Fungal Ecology 22 (2016) 43–51

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Seasonal prevalence of the insect pathogenic fungus *Colletotrichum nymphaeae* in Brazilian citrus groves under different chemical pesticide regimes☆

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ARTICLE INFO

Article history:
Received 5 August 2015
Received in revised form
8 March 2016
Accepted 13 April 2016
Available online 31 May 2016

Corresponding editor: Duur Aanen

Keywords:
Density-dependence
Natural epizootics
Entomopathogenic fungi
Praelongorthezia praelonga
Climatic factors
Conservational biocontrol

ABSTRACT

We report an endemic entomopathogenic fungus, known in Brazil as the 'salmão' fungus and identified here as *Colletotrichum nymphaeae* (Sordariomycetes: Glomerellales), infecting populations of citrus orthezia scale, *Praelongorthezia praelonga*. The seasonal prevalence of this pathogen in *P. praelonga* populations was investigated in three commercial citrus groves maintained under different pesticide regimes. Two citrus groves included inundative releases of another insect pathogenic fungus, *Lecanicillium longisporum*. Natural epizootics were consistently observed, with up to 84% infection rates being recorded during the warm rainy season. Temporal progression of *C. nymphaeae*-induced disease varied among the three pesticide regimes. Low infection levels from *C. nymphaeae* were associated with intensive application of broad spectrum pesticides. However, the prevalence of *C. nymphaeae* followed a density-dependent pattern with insect host abundance, irrespective of the pesticide regime. High proportions of *Lecanicillium*-infected insects were observed following infection peaks of *C. nymphaeae* and both fungi together contributed to 95% overall mortality of citrus orthezia during the wet season. Hence, the combined effect of both fungi considerably improves the biological control of citrus orthezia. We also surmise that the host abundance, environmental conditions, and application frequency of chemical pesticides in citrus groves exert a great influence in the seasonal prevalence of *C. nymphaeae*-induced disease. Altogether, these results suggest that *C. nymphaeae* is an important pathogen of *P. praelonga* and indicate that frequent use of synthetic pesticides may delay or reduce fungal epizootics.

1. Introduction

Brazil is the world's leading citrus producer with over 800,000 ha under production and an annual yield exceeding 19 million tons (IBGE, 2015). Phytophagous mites and phloem-feeding insects comprise a citrus pest complex associated with increased production costs (Smith and Peña, 2002). Chemical pesticides account for 32.5% of the total production costs in citrus produced in the state of São Paulo (Marzabal et al., 2004). The citrus orthezia, *Praelongorthezia praelonga* (Douglas) (Hemiptera: Ortheziidae), a

☆ Names are necessary to report factually on available data; however, we neither guarantee nor warrant the standard of the products, and the use of the name in this manuscript implies no approval of the product to the exclusion of others that may also be suitable. We dedicate this work to the late Dr. Sérgio Batista Alves due to his pioneering work on the 'salmão' fungus.

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http://dx.doi.org/10.1016/j.funeco.2016.04.005
1754-5048/

phloem-feeding scale insect, has emerged as an important pest throughout South America, the Caribbean Region and more recently the Africa-tropical region (Kondo et al., 2013). This noxious species causes severe yield losses to *Citrus* spp. due to direct feeding and indirectly through its honeydew secretions that support growth of *Capnodium* sp. (Ascomycota: Capnodiaceae), which reduces plant photosynthesis (Cesnik and Ferraz, 2003).

Endemic fungal pathogens that suppress arthropod pests play an important role in integrated pest management programs (Jaronski, 2010; Pell et al., 2010). Among these, entomopathogenic fungi are promising biocontrol agents for scale insects since they infect via the host cuticle (Hall and Papierok, 1982; Humber, 2008; Hesketh et al., 2010; Jaronski, 2010). In addition, the conidial stages of ascomycetes in the Hypocreales can be mass-produced and applied commercially. For example, *Lecanicillium* spp. have been used for many years in citrus orchards, as well as in propagation nurseries in glasshouses in Brazil and other countries, to control aphids, soft scale insects, thrips and whiteflies (Alves et al., 2002; Fargues et al., 2003; Faria and Wraight, 2007; Goettel et al., 2008).

Cesnik and Ferraz (2000) reported an ascomycetous fungus identified as *Colletotrichum gloeosporioides* f. sp. *ortheziidae* (Sordariomycetes: Glomerellales: Glomerellaceae) infecting *P. praelonga* in citrus in São Paulo state. This fungus was later identified as *Colletotrichum nymphaeae* by Damm et al. (2012). This observation is interesting since *Colletotrichum* species are known plant pathogens that cause anthracnose in several commercial crops worldwide (Horowitz et al., 2004; Sreenivasaprasad and Talhinhas, 2005). According to Teixeira et al. (2001), *C. nymphaeae* may have developed pathogenicity against *P. praelonga* based on its close association with the citrus pest complex. Owing to the importance of this scale insect as an emerging citrus pest, we are interested in the population dynamics of this entomopathogenic fungus with its host under field conditions.

There is little published information about *Colletotrichum* species infecting insects (Cesnik and Ferraz, 2000; Marcelino et al., 2008, 2009a, 2009b; Damm et al., 2012) nor epizootiology of the citrus orthezia/*Colletotrichum* association. Here we investigated the temporal dynamics of the 'salmão' (salmon) fungus, so called because of the orange-pink (salmon) color of its conidia on affected hosts, with the citrus orthezia population under field conditions and correlated with citrus groves under different pesticidal use practices in a commercial citrus farm in Matão, SP, Brazil, over 18 months between 2004 and 2006. Additionally, we ascertained the potential impact of agrochemicals and climate on the natural occurrence of this insect pathogen in citrus orthezia populations. *Lecanicillium longisporum* (Sordariomycetes: Hypocreales: Cordycipitaceae) was also applied as a microbial pesticide against this scale insect in two citrus groves. Therefore, we also assessed whether infection by *L. longisporum* influenced the *Colletotrichum* disease dynamics. We hypothesize that broad-spectrum chemical pesticides might disrupt the establishment of natural *Colletotrichum* epizootics in *P. praelonga*.

