Biotecnia
ISSN: 1665-1456
Universidad de Sonora, División de Ciencias Biológicas y de la Salud

Machín Suárez, A; del Pozo Núñez, EM; García Cruz, I; Héctor Ardisana, EF; Domínguez, Y; Guerrero-Morales, S; Chávez Medina, JA; Escobedo Bonilla, CM; Pérez-Álvarez, S

Isolation and characterization of one autochthonous *Beauveria* sp. isolate from Cuban fields

Biotecnia, vol. 24, no. 1, 2022, January-April, pp. 142-150
Universidad de Sonora, División de Ciencias Biológicas y de la Salud

DOI: https://doi.org/10.18633/biotecnia.v24i1.1553

Isolation and characterization of one autochthonous *Beauveria* sp. isolate from Cuban fields

Aislamiento y caracterización de un aislado autóctono de *Beauveria* sp. de los campos cubanos

Machín Suárez A[1], del Pozo Núñez EM[1], García Cruz I[1], Héctor Ardisana EF[2], Domínguez Y[3], Guerrero-Morales S[4], Chávez Medina JA[5], Escobedo Bonilla CM[5], Pérez-Álvarez S[4]*

[1] Universidad Agraria de la Habana "Fructuoso Rodríguez Pérez", Departamento Biología y Sanidad Vegetal. Carretera Tapaste y Autopista Nacional, San José de las Lajas, Mayabeque, C. P.32700, Cuba.

[2] Instituto de Posgrado, Universidad Técnica de Manabí. Portoviejo, Ecuador, CP EC13010.

[3] Universidade Estadual Paulista (UNESP), Instituto de Biociências, Câmpus São Vicente, Praça Infante Dom Henrique s/n, Parque Bitaru, São Vicente, SP, Brasil, CEP 11380–972.

[4] Facultad de Ciencias Agrícolas y Forestales, Universidad Autónoma de Chihuahua. Km 2.5, carretera Delicias-Rosales, campus Delicias, CD. Delicias, Chihuahua, Mèxico, CP 33000.

[5] Instituto Politécnico Nacional, CIIDIR Unidad Sinaloa. Blvd. Juan de Dios Bátiz Paredes 250, CP 81101 Guasave, Sinaloa, México.

SUMMARY

The genus *Beauveria* comprises a cosmopolitan teleomorphic and anamorphic group of entomopathogenic fungi. The aim of this study was to characterize a new isolate of *Beauveria* sp. and compare it to the LBB-1 commercial Cuban strain. The isolate was collected from ten individuals of the ant *Atta insularis* and ten adults of the coffee borer (*Hypotenemus hampei*). It was cultivated on solid SDAY medium enriched with 1% yeast extract. The biological effectiveness of the isolate was determined on *C. formicarius*. DNA was isolated using the CTAB method and the internal transcribed spacer region was amplified using the primers ITS1 and ITS 4. The consensus sequence obtained was deposited in GenBank (MK246940) and used for phylogenetic analysis. The isolate of *Beauveria* sp. showed differences in morphological characteristics; however, it showed low genetic variability regarding worldwide isolates of *B. bassiana*. Concerning its biological effectiveness, the isolate demonstrated to be virulent on the phytophagous adults, producing 50% mortality. Four clades were identified and the polytomous topology recovered for clade 1 indicates high genetic similarity (99-96%) with *Beauveria bassiana* from several countries. The morphological and molecular characterization made it possible to accurately identify the new isolate as *Beauveria bassiana*.

Key words: Autochthonous isolate, entomopathogen, enzymatic activity, morphology, pest control

RESUMEN

El género *Beauveria* comprende un grupo cosmopolita teleomórfico y anamórfico de hongos entomopatógenos. El objetivo de esta investigación fue caracterizar un nuevo aislado de *Beauveria* sp. en comparación con la cepa comercial cubana LBB-1. El aislamiento se obtuvo de diez individuos de *Atta insularis* y diez adultos del barrenador del café (*Hypotenemus hampei*), respectivamente. Se cultivó en medio sólido SDAY enriquecido con 1% de extracto de levadura. Se determinó la eficacia biológica del aislado sobre *C. formicarius*. El ADN se aisló usando el método CTAB y se amplificó la región de espaciador interno transcrito usando los cebadores ITS1 e ITS4. La secuencia consenso obtenida se depositó en GenBank (MK246940) y se utilizó para análisis filogenético. El aislado de *Beauveria* sp. mostró diferencias en sus características morfológicas; sin embargo, mostró una baja variabilidad genética con respecto a los aislamientos mundiales de *B. bassiana*. En cuanto a su eficacia biológica, el aislado demostró ser virulento sobre los adultos fitófagos, alcanzando el 50% de mortalidad. Se identificaron cuatro clados y la topología politómica recuperada para el clado 1 indica una alta similitud genética (99-96%) con *Beauveria bassiana* de varios países. La caracterización morfológica y molecular permitió identificar con precisión la especie del nuevo aislado.

Palabras clave: aislado autóctono, entomopatógeno, actividad enzimática, morfología, control de plagas

INTRODUCTION

The chemical control of agricultural pests has been the foremost used method in the last 70 years. During this time, enough evidence has been accumulated on the risks posed by the use of chemical pesticides, both on the environment and human health, and they also compromise the sustainability of agricultural systems. This situation has generated global concern, and has increased claims that the use of chemical pesticides should be reduced in favor of an integrated pest management (IPM) (Bueno *et al*., 2017). The alternative to chemical control is the application of biological control. This constitutes an environmentally safe method and it is proposed as a component of the IPM. This approach has gained momentum as a strategy to replace the chemical pesticides. In recent years, an increased interest has emerged to use entomopathogenic fungi as biological control, having reached a significant advance in the development and manufacture of these agents (Swiergiel *et al*., 2016).

*Autor para correspondencia: Sandra Pérez Álvarez
Correo electrónico: spalvarez@uach.mx
Recibido: 17 de agosto de 2021
Aceptado: 11 de enero de 2022

Several entomopathogenic fungi species, especially *Beauveria bassiana* (Bals.-Criv.) Vuill., have a wide host range, such as Coleoptera, Lepidoptera and Diptera. According to Humber (2012), this fungal species is filamentous, has chitinized cells, belongs to the class Ascomycetes, and like other entomopathogenic fungi, it is heterotrophic. *B. bassiana* parasites insects of great economic importance, due to its physical (action of pressure on the contact surface) and chemical (action of enzymes: proteases, lipases and chitinases) mechanisms of infection (Humber, 2012; Dhawan and Joshi, 2017). The usefulness of this microorganism in the biological control of insects has prompted its inclusion in several IPM programs.

Beauveria bassiana, like many other entomopathogenic fungi, shows a wide intraspecific variability. This feature allows its manipulation to obtain environmentally safe strains, characterization and their use to produce biopreparations. Basic taxonomy, ecology, behavior, population dynamics, population genetics and modelling research studies are needed to improve the procedures used in biological control agent exploration (Barrat *et al*., 2018).

