Fungal growth development index and ultrastructural study of whiteflies infected by three *Isaria fumosorosea* isolates of different pathogenicity

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Índice de crecimiento y desarrollo fúngico y estudio ultraestructural de mosquitas blancas infectadas por tres aislados de *Isaria fumosorosea* de diferente patogenicidad

Resumen. El proceso de infección por el cual *Isaria fumosorosea* coloniza a las ninfas de la mosquita blanca (*Trialeurodes vaporariorum*) fue investigado usando microscopía de luz y electrónica de barrido. El índice de crecimiento y desarrollo fúngico fue usado para determinar la patogenicidad de los aislados estudiados. Los hallazgos ultraestructurales nos permitieron examinar el curso de la colonización de este hongo en *T. vaporariorum* mediante la formación de estructuras de penetración en la cutícula. Los aislados de *I. fumosorosea* produjeron estructuras que recuerdan apresorios en su forma y causan serios daños cuticulares en el hospedante que sugieren la acción enzimática. Los resultados de este estudio sugieren que el aislado EH-506/3 es adecuado para el control biológico de la mosquita blanca.
Palabras clave: hongos entomopatógenos, *Trialeurodes vaporariorum*, proceso de infección y colonización, microscopía electrónica de barrido (MEB).

Abstract. The infection process whereby *Isaria fumosorosea* colonizes whitefly (*Trialeurodes vaporariorum*) nymphs was investigated using light and scanning electron microscopy. The fungal growth development index was used to determine pathogenicity of the isolates studied. The ultrastructural findings allowed us to examine the course of colonization of *T. vaporariorum* by this fungus through the formation of cuticular penetration structures. *I. fumosorosea* produced structures that resemble appressoria in shape, and isolates cause serious cuticular damage suggesting of enzymatic action. The results of this study suggest isolate EH-506/3 as suitable for whitefly biocontrol.
Keywords: entomopathogenic fungi, *Trialeurodes vaporariorum*, infection and colonization process, scanning electron microscopy (SEM).

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Introduction

Isaria fumosorosea is a fungal entomopathogen that has been used as a biocontrol agent against whiteflies (Hemiptera: Aleyrodidae) and other insects (Butt *et al.*, 2001; Shah and Pell, 2003; Meyer *et al.*, 2008; Cabanillas and Jones, 2009). Whiteflies are polyphagous insects of vegetables, ornamental crops and many cultivated plant species, both in greenhouses and in open fields worldwide. In Mexico, this insect is a significant agricultural pest (Ramírez-Villapudua, 1996).

An effective biocontrol strategy against this insect requires the selection of fungal isolates that combine desirable characteristics such as pathogenicity and conidia production to control the whiteflies (Pedrini *et al.*, 2007). Methods used to assess the different pathogenicity levels of isolates are time-consuming and laborious (Vidal *et al.*, 1997). Thus, in the present work was used Fungal Growth

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Development Index (FGDI), described by Landa *et al*. (1994) for determining pathogenicity of entomogenous fungi in whiteflies. During the bioassay, the nymphs induced fungal development, and the area of the inoculum drop closest to the nymph was the most interactive zone.

Nonspecific attachment of the conidia to the host surface is the first event of the infection process (Rangel *et al.,* 2008; Chouvenc *et al.,* 2009), and it is followed by conidial germination and the production of mycelia and different structures, such as appressoria, that colonize the insect's cuticle surface (Amóra *et al.*, 2010). A successful fungal invasion involves two main mechanisms: one is a mechanical force employing enormous turgor pressures (Fang *et al*., 2009), and the other is enzymatic digestion of the cuticle, mainly by proteases (Zhang *et al*., 2008; Khan Pathan *et al*., 2007) and chitinases (Staats *et al.*, 2013). The first mechanism is exerted by entomopathogenic fungal structures such as the appressoria, which have been observed with electron microscopy. *Metarhizium anisopliae* develop appressoria when grown on the western flower thrips (*Frankiniella occidentalis*) (Vestergaard *et al.*, 1999), as do *Lecanicillium* (=*Verticillium*) *dimorphum* or *L*. cf. *psalliotae* on red scale insects of palms (*Phoenicococcus marlatti)* (Asensio *et al*., 2005), and *L. lecanii* on brown scale (*Coccus hesperidum)* (Liu *et al*., 2011). These structures have also been observed in *Paecilomyces lilacinus* growing on nematode eggs (*Meloidogyne javanica*) (Holland *et al*., 2002). However, hyphae from other entomopathogenic fungi such as *Entomophaga maimaga* and *Beauveria bassiana,* penetrate the insect cuticle directly, without the need for appressoria formation, or through natural orifices as mouthparts (Hajek and Eastburn, 2003; Asensio *et al.*, 2005; Mauchline *et al.,* 2011).

Due to the relevance of both pathogenicity and the fungal structures in the infection processes of microbial agents used in biological control, in this paper, the FGDI was used to compare the pathogenicity of three *I. fumosorosea* isolates with different lethal median concentrations (LC_{50}). An ultrastructural study was carried out to examine the formation of infection structures and cuticular penetration during the infection process of *I. fumosorosea* in *T. vaporariorum* nymphs.

