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Increased *Hirsutella citriformis* Conidia Shelf Life in *Acacia* and *Hirsutella* Gum Formulations

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Abstract: Biological control by beneficial microorganisms is known to significantly reduce the effect of pests on crops yield. Among the biocontrol strategies is the use of entomopathogenic fungi such as *Hirsutella citriformis*, which has been applied to infect and kill hemipteran insect pests, including *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) and *Bactericera cockerelli* Sulc. (Hemiptera: Triozidae). These biological agents are applied in the form of conidia that are often combined with other inert materials to facilitate application, protect conidia, and improve their shelf life. The aim of this study was to implement strategies for developing formulations to increase conidia shelf life. We evaluated gum produced from one strain and conidia from two different *H. citriformis* strains. Conidia were formulated by evaluating different concentrations of *Acacia* and *Hirsutella* gums to enhance conidia viability during storage at 4 °C or 25 °C. Results indicated that formulations maintained conidia viability for at least 90 d after storage at 25 °C (≥70% viability) and at least 120 d after storage at 4 °C, which was significantly ($p \leq 0.05$) higher than that of the control, without observing changes in pH values. We also demonstrated 100% formulation purity from days 0 to 120, among all treatments. In conclusion, evaluated formulations maintained *H. citriformis* conidia viability for at least three months, when stored at 4 °C.

Keywords: biocontrol; *Acacia* gum; *Hirsutella* gum; entomopathogenic fungi; bioinsectides; shelf life; conidia formulation

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1. Introduction

Chemical pesticides currently account for 95% of the global pesticide market and prevent about 50% of crop losses, which have not decreased in the last decades. Reports of the undesirable effects of chemical pesticides on animal and human health have prompted the search for alternatives to chemical pesticides [1,2].

Biological control of insect pests reduces harmful chemicals and pests-associated crop yield loss by using beneficial organisms, including insects, plants, or microorganisms. One of the most relevant biological control strategies under development are biopesticides, involving formulations based on entomopathogenic bacteria, fungi, or viruses [3,4].

Entomopathogenic fungi are commonly used as biopesticides to control aphids, ticks, and other insect plague populations, affecting plants and animals [5]. Their application is in the form of conidia, which are commonly combined with other inert materials for protection from environmental changes and stabilization during storage [6]. Some polymers used as adherents in formulations have a dual purpose. For example, arabic gum (*Acacia* gum) is used as an emulsifier for safer and effective bioactive components delivery after an application [7] and to encapsulate entomopathogenic fungi [8]. It has also been reported that *Hirsutella* produces a protective exopolysaccharide against desiccation [9,10].

Among entomopathogenic fungi, *Hirsutella* is one of the most abundant and important fungal genera for pest insect control in the field. It includes about 90 species infecting and parasitizing a wide variety of invertebrates such as mites and insects, many of which are considered pests of economic importance [11]. In this regard, *Hirsutella citriformis* Speare is the only entomopathogenic fungus involved in *Diaphorina citri* Kuwayama natural epizootics, allowing for its dissemination [12–14]. *H. citriformis* has been isolated from *Diaphorina citri* carcasses in Mexican citrus localities [15–17], which has allowed for the identification of several strains of interest, as possible biocontrol agents [18]. It is a difficult-to-grow, complex fungus with a limited shelf life and well-known biological control potential against *Diaphorina citri*, which develops a low spore amount compared with other entomopathogenic fungi [19].

H. citriformis has been applied to control *Diaphorina citri* Kuwayama and *Bactericera cockerelli* Sulc. [13,15], which are *Candidatus* Liberibacter spp. bacterium vectors. This bacterium is associated with several diseases in tomato, chili, and potato and is the causing agent of the Huanglongbing disease (HLB) [18,20]. The main control method against *D. citri* and *B. cockerelli* is based on chemical insecticides. However, when the resistance of an insect pest to insecticides increases, the insecticide becomes ineffective and may also confer cross-resistance to other related chemical compounds [21,22]. For the biocontrol of such insects, high-efficacy formulations are based on entomopathogenic fungi, such as *Metarhizium robertsii* (formerly known as *Metarhizium anisopliae*) (Metchnikoff) Sorokin (1883), *Isaria fumosoroseus* Wize, and *Beauveria bassiana* (Bals. –Criv.) Vuill. [23,24]. The aim of the present study was to increase *H. citriformis* conidial viability, using formulations containing *Acacia* and *H. citriformis* gums as inert materials to improve the formulated conidia shelf life and support their stability for at least three months.

2. Material and Methods

2.1. Fungi

Hirsutella citriformis isolates (Table 1) were kept in the Departamento de Microbiología e Inmunología in the Facultad de Ciencias Biológicas (FCB) at Universidad Autónoma de Nuevo León (UANL), México. They were grown in potato dextrose agar (Difco Laboratories, Sparks, MD, USA), containing 1% yeast extract (PDAY) (Difco Laboratories) at 25 ± 2 °C and a relative humidity of ~80% [18].

Table 1. *Hirsutella citriformis* isolates.

H. citriformis Isolate	Isolation Location (MX)	Coordinates	Sampling Date	Host Plant
OP-Hir-3	Huimanguillo, Tabasco	17°51′14″ N, 93°25′13″ W	May 2018	Valence orange
OP-Hir-9	Othón P. Blanco, Quintana Roo	18°32′24″ N, 88°18′37″ W	August 2018	Persse lemon
OP-Hir-10	Xtepén, Uman, Yucatán	20°49′25″ N, 89°44′29″ W	November 2018	*Murraya paniculata*

2.2. Hirsutella Citriformis Gum Production

We produced gum from *H. citriformis* strain OP-Hir-9, which was cultured in 1000 mL flasks with 250 mL of potato dextrose broth (Difco Laboratories), containing 1% yeast extract (PDBY) (Difco Laboratories). Culture media were inoculated with 3 cm^2 agar-grown *H. citriformis*, and flasks were incubated for 14 d at 25 ± 2 °C under agitation at 180 rpm, after which the flask content was centrifuged for 5 min at 10,000× *g* rpm for gum separation. Supernatant was then collected in a beaker, and isopropanol:gum at a 3:1 ratio *(v/v)* was kept without shaking for 6 h at 25 ± 2 °C. Next, gum was dried in an infrared balance (AD-4715; A&D Weighing Co., Milpitas, CA, USA) at 121 °C to determine dry weight, and dried gum was crushed in a mortar for storage, until use.

2.3. Conidia Production by Biphasic Hirsutella citriformis Culture

Conidia production by biphasic culturing was performed as previously reported [25], using oat grains for solid fermentation, with some modifications. The first phase consisted

of a culture on PDAY in a Petri dish. Produced conidia were used as an inoculum for the second phase of the oat culture. Conidia were collected by adding 10 mL of sterile 0.85% saline solution to aerial mycelium, using a bacteriological loop. Collected conidia were then homogenized by stirring on a vortex for 5 min, and the inoculum was adjusted to 1×10^6 conidia/mL. For solid fermentation on oat grains, we used high-density poly paper bags (two kilograms capacity). Oat grains (200 g) were washed three times to remove foreign particles and soaked for 24 h in 400 mL of purified water (Laboratorios Monterrey, S.A. de C.V. Monterrey, Nuevo León, México). Purified grade water was obtained by a multimedia filter, an activated carbon filter, reverse osmosis, ozonation, and ultraviolet light, and was selected instead of distilled water for its higher content of minerals. Oats were then mixed with 400 mg of oxytetracycline (Forrajera San Carlos, Ciudad Victoria, Tamaulipas, México) to avoid bacterial growth during the 24 h of soaking period.

