natural enemies associated to the borer of branches of the avocado tree copturus aguacatae coleoptera curculionidae in tinguindin micho

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Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 6(4) pp. 089-096, April, 2017 Issue.
Available online http://garj.org/garjas/home

Full Length Research Paper

Natural Enemies Associated to the Borer of Branches of the Avocado Tree, *Copturus Aguacatae* (Coleoptera: Curculionidae) in Tingüindín, Michoacán, México

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Accepted 10 April, 2017

The borer of branches of the avocado tree, *Copturus aguacatae* Kissinger, 1957 (Coleoptera: Curculionidae) is one of the most important insect pests that directly affect the (*Persea americana* Mill.) cv. Hass tree. To control this insect, different methods have been used but they have been ineffective, however, studies of natural enemies of *C. aguacatae* have been limited in the avocado producing area of the Municipality of Tingüindín, Michoacán. In this study parasitoids of the genera *Euderus* Haliday and *Brasema* Cameron belonging to the Eulophidae and Eupelmidae families, respectively, with parasitism percentage of ≥1.0% to 16.0% were found. Twenty-eight isolates of entomopathogenic fungi were isolated and morphologically and molecularly identified, 26 belonging to *Metarhizium* genus, 13 corresponding to *M. anisopliae* and eight to *M. robertsii*, five remaining as *Metarhizium* spp., and two corresponding to *Beauveria* spp., according to their morphological characters. Pathogenicity tests with eight isolates of *M. anisopliae* and two *M. robertsii* caused a mortality ≥70%, considered with favorable pathogenic characteristics to be used in biological control programs of borer of avocado tree.

Keywords: Entomopathogens, Parasitism, Pathogenicity, *Euderus, Brasema, Metarhizium anisopliae, Metarhizium robertsii.*

INTRODUCTION

The trunk and branches borer of avocado tree *Copturus aguacatae* Kissinger, 1957 (Coleoptera: Curculionidae) is one of the most important insect pests that directly affect the (*Persea americana* Mill.) cv. Hass (Laurales:

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Lauraceae) tree, causing severe damage (Equihua *et al*., 2007). This borer is endemic to the most important producing areas of Mexico (Talabera and Padilla 2003; Engstrand *et al*., 2010). In the State of Michoacán has been reported its presence in all municipalities producing this fruit, except in Acuitzio (SAGARPA 2004). This borer destroys the middle medulla of the tissues of branches, which can subsequently be broken by wind, rain and fruit weight (Equihua *et al*., 2007). For the control of this insect, chemical insecticides, cultural practices and normative regulation (NOM-066-FITO 2002; USDA-APHIS 2011) have been used, which have been ineffective due to the ethology of the borer, since part of its cycle occurs within the branches and trunks of the tree, reducing the efficiency of the insecticides used for its control; Its excessive use has had consequences on the environment, preventing the establishment and increased populations of beneficial organisms that regulate the populations of this borer. There are currently studies on natural enemies and evaluations of biological products; however, the information generated in this regard is limited. In the States of Puebla and Nayarit, México Huerta *et al*., (1990), Hernández *et al*. (2009), De Dios-Ávila *et al*. (2016) have reported parasitoids that regulate borer populations naturally. Huerta *et al*. (1990), tested the entomopathogenic nematodes *Steinernema bibiones* and *Heterorhabdithis heliothidis* and Sánchez *et al*. (2012) to *H. indica*, as control agents of *C. aguacatae* larvae with satisfactory results. Given the importance of the borer of the branches of avocado tree in this country and the potential of biological control as a complementary strategy in integrated pest management schemes in the avocado region of the State of Michoacán, the aim was to study and identify natural enemies of *C. aguacatae* in commercial orchards of the Municipality of Tingüindín, Michoacán, with potential to be used as possible biological control agents.

