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Zoophthora radicans (Entomophthorales), a fungal pathogen of *Bagrada hilaris* and *Bactericera cockerelli* (Hemiptera: Pentatomidae and Triozidae): Prevalence, pathogenicity, and interplay of environmental influence, morphology, and sequence data on fungal identification

Reyna Ivonne Torres Acosta[a], Richard A. Humber[b], Sergio R. Sánchez-Peña[a,*]

[a] Departamento de Parasitología, Universidad Autónoma Agraria Antonio Narro, Saltillo, Coahuila C.P. 25315, Mexico
[b] Emerging Pests and Pathogens Research, USDA-ARS Robert W. Holley Center for Agriculture & Health, 538 Tower Road, Ithaca, NY 14853, USA

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ABSTRACT

The exotic bagrada bug or painted bug, *Bagrada hilaris*, and the native potato/tomato psyllid, *Bactericera* (=*Paratrioza*) *cockerelli*, are key pests of horticulture in western North America. In 2014–2015, adult and juvenile *B. hilaris* and *B. cockerelli* killed by fungi in the genus *Zoophthora* were detected near Saltillo, northeastern Mexico. We report the field prevalence and observations of *Zoophthora* on these hosts. The morphology and growth characteristics of field-collected specimens and pure *in vitro* cultures, as well as molecular markers (ITS1 and ITS4) were analyzed to identify these *Zoophthora* populations. Although there were morphological spore differences detected among field collections from both insect hosts, the fungi causing these mycoses can be identified as the same species (*Zoophthora radicans*), according to morphometric data from *in vitro* cultures (where differences observed in field material were attenuated) and sequence data (96–99% identity for ITS1 and 4). These results underscore the plasticity of field collections and *in vitro* cultures, and the relevance of comprehensive morphological and molecular analysis from cultures under standard conditions. Dose-response bioassays were conducted with one *Z. radicans* strain against bagrada bug nymphs. Exposure to conidial showers from cultures induced 30–90% mortality. This is the first report of a natural enemy of bagrada bug in Mexico, and the first published report of entomophthoralean fungi naturally attacking bagrada bugs and potato psyllids. *Z. radicans* should be further investigated as a tool in the biological control of hemipterans.

1. Introduction

Entomopathogenic fungi are important agents in biological control (Humber, 1991). Entomopathogenic fungi in the Entomophthorales (Zygomycota) demonstrate rapid sporulation and host invasion, high virulence, efficient dispersal from forcible conidial discharge, and they are often associated with epizootics in their host populations. A typical example of these fungi is *Zoophthora radicans*, which has been recorded as the causal agent of dramatic epizootics on insects across the world (Ullyett and Schonken, 1940; Velasco, 1983; Rietlmacher et al., 1992; Sosa-Gómez et al., 1994; Sánchez-Peña, 2000; Walter et al., 2003; Wraight et al., 2003; Barta and Cagán, 2006; Guzmán-Franco et al., 2008; Hassan, 2013). In some insect populations, *Z. radicans* is an important regulating agent; it can spread rapidly through a population and cause extensive mortality (Ullyett and Schonken, 1940; Manyangarirwa et al., 2011). The main host range of this fungus includes insects in the orders Coleoptera, Diptera, Lepidoptera and Hemiptera (Humber, 1991; Manyangarirwa et al., 2011).

The bagrada bug or painted bug is native to Africa and Asia; it was reported from California, USA in 2008 (Palumbo and Natwick, 2010), later on from additional states (Palumbo and Center, 2011; Bundy et al., 2012; Palumbo et al., 2015) and from Mexico in 2014 (Torres-Acosta and Sánchez-Peña, 2016); in both countries it is causing important damage to cruciferous crops. In the United States, the bagrada bug caused seedling stand losses as high as 70% (Palumbo et al., 2015). The potato/tomato psyllid, *Bactericera cockerelli* (Sulc.), is another primary pest native to North America, that causes significant economic damage in Mexico to potato, *Solanum tuberosum* L., tomato, *S. lycopersicum* L., and possibly pepper (chili), *Capsicum annum* L. The potato psyllid has recently been shown to transmit the bacterium "*Candidatus* Liberibacter solanacearum" that causes the often-catastrophic plant diseases: Zebra Chip or "papa manchada" (stained potato) in that plant (Lacey et al., 2011) and the disease known in Mexico as "permanente" on tomato. The potato psyllid has a short life cycle and can produce several generations per season, resulting in damaging densities. Its management often requires repeated applications of chemical pesticides (Goolsby et al., 2007; Sánchez-Peña et al., 2007; Mauchline et al., 2013; Tamayo-Mejía et al., 2014). We are conducting surveys of natural enemies of these pests in horticultural production systems and weeds near Saltillo, Coahuila, in northeastern Mexico. Preliminary observations indicated

* Corresponding author.
Email addresses: rita_taz84@hotmail.com (R.I. Torres Acosta); rah3@cornell.edu (R.A. Humber); sanchezcheco@gmail.com (S.R. Sánchez-Peña)

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the presence of entomopathogenic fungi infecting these hosts in the area; infections were observed first on *B. hilaris*, thus these observations are described first herein. The present work provides information on epizootics by and isolation of *Zoophthora* entomophthoralean fungi naturally infecting *B. hilaris* and *B. cockerelli* in the Saltillo area. We also analyzed the taxonomic identity of these *Zoophthora* isolates, and evaluated the pathogenicity of *Zoophthora* against bagrada bug in laboratory bioassays.

2. Material and methods

2.1. Observations on Bagrada hilaris

2.1.1. Field collection of bagrada bug

During a survey of natural enemies (parasitoids and pathogens) of bagrada bug, adults and nymphs of this insect were randomly sampled from host weeds in the Brassicaceae [rocket salad (*Eruca sativa* Mill.), pepperweed (*Lepidium latifolium* L.), common giant mustard (*Rapistrum rugosum* (L.) All.), and London rocket (*Sisymbrium irio* L.)] at the experimental fields of the Universidad Autónoma Agraria Antonio Narro (UAAAN), Saltillo, Coahuila, Mexico (N 25°21′12.09, W 101°1′49.097; 1743 m above sea level) from 7 October 2014 to 20 February 2015. Climatological data were obtained from a local meteorological station on campus (Saltillo).