Next, water was drained, and 4% wheat bran was added and sterilized twice in a 24 h period at 121 °C and 15 lb pressure for 30 min. After the second sterilization, and when oat/wheat bran temperature reached a temperature of 24 ± 2 °C, 16 mL of a suspension containing 1×10^6 conidia/g was added as an inoculum, which was homogenized with a sterile spatula. Containers with *H. citriformis*-inoculated oat/wheat bran were incubated at 25 ± 2 °C for 21 d.

Conidia were then mechanically detached from the substrate, using a sterile spatula, in 250 mL of sterile distilled water and concentrated by centrifugation (Thermo Fisher Scientific, Waltham, MA, USA) at 10,000 rpm for 10 min, using 50 mL conical tubes. Supernatant was removed, keeping 5 mL to 10 mL of the precipitate from each conical tube, after which conidia were quantified in a Neubauer chamber and adjusted to 1×10^7 conidia/mL with distilled water.

2.4. Effect of Formulation Ingredients on Hirsutella citriformis Conidia Viability

To evaluate each formulation ingredient's effect on conidia viability, *H. citriformis* conidia (1×10^7 conidia/mL) were cultured on PDAY medium plus 3% liquid vegetable oil (*v/v*) or 3% vegetable oil powder (*w/v*). We then determined conidia viability at least three times, as previously reported [25]. We also evaluated *Hirsutella citriformis* conidia viability, after adding 0.1% to 0.7% (*w/v*) of *Acacia* and 0.1% to 0.5% *(w/v)* of *Hirsutella* gums at 25 ± 3 °C or 4 ± 1 °C for 30 d. Treatments resulting in cytotoxicity at 30 d were discarded.

2.5. Formulations Preparation

Formulations were prepared as oil-in-gum emulsions, using conidia from OP-Hir-3 (isolated from Huimanguillo, Tabasco, México) and OP-Hir-10 (isolated from Xtepén, Uman, Yucatán, México) strains. Four different formulations were produced by combining different *Acacia* and *Hirsutella* gum concentrations (Table 2) plus 3% vegetable oil powder. For this, emulsions were autoclaved for 15 min at 121 °C, under 15 lb of pressure. The emulsion ingredients were homogenized by stirring on a vortex at speed 5, and after cooling at 24 ± 2 °C, *H. citriformis* conidia from selected strains were added at 1×10^7 conidia/mL (final concentration) [26].

2.6. Formulations' Shelf Life

We determined *Hirsutella citriformis* formulations' shelf life after storing at 25 ± 2 °C and 4 ± 1 °C. Evaluations were performed in triplicate at days 0, 30, 60, 90, 120, and 150 by counting germinated conidia on PDAY (72 h after sowing) [27].

2.7. Effect of pH on Formulated Conidia Stability

To determine the effect of pH changes on *Hirsutella citriformis* formulated conidia stability, samples from each formulation (Table 2) were mixed at a 1:10 ratio (*w/v*) with sterile distilled water, homogenized, and left to stand for one hour. The pH was determined from three different samples of each formulation, using a previously calibrated potentiometer. The pH evaluation of each sample was performed at least twice [28].

Table 2. *Hirsutella citriformis* conidia formulation ingredients.

Strain	*Acacia* Gum	*Hirsutella* Gum	Formulation Code
OP-Hir-3	0.50%	-	FH3-1
	-	0.50%	FH3-2
	0.30%	0.20%	FH3-3
	0.20%	0.30%	FH3-4
Control	Water		FH3 Control
OP-Hir-10	0.50%	-	FH10-1
	-	0.50%	FH10-2
	0.30%	0.20%	FH10-3
	0.20%	0.30%	FH10-4
Control	Water		FH10 Control

2.8. Purity Test

To evaluate the formulation purity after storage, PDAY were inoculated by extension with 100 µL of each formulation after storage and incubated at 25 ± 2 °C for 8 d. Evaluation was performed at least twice. In the event of the presence of other microorganisms, it was considered as a contaminated formulation. The contamination percentage [29] was determined using the following formula:

$$\%\ Sample\ purity = \frac{Number\ of\ colonies\ of\ interest}{Total\ colonies\ number} \times 100$$

2.9. Statistical Analysis

Results from different evaluations, except for storage temperatures comparison, were subjected to analysis of variance (ANOVA) (GraphPad Prism version 6.0; San Diego, CA, USA). Means were compared by the Tukey's test (α = 0.05%), using the software IBM SPSS Statistics Version 21 (SPSS, Inc., Chicago, IL, USA).

Differences in conidia viability after formulations storage at different temperatures were analyzed by the Student *t* test, where we compared conidia germination percentages among the formulation treatments after 120 d.

3. Results

3.1. Effect of Formulation Ingredients on Conidia Viability

Hirsutella citriformis conidia showed a viability of 79.7 ± 7.04% and 77 ± 3.6% after exposure to vegetable oil powder or liquid vegetable oil, respectively, as compared with the control (70.7 ± 3.025% viability) ($F_{2,6}$ = 2.65; $p \geq 0.05$). We discarded liquid vegetable oil to continue with the formulation elaboration, because it delayed conidia germination for up to 10 d.

3.2. Evaluation of Gum Concentration Regarding Conidia Viability

We evaluated *Hirsutella citriformis* OP-Hir-10 strain conidia viability at different concentrations of *Acacia* (from 0.1% to 0.7%) or *Hirsutella* (from 0.1% to 0.5%) gums and at 25 ± 3 °C and 4 ± 1 °C. Results showed that at 0 d of storage, conidia viability in all of the treatments and the control ranged from 90.44% to 93.49%. After 15 d, we observed 9.62% viability reduction (the lowest viability) with the 0.25% *Acacia* formulation stored at 4 °C (81.84 ± 2.29% viability), as compared with the control, whose viability was reduced by 19.85% (Table 3).

Table 3. Effect of gum and temperature on *Hirsutella citriformis* OP-Hir-10 strain conidia germination after storage for up to 30 d [1].

Treatments	Storage Period at 4 ± 1 °C			Storage Period at 25 ± 3 °C		
	0 d	15 d	30 d	0 d	15 d	30 d
0.1% *Acacia*	92.96 ± 0.26 a	87.34 ± 0.64 a	87.77 ± 1.1 a	92.09 ± 0.73 a	87.99 ± 0.69 a	64.0 ± 1.62 a
0.2% *Acacia*	90.29 ± 1.36 a	86.26 ± 0.84 a	90.28 ± 0.88 a	91.43 ± 1.57 a	87.47 ± 1.98 a	61.36 ± 2.57 a
0.25% *Acacia*	90.56 ± 0.99 a	81.84 ± 2.29 a	85.96 ± 1.29 a	90.94 ± 1.27 a	86.49 ± 1.16 a	61.03 ± 3.93 a
0.5% *Acacia*	90.69 ± 1.38 a	90.15 ± 1.25 a	89.63 ± 2.65 a	92.86 ± 0.87 a	84.31 ± 0.64 a	67.43 ± 2.59 a
0.7% *Acacia*	90.44 ± 1.51 a	88.26 ± 1.91 a	90.68 ± 0.85 a	93.5 ± 0.78 a	88.62 ± 1.08 a	66.32 ± 2.05 a
0.1% *Hirsutella*	92.47 ± 1.16 a	87.99 ± 0.84 a	80.30 ± 0.85 a	94.58 ± 0.05 a	81.93 ± 0.9 a	60.72 ± 1.48 a
0.2% *Hirsutella*	94.29 ± 0.32 a	90.47 ± 2.73 a	86.40 ± 2.14 a	93.49 ± 1.29 a	83.12 ± 1.74 a	59.44 ± 2.91 a
0.25% *Hirsutella*	92.5 ± 0.88 a	90.38 ± 1.5 a	86.91 ± 2.07 a	89.09 ± 0.31 a	86.99 ± 1.24 a	68.76 ± 1.83 a
0.5% *Hirsutella*	93.17 ± 1.13 a	91.31 ± 0.99 a	93.20 ± 0.8 a	91.95 ± 1.36 a	87.86 ± 1.65 a	72.13 ± 0.9 a
Control	92.68 ± 0.32 a	74.28 ± 1.63 b	53.36 ± 3.58 b	91.59 ± 0.73 a	77.5 ± 1.49 b	31.34 ± 3.47 b

