THE EFFECTS OF PLANT DOMESTICATION AND CULTIVATION ON NEMATODE COMMUNITIES AND BELOWGROUND TRITROPHIC INTERACTIONS IN THE HIGHBUSH BLUEBERRY (*Vaccinium corymbosum*) AGROECOSYSTEM

By

Monique Joy Rivera

A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Entomology

Written under the direction of

Dr. Albrecht M. Koppenhöfer

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

May 2016

ABSTRACT OF THE DISSERTATION

The effects of plant domestication and cultivation on nematode communities and belowground tritrophic interactions in the highbush blueberry (*Vaccinium corymbosum*) agroecosystem

by MONIQUE JOY RIVERA

Dissertation Director:
Dr. Albrecht M. Koppenhöfer

To address how plant domestication has impacted nematode populations and interactions, the highbush blueberry (*Vaccinium corymbosum*) agroecosystem within the Pinelands National Reserve was selected because of the availability of nearby wild-growing stands of *V. corymbosum*. Over 3 years, the rhizospheric soil of both wild and cultivated plants was sampled three times per year. Alongside sampling for entomopathogenic and plant parasitic nematodes, soil abiotic traits were measured. There was an overall trend for EPN abundance to be higher in cultivated soil than wild soil. The wild sites also showed seasonal variation in EPN abundance in 2011 but not in 2012 or 2013 while the cultivated environment had more consistent abundance. EPN diversity was higher in the wild environment, and isolates from the wild showed varying virulence to *Anomala orientalis* larvae. Conversely, plant parasitic nematodes were more diverse in the cultivated setting. The most prevalent isolate from cultivated plants was identified as *Steinernema glaseri* (B1) and used in subsequent laboratory studies. The attraction of two local EPN isolates, one from cultivated plants (B1) and one from wild plants (N9), to *A. orientalis* larvae and wild and cultivated *V. corymbosum* plant roots was assessed using two-choice

olfactometers. B1 was more attracted to the cultivated plants than the wild and insect feeding enhanced this attraction. N9 was more attracted to wild plants alone than wild plants with feeding larvae. From preliminary GC-MS analysis, it appears that the plant alone constitutively produces more volatiles without third instar *A. orientalis* feeding than with it.

To test if the B1 isolate attraction and infectivity could be increased by use of known chemicals [E)-β-caryophyllene and pregeijerene], we compared the two chemicals in the laboratory and field. In six-arm belowground olfactometers, B1 was significantly more attracted to (E)-β-caryophyllene. But in the field, the differences between the treatments were not significant. However, in counting the number of EPNs successfully establishing the host in the field, the blank treatment (without attractant) attracted 2× more EPNs. In conclusion, plant domestication in a perennial setting gives rise to selection pressures on the local EPN populations to the benefit of the attraction of EPNs.

Acknowledgements

I would first like to thank my advisor, Dr. Albrecht Koppenhöfer without who's unending support and confidence in me, I would not have been able to see it through.

I would also like to thank my committee, Drs. Peter Morin, John Dighton, and Cesar Rodriguez for their support and constructive criticism.

I also thank Dr. Olga Kostromystka, my labmate and dear friend, who has endured many inane questions about statistics and helped me execute my plans. I feel deeply thankful for your friendship and assistance.

I would like to thank my former and continuing mentor, Joanne Whalen. You are an inspiration to me and your persistent belief in me has supported me through all of graduate school.

In no particular order, the following people have been also been a large part of this process and deserve recognition here: Drs. Raquel Campos-Herrera, Patricia Stock, John Tooker, Anjel Helms, Tom Bentley, Dina Fonseca, and Andrea Egizi as well as the Rutgers University Department of Entomology. And previously, Dr. Hannah Burrack, Anna Chapman, NCSU Department of Entomology, Dr. Judith Hough-Goldstein and the University of Delaware Department of Entomology and Wildlife Conservation

Dedication

For my grandparents, John and Erma Offner

Table of Contents

List of Tables by Chapter

Introduction

Chapter 1

List of Figures by Chapter

Introduction

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Introduction

Plants are the connection between above and belowground ecosystems (Wardle et al. 2004, van Dam et al. 2011). This connection is formed by plant production of tissue that extends into both environments. Plant tissue serves as a primary source of nutrients for their consumers (pathogens and herbivores). In order to survive such attacks, plants have evolved many ways in which they can defend themselves. Despite the obvious bond of plants with both above and belowground environments, for over 50 years research on plant defense has mainly focused on the defense of aboveground plant parts. Recently, however, studies have expanded to investigate and integrate plant roots and the soil ecosystem to gain a greater understanding of how plants are able to survive relentless attacks on the very parts that are necessary for their survival.

Aboveground plant parts photosynthesize and produce reproductive structures while roots provide stability, water, and nutrient uptake, and are frequently the site of defensive chemical synthesis (Baldwin 1989). According to the optimal defense theory, plants should allocate defenses in a way that best protects and enhances their individual fitness. More specifically, when considering the whole plant, defenses should be allocated proportionally to the risk of attack and the value of the plant part to the fitness of the plant (Rhoades 1979). Based on the value of the roots to the plants overall survival and stability, it would be sensible to reason that both shoots and roots should be equally defended. Furthermore, belowground herbivory has been shown to cause reductions in plant fitness similar to that of aboveground herbivory (De Deyn et al. 2007; Karban 1980) and likely also activates the same plant defenses.

Because plants lack a true immune system, the foundation of their defense outside of physical structures is in secondary metabolites. These metabolites have been shown to mediate interactions with their biotic and abiotic environments. In particular, many of these chemicals are toxic and repellant to herbivores but can also be utilized by herbivore predators and parasites to locate hosts (Mumm and Dicke 2010). The optimal defense hypothesis predicts that there should be a negative correlation between the ability to be induced and the level of constitutive expression (Rhoades 1979; Karban and Baldwin 1997). Based on the metabolic cost to the production of secondary metabolites, many studies have investigated the variation in their production by classifying production strategies as "constitutive" or "inducible" despite evidence that these defensive styles are not mutually exclusive (e.g., Zangerl and Berenbaum 1990). Between the two ways of expressing ecologically relevant chemistry, inducible and constitutive, there has been a larger focus on induced chemistry likely because it is a measurable response variable.

Induced responses can be produced locally at the feeding site or the secondary chemistry can be changed throughout the whole plant, including belowground structures (Bezemer and Van Dam 2005; Kaplan et al. 2008). Within the plant, induced defenses are activated and regulated by phytohormones (Pieterse and Dicke 2007). Phytohormones (jasmonic acid, salicylic acid, ethylene) regulate a suite of induced plant responses, which includes herbivore-induced plant volatiles (HIPVs) (Walling 2000). Pathways that produce phytohormones are highly conserved among plants and often activated differentially depending on the type of harm being done to the plant. For example, insect feeding guild (i.e., piercing and sucking versus chewing) influences what phytohormone pathway is activated (Walling 2000; Zarate et al. 2007; Wu and Baldwin 2009).

Sensitivity to such differences suggests that there may also be the ability to distinguish above and belowground herbivores and choose how to allocate defenses.

The ability of plants to facilitate the attraction of herbivore predators and parasites through volatile production has been shown to occur across multiple plant genera. However, it is unclear if the "calling" for third trophic level assistance against an herbivore is truly beneficial to the plant and whether or not the sometimes very complex volatile blends are general or specific signals to the receivers. Predators and parasites can exhibit top-down control of insect herbivore populations (Preisser et al. 2006; Dicke and Baldwin 2010; van Dam et al. 2010) which regulates the impact of herbivory on plants. However, most of the studies showing the top down effect focus on more specialized herbivores that are likely to be highly adapted to their host plant (see Mumm and Dicke 2010). Nonetheless, this idea has subsequently raised interest in exploring the dynamics and applicability of analogous interactions in agricultural soil ecosystems. In agricultural or natural systems studied aboveground, the third trophic level is occupied mostly by highly mobile predators of insect herbivores, usually other insects, attracted indirectly by plant volatiles (as reviewed by Heil and Ton 2008). While not as well studied, this appears to also be true of belowground insect herbivory. In belowground arenas, the central focus has been on studying the behavior and response of entomopathogenic nematodes (EPN). However, the studies of belowground systems have thus far not addressed how EPN biology or plant domestication may alter such interactions to differ between agricultural and wild settings.

Previous studies of belowground tritrophic interactions involving EPN have focused on their attraction to domesticated plant varieties (Table 1(van Tol et al. 2001;

Rasmann et al. 2005; Ali et al. 2012). Plant domestication is the selection and enhancement of human-desired traits through plant breeding. The consequent changes to a plant's genome are substantial due to genetic drift (Chen et al. 2015), and this can cause indirect and sometimes negative effects on the plant's ability to respond and defend itself against belowground herbivore attacks (Degenhardt et al. 2009). Extending this idea, domestication also can impact the diversity and functionality of belowground communities (Sánchez-Moreno and Ferris 2007).

The objectives of this dissertation were to explore: 1. Explore variation in nematode community diversity (plant parasitic nematodes and EPN) between wild and cultivated highbush blueberry (*Vaccinium corymbosum* Authority) in the Pinelands National Reserve; 2. Test the differential attraction to signals from wild and cultivated *V. corymbosum* plants; 3. Compare the herbivore-induced volatiles found in other systems for their generality in the *V. coymbosum* agroecosystem.

Entomopathogenic Nematodes

EPNs, along with their symbiotic bacteria, blur the line between pathogen and parasite by forming a complex in the soil during the infective juvenile stage. Ultimately, EPNs are best described as obligate insect pathogens and are primarily found in the nematode families Steinernematidae and Heterorhabditidae. The third juvenile stage is called an "infective juvenile (IJ)" and is the only stage found outside the host. It does not feed and persists in the soil until a potential host is located and utilized (Figure 1). Gaining access through orifices or thin cuticle, the IJs release their symbiotic bacteria from their intestine, and both organisms cooperate to kill the host within 24-48 hours. The bacteria break down the host tissue, and the nematodes feed on both the bacteria and

digested tissue; this nutrient supply supports development through 1-3 nematode generations within the host. When the host cadaver is depleted, a new cohort of IJs emerges from the cadaver that carry with them cells of the bacterial symbiont. IJs from different EPN species exhibit different host foraging strategies ranging from widely searching (cruisers) to sit-and-wait (ambushers) (Lewis et al. 2009).

Commercial use of EPNs for the biological control of root feeding insect pests in the place of soil insecticides has received attention for over twenty years (Gaugler et al. 1997; Denno et al. 2008). However, in most cases where EPN have provided control of a target pest the effect has been short term, and there are only few examples of persistent EPN control (Gaugler et al. 1997; Georgis et al. 2006). The lack of persistent suppression of insect targets could be attributed to an array of interactive abiotic and biotic factors that limit EPN establishment and survivorship. Recent studies have examined the role of HIPVs in the attraction of EPN to plants and the role of EPN as an indirect mode of plant defense. HIPVs could potentially be used in an agricultural setting to enhance the attraction of EPN to their insect hosts and increase efficacy of EPN in the field.

The Highbush Blueberry (*Vaccinium corymbosum*) System in the Pinelands National Reserve and Oriental Beetle, *Anomala orientalis*

Highbush blueberry, *V. corymbosum*, is a perennial shrub that is both native to and cultivated within the eastern United States. New Jersey, home of the initial domestication of blueberry, is the fifth largest blueberry producing state in the United States; only Michigan, Georgia, Oregon, and Washington exceed in blueberry production acreage (USDA 2014). In New Jersey, blueberry is predominately cultivated on 8,800

acres throughout Burlington and Atlantic counties, a local industry valued at 79.5 million dollars (USDA 2014). Domesticated highbush blueberries are not far removed from the native blueberry species (wild *V. corymbosum* and lesser so, *V. angustifolium, V. vacillans, V. atrococcum,* and *V. stamineum*). Wild blueberry plants thrive over large areas in the neighboring understory of the heavily forested and protected 1.1 million acre area known as the "Pinelands National Reserve." The blueberry agroecosystem in this setting is particularly unique because the native plants found in an undisturbed, protected area provide an exemplification of the plant thriving in a natural setting. With regard to using EPNs for biological control, the naturally flourishing plants provide a reserve of information about the soil community left undisturbed and potentially EPN species that could be used to control a primary pest of blueberry, the root-feeding larvae of the oriental beetle (*Anomala orientalis* Waterhouse; Coleoptera: Scarabaeidae).

The oriental beetle, is an invasive, exotic pest in the United States. It was first found in Connecticut, USA in 1920. It has since spread around the Northeastern United States, including New Jersey. While the larvae are considered serious pests, the adults are not. Larvae are polyphagous and have been found to feed in turfgrass, ornamentals and vegetable crops. In New Jersey, oriental beetle has a 1 year life cycle with three larval instars. Adults emerge in from early June through mid-July, typically peaking in mid- to late June and larvae feed from mid to late July to October going through three larval stages in this time. To overwinter the third instars descend in the soil profile where they stay inactive and not feed until warming soil temperature in April bring them back closer to the surface to feed for another 4-6 weeks before pupating in the soil. The larvae are typical C-shaped white grub larvae and look very similar to Japanese beetle (*Popillia*

japonica Newman) larvae but are able to be separated by spine patterns on their raster; where Japanese beetle larvae have a V-shaped pattern of spines on their raster, oriental beetles have two parallel lines.

References Cited

Ali JG, Alborn HT, Campos-Herrera R, et al (2012) Subterranean, herbivore-induced plant volatile increases biological control activity of multiple beneficial nematode species in distinct habitats. PLoS One 7:1–7. doi: 10.1371/journal.pone.0038146

Bezemer TM, Van Dam NM (2005) Linking aboveground and belowground interactions via induced plant defenses. Trends Ecol Evol 20:617–624. doi: 10.1016/j.tree.2005.08.006

Chen YH, Gols R, Benrey B (2015) Crop domestication and naturally selected species interactions. Annu Rev Entomol. doi: 10.1146/annurev-ento-010814-020601

Degenhardt J, Hiltpold I, Köllner TG, et al (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proc Natl Acad Sci U S A 106:13213–8. doi: 10.1073/pnas.0906365106

Denno RF, Gruner DS, Kaplan I (2008) Potential for entomopathogenic nematodes in biological control: a meta-analytical synthesis and insights from trophic cascade theory. J Nematol 40:61–72.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the "cry for help". Trends Plant Sci 15:167–75. doi: 10.1016/j.tplants.2009.12.002

Gaugler R, Lewis E, Stuart RJ (1997) Ecology in the service of biological control: the case of entomopathogenic nematodes. Oecologia 109:483–489. doi: 10.1007/s004420050108

Georgis R, Koppenhöfer A. M, Lacey L A., et al (2006) Successes and failures in the use of parasitic nematodes for pest control. Biol Control 38:103–123. doi: 10.1016/j.biocontrol.2005.11.005

Heil M, Ton J (2008) Long-distance signalling in plant defence. Trends Plant Sci 13:264–

72. doi: 10.1016/j.tplants.2008.03.005

Kaplan I, Halitschke R, Kessler a, et al (2008) Constitutive and induced defenses to herbivory in above- and belowground plant tissues. Ecology 89:392–406. doi: 10.1890/07-0471.1

Karban R, Baldwin IT (1997) Induced responses to herbivory. University of Chicago Press

Lewis EE, Grewal PS, Gaugler R (2009) Hierarchical order of host cues in parasite foraging strategies. Parasitology 110:207–213. doi: 10.1017/S0031182000063976

Mumm R, Dicke M (2010) Variation in natural plant products and the attraction of bodyguards involved in indirect plant defenseThe present review is one in the special series of reviews on animal–plant interactions. Can J Zool 88:628–667. doi: 10.1139/Z10-032

Pieterse CMJ, Dicke M (2007) Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends Plant Sci 12:564–9. doi: 10.1016/j.tplants.2007.09.004

Preisser EL, Dugaw CJ, Dennis B, Strong DR (2006) Plant facilitation of a belowground predator. Ecology 87:1116–23.

Rasmann S, Köllner TG, Degenhardt J, et al (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434:732–7. doi: 10.1038/nature03451

Rhoades DF (1979a) Evolution of plant chemical defense against herbivores. In: Rosenthal GA, Janzen DH (eds) Herbivores: Their interactions with secondary plant metabolites. Academic Press, New York, pp 1–55

Sánchez-Moreno S, Ferris H (2007) Suppressive service of the soil food web: Effects of environmental management. Agric Ecosyst Environ 119:75–87. doi: 10.1016/j.agee.2006.06.012

USDA (2014) Blueberry Statistics 2014.

van Dam NM, Qiu B-L, Hordijk C a, et al (2010) Identification of biologically relevant compounds in aboveground and belowground induced volatile blends. J Chem Ecol 36:1006–16. doi: 10.1007/s10886-010-9844-9

van Tol RWHM, van der Sommen ATC, Boff MIC, et al (2001) Plants protect their roots by alerting the enemies of grubs. Ecol Lett 4:292–294. doi: 10.1046/j.1461-0248.2001.00227.x

Walling LLLL (2000) The myriad plant responses to herbivores. J Plant Growth Regul 19:195–216. doi: 10.1007/s003440000026

Wu J, Baldwin IT (2009) Herbivory-induced signalling in plants: perception and action. Plant Cell Environ 32:1161–74. doi: 10.1111/j.1365-3040.2009.01943.x

Zangerl AR, Berenbaum MR (1990) Furanocoumarin induction in wild parsnip: evidence for an induced defense against herbivores. 71:1933–1940.

Zarate SI, Kempema L a, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiol 143:866–75. doi: 10.1104/pp.106.090035

Table 1. Associations between point of origin among plants, insects, and entomopathogenic nematodes used in previous studies

Paper	Plant	Insect	Entomopathogenic Nematode
Van Tol et al. 2001	*Thuja occidentalis*, Northern White Cedar, North America	*Otiorhyncus sulcatus*, Black Vine Weevil, Europe	*Heterorhabditis megidis*
Rasman et al. 2005	*Zea mays*, Corn, North America	*Diabrotica virgifera virgifera*, Western Corn Rootworm, North America	*H. megidis*
Ali et al. 2010, 2012	*Poncirus trifoliata,* Trifoliate Orange, China	*Diaprepes abbreviatus*, Citrus Root Weevil, Caribbean	*Steinernema diaprepesi*, *S. carpocapsae*, *S. riobrave*, *H. indica*
Rivera et al. 2016	*Vaccinium corymbosum*, Highbush Blueberry, North America	*Anomala orientalis*, Oriental Beetle, Asia	*S. glaseri*

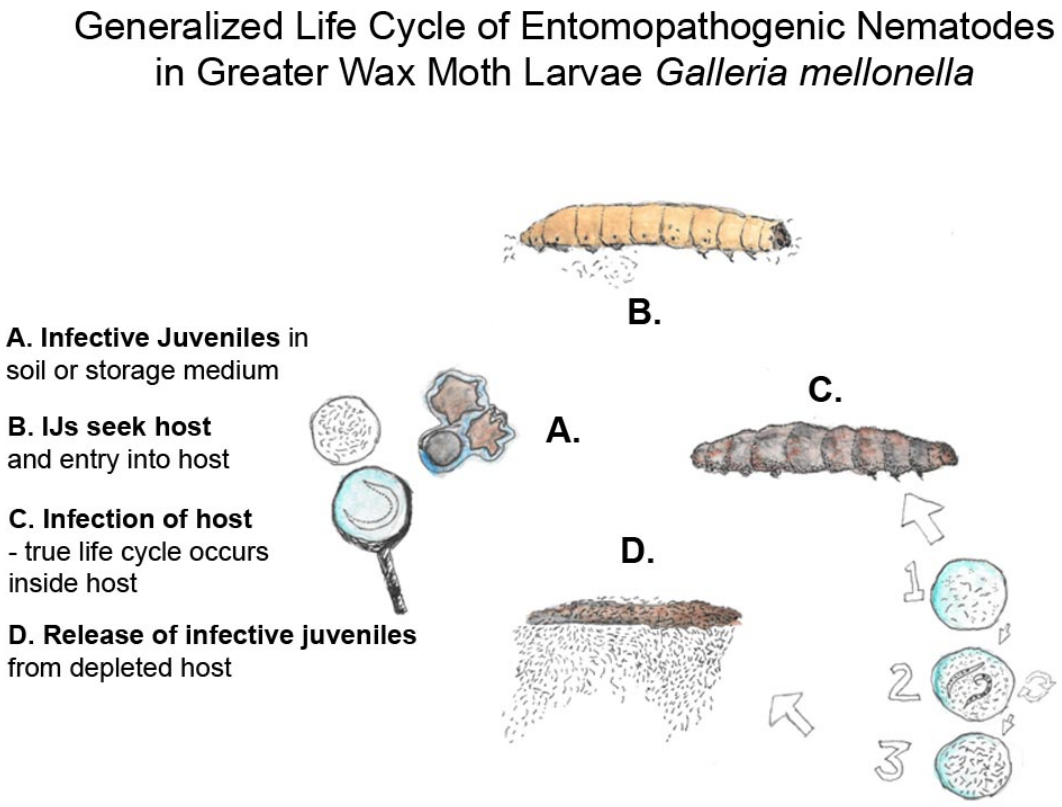
Generalized Life Cycle of Entomopathogenic Nematodes
in Greater Wax Moth Larvae *Galleria mellonella*
A. Infective Juveniles in soil or storage medium
B. IJs seek host and entry into host
C. Infection of host - true life cycle occurs inside host
D. Release of infective juveniles from depleted host
A.
B.
C.
D.
1
2
3

Figure 1.