2. Materials and methods

2.1. Molecular and morphological identification of Colletotrichum sp.

Two isolates of *Colletotrichum* sp. were originally collected from citrus orthezia scale in Matão (ESALQ-1393) and Cordeirópolis (ESALQ-1368), São Paulo state, Brazil. These fungal isolates were previously purified *in vitro* and subsequently deposited in the Insect Pathology and Microbial Control Laboratory from ESALQ-USP (Piracicaba, Brazil). Fungal inoculum was obtained from frozen stock cultures (−20 °C) plated on potato-dextrose-agar (PDA, Difco®) and grown for 10 d (26 °C with 12 h photoperiod) to further provide conidia for inoculation of 100 ml potato-dextrose broth (Difco®). Liquid cultures were grown for 5 d at 26 °C under total darkness to produce mycelia for DNA extraction. Fungal biomass was vacuum-filtered using filter paper (Whatman #1) and macerated in liquid nitrogen using a mortar and pestle. The powder was transferred to microcentrifuge tubes containing 0.6 ml lysis buffer (0.2 M Tris-HCl pH 8.0, 0.25 M NaCl, 0.025 M EDTA, 1% SDS). The samples were vortexed and incubated for 30 min at 65 °C. Phenol-chloroform (1:1 ratio) was added, mixed thoroughly and centrifuged. The aqueous layer was transferred to a new tube, mixed with an equal volume of chloroform, and the phases separated by centrifugation. Five hundred μl of the aqueous supernatant were transferred to a fresh tube, and the DNA was precipitated with isopropyl alcohol and 5 M potassium acetate (10% v/v). The DNA precipitate was collected by centrifugation, dried with 70% ethanol, and re-suspended in 0.05 ml nuclease-free water. RNA was removed by digestion with 3 μl RNase A (10 mg/ml) at 37 °C for 30 min. Genomic DNA was assessed with a 1% (w/v) agarose gel and compared to Lambda DNA/*Hin*d III Marker standards.

The beta-tubulin-encoding protein gene (*Tub2*), which is widely used in phylogenetic analyses of fungi (Sreenivasaprasad and Talhinhas, 2005), was amplified and sequenced using the primer pairs TBCA/TB5 (Talhinhas et al., 2002, 2005). Two extractions of DNA were used for each fungal isolate. PCR was performed using 10 μl buffer, dNTP (10 nM) 2 μl, primers (10 nM) 1.5 μl, Taq 5 U (Promega®) 0.25 μl and Milli-Q water to adjust final volume of 50 μl. We used 1 μl of DNA with concentration at 25 ng μl^{-1}. PCR conditions were 95 °C/2 min, 94 °C/1 min, 62 °C/1 min, and 72 °C/1 min (repeated 35×), and 72 °C/10 min. Products of PCR were purified by Wizard SV Gel and PCR clean-up system. GenBank data were used to compare *Tub2 Colletotrichum* sequences (Damm et al., 2012) with accession nos. EF593327 and EF593328 from strains ARSEF 4360 and EMA 26, respectively, originally obtained from *P. praelonga* in São Paulo, Brazil (Marcelino et al., 2008). To quantify similarity between DNA sequences, sequence alignment was performed in BLASTN 2.2.32+ (Zhang et al., 2000). Nucleotide sequences were deposited in the GenBank database (accession nos. KJ509199 and KJ509200).

For morphological characterization of *Colletotrichum*, conidiophores and aerial conidia were harvested from 10 d old sporulated PDA cultures, mounted on glass slides, stained with lactophenol cotton blue, and examined at 400× magnification with a phase-contrast microscope (Zeiss®, Germany). Furthermore, scanning electron microscopy was performed with mycosed insects (n = 10), collected from those samples of dead insects incubated in humid chambers for conidiation under laboratory (see subsection 2.4.). Additionally, cadavers were dried with ethanol, mounted on stubs, fixed in osmium tetroxide vapor, and sputter-coated with gold for 210 s before examination in a Zeiss 940A DSM scanning electron microscope.

2.2. Field sites

Studies were conducted between November 2004 and April 2006 in three commercial citrus groves (each 5 ha) planted with *Citrus sinensis* cv. Pera Rio (Rutaceae) in Matão, São Paulo, Brazil (21° 39.178′S/48° 32.626′W). Each survey area comprised 1000 mature orange trees (7–9 years old), planted with 6 × 8 m spacing. Climate data was obtained from a meteorological station <2 km from each of the citrus groves.

2.3. Pest management systems

Citrus groves receiving three differing phytosanitary practices

were categorized as 'intensive pesticide use' (area 21°38.193′S/48°29.726′W), 'limited pesticide use' (area 21°39.178′S/48°32.626′W), and 'low pesticide use' (area 21°39.134′S/48°32.634′W) (Table 1). Insecticides were applied against *P. praelonga* and other pests (e.g., phytophagous mites). A conidial formulation of *L. longisporum* (strain ESALQ-1300, collected from *P. praelonga* in a commercial citrus orchard in Bebedouro, SP, Brazil) was used as a supplement bioinsecticide in citrus groves with reduced ('low' and 'limited') chemical pesticides, and mechanical weed removal used in place of glyphosate was employed in these groves. All sprays of *L. longisporum* were applied in the evening (Jan, May, Jul, Aug, and Dec 2005) with a mist blower sprayer (2000 l capacity) at a label rate of 4 kg ha^{-1} (equivalent to 2×10^{12} viable conidia ha^{-1} in 2000 l of water). This fungus was grown on moist, pre-cooked rice (~45–50% initial moisture) at 26 °C and 12 h light regime for 10 d in polypropylene bags and further air-dried before storage. Conidial viability (i.e., germ tube exceeding the conidium diameter) was assessed upon inoculation on PDA medium after incubation for 24 h at 26 °C under total darkness. In all cases, inoculum exceeded 90% germination prior to each field application. Acaricides were used to manage phytophagous mites such as *Phyllocoptruta oleivora* and *Brevipalpus phoenicis* (Table 1). Other management practices, such as pruning, fertilization (foliar and soil), fungicidal treatments (e.g., copper, sulphur along with mineral oil) against citrus black spot and blossom blight diseases were conducted simultaneously in all citrus groves.

