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Species Diversity of Entomopathogenic Fungi Infecting the Sugarcane Aphid *Melanaphis sacchari*: A Recently Introduced Pest in Mexico

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Abstract

The sugarcane aphid, *Melanaphis sacchari*, is a key pest that affects sorghum in Mexico. During 2014 to 2016, in South of Tamaulipas sites and Bajio region in Guanajuato, the populations of this aphid were infected by different species of Hypocrealean fungi. Based in the morphometric identification and molecular characterization, the species associated with sugarcane aphid in South of Tamaulipas were *Lecanicillium longisporum*, *Beauveria bassiana* and *Isaria javanica*. In this region, the higher infection levels were caused by *L. longisporum*, mortality range from 30.0% to 50.0%. The presence of *I. javanica* and *B. bassiana* represented less than 26.0% and 10.0%, respectively. In Guanajuato, the species found corresponded to *L. longisporum* and *B. bassiana*. The infection levels of both species in sugarcane aphid populations in Guanajuato sites were less than 1.00%. The natural occurrence of entomopathogenic fungi on sugarcane aphid populations was associated with climactic factors such as temperature and relative humidity and development of infections was possibly affected by abiotic factors such as crop phenological stage and applications of chemical insecticides realized by farmers for control of this aphid. Further studies on the ecology and physiology of these fungi and trials to determine virulence and persistence in *M. sacchari* populations are needed. This is the first report on natural presence of *L. longisporum*, *B. bassiana* and *I. javanica* causing disease on *Melanaphis sacchari* in Guanajuato and Tamaulipas, Mexico.

Keywords

Aphid, Sorghum, Hypocrealean Fungi, Native Isolates, Identification

1. Introduction

The sugarcane aphid (SCA) *Melanaphis sacchari* (Zehntner) (Hemiptera: Aphididae) is an invasive species and the most important insect pest of *Sorghum* spp. in Mexico and the United States of America [1] [2] [3]. This aphid can infest sorghum in all its stages of development and cause production losses of 20 to 100% [1] [2] [4]. The foliar application of chemical insecticides such as imidacloprid, spirotetramat, thiamethoxam, sulfoxaflor, and fluopyradifurone is the main strategy to control SCA [1] [2] [5]. However, the inadequate management of these chemicals in the field can induce the development of resistance in *M. sacchari* populations. This situation has been documented in other Aphididae species with some of the insecticides used for the control of SCA [6] [7] [8]. Thus, other strategies such as sorghum genotypes tolerant to *M. sacchari* have been evaluated [9] [10] [11] [12]. Predators, parasitoids, and species of entomopathogenic fungi (EPF) such as *Lecanicillium lecanii* (Zimm.) Zare and Gams and *L. longisporum* (Petch) Zare and Gams can offer a sustainable control *M. sacchari* populations [2] [13] [14] [15]. EPF is the most important organism for the biological control of aphids; however their infection on insect populations can be regulated by climatic conditions such as temperature, relative humidity, rain, and wind [16] [17] [18]. Worldwide, *L. longisporum* and *L. lecanii* have been reported causing epizooties in SCA populations [15] [19]. Other genera such as *Beauveria*, *Isaria* and several species of Entomophthorales can infect other species of aphids [16] [20]. Given the recent introduction of this pest in Mexico, it is important to look for species of fungi that are infecting this pest in the most important sorghum producing areas, in order to look for (in future works) candidates for their microbial control. Thus, we identified the species of EPF which were infecting the sugarcane aphid in some regions of Tamaulipas and Guanajuato, in first states infested by *M. sacchari* in Mexico.

2. Materials and Methods

2.1. Study Area

The study was carried out on agricultural fields of commercial sorghum in different phenological stages planted in Tamaulipas and Guanajuato. For sampling in Tamaulipas, populations of the sugarcane aphid were monitored in December 2014 as well as February, March, and May 2015 in sorghum crops of the autumn-winter cycle located in the municipalities of Aldama, Altamira, and Mante. Samples in Guanajuato were collected during the spring to summer of 2016 in the municipalities of Irapuato, Jaral del Progreso, and Salvatierra (**Table 1**).

Table 1. Geographic location and phenological stage of the crops of sorghum with presence of entomopathogenic fungi associated with sugarcane aphid in Tamaulipas and Guanajuato.

