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Assessment of Two Novel Host-Derived *Beauveria bassiana* (Hypocreales: Cordycipitaceae) Isolates Against the Citrus Pest, *Diaphorina citri* (Hemiptera: Liviidae)

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Abstract

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), vectors '*Candidatus* Liberibacter spp.', the causative agent of Citrus Greening Disease (CGD) or Huanglongbing (HLB). Managing populations of psyllids in the Lower Rio Grande Valley (LRGV), TX, United States is imperative given a continuous increase in HLB-positive trees. A component of integrated pest management (IPM) program is the use of strains of entomopathogenic fungi for the biological control of *D. citri*. In an attempt to find endemic strains of entomopathogenic fungi that grow favorably under LRGV environmental conditions and naturally infect *D. citri*, psyllids were collected from local residential areas, surface sterilized, and plated on a semi-selective agar medium. Collection of over 9,300 samples from 278 sites throughout the LRGV led to the positive identification of two *Beauveria bassiana* (Balsamo-Crivellii) Vuillemin (Hypocreales: Cordycipitaceae) isolates, ACP18001 and ACP18002. Chi-square analysis of primary and secondary acquisition bioassays revealed that both field isolated strains outperformed *Cordyceps* (*Isaria*) *fumosorosea* (Wize) (Hypocreales: Cordycipitaceae) Apopka97 under both primary (direct spray) and secondary acquisition (adult exposure to sprayed foliage) bioassays with ACP18002 marginally outperforming ACP18001 under secondary acquisition. Slopes of the dose response regression lines for the three fungi were not significantly different. In addition, the thermal profiles for vegetative growth of each isolate indicated that the field isolates grew at higher rates than the standard at higher temperatures. The new isolates may prove to be good candidates for the management of *D. citri* populations in the LRGV.

Key words: Hypocreales, Cordycipitaceae, biological control, entomopathogenic fungi, integrated pest management

Citrus Greening Disease (CGD) has become a growing concern for the citrus industry worldwide, and management of the disease is at the forefront of citrus related research. While the bacteria belonging to the genera *Candidatus* Liberibacter are fastidious and therefore difficult to study directly, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), one of the bacterium's known vectors has become a target of IPM for pest population management. A component of IPM for this insect has been the use of entomopathogenic fungi as a biological control agent (Mudronček ová et al. 2021, Lezama-Gutiérrez et al. 2012, Avery et al. 2009, Gulzar et al. 2021). Numerous programs that have employed fungi have shown their efficacy against other pests (Wakil et al. 2021, Hong and Kim 2007) and the combined use of fungal pathogens with certain chemical pesticides has been found to have a synergistic effect (Zou et al. 2014). Additionally, entomopathogenic fungi are environmentally friendly and unlikely to induce resistance in target pests by evading innate humoral responses (Jiang et al. 2020), as some pesticides tend to do (Tiwari et al. 2011).

A recent study by Maina et al. (2018) showed that over 170 mycoinsecticides have been commercialized throughout multiple regions including North and South America, Europe, Asia, Africa, and the Middle East. Biopesticides in general have also seen a compound annual

growth rate from 12% to 14.1% from 2005 to 2016 (Zaki et al. 2020), indicating an increased interest for alternatives to chemical pesticides.

Mycoinsecticide selection is based not only on virulence but also on several other factors such as shelf life of the fungus, UV tolerance, thermal profile, and spore production of the fungus (Ghazanfar et al. 2020, Quesada-Moraga et al. 2006). As such, screening candidate fungi based on these characteristics prior to commercialization is an important step.

To identify a suitable fungus, a library of possible candidates must be assembled for screening. This task can be done by baiting soils with insects (Zimmermann 1986) or by surveying insect populations for the presence of established fungal infections (Wakil et al. 2014, 2013; Parker et al. 2003). Some researchers have opted to produce a suitable candidate instead, by editing the genome of a given strain to upregulate the production of proteolytic enzymes (Shin et al. 2020, St Leger et al. 1996). Others have characterized a given molecule; a protease, chitinase, or toxin directly involved in fungal virulence within a fungal genus and species looking for metabolites that could be used against a target pest (Proctor et al. 2018, Kang et al. 1999).

Regardless, field sampling remains an important aspect of the commercialization process. While searching for an efficacious fungus to be used against *D. citri*, some researchers have concentrated on collecting cadavers of *D. citri* from the field for the isolation of entomopathogenic fungi (Meyer et al. 2008, Meyer et al. 2007). Finding cadavers can prove to be a tedious and slow process, especially considering the pest's miniscule size (Awan et al. 2021). Therefore, the need for a streamlined process for screening of *D. citri* to isolate entomopathogenic fungi is still necessary.

The LRGV is a region of southern Texas that contains numerous acres of recreational vehicle (RV) parks on land that was previously used as citrus groves. These groves were primarily composed of grapefruit and orange trees and many citrus trees were left behind in the lawns of lots within these parks as unmanaged reservoirs of the insect as well as the bacterium, *Candidatus* Liberibacter. Though problematic for pest control programs against the *D. citri*, these RV parks provide a unique opportunity for screening large populations of *D. citri* for the presence of entomopathogenic fungi.