MATERIAL AND METHODS

Field Collect and Laboratory Processing

Avocado tree branches were collected from commercial orchards (≥ 50%) with *C. aguacatae* in the months of July, August, September and December 2012, and February-March 2013, in the municipality of Tingüindín, Michoacán, which is located in the geographical coordinates 19º44'19''N 102º28'56''W and at an altitude of 1,683 meters above sea level. Six trees/ha were selected randomly in different localities of the municipality; of each tree five segments of branches 30 cm long, 1.5 to 2.0 cm in diameter with indicators of the presence of the borer (crystallized sage, excreta and visible damage) were taken from the area with the highest incidence of sunlight, mainly in the stratum middle of the tree (NOM-066-FITO 2002).

The samples were transferred to the Department of Agricultural Parasitology (DAP) of the Universidad Autónoma Agraria Antonio Narro (UAAAN), where they were kept in a bioclimatic chamber at 20 ± 2 °C to keep them moist and turgid to allow the development of *C. aguacatae* larvae; the segments of branches were dissected at 20 ± 5 d after being cut and stored (optimal time of conservation of the vegetative material), to observe the presence of parasitoids and entomopathogens fungi of *C. aguacatae* inside the galleries and the number of larvae, pupae and adults. The larvae and pupae of parasitoids found inside the galleries were placed in Petri dishes with previously sterilized wet sawdust until adult emergence, which were individually preserved in 2 mL microtubes with 70% ethanol.

Preparation of Adult Specimens

The parasitoid specimens were sectioned (head, thorax, abdomen, antennae, anterior and posterior wings) and successively submerged in 5% KOH solution for 24 to 48 h, to decolor the chitinized sections except for the wings. After being discolored were washed with distilled water for 10-15 min with a piseta; subsequently, they were dehydrated in ethanol at different concentrations (50, 70, 90 and 100%) for 10 to 15 min in each step; at the end of this process, were placed in clove oil to soften and finally fixed on slides and with a mixture of clove oil and balsam of Canada according to the techniques described by Noyes (1982) and Castro (1996) for micro-hymenopterans.

Taxonomic Identification of Parasitoids

For the morphological identification of the specimens a stereo microscope and an optical microscope (Leica brand, model DM/LS, Germany) were used; using the taxonomic keys of Yoshimoto (1971); Gibson (1995); Gibson (1997) and Schauff *et al*. (1997) for identification at the family, subfamily and genus levels; then, adults parasitoids were reviewed by Dr. Alejandro González Hernández (Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León) and sent to Dr. Christer Hansson (Department of Zoology, Lund University, Sweden) to confirmation.

The percentage of parasitism was calculated based on the number of parasitized borer larvae, divided between the total number of larvae collected multiplied by 100 (Pair *et al*., 1986; Paiva and Parra 2012), using the formula: RP = (Np/Nt) × 100. Where Npi = number of parasitized individuals and Nt = total number of individuals collected.

Isolation of Entomopathogenic Fungi and Morphological Identification

Eighty soil samples of approximately 500 g c/u taken of the drip area of each tree were collected, as well as the sampling area sharing similar characteristics, 50% of the samples randomly selected were processed to isolate entomopathogenic fungi, according to the insect bait technique (Zimmermanin, 1986). For this purpose, 200 g of wet soil were placed into 250 mL containers, then five larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) were deposited. The containers were incubated at 25 ± 2 ° C for 15 d; and were systematically examined every 24 h, until the larvae showed symptoms of fungal infection. The symptomatic larvae were disinfected with 1% sodium hypochlorite for 3 min and washed three times with sterile distilled water, dried on sterile filter paper and subsequently incubated in humid chambers at 25 ° C for 7-15 d to stimulate sporulation of fungi; subsequently, these larvae were transferred to culture medium Potato Dextrose Agar (PDA) added with 1% soy broth for *Metarhizium* spp., and PDA supplemented with 2% V8 juice (vegetable juice) at pH 6.0 for *Beauveria* spp. The morphological identification of *Metarhizium* spp., and *Beauveria* sp., was performed according to their micro and macroscopic characters mainly by their typical reproduction structures such as colonies, phallids and conidia (Barnett and Hunter, 1999), assembled in slides, stained with lactophenol blue and observed at 400 and 1,000 magnifications in an optical microscope.