Isolate†	Geographic location	Collection date	Sampled sites	Phenological stage of crop¶
		***Lecanicillium* sp.**		
CP-Llon1PAS	20˚09'02.6"N 100˚53'06.9"W	01/09/2016	San Antonio Eménguaro, Salvatierra, Guanajuato	Boot stage**
CP-Llon2PAS	20˚25'21.6"N 101˚01'31.5"W	01/09/2016	Cerrito de Camargo, Jaral del Progreso, Guanajuato	Hard dough stage*
CP-Llon3PAS	20˚38'43.8"N 101˚17'59.7"W	25/08/2016	Distrito de Riego 011, Irapuato, Guanajuato	Soft dough stage*
CP-Llon4PAS	22˚31'57.9"N 98˚12'17.5"W	26/12/2014	Cuauhtémoc, Altamira, Tamaulipas (Plot A)	Flowering*
CP-Llon5PAS	22˚27'46.5"N 98˚04'58.6"W	26/12/2014	Cervantes, Altamira, Tamaulipas	Flag leaf visible*
CP-Llon6PAS	22˚36'09.6"N 98˚12'06.0"W	26/12/2014	Cuauhtémoc, Altamira, Tamaulipas (Plot B)	Soft dough stage**
		***Beauveria* sp.**		
CP-Bb1PAS	20˚25'21.6"N 101˚01'31.5"W	01/09/2016	Cerrito de Camargo, Jaral del Progreso, Guanajuato	Hard dough stage*
CP-Bb2PAS	20˚25'21.6"N 101˚01'31.5"W	01/09/2016	Cerrito de Camargo, Jaral del Progreso, Guanajuato	Hard dough stage*
CP-Bb3PAS	22˚32'22.1"N 98˚08'17.1"W	13/03/2015	Cuauhtémoc, Altamira, Tamaulipas (Plot C)	Hard dough stage**
CP-Bb4PAS	22˚30'01.8"N 98˚34'21.4"W	06/05/2015	Los Aztecas, Mante, Tamaulipas	Physiological maturity*
		***Isaria* sp.**		
CP-Ija1PAS	22˚45'28.1"N 98˚07'07.3"W	08/05/2015	Higinio Tanguma, Aldama, Tamaulipas	Flowering*
CP-Ija2PAS	22˚32'49.3"N 98˚34'13.6"W	06/05/2015	Tantoyuquita, Mante, Tamaulipas	Physiological maturity*

N = North latitude. W = West longitude. †Isolates obtained from naturally infected individuals of SCA. ¶It was determined at the time of sampling in field based in the established by Vanderlip and Reeves [21]. The samplings were carried on: *Commercials hybrids or varieties and **Soca of sorghum.

2.1.1. Sampling of Aphid Populations

Within each location, five sampling points were established (5 × 5 m at each point). There were four points in the corners and one in the middle of each plot. At each point, one sample of 10 sorghum leaves was randomly selected and the number of fungal infected and healthy aphids was recorded. Due to the difficulty of identifying fungal pathogens in the field, infected aphids were collected in plastic containers with an airtight lid (15 cm wide by 7 cm deep). These containers were labeled and then kept at 10˚C for 72 hours until processing. Growth stage of the crop was recorded on each sampling occasion using the growth stages for sorghum proposed by Vanderlip and Reeves [21].

2.1.2. Weather Data

The local temperature, precipitation, and relative humidity (RH) data before sampling were obtained from the local weather station of the National Institute of Forestry, Agriculture and Livestock (INIFAP) (Table 4).

2.2. Isolation of Entomopathogenic Fungi Infecting the Sugarcane Aphid

The EPF Hypocreales were isolated using the rain conidia descending method proposed by Papierok and Hajek [22] for Entomopthoralean fungi with some modifications. The mycosed aphids were adhered individually with a double-sided adhesive tape to the lid of a 60-mm-diameter Petri dish with 10 mL of Papa Dextrose Agar (PDA: BD Bioxon, Becton Dickinson of Mexico S. A. de C. V., Cuautitlán Izcalli, state of Mexico). The dishes were incubated at 25˚C ± 2˚C in the dark inside a bioclimatic chamber (Thermo Fisher Scientific, Model 3721. Marietta, OH, USA) until colonies developed. Each isolate was purified as monosporic culture and incubated for 15 days at the conditions described above.

2.3. Morphological Characterization

For morphological studies of EPF genus, isolates were grown on PDA at 25˚C ± 2˚C for 15 days. Common saprophytic species were excluded from this research. These included species from the genera *Penicillium*, *Cladosporium*, *Mucor*, and *Aspergillus*. For detailed morphological comparisons, the characteristics of the colony were described along with the size and shape of the conidia and conidiogenous cells. To determine the shape and size of conidia and conidiogenous cells, microcultures were grown on PDA incubated at 25˚C ± 2˚C in the dark for 10 to 15 days until sporulation was evident. After incubation, images of conidia and conidiogenous cells were photographed with a photomicroscope (Leica DM750; Leica Microsystems, Heerbrugg, Switzerland) at 40× with a Leica Application Suite program version 3.0.0. Images were processed with GIMP version 2.8 (GNU Image Manipulation Program). ImageJ version 3.00 was used to measure the structures. For each isolate, a total of 100 independent measurements of conidia and conidiogenous cells were made. All isolates were stored at −80˚C in 2 mL cryovials containing 10% sterile glycerol.

2.4. Genetic Characterization

2.4.1. DNA Extraction

For genomic DNA extraction, each isolate was grown on a layer of sterile sweet cellophane paper (Stationery Lumen S. A. de C. V. Mexico City, México) placed on top of PDA. Plates were incubated at 25˚C ± 2˚C for 15 days in complete darkness. After the incubation period, the mycelium was harvested and transferred to sterile 20 mL vials and lyophilized for 48 h using a freeze dryer (FreeZone 4.5, Labconco Coporation. Kansas City, Missouri, USA). For the DNA extraction, 0.2 g of lyophilized mycelium was deposited in 1.5 mL Eppendorf tube with 1 mL of liquid nitrogen and then was macerated using a No. 14 knitting

hook (Fabrics Parisina S. A. de C. V. Texcoco, State of Mexico, Mexico). Total genomic DNA was extracted with the DNeasy® PlantMinikit following the manufacturer's instructions with some modifications [23]. DNA concentration was quantified using a spectrophotometer NanoDrop™ 2000/2000 C (Thermo Fisher Scientific, Inc. Waltham, MA, USA), and the samples were stored at −20˚C until used.