The purpose of this work was to seek out and identify fungal entomopathogens actively infecting the LRGV *D. citri* population. Our work further focused on the biological characterization of two isolates, ACP18001 and ACP18002, by analyzing their infectivity and virulence in adult *D. citri*, and their thermal profiles.

Materials and Methods

Field Collections

Collections began in April 2018 by United States Department of Agriculture (USDA) Field Operations agents, in addition to our own field collections. Collections consisted of weekly trips to RV parks within the LRGV. Once a citrus tree was identified, individual branches and leaves were inspected for presence of *D. citri*. GPS coordinates were also annotated for each collection site, along with the number of insects collected at that site. Samples were initially sought in both residential areas and commercial citrus groves, but there was scarce *D. citri* presence in the groves, likely as a result of scheduled pesticide treatments. Additionally, the data collected at each site were reduced to the GPS coordinates, type of tree, and recently, temperature and humidity data at the time and site of collection (not shown). As the timeline of the project progressed, secondary collections from previously visited sites were made, particularly where isolates had been discovered.

Sample Processing

Collected field samples were immediately stored for 24 h at –20°C to freeze-kill all *D. citri* adults. Fungi within cadavers were protected from extreme temperature damage as demonstrated by Hoy et al. (2010). Once killed, samples were stored dry at 4°C in 1.5 ml Eppendorf tubes until processed to preserve any fungal pathogens within the insects. This also prevented any saprophytic growth on the outside of *D. citri*. During processing, approximately 3 ml of 10% sodium hypochlorite solution, with 1 ml Dawn soap (as a wetting agent) L^{-1}, were added to each individual vial and agitated via vortexing at 3,000 rpm for 30 s. The sample with sodium hypochlorite solution was decanted onto a Buchner funnel with #1 filter paper attached to a vacuum pump, and the insects were rinsed thoroughly with reverse osmosis H_2O. Once most of the H_2O had been evacuated, the filter was removed from the funnel, and psyllids were plated in rows onto 100 mm selective water agar composed of 0.23 mg/ml dodine and 0.015 mg/ml chloramphenicol (WA-DC). Plates were incubated at approximately 29°C and after a 7-day incubation period, cadavers were inspected for fungal growth. Conidia from potential presumptive entomopathogenic fungi were subcultured onto a selective medium (potato dextrose agar with 0.1% yeast extract, 0.23 mg/ml dodine, and 0.015 mg/ml chloramphenicol (PDAY-DC) to obtain single colonies for further culture. Plates were then incubated for 7 d at 29°C in complete darkness. Single colonies from the selective agar were transferred to 60 mm PDAY plates and allowed to grow to confluency. Mycelial growth on plates was transferred using a metal spatula to cryogenic vials containing 1 ml 30% glycerol and 3 glass beads. Samples were then vortexed at 3,000 rpm for approximately 30–45 s until relatively homogenous and then stored at –80°C for long-term preservation. Agar cultures of the isolates were submitted to the USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, NY) under the accession numbers ARSEF13765 and ARSEF13766.

Genetic Characterization

Characterization of the field isolates to the level of genus was determined via phylogenetic studies using multiple loci. Isolates grown up on 60 mm Sabouraud dextrose agar with 0.1% yeast extract (SDAY) plates were allowed to grow to the periphery of plates. Fungal mycelia were taken from plates using a sterile metal spatula and were added to 1.5 ml collection tubes. Genomic DNA was extracted using the DNEasy Plant Mini Kit (QIAGEN Inc., Germantown, MD) following the manufacturer's recommendations and DNA was eluted using 50 µL of EB buffer.

A fragment of four nuclear loci was amplified by Polymerase Chain Reaction (PCR) using the primers found in Table 1 (Rehner et al. 2011). PCR reactions were performed using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster, CA). Amplification reactions consisted of 25 µL volumes containing 16.4 µL of water, 2.5 uL of 10× buffer, 2.6 µL of dNTPs, 0.13 µL of DNA Taq polymerase, 1 µL of each primer (10 mM), and 2 µL of DNA template. PCR reaction conditions for the B locus and EF1-α gene loci were replicated as in Rehner and Buckley (2005). Thermocycler reaction conditions for the remaining sequences were performed as found in Rehner et al. (2011).

PCR products were visualized on 1% agarose gels using ethidium bromide to confirm the presence of the amplified fragment and then purified using ExoSAP-IT (Affimetrix, Santa Clara, CA) as per manufacturer's instructions prior to sequencing. Samples were sent to Functional Biosciences (Madison WI, USA), for bidirectional

Table 1. List of isolates with their respective accession numbers, host substrate, collection site, and collection date

Scientific name of fungus	Method of ID	ARSEF accession #	Isolate code	Host and life stage	Collection site	Collection date
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	13765	ACP18001	*Diaphorina citri*, Adult	Pharr, Texas, USA	4/27/2018
Beauveria bassiana	Bloc, EF1α, *rpbI*, *rpbII*	13766	ACP18002	*Diaphorina citri*, Adult	Pharr, Texas, USA	5/1/2018

sequencing. Sequences were edited on Sequencher 5.0 (Gene Codes Corp., Ann Arbor, MI).

