Spatial relationships between entomopathogenic nematodes and nematophagous fungi in Florida citrus orchards

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ARTICLE INFO

ABSTRACT

Relationships between entomopathogenic nematodes (EPNs), nematophagous fungi (NF) and soil physical and chemical properties were studied in a survey of 53 citrus orchards in central ridge and flatwoods ecoregions of Florida. Seven species of NF associated with nematodes were quantified directly using a real time qPCR assay. All nematophagous fungi studied except *Arthrobotrys musiformis* and *Hirsutella rhossiliensis* were frequently detected (24–56%) in both regions. *Paecilomyces lilacinus* and *Gamsylella gephyropagum* were encountered more frequently in the flatwoods ($P = 0.03$) and on the ridge ($P = 0.02$), respectively. Redundancy analysis revealed seven abiotic and biotic factors as significantly related to the NF occurrence. Multiple regression of fungi on these variables explained 78%, 66%, 48%, 36%, 23% and 4% of the variation in *Catenaria* sp., *A. musiformis*, *A. dactyloides*, *P. lilacinus*, *A. oligospora* and *G. gepharopagum*, respectively. When the data from citrus were pooled with those reported previously from natural areas and subjected to principle component analysis, the first two principle components explained 43% of the variation in NF communities. The surveys (citrus vs natural areas) were discriminated by PC2 ($P < 0.001$) and the ecoregion by PC1 ($P < 0.002$), and all but one NF species were related ($P < 0.01$) to one or both components. NF communities tended to have more species and greater diversity in the flatwoods, where EPN richness and diversity were the least. However, the strength of associations between individual EPN and NF species as measured by SADIE reflected the associations between each species and ground water depth, suggesting that ecoregion preferences affected the species associations. Within each ecoregion, significant relationships between the individual NF and EPN species measured by stepwise regression tended to be positive. The results did not support the hypothesis that NF modulate the spatial patterns of EPN species between or within these two ecoregions.

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1. Introduction

Entomopathogenic nematodes (EPNs) have been studied for more than a quarter century in Florida citrus orchards for their potential as components of root weevil IPM programs. Following the deregistration of soil-applied organochlorine pesticides in the mid-1970s, the invasive pest *Diaprepes abbreviatus* and other root weevils such as *Pachnaeus litus* became the most economically damaging arthropods in Florida citrus due to a lack of adequate control measures (Dolinski et al., 2012). Disrupting the weevil's long life cycle, which comprises both arboreal and subterranean stages, is difficult using non-persistent chemical and biological insecticides. The introduction of the bacterial disease huanglongbing (HLB) into the citrus industry, first detected in 2005 (Halbert, 2005), intensified the need for root weevil management because HLB destroys more than half of the fibrous roots on trees before aboveground symptoms become evident (Johnson et al., 2014). As a consequence, orchards damaged by both weevils and HLB decline much more rapidly than those affected by just the bacterium or weevil (Duncan, 2014).

The two primary genera of EPNs, *Steinernema* and *Heterorhabditis* (Dillman et al., 2012), are each represented by several native species in Florida. A survey of orchards (Campos-Herrera et al., 2013) and another of natural areas in the citrus growing regions of the state (Campos-Herrera et al., 2016), revealed similar associations between EPN communities and two ecoregions, the "central

ridge" running north to south in the middle of the peninsula and the "flatwoods" regions surrounding the central ridge and extending to the coasts. Orchards on the higher elevation, well drained sandy soils of the central ridge were almost always inhabited by both *H. indica* and *S. diaprepesi* and frequently also by *H. zealandica*. By contrast, EPN communities in orchards on lower elevation, shallow, less well drained soils of the flatwoods were almost always composed of *H. indica,* but only occasionally in combination with *S. diaprepesi* and/or an undescribed *Steinernema* species in the *S. glaseri* group proposed by Spiridonov et al. (2004). The population density of *D. abbreviatus* is larger in flatwoods orchards than in those on the central ridge (Futch et al., 2005), perhaps due in part to lower rates of biological control by EPNs in the flatwoods (Duncan et al., 2003). These patterns and their relationships suggest that natural control of weevils can be increased by discovering properties of habitats that can be modified in ways that conserve EPNs and enhance their services.