[1] Values followed by the same letter indicate no significant difference (Tukey $\alpha = 0.05$). Data represent the means ± SEM of triplicate determinations from three independent experiments.

3.3. Formulated Conidia Shelf Life

Shelf life analysis results showed that formulated conidia from OP-Hir-3 and OP-Hir-10 *Hirsutella citriformis* strains maintained a high viability, as compared with the unformulated conidia, after storage at 25 ± 3 °C for up to 120 d (Tables 4 and 5).

Table 4. Effect of formulations and storage periods at 25 ± 3 °C on *Hirsutella citriformis* OP-Hir-3 strain conidia germination percentage [1].

	Germination (Mean ± SEM)				
Days	FH3-1	FH3-2	FH3-3	FH3-4	FH3-Control
0	95.5 ± 0.41 a	95.8 ± 0.69 a	96.2 ± 0.53 a	94.7 ± 0.61 a	91.8 ± 0.86 b
30	94.0 ± 0.37 a	93.7 ± 0.41 a	92.8 ± 0.86 ab	92 ± 0.53 b	90.2 ± 0.53 c
60	89.0 ± 0.98 a	87.7 ± 2.24 a	87.8 ± 1.63 a	87.0 ± 1.67 a	83.7 ± 1.67 a
90	77.7 ± 1.67 ab	79.2 ± 1.31 ab	80.3 ± 0.73 a	76.0 ±2.12 b	68.3 ± 1.22 c
120	61.3 ± 3.34 a	64.0 ± 2.53 a	67.5 ± 1.63 a	66.7 ±2.28 a	60.3 ± 0.76 a

[1] Values followed by the same letter indicate no significant difference (Tukey $\alpha = 0.05$). Data represent means ± SEM of triplicate determinations from three independent experiments.

Table 5. Effect of formulations and storage periods at 25 ± 3 °C on *Hirsutella citriformis* OP-Hir-10 strain conidia germination percentage [1].

	Germination (Mean ± SEM)				
Days	FH10-1	FH10-2	FH10-3	FH10-4	FH10-Control
0	95.7 ± 0.21 a	95.0 ± 0.36 a	96.0 ± 0.36 a	95.7 ± 0.56 a	93.7 ± 0.21 b
30	93.2 ± 0.7 a	93.8 ± 0.48 a	94.0 ± 0.36 a	93.2 ± 0.6 a	92.0 ± 0.51 a
60	88.7 ± 1.88 a	86.0 ± 0.58 b	85.5 ± 0.43 b	85.5 ± 0.76 b	81.7 ± 1.14 c
90	81.3 ± 0.42 a	81.3 ± 0.42 a	80.8 ± 0.7 a	82.0 ± 0.63 a	69.2 ± 0.48 b
120	70.0 ±0.51 ab	69.8 ± 0.75 b	72.3 ±0.33 a	70.2 ± 0.6 ab	56.0 ± 1.53 c

[1] Values followed by the same letter indicate no significant difference (Tukey $\alpha = 0.05$). Data represent means ± SEM of triplicate determinations from three independent experiments.

After 90 d of storage, conidia from the FH3-3 formulation of the OP-Hir-3 strain achieved the highest and most significant ($F_{4,25} = 10.19$; $p \leq 0.05$) percentage of viability, as compared with other formulations and the control, whereas conidia from the FH10-4 formulation of the OP-Hir-10 strain reached a high but not significant ($F_{4,25} = 101.43$; $p \leq 0.05$) germination percentage, as compared with other treatments, but it was significant as compared with the control.

Hirsutella citriformis conidia viability of OP-Hir-3 (Table 6) and OP-Hir-10 (Table 7) strains was similar among all formulation treatments, after storage at 4 ± 1 °C. Only the control showed a significant ($F_{4,25}$ = 18.18; $p \leq 0.05$) and ($F_{4,25}$ = 118.2; $p \leq 0.05$) difference, respectively. At 120 d of storage, conidia viability of OP-Hir-3 and OP-Hir-10 strains decreased by 16.5% and 17.3%, respectively. Viability reduction of conidia control ranged from 23.16% to 34.3%.

Table 6. Effect of formulations and storage periods at 4 ± 1 °C on *Hirsutella citriformis* OP-Hir-3 strain conidia germination percentage [1].

	Germination (Mean ± SEM)				
Days	**FH3-1**	**FH3-2**	**FH3-3**	**FH3-4**	**FH3-Control**
0	95.50 ± 0.43 a	95.83 ± 0.7 a	96.17 ± 0.48 a	94.67 ± 0.71 a	91.83 ± 0.87 b
30	93.50 ± 0.76 a	94.50 ± 0.43 a	94.83 ± 0.48 a	93.50 ± 0.56 a	90.83 ± 0.48 b
60	92.83 ± 0.70 a	92.50 ± 1.06 a	92.33 ± 0.96 a	92.83 ± 0.70 a	85.33 ± 1.38 b
90	85.50 ± 1.33 a	84.83 ± 1.11 a	87.17 ± 0.83 a	85.50 ± 0.76 a	70.67 ± 1.28 b
120	79.50 ± 1.23 a	81.17 ± 0.60 a	82.00 ± 0.51 a	80.67 ± 0.56 a	68.67 ± 2.43 b

[1] Values followed by the same letter indicate no significant difference (Tukey α = 0.05). Data represent means ± SEM of triplicate determinations from three independent experiments.

Table 7. Effect of formulations and storage periods at 4 ± 1 °C on *Hirsutella citriformis* OP-Hir-10 strain conidia germination percentage [1].

	Germination (Mean ± SEM)				
Days	**FH10-1**	**FH10-2**	**FH10-3**	**FH10-4**	**FH10-Control**
0	95.67 ± 0.21 a	95.00 ± 0.36 a	96.00 ± 0.36 a	95.67 ± 0.56 a	93.67 ± 0.21 b
30	94.00 ± 0.26 a	94.33 ± 0.42 a	93.83 ± 0.31 a	93.83 ± 0.31 a	92.67 ± 0.49 b
60	92.00 ± 0.58 a	91.83 ± 0.48 a	91.67 ± 0.56 a	90.67 ± 0.49 a	85.67 ± 0.49 b
90	87.83 ± 0.48 a	87.33 ± 0.56 a	87.83 ± 0.75 a	86.67 ± 0.49 a	72.33 ± 0.33 b
120	80.67 ± 0.62 a	80.17 ± 0.91 a	82.17 ± 0.40 a	80.50 ± 0.67 a	61.50 ± 1.17 b

[1] Values followed by the same letter indicate no significant difference (Tukey α = 0.05). Data represent means ± SEM of triplicate determinations from three independent experiments.