2.4. Sampling method and determination of fungal infection rate

Ten trees per citrus grove (treatment) were sampled monthly between November 2004 and April 2006. Sampled trees were selected for high *P. praelonga* infestation level (>50 insects per leaf) and were separated by at least 12 m. Sampling units consisted of four branches per tree with a total of 12 leaves, which were collected from four different quadrants (N, S, W, E) at 1.5–2.0 m height. Samples were evaluated for the presence of *P. praelonga* nymphs and adults under a dissecting microscope (Zeiss®, Germany) at 40× magnification. To confirm mycosis, dead insects were incubated at 26 °C with 12 h photoperiod for 5 d in Petri dishes lined with wet filter paper (~100% RH). *L. longisporum* and *Colletotrichum* sp. were easily identified later during sample examination on the basis of their differing morphological features: citrus orthezia infected with *Colletotrichum* sp. typically presents hyphae and conidiophores emerging between insect segments and bearing orange salmon-pigmented conidial masses, and cadavers are generally found firmly attached to vegetation by rhizoid-like hyphal extensions. *L. longisporum* is recognized by its extensive external growth and bright white-pigmented conidiophores with clusters of conidia emerging from the insect intersegmental membranes (Mascarin et al., 2016 – Figure S1). Cadavers with no clear signs of mycosis were mounted on glass slides with lactophenol cotton blue to identify the fungal species by examining the morphologies of the conidiophores and conidia.

2.5. Quantification of secondary fungal inoculum from P. praelonga cadavers

Mycosed insect cadavers presenting profuse sporulation were collected during field surveys and subsequently assessed for conidial production by *C. nymphaeae* and *L. longisporum* under laboratory conditions. Batches of cadavers (n = 13–81 per leaf) were transferred to glass vials containing 10 ml of 0.1% Tween® 80 (Sigma®) solution, vortexed for 1 min and sonicated for 2 min. Conidial suspensions were quantified using a hemocytometer (Neubauer, Hauss Scientific®, Horsham, US) under a phase-contrast microscope (400× magnification). For *L. longisporum*, sporulated cadavers were separated by insect life stage (nymphs and adult females), whereas samples for *Colletotrichum* comprised of cadavers from all life stages except eggs. There were four to six independent samples per fungus, and conidial production was calculated by dividing the total number of conidia counted by the number of cadavers found per leaf.

Table 1
Chemical and biological treatments within three chemical pesticide regimes performed in commercial citrus groves in Matão, São Paulo state, Brazil (from November 2004 to April 2006).

System/Products applied[a]	Classification	Active ingredient	Frequency of application
Low pesticide use			
Lecanicillium longisporum[b]	Bioinsecticide	Aerial conidia	5
Envidor®	Acaricide	Spirodiclofen	1
Sipcatin®	Acaricide	Cyhexatin	1
Limited pesticide use			
Lecanicillium longisporum	Bioinsecticide	Aerial conidia	5
Envidor®	Acaricide	Spirodiclofen	1
Vertimec®	Acaricide/Insecticide	Abamectin	1
Match®	Insecticide	Lufenuron	1
Dimetoato®	Acaricide/Insecticide	Dimethoate	1
Intensive pesticide use			
Malathion®	Insecticide	Malathion	4
Talstar®	Acaricide	Bifenthrin	1
Dimetoato Nortox®	Acaricide/Insecticide	Dimethoate	1
Karate®	Insecticide	Lambda-cyhalothrin	1
Sipcatin®	Acaricide	Cyhexatin	1
Decis®	Insecticide	Deltamethrin	2
Savey®	Acaricide	Thiazolidin carboxamide	1
Dicofol®	Acaricide	Dicofol	1
Cefanol®	Acaricide/Insecticide	Acephate	1
Torque®	Acaricide	Fenbutatin oxide	1
Envidor®	Insecticide	Spirodiclofen	1
Calypso®	Insecticide	Thiacloprid	1

[a] Other recommended agricultural practices, such as pruning, fertilization (foliar and soil), fungicidal treatments (e.g., copper, sulphur along with mineral oil) were conducted concurrently in all three management systems.
[b] *Lecanicillium longisporum* (strain ESALQ1300) was used in this study as an experimental application, since there is no commercially registered product based on this fungus in Brazil.

2.6. Statistical analysis

We used proportional mortality rates determined by each entomopathogenic fungus, as previously described, to examine their temporal prevalence in citrus orthezia populations and host-pathogen density relationship. Temporal infection by *Colletotrichum* or *Lecanicillium* in field surveys was analyzed using binomial generalized additive mixed model (GAMM) with different smoothing functions for each pesticide application system (i.e., grove) and a random intercept per sampled tree. In the model, $Y_{ijk} \sim \text{Binomial}(m_{ijk}, \pi_{ijk})$ represents the number of fungus-infected individuals of all stages (nymphs + adult females). We used a logit link function to model the probability of fungus-induced mortality (mycosis):

$$log\left(\frac{\pi_{ijk}}{1-\pi_{ijk}}\right) = \beta_0 + \tau_i + T_j + f_j(w_k)$$

where β_0 is the intercept, $\tau_i \sim N(0, \sigma^2_\tau)$, $i = 1, \ldots,10$, is the random effect associated with the i-th citrus tree, Tj, j-th $= 1, 2, 3$, is the fixed effect of the j-th treatment, w_k, $k = 1, \ldots,18$, is the k-th month, and $f_j(.)$ is a smoothing function with a P-spline basis. To test whether trends of temporal infection rates differed between management practices (i.e., pesticide regimes conducted in different citrus groves), we compared nested models using the Akaike Information Criterion (AIC) using the 'gamm4' package (Wood and Scheipl, 2014) in software R (R Core Team, 2015). The AIC is a measurement of goodness-of-fit of a model that adjusts the value of the log-likelihood parameter estimates based on the number of model parameters. As a means of interpretation, models with lower AICs correspond to a better fit of the data (Bolker et al., 2009).

