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Pathogenicity of *Cordyceps javanica* (Hypocreales: Cordycipitaceae) to *Diaphorina citri* (Hemiptera: Liviidae) Adults, with Ultrastructural Observations on the Fungal Infection Process

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Abstract: Entomopathogenic fungi are proposed biological control agents against the Asian citrus psyllid (*Diaphorina citri*). We quantified the pathogenicity of *Cordyceps javanica* strain Apopka 97 strain (*Cja* Apopka 97) (blastospores and conidia) against *D. citri* using the spray technique. We also used light and scanning electron microscopy to observe the *Cja* Apopka 97, infection process against *D. citri* adults at different stages pre- and post-mortem. Our findings demonstrated that psyllid mortality in the blastospore treatment ranged from 8 to 25% within 24–48 h of exposure, compared to 0% in the conidial and control treatments. However, psyllid mortality rate had reached 100% by 7 days after exposure at a concentration of 10^7 spores/mL, under both fungal treatments compared to the controls (0%). SEM and light microscopy revealed several stages in in the *Cja* Apopka 97 infection process of *D. citri*, including spore adherence and germ tube formation within 24 h post-inoculation, penetration pegs and mycelia growth on wings after 72 h, rupturing of cuticle after 96 h and mycelial mass colonizing host body after 144 h. Our study findings provide basic information on the interaction of entomopathogenic fungi with *D. citri* which will assist in the understanding of the infection process and the potential roles of entomopathogenic fungi in its management.

Keywords: biocontrol agent; *Cordyceps javanica*; *Diaphorina citri*; entomopathogenic fungi; fungi-host interaction; scanning transmission electron microscopy; light microscopy; virulence

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1. Introduction

Diaphorina citri (Hemiptera: Liviidae) is an economically important pest of citrus worldwide and the vector for *Candidatus* Liberibacter asiaticus, a Gram-negative bacterium which is associated with citrus Huanglongbing [1,2]. Currently, *D. citri* management is mostly based on intensive use of synthetic foliar insecticides, sometimes in combination with soil-applied systemic insecticides, such as aldicarb or imidacloprid [3,4]. However, these treatments are not sustainable because *D. citri* has a rapid generation time and can

build up resistance to these chemical insecticides and can multiply very rapidly. Furthermore, broad spectrum synthetic chemical pesticides are becoming less acceptable because they have low selectivity and increasing safety issues [5]. Therefore, there is a clear need to develop alternative control and management strategies based on the use of biocontrol agents which are effective for *D. citri* management as well as ecologically and user friendly [6].

Currently under research are entomopathogenic fungi belonging to the Hypocreales: Cordycipitaceae [7]. *Cordyceps* species have a worldwide distribution with wide-ranging host associations among several insect orders. These fungi are being used or developed for the management of several economically important pests, such as lepidopterans, psyllids, thrips and whiteflies [8–10].

Previous authors investigated the virulence and host–pathogen associations with *Cordyceps* and *D citri* [2,11–14]. However, few quantified how the pathogenic mode of action relates to the ultrastructural investigations of fungal infection processes [15–17]. In this study, we describe the ultrastructural observations on fungal infection process of *Cordyceps javanica* (Frieder. & Bally) Kepler, B. Shrestha & Spatafora (Hypocreales: Cordycipitaceae) strain Apopka 97 (henceforth *Cja* Apopka 97) colonizing *D. citri* through SEM (scanning transmission electron microscopy) and light microscopy techniques. We also compared the relative virulence of *Cja* Apopka 97 blastospore and conidia, two infective propagule types that can be produced as inoculum for microbial insecticides.

2. Materials and Methods

2.1. Insects

A population of healthy *D. citri* adults were collected from a citrus orchard located in Guangzhou city, Guangdong province, China, and maintained on orange jasmine, *Murraya paniculata* (L.) Jack (Rutaceae). The insects were reared in the Institute of Zoology, Guangdong Academy of Sciences, Guangzhou, China. Psyllids were reared at 25 °C, with 60% relative under a 14-h photophase. Four successive generations were reared prior to the start of the study.

2.2. Plants and Fungus

Orange jasmine, *Murraya paniculata* (L.) leaves naturally infested with *Cja* Apopka 97 were sampled in the laboratory and subsequently maintained on Czapek Dox (CD) media (Thermo Fisher Scientific (China) Co., Ltd., Shanghai, China) for one month. For long-term storage (two months), conidial suspensions were prepared in 20–30% glycerol solution, and then stored at −80 °C in chamber (Jinan Qianshi Biotechnology Co., Ltd., Jinan, China) containing liquid nitrogen as stock cultures and sub-cultured in our laboratory until needed. Passaging was conducted on the *D. citri* (host insect) after every two to three months and the subculture was re-isolated and maintained on CD and preserved by lyophilization. The subculture stock of *Cja* Apopka 97 was used for all the experiments.