DNA sequences were compared to public GenBank records using NCBI default BLAST search option for nucleotide sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the highest genus and species matches per sequence. The sequences were also searched comparing them to records of *Beauveria*, *Isaria*, *Lecanicillium*, and *Fusarium*. Once genus-level identification was determined using the BLAST searches, a phylogenetic analysis of records was performed. An alignment was constructed for each locus using Clustal W (Thompson et al. 1994) and the program MEGA v.7.0 (Tamura et al. 2013). Aligned sequences were trimmed to the longest length of high-quality sequence reads for all specimens. Reference sequences were taken from Rehner et al. (2011) as a basis for the *Beauveria* phylogeny. In addition, the four single-locus alignments were concatenated into a multigene alignment for analysis in MEGA v.7.0. Tree reconstructions were performed for each data alignment in MEGA v.7.0 using the Neighbor-Joining algorithm, 100 bootstrap replicates, and default settings.

Primary (Direct) and Secondary (Indirect) Acquisition Bioassays

The two isolates that were molecularly characterized and submitted to ARSEF under the accession numbers ARSEF 13765 (ACP18001) and ARSEF 13766 (ACP18002) were cultured on solid substrate fermentation (SSF) as described in Jaronski and Jackson (2012) and harvested spores were used in primary and secondary dose-mortality bioassays, that is, with exposure of unsprayed *adult D. citri* to sprayed foliage. Stock solutions of approximately 1×10^9 ml^{-1} were suspended in 0.1% Tween 80 using a glass tissue homogenizer and 100× dilutions were subsequently counted on a hemocytometer to determine spore concentration of the stocks.

Spore viabilities were determined by plating the 100× dilutions onto PDA plates and incubating at 29°C in complete darkness for 16–18 h. Viabilities were read by staining an excised block of the inoculated PDA plates with cotton blue stain (MycoPerm; Fisher Cat # 23769320) and determining viable and nonviable spores under a microscope. At 16–18 h, any spore that had developed a germ tube was considered viable, while any spore lacking a germ tube was considered nonviable. Spore suspensions at concentrations of 3×10^6, 1×10^7, 3×10^7, 1×10^8, and 3×10^8 viable spores ml^{-1} were prepared. Reverse osmosis H_2O was used as an untreated control for the two isolates and Apopka97. Primary acquisition sprays were performed by taking approximately 20–35 unsexed *D. citri* in snap-cap, 9 dram vials, and cold stunning them at −15°C for approximately 3 min. Stunned insects were then placed onto a wax weigh paper placed inside of the spray tower and sprayed with 0.4 ml of a dose or control solution. For secondary acquisition bioassays, plant material was sprayed by placing the aqua tube with plant material in a tube rack inside the potter's spray tower and spraying each sample with 0.4 ml of the appropriate spore dilution. Inoculated insects were placed in fabricated acrylic flush shoot tubes with mesh tops and bottoms and plant material that was pruned from 160-liter pots of orange jasmine plants, *Murraya paniculata* (L.) Jack. The flush shoot samples measured approximately six to eight inches tall in aqua tubes (Syndicate Sales, Kokomo, IN) with 0.025% MaxiGro hydroponic solution (General Hydroponics, Sebastopoc, CA) and were wrapped at the base with parafilm. Flush shoots were incubated in environmental growth chambers at 28°C, 80% RH, and a photoperiod of 12:12 (L:D) h for 7 d. After the 7th day, live and dead insects were counted and mortalities were calculated.

Mortality data was analyzed via probit analysis using PoloPlus (LeOra Software LLC, Parma, MO) to calculate the median lethal concentration (LC_{50}) and 95% confidence intervals (CI) where available. Chi-square analysis was performed using Statistix (V1.0, Analytical Software Inc., Tallahassee, FL) in order to find significant differences between the field isolates and the standard at their respective doses. The 95% CI were also compared using the confidence interval overlap test to identify if there were any significant differences. Data was visualized using a box and whisker graph (Fig. 2).

Thermal Profiles

Rates of vegetative growth were determined for both fungal isolates and the standard, Apopka97 at 5, 10, 15, 20, 25, 28, 32, and 35°C to obtain a thermal profile for each strain. Aerial conidia from two-week-old Sabouraud dextrose agar (SDA) petri plates were suspended in sterile 0.1% sorbitan monooleate (Tween 80) (Fisher Scientific, Fair Lawn, NJ), using a sterile cotton swab. The approximate concentration of the suspensions was 5×10^7 conidia ml^{-1} based on turbidity. To each stock suspension, 20 µL of an aqueous gentamycin or chloramphenicol solution was added to inhibit any subsequent bacterial growth. Three replicate 100 mm diameter Petri plates of PDAY were each inoculated with the conidial suspensions of each strain. Inoculation consisted of a 10 µL droplet placed in the center of each plate. All plate cultures were incubated for three days at 28°C, until the colonies were generally 1 cm in diameter. At this point, the colony edges were marked along two perpendicular axes and the cultures transferred to the various temperatures. Colony diameters were marked daily (15, 20, 25, 28°C) or every other day (5, 10, 32, 35°C) until colonies had grown to within 2 cm of the Petri dish margin, or 14 d in the case of the temperature extremes. Subsequently, the distance between opposing markings was measured and exported to an Excel file, with a Mitutoyo Digimatic Caliper and Input Tool (Mitutoyo Corporation, Japan).