Chapter 1: Assessing the impact of cultivation and plant domestication of highbush blueberry (*Vaccinium corymbosum*) on soil properties and associated plant parasitic nematode communities

Abstract

We investigated the interactive effects of plant domestication and perennial cultivation on diversity and richness of the plant parasitic nematode (PPN) community associated with highbush blueberry (*Vaccinium corymbosum*). Rhizospheric soil of *V. corymbosum* plants was sampled in coupled wild and cultivated sites within the Pinelands National Reserve. Although PPN diversity was higher in cultivated plant soil samples, richness was higher in wild plant soil samples. The most important soil properties, iron and calcium, were associated with cultivated soil and were the best predictors of genera abundance patterns. PPN of the genus *Criconemoides* were 187× more abundant in wild sites than cultivated and thus, were significant indicators of wild sites. In this study cultivation of *V. corymbosum* appears to benefit the PPN community but alters the community composition considerably.

Introduction

Over the past decade, interest in the response of belowground communities to plant species and land management practices has grown considerably (Bever et al., 2012; Duncan et al., 2007; Maron et al., 2011; Monroy et al., 2012). Soil ecosystems can support an astounding diversity of microbes, microfauna, and mesofauna that provide multiple levels at which the impact of human intervention can be assessed. Nematode communities have been a focus of many studies because of their multi-faceted importance to the soil food-web (Yeates 2010). Both abundant and diverse, nematodes are responsive bioindicators which provide a measure of the impact of human landscape management on the soil system (Bongers and Ferris 1999). Furthermore, in specific trophic roles, nematodes are targeted as both beneficial, by providing ecosystem services (e.g., entomopathogenic nematodes (EPN)) (Denno et al. 2008) and as pest organisms such as plant parasites (Kandji et al. 2001).

Terrestrial plants are the primary source of organic carbon in both above and below-ground environments and thereby are directly linked to the soil food-web (Bukovinszky et al. 2008). The function and diversity of nematode communities within the soil food-web is mediated by plant species identity, soil amendments, and land management practices (De Deyn et al., 2004; Liang et al., 2009; Robertson and Freckman, 1995). While the impact of plant species identity on nematode communities has been addressed qualitatively (Palomares-Rius et al. 2012) and empirically (Bezemer et al. 2010), there has been no previous work investigating the interactive effects of plant species and land management. Nematode communities are generally assessed in either undisturbed wild settings or highly disturbed annual agricultural cropping system (e.g.,

Freckman and Ettema, 1993; Rahman et al., 2007). Plant parasitic nematodes (PPN) are of particular interest to this study because, unlike other trophic groups, they appear to have little to no spatial correlation across highly disturbed agroecosystems (Robertson and Freckman, 1995) indicating a potentially more connected relationship to plant identity.

Highbush blueberry (*Vaccinium corymbosum*) is a perennial woody shrub. Native to the eastern United States, it was first domesticated and cultivated in Chatsworth, New Jersey, USA in the early 20th century. To assess the impact of perennial cultivation on diversity and abundance of PPN, we compared soil characteristics and nematode communities associated with the roots of wild stands of undomesticated *V. corymbosum* plants with those from adjacent stands of cultivated *V. corymbosum*.

Materials and Methods

Cultivated fields with wild *V. corymbosum* in nearby wooded field border were selected within the Pinelands National Reserve (Burlington County, New Jersey, USA). At each of five sites, two 15.24 m by 15.24 m plots were established, one in the cultivated site, the other in an adjacent wild *Vaccinium* stand within the wooded field border at no more than 30 m distance. All plots were sampled three times during the 2011 season: 19 May, 13 July, and 13 September. Eight vertical soil cores (30.5 cm L × 2.1 cm D) were taken 15−30 cm from the basal point of the main trunk of each of ten randomly selected plants. The cores from each plant were combined (~800 g) and homogenized and nematodes extracted or baited from 100 g subsamples. All PPN counts are the sum the soil extractions (1000 g/site) from the ten plants within each plot to assess PPN communities (Wiesel et al. 2015). PPN were extracted, identified to genus and counted

with the help of the Plant Diagnostic Laboratory at Rutgers University. The centrifugal-flotation technique (Jenkins 1964) was used for extractions to ensure the retrieval of all life-stages as well as both dead and alive specimens; it should be noted that totals for endoparasites are likely underestimates as root-dwelling stages were not extracted in this method. A 500 g subsample of the soil from each plot was used by the Rutgers Soil Testing Laboratory to measure the following properties including: soil moisture, pH, cation exchange capacity, and total nutrients: P, K, Ca, Mg, B, Zn, Mn, Cu, and Fe. EPN abundance was also assessed by exhaustive baiting with 10 *Galleria mellonella* larvae of a 200 g subsample from the same plant soil samples (Koppenhöfer et al. 1998b).

Diversity was measured using the Shannon Index (*H*) (Shannon and Weaver 1949). We then used canonical correspondence analysis (CCA) to explore the relationships between PPN, soil properties, and EPN abundance. Significance was assessed with forward selection to identify the most parsimonious model (Borcard et al. 2011). Indicator species analysis (ISA) was used to determine which genus-treatment associations were greater than expected by chance (Dufrene and Legendre 1997). All analyses were conducted in R 3.1.2 (R Core, 2014).

Results

Community composition and diversity of PPN differed between wild and cultivated sites. PPN diversity was higher within cultivated sites ($H = 2.4$) compared to wild sites ($H = 2.0$), but more taxa were found in wild sites (9) compared to cultivated sites (6). The ISA revealed a significant positive association between *Criconemoides* nematodes and wild sites (indicator value = 99.5, $P = 0.013$). Moreover, 187x more *Criconemoides* individuals were extracted from wild plots than cultivated ones (Table 1).

These differences, however, were not reflected in the Plant Parasite Index (PPI) [t(2)=0.05, $P > 0.67$] (Bongers and Ferris 1999) used to assess soil ecosystems using PPN. CCA indicated that most of the soil properties measured were associated with cultivated sites along with *Trichodorus* and *Xiphinema* nematodes. In contrast, nematodes in the genera *Criconemoides* and *Helicotylenchus* tended to be more frequently found in wild sites. The most parsimonious model suggested that nematode diversity was positively related to the soil properties Fe and Ca (AIC = 53.49), and model fit was not significantly improved by the addition of any other soil properties or EPN abundance.

Discussion

This is the first time PPN and soil properties have been compared between plants in a cropping system and closely associated wild plants of the same species. Results from the analysis of diversity suggest that the perennial cultivation of blueberry plants enhances the PPN community but restructures its composition. Even though it is not reflected directly in the *H* values (Table 1), the wild plants surveyed are likely experiencing attacks from a broader range of PPN. This increased variation in attack (e.g., more variation in stylet characteristics) has implications for the selective pressures on wild plants beyond the scope of the present study (Wondafrash et al. 2013). The ability of cultivated sites to support a higher abundance of PPN in more genera could be explained by soil nutrients and plant size. The most important soil properties, Fe and Ca, were associated with the cultivated sites (Figure 1). Blueberries are best adapted to sandy acidic soils (Hayden 2001) which are common to the Pinelands National Reserve area. However, acidity, soil texture, and soil prep for cultivation affect the availability of micronutrients like iron and calcium, which can be supplemented by growers if

necessary. Cultivated plants also tend to be larger and less variable in size and age than wild plants due to genetic and nutrient differences and thus, are likely supplying a better quality food source for PPN.

Although the soil textures are similar (MJR, unpublished data), the stratification of organic matter in undisturbed soils has been eliminated in the cultivated soil by soil disturbance as part of the management. Due to this change in overall soil structure, even with the lack of annual disturbance, top-down PPN regulating forces, such as nematophagous fungi, may have been eliminated in cultivated soils (Sánchez-Moreno and Ferris 2007). Conversely, the success of *Criconemoides* at wild sites could be due to greater resistance to top-down forces because of their considerably slower-moving lifestyle (i.e., less exposure over time to pathogens) and thick, annulated body cuticle (Hoffmann 1973). Although genus level differences often reflect feeding strategy and can indicate much about the stress on plant roots (Bird and Koltai 2000), they provide a coarse measure of diversity, and diversity at the species level would be further descriptive of the differentiation in PPN community between wild and cultivated plants. Furthermore, despite these differences between environments at the genus level, intraspecies diversity explored through molecular and morphological features may provide even more interesting insights to the effects of cultivation on community composition. For example, the *Criconemoides* genus, which was very prevalent in this study, in particular is taxonomically controversial (Cordero et al. 2012) and even a subsample of molecular and morphological data may reveal cryptic species which are common within nematode taxa (Blouin 2002).

Table 2. Collective abundance of plant parasitic nematodes associated with cultivated and wild *Vaccinium corymbosum*

		Cultivated			Wild		
Genus	Feeding strategy	Total	Mean[1] (±SE)	Relative abundance	Total	Mean[1] (±SE)	Relative abundance
Criconemoides	Ectoparasites	8	0.53 (0.36)	0.77	1,492	99.47 (46.87)	74.45
Helicotylenchus	Ectoparasites	8	0.53 (0.36)	0.77	48	3.20 (1.88)	2.40
Hemicycliophora	Ectoparasites	432	28.80 (15.65)	41.54	200	13.33 (5.14)	9.98
Heterodera	Sedentary endoparasites	0	0.00	0.00	16	1.07 (0.83)	0.80
Hoplolaimus	Ectoparasites	0	0.00	0.00	4	0.27 (0.27)	0.20
Meloidogyne	Sedentary endoparasites	0	0.00	0.00	24	1.60 (1.34)	1.20
Pratylenchus	Migratory endoparasites	0	0.00	0.00	0	0.00	0.0
Trichodorus	Ectoparasites	212	14.13 (4.89)	20.38	148	9.87 (2.70)	7.38
Tylenchorhynchus	Ectoparasites	276	18.40 (11.77)	26.54	68	4.53 (3.12)	3.39
Xiphinema	Ectoparasites	104	6.93 (6.09)	10.00	4	0.27 (0.27)	0.20
Total		1,040			2,004		
Richness		6			9		
Diversity (*H*)		2.4			2.0		

[1]Average per site sampled (n=5).

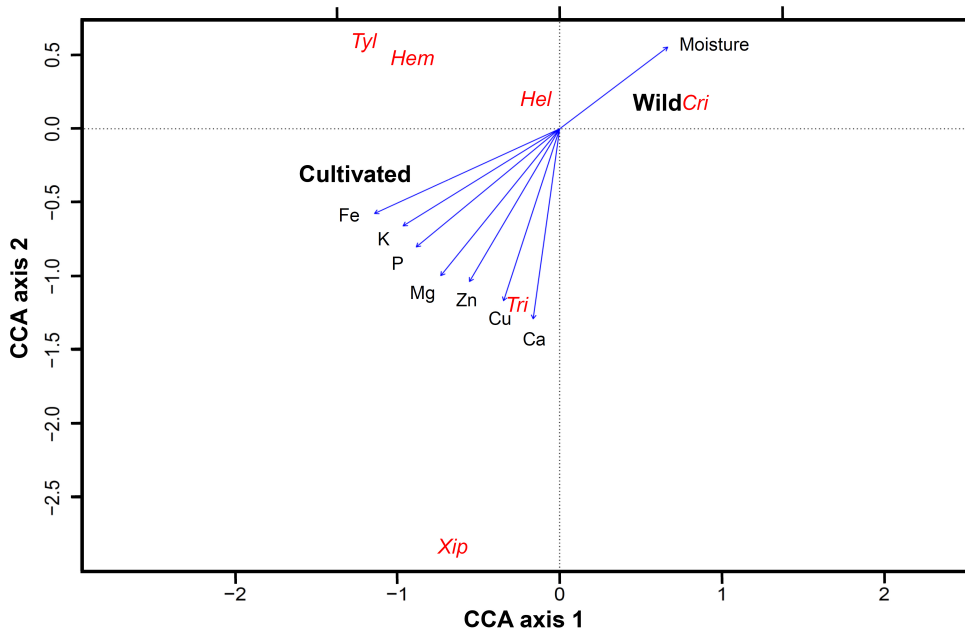

Figure 1. Canonical correspondence analysis (CCA) ordination plot of plant parasitic nematode abundances and soil properties. The length and angle of arrows indica the relative contribution of a particular soil property to the axes. To reduce clutt only the eight most important soil properties (as determined by forward selectic models) are shown. Nematode genera abbreviations are: Cri = *Criconemoides*, H *Helicotylenchus*, Hem = *Hemicycliophora*, Tri = *Trichodorus*, Tyl = *Tylenchorhynchus*, and Xip = *Xiphinema*.

Chapter 2: Cultivation and domestication of highbush blueberry (*Vaccinium corymbosum*) alters abundance, diversity, and virulence of entomopathogenic nematodes

Abstract

We investigated the interactive effect of plant domestication and perennial cultivation on abundance, diversity and virulence of entomopathogenic nematodes (EPN) associated with highbush blueberry (*Vaccinium corymbosum*). The rhizospheric soil of *V. corymbosum* plants was sampled in coupled wild and cultivated sites within the Pinelands National Reserve, New Jersey, USA from 2011-2013 at three points during each year. To isolate and assess abundance of EPN communities, soil samples were baited with wax moth larvae (*Galleria mellonella*). A subsample of EPNs isolated from the soil was identified using molecular barcoding. The virulence of three native isolates against oriental beetle, *Anomala orientalis*, larvae was assessed at multiple densities of nematodes per host. A cursory assessment of EPN diversity was based on variation in molecular barcodes because many sequences could not be identified in the genomic database GenBank. The variation among barcodes suggested that diversity was higher in rhizospheric soil associated with wild plants than that of cultivated fields. *Steinernema glaseri* was the only EPN species able to be identified using molecular methods and was the only species isolated from the six cultivated fields with EPN presence. An interaction of environment, sampling point, and year explained significant differences in EPN abundance in 2011 and 2012 when soil samples were assessed by plant. However, in 2013, soil was assessed by field rather than plant and our results show EPN abundance was significantly higher in cultivated than in wild sites. Soil nutrients and characteristics

were also assessed in 2013. A PCA analysis of soil characteristics linked higher plant nutrients to soil from cultivated fields and higher soil moisture, organic matter, boron and lower pH to wild plant-associated soil. In this study, cultivation of domesticated *V. corymbosum* appears to increase the abundance of one EPN species that is more virulent against the presumed host, *A. orientalis* but appears to eliminate genetic variability from the EPN community.

Introduction

The development of sustainable approaches to soil conservation necessitates an increased understanding of how land management practices impact the services soil can provide for humans and others (Doran 2002). Agricultural production of plants is known to change the abundance and diversity of communities of aboveground insect herbivores and their predators and parasitoids (Matson et al. 1997; Poelman et al. 2008). However, it is difficult to determine the true effect of plant domestication and altering of land for agricultural use on interactions in the agroecosystem without investigation of and comparison to undisturbed wild populations of plants; this is especially true for associated soil communities held in delicate balance with one another.

Nematodes are diverse and prevalent throughout soil communities worldwide. Within this taxon many feeding habits are found that occur at multiple trophic levels making nematodes an excellent bioindicator of disturbance to the soil ecosystem (Bongers 1990; Yeates et al. 1993; Bongers and Ferris 1999). Thus, the diversity of nematode species present is often used to evaluate the effects of land management practices (Freckman and Ettema 1993; Millar and Barbercheck 2002; Morise et al. 2012). The effect of altered soil structure through agriculturally focused land management on nematode communities is well studied and the effects of disturbance for this purpose (e.g., tillage, perennial or annual cultivation) are relatively clear (Erb and Lu, 2013; Govaerts et al., 2007; Sánchez-Moreno and Ferris, 2007; Vonk et al., 2013). In spite of this, the disturbance of soil is only a single factor in the alteration of land for agricultural production.

Alongside soil disturbance, plant domestication and the subsequent establishment of large plant monocultures are two other notable modifications to the land caused by agricultural production. Increasingly, plant genotype and species identity is being linked to the diversity and functionality of soil communities (Van der Putten et al. 2001; Wardle et al. 2003; Zak et al. 2003; Bezemer et al. 2010; Kostenko et al. 2015). Plant domestication is largely based on the artificial selection and enhancement of human-desired traits. The consequent changes to a plant's genome are substantial due to selection from a relatively small population of plants (i.e., genetic drift) which causes continued bottlenecking as a limited diversity of plant stock is used for breeding to accentuate specific traits (Chen et al. 2015). This can cause indirect and sometimes negative effects on the plant's ability to respond and defend itself against belowground attacks (Degenhardt et al. 2009; Chen et al. 2015). As knowledge of the connection between above and belowground interactions expands, the effect of cultivation and plant domestication on soil organisms has become a point of interest (Palomares-Rius et al., 2015; Rivera et al., 2015; Sánchez-Moreno and Ferris, 2007). While the impact of plant domestication has been assessed previously, usually indirectly, it is often hard to access wild populations of plants at their point of origin to directly study the effects of domestication and cultivation. This study investigates the effect of domestication and cultivation on nematodes that feed at the third trophic level, i.e., entomopathogenic nematodes (EPN) with the hypothesis that the dynamics of the belowground arena would mirror effects found in the aboveground arena with higher trophic levels being more responsive to disturbance (Jonsson et al. 2012).

EPNs are obligate parasites of soil-dwelling insects and other arthropods. In natural systems, EPNs can have significant top-down effects on insect populations (Strong et al. 1996) and thus, in agricultural systems, are considered promising biological control agents for insect pest control (Gaugler et al. 1997; Denno et al. 2008). EPNs are active in the soil only as infective juveniles (IJs) which carry with them a symbiotic bacterium released inside an insect host to cause septicemia. The IJ is a special dauer-like stage that is non-feeding but persists in the soil until a host is located. Upon locating a host, the IJ must then enter the host through any opening (e.g., mouth, anus, spiracles, etc.). Once inside the host's body cavity, the bacterium is released and the success of the EPN is dependent on the ability of the bacterium-nematode complex to overcome the insect's immune system. A successful infection will result in up to three EPN life cycles occurring in the cadaver as they feed on the multiplying symbiotic bacteria and host tissues broken down by the bacteria. Once resources in the cadaver are depleted, hundreds to hundreds of thousands of IJs exit into the surrounding soil and search for a new host. This search is directed by a variety of cues such as carbon dioxide and other volatile cues emitted by insects (Lewis et al. 1993; Lewis et al. 2009) and volatile organic compounds emitted from plant roots (Turlings et al. 2012). Because EPN respond to cues from soil insects and plant roots, we hypothesized that diversity and functionality of EPN communities would diverge between wild and cultivated plants of the same species.

We used the highbush blueberry (*Vaccinium corymbosum* L.) agroecosystem in southern New Jersey, USA. This system is quite unique in that *V. corymbosum* was first domesticated and grown commercially there in the early twentieth century. As a result of its recent domestication, wild and cultivated multispecies hybrids of *V. corymbosum* are

still considered the same species (Eck 1965; Bian et al. 2014). In contrast, many domesticated plants are such severe mutants of their parental lines they can no longer function as the same species (i.e., cannot successfully cross-pollinate one another to produce viable offspring). Relatively shortly after commercial production began, legislation was put into effect that protected the wild plants in the unique ecosystem surrounding this region. The legislation was established in 1978 as the United States National Parks and Recreation Act, which denoted the area as the "Pinelands National Reserve" (boundaries shown in Figure 1). This spurred the later and local 1979 New Jersey Pinelands Protection Act, which regulates the boundaries, land management and minimum standards for land use within the Pinelands National Reserve (Pinelands Comission, 2011; Pinelands Comprehensive Management Plan). This legislation acknowledges the economic importance and cultural connection of blueberry and cranberry production in this region by classifying areas within the reserve as "Special Agriculture Production Areas". These two acts mandated the preservation of wild stands in this region and uniquely allowed for the production of domesticated cultivars nearby protected wild stands, making this an ideal system to study the interactive effects of cultivation practices and plant domestication on EPN communities. The objective of this study was to compare EPN diversity and functionality of EPN communities in wild and domesticated *V. corymbosum* plants within the Pineland National Reserve according to plant phenology over multiple years.