2.1.2. Isolation of entomophthoralean fungi from bagrada bug

Fungi were isolated on agar culture medium (SDAYEYM): 40% Sabouraud dextrose agar (2 g Mycopeptone, 4 g dextrose, 4 g agar and 200 ml water), 40% yeast extract dextrose agar (1 g yeast extract, 2 g dextrose, 2 g agar and 100 ml water) (all reagents from Oxoid Ltd, Hampshire, UK) and 20% of a mixture of egg yolk (40%) and milk (60%) (i.e. 3 eggs yolks and 75 ml whole milk) (Papierok and Hajek, 1997; Papierok, 2007). Fungi were isolated from secondary conidia (obtained from fungal growth on field-collected insects) discharged onto agar plates as follows. Field-collected insects were placed individually inside the lid of 100-mm sterile petri dishes placed upside down. Each dish contained a 2 cm sterile cotton ball moistened with sterile water. After a 4–8 h period, primary conidia had been discharged onto the lid. The insect was remover and the lid with primary conidia was used to cover a petri dish bottom (normal position) containing SDAYEYM; secondary conidia were produced from primary conidia and discharged onto the agar below. After 3–5 days individual colonies of *Zoophthora* were aseptically transferred to fresh SDAYEYM media.

2.1.3. Identification of entomophthoralean fungi from bagrada bug

Infected bagrada bugs were examined using a Stemi DV4 stereomicroscope (Carl Zeiss AG, Oberkochen, Germany). Hyphae and conidiogenous material were taken from infected hosts, and stained in aceto-orcein (AO): 1 g orcein powder dissolved in 100 ml 1:1 acetic acid/water (v/v) (Keller, 1987) for observation of nuclei. Fungal structures in preparations (mycelium, conidiogenous cell, resting spores, and conidia) were measured using a Zeiss Standard 25 ICS microscope at 1000× with a Zeiss Eyepiece E-PL 10×/20, No. 444232. Fungal structures were also observed on a CKX41 microscope fitted with a IX2-SLP phase contrast slider (Olympus, Tokio, Japan). Photographs were taken with Nikon AW100 and D3200 cameras (Nikon, Tokio, Japan). Fungi were identified according to Humber (1989, 1997, 2012) and Keller (1987, 1991).

2.1.4. Zoophthora infection prevalence on field populations of bagrada bug

In the aforementioned sampling period (fall-winter, 7 October 2014–20 February 2015) 30–60 *B. hilaris* adults were randomly collected every week. Insects were placed individually in 30 ml cups and fed *S. irio* leaves in the laboratory. Insects were monitored daily for 96 h. Dead bagrada bugs with external evidence of mycelial growth were examined under the stereoscope for the presence of fungal rhizoids or conidia, and several of these insects (without conidia) were placed in moist chambers (24–72 h, 20 °C, 16L:8D photoperiod) to induce fungal sporulation. Infection data were obtained for each sampling date.

2.2. Observations on potato psyllid, B. cockerelli

2.2.1. Field collection of B. cockerelli and associated entomophthoralean fungi

Dead insects with apparent signs of fungal infection (Fig. 2A and B) were collected from "Habanero" pepper plants (*Capsicum annuum* var. *annuum*) grown in a 2-ha greenhouse located in Ramos Arizpe, Coahuila, Mexico (N 25°39′14″, W 101°6′47″). There was an ongoing fungal epizootic at the time of collection in the greenhouse.

2.2.2. Isolation and identification of entomophthoralean fungi from B. cockerelli

Isolation and identification methods are similar to those described in Sections 2.1.2 and 2.1.3 for *Z. radicans* from *B. hilaris*, following Keller (1987, 1991), Humber (1997, 2012), Papierok and Hajek (1997), Papierok (2007) and Hassan (2013).

2.2.3. Spatial distribution of infected B. cockerelli on plants in the greenhouse

Hundreds of fungus-killed insects were observed in the greenhouse attached to leaves and stems (Fig. 2A and B). To determine if there were spatial points with higher numbers of infected insects in the pepper plant canopy and greenhouse, the positions of infected insects on the canopy were registered. *B. cockerelli* adults and nymphs were sampled randomly cutting apical shoots (7–10 cm long) with leaves at different positions inside the greenhouse: Position (1) along plant rows, lower part (20–50 cm above ground); Position (2) along plant rows, higher part (200 cm above ground); Position (3) edges of rows, 20–200 cm high; and Position (4) center of rows, 20–200 cm high. An additional overall sample was made from shoots collected randomly through the crop. To prevent escape of nymphs, shoots and leaflets were placed in 10 × 10 cm Ziploc bags (S. C. Johnson, Racine, Wisconsin) along with a small, moistened cotton ball to induce fungal sporulation. Insects were kept at 25 ± 2 °C with 16L:8D photoperiod. To prevent inflated infection levels from horizontal transmission of fungus inside bags, plant material was examined (and numbers of infected nymphs and adults were obtained) under the microscope within 12 h of collection only. In this section, live adult insects were not considered because many escape during collection of shoots.

2.2.4. Prevalence of field infection in live B. cockerelli adults

Live adults of *B. cockerelli* (showing no signs of fungal infection) were also collected randomly from plants throughout the greenhouse with an aspirator, into a 300 ml plastic container with a small piece of moist cotton. The container was incubated in the laboratory and adult mortality was determined at 24, 48, 72 and 120 h. Dead individuals

were periodically removed from the container before sporulation occurred to avoid horizontal transmission to healthy insects.

2.3. Taxonomic resolution of Zoophthora isolates from B. hilaris and B. cockerelli: morphological methods and analysis

The *Zoophthora* isolates from *Bagrada* and *Bactericera* showed marked differences in conidial dimensions (see Section 3), indicating that fungal populations from these two hosts could belong to similar but distinct species. Therefore, isolates from each host insect were separately grown in the same artificial medium under identical laboratory conditions, in order to reevaluate the morphology of fungal structures *in vitro* and therefore resolve whether observed differences are taxonomically significant, or they result from environmental differences (i.e. host insects). One isolate from each insect host was grown in 100 ml liquid medium (6.5 g dextrose and 0.65 yeast extract/l) in 250 ml flasks; these were inoculated with 1 cm^2 agar piece (SDAYEYM) with active *Z. radicans* mycelial growth. The agar piece was placed inside the neck of the flask, allowing the discharge of conidia onto the broth below. Flasks were incubated on a shaker (150 rpm for 96 h under darkness). After 96 h, mycelial mats in the broth were harvested with a sterile loop and placed on 1.5% water agar in covered petri dishes. Dishes with mycelial mats were inverted and glass slides were placed inside dishes on the lid (below mats) once conidial production and discharge began. Conidia were discharged onto slides below for 1 h only, to avoid secondary conidial formation (resulting in conidia of different ontogeny and size) (Humber, 1989, 2012). Conidia on slides were immediately mounted in water and measured under the microscope at 1000× (Fig. 3A and B). Conidial measurements were: length (L), width (W) and L/W ratio. Measurements of primary conidia were statistically compared (Student's *t*-test, n = 22/strain) to detect significant differences. After measurements, conidia were stained in AO for nuclear visualization.