Colony diameters were converted into radii and subjected to least squares linear regression in Microsoft Excel to calculate growth rates in mm h^{-1}. In addition, the growth rates at suboptimal temperatures were calculated based on the temperature having the fastest growth for each strain. Slopes of the lines for the thermal profiles were calculated using Microsoft Excel and compared to the slopes of the lines from the first and last temperature points to identify discrepancies in the linearity of the repeated measurements. Two-Way ANOVA ($\alpha = 0.05$) (SigmaPlot V11.0) was used as done in Er and Gökçe (2004) and Gabiatti et al. (2006) to determine the effects of isolate strain and temperature on growth rate. All pairwise comparisons were subjected to Tukey's multiple-comparison test in order to identify significant differences between the isolates versus the standard, Apopka97.

Results

Field Collections and Processing

Since April 2018, approximately 9,305 *D. citri* samples from 278 sites were collected and processed. Of these samples collected, 2 of 9,305 processed insects resulted in positive identifications for entomopathogenic fungi. Genetic characterization of the isolates was determined by means of four gene loci and a phylogenetic tree was constructed based on this data (Fig. 1).

Genetic Characterization

NCBI BLAST searches for all genera in question were performed and their results could be found in Table 2. Secondary matches were provided where results of similar match percentages yielded contradictory results.

A phylogenetic tree for the concatenated alignments as well as for each individual locus for the two isolates that matched the *Beauveria* spp. revealed that both isolates belonged to the *B. bassiana* species clade. Only the tree for B locus is shown (Fig. 1) given the highest number of informative sites of all four loci used for this analysis. The BLAST results corroborated the findings that these isolates belonged to the genus *Beauveria*.

Primary and Secondary Acquisition Bioassays

The list of statistically acceptable bioassays as determined by the calculation of LC_{50} and 95% confidence interval (95CI) values by PoloPlus can be found in Table 3. In addition, complete datasets including bioassays that did not produce LC_{50} and 95CI were graphed using a bar and whisker plot in Fig. 2. Results of the bar and whisker graph revealed that the dataset had significant outliers, especially Apopka97 for both primary and secondary acquisition bioassays. In addition, chi-square analysis results on the lumped datasets for primary and secondary acquisition bioassays indicated there were significant differences between the field isolates and standard, the results of which can be found in Table 4.

Thermal Profiles

Thermal profiles were used to characterize the isolates by means of radial growth measurements, wherein the growth rates for each of the three fungal strains (Apopka97, ACP18001, ACP18002) were analyzed and compared. The plots for the data are shown in Fig. 3. Two-Factor ANOVA of all pairwise comparisons revealed there were significant differences between the two isolates and standard at various temperatures $F(2, 71) = 10.837$, $P < 0.001$ (Table 5). Tukey's post-hoc test revealed significant differences at the 10, 26, and 32°C temperature points between the field isolates and Apopka97. Specifically, ACP18001 outperformed the standard at the 10°C temperature point, while ACP18002 did so at the 26 and 32°C points. Similarly, ACP18001 did so at the 32°C point. Though not indicated in the graph, Apopka97 grew at faster rates than ACP18001 at the 20 and 26°C points, and greater than ACP18002 at the 30°C point.

Fig. 1. Phylogenetic tree of *Beauveria* illustrating species relationships inferred from Neighbor Joining (NJ) method of Bloc. Numbers above branches show boot support values for NJ methods (1,000 replicates). Species clades are in bold and indicated by vertical brackets.

Discussion

Field Collections and Processing

Based on our collections, the incidence of entomopathogenic fungi on live-collected *D. citri* was approximately 0.02%; a low rate, especially when compared to numbers found in other studies (Awan et al. 2021, Awan et al. 2021). Recovery of fungi from insect cadavers found in the field, a frequent tactic, is subject to the effects of environmental factors, especially humidity, on external fungal growth and sporulation (Wakil et al. 2014). In addition, collecting from a live host versus from soil samples would also select for an isolate that could likely be acclimated to the LRGV environment and a pathogen of the insect of interest.