2.3. Molecular Characterization of Entomopathogenic Fungi

Fungal identification was carried out using molecular analysis. The *Cja* Apopka 97 was grown in CD culture media and incubated for 10 days at 25 °C. DNA extraction was performed from the mycelium using the FastDNA® spin kit (MP Biomedicals, Illkirch, France). The precipitated DNA pellet was dissolved in sterile water (30 µL) and stored at −20 °C until needed. Additionally, a spectrophotometer was used at 260/280 nm to quantify the DNA concentration.

Detection and identification of *Cja* Apopka 97 was performed through polymerase chain reaction using a universal fungal primer designed for amplification of 18S rRNA gene using an Applied Biosystems 2720 thermal cycler. 18S (sequence: TCCGTCGGTGAACCTGCGG) was used as the forward primer and 18S (sequence: TCCTCCGCTTATTGATATGC) as the reverse primer [18,19]. The reaction was set up as follows: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 57 °C for 1 min, and 72 °C for

1 min, followed by a final extension step at 72 °C for 6 min. Gel Extraction Kit (E.Z.N.A®, Shanghai, China) was used to purify amplified DNA products separated on a 1% agarose gel stained with ethidium bromide solution.

2.4. Preparation of Conidia

Cja Apopka 97 was sub-cultured on CD culture media and incubated for 10 days at 27 °C to sporulate. For conidial production, a suspension of mycelia fragments with conidia was prepared by scraping the fungal growth with a sterile spatula and suspending in 10 mL of Tween 80 (*v*/*v*; 0.01%). The suspension was vortexed and filtered using sterile cotton for the elimination of mycelial fragments. Conidial concentration was finally adjusted with sterile 0.01% Tween 80 (*v*/*v*) to 1×10^7 conidia/mL using hemocytometer. Conidial viability was determined by plating 0.1 mL aliquots on CD plates and incubated at 27 °C with 65–70% RH under a light (14 h): dark (10 h) photoperiod for 24 h. The conidia were 99 viable after 24 h incubation period).

2.5. Inoculation of D. citri with Entomopathogenic Fungi

Murraya paniculata plants (2–3 months old) were selected at random for experimental use. Mated males and females (100) of *D. citri* were released into the center of each mesh cage. Females were allowed to oviposit for 24 h before adults were removed. All leaflets were subsequently observed under a stereomicroscope (SZ680, CNOPTEC) and the numbers of eggs on the abaxial surface were counted. The detached leaf petioles were covered with a piece of sterile cotton, moistened with 2 mL of sterile distilled water and placed in sterile 90 mm plastic Petri dishes containing three sterile Whatman filter papers, also moistened with sterile distilled water (2 mL) (Figure 1), based on earlier method [20]. Control leaflets were dipped into Tween 80 (0.01%; *v*/*v*). Both dishes (treated and control) were sealed and incubated in a growth chamber set at constant temperature (25 °C) and R.H. (70 ± 2%) with a photoperiod of 14 h (L):10 h (D) for 7 days.

Figure 1. *Murraya paniculata* leaf petioles covered with a piece of sterile cotton in a sterile plastic Petri dish used for inoculation of *D. citri* with *Cja* Apopka 97 strain.

Mated gravid female *D. citri* (100) were released into the center of new mesh cages. Females were permitted to oviposit (for 24 h) before adults were removed, as described above. The eggs were allowed to develop into third or fourth instar nymphs. Twenty

nymphs were separately kept in each Petri dish, and were treated topically, with a concentration of conidial suspension (10^7 conidia/mL) of *Cja* Apopka 97 strain or 0.01% *v*/*v* Tween 80 solution (control) for 30 sec. Following inoculation, leaf samples with nymphs were air dried at room temperature (27 ± 1 °C) for 10 min. Leaf petioles were wrapped in moistened cotton and then transferred into Petri dishes (Figure 1). Each fungal inoculation and the control were replicated 5 times. The Petri dishes were incubated in a growth chamber at 25 ± 1 °C and 70 ± 2.0% R.H. under a 14 h:10 h L:D photoperiod.

To rear *D. citri* adults, mated males together with females (100) were released into other insect cages for oviposition. After 24 h all the females were removed. The leaves were incubated for 16–17 days to generate fresh adult *D. citri*. Three to four day-old adults were used for pathogenicity test following the same procedure as described above for the eggs.