2.4. Taxonomic resolution of Zoophthora isolates from B. hilaris and B. cockerelli: molecular methods and analysis

Molecular identification of isolates proceeded as follows. For DNA extraction, *Zoophthora radicans* biomass (mycelium) from both strains (potato psyllid, ARSEF 13166 and bagrada bug, ARSEF 12081) was obtained from 10-day-old cultures grown on SDAYEYM agar (elaborated as described in Section 2) on 100-mm petri dishes. Genomic DNA was extracted from mycelial mats scraped off cultures, using the DNA Clean and Concentrator-25 (Zymoresearch, Irvine, CA, USA). DNA was quantified in a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

PCR amplification of internal transcribed spacers (ITS) 1 and 4 regions were carried out as described by White et al. (1990). Reactions were carried out in a volume of 45.3 µl consisting of 25 µl of 5× PCR buffer, 2.0 µl of genomic fungal DNA, 0.5 µl of primer ITS1, 0.5 µl of primer ITS4 R, 0.3 µl of MyTaqDNA polymerase 0.5 µl (all reagents from Bioline, Taunton, MA, USA) and 16.5 µl of MilliQ water. Primers utilized were:

ITS1: 5′-TCCGTAGGTGAACCTGCGG-3,
and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (White et al., 1990).

PCR amplifications were performed in a Veriti™ Thermal Cycler (Applied Biosystems, Waltham, Massachusetts, USA); conditions were: 94 °C (5 min), 35 cycles at 94 °C (45 s each), 55 °C (45 s), 72 °C (90 s) and 72 °C (5 min). Amplification products were visualized on 1.0% agarose gel stained with ethidium bromide. Nucleotide purification and sequencing of PCR products were performed in a 3130 Series Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). Marker sequences were compared by Basic Local Alignment Search Tool (BLAST) in GenBank looking for significantly homologous sequences. Extraction, PCR and sequencing were performed at the Molecular Services of LAMBAMA (Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental) of IPICYT (Instituto Potosino de Investigación Científica y Tecnológica), San Luis Potosí, Mexico.

2.5. Bioassay of Zoophthora radicans on second-instar Bagrada hilaris nymphs

The virulence of fungal conidia against bagrada bugs was assessed through exposure to conidial showers (Papierok and Hajek, 1997; Shah et al., 2004; Reyes-Rosas et al., 2012). F2 insects (second instar nymphs) used in bioassays were obtained from a laboratory colony (Bundy et al., 2012). Ten insects were placed in 30 ml P100 N plastic cups (33 mm tall and 43 mm mouth; Solo™, Dart Container Corp., Mason, Michigan). A window (12 × 12 mm) was cut on the lids of individual cups, and plastic mesh (1 × 1 mm grid) was glued with silicon to cover the window (Supplementary Material). Cups with insects were covered and small blocks (5 × 5 mm) of sporulating *Zoophthora* cultures on agar were placed (fungus culture down) on the screen on the covers of cups (outside). Cups with insects were placed inside disposable plastic lunch boxes (100 × 200 × 50 mm; Envases Cuellar, Saltillo, Mexico) lined with moistened paper towels (Supplementary Material). By removing the mycelial mats, insects were exposed to discharges of primary conidia from agar blocks for 6, 12, 18 and 24 h. Coverslips were placed on the bottom inside cups, to determine microscopically the inoculum density (conidia/mm^2) that insects were exposed to. Control insects were exposed to analogous fungus-free agar blocks for 24 h. There were three replicates/treatment (=conidial discharge time). Insects were incubated at 20–23 °C in a 16L:8D photoperiod. Mortality was recorded daily for six days.

Data analysis of bioassays: Mortality data from inoculation treatments were subjected to ANOVA, and treatment means were compared (Tukey's multiple range test). Statistical analyses were performed on SAS version 9.0 (2000).

3. Results

3.1. Observations on Bagrada hilaris

3.1.1. Field collection of bagrada bug

After its invasion of the Saltillo area (Sánchez-Peña, 2014) populations of the exotic bagrada bug were high during the sampled period (7 October 2014–20 February 2015); densities of 3–5 insects/m^2 were commonly observed. Bugs infected by entomopathogenic fungi were detected (Fig. 1A).

3.1.2. Identification of entomophthoralean fungi from bagrada bug

All entomophthoralean fungi from field-collected bagrada bugs were identified (by morphology) as *Zoophthora radicans*, according to the descriptions by Keller (1991) and Humber (2012). Depending upon relative humidity, mycelial development of *Z. radicans* on insects can display two different patterns. Under very high humidity or a saturated atmosphere, a granular, creamy-white mycelium (resembling sugar) emerges from insects (Fig. 1A). Production of distinctive conidiophores, primary and secondary conidia, capilliconidia and