Materials and methods

Fungal isolates and growth conditions

Isaria fumosorosea PFCAM, MBP, and PSMB1 were isolated from whiteflies and obtained from the National Center for Biological Control, Mexico (Centro Nacional de Referencia de Control Biológico-CNRCB). Single spore cultures numbered EH-506/3, EH-503/3 and EH-520/3 from PFCAM, MBP, and PSMB1, respectively, were prepared by the Goettel and Inglis method (1997) as modified by Cavallazzi-Vargas *et al*. (2001). The original and monospore cultures (isolates) were preserved in sterile water, mineral oil, and in liquid nitrogen cryopreservation at –196 °C and deposited in the fungal collection of the Laboratorio de Micología Básica, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, UNAM, registered at the World Federal Culture Collection (WFCC) as BMFM-UNAM 834, and in the Laboratorio de Micología, Departamento El Hombre y su Ambiente, Universidad Autónoma Metropolitana-Xochimilco (UAM-X). Isolates were maintained on culture medium slants containing 1 % sucrose, 0.5 % glucose, 0.05 % peptone, 0.5 % yeast extract, and 2.3 % agar (SGPYE medium) until used.

The pathogenicity of the three isolates used in this work had been tested previously by median lethal concentration (Castellanos-Moguel *et al.*, 2007). The high-pathogenicity isolate, EH-506/3, had an LC_{50} of 1.1×10^3, the medium pathogenicity isolate, EH-503/3, had an LC_{50} of 2.5×10^4, and the low pathogenicity isolate, EH-520/3, had an LC_{50} of 7.6×10^4 conidia/mL.

Insects

The whitefly nymphs (*Trialeurodes vaporariorum*) used for the FGDI and scanning electronic microscopy (SEM) protocols originated from colonies maintained at the greenhouse and experimental field of the Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos (CEIB-UAEM), México. Whiteflies were reared on aretillo (*Fuchsia* sp.) leaves.

Bioassay: Fungal growth development index (FGDI) procedure

We employed the method of Landa *et al*. (1994) with minor modifications. Fungal conidia were produced in SGPYE medium cultures, and a dose of 1 x 10^6 conidia/mL was used. *Fuchsia* leaves that were infested predominantly with early fourth instar nymphs were used. The nymphs were disinfected in a laminar flow hood by soaking them in the following solutions: 70 % alcohol for 5 s, sterile distilled water for 40 s, and 5 % sodium hypochlorite for 20 s, followed by rinsing in 3 changes of sterile distilled water for a total of 120 s. The leaves were air-dried on sterile filter paper and the nymphs were removed from the leaf surfaces with an entomological needle and transferred to 3.5 cm Petri dishes containing water-agar (2.3 % for light and 60 % for scanning microscopy).

The nymphs were infected by dropping 4 μL of the conidial suspension described above on the nymph vicinity. Control nymphs were treated with 0.05 % Tween 80. The Petri dishes were placed in an incubator at 28 ºC with a photoperiod of 16:8 hours light:dark, for a total of 96 h. In total, 75 nymphs were used for each method (FDGI and SEM): 25 for each replicate bioassay and another 25 nymphs as a control. For the SEM, only isolates that had shown high (EH-506/3) and low (EH-520/3) pathogenicity were used.

The nymphs were assessed by light microscopy and valued individually at 0, 6, 12, 18, 24, 36, 48, 60, 72 and 96 h, and the FGDI was calculated. The results were expressed as the means of the FGDI values for each isolate. The FGDI values were valued as in Landa *et al*. (1994). A value of 0 represented nymphs surrounded by nongerminated conidia. A value of 0.5 was equated to the germination of conidia with one or two germ tubes, especially in the area close to the nymph. A value of 1.0 corresponded to germinated and non-germinated conidia surrounding the nymph, while a value of 1.5 required initial fungal growth on the host. At this point, hyphae were oriented toward the nymph and the first contact between hyphae and nymph was noticed. At 2.0, mycelium growth was observed on the host, hyphal growth was observed on the surface of the nymph and in the area around the nymph, and the presence of dense mycelium was noted. A value of 2.5 denoted initial sporulation and the presence of the first conidiophore on the surface of the nymph. At 3.0, sporulation had completed, and the nymph was covered with mycelia and conidia.

Any nymphs covered with the fungus were assumed to be dead due to fungal infection. Photomicrographs were taken with an Olympus BX-40 microscope (Olympus Optical Co. Shibuya-Ku, Tokyo, Japan).

Statistical analysis

Analysis of variance (ANOVA, α=0.05) was calculated for the FGDI at the different assay times among the three monospore cultures studied and followed by a Tukey multiple means comparison test (Dowdy and Wearden, 1983). The statistical analyses were performed using the SPSS Program, version 10, 2003.