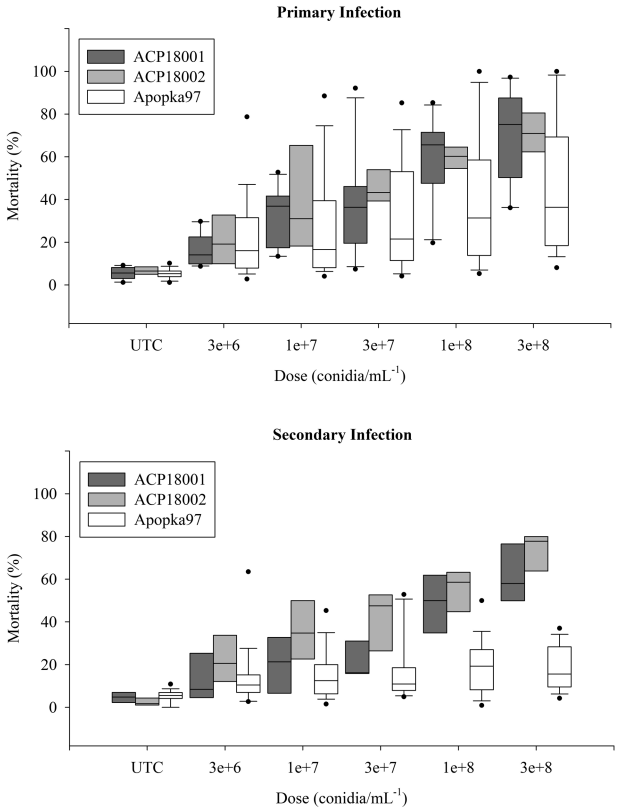

Fig. 2. Box and whisker graph of primary (top) and secondary (bottom) mortality bioassays. Figure indicates the median (center line), lower and upper quartiles (box), and lower and upper extreme values (whiskers) for all bioassay mortalities of ACP18001, ACP18002, and Apopka97. Asterisk indicates significant difference ($P < 0.05$) between field isolates and Apopka97 at each dose (Dunn's post hoc test).

Genetic Characterization

The primary goal of performing phylogenetic analyses and NCBI BLAST searches of the field isolates was to genetically characterize them for identification. Many entomopathogenic fungal genera are still under major revisions. For example, the genus *Isaria*, formerly known as *Paecilomyces*, was recently revised to *Cordyceps* and includes many new taxa that were not formerly classified as such (Kepler et al. 2017). In constructing the phylogenetic tree for the *Beauveria* isolates, the data from Rehner et al. (2011) was used in large part as a reference, given that this report is one of the few, if not only fully exhaustive characterization of the genus *Beauveria*. Four of the five loci referenced in the Rehner et al. 2011 were used to characterize the isolates and construct a phylogenetic tree. The sequences found within Rehner et al. 2011 were sufficiently informative to identify our isolates to the level of species, i.e., *B. bassiana* for both isolates. That study demonstrated that each locus can correctly identify *Beauveria* sp. Therefore, multiple loci and concatenation of data were not necessary for proper classification in this study, as the separate and concatenated data sets resulted in the same identifications. The Bloc data set had the most informative sites and the phylogenetic tree was reported.

Table 2. NCBI BLAST search results showing the highest identity percentage matches and their accession numbers. Alternative matches that had similar percentage similarities were also shown

Isolate code	Locus	BLAST results	Query cover	Max identity (%)	NCBI accession no.
ACP18001	Bloc	*Beauveria bassiana* isolate SASRI BWR1	87%	95.96%	MZ380362
ACP18002		*Beauveria bassiana* RCEF4425	99%	96.83%	MZ380357
ACP18001	EF1-α	*Beauveria bassiana* strain CHE-CNRCB 161	100%	99.31%	MZ380358
ACP18002		*Beauveria bassiana* strain CHE-CNRCB 161	100%	100.00%	MZ380359
ACP18001	RPB1	*Beauveria bassiana* strain ARSEF 1811	100%	99.65%	MZ380360
ACP18002		*Beauveria bassiana* strain ARSEF 1811	100%	99.65%	MZ380361
ACP18001	RPB2a	*Beauveria bassiana* strain ARSEF 1811	100%	99.91%	MZ380363
ACP18002		*Beauveria bassiana* strain ARSEF 1478	100%	99.91%	MZ380365
ACP18001	RPB2b	*Beauveria bassiana* strain ARSEF 1811	100%	99.78%	MZ380364
ACP18002		*Beauveria bassiana* strain ARSEF 300	100%	99.89%	MZ380366

Table 3. Results of the successful Probit analyses indicating the *n*, slope and SE, LC_{50} (conidia/ml^{-1}), lower and upper 95% confidence intervals (95CI), and heterogeneity values for each bioassay