2.6. Psyllid Mortality Bioassays

The mortality of the adult *D. citri* in the leaf disks bioassays was determined using the protocol described by Avery, et al. [21]. Treatments consisted of the following (1) blastospores or (2) conidia of *Cja* Apopka strain and (3) tween for the control. Adult *D. citri* mortality was assessed daily for 7 days.

2.7. Light Microscopy (LM) and Electron Microscopy (EM)

The morphological development of *Cja* Apopka-97 growth was observed on the premortem and dead *D. citri* over time as well as culture medium, using scanning electron microscopy (SEM), and light microscopy (LM).

(a) For light microscopic observations, a piece of PDA agar with grown fungus was collected using a sterile scalpel and mounted on a glass slide. A drop of lactophenol blue solution was used to enable observation under a bright field light microscope (OLYMPUS BX41, Shinjuku, Tokyo, Japan).

(b) For conventional SEM, at 24, 48, 72, 96, 120, 144, and 168 h, the infected *D. citri* were collected (post-inoculation) and observed using a Hitachi SU-3500 SEM (Hitachi, Tokyo, Japan) operating at 15 KV. Adult *D. citri* were prepared by first being immersed in 4% glutaraldehyde for 24 h, rinsed thrice for 10 min (each time) in phosphate buffer (0.2 M) and then dehydrated in an ethanol series (75, 80, 85, 90, 95, and 100% ethanol [*v*/*v*]). Samples were then immersed in chloroform (for 1 h) to dissolve the surface wax [13,16], followed by a final rinse in ethanol (100%). The ethanol was then exchanged for liquid (CO_2) and *D. citri* samples were prepared using a drying apparatus (K850). *D. citri* samples were mounted on a graphite pad and sputter coated with gold to a standard thickness (20 nm). Micrographs were taken with a digital camera (Canon EOS 350D).

(c) For SEM, fungal-treated psyllids were fixed for 2 h in a solution of 2% glutaraldehyde, 3% paraformaldehyde, in sodium cacodylate buffer 0.2 M, pH 7.4 (#11650 Electron Microscopy Sciences, Hatfield, PA, USA), followed by dehydration in an ethanol series of 35, 50, 70, 95, and three changes in 100%, each for 1 h. Dehydrated samples were lyophilized, and sputter coated with gold (at thickness of 20 mm). A Hitachi 4800, SEM microscope was used to examine the specimens (Tokyo, Japan).

2.8. Statistical Analysis

For mortality bioassays, within each treatment group (20 disks/treatment), each disk (Petri dish) was counted as an independent replication, and each of the three treatments was evaluated on three successive dates (n = 60/treatment) until the insects started dying (n = 60/treatment—# dying). Cumulative mean percent mortality was determined using Abbott's formula. An ANOVA was used to compare the means of mortality (%), followed by a Tukey's HSD test ($p < 0.05$). All statistical analyses were carried out on a WIN PRO platform using SAS Proc GLM routines (SAS 1999–2001).

3. Results

3.1. Percent Psyllid Mortality

Psyllids started dying earlier in the blastospore treatment, with initial mortality observed on day 2 post-inoculation (Figure 2). However, both blastospore and conidia treatments significantly affected cumulative percent mortality of adult psyllids that died after 4 days post-exposure (F = 7.82; df = 2, 8; p = 0.04). Both blastospore and conidial treatments had 91–97% mortality at 6 days. Seven days after exposure to *Cja* Apopka 97 blastospores or conidia, all psyllid adults died. No psyllids died in the control over the 7 days.