Materials and Methods

Field selection and soil sampling

Field sites were selected within the boundaries of the Pinelands National Reserve (Burlington and Atlantic Counties, New Jersey, USA) based on two criteria: 1. A history of high pheromone trap captures of adult male oriental beetles, *Anomala orientalis* Waterhouse (Coleoptera: Scarabaeidae), the most prevalent soil-dwelling pest of blueberry (Dean Polk, Rutgers Fruit IPM Coordinator, Rutgers Cooperative Extension), and 2. Presence of wild *V. corymbosum* growing in a wooded field border within 30 m of the edge of the commercial field.

At each of the five sites in 2011 and ten sites in 2012 and 2013, a 15 m × 15 m plot was selected at random in both the commercial and wild *V. corymbosum* stands. Sites with coupled sampling plots in both areas were established and sampled according to the phenology of the commercial plants: at bloom (early May), fruit set/harvest (early July) and post-harvest (early September). At each sampling point, eight vertical soil cores were taken with an Oakfield sampler (30.5 cm length × 2.1 cm diameter) 15–30 cm concentrically from the base of the main trunk of ten plants randomly selected within the plots. In 2011 and 2012, soil from each plant (~800 g per plant) was stored in individual plastic bags and held at 8 °C for no more than 48 h until processing. To further assess EPN diversity with molecular methods, a follow up sampling effort was conducted in 2013 using the same ten sites as in 2012 but combining the soil from the ten plants sampled per plot in one plastic bag (~8000 g).

Laboratory processing of soil samples

The soil from each plant (2011–2012) or field (2013) was homogenized by hand in the lab before experimental set up. Wax moth larvae (*Galleria mellonella* L., Lepidoptera: Pyralidae) used for baiting were obtained from a commercial source (Big Apple Pet Supply, Boca Raton, Florida, USA) and used within 10 days of receipt. To estimate the relative abundance of EPN, a 200 g subsample from each plant was exhaustively baited with five last instar wax moth larvae in a deep-well Petri dish (25 mm H × 100 mm D) (Koppenhöfer et al. 1998a) at room temperature (22–25 °C). Exhaustive baiting refers to the process of baiting subsamples and checking them until there are no infections in the baiting larvae. The baited subsamples were checked every 5 days, which included the removal of infected larvae and subsequent their replacement with fresh larvae until the dish produced no infected larvae for two consecutive checks. For each dish, the status of every larva was recorded during each check and marked as: infected (with EPN), alive, or dead but not infected with EPN (including fungal and bacterial infections). Infected *G. mellonella* larvae retrieved from baiting dishes were placed on modified White traps (Stock and Goodrich-Blair 2012) to collect emerging IJs for subsequent reinfection of *G. mellonella* larvae, in fulfillment of Koch's postulates. Infective isolates were stored in tissue flasks at 8 °C (Stock and Goodrich-Blair 2012).

In 2013, two 200 g subsamples from each plot were baited according to the same methods as described above and resulting fresh EPN isolates were used for molecular identification. After the post-harvest sample (early July), 500 g from each plot was taken to the Rutgers Soil Testing Laboratory to determine pH, soil moisture, organic matter,

soil textural class and total extractable (Mehlich 3 method) of NH_4NO_3, $NO3^-$, phosphorous, and potassium as well as plant micronutrients (Ca, Mg, B, Mn, Cu, Zn).

Estimating EPN diversity between wild and cultivated sites

Initially, characteristics of the infected cadaver (e.g., color and degradation process) and IJs were used for cursory identification to family. As this did not reveal notable differences in diversity (all appeared to be Steinernematids), it became necessary to use molecular methods. To address the genetic diversity of isolates, the molecular operational taxonomic unit (MOTU) (Floyd et al. 2002) was used to estimate diversity because it is suited to identification of EPNs extracted from soil as isolates through soil baiting with *G. mellonella* (Chen et al. 2010). This technique removes the need for explicit species level identification by additional biological or morphological means and instead, depends on differences in sequence identity (i.e., molecular barcodes). The resulting sequences are from a genomic region (18S ribosomal DNA) stable enough across life stages to use DNA from IJs rather than rearing to obtain adults from the dissection of infected insects. The downside to this technique is that in the case of a new species, additional sequencing and expert morphological and biological assessment is needed. Multiple representative isolates were chosen for sequencing from cultivated and natural sites with high EPN activity. The number of individual MOTUs was used to estimate diversity because a subsample of extracted EPNs were sequenced. A diversity index was not used because we were not able to identify all samples in the subsample to species.

To use this method, the 5' end of the small subunit ribosomal RNA (SSU) was amplified from individual IJs from a subset of isolates and sequenced. Then, NCBI's

GenBank BLASTn search (www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to identify species matches. Top matches from the search (≥ 99% match) were considered good candidates for taxonomic identification. One of the downsides of the baiting approach is that a multi-species complex may outcompete each other in the baiting dish. Thus, in 2015, the six sites with the most EPN activity in the wild and cultivated plots were screened for other known EPN species occurring in New Jersey using quantitative PCR to confirm there was no bias or outcompeting of species in the traditional baiting methods (Rivera et al., unpublished data).

Single nematode digestion and PCR

Methods of DNA extraction and PCR amplification of SSU rRNA were obtained from Floyd et al. (2002) with changes in PCR master mix according to available resources. To extract DNA, an individual IJ from a stored isolate was put in 20 µL of 0.25 M NaOH in a 0.2 mL tube and left at room temperature (24 °C ± 2) for 3–16 hours. Then, the tubes were heated at 99 °C for 3 min and then neutralized by adding 4 µl of 1 M HCl, 10 µl of 0.5 M Tris-HCl (pH 8.0), and 5 µL of 2% Triton X-100. A 20 µL polymerase chain reaction was used to amplify 18S: 200 µM dNTPs, 1X PCR reaction buffer (10 mM Tris-HCl, pH 8.3, and 50 mM KC), 2.5mM $MgCl_2$, 50 nM each primer, 1U Taq polymerase and 1 µL template DNA (or sterile water for negative control). The SSU primers used were: SSU18A-4F (5'-GCTTGTCTCAAAGATTAAGCCATGCATG-3') and SSU26Rplus4 (5'-AAGACATTCTTGGCAAATGCTTTCG-3') (Morise et al. 2012). Amplification of the 18S locus was initiated with a 2-min denaturation at 94 °C followed by 35 cycles each involving denaturation at 94 °C for 10 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min in an Applied Biosystems™ Veriti® 96-Well

Thermal Cycler. Products (5 μL) of this reaction were visualized on agarose gels stained with ethidium bromide.

DNA purification and sequencing

PCR products were purified using ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) for PCR product cleanup according to manufacturer protocol. Samples were prepared according to vendor guidelines and sent to GenScript (Piscataway, NJ, USA) for sequencing with the SSU18A-4F/SSU26Rplus4 previously mentioned. Sequencher 5.2 (Gene Codes, Ann Arbor, MI, USA) was used to align and edit sequences. A multiple sequence alignment containing 18S sequences from both wild and cultivated isolates ("cultivated", "NJXBB1", "NJXBB2") was created in BioEdit version 7.1.3.0 (Hall 1999) using the ClustalW algorithm version 1.8 (Thompson et al. 1994) with default parameters.

Virulence of native Steinernema glaseri and wild isolates against A. orientalis

Soil, insects and nematodes

Soil used in this assay was an acidic loamy sand (82% sand, 12% silt, 6% clay; 1.8% organic matter, pH 3.9) from a commercial blueberry farm in Burlington County, NJ, USA. The blueberry field soil was sieved (4 mm) to remove large particles, pasteurized for 3 hours at 70 °C and then allowed to air dry before use. To prepare for use, the dry soil was brought to 10% (w/w) moisture (-25 kPa soil water potential). After addition of nematode treatments the final soil moisture was 11% (w/w) (-10 kPa). Third-instar *A. orientalis* larvae used in this study were collected from turfgrass areas (Rutgers

Horticultural Farm II, North Brunswick, NJ) and kept individually in 24-well tissue culture plates at 8 °C for 6–14 weeks in pasteurized sandy loam soil at approximately 10% moisture. Four nematode isolates were used in this study. Because the sequences of the isolates from the cultivated areas were a very close match (≥ 99%) to *Steinernema glaseri* (Steiner) and very similar among one another, only one cultivated ("Cultivated") isolate was tested (Fig. 3.2). Due to variation in sequences among the isolates from the wild environments, two isolates ("Wild 1" and "Wild 2") from wild plots were used in this study for comparison; sequences of which are also shown in Figure 3.2. In addition to these, the *S. glaseri* NC (SgNC) strain (originally obtained from the lab of Dr. Harry Kaya in 1998 and kept in culture) was used as a positive control as it has been shown previously to be effective at parasitizing *A. orientalis* larvae in this same study set up (e.g., Koppenhöfer and Fuzy, 2006). All batches of native IJs were produced from the same original stock batch isolated from the field. SgNC was reinfected from a lab culture of this strain. All IJs were used within 1 week of harvest from reinfected *G. mellonella* and kept at room temperature (21–24 °C) until use. Third-instar plum curculio (*Conotrachelus nenuphar* Herbst; Coleoptera: Curculionidae) were obtained from the lab of Dr. Tracy Leskey.

Assay set up and experimental design

To assess virulence of the native isolates against third instar *A. orientalis* larvae, an assay was set up at room temperature (21 – 24 °C) in 30 mL plastic cups (34 mm inner diameter × 33 mm height). Each cup was filled with 25 g of blueberry field soil with perennial ryegrass, *Lolium perenne* L., in the soil for larval food. Larvae were held at

room temperature for at least 24 hours before placement in the cups and replaced if they did not completely bury themselves in the soil after 2 hours.

Each isolate including SgNC was represented by 60 cups with 20 cups at each of three densities of IJs per larva: 50, 150, 450 in 50 µL of water. An untreated control using only water (50 µL) was represented by 20 cups in each run of the experiment. The assay was run twice with a new batch of IJs each time. In a separate test, third instars of *C. nenuphar*, a native pest species prevalent in commercial *V. corymbosum* fields that is not a root-feeder but pupates in soil, were exposed to the same treatments using the same method but only replicated 10 times in pasteurized silica sand at 10% w/v moisture. This test was not repeated because no infections resulted.

Statistical Analysis

Analyses were performed using the statistical software packages SAS v. 9.3 (SAS Institute, Cary, NC, USA) and R (R Core Development Team 2014). The principal components analysis (PCA) was conducted using JMP software 3 (SAS Institute, Cary, NC, USA). To determine differences in EPN abundance between wild and cultivated plots in 2011 and 2012, a mixed linear model (Littell et al. 2006) was constructed with "environment" as the treatment (wild or cultivated), year, sample date and their interactions as fixed effects. Site was nested in treatment and considered as a random effect. The data was also sorted by year and sample to explore further the nature of the interactive effects.

The effect of isolate and IJ density on mortality of larvae was determined using the GLM procedure. In both analyses, a pairwise t-test using the Tukey-Kramer method was employed for least squares means separation when relevant effects were significant.

Data from 2013 were also analyzed with a mixed linear model. The model was constructed with treatment (wild or cultivated) and sample date as fixed effects, and site as a random effect, using the lme4 package in R. EPN abundance data were log transformed to improve normality, and significance was assessed using type II sums of squares. Effect of isolate and density on mortality of larvae was determined using the GLM procedure. In both analyses, a pairwise t-test using the Tukey-Kramer method was employed for least squares means separation when relevant effects were significant. Additionally, we were interested in the relationships between the abiotic soil characteristics measured (clay, sand, silt, organic matter, pH, soil moisture, B, Ca, Cu, Fe, K, Mg, Mn, NH_4^+, NO_3^-, P, Zn) and treatment. Toward that end, a PCA was used with data on soil characteristics first transformed into z-scores to standardize the units (Shaw 2003).

Results

EPN Abundance

In 2011 and 2012, the interactive effect of year, sample time, and environment (treatment) ($F_{(4/870)} = 4.99$, $P = 0.0006$) combined was highly significant (Fig. 2). EPN abundance showed a trend towards higher abundance in the soil of the cultivated plants than the soil of wild plants ($F_{(1/18)} = 4.35$, $P = 0.0516$). Despite the lack of significance of treatment overall, two major visual trends appear in the data, which is presented by sample, year and treatment in Figure 2. Firstly, even though overall EPN abundance was higher in 2011 ($F_{(1/870)} = 22.00$, $P \leq 0.0001$), seasonality had no effect on abundance in the cultivated environment regardless of year (Fig. 2), with no significant differences

between overall sample times when interactive effects of treatment and year were not included. In the wild environment, however, abundance tended to be higher (3× in 2011, 1.5× in 2012) in the first sample of year but this effect was statistically significant only in 2011 (Fig. 2). There was no interaction between year and environment ($F_{(1/870)} = 0.05$, $P = 0.8219$), but sampling time also interacted significantly with environment ($F_{(2/870)} = 8.15$, $P = 0.0003$).

To further describe the nature of these trends, the data was also analyzed by year and sample. Analysis of these data by sampling time showed no significant effect of environment in either year in the first sample (bloom) ($F_{(1/18)} = 0.63$, $P = 0.4394$), but significantly higher EPN abundance in the cultivated than the wild environment in samples 2 (fruit set) ($F_{(1/18)} = 16.91$, $P = 0.0007$) and 3 (post-harvest) ($F_{(1/18)} = 7.61$, $P = 0.0129$) (Fig. 2). When data were analyzed within each year, sampling time had no effect on abundance in the cultivated environment regardless of year; in the wild environment, however, abundance tended to be higher in the first sample of year (8–16× in 2011, 3-4× in 2012) but this effect was statistically significant only in 2011 ($F_{(2/286)} = 5.72$, $P = 0.0037$) (Fig. 2).

In 2013, when soil was combined by site rather than by plant, treatment had a significant effect on EPN abundance ($\chi^2 = 4.89$, $df = 1$, $P = 0.027$), with greater mean numbers of EPN infections per sample found in cultivated plots (0.97 ± 0.25) compared with wild plots (0.43 ± 0.22) (Fig. 3). However, there was no significant effect of sampling time on EPN abundance ($\chi^2 < 0.01$, $df = 1$, $P = 0.991$).

EPN Diversity

Cultivated soils had a marked lack of diversity of EPN. Of the 10 coupled sites sampled in 2012 and 2013, six cultivated plots and five of the wild sites were positive for EPN presence. In the subset of isolates sequenced, 100% from the cultivated fields were identified as *Steinernema glaseri* using a BLASTn search. Since the subset of isolates was selected based on morphological or life history characteristics that represented that variation seen over the three years of extracting from soil, we assume the most if not all of the isolates from the cultivated soils were *S. glaseri*, and this notion was later confirmed by screening for 10 other nematode species using quantitative-PCR (Rivera et al. unpublished data). Samples from cultivated fields being the same species, had very little variation between them and therefore, only a single representative sequence is presented in Figure 4. Isolates from the five wild sites positive for EPN presence had such distinct variability that none of them could be reliably identified using the BLASTn search. Although comment and analysis on the evolutionary implications are beyond the scope of the present study, shown also in Figure 4 is an example of variation between two sequences from EPN isolated from wild plants from two different sites. These could not be reliably identified using a BLASTn search because their first match was only 96% to *S. glaseri*. The potential divergence of cultivated and wild populations is evident in the multiple sequence alignment (Fig. 4) with the most marked differences occurring between positions 182 and 200, including a 8bp gap corresponding to a shift in the reading frame.

Virulence of native isolates

Mortality of third instar *A. orientalis* was significantly affected by EPN density and isolate. Density had a significant effect on mortality only for the 'Wild 2' isolate ($F= 261.15$, $df = 2$, $P \leq 0.0001$) (Fig. 5). Percent mortality did differ significantly, however, between isolates (Fig. 5). All the native EPN isolates caused less mortality in *A. orientalis* than the NC strain (78%). The cultivated strain caused greater mortality (46%) in *A. orientalis* than the 'Wild 1' strain (32%) but was equally as effective the 'Wild 2' strain (42%).

Effect of abiotic soil characteristics in wild and cultivated environments on EPN abundance

The PCA model used explained 58.4% of the variance (42.5% for axis 1, and 15.9% for axis 2) and revealed that the soil characteristics of sites were markedly clustered by environment (Fig. 6). More specifically, moisture, organic matter, and to a lesser extent silt, pH, and B, were correlated with wild sites, but the other 12 abiotic characteristics, particularly plant macro and micronutrients, were strongly positively associated with cultivated sites.

Discussion

To the best of our knowledge, the present study is the first to link the interactive effect of plant domestication and altered soil structure to the abundance, diversity and virulence of EPN. The overall conclusion from the present study is that the perennial cultivation of highbush blueberry in its primordial environment appears to promote EPN

abundance and to some extent EPN virulence against *A. orientalis* larvae but potentially decreases substantially the diversity of EPN communities.

EPN abundance in per-plant soil samples from cultivated fields showed a strong trend for higher abundance in 2011 and 2012. In 2013, when soil was consolidated by site rather than plant in this same study, EPN abundance also was higher in the cultivated fields. Although results from 2013 are consistent with trends from the previous years, it is our belief that it is more useful to examine variation on a per plant basis. In the case of this study, the significantly higher abundance of EPN in cultivated sites in 2013 could be the populations of just a few of the ten plants sampled in the site. If this is true, it falsely explains the nature of the variation across an area by favoring redundancy unlinked to a spatial area that is massive in scale for such small organisms; it also neglects the potential for indirect connection of EPN with plants (Dicke and Baldwin 2010; Turlings et al. 2012; Gols 2014; Hiltpold et al. 2014). Furthermore, evidence from previous studies suggests plants are the architects of soil communities (Bezemer et al. 2010; Eisenhauer and Reich 2012), especially with relation to EPN because they provide nutrition to their insect hosts (Barbercheck et al. 1995). This is also relevant when considering the life history of EPN and the exodus of tens of thousands (or more) of IJs into the soil from a depleted host cadaver; it would be more likely to find higher abundance in an area where a cadaver was recently depleted than one where a cadaver has not been present for an extended amount of time.

Since fields with high *A. orientalis* pressure were selected, the increase in abundance of EPNs in cultivated sites could be attributed to greater host availability. However, the number of larvae per plant was not measured to avoid destruction of

grower plants, and therefore this assumption remains speculative. Limited soil sampling around both wild and cultivated plants did not reveal any significant host numbers other than *A. orientalis* larvae (Rivera, pers. observations). Also, in a previous study investigating the efficacy of EPN for *A. orientalis* management in commercial *V. corymbosum* fields in the same area in 2003 and 2004, no other potential host species were detected among the roots even though the plants were uprooted and the entire root mass destructively searched (Polavarapu et al. 2007). Since that study was conducted, the plum curculio has also become established in the *V. corymbosum* growing areas in New Jersey. As it pupates in the soil, it could potentially serve as another host for EPN. But in our laboratory experiments, no EPN infections were observed. It is possible that the large IJ size may have contributed to the lack of infection in this host since mature *C. nenuphar* larvae are only 6 – 9 mm in length and the EPNs extracted from wild and cultivated *V. corymbosum* soil are quite large (average IJ length × maximum diameter: 1200 × 43 µm). However, *S. glaseri* readily infects second-instar *A. orientalis*, which are of similar size as mature *C. nenuphar* larvae, at a similar density as third-instar *A. orientalis* (Koppenhöfer & Fuzy, 2004).