Scanning Electron Microscopy (SEM)

The germination and development of EH-506/3 and EH-520/3 isolates were observed by SEM at the same times mentioned above for the FGDI assay. Ten infected nymphs were fixed at each time with 2.5 % glutaraldehyde (v/v) in 0.1 M phosphate buffer at pH 7.2 for 48 h at 4 ºC, and post-fixed with 1 % osmium tetroxide (v/v) in 0.1 M phosphate buffer at

pH 7.2 for 3 h at 4 ºC. The nymphs were then dehydrated with ethanol and desiccated at their critical point in a CO_2 chamber. The insects were mounted on aluminum studs, sputter-coated with silver and covered with coal and ionized gold. The samples were examined with a Carl Zeiss DSM 950 electron microscope operating at 15 Kv.

Chemicals

Unless otherwise stated, all chemicals used were from Sigma-Aldrich Química (Toluca, México).

Results

Fungal growth development index

The FGDI provided an overview of the chronological events of the whitefly infection by *I. fumosorosea*. Table 1 shows the FGDI for the three isolates of different pathogenicity. At 6 h into the bioassay, the FGDI values of EH-506/3 were significantly different ($P< 0.05$) from those observed for EH-503/3 and EH-520/3. The isolate with high pathogenicity, EH-506/3, was the only one that displayed germination of conidia with one or two germ tubes, particularly in the area close to the *T. vaporariorum* nymph (FGDI = 0.5). Similarly, after 12 h, the FGDI values of EH-506/3 were significantly different ($P< 0.05$) from those observed for EH-503/3 and EH-520/3. At the 18 and 24 h time points, the three isolates showed significant differences ($P< 0.05$). At 36 and 48 h, the FGDI values of EH-506/3 and EH-503/3 were the same, and they were significantly different ($P< 0.05$) from that of EH-520/3. At 60 h, the three isolates attained the same FGDI.

Isolate EH-506/3 germinated and colonized whitefly nymphs faster than the other two isolates tested, supporting our assessment of EH-506/3 as the most virulent isolate. At 6 h, this isolate showed conidia germination in the area near the nymph (FGDI = 0.5; data not shown), whereas this value was not attained by the other two isolates until 12 h (Table 1). Figure 1 shows the comparison of isolates EH-506/3 (left column), EH-503/3 (middle column), and EH-520/3 (right column) of *Isaria fumosorosea* by the Fungal Growth Development Index (FGDI), at 12 (a, f, k), 18 (b, g, l), 24 (c, h, m), 60 (d, i, n) and 96 (e, j, o) hours of incubation. At 12 h, EH-506/3 mycelial growth was observed on the host (FGDI = 2.0; Figure 1 a), suggesting hyphal emergence from the host's body. At the same time, the other two isolates, EH-503/3 and EH-520/3, had scarcely started germinating near the nymph (Figures 1f and 1k), and EH-520/3 showed mostly swollen conidia. At 18 h of incubation, EH-506/3-infected nymphs showed few conidiophores with the characteristic *Isaria* sporulation on the surface (FGDI = 2.5; Figure 1 b). At this time point, EH-503/3 showed nymph-orientated hyphae and the first contact between nymph and hyphae (FGDI = 1.5; Figure 1 g). At 18 h, isolate EH-520/3 developed one or more germ tubes near the nymph (FGDI = 0.5; Figure 1 l). At 24 h, EH-506/3 retained a constant FGDI value (2.5) with *Isaria* sporulation on the surface (Figure 1 c), while EH-503/3 developed dense mycelial growth on the nymph, suggesting that hyphae were emerging from the host (FGDI = 2.0), and nymph deformation and destruction were also evident (Figure 1 h). At this time, the FGDI value of EH-520/3 was 1.0; germinating conidia and some germ tubes scarcely showed the first contact with the nymph surface (Figure 1 m). At 36 h (data not shown), EH-506/3 continued to show sporulation, and EH-503/3 initiated sporulation on the nymph surfaces (FGDI = 2.5). EH-503/3 continued to display dense mycelial growth, and nymphs infected with this isolate showed structural damage and altered morphology (data not shown). Control nymphs and those infected with the other two isolates conserved their shapes even during the manipulation procedure at the equivalent time point. The EH-520/3-infected nymphs showed an FGDI of 1.5, and hyphae started to colonize the host. At 48 h of incubation (data not shown), EH-506/3-infected nymphs were completely covered with mycelia (FGDI = 3.0), but no further sporulation was

observed for the rest of the assay; in contrast, EH-503/3 (FGDI = 3.0) continued to display profuse sporulation (Table 1). At 60 h, EH-506/3-infected nymphs continued to have the maximum FGDI value (3.0), and growth became denser on the host (Figure 1 d). EH-503/3 exhibited profuse sporulation (Figure 1 i), and conidiophores were observed on nearly every surface of the nymph. The isolate EH-520/3 grew slower than the other two isolates, and it did not reach the maximum FGDI (3.0) value until 60 h, at which point it also showed profuse sporulation (Figure 1n). All of the nymphs were incubated until 96 h to follow the infection process, and, at this time, all of the infected nymphs showed complete mycelial colonization of their surfaces (Figures 1e, 1j and 1o). The EH-503/3-infected nymphs showed more evidence of cuticle damage, and some insects exhibited significant perforations. This phenomenon was not observed with the other two isolates.