After evaluating if a higher temperature decreases conidia viability after 90 d of storage, results indicated a significantly lower viability of conidia in those formulations stored at higher temperatures. This was observed with OP-Hir-3 (Tabasco; Figure 1A) ($F_{4,11}$ = 3.356; $p \leq 0.05$) and OP-Hir-10 (Yucatán; Figure 1B) ($F_{4,11}$ = 3.356; $p \leq 0.05$) strains, showing that storage at 4 ± 1 °C decreased the loss of viability of the active ingredient.

3.4. Effect of pH on Formulated Conidia Stability

After analyzing the pH values of the OP-Hir-3 (Tabasco) strain-formulated conidia, we observed that treatments stored at 25 ± 3 °C (Figure 2A) remained stable after 90 d, showing no significant ($F_{1,11}$ = 4.844; $p \geq 0.05$) difference as compared with the 0 d sample, except for the FH3-3 treatment. The FH3-3 treatment was formulated with both gums, which evidenced significant ($F_{1,11}$ = 4.844; $p \leq 0.05$) differences between 0 d and 90 d. Furthermore, treatments showed significant ($F_{1,11}$ = 4.844; $p \leq 0.05$) differences between 0 d and 120 d, except for the control, which evidenced no significant difference ($F_{1,11}$ = 4.844; $p \geq 0.05$).

All treatments remained stable after 90 d of storage at 4 ± 1 °C (Figure 2B), as there was no significant ($F_{1,11}$ = 4.84; $p \geq 0.05$) difference in pH values, compared with the initial sample. However, treatments presented significant differences between days 0 and 120, where pH values were lower, unlike the control, which did not show significant ($F_{1,11}$ = 4.844; $p \geq 0.05$) differences after storage.

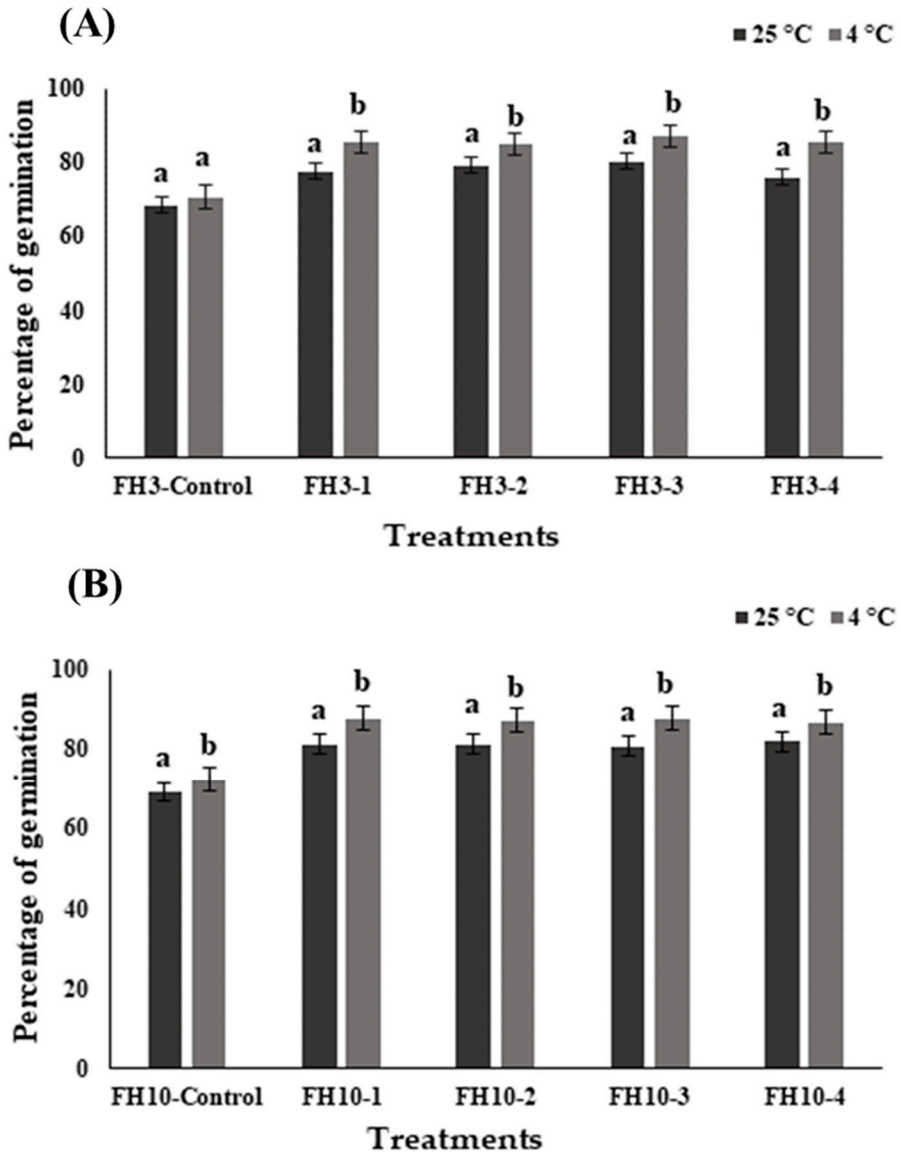

Figure 1. Hirsutella citriformis conidia shelf life after storage for 90 d at 25 ± 3 °C and 4 ± 1 °C. (**A**) OP-Hir-3 (Tabasco) and (**B**) OP-Hir-10 (Yucatán). Bars represent means + SD of triplicate determinations from three independent experiments. Different letters indicate significant differences at the 0.05 level (Student *t* test).

Furthermore, treatments of the OP-Hir-10 strain stored at 25 ± 3 °C remained stable after 90 d ($F_{1,11}$ = 4.844; $p \geq 0.05$), as compared with the 0 d sample, except for the treatments FH3-2 and FH3-4, which were significantly ($F_{1,11}$ = 4.844; $p \leq 0.05$) different after 0 d and 90 d of storage. All treatments, including the control, showed significantly ($F_{1,11}$ = 4.844; $p \geq 0.05$) lower pH values after 0 d and 120 d of storage (Figure 2C).

We observed that the pH of treatments remained stable after 90 d of storage at 4 °C, as there was no significant ($F_{1,11}$ = 4.84; $p \geq 0.05$) difference, as compared with the sample evaluated on the initial day. However, all treatments showed significantly lower pH values between 0 d and 120 d, including the control (Figure 2D).