2.4.2. Amplification and Sequencing

For the phylogenetic placement of the genus of each isolate, the ITS1/5.8S rDNA/ITS2 region was amplified using the Polymerase chain reaction (PCR) [24]. PCR conditions consisted of an initial denaturation at 94˚C for 5 minutes followed by 35 cycles of denaturation at 94˚C for 60 seconds, annealing at 55˚C for 60 seconds, and extension at 72˚C for 90 seconds; the final extension was at 72˚C for 7 minutes.

For *Lecanicillium* sp. isolates, the PCR mix for ITS region contained 30 ng of genomic DNA, 1× PCR reaction buffer, 3.0 mM $MgCl_2$, 0.6 mM dNTPs mix (QIAGEN GmbH, Hilden, Germany), 0.6 mM of each primer, and 2.5 U *Taq* DNA Polymerase (BIOLASE™).

The mixture reaction for ITS region of *Beauveria* sp. was performed with 20 ng of genomic DNA, 1× PCR reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP's mix, 0.4 mM of each primer, and 2 U *Taq* DNA Polymerase.

The PCR reaction mixture for *Isaria* sp. consisted of 80 ng of genomic DNA, 1× PCR reaction buffer, 2.0 mM $MgCl_2$, 1.0 mM dNTP's mix, 1.0 mM of each primer, and 2.5 U *Taq* DNA Polymerase.

For species identification were amplified the 18S rDNA region and Bt2 of the β-tubulin gen in the isolates of *Lecanicillium* [25]. The mitochondrial intergenic region atp9-nad3 and Bloc in the isolates of *Beauveria* [26] [27]. The small subunit ribosomal ribonucleic acid (SSU rRNA) was amplified in the isolates of *Isaria* [28]. The amplification of each region was realized using the primers indicated in **Table 2**.

The PCR mixture for 18S rDNA region and Bt2 of the β-tubulin were performed with 50 ng of DNA genomic, 1× PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's mix (QIAGEN GmbH, Hilden, Germany), 0.6 mM of each primer, and 1 U *Taq* DNA Polymerase. For both regions, the amplification conditions included an initial denaturation at 94˚C for 5 minutes; 35 cycles of denaturation at 94˚C for 60 seconds, annealing at 55˚C for 60 seconds, and extension at 72˚C for 60 seconds with a final extension at 72˚C for 10 minutes.

The mitochondrial intergenic region atp9-nad3 was amplified using 50 ng of genomic DNA, 1× PCR reaction buffer, 3.0 mM $MgCl_2$, 0.6 mM dNTPs mix, 0.6 mM of each primer, and 2 U *Taq* DNA Polymerase. The amplification program included an initial denaturation at 95˚C for 5 minutes, 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds, extension at 72˚C for 60 seconds, and a final extension at 72˚C for 7 minutes. The PCR reaction for Bloc region was performed with 50 ng of genomic DNA, 1× PCR reaction

Table 2. Primers used for PCR[¶] amplification of regions of genomic DNA of entomopathogenic fungi associated with the sugarcane aphid.

Region	Primers	Sequence (5'-3')
ITS1/5.8S rDNA/ITS2	ITS5	GGAAGTAAAAGTCGTAACAAGG
	ITS4	TCCTCCGCTTATTGATATGC
18S rDNA	NS1	GTAGTCATATGCTTGTCTC
	NS2	GGCTGCTGGCACCAGACTTGC
β-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC
intergenic mitochondrial region nad3-atp9	nad3F	GAATTAGGTAAAGGAGCC
	atp9R	GAGAATAATTGATTTTTTAATG
Bloc	B22U	GTCGCAGCCAGAGCAACT
	B822L	AGATTCGCAACGTCAACTT
SSU rDNA	NS1	GTAGTCATATGCTTGTCTC
	FS2	TAGGNATTCCTCGTTGAAGA

[¶]All reactions of PCR were carried out in at a final volume of 25 µl.

buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.6 mM of each primer, and 1 U *Taq* DNA Polymerase. The amplification conditions for this region consisted of an initial denaturation at 94˚C for 2 minutes; 40 cycles of denaturation at 94˚C for 30 seconds, annealing at 56˚C for 30 seconds, and extension at 72˚C for 60 seconds; and a final extension at 72˚C for 15 minutes.

The mixture reaction for SSU region was performed using the same mixture reaction described for amplification of ITS region for *Isaria* spp. isolates. Cycling conditions for the SSU rRNA region consisted of denaturation at 94˚C for 2 minutes followed for 30 cycles of denaturation at 94˚C for 60 seconds, annealing at 50˚C for 60 seconds, and extension at 70˚C for 3 minutes; the final extension was at 70˚C for 2 minutes.

PCR reactions were performed at a final volume of 25 µl using a MyCycler™ thermocycler (BIO-RAD Laboratories Inc. Hercules, California, USA). All amplification products were examined by electrophoresis in 1.5% agarose gel stained with ethidium bromide. Bands were visualized under UV light in a transluminator (Infinity-1000 WL/26 MX, Vilber Lourmat®, Marne la Vallée, France). Samples were sent to Macrogen Inc. (Seoul, Korea) for sequencing with the primers used in the initial amplification.