Scanning Electron Microscopy (SEM)

The FGDI assay allowed us to select the appropriate time points for SEM analysis of the two isolates tested: EH-506/3 (high pathogenicity and rapid nymph colonization) and EH-520/3 (low pathogenicity and slow nymph colonization) showing that at 6 h of incubation is a critical point for whiteflies infection. The SEM results showed that the conidia of both *I. fumosorosea* isolates were capable of adhering to any site on the nymph surface, but they mainly did so at the rachis and vasiform orifice areas. In general, EH-506/3 showed less mycelial growth but more severe cuticular damage when compared with EH-520/3. The control nymph maintained an intact shape, did not sustain cuticle damage after manipulation and no fungal growth was observed on it (Figure 2a). Moreover, almost all of the control nymphs reached the 3rd instar stage of insect development.

All of the EH-506/3 and EH-520/3-infected nymphs showed signs of infection; in most cases, a single germ tube emerged from each conidium and extended up to a certain distance before penetrating. At 0 h, conidia of EH-506/3 and EH-520/3 were found on the nymph surface (Figure 2b) and near the vasiform orifice of the insect. At this time, the nymph surface showed, naturally occurring microbiota (Figure 2c). At 0 h the vasiform orifice did not show any extracellular matrix (Figure 2d). At 6 h, conidia of both isolates showed an extracellular matrix. Figure 2e shows EH-506/3 conidia with extracellular matrix at the rachis area. The EH-520/3-treated nymphs showed large amounts of the matrix, even at a

Table 1. Fungal Growth Development Index (FGDI) of high (EH-506/3), medium (EH-503/3) and low (EH-520/3) pathogenicity *Isaria fumosorosea* isolates

Bioassay	EH-506/3	EH-503/3	EH-520/3
(h)	X ± SD	X ± SD	X ± SD
0	0^{a}	0^{a}	0^{a}
6	$0.5^{b} \pm 0.7$	$0^{a} \pm 0.14$	$0^{a} \pm 0.9$
12	$2.0^{b} \pm 0.3$	$0.5^{a} \pm 0.23$	$0.5^{a} \pm 0.18$
18	$2.5^{c} \pm 0.13$	$1.5^{b} \pm 0.21$	$0.5^{a} \pm 0.15$
24	$2.5^{c} \pm 0.1$	$2.0^{b} \pm 0.32$	$1.0^{a} \pm 0.21$
36	$2.5^{b} \pm 0.06$	$2.5^{b} \pm 0.18$	$1.5^{a} \pm 0.25$
48	$3.0^{b} \pm 0.13$	$3.0^{b} \pm 0.16$	$2.0^{a} \pm 0.34$
60	$3.0^{a} \pm 0.08$	$3.0^{a} \pm 0.09$	$3.0^{a} \pm 0.19$

X ± SD = median ± standard deviation.
Values are representative of three independent experiments run in triplicate.
Values in the same line marked with the same letter did not differ significantly according to Tukey´s test of multiple mean comparisons at a significance level of 5%.