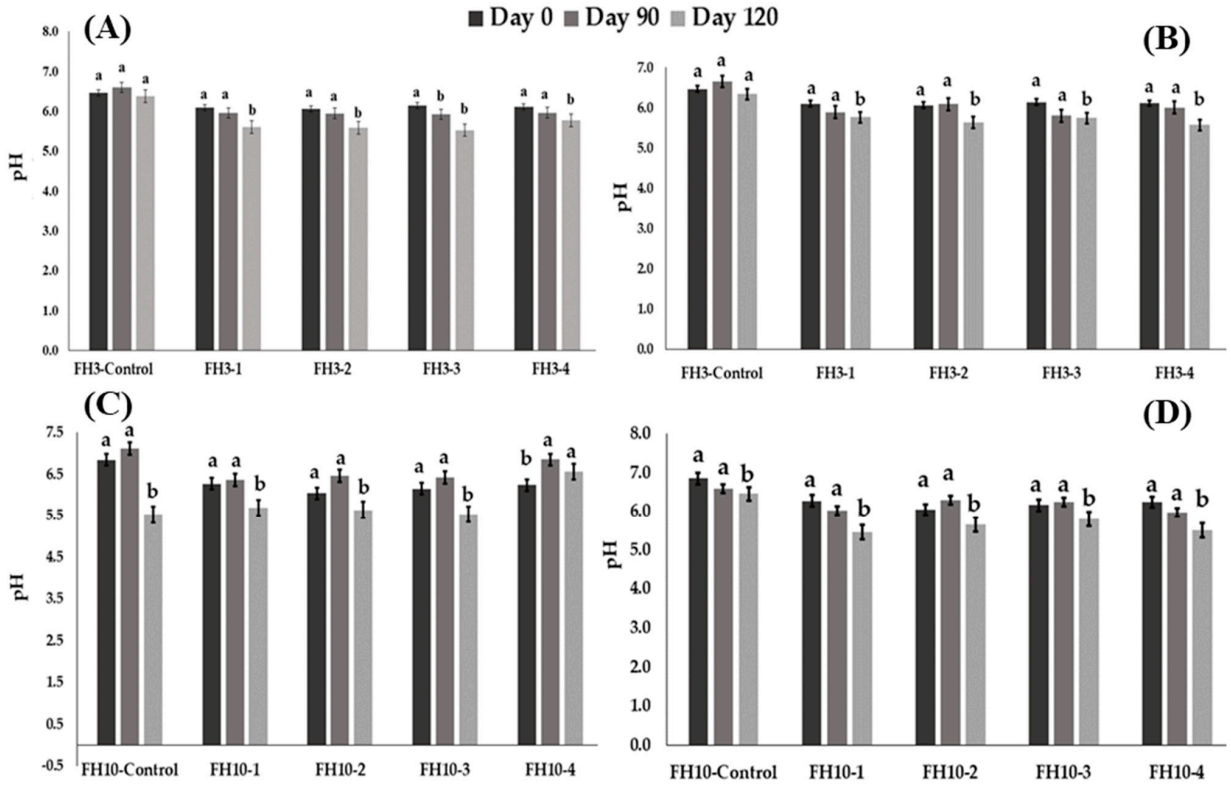

Figure 2. pH values of *Hirsutella citriformis*-formulated conidia after 0, 90, and 120 d of storage. (**A**) Strain OP-Hir-3 (Tabasco) at 25 ± 3 °C; (**B**) strain OP-Hir-3 (Tabasco) at 4 ± 1 °C; (**C**) strain OP-Hir-10 (Yucatán) at 25 ± 3 °C; and (**D**) OP-Hir-10 (Yucatán) strain at 4 ± 1 °C. Bars represent the means ± SD of triplicate determinations from three independent experiments. Different letters indicate significant ($p \leq 0.05$) differences within the same treatment (Day 0–Day 90; Day 0–Day 120) (Tukey's mean comparison analysis).

3.5. Formulation Purity Test

Based on purity evaluations, formulated conidia were not contaminated (*H. citriformis* conidia was only detected; Figure 3). Results indicated a purity of 100% from 0 d to 120 d of storage for all treatments at 25 ± 3 °C and at 4 ± 1 °C.

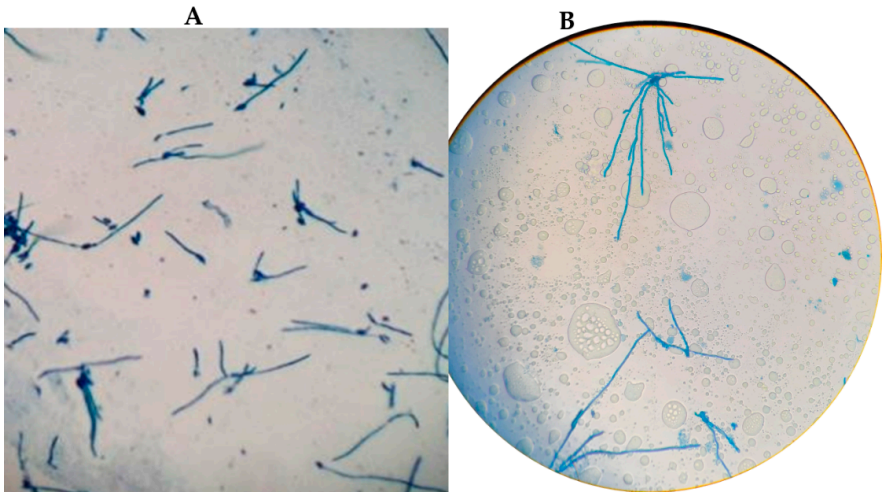

Figure 3. *Hirsutella citriformis* strain OP-Hir-3 (Tabasco) conidial germination stained with lactophenol-cotton blue dye and mounted for optical microscope observation (40×). (**A**) Unformulated conidia (control) and (**B**) formulated conidia.

4. Discussion

Biocontrol agents represent an alternative to chemical pesticides. They become natural enemies attacking pests, without affecting humans or animals. Entomopathogenic fungi are particularly important because of their pathogenicity routes, a variety of hosts, and their potential to control a wide range of pests. In this study, we observed that conidia viability and germination were not affected after analyzing conidia viability in response to formulation ingredients.

Some advantages of using oily formulations include the increase in the resistance to thermal changes, after evaluating different vegetable oils formulations, using the entomopathogenic fungus *Metarhizium robertsii* (Metchnikoff) Sorokin (1883), formerly known as *M. anisopliae* [30]. It has been reported that the use of oils in formulations favors spores' survival by maintaining their humidity [31]. However, we observed a delayed effect on *Hirsutella citriformis* mycelial growth in the presence of the maize-based liquid vegetable oil. The typical composition of corn oil is 11% palmitic, 2% stearic, 24.1% oleic, 61.9% linoleic, 0.7% linolenic, and to a lesser extent, other fatty acids (< 1%) [32]. A high concentration (3.9 mmol/L) of palmitic acid was shown to inhibit mycelial growth of fungi such as *Alternaria solani* (Cooke) Wint., *Colletotrichum lagenarium* (Pass.) Ellis & Halst. and *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen. Similarly, linoleic acid (2 mmol/L) was reported to reduce mycelium growth but oleic acid (3.2 mmol/L) did not inhibit mycelial growth of these fungi [33]. To date, studies on fatty acids antifungal mechanisms have focused on the inhibitory effect against human pathogenic fungi, whereas antifungal activities against entomopathogenic fungi are still unknown. Therefore, it can be inferred that fatty acids present in corn-based vegetable oils may interfere with *Hirsutella citriformis* mycelial growth.

Regarding conidia viability results, we showed that gums used in formulations at high concentrations reduced conidia viability at the two temperatures evaluated, in shelf life experiments. Number of viable conidia was initially calculated, inoculating a suspension of 1 cm^2 agar squares in Petri dishes. Conidia were incubated and the germination percentage was determined at least three times, thus assuring that conidia in the control were as viable as in all treatments. Nevertheless, each treatment was independently inoculated in Petri dishes. This may explain why the initial germination is different among treatments.