DNA extraction and Molecular Characterization of Entomopathogenic Fungi

The rDNA extraction was realized using young mycelium (7 d old) from purified cultures grown in PDA, macerated in a porcelain mortar, adding a buffer for the extraction of rDNA at 70 °C (200 mM Tris-HCl (pH = 8), 250 mM NaCl, 25 mM EDTA, 0.5% SDS), according to the protocol described by Raeder and Broda (1985). The rDNA obtained was examined by electrophoresis on a 1% agarose gel, which was then used to amplify the 18S RNA Internal Transcript Spacer (ITS) with the universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') by PCR technique, the expected fragments for *Metarhizium* spp. were approximately 600 to 710 bp.

The PCR products were separated on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. They were then sent to Company Macrogen (Maryland, USA) for sequencing. The sequences obtained were compared to the National Biotechnology Information Center (NBIC) database, using the BLAST algorithm (Altschul *et al*., 1990) to determine the genus and species of fungal isolates.

Pathogenicity Tests

Conidial suspensions of ten *M. anisopliae* and two *M. robertsii* isolates were prepared in distilled water added with 0.1% Tween-80 (Mochi *et al*., 2005). The number of conidia was determined under an inverse optical microscope (Olympus CKX41) with phase contrast at 1,000 magnifications using a Neubauer chamber (Blau Brand, Germany) and subsequently adjusted to a concentration of 1×10^7 conidia/mL. To determine the pathogenicity of the isolates, a completely randomized design was used with 12 treatments: ten isolates of *M. anisopliae* and two of *M. robertsii,* considering 10 replicates per treatment and an absolute control with distilled water alone added with Tween-80 at 0.1 %. Considering the low availability of third and fourth instar larvae, a larva was considered as an experimental unit. The larvae of *C. aguacatae* were individually immersed into 50 mL of conidia suspensions (maintained under constant stirring) for 60 s and immediately placed on filter paper to remove excess suspension (Butt and Goettel, 2000 and Fhiser *et al*., 2011) them placed Individually into 25 mL plastic containers, previously conditioned with sterile sawdust from avocado wood provided as a substrate and food source, incubated at 25 ± 2 °C for 10 d and checked every 24 h, until complete mycosis or sporulation. Data were processed using an analysis of variance (ANOVA) of the statistical program (SAS Institute, 1999), and the means were separated by the Tukey's test (P <0.05).

RESULTS AND DISCUSSION

Percentage of Natural Parasitism

From 917 segments of dissected branches, 1,685 specimens of *C. aguacatae* (1,577 larvae, 20 pupae and 88 adults) were found. From which, 112 immature parasitoids (hymenopterans) were obtained; the percentage of parasitism varied in the different samples, ≥1.0% to 16.0% (Table 1). A small number of parasitoid larvae emerged directly from larvae of *C. aguacatae*. In the absence of borer larvae, inside galleries, immature hymenopterans were found along with exuviae, cephalic capsules and mummified subimago of the borer.

Identification of Parasitoids Associated with *Copturus aguacatae*

Sixty seven percent of the specimens corresponded to the Eulophidae family (26♂ and 18♀ females) (Table 2), identified as *Euderus* sp. Haliday (Hymenoptera: Eulophidae) (Figure. 1a). This genus is easily recognized from other eulophids by the presence of three hairs lines radiating from the base of the stigmal and postmarginal vein of the anterior wing and presence of complete notauli. The species of *Euderus* are similar to each other, in color patterns, size and general appearance, making it difficult to identify (Yoshimoto, 1971; Schauff *et al*., 1997).

Table 1. Percentage of parasitism of *Copturus aguacatae* in the lower area of Tingüindín Michoacán in 2012 and 2013.