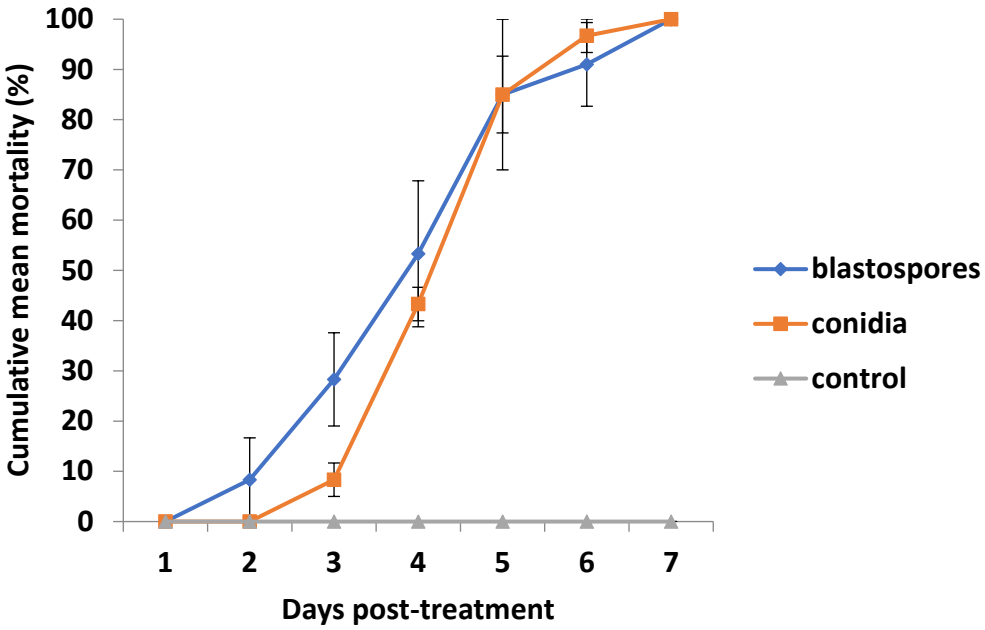

Figure 2. Mortality of *Diaphorina citri* adults after feeding on a leaf disk sprayed with blastospores, conidia of *Cja* Apopka or Tween 80 (control) over time.

3.2. Scanning Electron Microscopy

SEM observations revealed that the fungal spores were evenly distributed on the infected body of the adult *D. citri,* including leg and head area (Figure 3). In addition, the hyphae of *Cja* Apopka 97 strain grew and formed the conidia on the leaf surface (Figure 3F) which were similar to the conidia and hyphae observed on the wings and abdomen of infected psyllids). Blastospores germinated 6–8 h after the *D. citri* was inoculated, whereas only the conidia adhering to the legs (Figure 4A) successfully germinated and formed a germ tube after 24 h post-inoculation (Figure 4B). The penetration of the germ tube caused cracks in the *D. citri* cuticle (Figure 4C), while some hyphae developed into mycelial mass on antennae by 120 h post-inoculation (Figure 4D). After that time, it was observed that the germ tube elongated toward the body wall, and then penetrated the cuticle of the psyllids forming hyphae. Some hyphae grew upward while others grew parallel along the body surface (Figure 4E). Some hyphae colonized the wings after 72 h post-inoculation (Figure 4F). After that, the hyphae grew rapidly on the surface of the insect cuticle, intertwined to form mycelium (Figure 4E). Finally, mycelia colonized *D. citri* body after 144 h post-inoculation, and the insect body became desiccated.