	Bioassay	*n*	Slope ± SE	LC_{50}	U95CI	L95CI	Heterogeneity
Primary Apopka97	BA 4	726	0.66 ± 0.137	8.72×10^{8}	4.79×10^{9}	3.82×10^{8}	0.55
	BA 7	515	0.66 ± 0.162	1.08×10^{9}	8.83×10^{9}	4.33×10^{8}	0.12
	BA 10	669	0.59 ± 0.129	7.47×10^{8}	3.62×10^{9}	3.44×10^{8}	0.38
	BA 18	378	0.44± 0.106	1.65×10^{8}	8.09×10^{8}	7.11×10^{7}	0.55
	BA 19	271	2.35 ± 0.480	5.32×10^{6}	6.97×10^{6}	3.94×10^{6}	0.02
	BA 21	473	0.36 ± 0.095	1.31×10^{8}	7.62×10^{8}	5.25×10^{7}	0.41
	BA 22	276	1.382 ± 0.190	1.39×10^{7}	4.22×10^{7}	1.73×10^{6}	0.82
	BA 23	840	0.336 ± 0.077	2.38×10^{9}	0	6.14×10^{8}	2.84
	BA 25	690	0.89 ± 0.090	3.69×10^{7}	1.33×10^{8}	7.29×10^{6}	1.95
	BA 26	270	0.52 ± 0.170	7.64×10^{4}	8.25×10^{5}	3.10	0.27
	BA 29	499	0.577 ± 0.096	4.03×10^{7}	7.80×10^{7}	2.06×10^{7}	0.00
	BA 30	363	2.643 ± 0.520	3.43×10^{6}	4.37×10^{6}	2.35×10^{6}	1.04
	BA 33	560	0.450 ± 0.098	5.01×10^{7}	1.20×10^{8}	2.01×10^{7}	0.00
	BA 38	251	1.330 ± 0.226	6.36×10^{6}	1.12×10^{7}	2.54×10^{6}	0.78
	BA 47	657	0.79 ± 0.139	5.12×10^{8}	1.32×10^{9}	2.90×10^{8}	0.92
	BA 51	531	0.66 ± 0.180	3.76×10^{9}	0	9.53×10^{8}	0.85
Primary ACP18001	BA 42	407	0.538 ± 0.155	1.46×10^{9}	4.11×10^{9}	0	0.49
	BA 44	436	2.793 ± 0.376	8.34×10^{7}	1.44×10^{8}	3.50×10^{7}	2.19
	BA 46	537	1.292 ± 0.141	1.59×10^{7}	5.69×10^{7}	1.73×10^{6}	2.20
	BA 47	676	0.752 ± 0.092	2.78×10^{7}	4.46×10^{7}	1.61×10^{7}	0.20
	BA 50	577	1.318 ± 0.160	4.99×10^{7}	6.83×10^{7}	3.43×10^{7}	0.46
	BA 51	689	0.885 ± 0.093	7.99×10^{7}	3.48×10^{8}	2.82×10^{7}	3.08
Primary ACP18002	BA 52	931	0.834 ± 0.077	4.96×10^{7}	1.06×10^{8}	2.48×10^{7}	1.84
	BA 53	470	0.621 ± 0.129	1.10×10^{8}	2.70×10^{8}	5.27×10^{7}	0.86
	BA 54	1171	1.164 ± 0.103	1.04×10^{8}	2.65×10^{8}	4.69×10^{7}	3.55
	BA 95	513	1.271 ± 0.145	6.80×10^{7}	9.32×10^{7}	4.90×10^{7}	0.53
	BA 97	141	0.780 ± 0.241	1.56×10^{8}	5.44×10^{8}	3.10×10^{7}	0.58
Secondary ACP18001	BA 56	442	1.339 ± 0.208	2.13×10^{8}	3.70×10^{8}	1.41×10^{8}	0.35
	BA 60	468	0.888 ± 0.173	2.15×10^{8}	4.22×10^{8}	1.33×10^{8}	0.42
	BA 61	680	1.098 ± 0.251	5.14×10^{8}	1.55×10^{9}	2.99×10^{8}	0.15
Secondary ACP18002	BA 57	583	0.711 ± 0.091	4.85×10^{7}	2.61×10^{8}	1.13×10^{7}	1.35
	BA 61	631	0.538 ± 0.089	1.96×10^{7}	$3.40E \times 10^{7}$	9.49×10^{6}	0.95

Table 4. Chi-square analysis results for both primary and secondary acquisition bioassays, with their respective degrees of freedom, *P* values, and critical Q values for each dose. Significant differences at each group comparison are indicated by an asterisk

						Q Values		
Exposure	Dose	χ^2 (2 df)	*P*	Strain	% Mortality	ACP18001 vs Apopka97	ACP18002 vs Apopka97	ACP18001 vs ACP18002
Primary	3.00×10^6	23.55	<0.001	Apopka97	19%	1.8	4.47*	6.27*
				ACP18001	16%			
				ACP18002	25%			
	1.00×10^7	96.08	<0.001	Apopka97	22%	7.93*	8.09*	0.17
				ACP18001	34%			
				ACP18002	34%			
	3.00×10^7	132.84	<0.001	Apopka97	26%	7.74*	11.08*	3.34
				ACP18001	39%			
				ACP18002	45%			
	1.00×10^8	296.15	<0.001	Apopka97	39%	14.80*	14.40*	0.40
				ACP18001	71%			
				ACP18002	70%			
	3.00×10^8	473.35	<0.001	Apopka97	35%	18.65*	18.06*	0.59
				ACP18001	60%			
				ACP18002	60%			
Secondary	3.00×10^6	23.55	<0.001	Apopka97	14%	0.91	5.82*	4.91
				ACP18001	15%			
				ACP18002	22%			
	1.00×10^7	122.06	<0.001	Apopka97	14%	4.81*	15.58*	10.77*
				ACP18001	20%			
				ACP18002	37%			
	3.00×10^7	97.23	<0.001	Apopka97	20%	1.04	13.81*	12.77*
				ACP18001	21%			
				ACP18002	42%			
	1.00×10^8	284.5	<0.001	Apopka97	18%	17.66*	23.01*	5.35*
				ACP18001	46%			
				ACP18002	56%			
	3.00×10^8	583.83	<0.001	Apopka97	17%	26.47*	33.98*	7.51*
				ACP18001	60%			
				ACP18002	73%			

*Significant difference, $P = 0.01$; Critical Q = 4.12.