At present, little is known about the molecular characterization of *B. bassiana* and most of the studies are based on molecular markers (Dhar *et al*., 2019; Vargas-Martinez *et al*., 2019). Studies on the molecular features of this entomopathogenic species may help to determine its taxonomic, phylogenetic and biological properties.

Taking together all the above-mentioned elements, the aim of this research was to characterize by morphological, enzymatic, pathogenic and molecular means, an autochthonous *Beauveria* sp. Cuban isolate and compare it to the LBB-1 commercial Cuban strain.

MATERIALS AND METHODS

The *Beauveria* sp. isolate was collected in the province of Mayabeque, Cuba, from *Atta insularis* (Güerin) (Hymenoptera: Formicidae) and adults of the coffee borer (*Hypotenemus hampei* (Ferrari)) (Coleoptera: Scolytidae). The *Beauveria* sp. isolate and the LBB-1 strain were cultivated in the Sabouraud Dextrose Agar medium enriched with 1% of yeast extract (SDAY). The LBB-1 strain was provided by the Entomophagous and Entomopathogenic Reproduction Center, and it is conserved in the Phytopathology Laboratory of the Agronomy Faculty at the Agrarian University of Havana "Fructuoso Rodríguez Pérez".

Morphological characterization

The macroscopic characterization of the entomopathogenic fungi was done with 5 mm discs from the periphery of pure colonies cultivated in Petri dishes (90 mm) with SDAY medium. Cultures were incubated in the dark at 25 ºC. After 72 hours, appearance and colony color before and during sporulation, and characteristics of the reverse side of the colony were evaluated visually. Daily growth rate and production of conidia were determined (Martins *et al*., 2015).

The microscopic characterization was carried out from microcultures on slides covered with a thin layer of water Agar (WA), and incubated in a humid chamber for 72 hours in a Boxum incubator at 25 ºC in the dark. Subsequently, the morphology and disposition of the conidiogenic cells and the shape of the conidia were determined per isolate with an NLCD-307 (40X) microscope with Software-NMS designed for the Windows CE5.0 system. The macroscopic and microscopic characteristics obtained from the studied isolate were compared to those of the LBB-1 strain of *B. bassiana*, using the same procedure.

Spores were counted using a Neubauer's chamber in a NLCD-307 (40X) microscope with Software-NMS designed for Windows CE5.0 system, expressing the results in conidia mL^{-1}. The original data were transformed to their logarithms and then processed using a simple classification variance analysis, comparing the means through the Tukey test ($p<0.05$) (SAS Institute, 2016).

Enzymatic characterization

Enzyme activities were determined using 5 mm discs from the periphery of pure colonies obtained in Petri dishes (90 mm) with SDAY medium; the discs were cultivated in specific culture medium for the activity of each enzyme. The medium used for proteases determination was 0.5% peptone, 0.5% NaCl, 0.25% casein, and 0.05% $CaCl_2$ hydrated. In this medium, the hydrolysis halo formed by the degradation of the protein was measured. For the determination of lipase activity, a SDAY culture medium enriched with 5.0% butter was used and the opacity halo around the colony was measured. The determinations of lipase enzyme activity were done at 2, 4 and 6 days and protease at 3, 5 and 7 days. In the determination of the enzymatic activities of the fungi, a completely randomized design (three repetitions/treatment) was used. The data were transformed to $\sqrt{(x+1)}$ and were processed through variance bifactorial analysis (isolates x time), comparing the means through the Tukey test ($p<0.05$) (SAS Institute, 2016).

Pathogenic characterization

The biological effectiveness of the *Beauveria* sp. isolate and strain LBB-1 of *B. bassiana* on *Cylas formicarius* Fabricius was evaluated under laboratory conditions. Adult insects were collected in sweet potato fields (*Ipomoea batatas* (L.) Lam.) without biological or chemical treatments. They were immersed for one minute into a conidial suspension with Tween 80 (0.01%) at 107 conidia mL^{-1} from each isolate, and then transferred into jars with a diet based on 10 g of fresh sweet potato roots (Hua *et al*., 2021). After day 3 or 4 post-inoculation, the dead insects were collected daily until the growth of the fungus appeared on the body surface. The mortality data was obtained at seven days through direct observation method.

The experimental design was completely randomized; 30 insects were used for each isolate and 30 as a control (not immersed into the conidial suspension) distributed in three

replicates with 10 insects each. The data were transformed to arcsen(p)½ and then processed by simple classification analysis of variance, comparing the means through the Tukey test ($p<0.05$) (SAS Institute, 2016).

Molecular characterization

DNA extraction was done using the mycelium of each fungus, according to the method proposed by Polanco *et al*. (2019). The obtained DNA was visualized in a 1% agarose gel stained with ethidium bromide and stored at -20ºC for further use. Amplification of the internal transcribed spacer region (700–1050 bp) was performed using the ITS-1 (GGAAGTA-AAAGTCGTAACAAGG) / ITS-4 (TCCTCCGCTTATTGATATGC) primer pair (White *et al*., 1990). Polymerase chain reaction (PCR) was performed in a thermocycler (BioRad, CA, USA) and the amplification program comprised one cycle at 95 ºC, 4 min, 30 cycles at 95 ºC, 1 min, 60 ºC, 1 min and 72 °C by 2 min, with a final extension cycle at 72 °C for 5 min. The amplified fragments were visualized by 1% agarose gel electrophoresis. The PCR product was purified with a Wizard SV Gel Kit and PCR Clean-Up System (Promega) and sequenced at the Chemistry DNA laboratory in CINVESTAV-IPN Unit Irapuato using a kit Dye Terminator Cycle Sequencing, Ready Reaction, in an ABI PRISM 377 PERKIN-ELMER (Cetus, Norwalk, CT) sequencer. Forward and reverse sequences were examined and aligned in BioEdit (Hall, 1999) and ClustalW (Thompson *et al*., 1994) respectively, in order to obtain a consensus sequence for further analyses.

The ITS sequence obtained and submitted to GenBank (MK246940) was compared with selected sequences of *B. bassiana* from different locations worldwide available at GenBank® database (Benson *et al*., 2013). *Metharhizium rileyi* (Farlow) Kepler, (MG637450) and *Pochonia sp*. (DQ516081) were used as outgroup. Sequences were aligned with ClustalW (Thompson *et al*., 1994) and BioEdit 7.0.9 (Hall, 1999) and the final matrix containing 560 nucleotide positions was used for a phylogenetic analysis through Bayesian inference performed in MrBayes 3.2.6 (Ronquist *et al*., 2012). The analysis was carried out with two independent runs of four Metropolis-coupled Markov chain Monte Carlo $(MC)^3$ (Altekar *et al*., 2004) for a total of 3×10^6 generations, sampling one every 100 trees and discarding the first 25% of the trees as burn-in. Posterior probabilities were assessed from the 50% majority rule consensus and the tree was edited in FigTree 1.4.3 (Rambaut, 2016).

RESULTS AND DISCUSSION

Morphological characteristics

The *Beauveria* sp. isolate and LBB-1 strain macroscopic characteristics are shown in figures 1 and 2. The observed characteristics are very similar to those reported by Samson *et al*. (1988) and Humber (2012).