The formulation process may lead to an initial loss of viability, which is one of the disadvantages of using *Hirsutella* as a biological control agent, in addition to its slow growth and short lifetime [19]. This was shown in the germination at time 0 for both strains, where the germination percentage of the conidia was between 90.29% and 94.29% among treatments (Table 3). It was also observed that conidia germination in the untreated control was lower (viability between 91.8% and 93.7%), as compared with that of formulated treatments (Tables 4–7). However, by comparing this value with the initial assay, we did not observe significant differences. It is possible that formulation ingredients maintained conidia viability by protecting the mucilaginous layer that covers conidia, preventing their early germination in a nutrient-deficient medium (distilled water) [25]. This protective effect was observed with the addition of the vegetable oil in powder.

After testing *Trichoderma asperellum* Samuels, Lieckf & Nirenberg formulations at higher storage temperatures than those used in our study, it was observed that supersaturation of adherents on conidia completely inhibited their viability [34]. Due to the low levels of viability obtained with high concentrations of the gums used as adherents, it was necessary to make modifications by decreasing gum concentrations and achieving a higher stability of active ingredients, which allowed us to have a stable formula for up to one month. If we compared results obtained with our formulations with other reports, ours improved *H. citriformis* conidia stability [35,36]. This is a very important issue since *H. citriformis* conidia are very sensitive to temperature and humidity changes [18]. After evaluating different gums as adherents (bovine gelatin, lemon pectin, modified corn starch) at low concentrations on *Beauveria bassiana* fungus, similar viability results to those reported by our research team after 15 d of storage were observed [37]. In field tests, Pérez-González

et al. [25] reported that after applying conidia from *Hirsutella citriformis* strain INIFAP-Hir-2, which were formulated with *Acacia* and *Hirsutella* gums, the highest *Diaphorina citri* mortality was achieved with *Hirsutella* gum (80.0%), followed by the *Hirsutella* gum without conidia (control) (57.8%). In the same trial, conidia formulated with *Acacia* gum caused 37.8% mortality, whereas *Acacia* gum and negative controls induced 9% mortality. Therefore, this may indicate that conidia formulations including *Hirsutella* gums improve the efficacy of the active ingredient for *D. citri* biocontrol.

The most effective formulations were those that were kept stored at a cold temperature (4 °C), which maintained conidia viability above 70% after 120 d of storage, as compared with those stored at 25 °C, which remained viable for up to 90 d. This may be due to the use of additives to protect the active ingredient. *Acacia* and *Hirsutella* gums and vegetable oil powder may also provide protection to conidia. Interactions between storage temperature and bioformulate ingredients determine conidia longevity [37]. In a formulation of *Beauveria bassiana* conidia, in the presence of hydrogenated rapeseed oil granules, we obtained 84.7% viability when stored at 25 °C, and 92.3% when stored at 4 °C, after 45 d [38]. Furthermore, it was reported that after evaluating the efficacy of various sugars as additives in *B. bassiana* formulations, the use of gums or polysaccharides as additives for the active ingredient formulation, evidenced a nutritive effect and survival increase [38]. Formulations containing glucose as an additive were optimum for fungi growth and germination. Regarding shelf-life analysis, results revealed a significant variation in conidia germination, showing higher germination values (92%) after six months of storage at 30 °C. Similarly, a significant effect on the survival rate of polysaccharide-encapsulated *Trichoderma harzianum* Rifai conidia was previously reported [39].

Several studies have discussed the various storage conditions' impact on conidial stability. In our study, storage of formulated conidia at 4 °C reduced conidia viability loss for up to 120 d, which agrees with previous reports, where *B. bassiana* was formulated using oily formulations and conidia, showing a higher germination percentage when stored at 4 °C than at room temperature [40]. This may be due to the cellular metabolism, since, at room temperature, conidia remain active, producing metabolites related to germination and consuming internal nutrients, which affects conidia viability and germination. At storage temperatures from 3 °C to 8 °C, fungal cells maintain a low metabolic rate and remain viable for prolonged periods of time [41,42]. Unformulated conidia had a lower shelf life compared with formulated treatments at 25 °C and 4 °C. These results indicate that the additives present in the treatments provide a protective effect on the active ingredient, improving its stability during the storage time.

Other studies have shown that pH influences the active ingredient during conidia germination and growth [43,44]. The pH tolerance ranges from 6.5 to 7.5 of other entomopathogenic fungi, such as *Trichoderma* spp. Persoon is known to favor its growth [45]. Other fungi have a wider pH tolerance range, such as *Beauveria bassiana*, whose reported pH tolerance values are between 5 and 13 [46]. Similarly, pH values reported for *Hirsutella* spp. Brandy growth and sporulation are between 6.0 and 9.2 [47] or between 5.5 and 7 [48]. In our study, even with differences in the stability of some treatments, the optimal range of pH was maintained for up to 90 d in all formulations in both storage conditions, indicating that pH in the aforementioned tolerance range did not affect conidia germination. In addition, formulations may contaminate if good production practices are not followed. However, it is known that formulations that have more than 90% purity show a greater stability and viability of the active ingredient [29]. This is because contaminants may reduce active ingredients' efficacy due to the potential inhibitory effects of secondary metabolites [29,49,50].

5. Conclusions

The aim of the present study was to demonstrate that *Hirsutella* conidia shelf life increases after formulation. Vegetable oil powder used to formulate conidia did not reduce *Hirsutella citriformis*' viability, since their germination was not affected. Similarly, *Hirsutella* gum may be used as an ingredient for increasing *Hirsutella* conidia shelf life, along with

vegetable oil powder. In addition, we observed that formulations maintained conidia viability for at least 90 d after storage at 25 °C and at least 120 d after storage at 4 °C. It was evidenced that under storage at 4 °C, *H. citriformis* conidia loss of viability decreased. All formulations evaluated were within the optimum range of pH at 4 °C and 25 °C for 90 d. pH of the formulated conidia, which prevailed between 5.5 and 7.5, allowed for conidia germination, and all treatments maintained conidial purity (100%) up to 120 d at both storage temperatures.