2.4.3. DNA Sequences Analysis

For obtained the consensus sequences of amplified regions in each isolate, nucleotide sequences were edited and assembled with BioEdit v7.0.5 [29]. First, the consensus sequences of ITS region were compared with sequences from the GenBank database of the National Center for Biotechnology Information (NCBI) by Basic Local alignment search Tool (BLAST). For species identification, similar 18S rDNA, Bt2 of the β-tubulin gen, mitochondrial intergenic region atp9-nad3, Bloc, and SSU rRNA sequences were selected of the NCBI and the European Bioinformatics Institute (EMBL-EBI). The select sequences of da-

tabases and the amplified in the isolates were aligned with Mega 7.0 using Clustal W [30]. Phylogenetic analysis of aligned sequences was performed by Maximum Likelihood method (MLE), and the dendogram for each region was generated with a statistical analysis by Bootstrap [31] based on 1000 replications.

3. Results

3.1. Morphological Identification

The size of conidia and conidiogenous cells showed high variation between isolates of each genus (**Table 3**). Six isolates (CP-LlonPAS) were considered into the genus *Lecanicillium*; these had verticillated conidiogenous cells with a mucilaginous head and macro and micro ellipsoidal conidia with rounded ends. Conidial dimensions were of 3.25 - 7.91 × 1.15 - 3.95 µm (long × wide) and the size of conidiogenous cells was of 10.54 - 50.80 × 1.02 - 2.75 µm. These characteristics are similar to descriptions documented in the literature for *L. longisporum* [15] [18] [32].

Colonies of the CP-BbPAS isolates had white mycelium with a powdery aspect and abundant sporulation. The micro cultures had short globose to flask-sharped conidiogenous cells that were clustered or whorled. The rachis was elongated and long in shape of zigzag after each conidium. The detached conidiogenous cells had a succession of global to round and densely packed conidia with average dimensions of 1.97 to 2.30 × 1.65 to 2.02 µm (long × wide). The size of conidiogenous cells was of 3.84 to 3.94 × 1.32 to 1.49 µm (long × wide). The morphological characteristics and the dimensions of the conidia and conidiogenous cells were similar to *B. bassiana* (Blas.) Vuill [33] [34] [35].

The CP-IjaPASisolates were characterized by his powdery appearance and white to gray coloration that changed to pink during sporulation. The shape of conidia was elongated with dimensions of 2.61 to 5.24 × 1.35 to 2.22 µm (long × wide) and placed formed long chains. Conidiogenous cells have a globose basal portion with a narrow and long neck that connects with the conidia. These structures varying in size ranged of 3.63 to 8.37 × 1.17 to 2.55 µm (long × wide). The macro and microscopic characteristics of those isolates are similar to that described for the genus *Isaria*. The dimensions of conidia and conidiogenous cells are similar to descriptions reported by others authors for *I. javanica* (Frieder. & Bally) [36] [37].

3.2. Molecular Analyses

BLAST analysis showed that sequences ITS1/5.8S rDNA/ITS2 of the isolates CP-LlonPAS presented high similarity (94.0% to 99.0%) with some sequences corresponding to *Lecanicillium* species deposited in the GenBank. Phylogenetic tree for the sequences of the region Bt2 of β-tubulin gen showed that the CP-LlonPAS isolates were grouped within *L. longisporum* supported 80.0% bootstrap values (**Figure 1(a)**). Based in the phylogenetic analysis of 18S rDNA region, the six isolates CP-LlonPAS were grouped with *L. longisporum*

Table 3. Conidial and conidiogenous cells measurements (in µm ± SE) of *L. longisporum*, *B. bassiana* and *I. javanica* isolates of sugarcane aphid in Tamaulipas and Guanajuato, México.