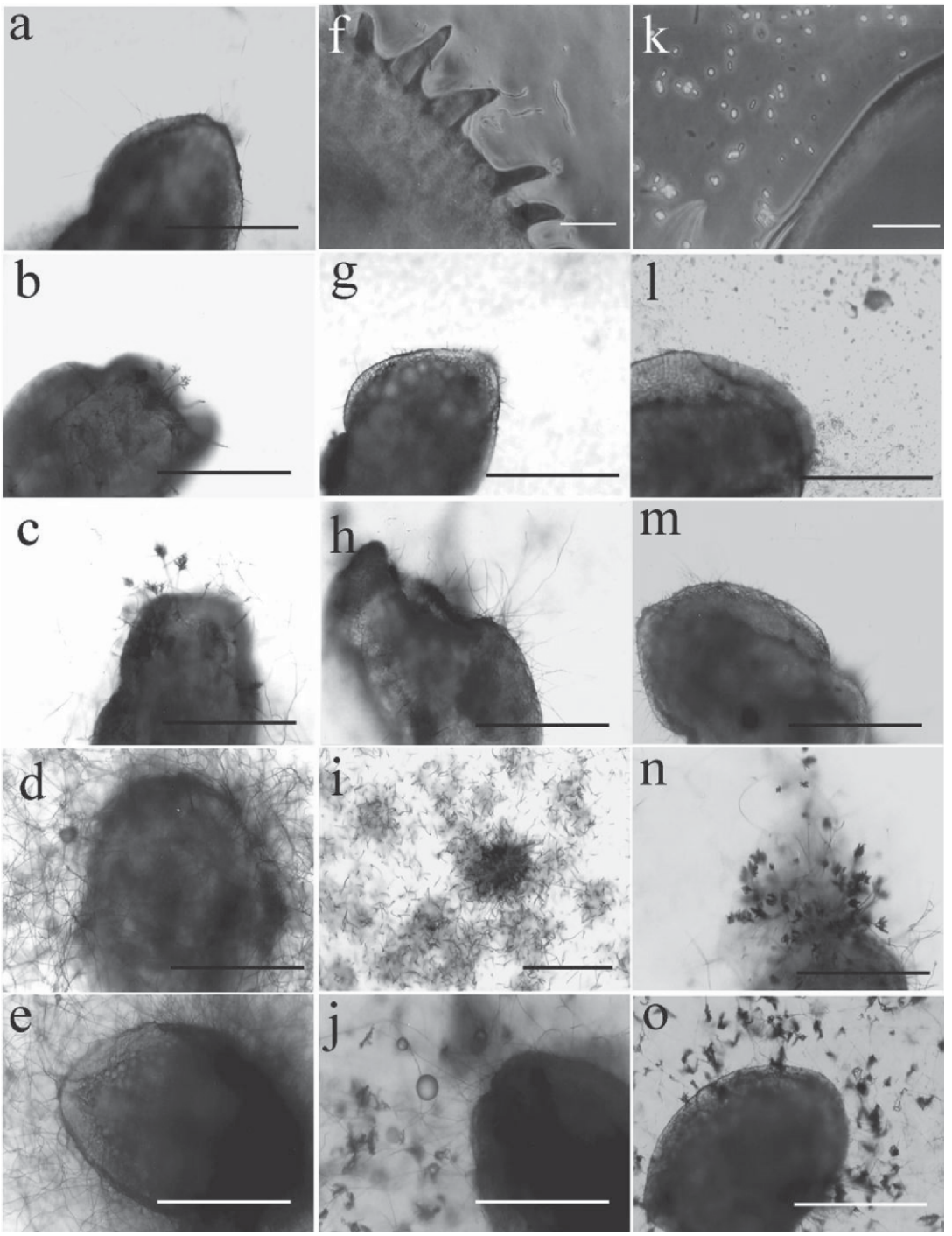

Figure 1. Comparison of isolates EH-506/3 (left column), EH-503/3 (middle column), and EH-520/3 (right column) of *Isaria fumosorosea* by the Fungal Growth Development Index (FGDI), at 12 (a, f, k), 18 (b, g, l), 24 (c, h, m), 60 (d, i, n) and 96 (e, j, o) hours of incubation. a) EH-506/3 hyphae emerging from a whitefly nymph at 12 h of incubation (FGDI = 2.0). f) EH-503/3 germ tube close to the nymph area at 12 h of incubation (FGDI = 0.5). k) EH-520/3 swollen conidia starting germination close to the whitefly area at 12 h of incubation (FGDI = 0.5). b) EH-506/3 conidiophores at the nymph surface at 18 h of incubation (FGDI = 2.5). g) EH-503/3 hyphae having first contact with the nymph at 18 h of incubation (FGDI = 1.5). l) EH-520/3 conidia with germ tubes closer to the nymph area at 18 h of incubation (FDGI = 0.5). c) EH-506/3 conidiophores sporulating over the nymph at 24 h of incubation (FGDI = 2.5). h) EH-503/3 hyphae on the surface with nymph deformation and destruction at 24 h of incubation (FGDI = 2.0). m) EH-520/3 initial colonization of the nymph at 24 h of incubation (FGDI = 1.0). d) EH-506/3 growing abundantly on the nymph at 60 h of incubation; conidiophores are not very evident (FGDI = 3.0). i) EH-503/3 conidiophores emerging from all nymph surface at 60 h of incubation (FGDI = 3.0). n) EH-520/3 conidiophores over the nymph at 60 h of incubation (FGDI = 3). e) EH-506/3 growth on the nymph at 96 h of incubation (FGDI = 3.0). j) EH-503/3 growth over the nymph at 96 h of incubation (FGDI = 3.0). o) EH-520/3 conidiophore production at 96 h of incubation (FGDI = 3.0). All bars = 250 mμ; with the exception of f) and k) with 100 μm, and i) = 400 μm.