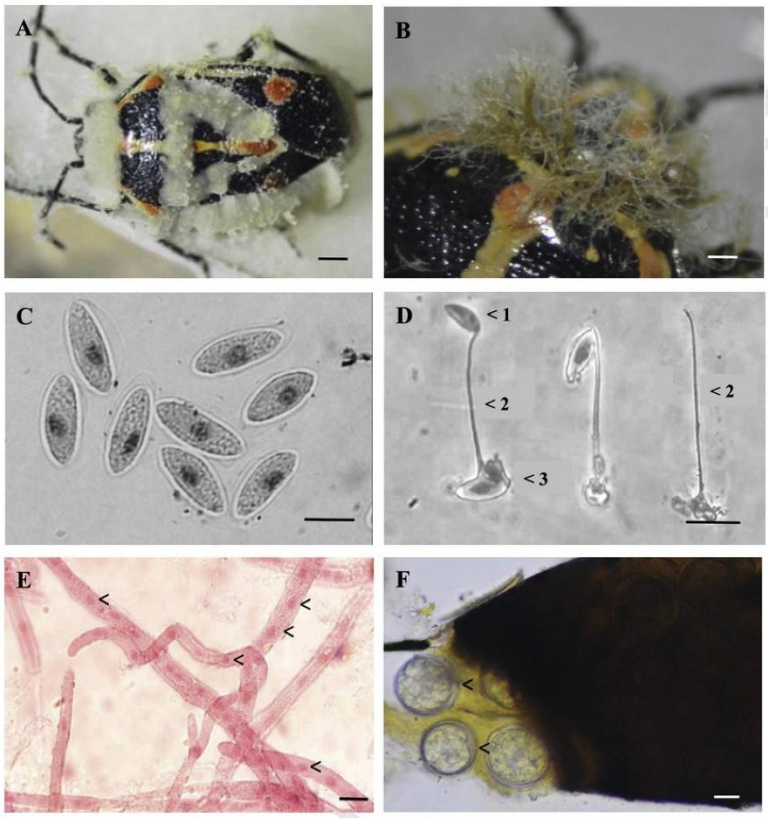

Fig. 1. *Zoophthora radicans* infection of *Bagrada hilaris*. (A) Adult of *B. hilaris* infected with *Z. radicans*. Mycelium and conidiophores emerging in high relative humidity. Bar = 1 mm. (B) Adult of *B. hilaris* infected with *Z. radicans*. Mycelium emerging under low relative humidity. Notice brownish color of mycelium. Bar = 0.3 mm. (C) Uninucleate primary conidia that are also bitunicate (thin outer layer or membrane separated from and surrounding main conidial body); aceto-orcein stain. Bar = 10 μm. (D) Capilliconidium (1) on capilliconidiophore (2) produced from primary conidium (3). Bar = 20 μm. (E) Hyphae with large nuclei (arrowheads); aceto-orcein stain. Bar = 5 μm. (F) Hialine resting spores inside the insect's abdomen. Bar = 10 μm.

capillary conidiophores is seen (Fig. 1C-D). Halos of conidia discharged from the branched conidiophores form around hosts. Under low humidities (near 60% RH), a second type of mycelial growth is seen on insects, characterized by thick hyphae that protrude through the exoskeleton proliferating as hirsute, branching hyphae that form a thick, tan-light brown thallus (Fig. 1B). In this mode of growth no conidia were observed, but these were produced within hours after transferring these infected insects to high humidity.

Measurements (in μm) for fungal structures listed below (from both field-collected hosts) are in Table 1. Vegetative hyphae are uniform in diameter, hyaline, multinucleate, coenocytic, with finely granular cytoplasmic contents, and vacuoles of variable sizes (Fig. 1E); through fungal cells (conidia and hyphae) nuclei are typical entomophthoraceous: granular, large, readily stained with aceto-orcein (Fig. 1C–E); sometimes they appear to be evenly spaced on a straight line in hyphae (Fig. 2E). The conidiophores are digitately branched. Primary conidia measure as follows (mean μm (minimum and maximum) ± standard deviation): length 17.8 (14–22) ± 2.4, width 7.5 (6–9) ± 0.7, length/width ratio 2.3 (2.0–2.4) ± 0.2. They are elongate-ellipsoid to short-cylindrical, with a conical to rounded papilla, conidial body demarcated by a very slight bulge from the papilla (Fig. 2C). All conidial types are uninucleate; typical entomophthoraceous, large, granular nuclei when stained with aceto-orcein (Fig. 1C and D). Conidia bitunicate (outer cell wall separates from inner wall, this separation or space appears like an almost complete, transparent halo around conidia: Fig. 1C, 3A). Both primary and forcibly discharged secondary conidia can germinate to produce hyphae, or they can form a single secondary or tertiary capilliconidium, respectively. Forcibly discharged secondary conidia are formed from primary conidia, similar to primary conidia but narrower. Capilliconidia are lunate, produced in line with, or at an angle to, the capillary conidiophore (Fig. 2D) and are passively dispersed when they adhere

Table 1
Main morphological features of *Zoophthora radicans* from field-collected *Bagrada hilaris* and *Bactericera cockerelli* hosts. Dimensions are mean (min.–max.) ± standard deviation (all in µm).

	Host	n	Length	Diameter	L/D ratio
Primary conidia	A	23	17.8 (14–22) ± 2.4	7.5 (6–9) ± 0.7	2.3 (2.0–2.4) ± 0.2
	B	15	22.7 (20–28) ± 2.04	7.6 (6–10) ± 0.7	2.9 (2.5–3.6) ± 0.29
Secondary conidia	A	23	18.2 (15–22) ± 1.8	6.5 (6–7) ± 0.5	2.8 (2.1–3.6) ± 0.3
	B	15	18.9 (15–22) ± 2.8	6.2 (5–8) ± 0.85	3.0 (2.5–3.6) ± 0.52
Mycelium	A	23	–	3.9 (2–5) ± 0.9	
	B	15	–	4.6 (4–5) ± 0.48	
Capillary conidia	A	2	16.0 (13–19) ± 3.0	6.5 (6–7) ± 0.5	2.4 (1.8–3.1) ± 6.0
	B	10	21.9 (21–23) ± 0.7	6 (5–7) ± 0.6	3.6 (3.0–4.6) ± 0.46
Capillary conidiophore	A	3	59 (53–66) ± 5.3	1.0	–
	B	17	49.11 (28–6) ± 10.8		

Resting spore			Wall thickness	Diameter
Mature	A	33	3.9 (2 − 5) ± 0.9	28.3 (25 − 35) ± 2.0
Immature	A	33	1.1 (1 − 2) ± 0.3	28.4 (26 − 31) ± 2.0

L/D ratio for capillary conidiophore is not provided because is not considered taxonomically relevant. A = *Bagrada hilaris*; B = *Bactericera cockerelli*. No resting spores were detected from field-collected *B. cockerelli*.

to passing arthropods. Each successive conidial generation is smaller in volume than the previous. Few specimens showed resting spores in the insect body; in these cases, no external mycelial growth was observed on insects. Resting spores are hyaline, spherical, and thick-walled (Fig. 2F) similar to those described by Sánchez-Peña (2000). Immature resting spores are thin-walled (Table 1). No discrete rhizoids but luxuriant mycelium growth attached some insects to the substrate.