Isolate	**Conidia ± SE		**Conidiogenous cells ± SE	
	Length† (Min - Max)¶	Wide† (Min - Max)	Length (Min - Max)	Wide (Min - Max)
		Lecanicillium longisporum		
CP-Llon1PAS	5.42 ± 0.08 (4.00 - 7.64)	1.96 ± 0.03 (1.34 - 2.66)	22.89 ± 0.47 (14.64 - 37.32)	1.69 ± 0.02 (1.08 - 2.26)
CP-Llon2PAS	5.04 ± 0.07 (3.57 - 7.91)	1.85 ± 0.03 (1.26 - 2.44)	24.72 ± 0.56 (16.53 - 48.44)	1.68 ± 0.03 (1.17 - 2.66)
CP-Llon3PAS	4.64 ± 0.07 (3.25 - 6.73)	1.74 ± 0.03 (1.15 - 2.36)	22.28 ± 0.60 (12.34 - 37.62)	1.52 ± 0.02 (1.02 - 2.28)
CP-Llon4PAS	5.19 ± 0.07 (3.51 - 6.89)	1.87 ± 0.04 (1.34 - 3.95)	25.53 ± 0.72 (13.28 - 50.80)	1.63 ± 0.02 (1.15 - 2.08)
CP-Llon5PAS	4.77 ± 0.05 (3.75 - 6.13)	1.95 ± 0.03 (1.53 - 2.62)	23.73 ± 0.50 (14.45 - 37.98)	1.81 ± 0.03 (1.20 - 2.75)
CP-Llon6PAS	5.05 ± 0.07 (3.67 - 7.16)	1.83 ± 0.03 (1.34 - 3.17)	17.63 ± 0.42 (10.54 - 32.06)	1.73 ± 0.02 (1.25 - 2.19)
[15]‡	7.54 ± 1.27	2.69 ± 0.47	25.87 ± 6.04	1.98 ± 0.33
[18]‡	4.50 - 8.50	1.50 - 2.50	NA	NA
[31]‡	5.00 - 10.5	1.50 - 2.50	20.00 - 40.00	1.00 - 3.00
		Beauveria bassiana		
CP-Bb1PAS	1.97 ± 0.03 (1.34 - 2.65)	1.65 ± 0.02 (1.04 - 2.30)	3.94 ± 0.09 (2.09 - 6.03)	1.49 ± 0.03 (1.00 - 2.36)
CP-Bb2PAS	2.03 ± 0.02 (1.48 - 2.71)	1.76 ± 0.02 (1.05 - 2.20)	3.84 ± 0.08 (2.27 - 5.66)	1.41 ± 0.03 (1.00 - 2.16)
CP-Bb3PAS	1.97 ± 0.03 (1.35 - 2.73)	1.76 ± 0.02 (1.35 - 2.39)	3.90 ± 0.06 (2.68 - 5.59)	1.32 ± 0.02 (1.02 - 1.99)
CP-Bb4PAS	2.30 ± 0.03 (1.77 - 2.93)	2.03 ± 0.02 (1.55 - 2.62)	3.92 ± 0.07 (2.51 - 5.52)	1.38 ± 0.02 (1.01 - 1.98)
[32]‡	1.81 - 3.10	1.10 - 2.10	NA	NA
[33]‡	2.00 - 3.00	2.00 - 3.00	3.00 - 6.00	NA
[34]‡	2.00 - 3.00	NA	3.00 - 6.00	NA
		Isaria javanica		
CP-Ija1PAS	4.03 ± 0.04 (3.09 - 4.96)	1.79 ± 0.02 (1.45 - 2.22)	5.71 ± 0.07 (3.94 - 6.94)	1.90 ± 0.02 (1.34 - 2.55)
CP-Ija2PAS	3.72 ± 0.06 (2.61 - 5.24)	1.71 ± 0.02 (1.35 - 2.21)	6.07 ± 0.09 (3.63 - 8.37)	1.75 ± 0.03 (1.17 - 2.54)
[35]‡	3.00 - 6.30	1.40 - 3.20	5.10 - 10.90	1.80 - 2.80
[36]‡	4.24 - 4.92	1.54 - 1.84	5.15 - 5.53	1.86 - 2.30

†Mean for length and wide of conidia and conidiogenous cells (n = 100). ¶Minimum and maximum values observed for each data set. **Obtained from micro cultures development in PDA and incubated at 25˚C ± 2˚C in darkness for 10 to 15 days. ‡Dimensions reported by other authors and used as comparison data with those obtained in the present study. SE = Standard error. NA = Data no available.