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References

1. De la Cruz, R.; Cruz, J.; De Jesús, M.; Torres, J.; Parra, R. Fungi-based biopesticides: Shelf-life preservation technologies used in commercial products. *J. Pest. Sci.* **2019**, *9*, 1003–1015. [CrossRef]
2. Silveira, M.; Aldana-Madrid, M.; Piri-Santana, J.; Valenzuela-Quintanar, A.; Jasa-Silveira, G. Plaguicidas agrícolas: Un marco de referencia para evaluar riesgos a la salud en comunidades rurales en el estado de Sonora, México. *Rev. Int. Contam. Ambient.* **2018**, *34*, 7–21. [CrossRef]
3. Flint, M.; Dreistadt, S. *Natural Enemies Handbook: The Illustrated Guide to Biological Pest Control*; Flint, M., Dreistadt, S., Eds.; University of California Press: Berkeley, CA, USA, 1998; ISBN 1-879906-4.
4. Rusch, A.; Valantin-Morison, M.; Sarthou, J.P.; Roger-Estrade, J. Biological control of insect pests in agroecosystems. *Adv. Agron.* **2010**, *109*, 219–259. [CrossRef]
5. Khan, S.; Guo, L.; Mamaiti, Y.; Mijit, M.; Qiu, D. Entomopathogenic Fungi as Microbial Biocontrol Agent. *Mol. Plant Breed.* **2012**, *7*, 63–79. [CrossRef]
6. Meikle, W.; Mercadier, G.; Holst, N.; Girod, V. Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on *Varroa mites* (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health. *Exp. Appl. Acarol.* **2008**, *46*, 105–117. [CrossRef]
7. Dhull, S.; Anju, M.; Kaushik, R.; Chawla, P. Application of gum Arabic in nanoemulsion for safe conveyance of bioactive components. In *Nanotechnology in the Life Sciences*; Prasad, R., Ed.; Springer Nature Switzerland AG: Cham, Switzerland, 2019; pp. 85–98. [CrossRef]
8. Tolun, A.; Altintas, Z.; Artik, N. Microencapsulation of grape polyphenols using maltodextrin and gum Arabic as two alternative coating materials: Development and characterization. *J. Biotechnol.* **2016**, *239*, 23–33. [CrossRef] [PubMed]
9. Nehad, E.A.; El-Shamy, A. Physiological studies on the production of exopolysaccharide by fungi. *Agric. Biol. J. N. Am.* **2010**, *6*, 1303–1308. [CrossRef]
10. Li, R.; Jiang, X.; Guan, H. Optimization of mycelium biomass and exopolysaccharides production by *Hirsutella* sp. in submerged fermentation and evaluation of exopolysaccharides antibacterial activity. *Afr. J. Biotechnol.* **2010**, *9*, 198–202.
11. Pérez-González, O.; Rodríguez-Guerra, R.; López-Arroyo, J.I.; Sandoval-Coronado, C.F.; Maldonado-Blanco, M.G. Effect of Mexican *Hirsutella citriformis* (Hypocreales: Ophiocordycipitaceae) strains on *Diaphorina citri* (Hemiptera: Liviidae) and the predators *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) and *Hippodamia convergens* (Coleoptera: Coccinellidae). *Fla. Entomol.* **2016**, *99*, 509–515. [CrossRef]
12. Subandiyah, S.; Nikon, N.; Sato, H.; Wagiman, F.; Tsuyumu, S.; Fakatsu, T. Isolation and characterization of two entomopathogenic fungi attacking *Diaphorina citri* (Homoptera, Psilloidea) in Indonesia. *Mycoscience* **2000**, *41*, 509–513. [CrossRef]

13. Meyer, J.M.; Hoy, M.A.; Boucias, D.G. Morphological and molecular characterization of a *Hirsutella* species infecting the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), in Florida. *J. Invertebr. Pathol.* **2007**, *95*, 101–109. [CrossRef] [PubMed]
14. Hall, D.G.; Hentz, M.G.; Meyer, J.M.; Kriss, A.B.; Gottwald, T.R.; Boucias, D.G. Observations on the entomopathogenic fungus *Hirsutella citriformis* attacking adult *Diaphorina citri* (Hemiptera: Psyllidae) in a managed citrus grove. *BioControl* **2012**, *57*, 663–675. [CrossRef]
15. Casique-Valdes, R.; Reyes-Martinez, A.Y.; Sanchez-Peña, S.R.; Bidochka, M.J.; Lopez-Arroyo, J.I. Pathogenicity of *Hirsutella citriformis* (Ascomycota: Cordycipitaceae) to *Diaphorina citri* (Hemiptera: Psyllidae) and *Bactericera cockerelli* (Hemiptera: Triozidae). *Fla. Entomol.* **2011**, *94*, 703–705. [CrossRef]
16. Pérez-Gonzalez, O.; Rodriguez-Villarreal, R.A.; Lopez-Arroyo, J.I.; Maldonado-Blanco, M.G.; Rodríguez-Guerra, R. Mexican strains of *Hirsutella* isolated from *Diaphorina citri* (Hemiptera: Liviidae): Morphologic and molecular characterization. *Fla. Entomol.* **2015**, *98*, 290–297. [CrossRef]
17. Suaste-Dzul, A.; Gallou, A.; Félix-Portillo, M.; Moreno-Carrillo, G.; Sánchez-González, J.; Palomares-Pérez, M.; Arredondo-Bernal, H. Seasonal incidence of '*Candidatus* Liberibacter asiaticus' (Rhizobiales: Rhizobiaceae) in *Diaphorina citri* (Hemiptera: Liviidae) in Colima, México. *Trop. Plant Pathol.* **2017**, *42*, 410–415. [CrossRef]
18. Pérez-González, O.; Gomez-Flores, R.; Tamez-Guerra, P. Insight into biological control potential of *Hirsutella citriformis* against Asian citrus psyllid as a vector of citrus Huanglongbing disease in America. *J. Fungi* **2022**, *8*, 573. [CrossRef] [PubMed]
19. Pérez-González, O.; Rodríguez-Guerra, R.; López-Arroyo, J.I.; Sandoval-Coronado, C.F.; Maldonado-Blanco, M.G. Radial growth, sporulation, and virulence of Mexican isolates of *Hirsutella citriformis* against *Diaphorina citri*. *Southwest. Entomol.* **2015**, *40*, 111–120. [CrossRef]
20. Melgoza, C.; Del Rosario, C.; López, J.; Hernández, L.; Velarde, S.; Garzón, J. Presencia de *Candidatus* Liberibacter solanacearum en *Bactericera cockerelli* Sulc asociada con enfermedades en tomate, chile y papa. *Rev. Mex. Cienc. Agríc.* **2018**, *9*, 499–509. [CrossRef]
21. Ramírez-Ahuja, M.; Rodríguez-Leyva, E.; Lomeli-Flores, J.; Torres-Ruiz, A.; Guzmán-Franco, A.W. Evaluating combined use of a parasitoid and a zoophytophagous bug for biological control of the potato psyllid, *Bactericera cockerelli*. *Biol. Control* **2017**, *106*, 9–15. [CrossRef]
22. Martínez-Carrillo, J. (Ed.) *Ficha técnica: Diaphorina citri Kuwayama, Psílido Asiático de los Cítricos*; Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), Unidad de Promoción y Vinculación—SENASICA: México City, México, 2009; pp. 10–12.
23. Saldarriaga, J.; D'Alessandro, C.; Conceschi, M.; Mascarin, G.; Delalibera, J. Efficacy of entomopathogenic fungi against adult *Diaphorina citri* from laboratory to field applications. *J. Pestic. Sci.* **2017**, *90*, 947–960. [CrossRef]
24. Lacey, A.; de la Rosa, F.; Horton, D. Insecticidal activity of entomopathogenic fungi (Hypocreales) for potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae): Development of bioassay techniques, effect of fungal species and stage of the psyllid. *Biocontrol. Sci. Technol.* **2009**, *19*, 957–970. [CrossRef]
25. Pérez-González, O.; Gomez-Flores, R.; Montesinos-Matías, R.; Mellín-Rosas, M.A.; Cantú-Bernal, S.H.; Tamez-Guerra, P. Improved *Diaphorina citri* (Hemiptera: Liviidae) adults biocontrol in citrus by *Hirsutella citriformis* (Hypocreales: Ophiocordycipitaceae) gum-enhanced conidia formulation. *Plants* **2023**, *12*, 1409. [CrossRef]
26. Pérez-González, O.; Sandoval-Coronado, C.; Maldonado-Blanco, M.G. Evaluation of Mexican strains of *Hirsutella citriformis* against *Diaphorina citri* in a semifield bioassay. *Southwest. Entomol.* **2016**, *41*, 361–372. [CrossRef]
27. Grijalba, E.P.; Espinel, C.; Cuartas, P.E.; Chaparro, M.L.; Villamizar, L.F. *Metarhizium rileyi* biopesticide to control *Spodoptera frugiperda*: Stability and insecticidal activity under glasshouse conditions. *Fungal Biol.* **2018**, *122*, 1069–1076. [CrossRef] [PubMed]
28. Tamayo-Mejía, F.; Tamez-Guerra, P.; Guzmán-Franco, A.; Gómez-Flores, R. Can *Beauveria bassiana* Bals. (Vuill) (Ascomycetes: Hypocreales) and *Tamarixia triozae* (Burks) (Hymenoptera: Eulophidae) be used together for improved biological control of *Bactericera cockerelli* (Hemiptera: Triozidae). *Biol. Control* **2015**, *90*, 42–48. [CrossRef]
29. Cardona, L.N.; Borrego, D.A.; Fernández, E.P.; Sánchez, J.; Cardona, V.; Montoya, G. Microbiological evaluation and pathogenicity of a liquid bioformulation of the fungus *Purpureocillium* sp. (strain UdeA 0109) on *Meloidogyne incognita-javanica* stages. *Biotecnol. Apl.* **2014**, *31*, 210–215.
30. Latifian, M. Physicochemical properties effects on different oil formulations on fungus *Metarhizium anisopliae* for control of *Oryctes elegans*. *J. Entomol.* **2018**, *15*, 83–92. [CrossRef]
31. Arbeláez-Tabares, M. Development of a W/O Emulsion for a Biocontrol Agent Bacillus velezensis UA2208. Master's Theses, Universidad Nacional de Colombia, Medellín, Colombia, 2020. Available online: https://repositorio.unal.edu.co/handle/unal/77791 (accessed on 24 March 2023).
32. Delucchi, C.; Percibaldi, M.; Trejo, M.; Eyhérabide, G. Genetic improvement of the fatty acid profile of corn oil. *Rev. Investig. Agropecu.* **2019**, *45*, 159–181.
33. Liu, S.; Li, J.; Hu, H.; Gao, Y.; Wang, J. Biological control of phytopathogenic fungi by fatty acids. *Mycopathologia* **2008**, *166*, 93–102. [CrossRef]
34. Perdomo, C. Desarrollo de Cuatro Prototipos de Bioformulaciones en Base a Conidias de Trichoderma asperellum. Master's Thesis, Universidad Central del Ecuador, Quito, Ecuador, 2018. Available online: http://www.dspace.uce.edu.ec/handle/25000/16686 (accessed on 20 March 2023).