To assess the correlation of *L. longisporum* infection rates with the proportion of insects infected by *Colletotrichum* over time, data were fitted using a logistic regression in a generalized linear mixed model (GLMM) and likelihood ratio test to compare nested models. The linear predictor may be written as

$$log\left(\frac{\pi_{ijk}}{1-\pi_{ijk}}\right) = \beta_0 + \beta_{1j} w_k + \beta_{2j} w_k^2 + \tau_i + w_{ijk}$$

where β_0 is the intercept,β_{1j} and β_{2j} are the linear and quadratic effects for the j-th treatment, j = 1, 2, 3, wk, k = 1,. . ., 18, is the k-th week, $\tau_i \sim N(0, \sigma^2_\tau)$, i = 1,. . ., 10, is the random effect associated with the i-th tree and $\omega ijk \sim N(0, \sigma^2_\omega)$, is the observational-level random effect included to model overdispersion.

The density dependence relationship between temporal *Colletotrichum* infection levels (cumulative proportion mortality calculated by aggregating mortality across monthly collections) and insect density (live + dead insects counted on 12 leaves per tree) was analyzed using quasi-binomial (overdispersed) generalized linear model with a logit link function:

$$Y_i = \frac{e^{(\beta_0 + \beta_1 X_i)}}{1 + e^{(\beta_0 + \beta_1 X_i)}}$$

where Y_i is the cumulative proportion of fungus-induced mortality, β_0 is the intercept, β_1 is the slope, and X_i is the insect density (in $\log_{10}$ scale), with $i = 1, \ldots,18$ as the i-th month. Finally, Spearman's rank test was carried out using R package 'HMISC' (Harrell, 2013) to compute correlations between biotic and abiotic variables.

We used a two-tailed Student–t test to compare secondary inoculum production in the field between *Colletotrichum* and *Lecanicillium*. To separate means of conidial production across insect life stages infected only with *Lecanicillium*, we used one-way analysis of variance (ANOVA) and Tukey HSD contrasts at $P < 0.05$.

3. Results

3.1. Molecular and morphological identification of Colletotrichum

The morphological and beta-tubulin 2 gene (*Tub2*) sequences of ESALQ1368 and ESALQ1393 (250 bp and 254 bp, respectively) were 100% identical to each other, and they were also consistent with *C. nymphaeae* sequences in GenBank (strains ARSEF 4360 and EMA), differing only by a single gap (99% BLAST match). The strains ESALQ1368 and ESALQ1393 produced dark orange aerial conidia measuring 4–19 μm in length and formed mucilaginous slime balls containing 2–50 conidia when cultures were grown on PDA for 10 d at 26 °C with 12 h photoperiod (Fig. 1). Sporulation on insect cadavers was characterized by orange salmon-colored masses of conidia emerging from the intersegmental membranes. Mycosis induced by *C. nymphaeae* in individuals of *P. praelonga* was distinguished from *L. longisporum* based on typical morphological features related to each fungal disease (Mascarin et al., 2016 – Figure S1).

3.2. Influence of abiotic conditions

Most rainfall occurred between November and March, when both temperature and relative humidity were relatively high (Fig. 2). The proportion of insect mortality induced by *C. nymphaeae* and scale density positively correlated with rainfall and temperature (Table 2). Intriguingly, neither insect density, fungal infection, nor precipitation correlated with relative humidity. Citrus orthezia infestation and *C. nymphaeae* disease prevalence were both highest during months characterized by rainy, warm environmental conditions (Figs. 2 and 3). Reductions in insect density and disease prevalence were observed during the winter and autumn seasons (April to September), characterized by cooler and drier conditions (Fig. 3).

3.3. Influence of biotic and management factors

The abundance of citrus orthezia (live + dead) varied across seasons as well as among citrus groves treated with differing pesticide regimes, and ranged from 24.8 to 176.7 insects per sample of 12 leaves. High insect densities (>100 insects/12 leaves) were associated with warm rainy conditions (Fig. 2). High *C. nymphaeae* infection levels in citrus orthezia populations (i.e., up to 84.1% infection in average for 10 trees) coincided with the rainy season (November to March) in citrus groves receiving low and limited pesticide applications (Fig. 3). The seasonal prevalence of *C. nymphaeae* in citrus orthezia populations was different among citrus groves under different pesticidal use regimes, as the AIC for the model which included three different smoothing functions was the smallest, indicating a better model fit (Table 3). Lower temporal *C. nymphaeae*-infection rates were found in the citrus grove under intensive pesticide regime rather than in the other citrus groves receiving low and limited pesticide treatment regimes (Fig. 3). Disease incidence due to *C. nymphaeae* varied greatly through time (0–100% infected insects per sample unit); the smoothing functions from fitted models were all significantly different from zero for the three pesticide regimes, indicating that these models explained well the temporal fluctuations of fungal disease (intensive: $F_{(6.4,\ 470.8)} = 15.70$, $P < 0.0001$, limited: $F_{(7.2,\ 470.8)} = 32.79$, $P < 0.0001$, low: $F_{(7.5,\ 470.8)} = 33.86$, $P < 0.0001$). In addition, *C. nymphaeae*-infection was positively correlated with host population size (i.e., total number of insects counted on 12 leaves) ($F_{(1,\ 47)} = 13.26$; $P = 0.0007$). This trend was independent of the