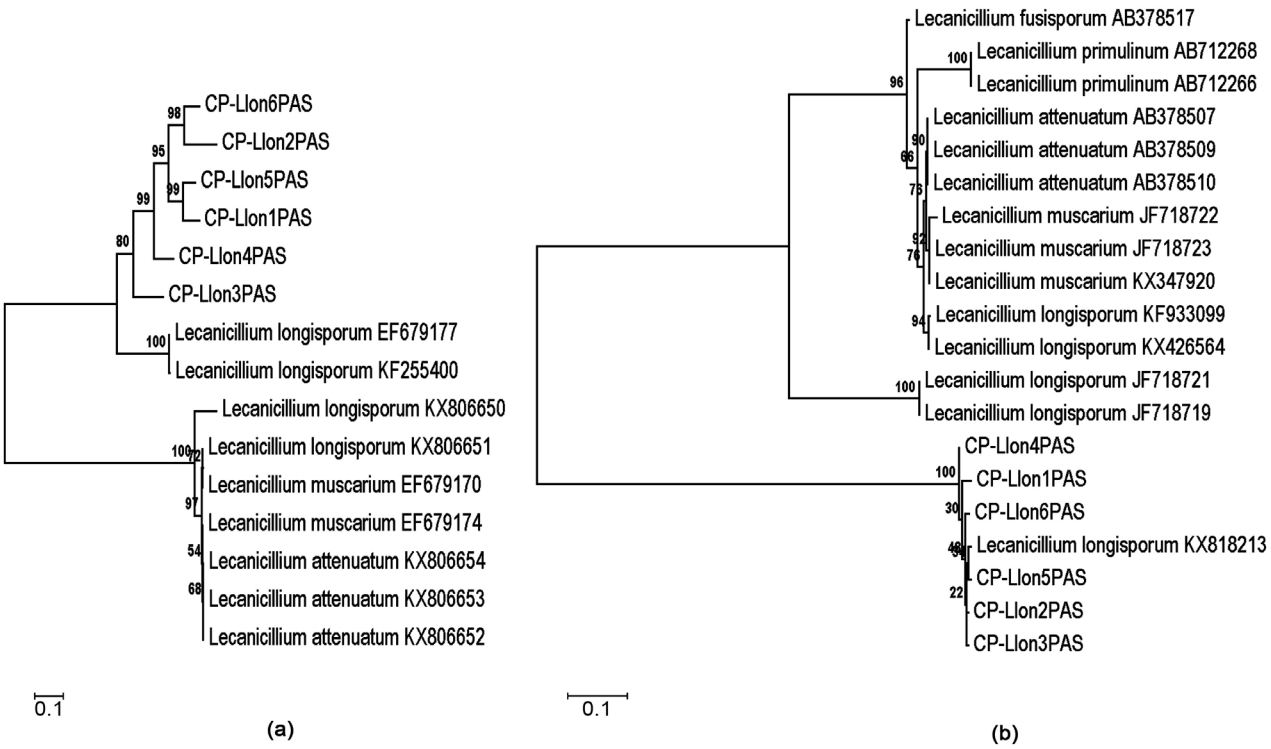

Figure 1. Phylogenetic trees for isolates of *Lecanicillium longisporum*: CP-Llon1PAS, CP-Llon2PAS, CP-Llon3PAS, CP-Llon4PAS, CP-Llon5PAS, and CP-Llon6PAS derived by Maximun Likelihood method with sequences of β-tubulina gen Bt2 region (a), and 18S rDNA region (b). Bootstrap values based on 1000 replications. The analysis involved nine nucleotide sequences of β-tubulina gen Bt2 region (Bt2a-Bt2b) (a), and 14 sequences 18S rDNA region (NS1-NS2) (b).

(Figure 1(b)).

The sequences ITS1/5.8S rDNA/ITS2 of the isolates CP-BbPAS exhibited high similarity with others homologous sequences of the specie *B. bassiana* placed in GenBank. Morphological identification of specie was confirmed with the phylogenetic analyses of the Bloc region, which showed that the four isolates were related with *B. bassiana* and a separated clade of other species such as *B. brongniartii* and *B. caledonica* (Figure 2(a)). This result was similar with the phylogenetic analyses of mitochondrial intergenic region nad3-atp9 in which isolates of *Beauveria* were grouped within the specie *B. bassiana* (Figure 2(b)).

The results of the BLAST analysis showed that sequences ITS1/5.8S rDNA/ITS2 of the isolates CP-Ija1PAS and CP-Ija2PAS presented between 97.0% to 99.0% of similarity with several species of genus *Isaria.* Phylogenetic tree indicated that both isolates were grouped within *I. javanica* supported by bootstrap value of 100.0% (Figure 3).

3.3. Natural Incidence of Mycosed Aphids

The highest aphid mortality was caused by *L. longisporum* in the Tamaulipas sites, where the infection levels were 30.0% to 50.0%, while in Guanajuato this specie represented less than 1.00%. *B. bassiana* infected SCA populations in ranges of 6.0% to 10.0% in Tamaulipas sites; while this pathogen in Guanajuato

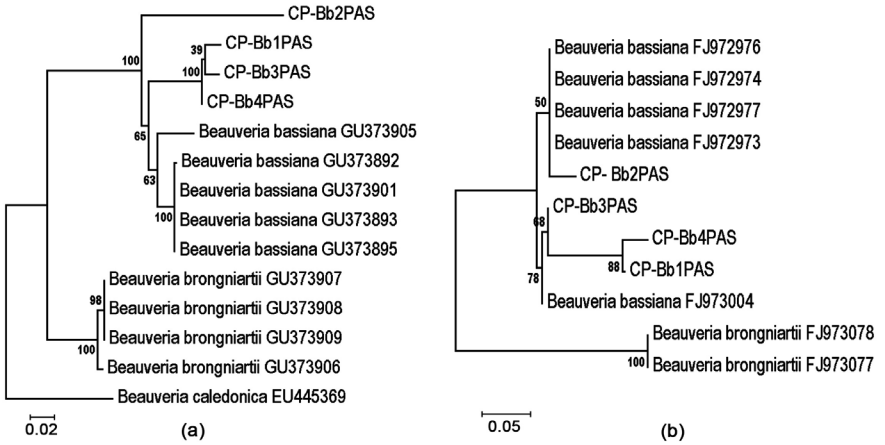

Figure 2. Phylogenetic trees for isolates of *Beauveria bassiana*: CP-Bb1PAS, CP-Bb2PAS, CP-Bb3PAS, and CP-Bb4PAS derived by maximum likelihood method with sequences of Bloc (a) and mitochondrial intergenic nad3-atp9 (b) regions. Bootstrap values based on 1000 replications. The analysis involved 10 nucleotide sequences of Bloc region (B22U-B822L) (a) and seven nucleotide sequences of mitochondrial intergenic nad3-atp9 region (nad3F-atp9R) (b).

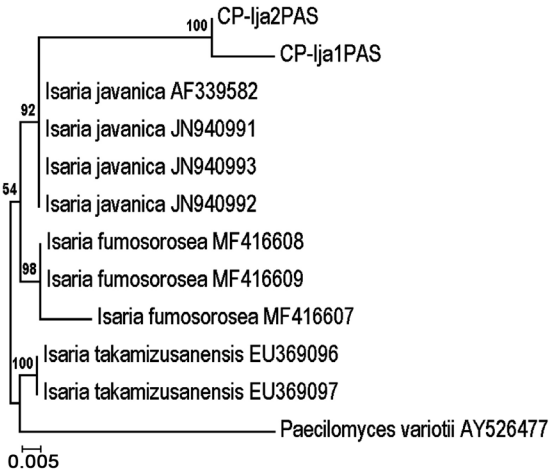

Figure 3. Phylogenetic trees for isolates of *Isaria javanica*: CP-IjaPAS and CP-Ija2PAS derived by Maximum Likelihood method with sequences of SSU region. Bootstrap values based on 1000 replications. The analysis involved 10 nucleotide sequences of SSU region (NS1-FS2).

showed low levels of infection. The presence of *I. javanica* was specific for Mante and Aldama, Tamaulipas; with infection range from 19.5% to 26.0% (**Figure 4**).