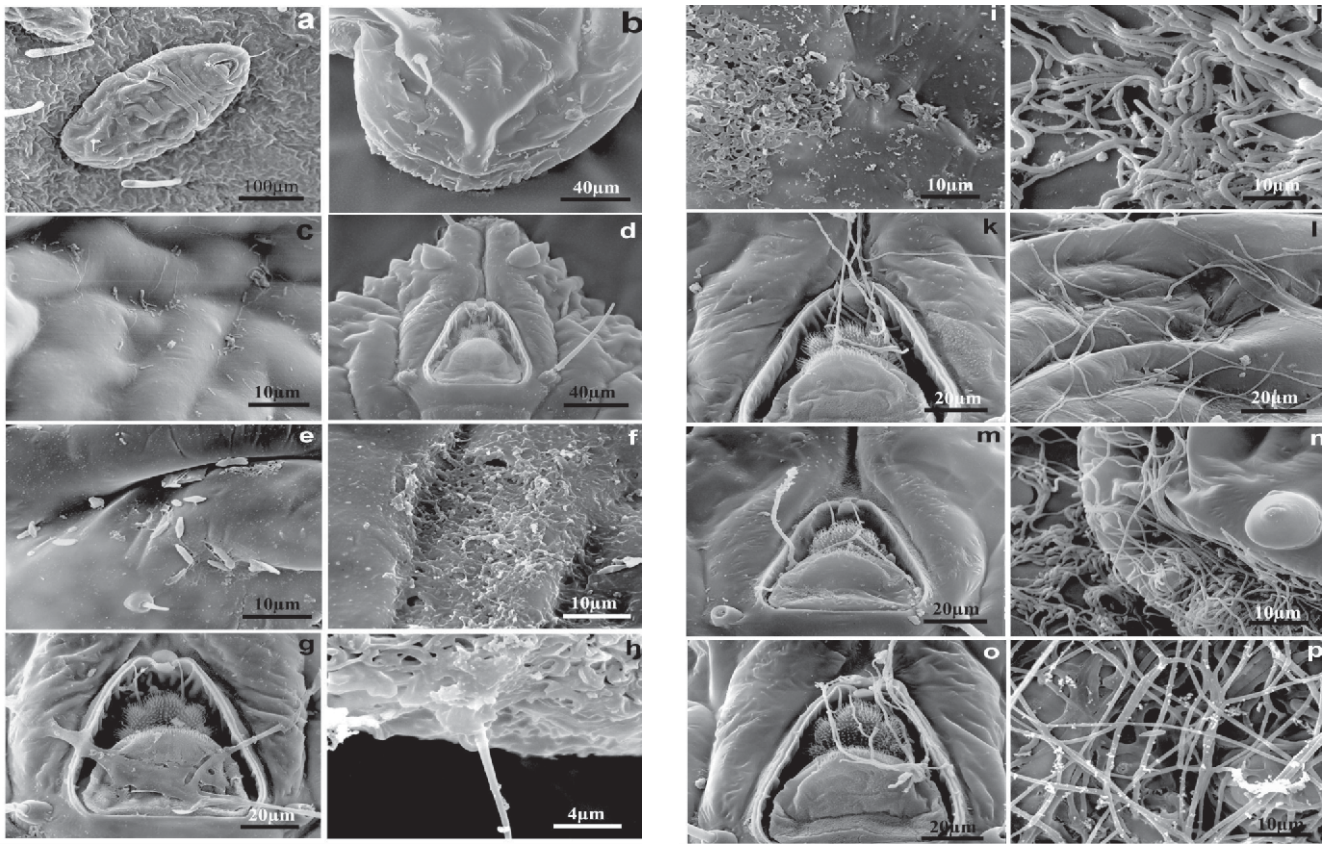

Figure 2. Scanning Electron Microscopy of *Isaria fumosorosea* EH-506/3 and EH-520/3 infected nymphs at 0, 6, 12, 18, 24 and 48 h of incubation. a) Intact whitefly control nymph on *Fuchsia* spp. b) EH-506/3 treated nymph showing a large amount of conidia at 0 h of incubation c) Naturally occurring microbiota at the nymph surface at 0 h of incubation. d) Vasiform orifice of EH-520/3 infected nymph at 0 h of incubation. e) EH-506/3 conidia adhering to the nymph surface with an extracellular matrix at 6 h of incubation. f) Ample cuticular damage on the EH-506/3 treated nymph surface after 6 h of incubation. g) EH-520/3 treated nymph with an extracellular matrix covering the vasiform orifice area. h) Cuticular damage on nymphs treated with EH-506/3 at 12 h of incubation, the photo shows gaps on the cuticle. i) EH-506/3 treated nymph covered with debris, most likely originating from cuticle degradation, at 12 h of incubation. j) EH-506/3 mycelial growth with mucilaginous matrix on the nymph at 18 h of incubation. k) Vasiform orifice of the nymph with EH-520/3 appressoria–like structures at 18 h of incubation. l) EH-520/3 hyphae at 18 h of incubation. The photo shows the extracellular matrix surrounding the hyphae. Direct penetration-like of the cuticle at the end of germ tubes is observed. m) EH-506/3 penetration-like hyphae with appressoria at the vasiform orifice, at 24 h of incubation. n) EH-520/3 mycelial growth on the nymph surface at 24 h of incubation. o) EH-520/3 hyphae penetration-like through the vasiform orifice at 24 h of incubation. p) EH-506/3 growing on the nymph surface at 48 h of incubation. The photo shows a gap on the cuticle and the mucilaginous matrix.