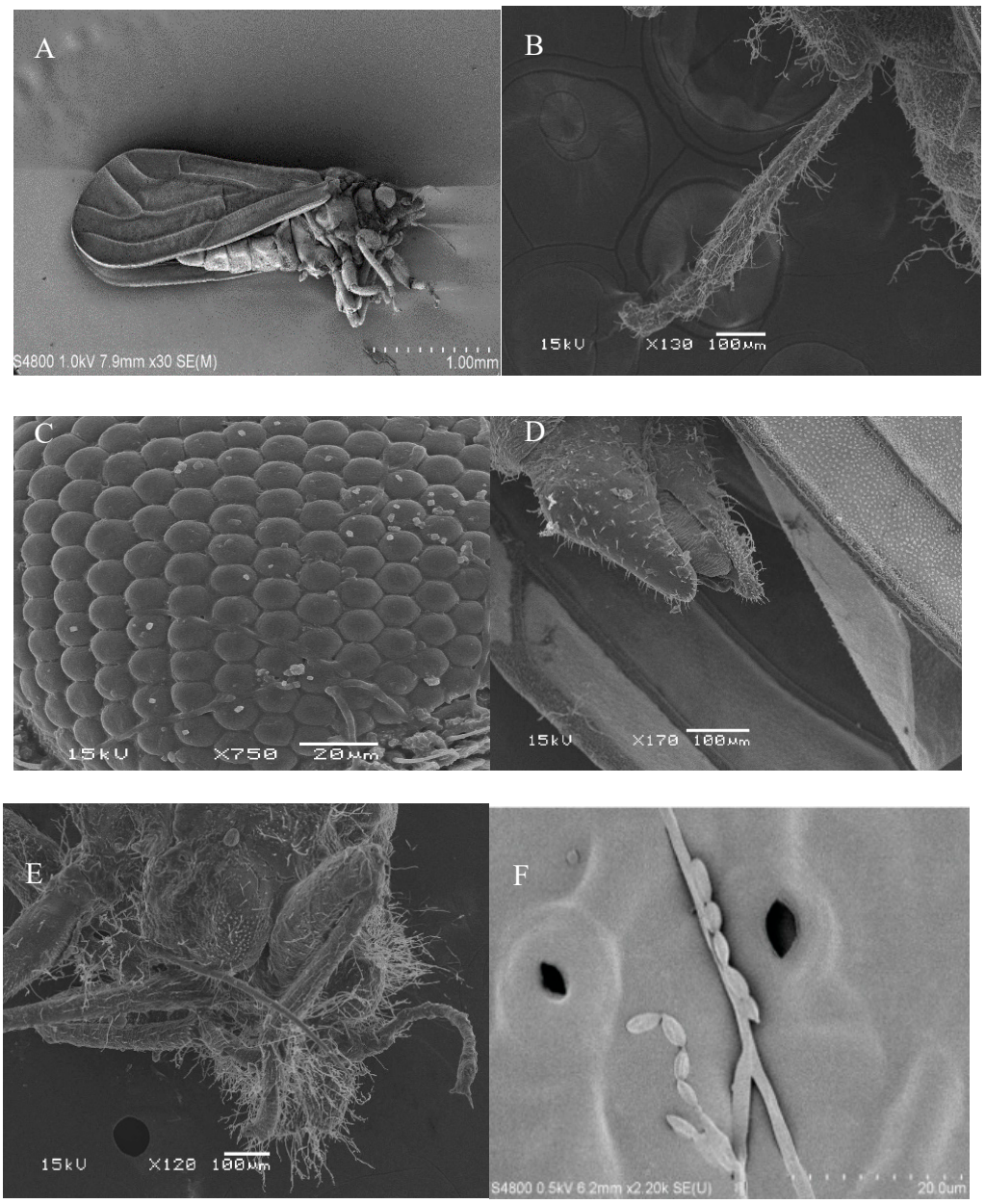

Figure 3. (**A**) SEM photomicrographs of *Diaphorina citri* uninfected adult; (**B**) *Cja* Apopka 97 hyphae growing on leg; (**C**) *Cja* hyphae growing on compound eyes; (**D**) *Cja* hyphal growth on genitalia; (**E**) head area covered with hyphae; (**F**) hyphae growing and forming conidia on the leaf surface.