3.4. Climatic Conditions in the Sampled Sites

In Guanajuato, the infections for *L. longisporum* occurred after eight days with a mean value of RH of 68.0% to 88.0% and medium temperature of 19˚C (**Table 4**). The presence of *L. longisporum* in the Tamaulipas sites was preceded by a period of RH higher than 87.0% and temperature range of 15˚C to 24˚C (**Table 4**). The natural presence of *B. bassiana* in Guanajuato and Tamaulipas was

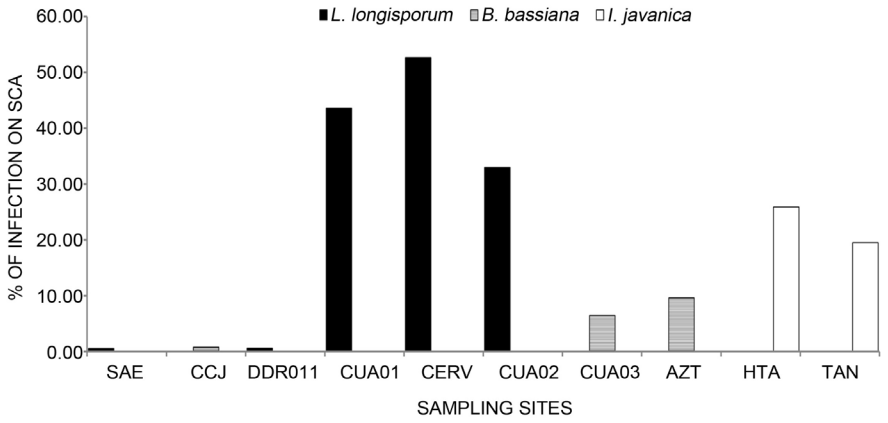

Figure 4. Natural presence of *L. longisporum*, *B. bassiana*, and *I. javanica* on populations of sugarcane aphid in Guanajuato and Tamaulipas. Sampled sites in Guanajuato: SAE (San Antonio Emenguaro), CCJ (Cerrıto de Camargo), DDR011 (Dıstrıto de riego 011). Sampled sites in Tamaulipas: CUA01 (Cuahutémoc, Plot A), CERV (Cervantes), CUA02 (Cuahutémoc, Plot B), CUA03, (Cuahutémoc, Plot C), AZTE (Los Aztecas), HTA (Hıgınıo Tanguma), TAN (Tantoyuquıta).

Table 4. Meteorological recorded in the sites with presence of infected sugarcane aphid by entomopathogenic fungi in Guanajuato and Tamaulipas, México.

Sampled site	Meteorological data†			
	Max Tem	Min Tem	Med Tem	HR
Guanajuato				
San Antonio Emenguaro[a]	26.64	13.75	19.00	77.05
Cerrito de Camargo[a,b]	26.51	14.45	19.36	68.30
Distrito de riego 011[a]	27.28	15.10	19.78	88.60
Tamaulipas				
Cuahutemoc (Plot A and B)[a]	24.21	15.15	18.98	87.24
Cervantes[a]	23.00	15.80	18.96	88.67
Cuahutemoc (Plot C)[b]	23.39	13.24	17.50	81.36
Los Aztecas[b]	30.75	18.84	24.43	69.88
Tantoyuquita[c]	30.75	18.84	24.43	69.88
Higinio Tanguma[c]	30.90	20.89	25.34	77.60

†Mean values of eight days before detection of *L. longisporum*, *B. bassiana*, and *I. javanica* on sugarcane aphid populations. Max Tem = Maximum temperature (˚C). Min Tem = Minimum temperature (˚C). Med Tem = Medium temperature (˚C). RH = Relative humedad (%). Sites with presence of: *L. longisporum*[a], *B. bassiana*[b] and *I. javanica*[c].

observed when the RH was from 68.0% to 81.0% with a mean temperature of 13˚C to 30˚C in the days before detection. The presence of aphids infected by *I. javanica* in Tamaulipas was remarkable when the temperature was 18˚C to 30˚C and RH from 69.0% to 77.0%.

4. Discussion

The search for native species of EPF is important for implementing a microbial

control strategy of the SCA. However, to establish a successful strategy it is important to know the exact identity of the EPF collected [37] [38]. Based on the morphological study (size and shape of the conidia and conidiogenous cells and characteristics of the colonies), the EPF isolates obtained in the Bajío region and southern Tamaulipas were grouped into three genera *Lecanicillium* (six isolates: CP-LlonPAS), *Beauveria* (four isolates: CP-BbPAS) and *Isaria* (two isolates: CP-IjaPAS). This classification was validated with a BLAST analysis that confirmed that the sequences ITS1/5.8S rDNA/ITS2 of the CP-LlonPAS, CP-BbPAS and CP-IjaPAS isolates showed high similarity with the homologous sequences of *Lecanicillium*, *Beauveria* and *Isaria* species deposited in the GenBank. For the morphological identification of the species, the dimensions of the conidia and the conidiogenous cells were determined. The dimensions of these structures in the isolates CP-LlonPAS, CP-BbPAS and CP-IjaPAS were similar to those documented by other authors for *L. longisporum*, *B. bassiana* and *I. javanica* or *I. fumosorosea*, respectively. However, morphological characteristics and classical taxonomic studies often do not provide sufficient evidence for species differentiation within each genus of EPF [39] [40] [41].