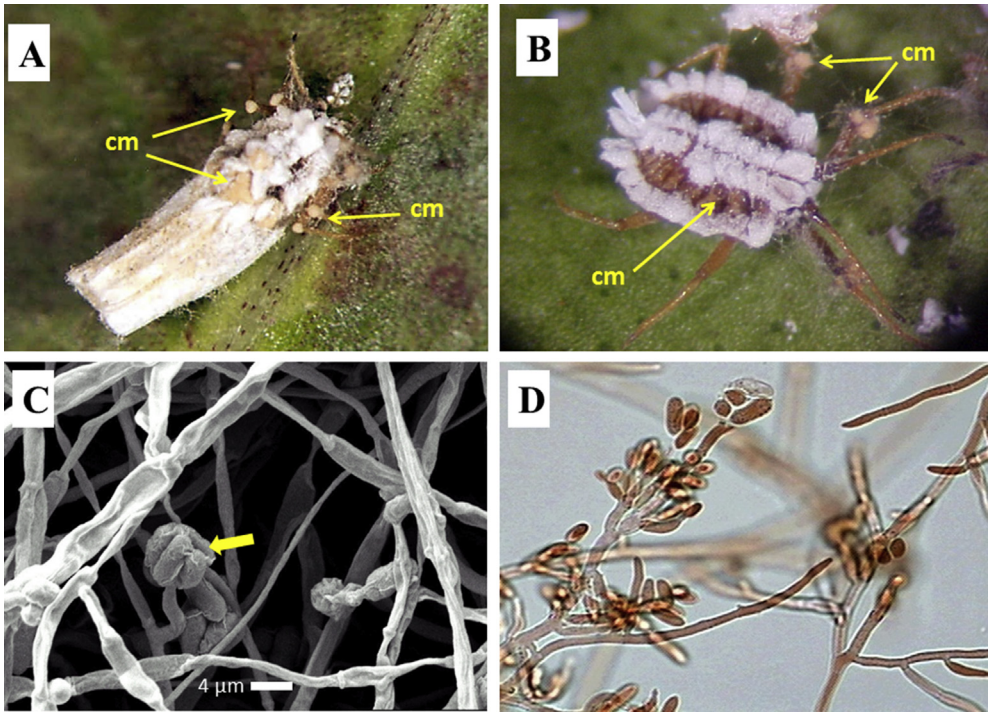

Fig. 1. Macro- and microscopic characterization of *Colletotrichum nymphaeae* mycosis. External growth of *C. nymphaeae* in *Praelongorthezia praelonga* adult female (A) and mycosed nymph (B) with typical cluster of orange salmon-colored conidia produced between the body segments such as thorax, abdomen, legs and antennae; (C) Scanning electron microphotograph depicting the mucilaginous matrix containing conidia (arrow); and (D) Microphotograph of conidiophores producing conidia on phialides (magnification 400×).

pesticide regime practiced due to the non-significance outcome for the interaction term (insect density × treatment) ($F_{(2,\ 45)} = 0.192$; $P = 0.83$) (Fig. 4). Infection levels by *L. longisporum* also varied considerably through time within the range of 0–100% for sample units and maximum average of 61.1% infection observed across months (n = 10 trees) (Fig. 3). Likewise the *C. nymphaeae* temporal trends, the generalized additive mixed models with different smoothing functions also described properly the temporal disease progression induced by *L. longisporum* in populations of citrus orthezia for all the three systems (intensive: $F_{(7.0,\ 473.2)} = 10.90$, $P < 0.0001$, limited: $F_{(5.8,\ 473.2)} = 14.85$, $P < 0.0001$, low: $F_{(5.9,\ 473.2)} = 11.91$, $P < 0.0001$). Furthermore, temporal *L. longisporum* disease trends were substantially different between the three pesticide regimes according to the smallest AIC recorded (Table 3).

Inundative applications of *L. longisporum* in citrus groves under low and limited pesticide regimes did not seem to enhance infection levels during drier months (April to September). During wetter periods an unidentified *Lecanicillium* species appeared consistently to cause high mortality in citrus orthezia in the citrus grove which was intensively managed solely with synthetic pesticides (i.e., intensive pesticide regime). High rates of infection by *L. longisporum* usually followed the occurrence of infection peaks due to *C. nymphaeae* (Fig. 3). We observed a significant quadratic relationship for all three pesticide regimes ($\chi^2_{(3)} = 19.02$, $P = 0.0003$), and fitted curves differed significantly between these pest management systems ($\chi^2_{(4)} = 21.96$, $P = 0.0002$). In all pesticide regimes, correlation trends indicated that increased infection rates by *L. longisporum* was also associated with decreases in *C. nymphaeae* infection rates, which implies an interespecific competition for the same host resource (Fig. 5). Overall, taking together both fungal infections over the entire period of survey, infection levels averaged from 1.2 to 94.7% and infection peaks consistently coincided with wetter and warmer months (data not shown).

3.4. Secondary inoculum production on cadavers from field surveys

Citrus orthezia attached to the leaves produced 2.8 times more conidia of *C. nymphaeae* compared with *L. longisporum* (i.e. $3.9 \pm 0.1 \times 107$ versus $1.4 \pm 0.3 \times 10^7$ conidia/insect) ($t_{(12)} = 7.93$, $P < 0.0001$). Significantly more conidia were produced by third instar cadavers ($2.4 \pm 0.1 \times 10^5$ conidia/insect) and adults ($2.6 \pm 0.1 \times 10^5$ conidia/insect) infected with *Lecanicillium* than by cadavers of younger nymphs (first instar: $3.7 \pm 0.3 \times 10^4$ and second instar: $4.4 \pm 0.1 \times 10^4$ conidia/insect) ($F_{(3,\ 12)} = 381.81$, $P < 0.0001$). We observed that both fungi were also able to colonize and to sporulate on the leaf surface surrounding the cadavers and usually accompanied by conidial production on these hyphal extensions.

4. Discussion

There is limited published information about *Colletotrichum* species infecting insects (Marcelino et al., 2008, 2009a, 2009b) and scarce knowledge concerning the temporal dynamics of citrus orthezia/*Colletotrichum* pathogen populations. In this study, we confirmed the identity of an endemic insect pathogen (identified here as *C. nymphaeae*) infecting the scale *P. praelonga* in citrus groves under different regimes of chemical pesticide use. Our model showed that *C. nymphaeae* epizootics were positively associated with humid and warm environmental conditions that also coincided with higher host densities. We noted *C. nymphaeae* always occurred in a density-dependent pattern, which agrees with epizootiological theory (Fuxa, 1987; Meyling and Hajek, 2010). Therefore, *C. nymphaeae* epizootics are more likely to coincide with high citrus orthezia outbreaks during the rainy warm season (November to March) in this region. Host/pathogen models accounting for pathogen dispersion, persistence, and host range in addition to seasonality may allow better predictions of this system.