3.1.3. Isolation of entomophthoralean fungi

Two *Z. radicans* strains (91 and 428) isolated from bagrada bug are deposited at the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Emerging Pests and Pathogens Research Unit, Ithaca, NY) as ARSEF 12800 and ARSEF 12801, respectively.

3.1.4. Zoophthora infection prevalence on field populations of bagrada bug

Zoophthora was detected on *B. hilaris* in the field in the following months (total number of infected insects/month− % infection): In 2014, October (8–3.8%), November (6–5%) and December (1–3.3%), and 2015, February (1–3.3%). A total of 450 insects were collected, of which 16 (3.6%) showed signs of *Zoophthora* infection. There were relatively cool temperatures (2–29 °C) for Saltillo during these months.

3.2. Observations on Zoophthora infections on Bactericera cockerelli

3.2.1. Field collection of B. cockerelli and associated entomophthoralean fungi

On 28 January 2015 there was an ongoing fungal epizootic caused by an entomophthoralean fungus in the commercial greenhouse grown to Habanero pepper (Fig. 2A and B). This greenhouse is 50 km on a straight line from the *Zoophthora*-infected populations of bagrada bug on field crucifers (Section 3.1.1). Infected specimens from the greenhouse showed spongy white mycelium protruding from the exoskeleton (Fig. 2A and B). Fungal infections were observed in adults (Fig. 2A) and in all five nymphal instars (Fig. 2B). No fungus-infected eggs were found.

3.2.2. Morphological description and isolation of Zoophthora from B. cockerelli

All entomophthoralean fungi from potato psyllids were identified as belonging to the genus *Zoophthora*, according to descriptions by Keller (1991) and Humber (2012). The conidiophores, primary conidia (Fig. 2C), capilliconidia (Fig. 2D) and mycelium (Fig. 2E) from the infected potato psyllids were similar to those described from bagrada bugs. Measurements (in µm) for fungal structures listed below (from both field-collected hosts) are in Table 1. Vegetative hyphae are uniform in diameter, hyaline, multinucleate, coenocytic, with finely granular cytoplasmic contents, and vacuoles of variable sizes; fungal cells (conidia and hyphae) possess typical entomophthoraceous nuclei: granular, large, readily stained with aceto-orcein (Fig. 2C–E). The conidiophores are digitately branched. Conidial dimensions were different and almost non-overlapping between *Bagrada* and *Bactericera* fungi; see list of morphological features in Table 1. Dimensions for primary conidia from *Bactericera* were (mean µm (minimum and maximum) ± standard deviation): length 22.7 (20–28) ± 2.04, width 7.6 (6–10) ± 0.7, and a length/width ratio of 2.9 (2.5–3.6). Primary conidia bitunicate, i.e. they present a thin outer layer (wall) separated from main conidial body and appearing like a transparent halo: this halo is not apparent in Fig. 2C, but it does surround the conidia partially except for the tip, as in Fig. 3. Secondary conidia as in *Bagrada*. Capilliconidia are lunate, produced in line with, or at an angle to, the capillary conidiophore (Fig. 1D) and are passively dispersed when they adhere to passing arthropods. Each successive conidial generation is smaller in volume than the previous. All conidial types are uninucleate; typical entomophthoraceous, large, granular nuclei when stained with aceto-orcein (Fig. 2C and D). Resting spores were not found in this host. On *B. cockerelli*, cadavers showed fasciculate rhizoids: specialized bundles of dozens of hyphae not thicker than vegetative hyphae, emerging ventrally from the abdomen and terminating in an anchor- or fan-like pad that adheres the insect to the substrate (Fig. 2F). One strain (451) was isolated and deposited at the USDA-ARS Collection of Entomopathogenic Fungal Cultures as ARSEF 13166.

In the same place and time we observed the presence of aphids (Hemiptera: Aphididae) infected by other entomophthoralean fungi (*Entomophthora* cf. *chromaphidis* and *Pandora neoaphidis*). No aphids were found infected by *Zoophthora* (n = 18) despite seemingly high levels of *Z. radicans* inoculum in the greenhouse environment. Aphids are (uncommonly) natural hosts of *Z. radicans* (Humber, 1991).

3.2.3. Spatial distribution of infected B. cockerelli on plants in the greenhouse

Infected *B. cockerelli* nymphs and adults were found on stems, buds, and on both upper and lower surfaces of leaves. Infected insects were also found attached to the greenhouse mesh, support poles, and other surfaces. The percentages of infection in adults and nymphs at different locations in the greenhouse crop are presented in Table 2. The highest infection rate on *B. cockerelli* nymphs was found on apical shoots and leaves at 50 cm from the floor (32.3%) as opposed to