The colonies of *Beauveria* sp. isolate have regular edges, with white color, velvety, they form striate that do not cover the whole colony, they are elevated (synnemata formation). At the beginning of sporulation, colonies acquire

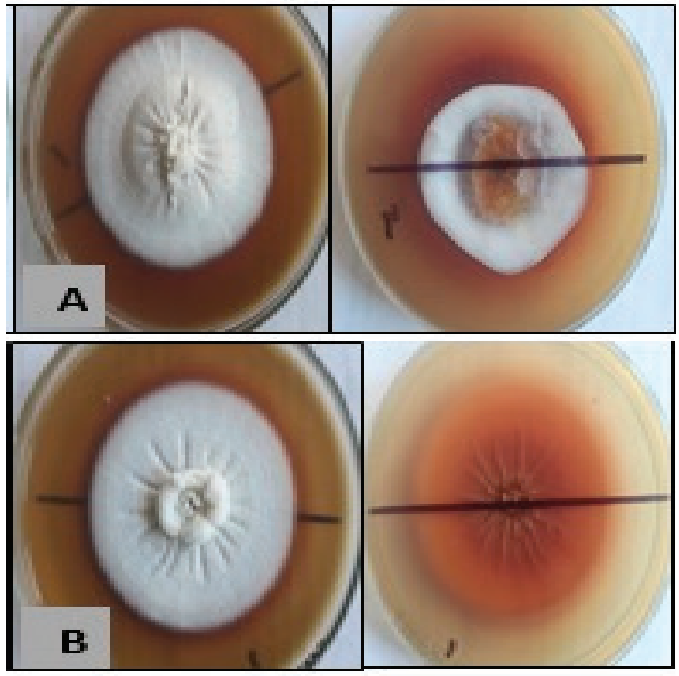

Figure 1. Colonies of the *Beauveria* sp. isolate and the commercial strain after 15 days of inoculation in culture medium**.** A: *Beauveria* sp. isolate; B: LBB-1 strain (Photo: NLCD-307 microscope (40X) with Software-NMS designed for the Windows CE5.0 system).
Figura 1. Colonias del aislado de *Beauveria* sp. y de la cepa comercial después de 15 días de su inoculación en medio de cultivo. A: Aislado de *Beauveria* sp.; Cepa LBB-1 (Foto: Microscopio NSZ-808 con Software-NMS diseñado para el sistema Windows CE5.0).

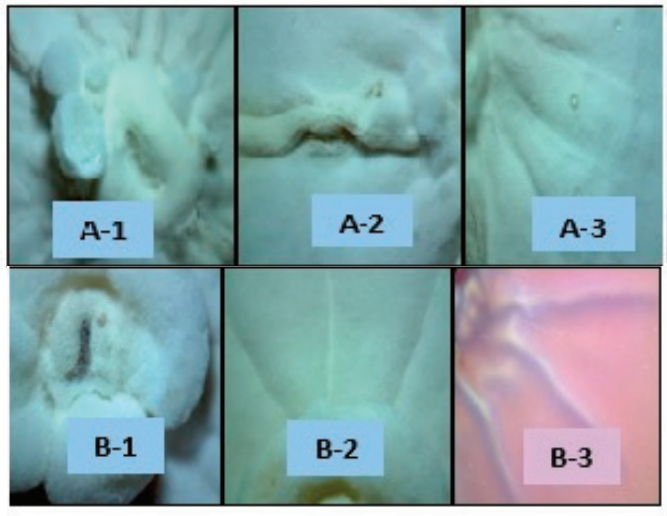

Figure 2. Colony images. A-1: Synnemata in *Beauveria* sp. isolate; A-2 and A-3: Stretch marks in *Beauveria* sp. isolate; B-1: Synnemata in LBB-1 strain; B-2: Stretch marks in LBB-1 strain; B-3: Color and stretch marks in the reverse of LBB-1 strain colonies (Photo: NLCD-307 microscope (40X) with Software-NMS designed for the Windows CE5.0 system).
Figura 2. Imagen de las colonias. A-1: Sinemas en el aislado de *Beauveria* sp.; A-2 y A-3: Estrías en el aislado de *Beauveria* sp.; B-1: Sinemas en la cepa LBB-1; B-2: Estrías en la cepa LBB-1; B-3: Coloración y estrías del reverso de la colonia de la cepa LBB-1 (Foto: Microscopio NSZ-808 con Software-NMS diseñado para el sistema Windows CE5.0).

a yellowish coloration, on the reverse side they are red but there comes a time when the medium is broken and the mycelium grows below (Figure 2 A-1 to A-3).

The LBB-1 strain used in the Entomophagous and Entomopathogenic Reproduction Centers of Mayabeque Cuban province for the production of biological media, showed the following characteristics: white colonies with powdery appearance, regular edges, elevated in the center (synnemata formation), although this strain rarely forms them, striated, and they acquire a creamy coloration at the beginning of sporulation; on the reverse they are red, and stretch marks do not cover the entire colony (Figure 2 B-1 to B-3).

This *Beauveria* sp. Cuban isolate showed a slow growth (Afifah and Saputro, 2020). Other observed morphological characteristics of the isolate are very similar to those reported by Samson *et al*. (1988) and Humber (2012), thus confirming that the isolate belongs to *B. bassiana*. Gebremariam *et al*. (2021) found that all of *B. bassiana* isolates showed disseminated growth patterns, with white to yellowish-white colony color, smooth powdery to cottony texture, raise to flat elevation, and round shape, some of these characteristics are different to those reported in the isolate from the present study.

The colonies daily growth under a temperature of 25 ºC showed a uniform rate (Figure 3). The colonies of the *Beauveria* sp. isolate grew well at the experimental conditions. The temperature conditions can be considered favorable for mycelial growth within the range of 20 to 30 ºC. According to Gebremariam *et al*. (2021), Qiu *et al*. (2019) and Chen *et al*. (2018), the optimal temperature ranges for the development of pathogenic effect and survival of *B. bassiana* and other

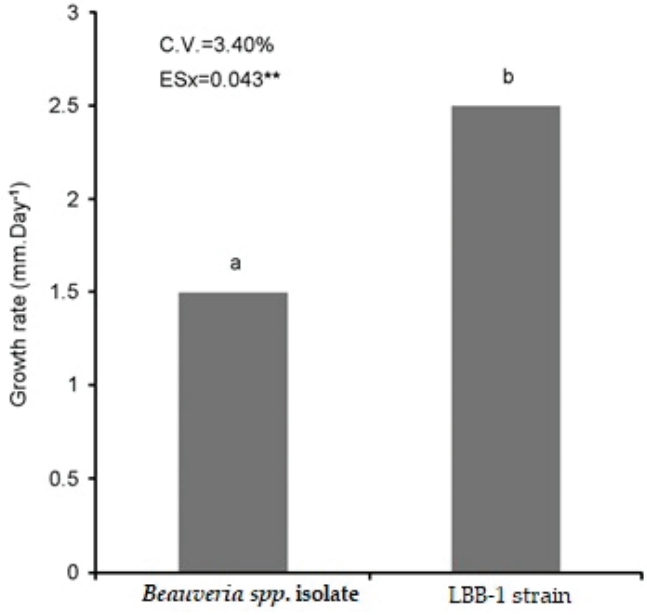

Figure 3. Growth rate of *Beauveria* sp. isolate and *B. bassiana* LBB-1 strain at 25 °C after 15 days (mm day^{-1}). Means with different letters are significantly different according to Tukey ($p \leq 0.05$).
Figura 3. Tasa de crecimiento del aislado de *Beauveria* sp. y la cepa LBB-1 de *B. bassiana* a 25 °C después de 15 días (mm día^{-1}). Medias con letras diferentes son significativamente diferentes según Tukey ($p \leq 0.05$).

entomopathogenic fungi fluctuates around 25 ºC. According to Yu *et al*. (2020) for a better practical application of fungi in agriculture a higher thermotolerance of entomopathogenic fungus is essential.