As with the higher abundance and diversity of plant parasitic nematodes associated with cultivated plants in this system (Rivera et al. 2015), it is similarly likely the EPN abundance is indirectly linked to increased plant health. The cultivated highbush blueberry sites sampled are genotypic monocultures, propagated from the same rootstock, that are irrigated and fertilized for fruit production and thus, plants in the cultivated arena are larger and more uniform (i.e., provide more of a better resource for insect herbivores). Evidence for this was found in the PCA analysis (Fig. 6) where the majority of the plant

nutrients assessed were correlated with the cultivated sites rather than the wild sites. Soil moisture and organic matter explained a significant amount of variation attributed to wild sites (Fig. 6) and soil moisture is very important to EPN function. However, since the agricultural fields are irrigated, the moisture level in soil of cultivated fields is unlikely fall below what is needed for EPN activity during the growing season.

The increased abundance of EPNs in the cultivated environment appears to be negatively related to diversity. While cultivated fields had more abundant populations of EPNs, *S. glaseri* was the only speciesidentified from cultivated fields and was isolated from six of the ten cultivated sites sampled. Conversely, isolates from the five wild sites positive for EPN presence had such distinct variability that none of them could be identified using the BLASTn search. They are likely undescribed species and have been submitted for description to taxonomists. The higher diversity of EPN seems to mirror the variability in virulence as the two wild isolates tested differed significantly in their ability to infect *A. orientalis* larvae, the presumed host (Fig. 5). This variability could impact the function of EPN communities beyond just what was tested here. For example, the coexistence of such a diverse EPN assemblage could be attributed to niche partitioning by factors not tested such as larval age and presence of other potential hosts (Hutchinson 1959; MacArthur 1970; Ettema 1998; Finke and Snyder 2008).

The presence, high abundance and distribution of *S. glaseri* among cultivated fields is both surprising and not. *S. glaseri* was first discovered in a golf course approximately 50km away in Haddonfield, NJ, USA (Glaser and Fox 1930). A large-scale, statewide effort followed to inoculate *S. glaseri* as a means to limit the spread of the then recently introduced Japanese beetle, *Popillia japonica* Newman. It is, however,

likely that this massive undertaking was doomed from the start as the researchers, at the time still unaware of the nematode's symbiotic bacterium, *Xenorhabdus poinarii*, use of antibiotics in the nematode's mass-production that probably eliminated the symbionts. However, both the initial inoculation and the subsequent sampling efforts to monitor *S. glaseri* establishment 50 years later (Gaugler et al. 1992) seem to have overlooked the Pinelands National Reserve. The sites sampled in the present study and the sites with previously confirmed *S. glaseri* populations are shown in Figure 1A. Thus, the prevalence of *S. glaseri* in this area was surprising as it was one of the species found less frequently in extensive sampling of the state of New Jersey (Gaugler et al. 1992). Taking into account the biology and ecology of *S. glaseri*, this finding is not surprising as the species is very competitive with other EPN species (Koppenhöfer et al., 1996) and highly mobile (Grewal et al. 1994; Lewis et al. 1995) and persistent in soil (Koppenhöfer and Fuzy, 2007).

The poor virulence of the isolate from cultivated blueberry fields when compared to the much-studied SgNC strain against *A. orientalis* larvae was unexpected (Fig. 5) mostly because it was assumed EPN of the same species, which carry the same bacterial symbiont, would be more similar in their ability to infect a specific insect. There is, however, evidence for differences among *S. glaseri* strains in their ability to infect *A. orientalis* larvae. This variability is, at least in part, due to the presence or absence of surface coat proteins that aid in overcoming the encapsulation by the immune system of *A. orientalis* larvae (Li et al. 2007). These results beget speculation on what benefit these *S. glaseri* isolates may gain from being only marginally effective at killing what appears to be the most common potential host.

It seems unlikely that evolutionary pressures on EPN would select for both higher virulence and higher persistence of *S. glaseri*. After all, this would mean the EPN would quickly exhaust their host resource population and have to persist long enough for the area to be recolonized by another host or adapt to another host. Perhaps the transitioning of once wild land into land for cultivation causes genetic drift in local EPN populations and in this case, selected for higher persistence but lower virulence. In fact, isolates extracted from soil samples in 2012 are still infective three years later in 2015 (Rivera, personal observation). Otherwise, the cultivated isolate has maybe adapted to the cultivated arena over time and thus, become more virulent to *A. orientalis* larvae than the wild isolates. EPN populations that are highly persistent and virulent to a specific insect host may also suggest a linkage of such a populations to a specific and highly linked insect-plant dynamic (Preisser et al. 2006). Additional research is needed to elucidate the connections between the belowground community at large and EPN in order to understand what drives the persistence, virulence and diversity of EPN communities.

Acknowledgements

We would like to especially thank the 10 landowners who allowed us to use their property for this survey as well as Dean Polk for his assistance in our selection of sites. Eugene Fuzy, Richard Keller, Wlodek Lapkiewicz, Robert Holdcraft and many others provided technical assistance in the field and lab. We also would like to thank Patricia Stock and Raquel Campos-Herrera for their helpful suggestions over the three years of surveying. Comments from Elvira De Lange and John Dighton improved a previous version of the manuscript. This research was supported by a grant from USDA Sustainable Agriculture Research and Education (GNE11- 027).

References Cited

Barbercheck ME, Wang J, Hirsh I. (1995) Host plant effects on Entomopathogenic Nematodes. J Invertebr Pathol 66:169–177.

Bezemer TM, Fountain MT, Barea JM, et al (2010) Divergent composition but similar function of soil food webs of individual plants: plant species and community effects. Ecology 91:3027–36.

Bian Y, Ballington J, Raja A, et al (2014) Patterns of simple sequence repeats in cultivated blueberries (Vaccinium section Cyanococcus spp.) and their use in revealing genetic diversity and population structure. Mol Breed 675–689. doi: 10.1007/s11032-014-0066-7

Bongers T (1990) The maturity index: an ecological measure of environmental disturbance based on nematode species composition. Oecologia 83:14–19. doi: 10.1007/BF00324627

Bongers T, Ferris H (1999) Nematode community structure as a bioindicator in environmental monitoring. Trends Ecol Evol 14:224–228. doi: 10.1016/S0169-5347(98)01583-3

Chen XY, Daniell TJ, Neilson R, et al (2010) A comparison of molecular methods for monitoring soil nematodes and their use as biological indicators. Eur J Soil Biol 46:319–324. doi: 10.1016/j.ejsobi.2010.05.002

Chen YH, Gols R, Benrey B (2015) Crop domestication and naturally selected species interactions. Annu Rev Entomol. doi: 10.1146/annurev-ento-010814-020601

Degenhardt J, Hiltpold I, Köllner TG, et al (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proc Natl Acad Sci U S A 106:13213–8. doi: 10.1073/pnas.0906365106

Denno RF, Gruner DS, Kaplan I (2008) Potential for entomopathogenic nematodes in biological control: a meta-analytical synthesis and insights from trophic cascade theory. J Nematol 40:61–72.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the “cry for help”. Trends Plant Sci 15:167–75. doi: 10.1016/j.tplants.2009.12.002

Doran JW (2002) Soil health and global sustainability: translating science into practice. Agric Ecosyst Environ 88:119–127. doi: Doi 10.1016/S0167-8809(01)00246-8

Eck P (1965) Blueberry Culture. Rutgers University Press, New Brunswick, NJ

Eisenhauer N, Reich PB (2012) Above- and below-ground plant inputs both fuel soil food webs. Soil Biol Biochem 45:156–160. doi: 10.1016/j.soilbio.2011.10.019

Erb M, Lu J (2013) Soil abiotic factors influence interactions between belowground herbivores and plant roots. J Exp Bot 64:1295–303. doi: 10.1093/jxb/ert007

Ettema CH (1998) Soil Nematode Diversity: Species Coexistence and Ecosystem

Function. 30:159–169.

Finke DL, Snyder WE (2008) Niche partitioning increases resource exploitation by diverse communities. Science 321:1488–1490. doi: 10.1126/science.1160854

Floyd R, Abebe E, Papert A, Blaxter M (2002) Molecular barcodes for soil nematode identification. Mol Ecol 11:839–850. doi: 10.1046/j.1365-294X.2002.01485.x

Freckman DW, Ettema CH (1993) Assessing nematode communities in agroecosystems of varying human intervention. Agric Ecosyst Environ 45:239–261. doi: 10.1016/0167-8809(93)90074-Y

Gaugler R, Campbell JF, Selvan S, Lewis EE (1992) Large-scale inoculative releases of the entomopathogenic nematode Steinernema glaseri: Assessment 50 years later. Biol Control 2:181–187. doi: 10.1016/1049-9644(92)90057-K

Gaugler R, Lewis E, Stuart RJ (1997) Ecology in the service of biological control: the case of entomopathogenic nematodes. Oecologia 109:483–489. doi: 10.1007/s004420050108

Glaser RW, Fox H (1930) A nematode parasite of the Japanese beetle (Popillia japonica). Science (80-) LXXI:343–344.

Gols R (2014) Direct and indirect chemical defences against insects in a multitrophic framework. Plant Cell Environ 37:1741–52. doi: 10.1111/pce.12318

Govaerts B, Fuentes M, Mezzalama M, et al (2007) Infiltration, soil moisture, root rot and nematode populations after 12 years of different tillage, residue and crop rotation managements. Soil Tillage Res 94:209–219. doi: 10.1016/j.still.2006.07.013

Grewal PS, Lewis EE, Gaugler R, Campbell JF (1994) Host finding behaviour as a predictor of foraging strategy in entomopathogenic nematodes. Parasitology 108:207–15. doi: 10.1017/S003118200006830X

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98.

Hiltpold I, Jaffuel G, Turlings TCJ (2014) The dual effects of root-cap exudates on nematodes: from quiescence in plant-parasitic nematodes to frenzy in entomopathogenic nematodes. J Exp Bot. doi: 10.1093/jxb/eru345

Hutchinson GE (1959) Homage to Santa Rosalia or Why Are There So Many Kinds of Animals? Am Nat 93:145. doi: 10.1086/282070

Jonsson M, Buckley HL, Case BS, et al (2012) Agricultural intensification drives landscape-context effects on host-parasitoid interactions in agroecosystems. J Appl Ecol 49:706–714. doi: 10.1111/j.1365-2664.2012.02130.x

Koppenhöfer AM, Campbell JF, Kaya HK, Gaugler R (1998) Estimation of entomopathogenic nematode population density in soil by correlation between bait insect mortality and nematode penetration. Fundam Appl Nematol 21:95–102.

Koppenhöfer AM, Fuzy EM (2006) Effect of soil type on infectivity and persistence of the entomopathogenic nematodes Steinernema scarabaei, Steinernema glaseri,

Heterorhabditis zealandica, and Heterorhabditis bacteriophora. J Invertebr Pathol 92:11–22. doi: 10.1016/j.jip.2006.02.003

Koppenhöfer AM, Kaya HK, Shanmugam S, Wood GL (1995) Interspecific competition between Steinernematid nematodes within an insect host. J Invertebr Pathol 99–103.

Kostenko O, Duyts H, Grootemaat S, et al (2015) Plant diversity and identity effects on predatory nematodes and their prey. Ecol Evol 5:836–847. doi: 10.1002/ece3.1337

Kung S-P, Gaugler R, Kaya HK (1991) Effects of soil temperature, moisture, and relative humidity on entomopathogenic nematode persistence. J Invertebr Pathol 57:242–249. doi: 10.1016/0022-2011(91)90123-8

Lewis EE, Gaugler R, Harrison R (1993) Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues. Can J Zool 71:765–769. doi: 10.1139/z93-101

Lewis EE, Grewal PS, Gaugler R (2009) Hierarchical order of host cues in parasite foraging strategies. Parasitology 110:207–213. doi: 10.1017/S0031182000063976

Lewis EE, Selvan S, Campbell JF, Gaugler R (1995) Changes in foraging behaviour during the infective stage of entomopathogenic nematodes. Parasitology 110:583–590. doi: 10.1017/S0031182000065306

Li X-Y, Cowles RS, Cowles EA, et al (2007) Relationship between the successful infection by entomopathogenic nematodes and the host immune response. Int J Parasitol 37:365–74. doi: 10.1016/j.ijpara.2006.08.009

Littell R, Milliken G, Stroup W, Wolfringer R (2006) SAS System for Mixed Models, 2nd edn. SAS Institute, Cary, NC, USA

MacArthur R (1970) Species packing and competitive equilibrium for many species. Theor Popul Biol 1:1–11.

Matson P a., Parton WJ, Power a. G, Swift MJ (1997) Agricultural intensification and ecosystem properties. Science (80-) 277:504–509. doi: 10.1126/science.277.5325.504

Millar LC, Barbercheck ME (2002) Effects of tillage practices on entomopathogenic nematodes in a corn agroecosystem. Biol Control 25:1–11. doi: 10.1016/S1049-9644(02)00042-7

Morise H, Miyazaki E, Yoshimitsu S, Eki T (2012) Profiling Nematode Communities in Unmanaged Flowerbed and Agricultural Field Soils in Japan by DNA Barcode Sequencing. PLoS One. doi: 10.1371/journal.pone.0051785

Palomares-Rius JE, Castillo P, Montes-borrego M, et al (2015) Soil Properties and Olive Cultivar Determine the Structure and Diversity of Plant-Parasitic Nematode Communities Infesting Olive Orchards Soils in Southern Spain. PLoS One 1–20. doi: 10.1371/journal.pone.0116890

Poelman EH, van Loon JJ a, Dicke M (2008) Consequences of variation in plant defense for biodiversity at higher trophic levels. Trends Plant Sci 13:534–41. doi:

10.1016/j.tplants.2008.08.003

Polavarapu S, Koppenhöfer AM, Barry J, et al (2007) Entomopathogenic nematodes and neonicotinoids for remedial control of oriental beetle, Anomala orientalis (Coleoptera: Scarabaeidae), in highbush blueberry. Crop Prot 26:1266–1271. doi: 10.1016/j.cropro.2006.10.026

Preisser EL, Dugaw CJ, Dennis B, Strong DR (2006) Plant facilitation of a belowground predator. Ecology 87:1116–23.

R Core Development Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Rivera MJ, Rodriguez-Saona C, Jennings DE, Koppenhöfer AM (2015) Assessing the impact of cultivation and plant domestication of highbush blueberry (Vaccinium corymbosum) on soil properties and associated plant-parasitic nematode communities. Soil Biol Biochem 88:25–28. doi: 10.1016/j.soilbio.2015.05.010

Sánchez-Moreno S, Ferris H (2007) Suppressive service of the soil food web: Effects of environmental management. Agric Ecosyst Environ 119:75–87. doi: 10.1016/j.agee.2006.06.012

Shaw PJ. (2003) Multivariate statistics for the environmental sciences. Wiley, Chichester, UK

Stock SP, Goodrich-Blair H (2012) Nematode parasites, pathogens and associates of insects and invertebrates of economic importance. In: Lacey LA (ed) Manual of Techniques in Invertebrate Pathology, 2nd edn. Elsevier, London, pp 373–426

Strong DR, Kaya HK, Whipple A V., et al (1996) Entomopathogenic nematodes: natural enemies of root-feeding caterpillars on bush lupine. Oecologia 108:167–173. doi: 10.1007/BF00333228

Thompson J, Higgins D, Gibson T (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4690.

Turlings TCJ, Hiltpold I, Rasmann S (2012) The importance of root-produced volatiles as foraging cues for entomopathogenic nematodes. Plant Soil 358:51–60. doi: 10.1007/s11104-012-1295-3

Van der Putten WH, Vet LEM, Harvey JA, Wäckers FL (2001) Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. Trends Ecol Evol 16:547–554. doi: 10.1016/S0169-5347(01)02265-0

Vonk JA, Breure AM, Mulder C (2013) Environmentally-driven dissimilarity of trait-based indices of nematodes under different agricultural management and soil types. Agric Ecosyst Environ 179:133–138. doi: 10.1016/j.agee.2013.08.007

Wardle D a, Yeates GW, Williamson W, Bonner KI (2003) The response of a three trophic level soil food web to the identity and diversity of plant species and functional groups. Oikos 102:45–56. doi: 10.1034/j.1600-0706.2003.12481.x

Yeates GW, Bongers T, Goede RGMDE, et al (1993) Feeding Habits in Soil Nematode Families andGenera--An Outline for Soil Ecologists. 25:315–331.

Zak DR, Holmes WE, White DC, et al (2003) Plant diveristy, soil microbial communities, and ecosystem function: are there any link? Ecology 84:2042–2050. doi: 10.1890/02-0433

Pinelands Comprehensive Management Plan. http://www.co.cumberland.nj.us/filestorage/173/251/761/pinelands_with_maps.pdf. Accessed 10 Jun 2012a

Pinelands Facts. http://www.state.nj.us/pinelands/infor/fact/Pinelands Facts.pdf. Accessed 6 Jun 2012b

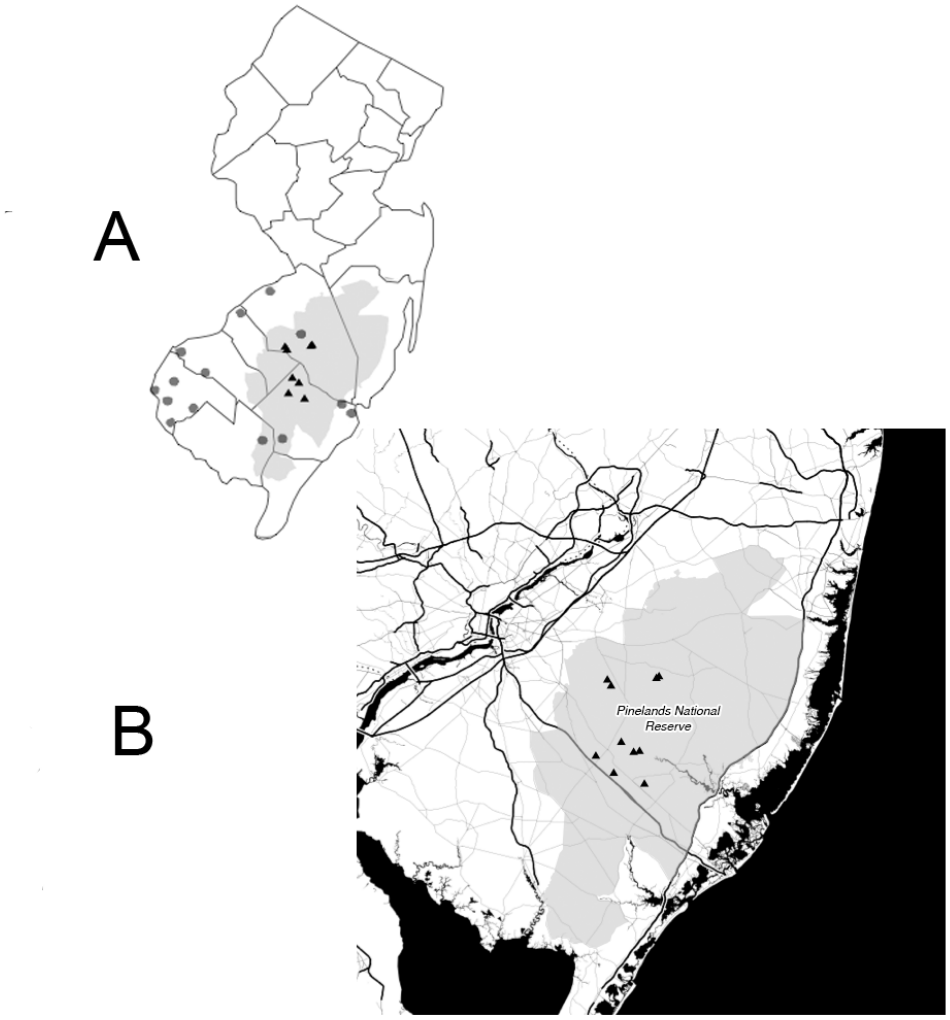

Figure 1. Distribution of entomopathogenic nematode sampling sites in wild and domesticated stands of *Vaccinium corymbosum* within the Pineland National Reserve (PNR) in New Jersey, USA. Panel A shows sites positive for *Steinernema glaseri* (solid black circles) in an extensive sample of the state of New Jersey (Gaugler et al. 1992), and sites used in this study with positive infections (solid black triangles). Panel B shows a more detailed map of the location within the PNR.