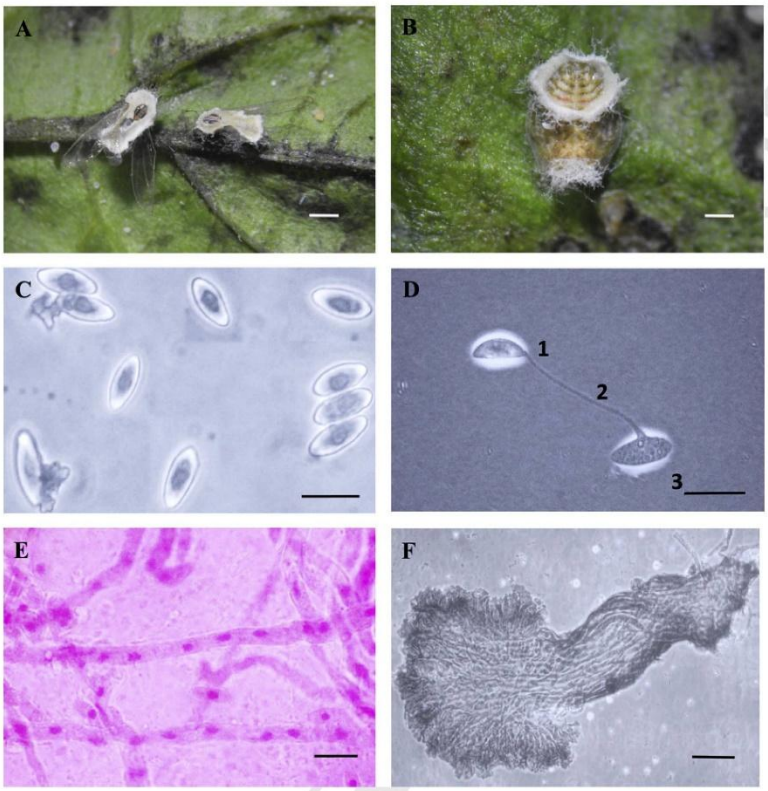

Fig. 2. *Zoophthora radicans* infection of *Bactericera cockerelli*. (A) Adults of *B. cockerelli* infected by *Z. radicans*. Bar = 2 mm. (B) Nymph of *B. cockerelli* infected by *Z. radicans*. Bar = 0.5 mm. (C) Uninucleate primary conidia, which are also bitunicate (thin outer layer or membrane separated from main conidial body; this layer is partial in photo, but it should surround the conidia except for the tip, as in Fig. 3A); aceto-orcein stain. Bar = 20 µm. (D) Capilliconidia (1) on capilliconidiophore (2) produced from primary conidium (3). Bar = 20 µm. (E) Hyphae with large nuclei; aceto-orcein stain. Bar = 10 µm. (F) Fan-shaped fasciculate rhizoid, made up of relatively undifferentiated hyphae growing from the insect's abdomen; aceto-orcein stain. Bar = 50 µm.

Table 2
Mean *Zoophthora radicans* infection on nymphs and adults of *Bactericera cockerelli* collected on leaves of greenhouse pepper plants, Ramos Arizpe, Coahuila, Mexico. Data are mean ± standard deviation.

Position in Greenhouse	n	Mean infected		Infected insects/ leaf[a]	Healthy nymphs/ leaf	Total nymphs/ leaf	Percent infected insects/ leaf[a]
		Adults	Nymphs				
1. Along rows, lower part of the crop (20–50 cm above ground)	7	1.15 ± 0.8	2.77 ± 3.8	3.9 ± 4.6	8.2 ± 6.8	12.1 ± 11.4	32.3
2. Along rows, high part of the crop (200 cm above ground)	14	2.64 ± 2.1	2.21 ± 1.5	4.8 ± 3.6	15.8 ± 8	20.7 ± 11.6	23.4
3. Edges of rows, 20–200 cm high	14	0.57 ± 0.7	5.14 ± 4.2	5.7 ± 4.9	9.7 ± 4.7	15.4 ± 9.6	37.0
4. Center of rows, 20–200 cm high	14	0.79 ± 1.2	0.93 ± 1.5	1.7 ± 2.7	8.5 ± 5.3	10.2 ± 8	16.7
Throughout the crop	49	5.15 ± 0.6	11.05 ± 1.4	16.2 ± 2.0	42.3 ± 1.5	58.5 ± 3.5	27.1

[a] Infected adults and nymphs.

23.4% infections on tips collected at 200 cm. Likewise, a higher percentage of infection was found on leaves from the edges of rows (37%) than on leaves collected inside the rows (16.7%).

3.2.4. Zoophthora infection prevalence on greenhouse populations of potato psyllid

A sample of 176 adult live insects collected in the greenhouse without apparent signs of infection upon collection, had cumulative *Zoophthora* infection rates of 40%, 74% and 76%, after 24, 72 and 120 h of incubation in the laboratory, respectively.

3.3. Taxonomic resolution of Zoophthora isolates from B. hilaris and B. cockerelli: morphological and molecular analysis

3.3.1. Morphological analysis

As mentioned, the morphological features [conidiophores, primary conidia (Fig. 1C and 2C), capilliconidia (Fig. 1D and 2D) and mycelium (Fig. 1E and 2E)] from field collections on *Bagrada* and *Bactericera* were similar when comparing material from both insect hosts, but primary conidial dimensions were different, essentially non-overlapping (Table 1). While conidial morphology from field *Bagrada* samples conformed well to the morphospecies concept of *Z. radicans* (Table 1) the *Bactericera* samples from the field were similar to *Zoophthora* species with larger conidia, like North American and Korean (small-spored) forms of the aphid pathogen *Zoophthora phalloides* (Glare et al., 1987; Yoon et al., 1998). Dimensions for primary conidia form the *Bactericera* fungus were: length 22.7 (20–28) ± 2.04, width 7.6 (6–10) ± 0.7, and a length/width ratio of 2.9 (2.5–3.6). The reported dimensions for the broad species concept of *Z. phalloides* are 24 48 × 6 14 µm, with a length/width ratio of 3.1 5.4 (Wilding and Brady, 1984).

Mycelium of *Bagrada* and *Bactericera* isolates grew in liquid medium and, when placed on water agar for 4 h, produced abundant primary conidia, secondary and tertiary fusiform conidia, and capillary conidia. Timing of spore discharge and development avoided mixing measurements of different conidial types. Measurements of primary and capillary conidia from *B. hilaris* and *B. cockerelli* are listed in Table 3. Student's *t*-tests detected no significant differences in length ($p = 0.4115$) or diameter ($p = 0.3087$) of primary conidia of *Zoophthora* among the isolates from the different hosts. These data suggest there is only one morphospecies, *Z. radicans*, infecting both hosts. On the other hand, the length/width (L/W) ratios of conidia from these hosts were significantly different ($p = 0.0001$); the isolates from *B. cockerelli* produced conspicuously more rotund conidia, and this feature even allowed the unambiguous assignment of each isolate to its original insect host after visual inspection of conidia under the microscope.

3.3.2. Molecular analysis

BLAST analysis resulted in the following maximum identity values with other *Z. radicans* accessions in GenBank: For the potato psyllid fungus (ARSEF 13166): 98% (ITS 1) and 96% (ITS 4). For the *Bagrada* fungus (ARSEF 12801): 96% (ITS 1) and 99% (ITS 4). Identity values for these Mexican strains with other GenBank accessions fall within values similar to those found when comparing other *Z. radicans* accessions among themselves. For example, BLAST analysis of the ITS 1 region of a randomly selected *Z. radicans* GenBank accession (strain NW323, accession EF137936.1) resulted in identity values of 95–99% with other *Z. radicans* accessions. These identity values confirm the assignment of these Mexican isolates to *Z. radicans*, as indicated firstly by morphological analysis. Identity values of 99 and 88% were obtained when comparing ITS1 and ITS4 sequences among both *Zoophthora* isolates, respectively. Table 4 shows GenBank accession numbers of sequences compared.