35. Pérez-González, O.; Maldonado-Blanco, M.; Valdez-González, A. Conidia shelf-life and pathogenicity of strains of *Hirsutella citriformis* Speare. *Southwest. Entomol.* **2019**, *44*, 165–171. [CrossRef]
36. Rosas-García, N. Desarrollo de formulados asperjables de Beauveria bassiana (Bálsamo) Vuillemin utilizando diversos polímeros. Ph.D. Thesis, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, 1999. Available online: http://eprints.uanl.mx/7235/1/1080087092.PDF (accessed on 8 August 2022).
37. Hidalgo, E.; Moore, D.; Le Patourel, G. The effect of different formulations of *Beauveria bassiana* on *Sitophilus zeamaisin* stored maize. *J. Stored Prod. Res.* **1998**, *34*, 171–179. [CrossRef]
38. Mishra, S.; Kumar, P.; Malik, A. Preparation, characterization, and insecticidal activity evaluation of three different formulations of *Beauveria bassiana* against *Musca domestica*. *Parasitol. Res.* **2013**, *112*, 3485–3495. [CrossRef] [PubMed]
39. Jin, X.; Custis, D. Microencapsulating aerial conidia of *Trichoderma harzianum* through spray drying at elevated temperatures. *Biol. Control* **2011**, *56*, 202–208. [CrossRef]
40. Morales, A.; Jarquín, R.; Gómez, J.; Díaz, O.; Marín, J. Evaluation of a vegetable oil formulation of *Beauveria bassiana* under laboratory conditions to control the coffee berry borer. *Fitosanidad* **2014**, *18*, 5–10. Available online: https://www.redalyc.org/articulo.oa?id=209131412001 (accessed on 21 September 2022).
41. Montesinos, R.; Ayala, M.; Berlanga, A. *Manual para la Conservación y Mantenimiento de Hongos Entomopatógenos*; Unidad de Promoción y Vinculación—SENASICA: México City, México, 2015; ISBN 978-968-5384-08-7.
42. Lopes, R.B.; Faria, M. Influence of two formulation types and moisture levels on the storage stability and insecticidal activity of *Beauveria bassiana*. *Biocontrol Sci. Technol* **2019**, *29*, 1–14. [CrossRef]
43. Vélez, P.; Posada, F.; Marín, P.; González, M.; Osorio, E.; Bustillo, Á. *Técnicas para el Control de Calidad de Formulaciones de Hongos Entomopatógenos*; CENICAFÉ: Caldas, Colombia, 1997. Available online: https://biblioteca.cenicafe.org/handle/10778/709 (accessed on 20 March 2023).
44. Abadías, M.; Teixido, N.; Usall, J.; Benabarre, A.; Viñas, I. Viability, efficacy, and storage stability of freeze fried biocontrol agent *Candida sake* using different protective and rehydration media. *J. Food Prot.* **2001**, *64*, 856–861. [CrossRef]
45. Gurumurthy, S.; Kumar, R.; Saabale, P.R.; Meena, S.K.; Mukesh, S. Effect of temperature, pH and various media on growth and sporulation of *Trichoderma* spp. isolates from Uttar Pradesh. *J. Plant Dev. Sci.* **2016**, *8*, 615–618.
46. Padmavathi, J.; Devi, U.; Rao, M. The optimum and tolerance pH range is correlated to colonial morphology in isolates of the entomopathogenic fungus *Beauveria bassiana*—A potential biopesticide. *World J. Microbiol. Biotechnol.* **2003**, *19*, 469–477. [CrossRef]
47. McMacoy, C.W.; Hill, A.J.; Kanavel, R.F.A. liquid medium for the large-scale production of *Hirsutella thompsonii* in submerged culture. *J. Invertebr. Pathol.* **1972**, *19*, 370–374. [CrossRef]
48. Jaffee, B.A.; Zehr, E.I. Sporulation of the fungus *Hirsutella rhossiliensis* from the nematode *Criconomella xenoplax*. *Plant Dis.* **1983**, *67*, 1265–1267. Available online: http://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=9466547 (accessed on 20 March 2023). [CrossRef]
49. Jenkins, N.; Grzywacz, D. Quality control of fungal and viral biocontrol agent- assurance of product performance. *Biocontrol Sci. Technol.* **2000**, *10*, 753–777. [CrossRef]
50. Torres, E. Study of Compatibility of the Yeast Pichia onychis LV027 with Excipients and Characterization of Formulated Media. Master's Thesis, Pontifica Universidad Javeriana de Bogotá, Bogotá, Colombia, 2009. Available online: https://repository.javeriana.edu.co/handle/10554/8245 (accessed on 7 August 2022).