in Colombia oil palm production, a factor that has not been indicated in previous studies (Aldana et al. 2003, 2004, Valencia and Benítez 2004, Aldana-De La Torre et al. 2010). Furthermore, this is the first use of molecular analyses to compliment the identification of an entomogenous fungus by morphology in Colombia. The molecular identification was included with the information of the CPMa1502 isolation previously deposited into Cenipalma's entomopathogenic collection at Entomopathogenic Microorganism Laboratory in Bogotá (Montes-Bazurto et al. 2019).

Table 6. *Demotispa neivai* larval mortality caused by the *Metarhizium anisopliae* CPMa1502 fungal strain, 14 days after spraying at different dosages in oil palm plantation conditions (27.6 ± 4.0°C; 85.6 ± 18.2% RH, and 57-mm rainfall during the experiment).

Entomopathogenic Fungus	Strain	Dosage (spores/ha)	No. of Bunches Evaluated	Mortality (%)*	Corrected Mortality (%)**
Metarhizium anisopliae	CPMa1502	5.0×10^{12}	14	85.0 a	80.0
		7.5×10^{12}	14	89.6 a	86.1
		1.0×10^{13}	14	77.6 a	70.1
None		Control	14	25.0 b	-

* Data followed by the same lowercase letter in the same column were not significantly different according to Tukey's test ($P = 0.05$).
** According to the Schneider–Orelli formula (Püntener 1981).

Table 7. *Demotispa neivai* larval mortality caused by the *Metarhizium anisopliae* CPMa1502 fungal strain, 11 days after spraying at a dosage of 1×10^{13} conidia/ha in oil palm plantation (27.6 ± 3.5°C; 92.0 ± 11.7% RH).

Treatment	Strain	*n**	Mortality (%)	Confidence Interval ($P = 0.05$)	Corrected Mortality (%)**
Metarhizium anisopliae	CPMa1502	6	68.2	12.6	67.9
Control		6	0.8	1.3	

* Number of bunches evaluated.
** According to Schneider–Orelli (Püntener 1981).

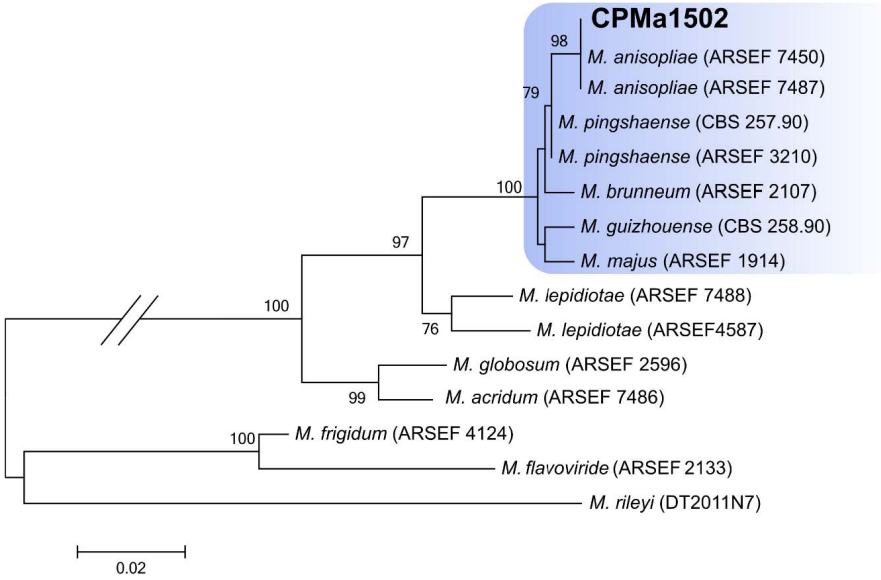

Fig. 5. Phylogenetic tree obtained by maximum likelihood of the consensus of the ITS region and β-tubulin of some species of the genus *Metarhizium*. The isolate corresponding to this work is indicated in bold. The numbers on the nodes indicate bootstrap values of >70 %. The species *M. frigidum* (ARSEF 4124), *M. flavoviride* (ARSEF 2133), and *M. rileyi* (DT2011N7) were included as outgroups.

Table 8. *Demotispa neivai* larval mortality caused by the *Metarhizium anisopliae* CPMa1502 strain, 15 and 30 days after spraying at a dosage of 5×10^{12} conidia/ha in an oil palm plantation (27.5 ± 3.2°C, 92.7 ± 11.6% RH, and 395-mm rainfall during the experiment).

Treatment	Strain	No. of Days After Spraying	*n**	Mortality (%)	Confidence Interval ($P = 0.05$)	Corrected Mortality (%)**
Metarhizium anisopliae	CPMa1502	15	6	62.0	29.5	62.0
		30	6	80.8	24.4	78.1
Reference control (no spraying)		15	6	0.0	0.0	
		30	6	12.2	11.0	

* Number of bunches evaluated.
** According to Schneider–Orelli (Püntener 1981).