Date	**MASL**	**Segments of the branches**	***C. aguacatae***	**# Total Parasitoids**	**Parasitism (%)**
July-12	1,743	240	100	16	16.0
Aug.-12	1,747	202	171	15	8.8
Sept.-12	1,657	118	362	37	10.2
Dec.-12	1,751	116	303	13	4.3
Feb.-13	1,724	119	364	27	7.4
Mar.-13	1,731	122	385	4	1.0

MASL: Meters above sea level

Table 2. Family, subfamily and genus of parasitoids associated with *Copturus aguacatae* larvae in Tinguindin, Michoacan in 2012 and 2013.

Family	Subfamily	Genus	Proportion %	Emerged parasitoids	Sex ♂	Sex ♀
Eulophidae	Euderinae	*Euderus* sp.	67	44	26	18
Eupelmidae	Eupelminae	*Brasema* sp.	33	22	1	21

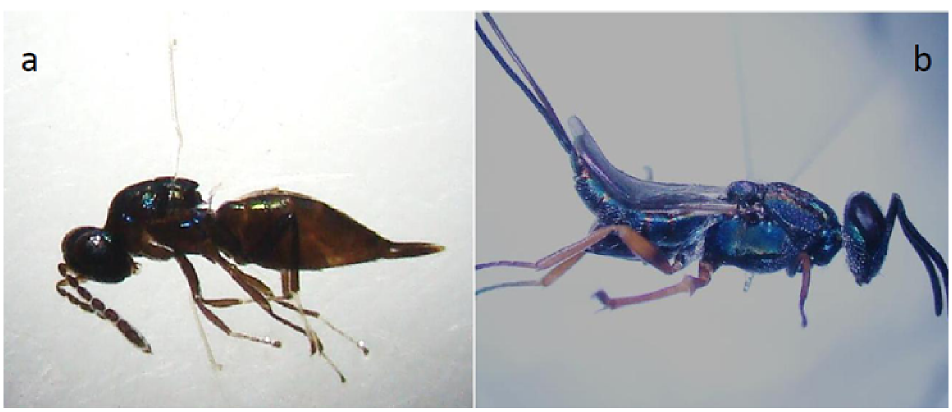

Figure. 1 a) ♀ *Eudurus* sp. Dark brown body with metallic blue green chandeliers
b) ♀ *Brasema* sp. completely shiny blue metallic body and ovipositor of equal length as head, thorax and abdomen together.

In addition Gibson *et al*. (2006); Dosdall *et al*. (2009) reported the species *Euderus glaucus* and *Euderus albitarsus* parasitizing *Ceutorhynchus obstrictus* (Marsham) (Coleoptera: Curculionidae) in Canada and USA, respectively. Huerta *et al*. (1990) reported to *Euderus* sp., parasitizing larvae of *C. aguacatae* in the State of Puebla, Mexico, an evidence that species of this genus specialize in parasitizing curculionids. On the other hand, Hernández *et al*. (2009) reported to *Urosigalphus avocadoae* Gibson (Hymenoptera: Braconidae) in the state of Mexico and De Dios-Ávila *et al*. (2016) to *Neocatolaccus*