Soil properties that affect soil water potential explained the greatest amount of EPN variation in each of the two surveys by Campos-Herrera et al. (2013, 2016) and results of subsequent experiments supported the possibility that different EPN spatial patterns in Florida result partly from differential species adaptation to soil moisture (Duncan et al., 2013; El-Borai et al., 2016). Additionally, natural enemies such as nematophagous fungi (NF) and micro-arthropods regulate EPN numbers locally (Ekmen et al., 2010; Hodson et al., 2012) and could also modulate EPN spatial patterns in the landscape if their effects differ by region. NF comprise a guild of diverse fungi that include saprophyte/facultative predators (most notably the 'trapping fungi') and obligate predators of nematodes and other soil fauna such as Chytridiomycete species that produce infective zoospores. Because many members of the guild can function at different trophic levels, conventional methods of quantifying these fungi in soil samples do not discriminate propagules derived from nematode prey from those derived from other nutrient sources. The development of molecular tools that can quantify NF associations with nematodes in nature, without the artifacts introduced by culturing methods, provides a means to study the importance of habitat properties on those associations (Pathak et al., 2012). Campos-Herrera et al. (2016) detected primarily positive associations between NF and either free-living bactivorous nematodes or EPNs from nematode samples taken at 91 natural (non-agricultural) sites. Those relationships suggested that r-selected, rapidly increasing nematode species provided local resources for NF populations. The relative lack of negative associations might mean that NF predation of nematodes did not vary much across regions and did not affect EPN spatial patterns at the landscape scale. Jaffee et al. (1996) similarly concluded that NF were unrelated to the spatial pattern of *Heterorhabditus marelatus* that regulate subterranean ghost moth larvae and the damage they do to the roots of bush lupines in coastal California. Relationships between EPNs and NF in different habitats were also reported from field plots of citrus trees planted into holes filled either with sand or the native loam soil, thus creating well-drained or poorly drained conditions (Duncan et al., 2013). EPNs that were introduced into the plots persisted and killed sentinel weevil larvae at higher rates in the sand than the loam during four years. However, real time PCR assays of DNA from nematodes isolated from soil samples showed that the NF species *Purpureocillium lilacinus* and *Catenaria* sp. consistently infected nematodes at higher rates in sand than loam, whereas no NF species appeared to be favored by conditions in the loam soil. Consequently, there is little evidence to date that the spatial patterns of EPN species at a broad, landscape scale are modulated by variation in susceptibility to NF across habitats.

The DNA of seven NF species recovered from samples of nematodes was identified and quantified, but not reported, in a survey of EPNs in Florida citrus orchards (Campos-Herrera et al., 2013). The agricultural inputs in those orchards created soil conditions very different than those of natural areas in the same region reported by Campos-Herrera et al. (2016). Here we report the relationships between NF and soil properties in the citrus orchards and compare them with those detected in the natural areas. Results from both surveys were also combined to explore spatial relationships between NF, members of the EPN soil food web and abiotic factors. Our hypotheses were that (1) the occurrence frequency of some NF species would differ between orchards and natural areas due to differences in soil chemistry and management, (2) some spatial associations between species of EPN and NF would be due to similar habitat adaptations and therefore, (3) associations between NF and EPNs within a habitat might provide better insights into the role played by NF in modulating EPN occurrence.

2. Materials and methods

2.1. General survey design, sampling and soil processing

The survey area extended nearly 200 km east to west (from the gulf coast to the Atlantic coast) and more than 300 km north to south (from Umatilla to Immokalee). The orchards were selected on the basis that they were known to be infested by *Diaprepes abbreviatus* and that representative numbers were located in each of the two ecoregions (23 sites in the central ridge and 30 in the flatwoods) in which Florida's orchards occur. Sampling and sample processing methods are described in detail by Campos-Herrera et al. (2013). Briefly, two samples (30 cores, 2.5-cm diam. × 30 cm deep per sample) were obtained systematically at each 2–3 ha site during summer-early autumn 2009–2010. Samples were mixed and nematodes extracted (Jenkins, 1964) from 2, 500-cm^3 subsamples (i.e., 2 L of soil per site). DNA was extracted using the Ultra-Clean Soil DNA Extraction Kit (MoBio), quality was ensured (Nanodrop system 1000 v.3.3.0, Thermo Scientific, Wilmington, DE, USA), and the quantity adjusted for further molecular analysis. Following previous optimized protocols, seven species of NF were identified and quantified using real-time qPCR approaches (Atkins et al., 2005; Zhang et al., 2006; Pathak et al., 2012). Selected organisms previously reported in this survey by Campos-Herrera et al. (2013) were also combined in the evaluation, which included the species specific qPCR quantification of EPNs (13 species), and several other nematodes, bacteria and oomycete phytopathogens, all of which are known to interact with EPNs (Huang et al., 2010; Campos-Herrera et al., 2011a,b, 2012, 2013) (Supplementary data 1). In all the cases, primers and probes were synthesized by Integrated DNA Technologies Inc. (IDT, San Diego, CA), with all TaqMan® PCR probes labelled with the fluorogenic reporter dye FAM, the quencher Iowa Black™ FG and the ZEN molecule in the middle of the probe. Real time qPCR runs were performed in ABI Prism 7000 (Applied Biosystem), in optical-well reaction plates (USA Scientific, Orlando, FL, USA) with a final volume of 20 μL, and employing those reactive materials and primers/probe concentrations, annealing temperature and number of cycles described previously (Supplementary data 1). For each of the species, the identification and quantification was performed using positive (standard curve), negative (sterile de-ionized water) controls and the corresponding unknowns, with two technical replicates in each run.

Soil characteristics were described for the citrus survey (Campos-Herrera et al., 2013) and the natural areas study (Campos-Herrera et al., 2016). Briefly, remaining soil was air dried and sand fractions were determined by wet sieving (53 mm) followed by re-drying and processing on stacked sieves on a mechanical shaker (W.S. Tyler Co., Mentor, OH). Soil pH, organic matter (OM), percentage sand, silt and clay, electric conductivity (EC),