The production of conidia by the *Beauveria* sp. isolate and the LBB-1 commercial strain is shown in Table 1. The *Beauveria* sp. autochthonous isolate produced more conidia that the commercial LBB-1 strain, which means that the infection of this isolate will be greater since conidia represents infectious units necessary for the production of a biopesticide.

Table 1. Conidia production by *Beauveria* sp. isolate and the *B. bassiana* LBB-1 strain at 25 °C.
Tabla 1. Producción de conidios por el aislado de *Beauveria* sp. y la cepa LBB-1 de *B. bassiana* a 25 °C.

Isolates	Conidia production (conidia mL^{-1})	
	X orig.	X transf.
Beauveria sp.	2.95×10^8	8.4593[a]
LBB-1	2.78×10^8	8.3843[a]
C.V (%)	2.34.	
ESx	0.10^{NS}	

Means with the same letters do not differ significantly, according to Tukey ($p \leq 0.05$).

The conidia production by *Beauveria* sp. isolate (2.95×10^8 conidia. mL^{-1}) is higher than those observed by Dhar *et al*. (2016) (2.66×10^7 conidia mL^{-1}) and Jordan *et al*. (2021) (2.8×10^7 conidia adult^{-1}) showing that sporulation depends, among other factors, on the isolate. The size of the conidia is also a feature that may influence its utility as control agent.

The microscopic observations revealed that *Beauveria* sp. isolate microcultures had branches of mycelium and conidiophores forming clusters (Figure 4), and showed an irregular (zigzag) shape. The designated features were similar for the isolate under study and the LBB-1 strain. The conidia of the studied isolates are hyaline, round to ovoid, born individually from a single cell (Figure 5). The average size of the conidia varies from 2.9 ± 0.40 µm and 2.3 ± 0.4 to 2.4 ± 0.30 µm long and wide respectively (Table 2).

According to Kirubhadharsini *et al*. (2017), the microscopic observations of the conidia showed that they were single cells, round to oval in shape with 2-3 µm length, with hyaline hyphae and zigzag rachis in all the isolates. These characteristics were present in the *Beauveria* sp. isolate from Cuba. The conidia characteristics (hyaline, round) of the *Beauveria* sp. isolate are also similar to those reported by Chen *et al*. (2018) for *B. majiangensis*, who also described that the conidiophores of this species form clusters. This isolate also overlaps with the features and keys described by Samson *et al*. (1988) for *B. bassiana*.

The Cuban isolate showed conidia of slightly larger size to that of the commercial LBB-1 strain (Table 2). In terms of the biological product dosage for application in the field, larger conidia mean a lower number of conidia per volume.

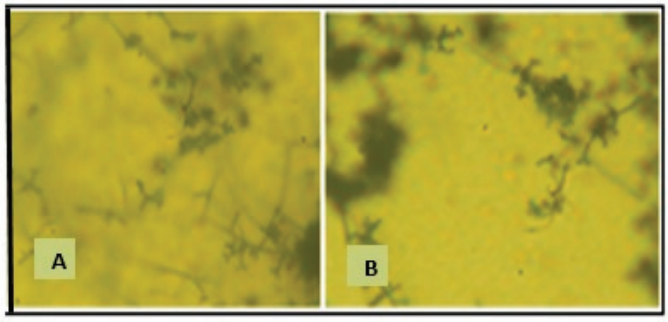

Figure 4. Morphology and outlook of the conidiogenic cells of the *Beauveria* sp. isolate and the *B. bassiana* LBB-1 strain. A: *Beauveria* sp. isolate; B: LBB-1 strain (Photo: NLCD-307 microscope (40X) with Software-NMS designed for the Windows CE5.0 system).
Figura 4. Morfología y panorama de las células conidiógenas del aislado de *Beauveria* sp. y la cepa LBB-1 de *B. bassiana*. A: aislado de *Beauveria* sp.; B: cepa LBB-1 (Foto: Microscopio NSZ-808 con Software-NMS diseñado para el sistema Windows CE5.0).

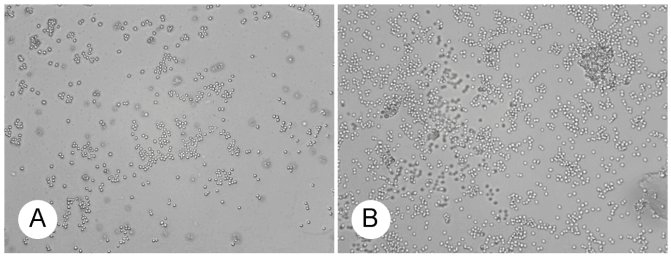

Figure 5. Conidia shape. A: *Beauveria* sp. isolate; B: *B. bassiana* LBB-1 strain (Photo: NLCD-307 microscope (40X) with Software-NMS designed for the Windows CE5.0 system).
Figura 5. Forma de conidios. A: aislado de *Beauveria* sp; B: cepa LBB-1 de *B. bassiana* (Foto: Microscopio NSZ-808 con Software-NMS diseñado para el sistema Windows CE5.0).

Table 2. Conidia size of the *Beauveria* sp. isolate and *B. bassiana* strain LBB-1 (µm) (n= 50).
Tabla 2. Tamaño de los conidios del aislado *Beauveria* sp. y cepa LBB-1 de *B. bassiana* (µm) (n = 50).

	Conidia size (µm)	
	Length	Width
B. bassiana isolates	2.9 ± 0.4	2.4 ± 0.3
Strain LBB-1	2.7 ± 0.2	2.4 ± 0.3

Means with the same letters do not differ significantly, according to Tukey (p≤0.05).

Enzymatic characteristics

No differences were observed when the protease enzyme activity was evaluated (Table 3). The isolate and the commercial strain produced a great hydrolysis halo in the three evaluated moments. From such result, it was inferred that both fungi had large enzymatic synthesis, which indicates a good mechanism of infection. On the other hand, lipase enzyme activity showed significant differences between the two fungi (Table 4). In the second observation, both the autochthonous isolate and LBB-1 strain produced similar large hydrolysis halos. However, in the third evaluation (7 days), the autochthonous isolate produced a larger halo of hydrolysis, being statistically different from the commercial strain.