Acknowledgments

We thank the staff at Palmeras Yarima Plantation and Hacienda Zamarkanda oil palm plantation for their support, the Oil Palm Development Fund of Colombia for funding this study, Hanna Alvarado and Angie Barragan for collaborating on the bioassays. Also, we thank students Eider Cabezas and Yamid García of the Universidad Nacional de Colombia (Palmira) and Alfonso Enrique González of the Universidad de Córdoba for their collaboration in conducting the bioassays.

References Cited

Aldana-De La Torre, R.C., J. Aldana, H. Calvache and P. Franco. 2010. Manual de Plagas de la Palma de Aceite en Colombia, Cuarta. ed. Cenipalma, Bogota, Colombia.

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

Aldana, J., J. Cataño and H. Calvache. 2003. Avances en el conocimiento de la biología y del control de Imatidium neivai Bondar, raspador de los frutos de la palma de aceite. Ceniavances. 107: 1–4.

Bustillo-Pardey, A.E. 2014. Manejo de insectos-plaga de la palma de aceite con énfasis en el control biológico y su relación con el cambio climático. Palmas (Colombia) 35: 66–77.

Calvache, H. 2004. La identificación y el control de los focos iniciales en el manejo eficiente de plagas en palma de aceite. Palmas (Colombia) 25: 186–193.

Corley, R.H.V. and P. Tinker. 2003. The Oil Palm, Fourth ed., Blackwell Science, Oxford, United Kingdom.

Edgar, R.C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32: 1792–1797.

Forero, D., P. Hormaza and H.M. Romero. 2012. Phenological growth stages of African oil palm (*Elaeis guineensis*). Ann. Appl. Biol. 160: 56–65.

Forero, D., and H.M. Romero. 2012. Escala BBCH para la descripción del desarrollo reproductivo de Elaeis guineensis Jacq., pp. 73–89. *In* Romero, H. (ed.), Gen. Sobre La Morfol. y Fenol. La Palma Aceite. Cenipalma, Bogota.

Genty, P., R. Desmier de Chenon and J.P. Morin. 1978. Oil palm pest in Latin America. Oleagineux. 33: 334–335.

Genty, P. and D. Mariau. 1973. Les ravageurs et maladies du palmier à huile et du cocotier Le genre Himatidium. Oleagineux 28: 513–515.

Glass, N.L. and G.C. Donaldson. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61: 1323–1330.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95–98.

Hormaza, P., D. Forero, R. Ruiz and H.M. Romero. 2010. El fruto y el racimo, pp. 65–83. *In* Fenol. La Palma Aceite Africana (Elaeis Guineensis Jacq.) y Del Híbrido Interes. (Elaeis Oleifera [Kunt] Cortes x Elaeis Guineensis Jacq.). Cenipalma, Bogota.

Keane, T.M., C.J. Creevey, M.M. Pentony, T.J. Naughton and J.O. Mclnerney. 2006. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol. Biol. 6: 1–17.

Kepler, R., J.J. Luangsa-Ard, N. Hywel-Jones, A. Quandt, G.-H. Sung, S. Rehner, M.C. Aime, T.W. Henkel, T. Sanjuan, R. Zare, M. Chen, Z. Li, A. Rossman, J.W. Spatafora, B. Shrestha. 2017. A phylogenetically-based nomenclature for Cordycipitaceae (Hypocreales). IMA Fungus 8: 335–353.

Montes-Bazurto, L.G., Y. Peteche and A.E. Bustillo-Pardey. 2019. Life Cycle and Natural Enemies of *Demotispa neivai* (Coleoptera: Chrysomelidae). J. Entomol. Sci. 54: 213–222.

Püntener, W. 1981. Manual for field trials in plant protection, Second. ed. Agricultural Division, Ciba-Geigy Limited, Basle (Switzerland).

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Teixeira, M.L. and A.A. Franco. 2007. Susceptibilidade de larvas de Cerotoma arcuata Olivier (Coleoptera: Chrysomelidae) a Beauveria bassiana (Bals.) Vuillemin, Metarhizium anisopliae (Metsch.) Sorokin e Bacillus thuringiensis Berliner. Ciência Rural. St. Maria. 37: 19–25.

Urueta, E. 1975. Insectos asociados con el cultivo de Palma Africana en Uraba (Antioquia) y estudio de su relación con la Pudrición de la flecha—Pudrición del cogollo. Rev. Colomb. Entomol. 1: 15–31.

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

Valencia, C. and E. Benítez. 2004. Evaluación del efecto de cuatro aislamientos de hongos entomopatógenos en el control de Imatidium neivai. Ceniavances 117: 1–4.

White, T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. *In* Innis, M., Gelfand, D., Sninsky, J., White, T.J. (eds.), PCR Protoc. a Guid. to Methods Appl. Academic Press, New York.

Zenner de Polanía, I. and F. Posada. 1992. Manejo de insectos plaga y benéficos de la Palma Africana, Man. Asist. Tec. Instituto Colombiano Agropecuario - ICA, Bogota.