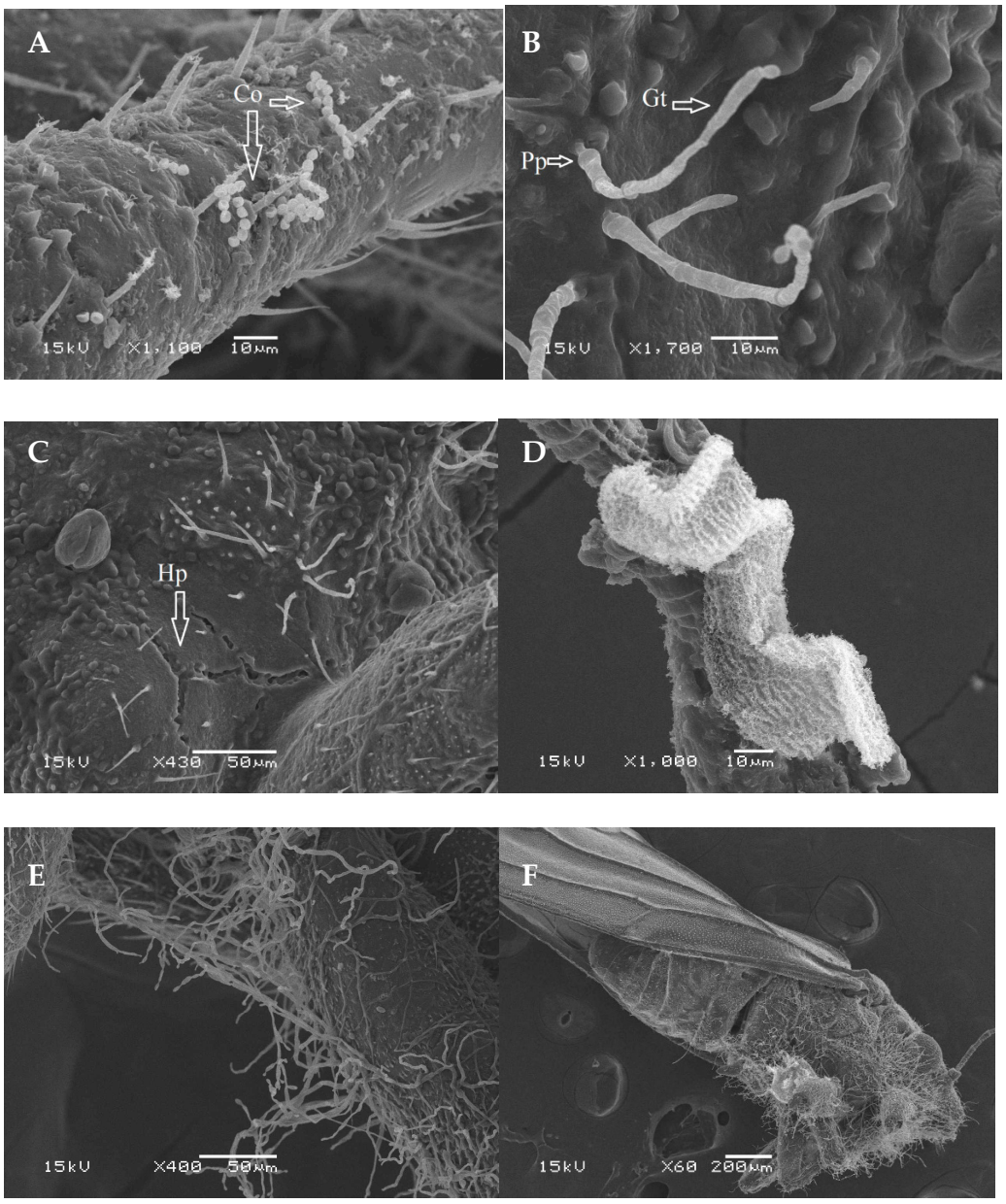

Figure 4. Ultrastructural observations of *Diaphorina citri* infected with *Cja* Apopka 97; (**A**) conidia (Co) adhering to the legs after 24 h post-inoculation; (**B**) germ tube formation (Gt) after 24 h post-inoculation formation of penetration pegs (Pp), and mycelia growth after 72 h post-inoculation; (**C**) rupturing of cuticle due to hyphal penetration (Hp) after 96 h post-inoculation; (**D**) mycelial mass on antennae surrounded after 120 h post-inoculation; (**E**) hyphae growing on the wings after 72 h post-inoculation; (**F**) mycelia colonizing *D. citri* body after 144 h post-inoculation.

3.3. Light Microscopy

We observed that after infection with *Cja* the psyllid remained at the same angle while the fungus penetrates through the tarsi and cements it to the leaf surface (Figure 5). Additionally, we observed *Cja* hyphae and conidia growing on phialides and dispersing from the infected host. Branched hyphae with conidia were observed (Figure 6).

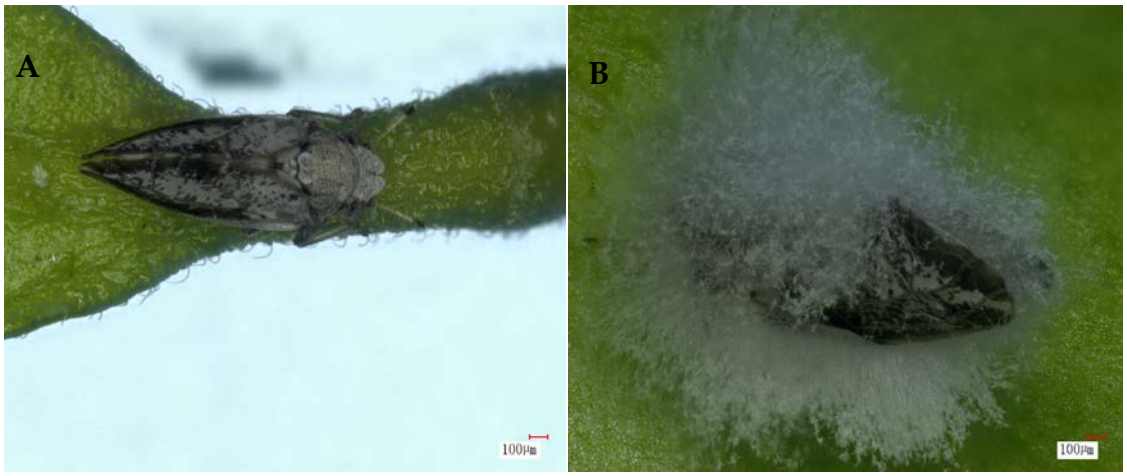

Figure 5. (**A**) Healthy *Diaphorina citri* adult (**B**) *D. citri* adult colonized with *Cordyceps javanica*.