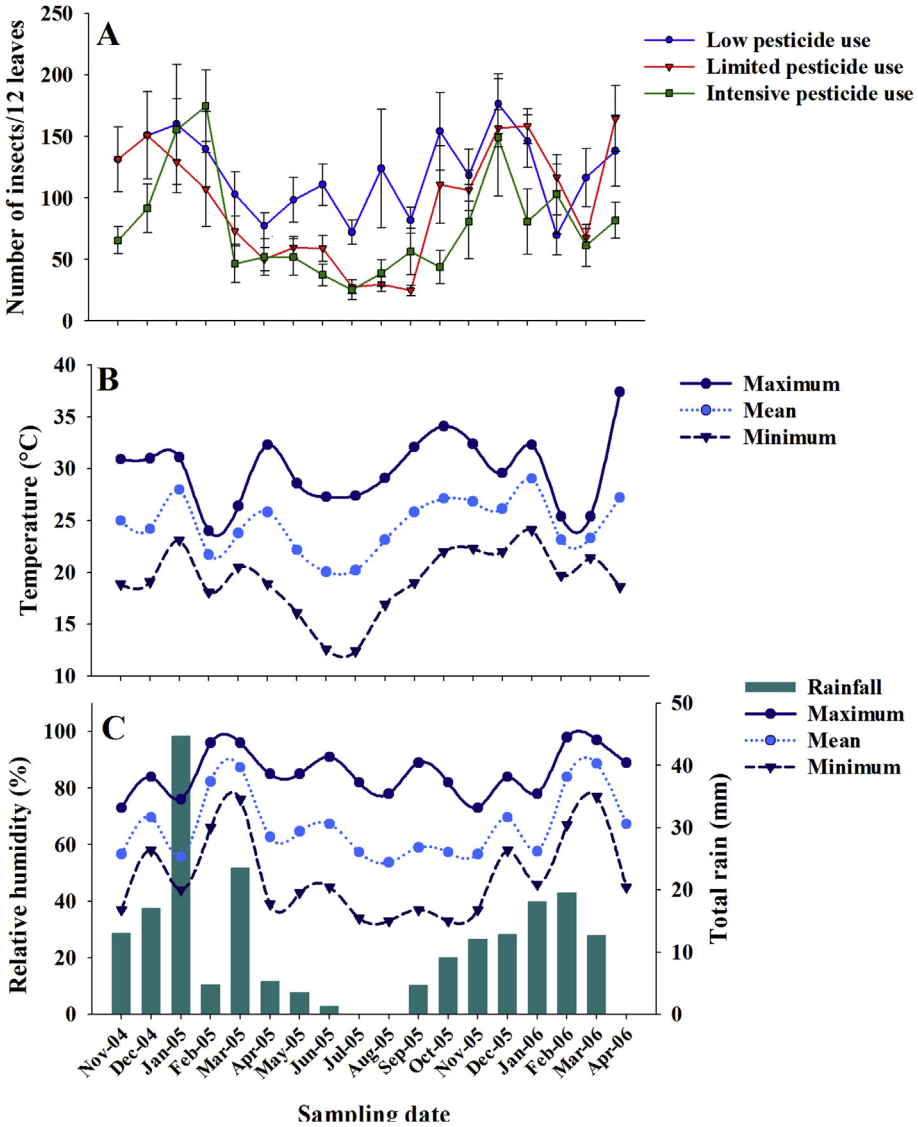

Fig. 2. Mean (±SE) population density (live + dead) of citrus orthezia (*Praelongorthezia praelonga*) under different pesticide application regimes (A) and climate data (B, C) recorded over 18-month period of survey in commercial citrus groves located in Matão, SP, Brazil (2004–2006). Lines in (B) and (C) represent maximum, mean, and minimum temperature or relative humidity.

Table 2
Spearman's correlation coefficients are presented to measure the relationship between abiotic (climatic conditions) and host/pathogen variables using data pooled across treatments.

Variables[a]	Collet	Temp	RH	Rain
N	0.51**	0.39**	0.07^{ns}	0.42**
Collet	–	0.38**	0.03 ^{ns}	0.35**
Temp	–	–	−0.37**	0.45**
RH	–	–	–	0.11 ^{ns}

[a] N = total number of insects (abundance); Collet = proportion of infected insects by *Colletotrichum nymphaeae*; Temp = monthly mean temperature in °C; RH = monthly mean relative humidity (%); Rain = monthly mean precipitation (mm). Significant correlations (r) with $n = 54$ at $P < 0.05$ (*), $P < 0.01$ (**), or not significant (ns).

The prevalence of this endemic fungal pathogen naturally infecting citrus orthezia under different managed field scenarios supports previous studies (Cesnik and Ferraz, 2000, 2003). More importantly, our data indicate that synthetic pesticides, namely insecticides, acaricides, fungicides, and herbicides can cause detrimental effects to the pest control capabilities of *C. nymphaeae* leading to reduced levels of infection. Our study suggests that chemical pesticide regimes may account for variation in the population dynamics of *C. nymphaeae* and citrus orthezia abundance across time. These results establish a host density and environmental relationship for the natural incidence of *C. nymphaeae*. Particularly, we observed that *C. nymphaeae* obtained >50% infection for three consecutive months (Dec 2005 to Feb 2006) in citrus groves receiving few and moderate pesticide inputs, but not in cases where more pesticides were applied (i.e., intensive application of pesticides). This finding may reflect direct toxicity and reduced host availability due to the frequent use of chemical pesticides. Previous reports have also documented reduced fungal epizootics due to synthetic insecticides lowering host density (Mietkiewski et al., 1997; Klingen and Haukeland, 2006; Wekesa et al., 2008). Cultural manipulations and reductions of unnecessary pesticide applications in citrus agroecosystems may favor the occurrence of *C. nymphaeae* and a subsequent reduction of