Table 3. Protease enzyme activity (halo in mm).
Tabla 3. Actividad de la enzima proteasa (halo en mm).

Isolates	Time (days)					
	2		4		6	
	X orig.	X transf.	X orig.	X transf.	X orig.	X transf.
Beauveria sp. isolate	2.666	1.908[a]	5.633	2.576[a]	4.550	2.354[a]
LBB-1	1.666	1.728[a]	3.333	2.081[a]	3.250	2.061[a]
C.V. (%)	4.79					
ESx	0.065^{NS}					

Means with the same letters do not differ significantly, according to Tukey (p≤0.05).

Table 4. Lipase enzyme activity (halo in mm).
Tabla 4. Actividad de la enzima lipasa (halo en mm).

Isolates	Time (days)					
	3		5		7	
	X orig.	X transf.	X orig.	X transf.	X orig.	X transf.
Beauveria sp. isolate	6.666	2.769[a]	9.500	3.230[a]	13.333	3.786[a]
LBB-1	5.833	2.444[b]	6.833	2.801[a]	9.333	3.163[b]
C.V. (%)	8.75					
ESx	0.120^{NS}					

Means with the same letters do not differ significantly, according to Tukey (p≤0.05).

The analyses done on protease enzyme activity showed no significant differences between the isolate and the commercial strain. Regarding the lipase enzyme activity at day seven, significant differences were found between the fungi, with the Cuban isolate having higher activity. The proteolytic and lipolytic activities were shown to be essential factors in the pathogenic activity of *B. bassiana* (Borgi *et al*., 2016). Pelizza *et al*. (2020) correlated the proteolytic activity of one of the studied isolates of *B. bassiana* (LPSc 1227) with the highest mortality of *Schistocerca cancellata*, also, Khosravi *et al*. (2015) and Dhawan and Joshi (2017) reported that fungal isolates which produced the maximum values of proteolytic and chitinolytic activity were those that produced higher mortality on the rose sawfly, *Arge rosae* and the caterpillar *Pieres brassicae*, respectively.

Regarding to lipase activity, Seyed-talebi *et al*. (2020) found a positive correlation between virulence of several *B. bassiana* isolates (TV, OZ, UN, DV and DE) with *Eurygaster integriceps* cuticle. Kaur and Padmaja (2009) and Robledo-Monterrubio *et al*. (2009) reported similar results against *Helicoverpa armigera*, *Sphenarium purpurascens*, *Acheta domesticus*, and *Tenebrio molitor* larvae. Palem and Padmaja (2013) showed that virulence of *B. bassiana* isolates against *Helicoverpa armigera* and *Spodoptera litura* was not correlated with lipase activity.

The results obtained in this investigation related to enzymatic activity are in agreement with all the mentioned research, since the proteolytic and lipolytic activities of the new isolate are related directly to *C. formicarius* mortality.

The production of extracellular enzymes by entomopathogenic fungi, according to Gomes *et al*. (2012), reveals their physiological and biological apparatus, showing their specialization in the degradation of numerous substances found in different hosts or environments. This knowledge of the enzymatic characteristics not only gives the idea of the pathogenic capabilities, but also indicates that substrates rich in lipids and proteins can be used for their reproduction.

Pathogenic characteristics

The results of *C. formicarius* mortality caused by the autochthonous isolate and the commercial strain (LBB-1) of *B. bassiana* under laboratory conditions, showed no significant differences between them (Table 5).

Table 5. *C. formicarius* Mortality caused by *B. bassiana* isolate and the *B. bassiana* LBB-1 strain (%).
Tabla 5. Mortalidad de *C. formicarius* causada por el aislado de *Beauveria* sp. y la cepa LBB-1 de *B. bassiana* (%).

Isolates	X orig.	X transf.
LBB-1	48.3	0.63[a]
Beauveria sp. isolate	50.0	0.63[a]
CV (%)		13.73
ESx		1.73[NS]

Means with the same letters do not differ significantly, according to Tukey ($p \leq 0.05$) test.

The results of the mortality experiments showed no intraspecific variability in the biological effectiveness of the *Beauveria* sp. isolate and the LBB-1 strain on *C. formicarius*. Usually, different isolates of the same entomopathogenic microorganism show differences in their virulence on the same insect host (Sung, 2008), but in the present study, differences between the two isolates were not significant. Similar results were reported by Seyed-talebi *et al*. (2020) in the control of *Hedypathes betulinus* (Klug) (Coleoptera: Cerambycidae). Bagus *et al*. (2019) found that *B. bassiana* effectively eliminate the pest (*C. formicarius*) with a mortality rate of 91.67%. In a similar experiment, Hlerema *et al*. (2017) described promising results with the use of *B. bassiana* in the control of *C. formicarius* with significantly reduction in the percentage of insect damaged roots (5.31%). In the present research, the *C. formicarius* mortality induced by the Cuban isolate and the commercial strain was similar but the Cuban isolate showed the potential to become an agent for biological control.

Molecular characteristics

The PCR amplification of the autochthonous isolate with primers ITS-1 and ITS-4 is shown in Figure 6.

The PCR fragment amplified of the *Beauveria* sp. isolate using the ITS1-ITS4 primers was of 600 bp. This fragment size is similar to that found in four strains of *B. bassiana* (BBPTG1, BBPTG2, BBPTG4, BBPTG6), where the amplification of the ITS regions revealed bands of around 600 bp (López, 2015). Gebremariam *et al*. (2021) identified seven *B. bassiana* isolates using the same primers and a single product of 545

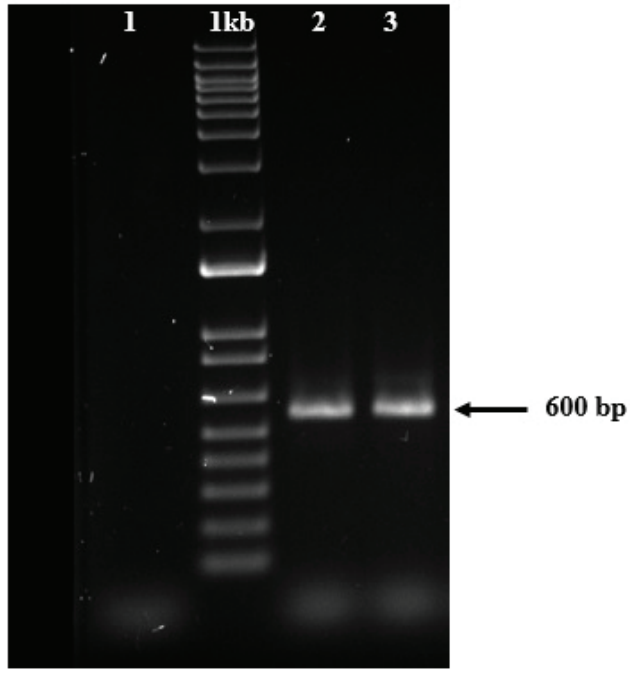

Figure 6. ITS amplification of the isolate by PCR. (1): negative control; (1 kb): marker; (2) positive control and (3): isolated fragment.
Figura 6. Amplificación ITS del aislado por PCR. (1): control negativo; (1 kb): marcador; (2) control positivo y (3): fragmento aislado.

bp fragment size was obtained. Some other results showed fragments size between 500-560 bp for eight *B. bassiana* isolates (Mora *et al*., 2016; Belay *et al*., 2017). As a conclusion isolates fragments sizes of *Beauveria* sp. using the ITS1-ITS4 primers are between 500 and 600 bp.