4. Discussion

In the mortality bioassay, we quantified the pathogenicity of *Cja* Apopka 97 spores against *D.* citri. The corrected mortality for *D. citri* treated with 10^7 spores/mL were higher than control, while we also observed faster initial mortality from *D. citri* exposed to the blastospore formulation, suggesting these germinate more quickly compared with the conidia (Figure 3). Our results revealed the ability of the fungus to infect different regions of the host, leading to an increase in host mortality and conidial production via colonization of the cadaver. In addition, our results indicate that infection of *D. citri* was dependent on the spore residual concentration on the leaf disk surface under high R.H. However, when *D. citri* adults were exposed to the spore suspension, rapid fungal growth on the *D. citri* body parts began after 24 h to 72 h post-inoculation, which contributed to higher mortality (Figure 5). After the adult psyllid becomes infected, the antennae and legs begin to twitch, and the body becomes unstable (see Supplementary Video S1).

Our ultrastructural studies illustrate the successful invasion of *D. citri* by the fungal entomopathogen *Cja* Apopka 97 (Figures 3–6). Our results expand on observations with the entomopathogenic fungal infection process in whiteflies [10] and other insects [21–24]. We observed that *Cja* Apopka 97 infect *D. citri* (Figures 3 and 4), with the infection process, similar to *Cja* Apopka 97 infecting other insects, such as whitefly, scale insects, and mites [25–27]. Our studies show how *Cja* Apopka 97 infected *D. citri* integument. It was observed that the infection process involved adherence of spores to the host body after 24 h post-inoculation (Figure 4A), germ tube (Gt) formation after 24 h post-inoculation and penetration pegs (Pp) along with mycelia growth out of host body after 72 h post-inoculation (Figure 4B); rupturing of cuticle due to hyphae growth (Hp) after 96 h post-inoculation (Figure 4C), surrounding of fungal mycelial mass on host antennae after 120 h post-inoculation (Figure 4D), growing of fungal hyphae on the wings after 72 h post-inoculation (Figure 4E), and fungal mycelia colonizing host body after 144 h post-inoculation (Figure 4F).