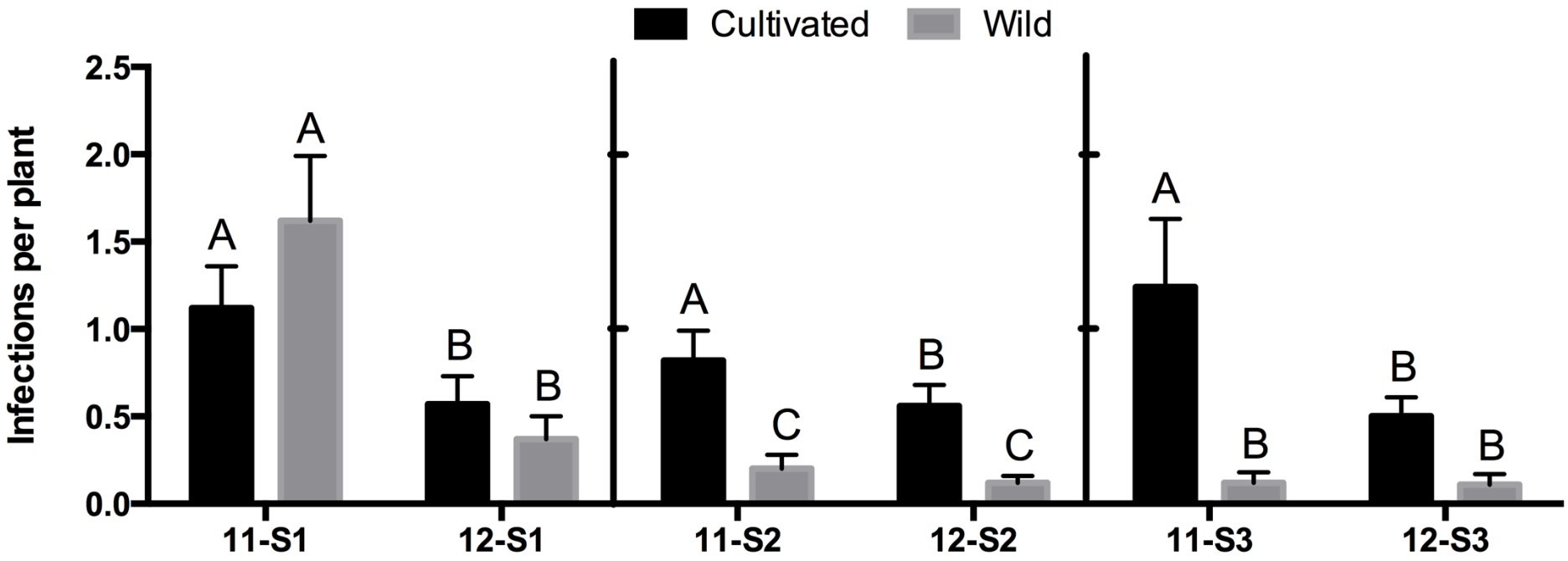

Figure 2. Abundance (mean +SEM) of entomopathogenic nematodes (number of infected bait insects) in rhizospheric soil of cultivated and wild highbush blueberry (*Vaccinium corymbosum*) in soil sampled in 2011 (11) and 2012 (12). Soil sampling occurred at three different times correlating with the phenology of the cultivated plants: bloom (S1), fruit set (S2), and post-harvest (S3). Means with same letter within sampling time did not differ significantly ($\alpha = 0.05$).

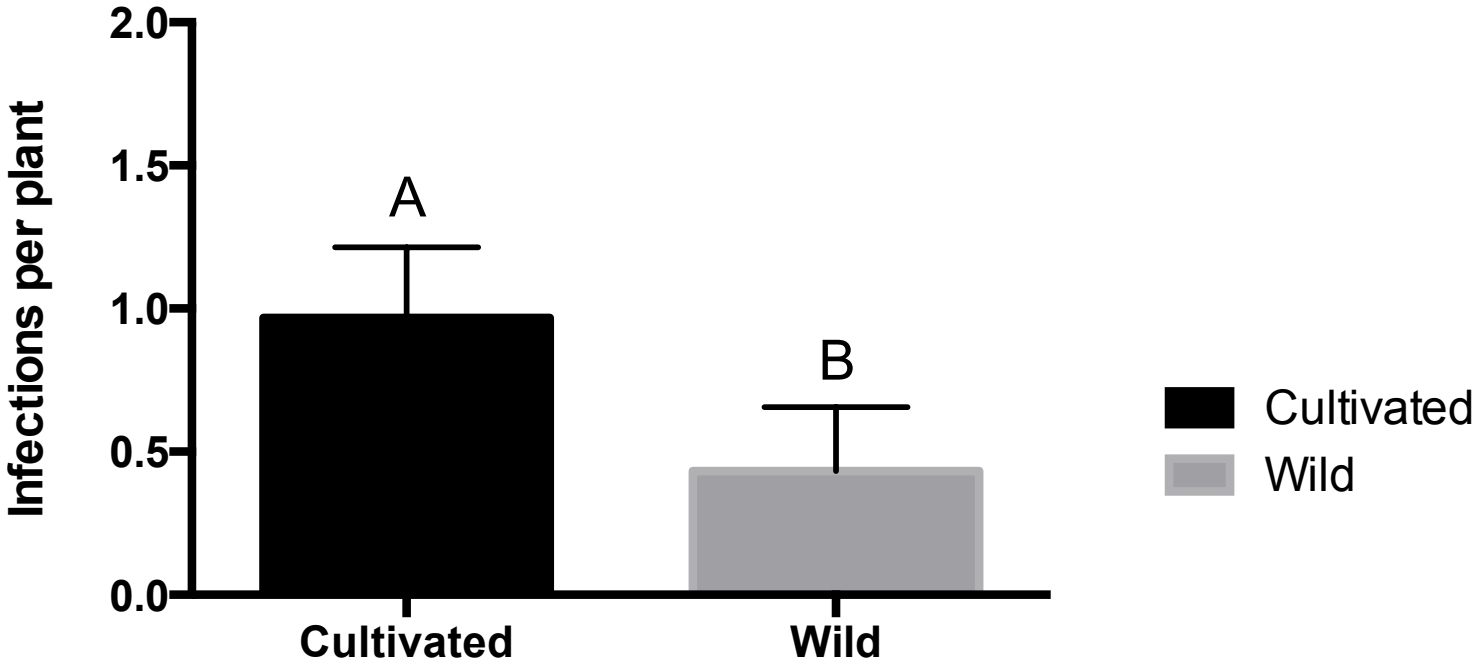

Figure 3. Abundance (mean +SEM) of entomopathogenic nematodes from rhizospheric soil associated with sites containing wild and cultivate highbush blueberry (*Vaccinium corymbosum*) in 2013. Means with same letter did not differ significantly ($\alpha = 0.05$).

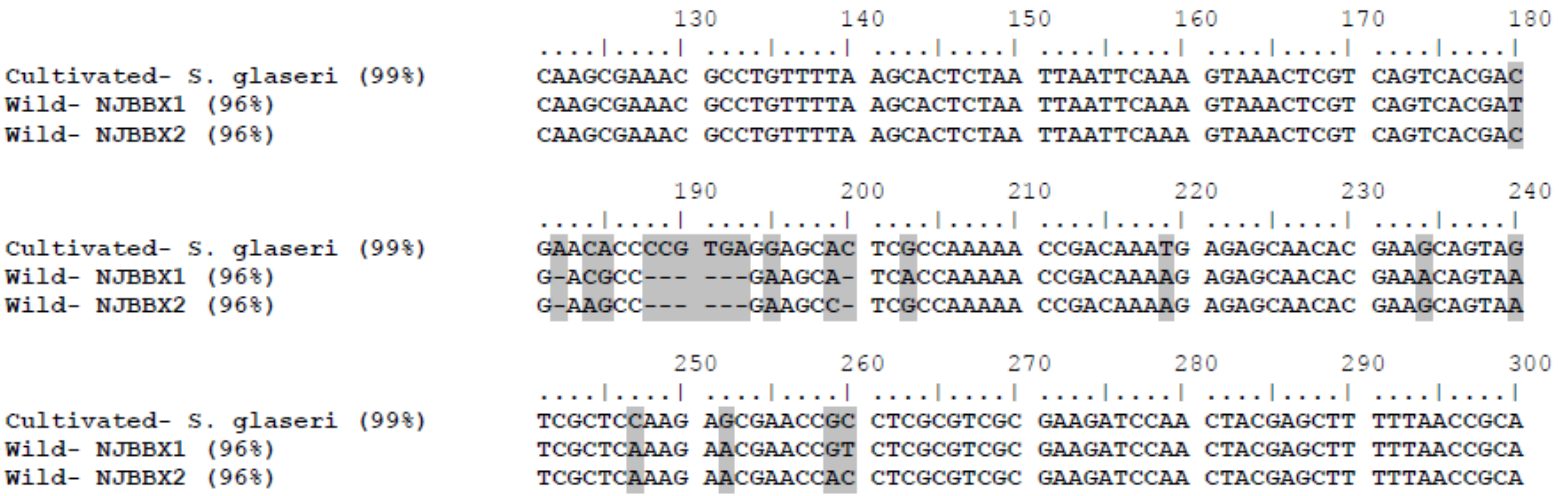

Figure 4. Portion of the multiple sequence alignment comparing 18S sequences from nematodes collected in cultivated vs. wild soil. Alignmen exported from BioEdit version 7.1.3.0. Polymorphic positions are highlighted in gray. Percentages represent sequence similarity to *S. glaseri* (GenBank Accession# FJ040422.1) obtained from a BLASTn search.

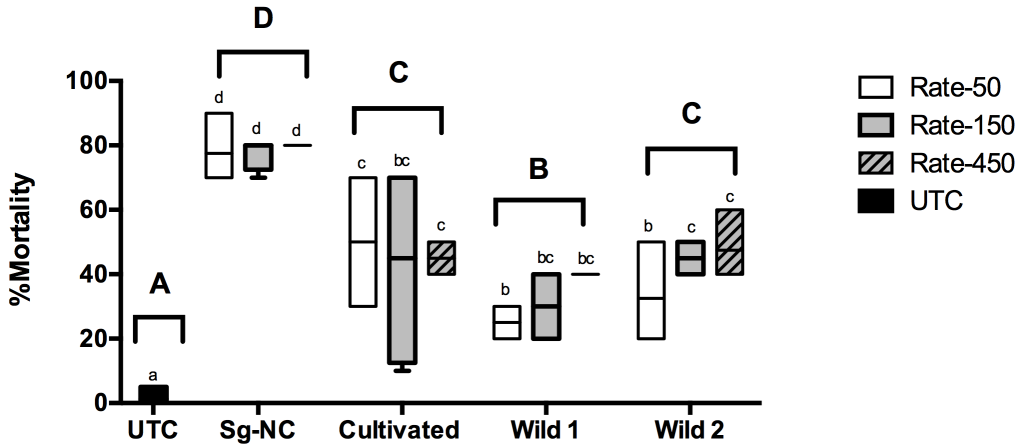

Figure 5. Percent mortality of *Anomala orientalis* third instar larvae when exposed to different rates and strains of entomopathogenic nematodes endemic to wild and cultivated highbush blueberry (*Vaccinium corymbosum*) in the Pineland National Reserve. Three native isolates were tested. *Steinernema glaseri* (NC Strain) was also included as a positive control. Means with the same letter did not differ significantly ($\alpha = 0.05$). Panel shows larval mortality when exposed to three densities of three native EPN isolates (lowercase letters) and shows larval mortality by isolate (uppercase letters).

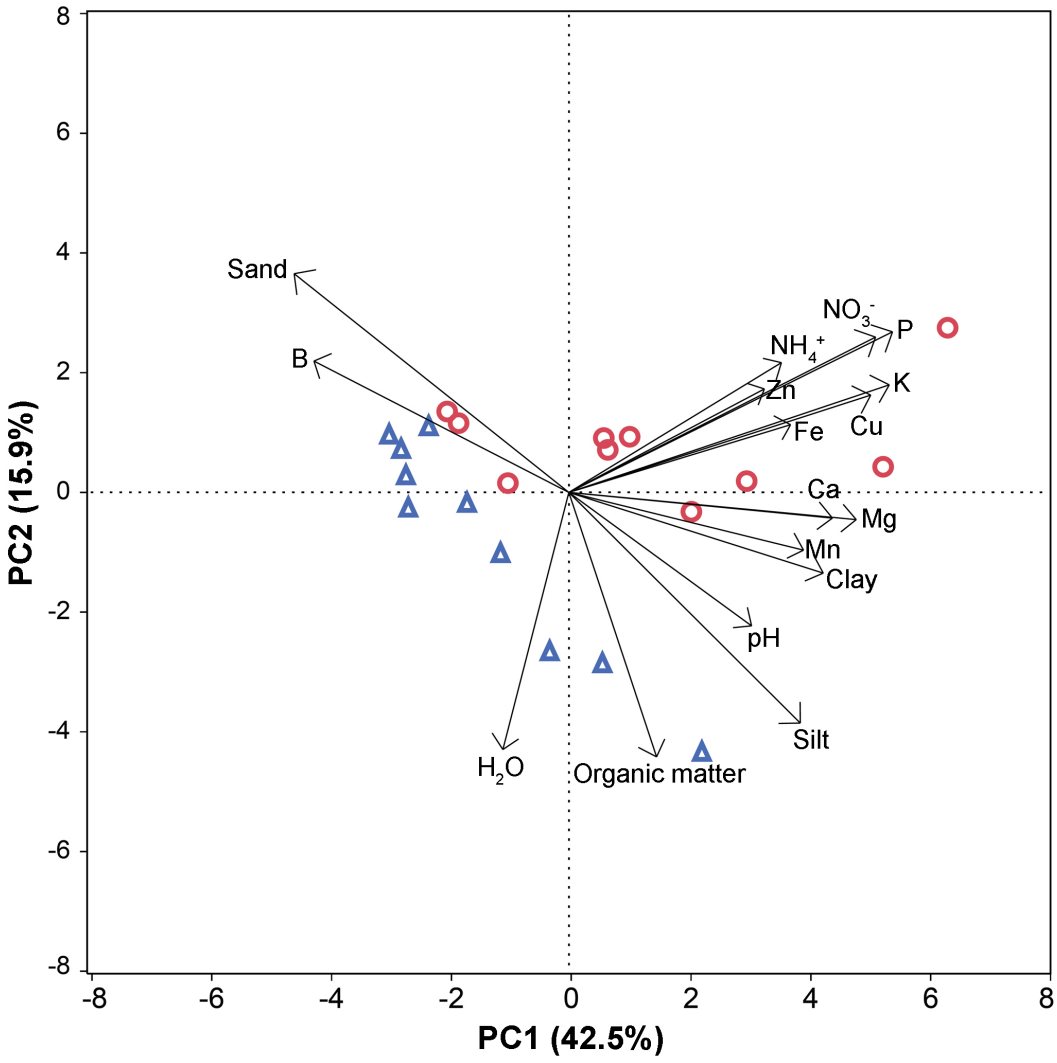

Figure 6. Biplot showing the first and second axes of ordination from the principal components analysis of soil characteristics and total extractable nutrients (loadings represented by arrows) by treatment (circles = cultivated sites, triangles = wild sites).

Chapter 3: Entomopathogenic nematode isolates associated with domesticated and wild *Vaccinium corymbosum* are more attracted to the plant type from which they were isolated

Abstract

Over the past decade, interest in belowground tritrophic interactions involving a plant-root-feeding insect herbivore and entomopathogenic nematodes (EPN) has surged. The research focus has been on domesticated plant varieties because of their relevance in agricultural production. The objective of this study was to investigate differences in this interaction when comparing wild and domesticated plants. We used highbush blueberry (*Vaccinium corymbosum*) as a study system because it was domesticated locally and is produced locally. This allowed us access to wild plants for propagation and native EPN isolates to both environments. Two-choice belowground olfactometers were used to examine the preferential attraction of EPN when given three sets of choices: 1. *Anomala orientalis* larvae versus sand only, 2. plants alone versus sand only and 3. plants with feeding larvae compared to plants alone. Additionally, volatiles were collected from the roots of wild and domesticated plants to examine potential signals and variation in volatile profiles between plants with and without feeding larvae. Overall, EPN from domesticated plants (B1) and wild plants (N9) were more attracted to the plant types with which they were originally associated. EPN were not attracted to the larvae alone. Specifically, B1 was not attracted significantly to domesticated or wild plants alone but was attracted to domesticated plants with feeding larvae. In contrast, N9 was more

attracted to the wild plants alone but the addition of larvae did not significantly enhance this attraction. Despite some promising leads, volatile analysis did not show significant induction of chemicals from insect feeding on the roots of *V. corymbosum*. Our results suggest that nematodes are locally adapted to the system from which they were isolated but this effect was stronger for domesticated plants and the domesticated nematode isolate.

Introduction

Over the past decade, research focus has shifted from solely aboveground tritrophic interactions to the integration and investigation of belowground interactions (van Dam and Heil 2011). Thus, concepts developed in aboveground interactions are being explored in relation to belowground systems, namely, the idea that herbivore feeding induces the plant to release a different blend of volatiles that thereby "calls" the herbivore's natural enemies (Dicke and Baldwin 2010) and that this is another way plants can defend themselves (Price et al. 1980). In belowground studies, the central focus has been on studying the behavior and response of entomopathogenic nematodes (EPN) to domesticated agricultural plant varieties (Chen et al. 2015). Domesticated plant varieties are very easy to access and are generally widely available for purchase. However, natural soil ecosystem are so complex that the interpretation of field study results is very challenging. While this complexity makes it also nearly impossible to replicate natural soil ecosystems in a controlled lab study (Coleman et al. 2004), laboratory studies still have great value in quantifying the importance of various factors that may be relevant in natural field interactions.

Compared to many of the well-studied aboveground third trophic level predators, EPN introduce even more factors to the interaction. EPN have evolved the ability to carry with them highly species-specific symbiotic bacteria and use them to infect and kill an insect host, thus providing unprecedented mobility to these entomopathogenic bacteria. The host provides a place and a nutrient source for bacteria, and the nematodes feed on the proliferating bacteria and broken down host tissues inside the body cavity. Once the resources in the host cadaver have been depleted after 1-3 life cycles inside the host, third instar non-feeding infective juveniles (IJs) leave the cadaver and seek out a suitable host.

The attraction of EPN to insect damaged roots has been shown in multiple systems (Van Tol et al. 2001; Rasmann et al. 2005; Ali et al. 2011). In each system, the insect damaged roots with or without insects are more attractive than the insect larvae alone which has lead to the conclusion that EPN use the change in volatile profile being released from the roots as a cue in the location of potential insect hosts. However, it is unclear how general these signals are and if they work for all EPN. It also remains unclear if genotypic variation in plants and EPN strains affect the quality and degree of these interactions.

In the study of soil systems, plant domestication presents an interesting research challenge. The domesticated plant is wed to the domesticated environment, which includes the soil itself, whereas the wild plant is a survivor of its own environment and adept at thriving there. Thus, studying the interactions related to domesticated and wild plants in the laboratory could not possibly replicate the differences in the environments in which they are grown. Laboratory studies, however, allow for such differences to be controlled. The impact of intense domestication has been shown in previous studies of

maize's ability to respond to belowground herbivory (Rasmann et al. 2005; Degenhardt et al. 2009). But this leads us to the questions of just how different are such abilities between purely wild and domesticated plants and how much of any such differences is based on variation in the communities of plant, the insect, and/or the predator/parasite. The objective of this research was to address how plant domestication changes tritrophic interactions in the rhizosphere and their specific dynamics.

We used the highbush blueberry (*Vaccinium corymbosum* L.) agroecosystem in the Pinelands National Reserve in southern New Jersey, USA to explore the effects of plant domestication on belowground interactions. This system is ideal in that *V. corymbosum* was very recently domesticated (i.e., early twentieth century) and remains the same species (i.e., can interbreed with) as its wild relative (Eck 1965; Bian et al. 2014). In contrast, many domesticated plants are such severe mutants of their parental lines they can no longer function as the same species (i.e., cannot successfully cross-pollinate one another to produce viable offspring). Moreover, *V. corymbosum* fields with domesticated cultivars in the Pinelands National Reserve are typically surrounded by woody borders that contain stands of wild *V. corymbosum*.

We focused on the oriental beetle (*Anomala orientalis*) as the insect species because it is a generalist, root-feeding pest of commercial blueberry that can cause problems ranging from yield loss to plant death (Polavarapu et al. 2007). We have also used this system to study the distribution of plant parasitic nematodes and EPN within the domesticated and natural environments (Rivera et al. 2015; Rivera et al. 2016) thereby allowing us to select EPN isolates that are relevant to the system rather than laboratory colonies of various EPN strains. In our previous studies, we found new EPN species

associated with the wild environment but found *Steinernema glaseri* to be dominant in production fields (Rivera et al. 2016). Both of these EPN are large, cruising foragers able travel relatively large distances to find hosts (Koppenhöfer and Kaya 1996; Lewis et al. 2009).