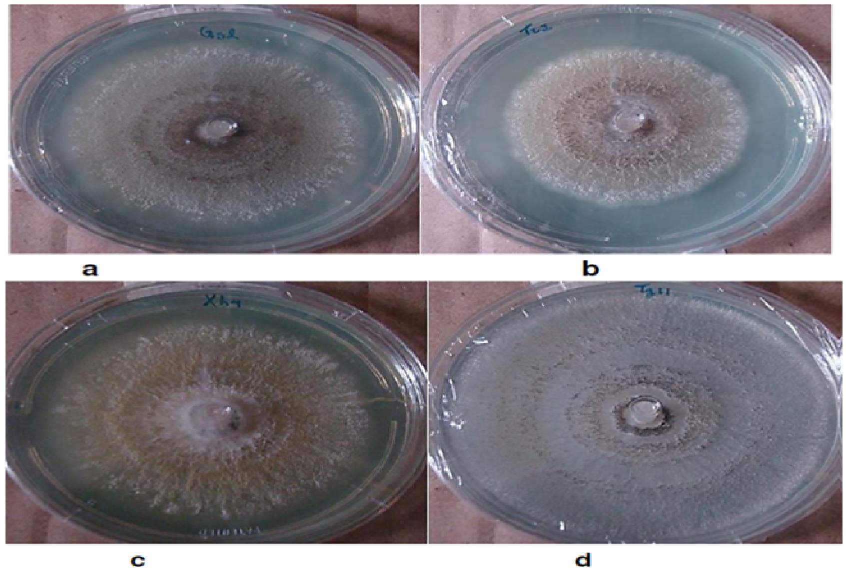

Figure. 2 Macroscopic growth of *Metarhizium* spp., in PDA culture medium. (a-b) formation of aerial mycelium and growth in concentric rings of *Metarhizium anisopliae*. (c-d) growth and uniform sporulation of *Metarhizium robertsii*.

tylodermae Ashmead (Hymenoptera: Pteromalidae) in Nayarit, Mexico as natural enemies of *C. aguacatae*.

Twenty two (1♂ and 21♀) specimens of the parasitoid *Brasema* sp. Cameron (Hymenoptera: Eupelmidae) were found, parasitizing immature individuals of *C. aguacatae* in commercial orchards of avocado tree of Michoacán (Table 2). *Brasema* is a small genus of Eupelmidae family, with about 25 species described in North America and Northern Mexico, some were originally described within the genus *Eupelmus* (Gibson, 1995). However, Gibson, (2011) relocated 15 species of *Eupelmus* to *Brasema.* This genus is distinguished by a combination of the following features: tridentate mandibles, a mesotibial apical groove, mesotibial apical pegs above the tibial spur ventral to groove and absence of a syntergal flange (Gibson, 1995). *Brasema* species parasitize insects that develop inside plants (Hanson and Gauld, 1995), are mainly ectoparasites of lepidopteran and coleopteran larvae (Gibson *et al*., 2006), also of hemipteran, mantis and orthopteran eggs (Gibson, 1995; Marchiori *et al*., 2002).This genus is reported for the first time as a natural enemy of *C. aguacatae* larvae in Michoacan, Mexico in this study (Figure. 1. b).

The identification of species of *Metarhizium* based on their morphological characters is difficult due to their similarity (Crous *et al*., 2005; Rehner and Buckley, 2005; Tsui *et al*., 2006); However, it has been facilitated with molecular techniques (Driver *et al*., 2000). Twenty-six isolates of *Metarhizium* spp., and two of *Beauveria* sp. were identified morphologically, only 21 isolates of *Metarhizium* were molecularly characterized (Entz *et al*., 2005). According to the comparison of their sequences in the BLAST GenBank database, 13 isolates corresponded to *M. anisopliae* and eight to M*. robertsii,* respectively (Table 3). Most isolates of entomopathogenic fungi isolated from soil in orchards of avocado tree corresponded to the genus *Metarhizium.* In studies of natural occurrence, Keller *et al*. (2003) mention that *M. anisopliae* is common in agricultural soils, similar to that found in this study; in contrast Bidochka *et al*. (1998) and Vänninen (1996) reported that they are less occurrence in forest soils, so that this characteristic gives *Metarhizium* a broad spectrum of development in disturbed ecological environments unlike *Beauveria* species, which is more common in forest soils. According to the dendrogram based on the ITS sequences, the isolates were associated in four phylogenetic groups, with *M. anisopliae* being more frequently present in groups one and four, *M. robertsii* within groups two and three (Figure. 3), although the isolates (TZ1, TG4, TG10, GS2, and XH2) belong to *M. robertsii* but genetically related to *M. anisopliae*, in accordance with Bischoff *et al*. (2009) who mention that *M. robertsii* is morphologically indistinguishable from *M. anisopliae* (Figure. 2), however, is molecularly differentiated by the position of its nucleotides. The 12 *Metarhizium* isolates tested in the study were pathogenic against *C. aguacatae* larvae, with a mortality of ≥70%, where eight of these reached 100% on

Table 3. *Metarhizium* species isolated from soil in commercial avocado tree orchards of Tingüindín, Michoacán, México.