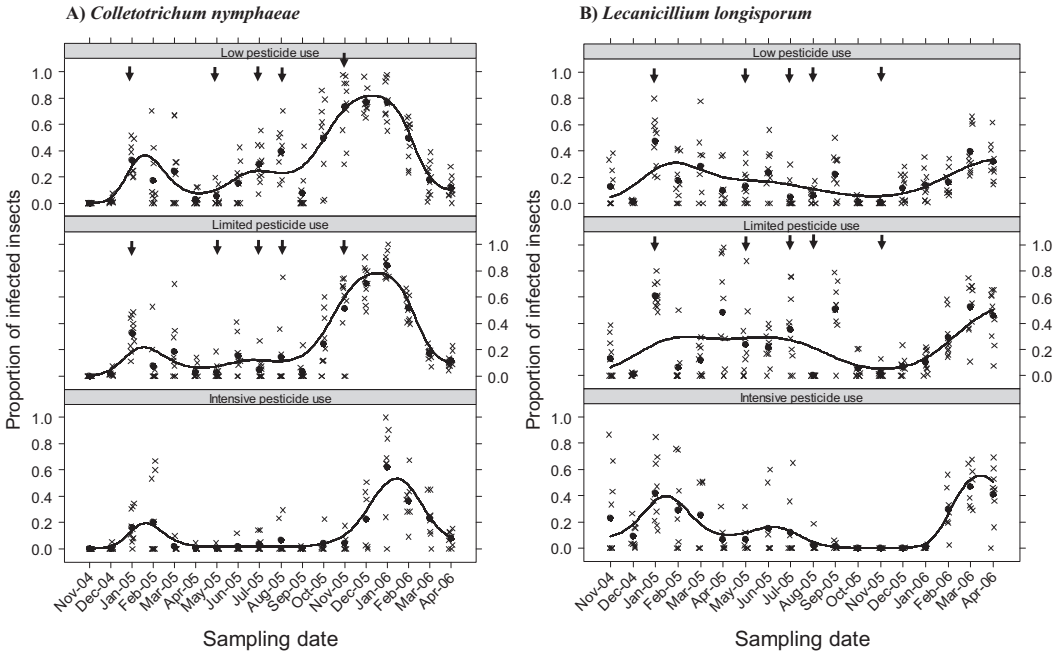

Fig. 3. Prevalence of *Colletotrichum nymphaeae* (A) and *Lecanicillium longisporum* (B) in the citrus orthezia (*Praelongorthezia praelonga*) population monitored in three pest management systems over 18 months. Cross symbol represents observed data, solid points are the estimated mean values, and solid lines denote the fitted curves by the binomial generalized additive mixed model (GAMM) (Adjusted $R^2 = 0.66$ [$P < 0.0001$] for *C. nymphaeae* and $R^2 = 0.23$ [$P < 0.0001$] for *L. longisporum*). Vertical arrows indicate repeated applications of *L. longisporum* (ESALQ-1300) in groves with low pesticide use and with limited pesticide use.

Table 3
Nested models were fitted by binomial generalized additive mixed models to compare treatments (chemical pesticides regimes in citrus groves) and model selection was performed based on the Akaike Information Criterion (AIC).

Model	AIC[a]	
	Colletotrichum prevalence	*Lecanicillium* prevalence
All treatments are different	8892.34	11536.37
Intensive and Limited are equal	9911.17	12324.04
Intensive and Low are equal	10014.12	12284.88
Limited and Low are equal	8979.75	11728.57
All treatments are equal	10209.31	12537.93

[a] Smaller AIC indicates a better model fit.

citrus orthezia outbreak levels on the trees.

C. nymphaeae and *L. longisporum* are both pathogenic to citrus orthezia under field conditions. Although we have applied *L. longisporum* in citrus groves under 'low' and 'limited' pesticide management, we observed that *C. nymphaeae* and *L. longisporum* occurred simultaneously among citrus orthezia from a given site, although no signs of dual infection were recorded in any individual insects. Our data suggest that inundative applications of *L. longisporum* may not reduce the prevalence of *C. nymphaeae* in citrus orthezia populations. Disease levels (as high as 95% infection) caused by both fungi improved overall biological control of citrus orthezia. Unexpectedly, we have found natural *Lecanicillium* infection levels up to 63% in citrus groves under intensive pesticidal use regime that did not receive any application of entomopathogenic fungi. This outcome could be attributed to an indigenous isolate of *Lecanicillium* or dissemination of *L. longisporum* from the other citrus groves sprayed with the fungus. Biotic factors that influence disease dynamics, such as the dispersal of entomopathogenic fungi by predators (Meyling and Hajek, 2010) or competition between predators and entomopathogenic fungi for the same host (Ong and Vandermeer, 2014), have been poorly studied in citrus agroecosystems.

Horizontal transmission from infected cadavers to healthy hosts allow sustaining fungal epizootics in arthropod populations (Fuxa, 1987). Our data demonstrate that *C. nymphaeae* produced many conidia on citrus orthezia cadavers. Sporulation on insect cadavers in field conditions has been associated with moisture, temperature, and host body size (Tanada and Kaya, 1993). Moisture is a key limiting abiotic factor for sporulation on host cadavers in some regions and seasons. Nonetheless, some fungal pathogens, such as *Entomophthora muscae* (Entomophthorales: Entomophthoraceae), have evolved the ability to sporulate under low ambient moisture (Kramer, 1980). Horizontal transmission of the fungus *Metarhizium rileyi* (Farlow) (formerly *Nomuraea rileyi*) (Ascomycota: Clavicipitaceae) on *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae requires an alternation of wet and dry environmental conditions (Kish and Allen, 1978). *C. nymphaeae*-induced disease was also reduced during drier environmental conditions. Despite the non-significant correlation of *C. nymphaeae* prevalence and ambient RH, microclimatic conditions on the leaf surface should be more important to fungal development, and these factors may not be revealed by ambient RH measurements (Boulard et al., 2002; Jaronski, 2010). We hypothesize that this ability of *C. nymphaeae* to persist during dry periods is key to its prevalence and