We hypothesized that as in previous studies (van Tol et al. 2001; Rasmann et al. 2005; Ali et al. 2011) local strains of EPN would be more attracted to plants with root-feeding *A. orientalis* larvae because of volatile cues from the plant. We also hypothesized that there would be differences in the volatile blends of wild and domesticated plants and that those from domesticated varieties may have lost the ability to activate an induced response to insect feeding.

Materials and Methods

Nematodes, plants and insects

All batches of infective juvenile nematodes (IJs) were produced from the original stock batch isolated from populations in commercial *V. corymbosum* fields (B1: Hammonton, NJ, USA, 39.59254, -74.77338) and in wild stands (N9: Tabernacle, NJ, USA 39.825374, -74.634985). These isolates were selected based on our previous survey of EPN in blueberry because they represent the commonly found isolates in the cultivated and wild environments respectively (Rivera et al. 2016). All IJs were used within 2 weeks of harvest from reinfected *Galleria mellonella* and kept at room temperature (21 – 24 °C) until use.

Wild and domesticated (var. Bluecrop, abbreviated as "BC") *V. corymbosum* plants were hand propagated from soft cuttings (Eck 1965) in the spring of 2011 and the spring of

2012. Wild plants originated from three different populations within the Pine Barrens National Reserve. Propagation material was collected from at least ten plants per site. All plants were kept in the breeding greenhouse at the Philip E. Marucci Center for Blueberry and Cranberry Research where they were treated in accordance with production standards including fertilization and winter chilling periods. Plants were grown for 3 years after propagation to ensure substantial root development. For experiments, plants were potted in glass treatment chambers with pasteurized sand (3 hours at 60 °C) and allowed to adjust for at least 48 hours. Three third-instar *A. orientalis* larvae were released into the chambers 24 hours before the start of all experiments to allow for feeding on the roots.

Two-choice olfactometer

A glass two-choice olfactometer was used. The set up consisted of a horizontal central chamber, two removable connector pieces on opposite sides of the central chamber, and two treatment chambers that were attached to the two connector pieces. The central chamber (25 mm diameter, 152 mm length) had an opening in the middle to place IJs in the substrate. The connector pieces (25 mm diameter, 76 mm length) contained nematode-impermeable borosilicate glass frits (medium porosity, 6.35 mm thick) at 30 mm from the end facing the central chamber. The end of the treatment chambers fit into the glass ends of the central chamber with a ground glass joints (24/25). The center of the central chamber and the opening of the treatment chamber were hence separated by 152 mm distance, and IJs had to travel a minimum of 76 mm to reach the connector pieces from which they were later extracted.

In choice tests, 1000 IJs (B1 or N9) were applied to the central chamber and allowed to move freely at room temperature for 40 hours. Each experiment used a fresh batch of IJs. During each run, six two-choice olfactometers were set up on the lab bench. Experiments involving cultivated and wild plants were run with three wild and three cultivated plant set-ups simultaneously. The replicates for the choice tests were: grubs vs sand alone (N = 12), plants vs. sand alone (N=10) and plants vs. plants with grubs (N=10). The difference in replicate numbers is due to the limitation of the number of olfactometers, time of the year (July and August) and the running of experiments with both wild and cultivated plants at once.

To determine IJ attraction, the two connector pieces were removed carefully removed from the central and treatment chambers, and the substrate emptied in separate beakers for each connector. IJs were then extracted using the sieve and decant method and counted using a dissecting microscope. To do this, the sand removed from the choice chambers was submerged in 1000 mL of water and agitated for one minute. Then, the solution was decanted over a (no. 400) mesh sieve and then the contents washed into a 100 mL beaker. This process was repeated twice for each sample to ensure the removal of all nematodes from the sample.

Volatile collection

Volatiles were collected from infested and uninfested plants to determine differences in volatile release from *V. corymbosum* roots damaged by third-instar *A. orientalis* larvae. Volatiles were collected using volatile organic compound (VOC) traps containing 30 mg HayeSep Q (Volatile Assay Systems, Rensselaer, NY, USA). When plants were repotted,

a glass Pasteur pipette was placed in the sand alongside it so that the roots grew with a space to place the soil probe; otherwise, probe placement can cause unwanted damage to the roots. Soil probes for volatile collection were made of glass Pasteur pipettes with three holes drilled in the side of them. After placement in the pots, the VOC traps were inserted into to the upper opening of the probes and secured using Teflon tape. The traps were then connected to vacuum pumps pulling at 2 L/min for 18 hours. Each infestation treatment × plant type was replicated ten times. Filters were eluted with 150 µL dichloromethane into 2 mL clear glass vials with 500 µL inserts. An internal standard was added to each sample (400 ng nonyl acetate).

Volatile Analysis

A 2-µL aliquot of dichloromethane extract was injected onto a gas chromatograph (Agilent 5980) in electron impact (EI) mode fitted with a flame ionization detector (FID) using a splitless injector held at 250 °C. The column (HP-5 30 m × 320 µm × 0.25 µm film thickness) was maintained initially at 40 °C for 1 minute and then ramped up 10 °C /min to 280 °C and kept at 280 °C for 5 minutes. Quantifications of compounds were made relative to the nonyl acetate standard using ChemStation software (Agilent Technologies, Wilmington, DE, USA).

Samples were also run in a gas chromatograph fitted with a mass spectrometer also in EI mode to do a preliminary identification of the compounds. Input to this machine was the same as above although the column was different (HP-1 15 m × 250 µm × 0.1 µm film thickness). The machine ran the same program as in the quantification run.

Preliminary compound identification was done using through an EI spectra library search using the NIST08 library.

Statistical analysis

Resulting counts of IJs from the two choice olfactometer tests were recorded along with the total number of IJs extracted from the central chamber where they were applied. The central chamber data and data from the two choice chambers were used to correct the data for extraction efficiency by converting the choice numbers into percentages of the total number of IJs extracted. The percentages in either choice section were then analyzed. First, the data was analyzed for normality (SAS 9.3, Proc Univariate). Because not all the datasets aligned with the normal distribution, both parametric and non-parametric methods were necessary. We used one-tailed paired T-Tests and Wilcoxon matched pairs signed rank tests where appropriate (GraphPad Prism 6). To compare the amount of total movement towards the treatments, unpaired nonparametric Mann-Whitney tests were used.

Multivariate statistics were necessary to assess volatile production by *V. corymbosum* roots due to the large amount of compounds emitted many of which are produced by highly conserved pathways. A principal component analysis and MANOVA were performed to analyze fifty-six relevant compounds using SAS (Proc Princomp and GLM).

Results

When comparing IJs attraction to three *A. orientalis* larvae in absence of plants, about 1.8× more B1 (from commercial fields) IJs were attracted to the larvae than the sand alone but the effect was not statistically significant ($P = 0.0739$). N9 (from wild stands) IJs were not attracted to larvae ($P = 0.1269$) (Figure 1).

When given the choice between a plant or sand, 2.2× more B1 IJs were attracted to domesticated plants but the effect was not statistically significant ($P = 0.0809$); B1 IJs were not attracted to wild plants ($P = 0.4551$). When the overall attraction (movement towards a choice in either two choice set ups) to the domesticated and wild plants was analyzed, more B1 IJs moved towards domesticated plants than wild ones (Figure 2, $U = 9.00$, $P = 0.0040$). N9 IJs were not attracted to domesticated plants ($P = 0.2480$) but were attracted to wild plants (Figure 3, $t_8 = 2.936$, $P = 0.0094$). When *A. orientalis* larvae were added to the plants, B1 IJs were more attracted to domesticated plants with the grubs than the domesticated plant alone (Figure 4, $P = 0.0039$), but there was no significant preference when exposed to wild plants with larvae or wild plants alone. N9 IJs showed no preference between cultivated plants with our without larvae ($P = 0.1705$). With wild plant, 2× more N9 IJs were attracted to plant with larvae than plants without larvae but the effect was not statistically significant ($P = 0.0645$) (Figure 5). When comparing just the number of IJs moving in response to the treated plant, ~2× more N9 IJs moved towards the wild plant with larvae than towards the domesticated plant with larvae (Figure 5, $U = 0.0383$, $P = 0.0383$).

Overall, volatile blends were not different between plants with and without feeding larvae as well as between wild and cultivated plants (Figure 6). However, one

compound yet to be identified was produced more from domesticated plants with feeding grubs than wild plants ($F = 8.27$, $P = 0.0100$). But in order to identify it, more replication and analysis is necessary to further elucidate this potential difference. The preliminary library was not able to dependably identify the compound.

Discussion

Our results suggest that EPN IJs tend to respond more to the plant types with which they have been associated. Thus, EPN isolated from domesticated plants/environment (B1) responded more to domesticated plants and EPN isolated from wild plants/environment (N9) responded more to wild plants. Following the same pattern, IJ attraction tended to be strengthened when larvae were feeding on the plant roots, but only in plant types with which they have been associated.

Nematodes from both the wild and domesticated environments were not significantly more attracted to third instar *A. orientalis* larvae in sand than to sand alone (Figure 1). Despite the similarity of this result to that of other studies (the EPN is not attracted to the larvae alone), the result when plants and plants with insect larvae were combined was not wholly consistent with the idea that EPN would use the feeding larvae as a long distance cue (Rasmann et al. 2012).

When given the choice between plant or sand alone, N9 was more attracted to wild plants than sand alone (Figure 3) whereas B1 only moved more to domesticated plants versus wild plants (Figure 2). When *A. orientalis* larvae were added to the plants, B1 nematodes were more attracted to domesticated plants with feeding larvae but were not significantly attracted to the wild plant with larvae. Conversely, N9 nematodes only

moved more in response to the wild plants with larvae versus the domesticated plants with larvae but did not have significant directional movement to any of the two-choice set ups.

N9 IJ attraction to wild plants with feeding larvae may not be significant due to genetics variation among propagated wild plants. Like in a natural setting, the wild plants we studied were not a single genotype (i.e., not propagated from a single wild plant or population). Based on the significant results when looking at the domesticated plants and isolate from a domesticated environment, it may be that N9 nematodes would respond more clearly to a single genotype of plants that they were exposed to previously.

Recent studies show how EPN can be selected for attraction to and are more likely to hone in on familiar chemical signals (Hiltpold et al. 2010; Willett et al. 2015). Based on this, perhaps our results reflect the differences in the dynamics of the two environments. In a wild environment, there is more diversity in the plant community. So, in order to hone into where potential insect hosts might be, it may be more advantageous to move toward signals from the plant itself regardless of insect feeding because the chance of coming in contact with a host is higher. Conversely, in a domesticated environment, the diversity of the plant community is purposely limited and as a result, the insect community diversity can also be reduced. This uniform plant genotype and herbivore population over a large space may enhance the B1 nematode's ability to learn and use the complex blend of volatiles coming from the roots.

That being said, the results of the volatile analysis were not conclusive. Compared to other similar studies done (Rasmann et al. 2005; Ali et al. 2012), the volatile profile coming from *V. corymbosum* roots appears to be more complex with no chemicals

induced by insect feeding. One difference in our studies that may possibly contribute to this is that our plants are three years old whereas in previous studies seedlings or very young plants were used. Because there are so many compounds present, it will be necessary for more replication and identification of various compounds by a chemist. Interestingly, when visually assessing the chromatograms, it appears that undamaged roots are expressing some compounds more than those with feeding larvae. If true, this could have implications for importance of silencing as a signal.

There are many more questions that need to be addressed in the laboratory and field before the idea can be fully confirmed that induced chemistry in the roots is a primary signal for EPN in natural environments. An interesting question would be how plant age impacts root emissions and nematode attraction. As for foliage, it is known that plant developmental age and time of day can alter volatile released (Agelopoulos et al. 2000). Because time of day should impact roots less, constitutively produced chemicals may "define" the scent of roots more than induced chemicals. Additionally, there may be differences in the constitutive and induced expression of root chemicals by root type (primary or secondary) and presence or absence of vascular cambium.

Acknowledgements

We would like to acknowledge KJ Brown for advice in the design and set up of vacuum pumps for volatile collection.

References Cited

Agelopoulos NG, Chamberlain K, Pickett JA (2000) Factors affecting volatile emissions of intact potato plants, Solanum tuberosum: Variability of quantities and stability of ratios. J Chem Ecol 26:497–511. doi: 10.1023/A:1005473825335

Ali JG, Alborn HT, Campos-Herrera R, et al (2012) Subterranean, herbivore-induced plant volatile increases biological control activity of multiple beneficial nematode species in distinct habitats. PLoS One 7:1–7. doi: 10.1371/journal.pone.0038146

Ali JG, Alborn HT, Stelinski LL (2011) Constitutive and induced subterranean plant volatiles attract both entomopathogenic and plant parasitic nematodes. J Ecol 99:26–35. doi: 10.1111/j.1365-2745.2010.01758.x

Bian Y, Ballington J, Raja A, et al (2014) Patterns of simple sequence repeats in cultivated blueberries (Vaccinium section Cyanococcus spp.) and their use in revealing genetic diversity and population structure. Mol Breed 675–689. doi: 10.1007/s11032-014-0066-7

Chen YH, Gols R, Benrey B (2015) Crop domestication and naturally selected species interactions. Annu Rev Entomol. doi: 10.1146/annurev-ento-010814-020601

Coleman DC, Crossley DA, Hendrix PF (2004) Fundamentals of Soil Ecology, 2nd Editio. Elsevier Inc.

Degenhardt J, Hiltpold I, Köllner TG, et al (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proc Natl Acad Sci U S A 106:13213–8. doi: 10.1073/pnas.0906365106

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the “cry for help”. Trends Plant Sci 15:167–75. doi: 10.1016/j.tplants.2009.12.002

Eck P (1965) Blueberry Culture. Rutgers University Press, New Brunswick, NJ

Hiltpold I, Baroni M, Toepfer S, et al (2010) Selective breeding of entomopathogenic nematodes for enhanced attraction to a root signal did not reduce their establishment or persistence after field release. Plant Signal Behav 5:1450–2. doi: 10.1242/jeb.041301

Koppenhöfer AM, Kaya HK (1996) Coexistence of two steinernematid nematode species (Rhabditida: Steinernematidae) in the presence of two host species. Appl Soil Ecol 4:221–230. doi: 10.1016/S0929-1393(96)00121-7

Lewis EE, Grewal PS, Gaugler R (2009) Hierarchical order of host cues in parasite foraging strategies. Parasitology 110:207–213. doi: 10.1017/S0031182000063976

Polavarapu S, Koppenhöfer AM, Barry J, et al (2007) Entomopathogenic nematodes and neonicotinoids for remedial control of oriental beetle, Anomala orientalis (Coleoptera: Scarabaeidae), in highbush blueberry. Crop Prot 26:1266–1271. doi: 10.1016/j.cropro.2006.10.026

Price PW, Bouton CE, Gross P, et al (1980) Interactions Among Three Trophic Levels: Influence of Plants on Interactions Between Insect Herbivores and Natural Enemies. Annu Rev Ecol Syst 11:41–65. doi: 10.1146/annurev.es.11.110180.000353

Rasmann S, Ali JG, Helder J, van der Putten WH (2012) Ecology and evolution of soil nematode chemotaxis. J Chem Ecol 38:615–28. doi: 10.1007/s10886-012-0118-6

Rasmann S, Köllner TG, Degenhardt J, et al (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434:732–7. doi: 10.1038/nature03451

Rivera MJ, Rodriguez-Saona C, Egizi A, et al (2016) Cultivation and domestication of highbush blueberry (Vaccinium corymbosum) alters abundance, diversity and virulence of entomopathogenic nematodes. Agric Ecosyst Environ 222:148–155. doi: 10.1016/j.agee.2016.02.013

Rivera MJ, Rodriguez-Saona C, Jennings DE, Koppenhöfer AM (2015) Assessing the impact of cultivation and plant domestication of highbush blueberry (Vaccinium corymbosum) on soil properties and associated plant-parasitic nematode communities. Soil Biol Biochem 88:25–28. doi: 10.1016/j.soilbio.2015.05.010

van Dam NM, Heil M (2011) Multitrophic interactions below and above ground: en route to the next level. J Ecol 99:77–88. doi: 10.1111/j.1365-2745.2010.01761.x

van Tol RWHM, van der Sommen ATC, Boff MIC, et al (2001) Plants protect their roots by alerting the enemies of grubs. Ecol Lett 4:292–294. doi: 10.1046/j.1461-0248.2001.00227.x

Willett DS, Alborn HT, Duncan LW, Stelinski LL (2015) Social Networks of Educated Nematodes. Sci Rep 5:14388. doi: 10.1038/srep14388

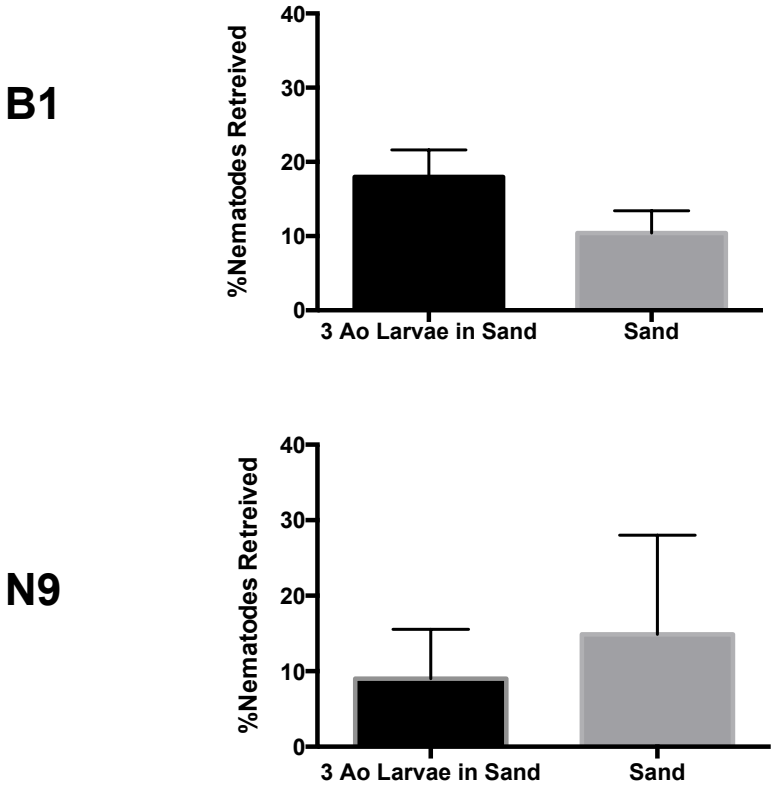

Figure 1. Mean (+SEM) percentage of entomopathogenic nematodes isolates from cultivated (B1) and wild (N9) fields attracted over 40 hours to three third-instar *Anomala orientalis* larvae in sand versus sand alone.