A consensus sequence was obtained by alignment of the forward and reverse sequences of this region and deposited in GenBank with the accession number MK246940.

From the phylogenetic analysis, this isolate was identified as *B. bassiana*, as it constitutes a monophyletic group with other B. bassiana's accessions retrieved from GenBank. The analysis was done using sequences from different parts of the world, giving identities between 96 and 99% (Figure 7).

Using the ITS sequence information, four clades have been identified, and the polytomous topology suggests a high genetic similarity among *Beauveria* isolates (clade 1). However, other studies pointed out a high genetic differentiation among *Beauveria* lineages with intercontinental distribution (González-Mas *et al*., 2019; Kazartsev and Lednev, 2021). Moreover, Ramos *et al*. (2017) identified five putative lineages of *B. bassiana* occurring naturally in Cuban fields, and the phylogenetic results of the present study are in agreement with their finding.

Recently, some researches have investigated the efficiency of *B. bassiana* isolates in the control of several insects (Toledo *et al*., 2017; Yuan *et al*., 2017); however, this is the first report with a molecular characterization of an autochthonous isolate of *B. bassiana* in Cuba.

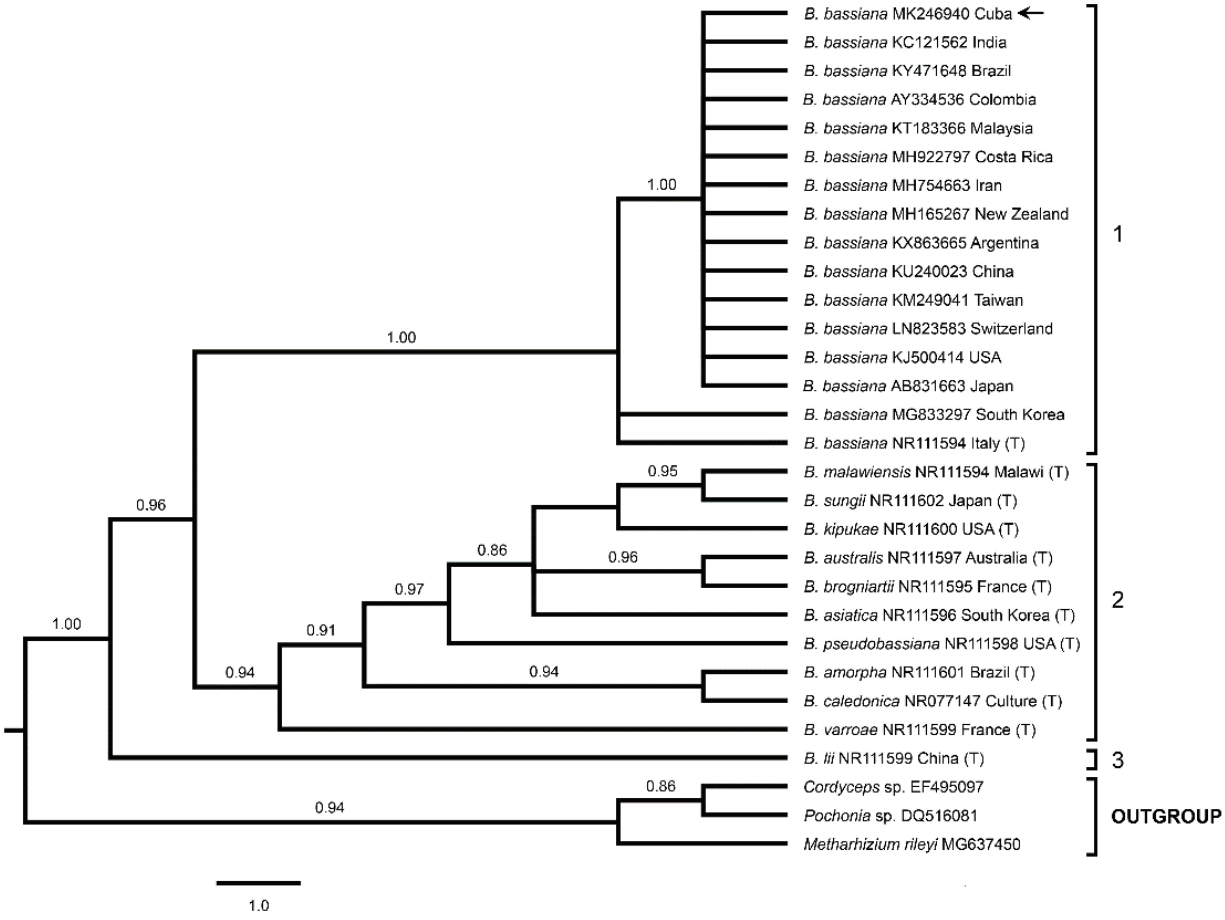

Figure 7. Phylogenetic analysis of the *Beauveria* genus, including different *B. bassiana* isolates from different regions. The autochthonous isolate is indicated by an arrow and (T) indicates *Beauveria* species' type materials (Numbers above branches indicate posterior probabilities).
Figura 7. Análisis filogenético del género *Beauveria* incluyendo diferentes aislados de *B. bassiana* de diferentes regiones. El aislado autóctono se indica con una flecha y (T) indica los materiales tipo de la especie *Beauveria*. (Los números sobres los ramos indican probabilidades posteriores).

CONCLUSIONS

The results of this investigation demonstrate that under the natural conditions of Mayabeque province, it is possible to obtain autochthonous entomopathogenic fungi isolates from several pest hosts, such as *A. insularis* and adults of the coffee borer (*H. hampei*). The morphological characteristics described for the entomopathogenic fungus are consistent with the morphology of *B. bassiana*. The molecular characterization of the *Beauveria* sp. Cuban isolate showed a band of 600 bp and through the phylogenetic study, four clades were identified and the polytomic topography recovered for clade 1 indicates a high genetic similarity (99-96%) with *B. bassiana* from several countries. Morphological, pathogenic and molecular characterization allowed the exact species of the new isolate to be identified as *Beauveria bassiana* (Bals.-Criv.) Vuill.

REFERENCES

Afifah, L. and Saputro, N. W. 2020. Growth and viability of entomopathogenic fungus *Beauveria*

bassiana (Balsamo) Vuillemin in different alternative media. Earth and Environmental Science. 468: 1-8. https://doi.org/10.1088/1755-1315/468/1/012037.

Altekar, G., Dwarkadas, S., Huelsenbeck, J. P. and Ronquist, F. 2004. Parallel Metropolis coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. Bioinformatics. 20:407-415. https://doi.org/10.1093/bioinformatics/btg427.