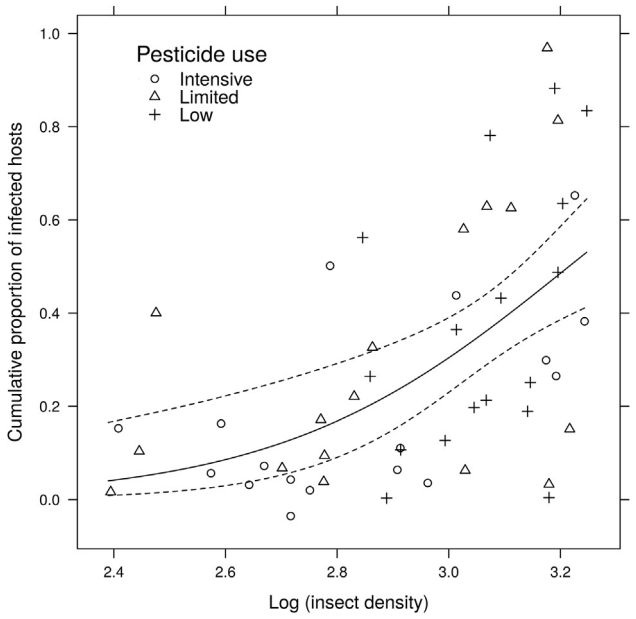

Fig. 4. Density dependent relationship for *Colletotrichum nymphaeae* based on cumulative proportion of infected hosts (*Praelongorthezia praelonga*) against host density. Data points (triangles) were pooled across ten trees and represent all pest management systems. Logistic regression model fitted to the experimental data (solid line trend): $Y = e^{(-12.4 + 3.8X)}/(1 + e^{(-12.4 + 3.8X)})$, adjusted $R^2 = 0.25$, $P < 0.001$; where Y is the proportion of cumulative mortality caused by *C. nymphaeae* and X is the insect density (in log scale). Symbols (triangle, circle, and cross) represent data points, solid line is the fitted logistic model, and dashed lines correspond to 95% confidence interval.

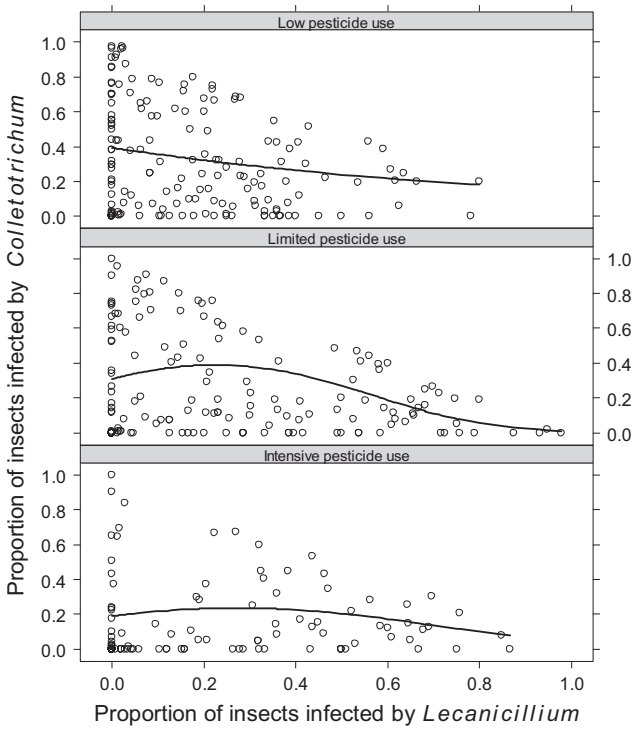

Fig. 5. Correlation between *Lecanicillium longisporum* and *Colletotrichum nymphaeae* temporal infection rates for three chemical pesticide regimes. Quadratic curves were significantly different among the three pesticide regimes and indicate that *L. longisporum* infection rates were correlated in a quadratic manner with *C. nymphaeae* temporal occurrence ($\chi^2_{(4)} = 21.96$, $P = 0.0002$).

establishment in citrus orthezia populations.

Colletotrichum species are generally documented as major plant pathogens with few reports from insects. However, it appears that some species of this group have evolved to infect insects (Marcelino et al., 2008; Damm et al., 2012). The first report of a *Colletotrichum* species infecting *P. praelonga* in Brazil was presented by Cesnik and Ferraz (2000), who referred to the fungus as *C. gloeosporioides* f. sp. *ortheziidae*, but Damm et al. (2012) later suggested that this fungus would be better identified as *C. nymphaeae*; however, intra-specific variability within *C. nymphaeae* that may be taxonomically significant has been found, and the overall taxonomy of this species remains unresolved. Although no natural epizootics of *C. nymphaeae* have been previously documented, Cesnik and Ferraz (2000) reported reductions of *P. praelonga* populations by 80–90% after applications of this fungus in a citrus field trial. More recently, *Colletotrichum acutatum* var. *fioriniae* was noted to cause epizootics on the elongate hemlock scale, *Fiorinia externa* (Hemiptera: Diaspididae), in the United States (Marcelino et al., 2008, 2009a, 2009b). To date, it appears that these are the two exceptional cases in which *Colletotrichum* was found associated with insects. Entomopathogenic fungi in the genus *Colletotrichum* might have been overlooked or misdiagnosed as plant pathogens, possibly because their different life histories and interactions with their insect hosts are complex or poorly understood, and because the general morphology of these fungi closely resembles that of such genera in the Hypocreales as *Aphanocladium*, *Synglyocladium*, and *Lecanicillium*. As a consequence, it is possible that some fungal infections in insects may have been misdiagnosed as caused by clavicipitoid fungi (Hypocreales), with a broad taxonomic diversity of entomopathogenic species, when in fact these infections might have been caused by *Colletotrichum* species.

In summary, this study reports *C. nymphaeae* as an endemic natural pathogen of an economically important scale insect pest in commercial citrus groves in Brazil. Conservational biological control strategies might be employed to increase inoculum levels of *C. nymphaeae* through crop irrigation or manipulation of cultural practices for enhancing natural control of citrus orthezia. Further studies of insect host and pathogen relationships in this group are needed.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

Our sincere gratitude to Cambuhy Agricola Ltd. through Dr. Fernando Amado Tersi and Paulo Sperandio for providing support for field studies. The second author was granted with a scholarship from CORPOICA (Corporación Colombiana de Investigación Agropecuaria). We are also grateful to anonymous reviewers for their insightful suggestions which improved this manuscript.

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