distance from the conidia. The EH-506/3-treated nymphs showed ample cuticular damage on the nymph surface after 6 h (Figure 2f). The vasiform orifice was occupied by this matrix, which trapped the small pieces of cuticle debris that were produced as a result of cuticle degradation (Figure 2g). Conidia that were starting to germinate were also observed. At this same time (6 h), the EH-520/3-treated nymphs also showed cuticular damage, but it was less severe than that of the EH-506/3-treated nymphs. After 12 h of incubation, the EH-506/3-treated nymphs displayed sustained cuticular damage and gaps on the nymph surface (Figure 2h). This surface was covered with an extracellular matrix, which was most likely produced by the isolate, as well as a large amount of cuticle debris deposited on the nymph surface, even at a distance from the fungal structures (Figure 2i). The development of hyphae continued and was faster and denser than that of EH-520/3. At this time (12 h), EH-520/3 formed structures at the tip of the germ tubes whose shapes resembled appressoria. These structures were observed mainly when hyphae developed at the rachis, where a thin extracellular matrix was also observed near the hyphae, appressoria and the vasiform orifice. When appressoria-like structures were at the

rachis, gaps with germ tubes orienting towards them were also observed. Apparent penetration of the cuticle and a slight clear zone at the appressorium tip were also observed. At 18 h, EH-506/3 showed dense mycelial growth and conidia with elongated germ tubes embedded in a thin mucilaginous matrix (Figure 2 j). Hyphal growth toward the vasiform orifice was also observed (data not shown). At this time, hyphae with appressoria-like structures were also observed. Hyphae developed and appeared to penetrate the insect in the area of the vasiform orifice in EH-520/3-treated nymphs (Figure 2k). Direct penetration of the cuticle (without appressoria-like structures) by the end of the germ tube was also observed. A mucilaginous matrix was seen to cover hyphae (in some instances) or other sites, suggesting the existence of an adherence mechanism between hyphae and the nearest cuticle (Figure 2 l).

At 24 h, the penetration of hyphae through the vasiform orifice (Figure 2m) was observed in EH-506/3-treated nymphs. EH-520/3-treated nymphs showed a dense hyphal layer covered with the same extracellular mucilaginous matrix observed on the nymph's surface (Figure 2n). At the vasiform orifice area, well-developed appressoria-like structures (Figure 2 o) and penetration-like of the hyphae through the orifice were observed. Structures that resembled developing phialides were observed at the hyphal tips (Figure 2o).

At 48 h, EH-506/3-treated nymphs were densely colonized with mycelia (Figure 2 p); under the mycelial mat, a thin mucilaginous matrix, cuticular damage and gaps could be observed. Conidia produced by mycelia were also observed. This same type of growth was observed on EH-520/3-treated nymphs (data not shown). At 60 h, all of the isolates had completed their life cycles, and the fungus had emerged from dead whitefly nymphs.

Discussion

The three isolates tested were shown to be virulent against *T. vaporariorum* whitefly nymphs. In this study, use of the FGDI permitted us to perform a detailed observation of the induction of fungal growth on the nymphs, as reported by Landa *et al*. (1994) regarding *P. fumosoroseus* (now *Isaria fumosorosea*) infecting *Bemisia argentifolii*. Conidia of EH-506/3, EH-503/3 and EH-520/3 were capable of adhering to the host's cuticle, germinating and producing infections. The EH-506/3 strain displayed the highest values of FGDI within the shortest time period, highlighting the differing pathogenicity of the isolates tested. The FGDI values and the previous CL_{50} data (Castellanos-Moguel, 2002) show that EH-506/3 is the most virulent isolate (FGDI = 2.5 at 18 h of incubation; CL_{50} = 1.1 x 10^3 conidia/mL), followed by EH-503/3 (FGDI = 1.5 at 18 h of incubation; CL_{50} = 2.5 x 10^4 conidia/mL) and EH-520/3 (FGDI = 0.5 at 18 h of incubation, CL_{50} = 7.6 x 10^4 conidia/mL). Between 60 and 96 h, the three tested isolates adhered, germinated, penetrated the nymphs and later sporulated on their surface. This development was faster than that reported by Landa *et al*. (1994) for *P. fumosoroseus* growing in *B. argentifolii* nymphs. Previous work regarding *Verticillium lecanii* (now *Lecanicillium lecanii*) growing on the aphid *Macrosiphum euphorbiae* reported the development of dense hyphal growth at 120 h of incubation (Askary *et al.,* 1999), and *M. anisopliae* var. *acridum* growing on *Lutzomya longipalpis* formed mycelia, anastomosis, appressoria and conidiophore primordia at 96 h of incubation (Amóra *et al*., 2010).

EH-506/3-treated nymphs showed mycelial growth that had apparently emerged from the nymphal body at 12 h of incubation, suggesting that penetration was achieved between 6 and 12 h of incubation. The fungus emerged from the whitefly to start the sporulation process at 18 h (Figure 1b).

Although this isolate infected whiteflies with a low apparent hyphal production, it grew abundantly on the insect's surface after host death, suggesting saprobic growth.

The EH-503/3-treated nymphs showed more abundant mycelial growth than EH-506/3. Moreover, the cuticle of these whiteflies was severely deformed (36 h post-incubation) or completely destroyed after 96 h of incubation. An enzymatic hydrolysis (Charnley, 2003) mechanism could lead to the cuticle damage induced by EH-503/3 because it has high chitinase production (Castellanos-Moguel *et al*., 2001). Nymphs treated with the other two isolates did not show this particular cuticle damage. The infection patterns we observed correspond to those reported by James *et al*. (2003) for *P. fumosoroseus* growing on third-instar silverleaf whiteflies.