Bagus, T.S., Prayogo, Y., Lazuardi, F. R., Hidayatul, N.A. 2019. The virulence improvement of *Beauveria bassiana* in infecting *Cylas formicarius* modulated by various chitin based compounds. Biodiversitas. 20(9): 2486-2493. https://doi.org/10.13057/biodiv/d200909.

Barrat, B. I. P., Moran, V. C., Bigler, F. and Van Lenteren, J. C. 2018. The status of biological control and recommendations for improving uptake for the future. BioControl. 63:155-167. https://doi.org/10.1007/s10526-017-9831-y.

Belay, Y.C., Meressa, B.H., Alemu, T. and Hallmann, J. 2017. Molecular detection of the entomopathogenic fungus *Beauveria bassiana* isolates from soils of coffee growing areas in Ethiopia using rDNA-ITS. Journal of Applied Biosciences. 119:11943-11953. https://www.researchgate.net/publication/333930208_Published_online_at_wwwmelewaorg_on.

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Sayers, E.W. 2013. Genbank. Nucleic Acid Research. 41: D36-D42. https://doi.org/10.1093/nar/gkw1070.

Borgi, I., Dupuy, JW., Blibech, I., Lapaillerie, D., Lomenech, A M., Rebai, A., Ksantini, M., Bonneu, M. and Gargouri, A. 2016. Hyper-proteolytic mutant of *Beauveria bassiana*, a new biological control agent against the tomato borer. Agronomy for Sustainable Development. 36:1-9, https://doi.org/10.1007/s13593-016-0394-6.

Bueno, A. F., Carvalho, G. A., Dos Santos, A. E., Sosa-Gómez, D. R. and Da Silva, D. M. 2017. Pesticide selectivity to natural enemies: challenges and constraints for research and field recommendation. *Ciência* Rural. 47(6):1-10. https://doi.org/10.1590/0103-8478cr20160829.

Chen, W-H., Liu, M., Huang, Zh-X., Yang, G-M., Han, Y-F., Liang, J-D. and Liang, Z-Q. 2018. *Beauveria majiangensis*, a new entomopathogenic fungus from Guizhou, China. Phytotaxa. 333(2): 243-250. https://doi.org/10.11646/phytotaxa.333.2.8.

Dhar, S., Jindal, V., Jariyal, M. and Gupta, V.K. 2019. Molecular characterization of new isolates of the entomopathogenic fungus *Beauveria bassiana* and their efficacy against the tobacco caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). Egyptian Journal of Biological Pest Control. 29(8):1-9. https://doi.org/10.1186/s41938-019-0110-3

Dhar, S., Jindal, V. and Gupta, V. K. 2016. Optimization of growth conditions and medium composition for improved conidiation of newly isolated *Beauveria bassiana* strains. Journal of Experimental Biology. 54(10):634-643. https://pubmed.ncbi.nlm.nih.gov/30084563/.

Dhawan, M. and Joshi, N., 2017. Enzymatic comparison and mortality of Beauveria bassiana against cabbage caterpillar Pieris brassicae. Brazilian Journal of Microbiology, 48, 522-529. https://doi.org/10.1016/j.bjm.2016.08.004.

Gebremariam, A., Chekol, Y. and Assefa, F. 2021. Phenotypic, molecular, and virulence characterization of entomopathogenic fungi, *Beauveria bassiana* (Balsam) Vuillemin, and *Metarhizium anisopliae* (Metschn.) Sorokin from soil samples of Ethiopia for the development of mycoinsecticide. Heliyon. 7: 1-12. https://doi.org/ 10.1016/j.heliyon.2021.e07091.

Gomes, E., Maia, H., Feltrin, T. and Van Der, S.T. 2012. Variability in the production of extracellular enzymes by entomopathogenic fungi grown on different substrates. Brazilian Journal of Microbiology. 43(2):827-833, https://doi.org/10.1590/S1517-83822012000200049.

González-Mas, N., Ortega-García, L., Garrido-Jurado, I., Dembilio, O., Jaques, J.A., Quesada-Moraga, E. 2019. Which came first: The disease or the pest? Is there a host mediated spread of *Beauveria bassiana* (Ascomycota: Hypocreales) by invasive palm pests?. Journal of Invertebrate Pathology. 162: 26-42. https://doi.org/10.1016/j.jip.2019.01.007.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT (ver. 7.0.9.0. 2007). *Nucleic Acids Symposium Series*. 41:95-98. https://www.academia.edu/2034992/BioEdit_a_user-friendly_biological_sequence_alignment_editor_and_analysis_program_for_Windows_95_98_NT.

Hlerema, I., Laurie, S. and Eiasu, B. 2017. Preliminary observations on use of *Beauveria bassiana* for the control of the sweet potato weevil (*Cylas* sp.) in South Africa. Open Agriculture. 2: 595-599. https://doi.org/10.1515/opag-2017-0063.

Hua, J., Fu, Y., Zhou, Q., Huang, Y., Li, H., Chen, T., Ma, D. and Li, Z. 2021. Three chemosensory proteins from the sweet potato weevil, *Cylas formicarius*, are involved in the perception of host plant volatiles. Pest Management Science. 77(10): 4497-4509. https://doi.org/10.1002/ps.6484.

Humber, R.A. 2012. Identification of entomopathogenic fungi. In: Lawrence AL (Ed.) "Manual of Techniques in Invertebrate Pathology" Second Edition. Academic Press, New York. https://doi.org/10.1016/B978-0-12-386899-2.00006-3.

Jordan, C., dos Santos, P.L., Oliveira, L.R., Magalhães, D. M., Costa, G.B.C., Fonseca, R. M., Moura, M. G. and Frederico, W. C. 2021. Entomopathogenic fungi as the microbial frontline against the alien Eucalyptus pest *Gonipterus platensis* in Brazil. Scientific Reports. 11: 7233. https://doi.org/10.1038/s41598-021-86638-9.

Kaur, G. and Padmaja, V. 2009. Relationships among activities of extracellular enzyme production and virulence against *Helicoverpa armigera* in *Beauveria bassiana*. Journal of Basic Microbiology. 49: 264-274. https://doi.org/10.1002/jobm.200800156.

Kazartsev, I.A. and Lednev, G.R. 2021. Distribution and Diversity of *Beauveria* in Boreal Forests of Northern European Russia. Microorganisms. 9: 1409. https://doi.org/10.3390/microorganisms9071409

Khosravi, R., Sendi, J.J., Zibaee, A. and Shokrgozar, M.A., 2015. Virulence of four *Beauveria bassiana* (Balsamo) (Asc., Hypocreales) isolates on rose sawfly, *Arge rosae* under laboratory condition. Journal of King Saud University-Science. 27, 49-53. https://doi.org/10.1016/j.jksus.2014.04.003.

Kirubhadharsini, B.L., Nakkeeran, S., Kennedy, J.S. and Manoharan, T. 2017. Morphological and molecular characterization of entomopathogenic fungi, *Beauveria bassiana* isolated from different insects in India. Green Farming. 8(4):940-944. https://www.researchgate.net/publication/319976659_Morphological_and_molecular_characterization_of_entomopathogenic_fungi_isolated_from_different_Beauveria_bassiana_insects_in_India.