Isolated	Morphological identification	Molecular identification	Coordinates		GenBank		
					Maximum score	% Similarity	ITS Sequence
TG7	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°43´53.8" N	102°30´12.2" W	989	99	KU983799
TG8	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°43´51.5" N	102°30´04.1" W	595	87	FJ545310
TG1	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°43´53.7" N	102°30´16.4" W	987	99	FJ545279
TG15	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°43´50.7" N	102°30´06.1" W	983	99	FJ545279
ES7	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°42´40.1" N	102°28´28.5" W	972	100	FJ177473
ES1	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°42´37.4" N	102°28´33.2" W	985	99	FJ545279
TZ1	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°46´15.7" N	102°34´02.0" W	985	99	KU983799
TZ5	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°45´06.9" N	102°33´43.5" W	972	99	FJ545279
TZ10	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°45´50.4" N	102°33´22.9" W	977	99	FJ545279
GS2	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°46´56.1" N	102°30´18.2" W	990	99	KU983799
TG10	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°43´54.6" N	102°30´15.5" W	992	99	KU983799
CQ1	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°41´58.4" N	102°27´25.8" W	985	99	KU983799
XH4	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°41´59.4" N	102°26´46.3" W	990	99	FJ545279
TG3	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°43´51.8" N	102°30´05.6" W	981	99	FJ545279
TG13	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°43´50.7" N	102°30´06.1" W	972	99	KM117232
TG16	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°43´50.3" N	102°30'5.9" W	966	99	KU983799
TG11	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°43´54.5" N	102°30´1.4" W	968	99	FJ177473
XH2	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°41´39.0" N	102°25´57.9" W	981	99	KU983799
XH3	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°41´55.2" N	102°25´07.9" W	976	99	FJ177473
GS1	*Metarhizium*sp.	*Metarhiziumanisopliae*	19°46´57.6" N	102°30´21.7" W	972	100	FJ177473
TG4	*Metarhizium*sp.	*Metarhiziumrobertsii*	19°44´55.1" N	102°30´34.9" W	994	99	KU983799
XH7	*Metarhizium*sp.	----------- ----------	19° 41´ 57.7" N	102°26´46.9"W	---	--	--------
XH1	*Metarhizium*sp.	----------- ----------	19° 41´ 59.0" N	102°26´00.1"W	---	--	--------
ES3	*Metarhizium*sp.	----------- ----------	19° 42´ 37.5" N	102°28´30.2"W	---	--	--------
GS3	*Metarhizium*sp.	----------- ----------	19° 46´ 55.5" N	102°30´12.0"W	---	--	--------
ZP1	*Metarhizium*sp.	----------- ----------	19° 44´ 35.0" N	102°30´51.6"W	---	--	--------
BB1	*Beauveria*sp.	----------- ----------	19° 45´ 06.9" N	102°33´43.5"W	---	--	--------
BB2	*Beauveria*sp.	----------- ----------	19° 47´ 23.4" N	102°33´50.1"W	---	--	--------

Comparison of sequences in the NCBI database, using the BLAST algorithm with GenBank sequences, according to the maximum percentage of similarity.