The identification of CP-LlonPAS isolates was confirmed with phylogenetic analysis of the regions 18S rDNA and Bt2 of the *β*-tubulin gen. The combination of morphometric studies and phylogenetic analyses of the amplified regions confirm that the six CP-LlonPAS isolates belong to *L. longisporum*. This specie was previously reported as a natural regulator of SCA populations in Tecomán, Colima and on others aphids species in different regions worldwide [15] [18] [42]. Molecular identification of species within the *Beauveria* genus may be possible by sequencing of the mitochondrial intergenic region nad3-atp9 and Bloc [26] [27] [41]. The four CP-BbPAS isolates were identified as *B. bassiana* based in the combination of morphometric characteristics and molecular analysis of intergenic mitochondrial region nad3-atp9 and Bloc. The genus *Beauveria* is a common pathogen among several insect species of agricultural importance [34] including SCA. Based in the morphological studies, the isolates CP-IjaPAS belong to *I. javanica* or *I. fumosorosea*. BLAST analysis indicated that the isolates CP-IjaPAS presented high similarity with some sequences corresponding to *I. javanica* or *I. fumosorosea*. However, phylogenetic tree constructed with SSU rRNA region sequences grouped both isolates within *I. javanica*.

EPF natural infections on insect populations are regulated by biotic and abiotic factors, such as temperature, relative humidity, rain, wind, and cultural practices [16] [43] [44]. The climate in Tamaulipas is usually warm, dry, and temperate [45], the municipalities in which sampling was performed are located in the southern region near the Gulf of Mexico. This zone is characterized by tropical weather (Aw) with an annual average temperature highest to 18˚C and a high percentage of humidity coming from the sea [45] [46]. In Guanajuato state, the aphid collections were from Celaya and Jaral del Progreso; these municipalities are part of the Bajio region that has a humid and temperate climate (Cw) with an average annual rainfall of 600 to 800 mm [46]. It has been documented

that *L. longisporum* causes infection in an average temperature range of 20˚C to 25˚C with a relative humidity greater than 70.0% in populations of *M. sacchari*, *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulzer) [15] [18]. Similar conditions were observed before the detection of aphids with mycosis in the sampling sites of Guanajuato and Tamaulipas. However, in Guanajuato infection levels were lower than 1.0%. A similar situation was observed with the incidence of *B. bassiana* in Guanajuato and Los Aztecas, Tamaulipas; in these sites RH was less than 70.0%.Conidia of *B. bassiana* require a relative humidity greater than 95.0% to germinate [44]. The highest incidence of *B. bassiana* was observed in Cuahutemoc, Tamaulipas, where the relative humidity was higher than 80.0%, while that in the sites with low presence of infected aphids by *B. bassiana* the RH was less than 70.0%. The presence of aphids infected by *I. javanica* in Tamaulipas was remarkable when the temperature was between 15˚C to 33˚C and the RH was 65.0% to 82.0%. Some species of *Isaria* have thermotolerance, for example, *I. javanica* can develop at 35˚C [47]. The CP-IjaPAS isolates, collected in Tamaulipas, might have this characteristic because they were collected in areas where the maximum temperature fluctuated from 30˚C to 35˚C.

Others factors that may be related to the low levels of infection observed in Guanajuato are cultural practices and agrochemicals applications. The use of chemical fertilizers and insecticides affect the survival of EFP [16] [17]. Generally, when aphid population increases, producers apply insecticides by reducing the population of SCA. These control actions can prevent the propagation of the EFP species associated with this aphid, since low density can affect the potential of transmission and development of epizootics [16] [48]. Also, in some sites the detection of infected aphids was carried out during the physiological maturity stage of the crops when the presence of SCA was observed in isolated colonies on the leaves.

Adoption of these species for control of SCA will rely on achieving efficacy, cost reduction, and an ability to broaden the range of pest species that may be targeted. Detailed knowledge of fungal ecology is needed to better understand their role in nature and limitations in biological control. Testing under field conditions is required to identify effects of biotic and abiotic factors and their interactions on efficacy, persistence, and potential limitations to the use of these biocontrol agents in certain crops or locations. There are great opportunities to use these fungi in classical and conservation biological control approaches that can improve environmental stability, efficacy and the cost effectiveness. Temperature is one of the most important abiotic factors affecting the biology and ecology of entomopathogenic fungi [49] [50]. Therefore, the estimation of the *in vitro* growth profiles of entomopathogenic fungi is important in order to gain more information towards the understanding of the abiotic factors affecting specific diversity and distribution of fungal species.

5. Conclusion

The diversity and presence of EF associated with SCA was highest in Tamauli-

pas, and wherein the species *L. longisporum*, *B. bassiana* and *I. javanica* were isolated. In Guanajuato, only the species of *L. longisporum* and *B. bassiana* were detected. *B. bassiana* and *I. javanica* represent the first record of other native species associated with *M. sacchari*. A more detailed study of the ecology of *L. longisporum*, *B. bassiana* and *I. javanica* within this agroecosystem is needed to analyze their possible role in the insect population dynamics.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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