Table 5. ANOVA results for the analysis performed on the thermal profile data. Comparisons shown for each field isolate in comparison to the standard, Apopka97 at each temperature point tested where $P < 0.05$ indicates a significant difference

Isolate vs Apopka97		Diff of means	*P*	q	*P*	*P* < 0.05
5°C	ACP18001	0.00776	3	3.122	0.080	Do not test
	ACP18002	0.00000222	3	0.000893	1.000	Do not test
10°C	ACP18001	0.0116	3	4.675	0.005	Yes
	ACP18002	0.00765	3	3.078	0.086	No
15°C	ACP18001	0.00717	3	2.885	0.114	No
	ACP18002	0.00432	3	1.738	0.442	No
20°C	ACP18001	0.0425	3	17.122	<0.001	Yes
	ACP18002	0.0501	3	20.184	<0.001	Yes
26°C	ACP18001	0.0325	3	13.072	<0.001	Yes
	ACP18002	0.00958	3	3.855	0.024	Yes
30°C	ACP18001	0.00356	3	1.433	0.572	No
	ACP18002	0.00871	3	3.508	0.043	Yes
32°C	ACP18001	0.0205	3	8.268	<0.001	Yes
	ACP18002	0.0419	3	16.852	<0.001	Yes
35°C	ACP18001	0.000	3	0.000	1.000	Do not test
	ACP18002	0.0000134	3	0.00537	1.000	No

Primary Acquisition Bioassays

It was evident from the number of bioassays with excessive heterogenity in probit analysis, and examination of the bar and whisker graph (Fig. 2) that there were issues with the heterogeneity in the mortality responses. As observed in Fig. 2, the mortality data for the isolates and Apopka97 was highly heterogenous, with many outliers present in the dataset. One likely explanation for this was the variability in spore deposition rates when using the sprayer

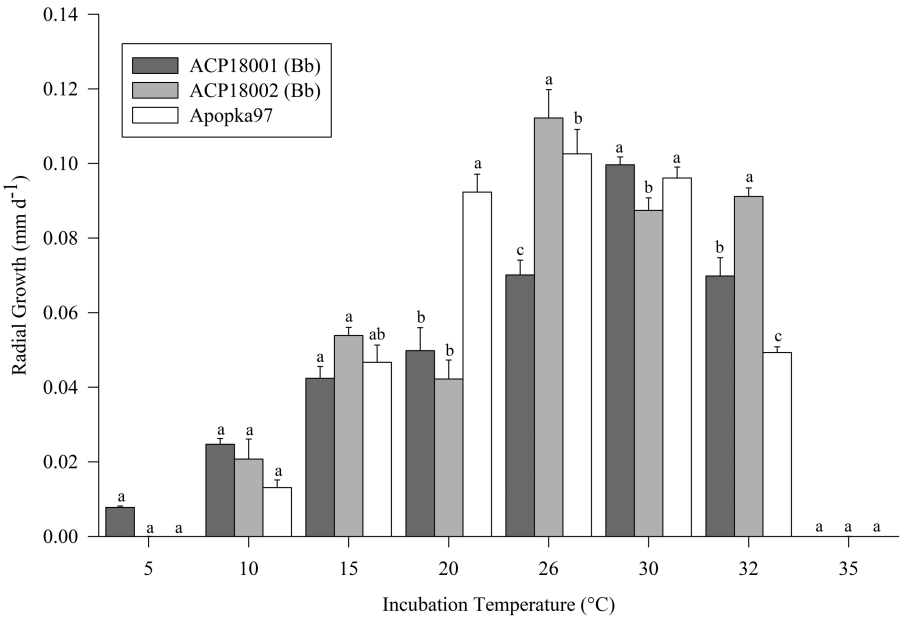

Fig. 3. Mean radial growth (mm d^{-1}) with standard error for field isolates, ACP18001, ACP18002, and Apopka97 at the tested constant temperatures. Significance ($P < 0.05$) is indicated by mean separation letters within each incubation temperature.

system, a factor possibly ignored in some published reports using a spray application method (Erdos et al. 2020). Errors during the actual sprays, preparations of spore dilutions, among other factors, can result in highly variable spore depositions. Some studies have circumvented this issue by counting deposited spores on agar cubes or cover slips, essentially producing a spores/mm^2 value for each spray and adjusting the actual dose sprayed accordingly (Wraight et al. 1998, 2010). In hindsight, this would have been a more efficient way of developing and performing the bioassay but the labor was beyond our capability. Nevertheless, we obtained sufficiently acceptable replicate bioassays to obtain information about the relative efficacy of the ACP isolates relative to Apopka97. In addition, chi-square analysis results on the lumped datasets for primary acquisition bioassays revealed significant differences at the 1×10^7, 3×10^7, 1×10^8, and 3×10^8 doses between ACP18001 and Apopka97 and at all doses for ACP18002 and Apopka97 (Table 4).

Based on the statistical analyses performed in addition to the data distribution from the bar and whisker graph, ACP18001 and ACP18002 mortality rates were significantly different, and greater than those of Apopka97. Further studies with an optimized bioassay protocol could possibly provide further differentiation in the performance of these insect pathogens.