Table 3
Measurements of primary conidia, capillary conidia and capillary conidiophores produced by *Zoophthora radicans* strains grown in artificial medium; strains isolated from natural infections on *Bactericera cockerelli* and *Bagrada hilaris*. Dimensions are mean (min.–max.) ± std. dev. (all in µm). Length/Diameter (L/D) ratios are provided for primary conidia and capillary conidia; L/D ratios are not provided for capillary conidiophores because these data are not considered relevant.

Strain	n	Length	Diameter	L/D ratio
Measurements of primary conidia				
B. hilaris ARSEF 12801	23	21.70 (17–25) ± 2.15	10.71 (9–13) ± 2.51	2.12 (0.2–2.4) ± 1.83
B. cockerelli ARSEF 13166	24	22.20 (18–25) ± 2.02	8.9 (8–13) ± 1.1	2.51 (1.8–3.0) ± 0.29
P-value*		0.4115	0.3087	0.0001

ARSEF strain+	Fungal structure	n	Length	Diameter	L/D ratio of conidia
Measurements of capillary conidia and conidiophores					
13166	C-conidia[a]	14	19.2 (13–25) ± 2.9	5.2 (4–8) ± 0.8	3.6 (3.1–3.2)
12801	C-conidia	14	21.3 (12–26) ± 4.2	4.8 (4–5) ± 0.4	4.4 (3–5.2)
13166	C-conidiophore[b]	10	67.1 (38–80) ± 11.2	1.0	
12801	C-conidiophore	11	106.0 (65–141) ± 20.8	1.0	

* P-value for comparisons of conidial length, width and length/width ratio; for *Zoophthora* strains of *B. hilaris* vs. *B. cockerelli*; Student's *t*-test.

+ ARSEF 13166, *Z. radicans* strain from *Bactericera cockerelli*; ARSEF 12801, *Z. radicans* strain from *Bagrada hilaris*.

[a] Capillary conidia.

[b] Capillary conidiophore.

Table 4
ARSEF strains isolated from bagrada bug and potato psyllid, molecular markers (ITS) amplified, and sequence accession numbers of markers (GenBank).

Zoophthora Host	ARSEF accession	Sequence obtained	GenBank accession
Bagrada hilaris	ARSEF 12801	ITS1	KX267761
		ITS4	KX267762
Bactericera cockerelli	ARSEF 13166	ITS1	KX267763
		ITS4	KX267764

3.4. Bioassay of Z. radicans on second-instar B. hilaris nymphs

On all treatments the mortality of *B. hilaris* was rather uniform and progressive for six days after exposure to conidial showers (Table 5). Mortality was clearly dose-dependent; the highest mortality was recorded under the highest conidial concentration (Group 4: 24 h of deposition of a conidial shower resulting an inoculum density

Table 5
Cumulative percent mortality of second-instar nymphs of *Bagrada hilaris* exposed to conidial showers of *Zoophthora radicans* for different time periods.

Exposure[a]	Conidia/mm^2	Day 2[b]	Day 3	Day 4	Day 5	Day 6
24	1665	13.0 a[c]	16.6 a	36.6 a	56.6 a	90.0 a
18	483	10.0 a	30.0 a	36.6 a	46.6 ab	70.0 ab
12	171	0.0 a	16.6 a	30.0 ab	36.6 abc	53.0 bc
6	26	3.3 a	6.6 a	10.0 ab	16.6 bc	30.0 cd
Control	0	0.0 a	0.0 a	3.3 b	6.6 c	6.6 e

[a] Hours of exposure to conidial showers from *Z. radicans* cultures.

[b] Days after exposure to specified conidial shower.

[c] Means within a column followed by the same letter are not significantly different (Tukey test, $P < 0.05$).

of 1665 conidia/mm^2), followed by Group 3 (16 h; 483 conidia/mm^2), Group 2 (12 h; 171 conidia/mm^2) and finally Group 1 (6 h; 26 conidia/mm^2). After exposure to conidial showers, there were significant differences in mortality among treatments for the incubation periods as follows: day 4 ($F = 5.24$; gl = 4, 14; p = 0.0001), day 5 ($F = 7.44$; gl = 4,14; p = 0.0048) and day 6 ($F = 18.5$; gl = 4, 14; p = 0.0001) post-exposure to conidial showers. With the exception of the control groups, all dead insects from treatments were densely covered (dorsally and ventrally) by *Zoophthora* mycelium and sporulation was profuse, thus confirming the susceptibility of *B. hilaris* to *Z. radicans*. Control mortality was attributed to handling of insects.

4. Discussion

The present report describes the morphology, identification and bioassay of the fungal morphospecies *Z. radicans*, from two hemipteran host species field-collected at Saltillo, Mexico. It also provides ecological observations on these pathogen-host associations. In the case of the bagrada bug, Koch's postulates were fulfilled. In the Saltillo area, *B. hilaris* is an exotic (invasive) pest, while *B. cockerelli* is considered an indigenous pest.

The presence of *Z. radicans* infections in these populations was probably favored by the cool temperatures prevalent locally at the time of collection; also, by the relatively humid conditions in the respective crops, although both localities are located in Chihuahuan desert habitats. In the case of the bagrada bug, infections occurred on relatively high insect populations (mean of 3–5 insects/m^2) when temperatures from 7 p.m. to 8 a.m. were cool (down to 2° C). In the greenhouse, conditions of 20–30 °C and 70% RH, the pepper plants' dense foliage that favored a humid microclimate, and the high population of *B. cockerelli* (10.3–21.7 nymphs and adults/leaf) probably sustained the development of the observed epizootic. Ullyett and Schonken (1940) discussed epizootics of this fungus on larvae of diamondback moth (*Plutella xylostella*) and indicated that fungal outbreaks depended on high host population densities and favorable weather conditions: periods of rain and/or high relative humidity.