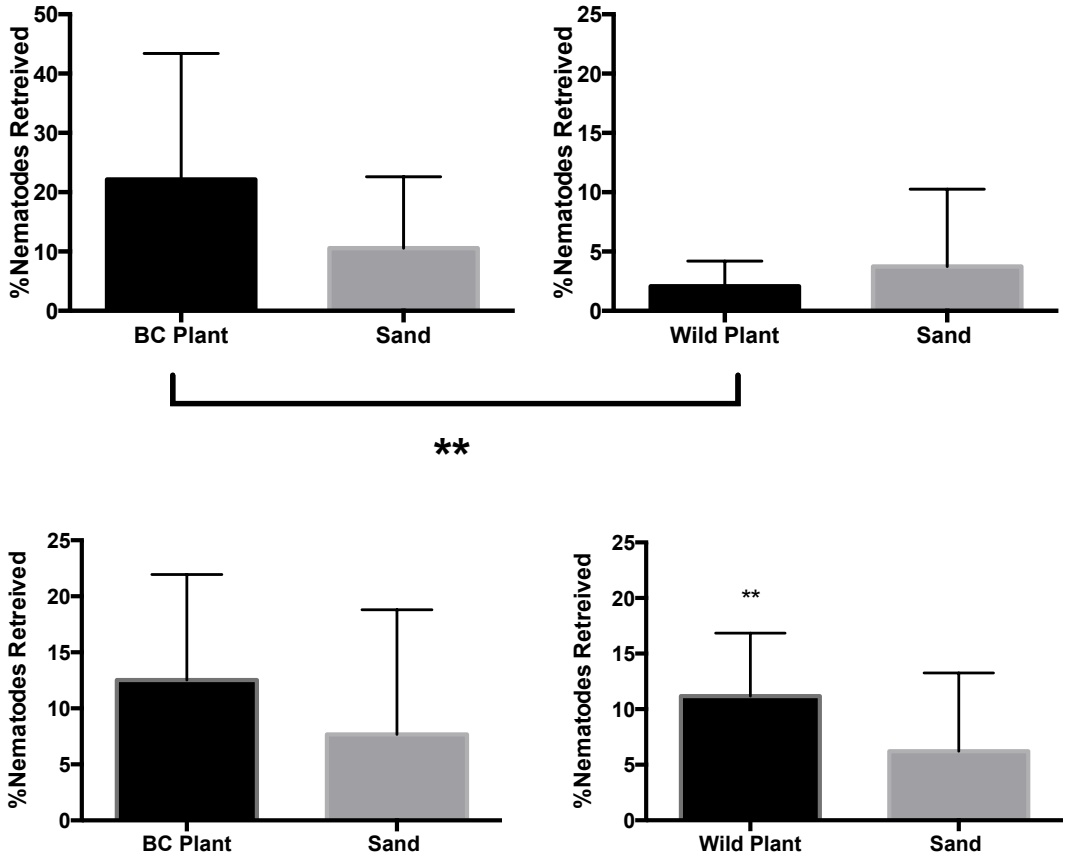

Figure 2. Mean (+SEM) percentage of entomopathogenic nematodes isolates from domesticated (B1) and wild (N9) environments attracted over 40 hours to *Vaccinium corymbosum* (BC= "Blue Crop" var or wild) versus sand alone in two-choice olfactometers. ** = $P \leq 0.01$

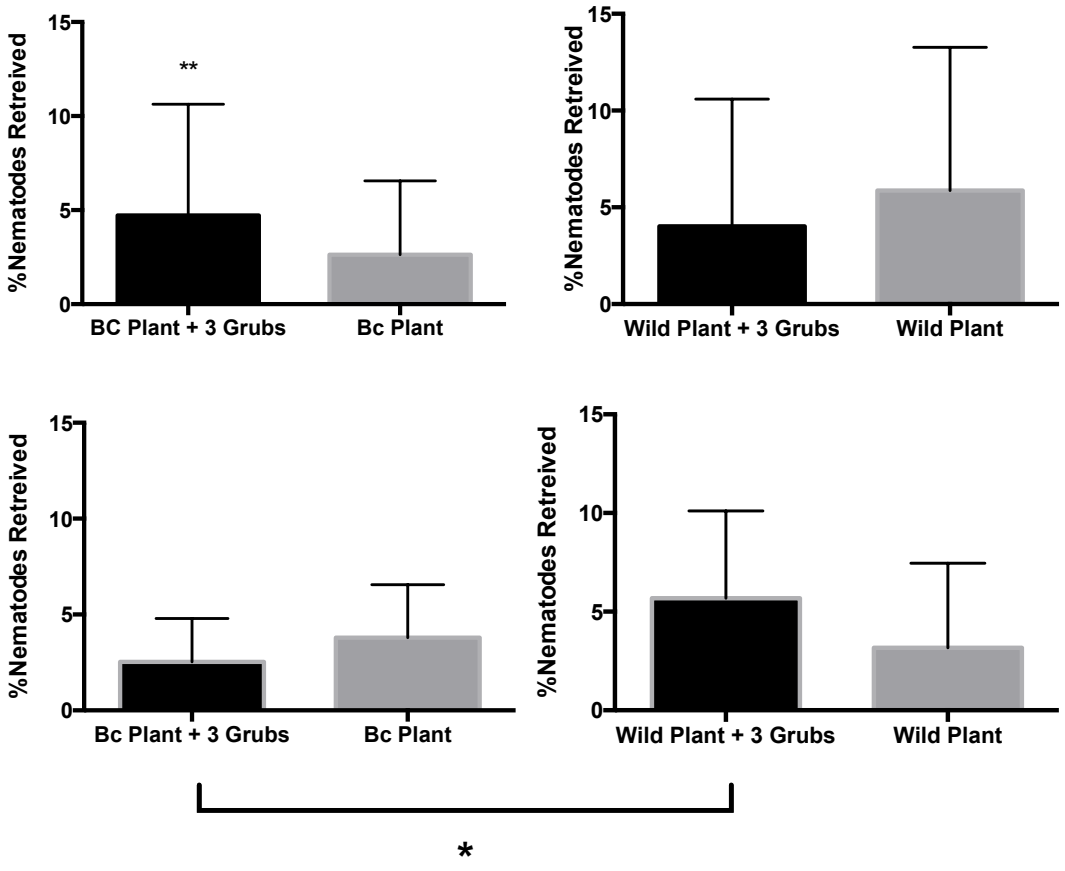

Figure 3. Mean (+SEM) percentage of entomopathogenic nematodes isolates from domesticated (B1) and wild (N9) environments attracted over 40 hours to *Vaccinium corymbosum* (BC= "Blue Crop" var. or wild) with or without three feeding, third-instar *Anomala orientalis* larvae in two-choice olfactometers. ** = $P \leq 0.001$

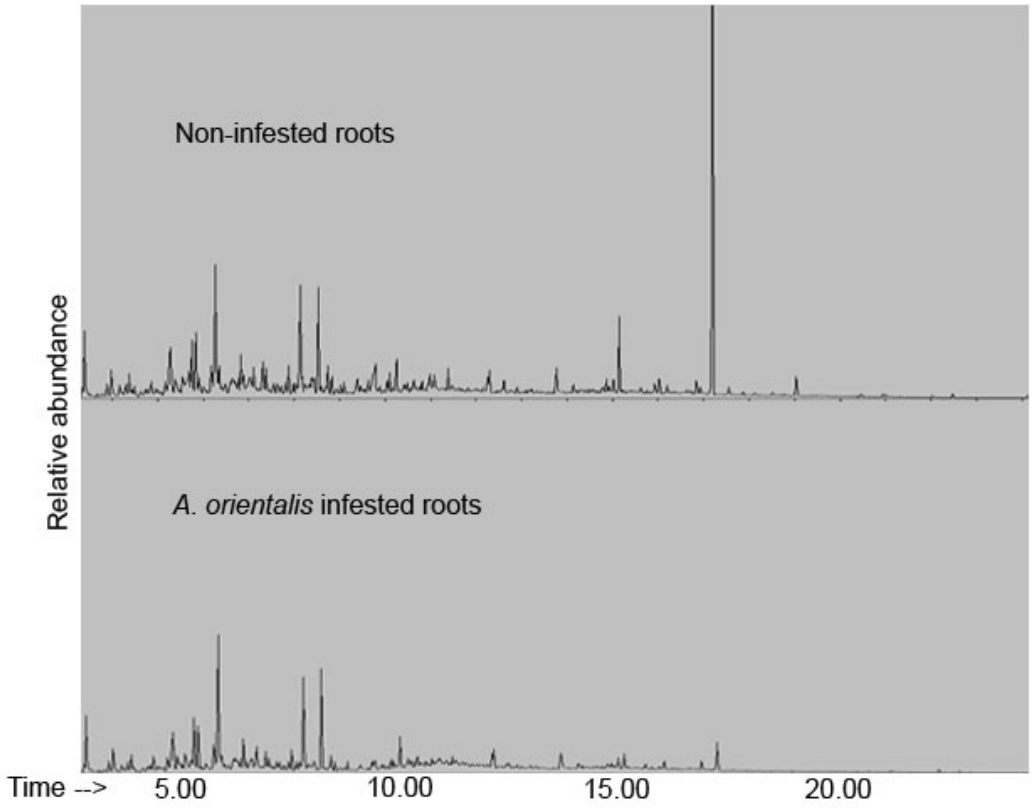

Figure 4. Example chromatogram showing volatile profiles from Blue Crop variety *Vaccinium corymbosum* with infested roots (three third instar *Anomala orientalis* larvae) and uninfested roots. All samples were collected for 18 hours.

Chapter 4: Differential response of a local entomopathogenic nematode population to non-native herbivore-induced plant volatiles (HIPV) in the laboratory and field

Abstract

Recent work has shown the potential for enhanced efficacy of entomopathogenic nematodes (EPN) through their attraction to herbivore induced plant volatiles. However, there has been little investigation into the utilization of these attractants in systems other than in those in which the compounds were identified. We compared (*E*)-β-caryophyllene (maize) and pregeijerene (citrus) in the highbush blueberry (*Vaccinium corymbosum*) system in their ability to enhance the attractiveness and enhanced efficacy of EPN against the system's herbivore, oriental beetle (*Anomala orientalis*). Using an endemic strain of the EPN, *Steinernema glaseri*, in a six-arm olfactometer, the relative attractiveness of (*E*)-β-caryophyllene and pregeijerene was tested in the laboratory to gather baseline values of attraction to the chemicals alone in sand substrate. In a field study, an arrangement similar to the 6-arm olfactometer was used. One week before the field study, the soil of 30 plants was sampled and baited with *G. mellonella* larvae to select plants with the highest *S. glaseri* activity. Six cages containing third-instar *A. orientalis* larvae or late instar greater wax moth, *Galleria mellonella*, larvae with and without compound were placed into the soil around the base of 10 plants. The gaskets were removed after 72 hours and insect baits retrieved and assessed for EPN infection. After removal of the insect bait from the gasket, the remaining clean sand packed in the gasket along with the insect was exhaustively baited with *G. mellonella* larvae to assess EPN density. The lab results indicate that in sand alone (*E*)-β-caryophyllene is significantly more attractive than pregeijerene to endemic *S. glaseri*. Conversely, there was no difference in

attractiveness or efficacy in the field study but rather, endemic *S. glaseri* were more attracted to cages with *G. mellonella* larvae, no larvae, and cages with the blank control and *G. mellonella* larvae.

Introduction

The release of plant compounds in response to insect feeding has been shown to modify behavior and community composition (Price et al. 1980; Karban and Baldwin 1997; Underwood and Rausher 2002). Although the general theories and patterns of chemically mediated multi-trophic interactions were developed in aboveground arenas, research over the last decade has started to investigate the application of these ideas to belowground interactions (van Tol et al. 2001; Rasmann et al. 2005; Preisser et al. 2006; Rasmann and Turlings 2008; Ali et al. 2012). Investigating the specificity of relationships within these interactions is needed to advance the understanding of how these ideas could be applied in field arenas and if the dynamics of chemical cues from plants are system specific.

The primary focus at the third trophic level in belowground systems has been on entomopathogenic nematodes (EPNs). EPN are obligate parasites of insects and occur in two families, Steinernematidae and Heterorhabditidae. EPN have evolved the ability to carry and release highly specific symbiotic bacteria and use them to infect and kill an insect host. The host provides a place and a nutrient source for these highly evolved bacterivorous nematodes to introduce and feed on the proliferating symbiotic bacteria inside the body cavity. Once the resources in the host cadaver have been depleted after 1-

3 life cycles inside the host, third stage non-feeding infectivejuveniles (IJs) leave the cadaver and seek out a suitable host.

The soil provides an arena for host-seeking, non-feeding third instar IJs. The soil matrix and the properties thereof can slow movement through dense mixture of solid, liquid, and gas with high retention capacity, which can impede diffusion of chemical metabolites and restrict mobility of soil organisms (Coleman et al. 2004). IJs have been shown to respond to cues from their host (e.g., CO_2, movement, feces) and chemical compounds often emitted from the plants on which their host is feeding (as reviewed by Rasmann et al. 2012). But it remains unclear if the relationship between attraction to a volatile compound and attraction to the host itself is synergistic in a general sense or adaptive to specific situations.

The objective of this study was to determine how two individual herbivore-induced plant volatiles (HIPVs) found to attract EPN work with a local system that does not express either of the compounds (Rivera, unpublished data). (*E*)-β-caryophyllene, isolated from maize roots (Rasmann et al. 2005) and pregeijerene isolated from citrus roots (Ali et al. 2011), have been shown to attract various species of EPN in laboratory and field studies alike (Hiltpold et al. 2010; Willett et al. 2015). It is important to note that (*E*)-β-caryophyllene has not been attractive to EPN in all laboratory studies (Anbesse and Ehlers 2013). And to the best of our knowledge, the two compounds have never been found to be expressed together in the same plant and have not yet been tested together. The local population of EPN used in this study has been studied since 2011, was identified as *Steinernema glaseri,* and was found to be prevalent and abundant in commercial blueberry (*Vaccinium corymbosum* L.) fields in southern New Jersey, USA

(Rivera et al. 2016). We hypothesized that these HIPVs would enhance the attraction of EPN in both the laboratory and field. To test this hypothesis, we used a six-arm olfactometer in the laboratory to compare the baseline attraction to (*E*)-β-caryophyllene and pregeijerene and tested the same idea in the field using baited traps to assess the enhancement of insect infection when using the attractants.

Materials and Methods

Nematodes, insects, and attractants

All batches of IJs were produced from the original stock batch isolated from the population at the site selected for field-testing (Hammonton, NJ, USA, 39.59254, -74.77338). All IJs were used within 1 week of harvest from reinfected wax moth (*Galleria mellonella* L., Lepidoptera: Pyralidae) late instar larvae and kept at room temperature (21 – 24 °C) until use.

Third-instar oriental beetle, *Anomala orientalis*, larvae used in field experiments were collected from turfgrass areas (Rutgers Horticultural Farm II, North Brunswick, NJ, USA) and kept individually in 24-well tissue culture plates at 8 °C for 1-6 weeks in pasteurized sandy loam soil at approximately 10% moisture.

Galleria mellonella larvae used for baiting in the field and laboratory were obtained from a commercial source (Big Apple Pet Supply, Boca Raton, Florida, USA) and used within 10 days of receipt.

Pregeijerene was purified from the roots of common rue (*Ruta graveolens*) as in Ali et al. (2012) by Hans Alborn and (*E*)-β-caryophyllene was purchased from a commercial source (Sigma Aldrich, Saint Louis, MO, USA). Both pregeijerene and *(E)*-

β-caryophyllene were diluted in pentane to 8 ng/μL and kept in a freezer (-20 °C) until use. The dilution to 8 ng/μL was based on previous studies using pregeijerene and was found to be most attractive at this amount (Ali et al. 2012). Pregeijerene was diluted from a stock solution that was sealed in a glass ampule to maintain purity. During field studies, both attractants and control (pentane only) were kept on ice and out of the sun while pipetting.

Laboratory experiment

EPN attraction to pregeijerene, (*E*)-β-caryophyllene, and the control was first assayed in the lab with a root-zone six-arm olfactometer (Analytical Research Systems/Southern Scientific, Gainesville, FL, USA) modeled after the one used in Rasmann et al. (2005). Instead of Teflon joints with nematode-impermeable mesh, nematode-impermeable borosilicate glass frits (medium porosity, 6.35 mm thick) separate the side chambers from the central chamber. The connector pieces (25 mm diameter, 76 mm length) contain nematode-impermeable borosilicate glass frits (medium porosity, 6.35 mm thick) at 30 mm from the end facing the central six-choice chamber. The side chambers and central chamber were larger (165 mm H×W 127 mm) than the ones used in Rasmann et al. (2005) to contain older, whole plants completely within the side chamber as well as to run a push air system through the side chambers towards the central chamber. However, those features of the olfactometer were not used in this study aside from closing the tops of the chambers after application of the attractant to the sand. Pasteurized (60 °C for 3 hours) subangular, kiln-dried silica sand (bulk density of 1.56 g/cm^3, U.S. Silica Co., Mauricetown, NJ) was used in all experiments. Sand was adjusted to 10% moisture (w/v)

before use and 50 μL of each assigned treatment was injected into the sand (12-15 mm depth) using a pipetman. The assay was repeated ten times. To set up the assay, the central chamber(s) (800 g) and side chambers (300 g) were filled with the clean sand. The olfactometer was always placed on the lab bench so that the alignment of the chambers with the area around it was haphazardly placed and inconsistent to prevent any potential directional bias. Then, treatments were applied to the side chambers and the tops of the chambers were closed. Each treatment was applied to two of six chambers and both chambers of the treatment were always across from one another to prevent a stronger pull of one of the treatments by proximity to eachother. Finally, 5000 nematodes (endemic *Steinernema glaseri* strain) were applied to the center of the central six-choice chamber and then closed. This assay was left out in the bench for 72 hours until it was disassembled for extraction and counting. The sand from the connector pieces was washed from the connector into a 1000 mL beaker and the sieve and decant method used to extract the nematodes for counting under a dissecting microscope.

Field Testing

A field site with high *S. glaseri* activity was selected for the comparison of the attractants using a local EPN population. This site (Hammonton, NJ, USA, 39.59254, -74.77338) had been surveyed three times a year since 2011 and showed consistent *S. glaseri* activity throughout sampling efforts (Rivera et al. 2016). Field studies were conducted in the early season, May of 2014 and 2015, because EPN activity had been shown to be highest at this point in the season.

Before starting each experiment, up to 30 blueberry bushes in a 15 m × 15 m plot were pre-sampled to confirm *S. glaseri* presence by taking eight vertical soil cores with an Oakfield sampler (30.5 cm length × 2.1 cm diameter) 15−30 cm concentrically from the base of the main trunk of each plant. The resulting soil (~800 g) was combined in a plastic bag and baited once in the bag with 10 *G. mellonella* larvae to confirm EPN activity. The bags were placed in a container with wet paper towels (to ensure high enough humidity) at room temperature; the bags were not closed. After 4 days, the larvae were removed from the soil and the number of infected larvae was counted. Bushes with the highest proportion of infections in the bait larvae, were selected, and the field study began in the next week.

To compare the attractiveness of pregeijerene and (*E*)-β-caryophyllene in the field, mesh cages were used to place the attractant and/or bait insect in the root-zone arena (McCoy 2000; Duncan et al. 2009; Ali et al. 2012). Cages were made using 225-mesh stainless steel cylinders (Chemical Containers, Lake Wales, FL, USA) with two polypropylene caps (PGC Scientific, Palm Desert, CA, USA) on both ends and a wire through the caps and center of the cylinder for in-field removal from soil. The cages were filled with pasteurized sand (10% moisture w/v) with or without a bait larva (third-instar *Anomala orientalis* or late instar *Galleria mellonella*), and the bottom cap lined with filter paper the night before the field study was placed; cages were left in this state and in a cooler until arrival at the field site. The cages were buried 25−30 cm from the base of 10 bushes. The same holes created by the pre-sampling were used but made slightly larger to fit the cages by reinserting the Oakfield sampler and widening the holes. Around each

blueberry bush, six cages were buried to mimic the set up of the 6-arm olfactometer assay.

Field studies were conducted in May of 2014 and 2015. Each of the field studies included the two chemicals alongside a blank (pentane). In 2014, five of the ten bushes had cages that included volatile treatments plus *A. orientalis* larva to account for the attractiveness the insect (e.g., CO_2, movement, and insect-related scents) may add; the remaining five bushes had cages with volatile treatments but no insect; this experiment was conducted twice. In 2015, due to lack of infection of *A. orientalis* larvae in 2014, five bushes had cages with volatile treatments plus *G. mellonella* larvae while the other five had cages with volatile treatments plus *A. orientalis* larvae; there were not cages without insects. This study was conducted once. For all experiments, cages remained in the field for 72 hours before they were recovered, placed in plastic bags in a cooler and returned to the laboratory where they were processed immediately. Cages were labeled by treatment, always reused for the same treatment, and between experiments washed with Alconox® (Fisher Scientific, USA), triple rinsed, and allowed to air dry.

Lab processing of samples

Cages were opened and processed upon returning to the laboratory. First, cages were carefully opened over deep-well Petri dishes (25 mm H × 100 mm D) that were labeled with plant number and treatment to collect sand and/or to assess infection in the bait insect and to ensure containers were not reused for a different treatment. In 2014, bait insects that were dead with symptoms of infection were moved to a modified White trap (Stock and Goodrich-Blair 2012) to collect IJs, and any insects that were alive were kept

in the sand used in the field for an additional 48 hours to check for later infections. The remaining sand was baited exhaustively with five *G. mellonella* larvae to assess abundance of EPNs (Koppenhöfer et al. 1998b). In 2015, the procedure was modified because all of the *A. orientalis* larvae were alive upon recovery and all of the *G. mellonella* larvae were dead and with signs of infection. Hence, sand from cages with *A. orientalis* was baited as in 2014. But for cages baited with *G. mellonella*, rather than baiting the soil, the number of nematodes established per cadaver was determined. The infected insect was rinsed in tap water, dissected in a 0.5% pepsin solution and incubated for 2 hours at 37 °C to digest the insects' tissues (Mauléon et al. 1993) to facilitate the subsequent determination of the number of nematodes that had successfully established in the insect under a dissecting microscope.