López, MOT. 2015. Empleo de marcadores moleculares para el análisis de la prevalencia y diseminación de cepas de *Beauveria bassiana*. Tesis presentada como requisito parcial para obtener el grado de Maestría en Ciencias con acentuación en Microbiología. Universidad Autónoma de Nuevo León, México. https://1library.co/document/1y98e8lz-empleo-marcadores-moleculares-analisis-prevalencia-diseminacion-beauveria-bassiana.html.

Martins, C.C., Alves, L.F.A., Mamprim, A.P. and Souza, L.P.A. 2015. Selection and characterization of *Beauveria* spp. isolates to control the broad mite *Polyphagotarsonemus latus* (Banks, 1904) (Acari: Tarsonemidae). Brazilian Journal of Biology. 76(3):629-637. https://doi.org/10.1590/1519-6984.22614.

Mora, M.A.E., Chacon-Orozco, J.G., Harakava, R., Rouws, J.R.C. and Fraga, M.E. 2016. Molecular characterization and virulence of *Beauveria bassiana* and *Metarhizium anisopliae* against *Galleria mellonella* (Lepidoptera: Pyralidae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. African Journal of Microbiology Research. 10(19):662-668. https://doi.org/10.5897/AJMR2016.7973.

Palem, P. C. and Padmaja, V. 2013. Functional interplay of certain pathogenicity determinant factors governing virulence of the entomopathogenic fungal species *Beauveria*. International Journal of Research in Biosciences. 2 (2): 83-92. https://www.ijrbs.in/index.php/ijrbs/article/view/82.

Pelizza, S.A., Medina, H., Ferreri, N. A., Elíades, L. A., Pocco, M.E; Stenglein, S.A. and Lange, C.E. 2020. Virulence and enzymatic activity of three new isolates of *Beauveria bassiana* (Ascomycota: Hypocreales) from the South American locust *Schistocerca cancellata* (Orthoptera: Acrididae). Journal of King Saud University-Science. 32: 44-47. https://doi.org/10.1016/j.jksus.2017.11.006.

Polanco, F.L.G., Alvarado, G.O.G., Pérez, G.O., González, G.R. and Olivares, S. E. 2019. Fungi associated with the regressive death of citrus fruits in Nuevo Leon and Tamaulipas, Mexico. Revista Mexicana Ciencias Agrícolas. 10(4): 757-764. https://doi.org/10.29312/remexca.v10i4.1417.

Qiu, L., Li, J.J., Li, Z. and Wang, J.J. 2019. Production and characterization of biocontrol fertilizer from brewer's spent grain via solid-state fermentation. Scientific Reports. 9(480): 1-9. https://doi.org/10.1038/s41598-018-36949-1.

Rambaut, A. 2016. *FigTree v 1.4.3*. [Access 20 January 2019]. Available at: http://tree.bio.ed.ac.uk/software/figtree/

Ramos, Y., Portal, O., Lysøe, E., Meyling, N.V. and Klingen, I. 2017. Diversity and abundance of Beauveria bassiana in soils, stink bugs and plant tissues of common bean from organic and conventional fields. Journal of Invertebrate Pathology. 150:114-120, https://doi.org/10.1016/j.jip.2017.10.003.

Robledo-Monterrubio, M., Alatorre-Rosas, R., Viniegra-González, G. and Loera, O. 2009. Selection of improved *Beauveria bassiana* (Bals.) Vuill. strains based on 2-deoxy-D-glucose resistance and physiological analysis. Journal of Invertebrate Pathology. 101: 222-227. https://doi.org/10.1016/j.jip.2009.05.007.

Ronquist, F., Teslenko, M., Van Der, M.P., Ayres, D., Darling, A., Höhna, S., Larget, B., Liu, L., Suchar, M A. and Huelsenbeck, J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic biology. 61:539-542. https://doi.org/10.1093/sysbio/sys029.

Samson, R.A., Evans, H.C. and Latgé, J.P. 1988. Atlas of Entomopathogenic Fungi. Springer-Verlag, Berlin. ISBN: 9783540188315.

SAS Institute Inc. 2016. SAS® 9.4 Language Reference: Concepts, Sixth Edition. SAS Institute Inc., Cary, NC, USA. ISBN: 978-1-62960-822-8.

Seyed-talebi, F.S., Safavi, S.A., Talaei-Hassanloui, R. and Bandani, A. 2020. Variable induction of cuticle-degrading enzymes of *Beauveria bassiana* isolates in the presence of different insect cuticles. Journal of Crop Protection. 9(4): 563-576. https://jcp.modares.ac.ir/article-3-40182-en.html.

Sung, G.H., Poinar, G.O. and Spatafora, J.W. 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. Molecular Phylogenetics and Evolution. 49:495-502. https://doi.org/10.1016/j.ympev.2008.08.028.

Swiergiel, V., Meyling, N.V., Porcel, M. and Rämert, B. 2016. Soil application of *Beauveria bassiana* GHA against apple sawfly, *Hoplocampa testudinea* (Hymenoptera: Tenthredinidae): Field mortality and fungal persistence. Insect Science. 23:854-868. https://doi.org/10.1111/1744-7917.12233.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. ClustalW: improving the sensivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research. 22:673-4680, https://doi.org/10.1093/nar/22.22.4673.

Toledo, J., Flores, S., Campos, S., Villaseñor, A., Enkerlin, W., Liedo, P., Valle, A. and Montoya, P. 2017. Pathogenicity of three formulations of *Beauveria bassiana* and efficacy of autoinoculation devices and sterile fruit fly males for dissemination of conidia for the control of *Ceratitis capitata*. *Entomology* Experimental Applied Journal. 164:340-349. https://doi.org/10.1111/eea.12608.

Vargas-Martínez, A., Salazar-Blanco, J.D., González-Herrera, A. y Molina-Bravo, R. 2019. Análisis genético de Beauveria y Metarhizium tropicales asociados a insectos en caña de azúcar. Agronomía Mesoamericana. 30(1):267-280, http://dx.doi.org/10.15517/am.v30i1.32307.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: "PCR Protocols: A guide to methods and applications", (Eds.) Innis, M., Gelfand, D., Sninsky, J. and White, T. pp 315-322. Academic Press, San Diego. https://www.researchgate.net/publication/262687766_Amplification_and_Direct_Sequencing_of_Fungal_Ribosomal_RNA_Genes_for_Phylogenetics.

Yu, J.S., Lee, S.J., Shin, T.Y., Kim, W.J. and Kim, J.S. 2020. Enhanced thermotolerance of entomopathogenic *Beauveria bassiana* and *Metarhizium anisopliae* JEF-isolates by substrate modification. International Journal of Industrial Entomology. 41(2): 28-35. http://dx.doi.org/10.7852/ijie.2020.41.2.28.

Yuan, H., Lei, Z., Rondón, S.I. and Gao, Y. 2017. Potential of a strain of *Beauveria bassiana* (Hypocreales: Cordycipitaceae) for the control of the potato tuberworm, *Phthorimaea operculella* (Zeller). International Journal of Pest Management. 63(4):352-354. https://doi.org/10.1080/09670874.2016.1269975.