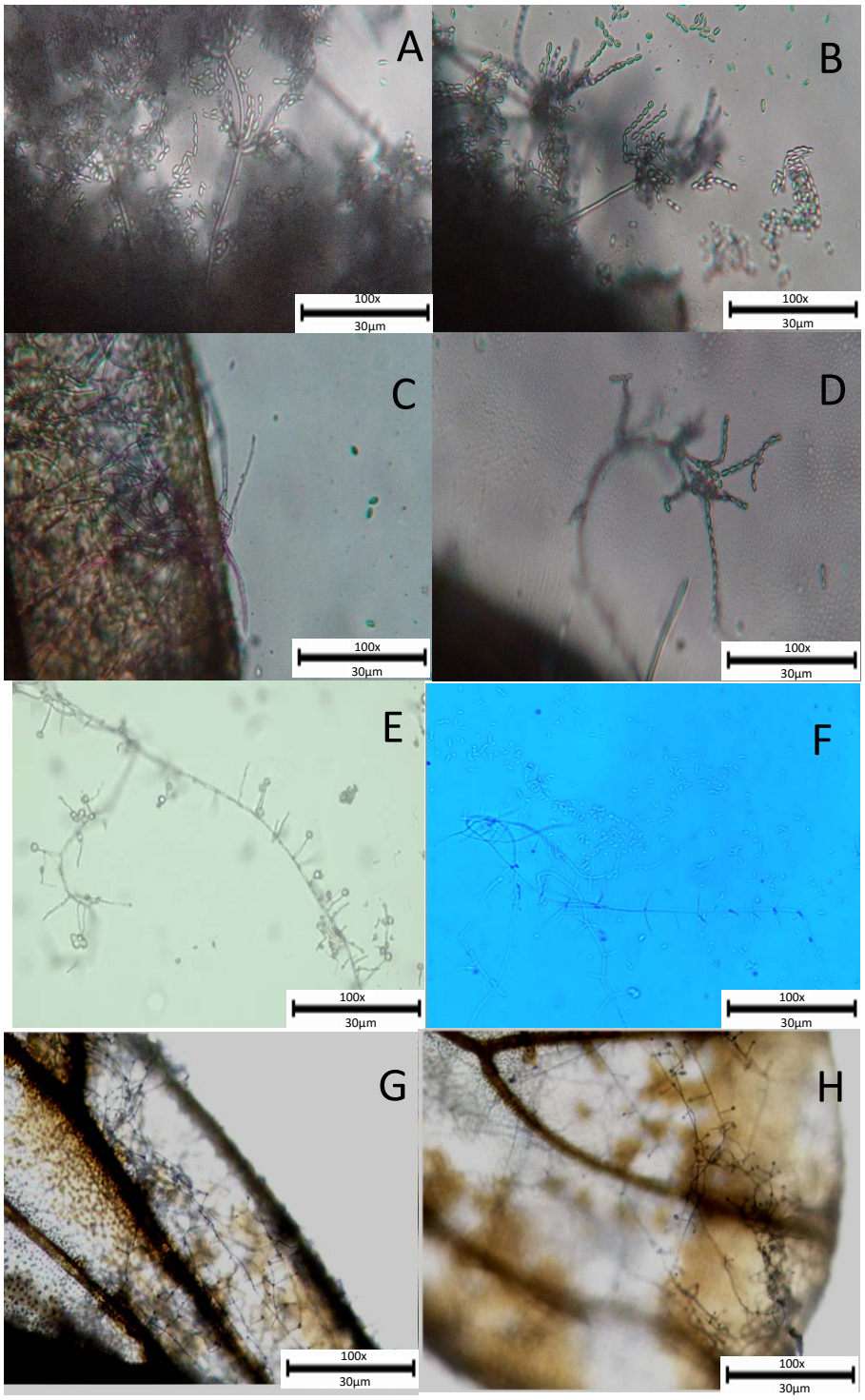

Figure 6. Light microscopic picture of *Cja* Apopka 97 hyphae and conidia, outgrowth from infected adult *Diaphorina citri* (**A**) vertically branched conidiophore with conidia dispersing from *D. citri* adult cadaver; (**B**) dense conidia and hyphae extending from the edge of the psyllid body (aerial conidial dispersal); (**C**) hyphae growing on the wing surface of infected psyllid; hyphae growing through the veins and forming mycelium; (D) hyphae and conidia growing on phialides extending from infected insect; (**E, F**) hyphae and conidia growing on phialides and dispersing from infected insect (aerial conidial dispersal); (**G**) hyphae growing on the infected psyllid wing surface; (**H**) hyphae with conidia growing on phialides on wing surface.

Our results showed that under our experimental conditions, *Cja* Apopka 97 can also colonize the leaf surface (Figure 3F). This observation suggests that under certain conditions, *Cja* Apopka 97 may persist in the environment even without an insect host, potentially contributing to later epizootics. This aspect of fungal colonization on plants by *Cordyceps* species expands on previous observations [21,28] but also requires further study. Pick et al. 2021 [29] also noted that *Cja* Apopka 97 may also colonize the phylloplane of citrus leaves under field conditions following rainfall events. On psyllids, *Cja* Apopka 97 fungal adherence and growth also seems to grow in the wax layer [30]. A thorough understanding of the interactions between entomopathogenic fungi and their potential hosts is required before they can be developed as biocontrol agents [24]. Appropriate targets on the host insects must be identified in such studies; as a result, the efficacy of formulation and application methods will be improved. For understanding how different fungi infect and colonize insect pests, light and scanning electron microscopy are valuable tools for studying the entomopathogenic fungus mode of action [30–32].

Previous studies have investigated fungal adherence and penetration structures on other insects using scanning electron microscopy [33,34]. These fungi could be effective biological control agents for insect pest populations on various host plants. Therefore, microscopic investigations have been helpful in determining how entomopathogenic fungi, such as *Cja* Apopka 97, interact with the *D. citri*. This research has led to a better understanding of the fungal infection process on adult psyllids with *Cja* Apopka 97 and the potential for using entomopathogenic fungi to control it.

5. Conclusions

In conclusion, our study provides basic and applied data on the interaction of *Cja* Apopka 97 with *D. citri*. We noted several new observations on the differences between infective propagules and the progression of the disease in *D. citri*. Therefore, the findings of the present study will help to increase the available data concerning the efficacy of using entomopathogenic fungi as potential biological agents against the *D. citri*. In the long run, the use of entomopathogenic fungi can serve as a sustainable alternative to the broad spectrum synthetic chemical insecticides commonly used to regulate and manage the *D. citri* populations in the field.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11122476/s1, Video S1: Effect of blastospore and conidial *Cordyceps javanica* formulation in inoculated *Diaphorina citri* displayed disease symptoms including twitching of legs, antennae, and wing. Prior death inoculated psyllids present fungal hyphae emerging from the body. Note: The progression of the disease on adult *D. citri* included moribund behaviors, one week after inoculation infected insects form a cemented sporulating cadaver on the surface of the leaf with the hyphae colonizing on the entire exoskeleton.

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