Secondary Acquisition Bioassays

Secondary acquisition bioassays revealed that ACP18001 and ACP18002 were more effective at inducing mortalities in *D. citri* than Apopka97. The chi-square analysis results indicated there were significant differences at the 1×10^7, 1×10^8, and 3×10^8 doses for ACP18001 vs Apopka97, and at all doses for ACP18002 vs Apopka97. Both field isolates induced greater levels of mortality than the standard at both primary and secondary acquisition bioassays. Results also indicated that ACP18002 significantly outperformed ACP18001 at almost all secondary acquisition doses.

Secondary acquisition bioassays assume that the target pest must come into contact with spores on a leaf surface in order to initiate infection. As could be seen in Fig. 2, the efficacy of the field isolates was robust in comparison with Apopka97. Our results also revealed mortality rates from secondary acquisition were comparable to those found in primary acquisition (direct spray), likely in part due to the persistence of spores on leaf surfaces where no UV radiation was present, in addition to the insect's feeding behaviors. These findings were supported by those reported by Behle (2006), wherein *Trichoplusia ni* larvae that were sprayed with *B. bassiana* spores on field-grown cabbage and bean plants showed similar mortalities to residual contact exposure bioassays on leaf disks. ACP18001 and ACP18002 were readily capable of infecting and killing *D. citri* by means of secondary acquisition of the spores during the insect's movement on the plant surface as well as direct application. Our findings suggest that these field isolates could be efficacious in operational use, where direct spore deposition conditions directly onto insect pests are not optimal.

There was a correlation between the dose and respective response for all strains, though it was more evident for the field isolates tested than for Apopka97. Future studies will include the testing of *D. citri* nymphs under primary acquisition bioassays to assess the extent to which ACP18001 and ACP18002 can induce mortality in these stages. There are several factors to be considered when testing these isolates, or any other fungi on nymphs. *Diaphorina citri* nymphs have five instars, lasting approximately 2.46 d (59 h), 2.13 d (51 h), 2.39 d (75 h), 2.73 d (66 h), and 5.04 d (121 h) between each instar, respectively (Nava et al. 2007). In contrast, *Beauveria* conidial germination and penetration through the insect cuticle can take 18–24 h (Maistrou et al. 2020). As such, a critical aspect impacting the capacity to which entomopathogenic fungi can kill *D. citri* nymphs is the speed with which it can penetrate the insect cuticle versus proximity in time to the next molt. A study by Yinquan et al. (2003), found that green peach aphid, *Myzus persicae* nymph mortality was significantly impacted by timing of inoculation and ecdysis, wherein nymphs that were inoculated early on in their molting process exhibited higher mortality rates than those closer to a molt. A similar study by Reingold et al. (2021) demonstrated that the variation in mortality of *M. persicae* nymphs induced by two different *Metarhizium brunneum* strains was in part due to the speed of conidial adhesion and haemocoel colonization of the insect.

The combined results of these studies demonstrated that nymphal ecdysis was an effective defense mechanism against fungal infection, and that finding ways to circumvent this, as was exemplified by the faster disease progression of the two *M. brunneum* strains, could be the key to higher nymph mortality. In addition, chemical pesticides like Neem oil have been extensively studied and found to induce disruptive damage in nymphs and adults of various insects, leading to prolonged developmental periods, early mortality, reduced fecundity, among others (Kraiss and Cullen 2008, Ahmad et al. 2015, Martinez and Van Emden 2001). A combination of a virulent entomopathogenic fungus with rapid germination and penetration and a Neem oil adjuvant that could extend a nymph's time to next ecdysis could prove to be an effective combination for the control of *D. citri* nymphs. As such, testing field isolates on nymphs would be crucial to further characterize the extent to which these entomopathogenic fungi could induce mortalities in the various life stages of *D. citri*.

The fact that Apopka97 is a currently established mycoinsecticide, and that ACP18001 and ACP18002 performed comparable to or better than this strain under several circumstances indicate these two newly identified strains could be considered viable alternative candidates for use in the management of *D. citri* and potentially other pests.

Thermal Profiles

Temperature can be a major factor limiting the efficacy of a fungus. The thermal profile of fungal isolates is an indirect metric of an isolate's fitness at a given temperature point, with radial colony growth on an agar medium reflecting vegetative growth within an insect. The slope of linear regressions of the isolates' radial growths was used to identify their optimal growth rates, as well as determine whether the isolates grew at rates greater than the standard, Apopka97. The results of this analysis showed that there were some significant differences in the growth rates of the isolates in comparison to Apopka97. For example, there was a significant difference between ACP18001 and Apopka97 at the 10°C and 32°C temperatures ($P < 0.05$). Significant differences were also identified for ACP18002 at the 26°C and 32°C temperatures ($P < 0.001$).

Our study indicates that the isolates found in the *D. citri* population were better suited for the LRGV environment, specifically at higher temperatures than Apopka97, given the high temperatures and humidity found in the LRGV throughout most of the year (Ghazanfar et al. 2020). Apopka97 was capable of producing growth comparable to the field isolates at several of the temperature points, but its growth rate tapered off significantly at the higher temperature range while the two field isolates continued to grow at significantly greater rates. These differences infer that ACP18001 and ACP18002 could be more efficacious than Apopka97 under LRGV conditions. Persistence of either *Beauveria* isolate in the face of UV could be an adverse factor. UV tolerance of these fungi remains to be measured.

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