The morphological and molecular analysis of fungal isolates grown *in vitro* indicated that infections on different host species at sites separated by 50 km were caused by isolates of *Z. radicans*. It must be recalled that the conidial morphology of fungi initially observed from field-collected hosts was similar but distinctly different among *B. hilaris* and *B. cockerelli* samples (Table 1). In fact, the fungus from *B. hilaris* was similar to *Z. radicans*, while the conidial dimensions of the *B. cockerelli* fungal samples were similar to *Z. phalloides*; for example, seeYoon et al. (1998). However, this possible identification was rejected by data from artificial culture and sequencing. After identical growth conditions in the laboratory on the same artificial medium, the isolates from different hosts morphologically converged towards the production of very similar primary and capillary conidia that conform to the morphological concept of *Z. radicans* (Humber, 1989; Keller, 2007; Moura-Mascarin et al., 2012). These observations emphasize the natural variability of these fungi and the less-than-clear, but significant, influence of environment upon fungal morphology. Regarding entomophthoralean fungi, Lin and Harper (1984) observed a considerable gradual increase in conidial size of *Pandora gammae* (=*Entomophthora gammae*) after repeated subculturing on artificial medium. After inoculation of *Furia pieris* (=*Erynia pieris*) on different insect host species, Li and Humber (1984) reported significant variation on rhizoid morphology (sparse, monohyphal vs. profuse, fasciculate) to the point that these morphologies could probably be identified as different species if collected in the field. Growth under controlled conditions on artificial culture controls to some extent variation due to environmental (including host) influences on growth and development. Relatively unambiguous information for identification can thus be obtained and this should be complementary to nucleic acid sequence data (Guzmán-Franco et al., 2008). In summary, the available information indicates that the differences detected, in particular the conidial length/width ratio, should be attributed to intraspecific variation. However, additional observations might detect significant biological differences between isolates that could support the existence of a *Z. radicans* species complex, as has been suggested due to the extremely broad host range of this morphospecies (Balazy, 1993; Sánchez-Peña, 2000; Humber, 2016).

Molecular data have become universal tools for taxon identification. Unfortunately, the building of taxon-specific molecular databases (from sequencing or -omics) often proceeds *after* the organisms have been assigned to species based upon morphological characters, potentially leading to erroneous circular references. This situation underscores the importance of comprehensive analyses before particular fungal isolates or specimens are assigned to species during identification.

In addition to *Z. radicans*, *B. hilaris* individuals were also infected in the field by *Beauveria* cf. *bassiana*, *Metarhizium cf. anisopliae*, *Isaria fumosorosea* and *Fusarium* sp. (data not shown). All these fungi can be considered generalist insect pathogens (Humber, 1997). They are often important natural mortality factors, and can cause epizootics that significantly reduce arthropod host populations (Ullyett and Schonken, 1940; Sánchez-Peña, 2000; Barta and Cagán, 2006; Guzmán-Franco et al., 2008). The combined effect of multiple natural enemies like fungi might result in economically significant control of this pest. However, despite the importance of this pest in many countries (Palumbo and Natwick, 2010) very little is known about the ecology of entomopathogenic fungi that attack *B. hilaris*. At the localities reported herein, native entomopathogenic fungi were able to quickly exploit the sudden abundance of this invasive, newly arrived insect host. Regarding other natural enemies, initial observations on thousands of bagrada individuals detected no parasitic insects attacking the eggs, nymphs, or adults of bagrada bugs at Saltillo, Mexico (Torres-Acosta and Sánchez-Peña, 2016; unpublished observations).

The bioassay data showed the susceptibility of bagrada bug to *Z. radicans*. While there are number of reports on the screening of fungi in the Ascomycota as pathogens of pentatomid bugs (i.e. Ihara et al., 2001), there are few data on the susceptibility of bagrada bug to entomopathogenic fungi. After topical application in the laboratory of commercial products (conidia) of *M. anisopliae*, *I. fumosorosea* and *B. bassiana* against *B. hilaris*, Dara (2013) reported mortality levels of 45, 60 and 90% respectively, and indicated the potential of fungi in management of this pest. Regarding insect-pathogenic Entomophthorales, there are even fewer publications and no reports on *Z. radicans* tests against Pentatomidae. Hannam and Steinkraus (2010) reported very low field prevalence (<0.01%) of *Pandora heteropterae* (Entomophthoraceae) on *Lygus lineolaris* (Hemiptera: Miridae); in the laboratory, this fungus infected other bug families including Pentatomidae. There are several reports on bioassays of Entomophthorales against aphids (Hemiptera: Aphididae). Working with the aphid pathogen *Pandora neoaphidis* against the English grain aphid, *Sitobion avenae*, Nielsen et al. (2001) obtained a median lethal concentration (LC_{50}) of 1–5 conidia/mm^2; notwithstanding differences on insects and fungi, this is a seemingly lower value that those reported herein (i.e. 171 conidia/mm^2 resulted in 53% mortality after 6 days) (Table 5). Likely factors that influence mortality values in bagrada bugs compared to aphids include the much larger size of the bugs, their relatively sclerotized cuticle, and the remarkable production of defensive chemicals by stink bugs (pentatomids), several of which are antifungal (Lopes et al., 2015).

Concerning *B. cockerelli*, the activity of native and commercial fungi in laboratory bioassays has been reported. Sánchez-Peña et al. (2007), Mauchline et al. (2013), Tamayo-Mejía et al. (2014) and Villegas-Rodríguez et al. (2014) reported significant mortality of this insect after applications of *B. bassiana* and *M. anisopliae* conidia. Although *B. cockerelli* is a primary pest, there are no reports of epizootics in this insect caused by entomophthoralean fungi (Lacey et al., 2009). There are images of fungal infections attributed to *Z. radicans* on *B. cockerelli*, taken at Lincoln, New Zealand (Cranshaw, 2013, at bugwood.org) but no further information is provided on this insect-fungal pathogen combination.

Considerable research is required on pathobiology, ecology and application methods before considering *Z. radicans* as a biological control tool against hemipterans (Wraight et al., 2003). The observations reported herein seem to indicate that the greenhouse environment might be favorable for manipulating the pathogenic action of this fungus. .

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2016.07.017.

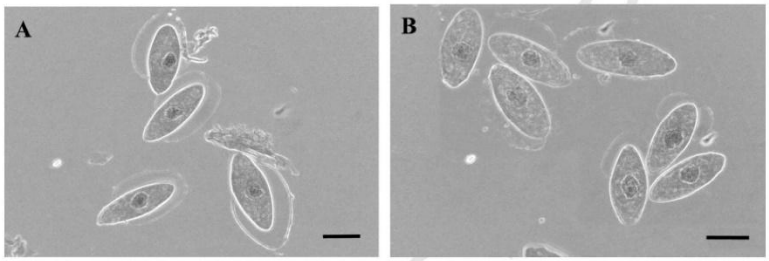

Fig. 3. Primary conidia of *Zoophthora radicans* obtained from mycelium grown in liquid medium in the laboratory. A, *Bagrada hilaris* strain. B, *Bactericera cockerelli* strain. Bars = 10 μm

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