Interestingly, more evident cuticle damage (and, in some insects, large perforations) was not observed with EH-520/3 and EH-506/3 isolates, suggesting that this damage was caused only by the fungal action of the EH-503/3 isolate. Cabanillas and Jones (2009) reported that *I. fumosorosea* isolates caused fungal-produced patches and collapse of the body wall of whitefly nymphs.

The SEM experiments were performed with EH-506/3 (high pathogenicity) and EH-520/3 (low pathogenicity) to compare the structure formation time and the mode of action of the two isolates. EH-506/3 has a high protease production, specifically Pr1 and Pr2 (Castellanos-Moguel *et al*., 2007), which may explain the intense and wide cuticular damage observed and most likely facilitated fungal penetration. These enzymes could also be responsible for the rapid emergence of the fungus after colonizing the insect (Small and Bidochka, 2005), a phenomenon that was observed for EH-506/3. These histolysis zones were covered with EH-506/3 mucilage, even far from the vicinity of the fungus. The EH-520/3-treated nymphs also displayed cuticular damage, but it was less extensive and only in the vicinity of the conidia. A similar phenomenon was observed as egg shell degradation associated with hyphae in *L. dimorphum* infection of the red scale insect of palms (*Phoenicoccus marlatii*) (Asensio *et al*., 2005), as well as in *M. anisopliae* infection of the western flower thrips (*Frankliniella occidentalis*) (Vestergaard *et al*., 1999).

In the present study, both tested isolates adhered to the cuticle in groups, consistent with the report of Yanagawa *et al*. (2008) for *P. fumosoroseus* in *Coptoptermes formosanus* cuticle; they then showed hyphal swelling, globose at the tip, that resembled appressoria in shape, which occurred mainly at the vasiform orifice and rachis areas of the nymph. In addition to the fact that the humidity of these areas promotes fungal germination, the presence of appressoria-like structures could be relevant because these structures have been implicated in cuticle penetration through exertion of mechanical forces by the rice blast fungus *Magnaporthe grisea* (Howard *et al*., 1991). However, these types of structures have not been observed by other authors in assays of *P. fumosoroseus* infection of *Plutella xylostella* (Altre and Vandenberg, 2001). These researchers reported that the fungi appeared to penetrate the cuticle directly with undifferentiated germ tubes within 22 h of inoculation.

The EH-506/3 and EH-503/3 isolates produced a mucilaginous extracellular matrix, which was more evident in isolate EH-520/3. This isolate also displayed a slight discoloration of the cuticle at the hyphal tips, which was likely caused by enzyme production. The extracellular matrix observed at the 6 h time point near conidia of both isolates EH-506/3 and EH-520/3 apparently conferred adhesion properties to the fungus. As mentioned by Askary *et al*. (1999), the mucilage matrix could have adhesive properties that facilitate penetration of *V. lecanii* during aphid invasion.

Invasive fungal growth was also observed in the two isolates. EH-506/3 showed faster but less abundant growth than EH-520/3, which is coincident with the development observed in the FGDI experiments. Our SEM observations showed that conidia formed a unipolar germ tube that

extended over a distance before penetration, as has been described by Askary *et al*. (1999) for *V. lecanii* invasion of the potato aphid *Macrosiphonella sanbornii*. However, these last authors mention that it was not possible to obtain clear evidence of the fungal entrance with SEM. In contrast, our observations showed that *I. fumosorosea* appears to penetrate directly through the cuticle (Figure 2l) at the vasiform orifice (Figure 2m) and rachis areas where appressoria-like structures were observed, suggesting that the penetration may be mediated by these structures. Liu *et al.* (2011) observed that *L. lecanii* can invade through the anus (the vasiform orifice in whiteflies) in nymphs of *Coccus hesperidium*. In other cuticle sections, we observed direct penetration of the fungus. This may be because the particular humidity conditions of the rachis and vasiform orifice create a microclimate that promotes appressoria development (Charnley, 2003) as well as cuticular toughness, as has been demonstrated for phytopathogenic fungi (Howard *et al*., 1991). In the rachis area, structures that resembled penetration pegs were observed; these structures have also been observed when *L. lecanii* is penetrating *C. hesperidum* nymphs (Liu *et al*., 2011). These authors also mentioned that this fungus forms a thick mycelial layer that covers the insect body, similar to that which we observed in both isolates of *I. fumosorosea* infecting whiteflies. The presence of phialides in both isolates after 24 h of incubation is intriguing, but abundant sporulation was not observed. Our results suggest that EH-506/3 is a suitable candidate for biocontrol due to the fungus's ability to rapidly colonize and emerge from the nymphs. However, this isolate has always shown a very low conidial production on slant and rice cultures (unpublished data). Therefore, we suggest the combined use of EH-506/3 with EH-503/3, an isolate with medium pathogenicity and high conidia production on slant cultures, for biocontrol purpose.

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