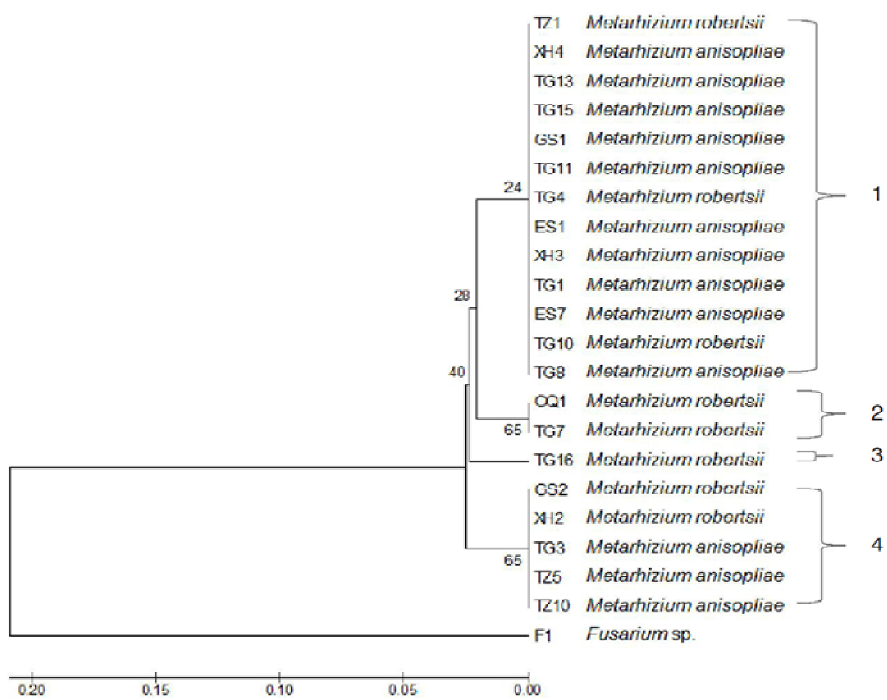

Figure. 3 Phylogenetic analysis with the UPGMA algorithm method of *Metarhizium* species at 1,000 replicates.

Table 4. Mortality rate of *Copturus aguacatae* on the tenth day after being treated with *Metarhizium* spp., under laboratory conditions.

Entomopathogenic fungi	Code of the isolated	Mortality (%)
M. robertsii	TG7	100 a
M. anisopliae	GS1	100 a
M. anisopliae	TZ5	100 a
M. anisopliae	TG1	100 a
M. anisopliae	XH4	100 a
M. anisopliae	TG8	100 a
M. anisopliae	TG3	100 a
M. robertsii	TG4	100 a
M. anisopliae	ES1	90 a
M. anisopliae	XH3	80 a
M. anisopliae	TG11	70 a
M. anisopliae	TG15	70 a
Testigo		10 b

Bioassay performed at a concentration of 1×10^7 conidia/mL. Percentages with the same letter are statistically similar according to Tukey's test (P <0.05).

the tenth day after inoculation. The TG11 and TG15 isolates being the ones that showed the lowest percentages of mortality, however, there was no significant difference (P <0.05) between the mortality rates of isolates, but there was with respect to the control (Table 4). From the 4th d of inoculation mortality occurred in all treatments except in the control. However, after the sixth day, mycosis and sporulation typical of green muscardine caused by *Metarhizium* were observed. The species of this fungus are considered endophyte with evolutionary adaptations (Vega *et al*., 2009), especially *M. robertisii* (Sasan and Bidochka, 2012), which are insect pathogens even in different habitats (Fisher *et al*., 2011), contrasting with this study where *C. aguacatae* and the isolates of *M. anisopliae* and *M. robertsii* do not interact, however, the borer was susceptible to both *Metarhizium* species acting in different absolute media.

CONCLUSIONS

The association of *Brasema* genus as parasitoid of *C. aguacatae* immature is reported for the first time and is the first report for the state of Michoacán of the presence of *Eudurus* and *Brasema,* as natural enemies of *C. aguacatae* and native isolates of entomopathogenic fungi, belonging to *Metarhizium* and *Beauveria* genera. Some isolates of *M. anisopliae* and *M. robertsii* showed pathogenic characteristics desirable to be considered in programs of integrated management of the borer of the branches of the avocado tree.

ACKNOWLEDGMENTS

We are gratefully acknowledge the financial support from Consejo Nacional de Ciencia y Tecnología (CONAYT), the Junta Local de Sanidad Vegetal (JLSV) Gral. Francisco J. Mújica personnel for their valuable help collecting Avocado tree branches, the Universidad Autónoma Agraria Antonio Narro who financed of the research project and, we are grateful assistance in the molecular test at Centro de Investigación en Alimentación y Desarrollo, A.C., Campus Cuauhtémoc.

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