Statistical analysis

The number of nematodes responding to pregeijerene, (*E*)-β-caryophyllene, and the control (pentane only) in the olfactometer experiment was compared using a *G* test for independence with William's correction and the resulting values of *G* compared with the critical values of χ^2 (Rohlf and Sokal, 1995).

The effect of pregeijerene, (*E*)-β-caryophyllene, and the control on in-field mortality of *G. mellonella* and *A. orientalis* was determined by fitting the generalized linear model (Proc GENMOD; SAS Institute, 2015) assuming binomial distribution. Data from 2014 and 2015 were analyzed separately due to differences in design. Although the chemical treatments were the same, in 2014 only *A. orientalis* was used as insect bait, but in 2015, both *A. orientalis* and *G. mellonella* were used.

In both analyses of in-laboratory baiting after the field experiments with *G. mellonella* larvae (2014, 2015) and numbers of nematodes entering 30 field baits (2015), an analysis of variance and pairwise t-tests using the Tukey-Kramer method was employed for least squares means separation when relevant effects were significant (Proc GLM; SAS Institute, 2015).

Results

In the olfactometer assays, (*E*)-β-caryophyllene was significantly more attractive than pregeijerene or the control ($G = 19.99$; df = 2; $P < 0.001$, Figure 1). Conversely, in the 2014 field study, volatile treatment had no significant effect on the mortality of the larvae (none was observed) and *S. glaseri* abundance (i.e, number of *G. mellonella* larvae infected during baiting). However, *S. glaseri* was more than twice as abundant in samples without *A. orientalis* larvae than in samples with larvae ($F = 40.78$; $P < 0.001$; Figure 2).

To further investigate these results, in 2015, the study was repeated using *G. mellonella* larvae alongside *A. orientalis* larvae. Similar to 2014 results, in the field, there were no infections in the *A. orientalis* larvae and thus, there were significantly more infections in *G. mellonella* than in *A. orientalis* larvae ($P < 0.001$; Figure 3a); volatile treatment, however, had not significant effect. Because there were no *A. orientalis* infections in the field, the sand substrate in the cages that had *A. orientalis* larvae was assessed for EPN abundance by treatment and there were no differences by treatment (Figure 3b). Finally, the counts of *S. glaseri* established in the larvae in the field (only *G. mellonella*) were significantly different by treatment. Twice as many *Steinernema glaseri* had established in infected *G. mellonella* larvae in cages with the control treatment than

in larvae from cages with either (*E*)-β-caryophyllene or pregeijerene ($F = 13.51$; $P < 0.001$; Figure 3c).

Discussion

Contrary to our hypothesis, the lab assays were not relatable to the field data. The attractiveness of (*E*)-β-caryophyllene found in the less complex sand matrix in the olfactometer assays was not reflected in the field study when the attractant was placed with and without the insect larvae. We suspect there are two reasons for this.

Firstly and most obviously, the laboratory setting is extremely simplified and did not have the natural background noise of a plant or living soil. While (*E*)-β-caryophyllene is attractive alone in this simplified setting, when it is among the natural blend of scents in the field at the rate at which we applied it, our results suggest it is not more attractive to the local EPN population. However, in the lab, it may have been used as a long-range cue as the nematodes traveled from the treatment arms (a distance of 16 cm) whereas, this could not be controlled for in the field and potentially skewed their response (Rasmann et al. 2012). On the other hand, this could also mean it is not associated with the plant by local EPN and they have not "learned" to recognize it as a signal for location of their host (Hiltpold et al. 2010; Willett et al. 2015). Therefore, it appears these signals are not general and have to do with the adapted synergy of a system (i.e., relationship between insect and plant and EPN and host). Another way to test these ideas in the field would be using a suitable insect host (highly susceptible to infection by local EPN) feeding on a plant expressing a known chemical signal with a local population of EPN like in Degenhardt et al. (2009). Or conversely, testing the idea with a lab colony of nematodes

selected for responsiveness to such chemistries (e.g. Hiltpold et al. 2010; Willett et al. 2015). However, this approach would supplement the local population of EPN and risk a shift in the community structure.

Our results suggest that either attractants did not enhance the EPN attraction to or infection of *G. mellonella*. This result is contrary to results in Ali et al. (2012) with pregeijerene in blueberry in New Jersey. In that study, pregeijerene enhanced the mortality of *G. mellonella* but similarly to our study, not *A. orientalis*. Like the simplified laboratory setting, the relationship between *G. mellonella* is simplified; these are not soil-dwelling insects. In their natural environment, they would not come in contact with EPNs and thus, lack defensive mechanisms, structures and behaviors capable of repelling EPN attack. Thus, the difference in results may lie in the methods. In addition to addressing mortality, we also looked at the number of nematodes successfully establishing in the host in the field through use of a pepsin digestion. This provided finer scale detail in the attraction to the insect and attractant and showed that the blank was 2x more attractive than either of the attractants in the field (Figure 3C). As shown in Figure 3A, although not significant, the trend of the data collected from mortality alone skews towards pregeijerene being the most attractive which if significant, would have been consistent with the previous field 2012 study in blueberry.

Secondarily, the relationship between the EPN and the *A.orientalis* larvae may have muddled the results. We suspect that it was difficult to get field data about attraction using *A. orientalis* in the cages because they are highly evolved to resist such attacks. Attraction of EPN to scarab larvae maybe reduced by their ability to regulate and reduce other attractive signals they may be putting into the soil (Gaugler et al. 1994). Some IJs

may have been killed by aggressive behaviors of the *A. orientalis* larvae when attempting to enter the larvae's body while others that managed to enter the larvae may have been eliminated by the larvae's immune response (Gaugler et al. 1994). It is known that various strains of *S. glaseri* can, due to the presence or absence of surface coat proteins, overcome the encapsulation by the immune system of *A. orientalis* larvae (Li et al. 2007). We suspect that the local strain lacks the ability to overcome the immune system of the *A. orientalis* larvae and this factor potentially influenced evenness of the mortality data by reducing the numbers of nematodes in samples (i.e. the treatments with more nematodes attracted probably had more nematodes destroyed by the insect immune system).

In conclusion, induced defensive chemistries may be system-specific belowground and there may not be a direct relationship between attractant and attraction in all systems. It is not clear in both aboveground and belowground settings if HIPVs are truly beneficial to the plant as they appear to attract indirect “helpers” (e.g., predators and parasites) of herbivores as well as attract herbivores (Unsicker et al. 2009). For example, in Ali et al. (2011), plant parasitic nematodes were also attracted to pregeijerene. The belowground environment is very densely populated, highly competitive and has many trophic cascades (Wardle 2002). In our study, the cages with *G. mellonella* also occasionally attracted the thief ant, *Solenopsis molesta*, which appeared to have devoured the *G. mellonella* either before or after EPN infection, but this did not happen at all with *A. orientalis*. Ants seem to be a common and quick responder in field baiting studies (Larry Duncan, personal communication) but it is unclear if they would use the same signals as EPN. It is clear, however, that insects suited to soil environment are well defended against the many potential attacks of viruses, bacteria, and EPNs and this

should be observed with virulence bioassays before moving to the field. But even then, the limitations of laboratory bioassays should seriously considered when using them to estimate what may happen under field conditions.

Acknowledgements

We would like to thank Eugene Fuzy, John Sanders, and Rich Keller for technical assistance. We would also like to thank Kaitlyn Mathis for identifying *Solenopsis molesta* for us.

References Cited

Ali JG, Alborn HT, Campos-Herrera R, et al (2012) Subterranean, herbivore-induced plant volatile increases biological control activity of multiple beneficial nematode species in distinct habitats. PLoS One 7:1–7. doi: 10.1371/journal.pone.0038146

Ali JG, Alborn HT, Stelinski LL (2011) Constitutive and induced subterranean plant volatiles attract both entomopathogenic and plant parasitic nematodes. J Ecol 99:26–35. doi: 10.1111/j.1365-2745.2010.01758.x

Anbesse S, Ehlers R-U (2013) Heterorhabditis sp. not attracted to synthetic (E)-β-caryophyllene, a volatile emitted by roots upon feeding by corn rootworm. J Appl Entomol 137:88–96. doi: 10.1111/j.1439-0418.2012.01753.x

Coleman DC, Crossley DA, Hendrix PF (2004) Fundamentals of Soil Ecology, 2nd Editio. Elsevier Inc.

Degenhardt J, Hiltpold I, Köllner TG, et al (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proc Natl Acad Sci U S A 106:13213–8. doi: 10.1073/pnas.0906365106

Duncan L, El-Borai F, Cubero J, et al (2009) Differential susceptibility of entomopathogenic nematodes to nematophagous fungi from Florida citrus orchards. Nematology 11:231–241. doi: 10.1163/156854109X429565

Gaugler R, Wang YI, Campbell JF (1994) Aggressive and evasive behaviors in Popillia japonica (Coleoptera: Scarabaeidae) larvae: Defenses against entomopathogenic nematode attack. J Invertebr Pathol 64:193–199. doi: 10.1016/S0022-2011(94)90150-3

Hiltpold I, Baroni M, Toepfer S, et al (2010) Selective breeding of entomopathogenic nematodes for enhanced attraction to a root signal did not reduce their establishment or persistence after field release. Plant Signal Behav 5:1450–2. doi: 10.1242/jeb.041301

Karban R, Baldwin IT (1997) Induced responses to herbivory. University of Chicago Press

Koppenhöfer AM, Cmvipbell JF, Kaya HK, Gaugler R (1998) Estimation of entomopathogenic nematode population density in soil by correlation hetween hait insect mortality and nematode penetration. 21:95–102.

Li X-Y, Cowles RS, Cowles EA, et al (2007) Relationship between the successful infection by entomopathogenic nematodes and the host immune response. Int J Parasitol 37:365–74. doi: 10.1016/j.ijpara.2006.08.009

Mauléon H, Briand S, Laumond C, Bonifassi E (1993) Utilization d'enzyme digestives pour l'etude du parasitisme des Steinernematidae et Heterohabditae enverse les larves d'insectes. Fundam Appl Nematol 16:185–186.

McCoy C (2000) Entomopathogenic Nematodes and Other Natural Enemies as Mortality Factors for Larvae of Diaprepes abbreviatus (Coleoptera: Curculionidae). Biol Control 19:182–190. doi: 10.1006/bcon.2000.0852

Preisser EL, Dugaw CJ, Dennis B, Strong DR (2006) Plant facilitation of a belowground predator. Ecology 87:1116–23.

Price PW, Bouton CE, Gross P, et al (1980) Interactions Among Three Trophic Levels: Influence of Plants on Interactions Between Insect Herbivores and Natural Enemies. Annu Rev Ecol Syst 11:41–65. doi: 10.1146/annurev.es.11.110180.000353

Rasmann S, Ali JG, Helder J, van der Putten WH (2012) Ecology and evolution of soil nematode chemotaxis. J Chem Ecol 38:615–28. doi: 10.1007/s10886-012-0118-6

Rasmann S, Köllner TG, Degenhardt J, et al (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. Nature 434:732–7. doi: 10.1038/nature03451

Rasmann S, Turlings TCJ (2008) First insights into specificity of belowground tritrophic interactions. Oikos 117:362–369. doi: 10.1111/j.2007.0030-1299.16204.x

Rivera MJ, Rodriguez-Saona C, Egizi A, et al (2016) Cultivation and domestication of highbush blueberry (Vaccinium corymbosum) alters abundance, diversity and virulence of entomopathogenic nematodes. Agric Ecosyst Environ 222:148–155. doi: 10.1016/j.agee.2016.02.013

Stock SP, Goodrich-Blair H (2012) Nematode parasites, pathogens and associates of insects and invertebrates of economic importance. In: Lacey LA (ed) Manual of Techniques in Invertebrate Pathology, 2nd edn. Elsevier, London, pp 373–426

Underwood N, Rausher M (2002) Comparing the consequences of induced and constitutive plant resistance for herbivore population dynamics. Am Nat 160:20–30.

Unsicker SB, Kunert G, Gershenzon J (2009) Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. Curr Opin Plant Biol 12:479–485. doi: 10.1016/j.pbi.2009.04.001

van Tol RWHM, van der Sommen ATC, Boff MIC, et al (2001) Plants protect their roots by alerting the enemies of grubs. Ecol Lett 4:292–294. doi: 10.1046/j.1461-0248.2001.00227.x

Wardle DA (2002) Communities and Ecosystems: Linking the Aboveground and

Belowground Components. Princeton University Press, Princeton, NJ

Willett DS, Alborn HT, Duncan LW, Stelinski LL (2015) Social Networks of Educated Nematodes. Sci Rep 5:14388. doi: 10.1038/srep14388

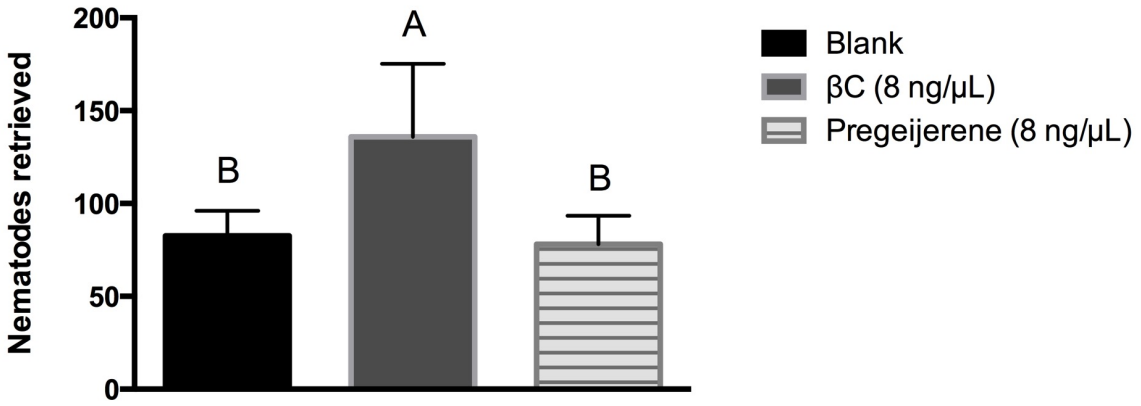

Figure 1. Mean (+SEM) *Steinernema glaseri* nematodes retrieved in comparison of (E)-β −caryophyllene and pregeijerene in a 6-arm olfactometer. Means with same letter did not differ significantly ($\alpha = 0.05$).

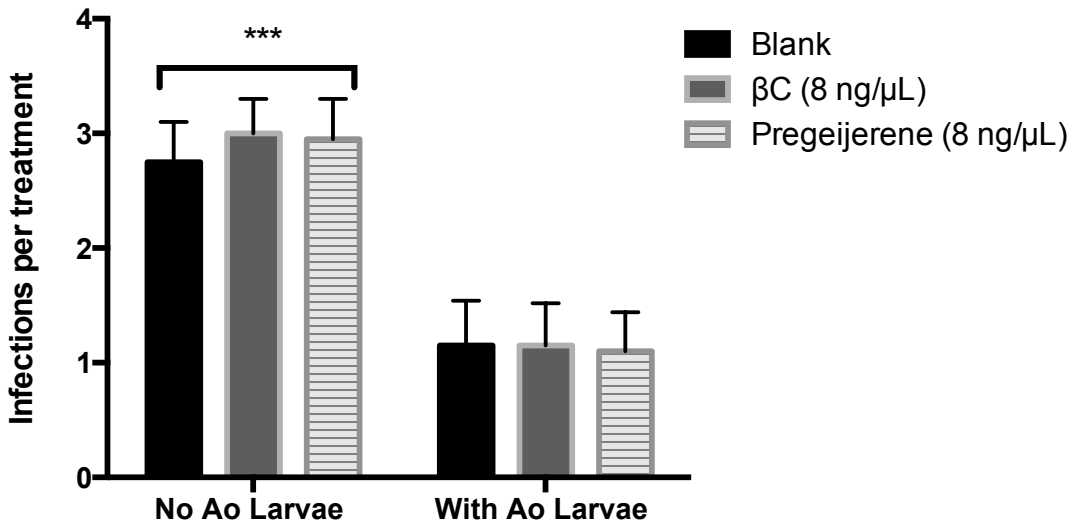

Figure 2. Mean (+SEM) *Galleria mellonella* larvae infected with entomopathogenic nematodes after 72 hours of placement in field (2014) with treatment and with or without third-instar *Anomala orientalis* larvae. Means by treatment did not differ significantly. *** = $P \leq 0.001$ ($\alpha = 0.05$).

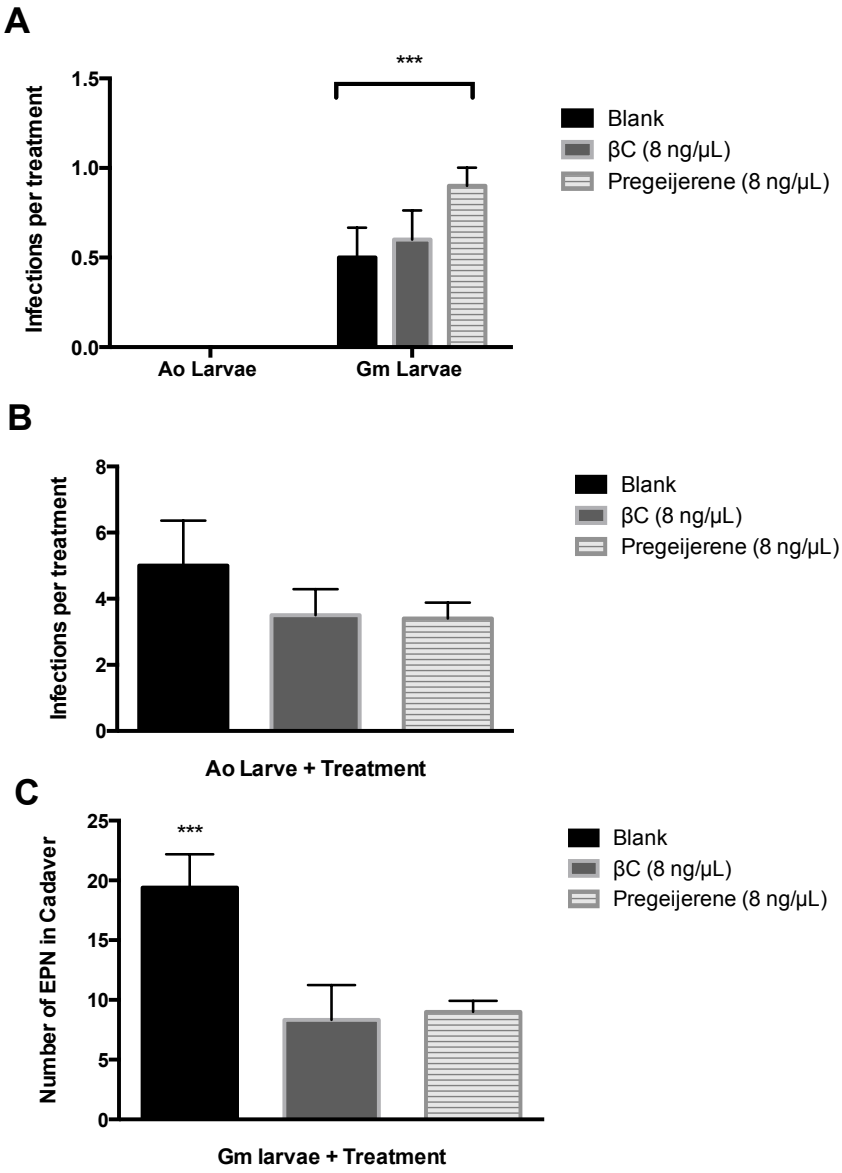

Figure 3. Results of field comparisons of attractiveness of herbivore-induced plant volatiles to entomopathogenic nematodes (EPNs) using baiting in the field, baiting in the lab, and pepsin digestion of field baits. Ao= *Anomala orientalis* larvae, Gm= *Galleria mellonella* larvae. (A) Mean (+SEM) of larvae infected during the 72 hour field experiment. Means did not differ by treatment but did by bait type. (B) Mean (+SEM) of infected Gm larvae placed in the sand after removal from field where the sand contained Ao larvae. (C) Mean (+SEM) of EPN