UNDERSTANDING THE PARASITIC VARIABILITY OF THE NORTHERN ROOT KNOT NEMATODE (*MELOIDOGYNE HAPLA*) THROUGH MULTIDISCIPLINARY APPROACHES OF SOIL BIOME AND ENVIRONMENT

By

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ABSTRACT

UNDERSTANDING THE PARASITIC VARIABILITY OF THE NORTHERN ROOT KNOT NEMATODE (*MELOIDOGYNE HAPLA*) THROUGH MULTIDISCIPLINARY APPROACHES OF SOIL BIOME AND ENVIRONMENT

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Managing *Meloidogyne hapla* remains challenging due to the ban of broad-spectrum nematicides, lack of resistant crops and its broad host range. It also has parasitic variability (PV) where populations (pop) are morphologically and genetically similar but vary in pathogenicity and reproductive potential. Although PV in *M. hapla* appears to have some relationship to soil types, what soil conditions favor its PV and/or its distribution are unknown. The goal of my research was to understand the soil conditions where *M. hapla* PV exist by quantifying the biophysicochemical (BPC) conditions from the ecosystem down to microbiome level. I designed observational and experimental approaches and tested four objectives. First, was to evaluate the association between soil conditions and *M. hapla* distribution at the ecosystem level. My hypothesis was that the presence of *M. hapla* will be associated with degraded soil conditions. I selected 15 (6 muck and 9 mineral soil) agricultural fields with adjacent natural vegetation in southwest, northwest and eastern regions of the lower peninsula of Michigan as study sites. I collected a total of 75 (5 per field) georeferenced soil samples from agricultural fields and equal number from adjacent natural vegetation soils, quantified the soil food web (SFW) conditions using the Ferris SFW model, and screened for *M. hapla* presence or absence. The fields were described either as disturbed, degraded (worst-case) or maturing (best-case). *Meloidogyne hapla* was present in 3 mineral (2, 8 and 13) and 6 muck (4, 5, 6, 10, 14 15) agricultural fields with degraded and/or disturbed soil conditions and absent from maturing soils, partially supporting the hypothesis. Degraded soils had low nitrogen content in both soil groups. The second objective was to

isolate and culture the 9 *M. hapla* populations to test a hypothesis that PV is related to specific SFW conditions. I found three categories of reproductive potential: the highest (Pop 13), medium (Pop 8), both from degraded mineral soils, and lowest from disturbed mineral (Pop 2) and disturbed (Pops 4, 6 and 10) and degraded (Pops 5, 14 and 15) muck soils. Thus, the hypothesis was not supported. The third objective~~,~~ was to determine relationships between microbial community structure and *M. hapla* distribution. My working hypotheses were that there is a relationship among microbiome, soil health and *M. hapla* occurrence~~.~~ Microbial community structure in the fields was determined from sub-samples of the same samples where the nematodes were isolated. I used 16S (bacteria) and ITS (fungi) rDNA analysis and characterized the microbial composition, core- and indicator-microbes co-existing with *M. hapla* pop in the field soils and soil conditions relative to the Ferris SFW model description. The results showed that bacterial and fungal community abundance and composition varied by soil group, SFW conditions and/or *M. hapla* occurrence. I found that a core of 39 bacterial and 44 fungal sub-operational taxonomic units (OTUs) were found variably, 25 bacterial OTUs associated with presence or absence of *M. hapla*, and 1,065 OTUs were associated SFW conditions. All three hypotheses were supported. The final objective was to determine the relationship between PV and the microbes associated with *M. hapla* pop. I compared bacteria present in *M. hapla* pop isolated from the field and greenhouse cultures. The hypothesis was that either presence and/or absence of specific bacteria are associated with *M. hapla* population. Population 8 shared more bacteria with the lowest reproductive potential pop than Population 13. Presence of several bacteria was unique to Population 8 as was the absence of other bacteria to Pop 13 in either field or greenhouse nematodes. Therefore, the hypothesis was supported. My research findings provide a foundation for: a) testing the relationship between *M. hapla* PV and the BPC conditions and b) designing soil health-based management strategies.

I dedicate this to my beloved family
Samariyan, and Kimberly and Kamilla

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KEY TO ABBREVIATIONS

Quadrant A	disturbed conditions
ACT	alignment, classification, tree service
ANOVA	analysis of variance
b	basal
Quadrant B	maturing conditions
BS	bulk soil
CABI	Center for Agriculture and Bioscience International
CONSTAX	consensus taxonomy
c-p	colonizer-persister
CCA	canonical correspondence analysis
DCA	detrended correspondence analysis
Quadrant D	degraded conditions
DD	degree day
DNA	deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
e	enrichment
EI	enrichment index
F	forward primer
FD	Fields
FDR	false discovery rate
FMH	field *Meloidogyne hapla*
FV	fungivore
GMH	greenhouse *Meloidogyne hapla*
GPS	global positioning system
hr	hour

HV	herbivore
IN	inoculum
ITS	internal transcribed spacer
J2	second stage juvenile
J3	third stage juvenile
J4	fourth stage juvenile
k	weight of nematode guild
LU	land use
MH	*Meloidogyne hapla* population
Mh+	*Meloidogyne hapla* presence
Mh-	*Meloidogyne hapla* absence
Mi	mineral
MSU	Michigan State University
Mu	muck
N	relative frequency of each functional guild
NCBI SRA	national center for biotechnology information sequence read archive
NE	Nebraska
N-P-K	Nitrogen-Phosphorus-Potassium
NRKN	northern root knot nematode
OM	organic matter
OTU	sub-operational taxonomic sequence
OV	omnivore
PCA	principal component analysis
PC	principal component
PCoA	principal coordinate analysis
PCR	polymerase chain reasction
PERMANOVA	permutational multi variate analysis of variance

PNA	peptide nucleotide acid
Pop	Population
PR	predator
PPN	plant parasitic nematode
PV	parasitic variability
q2	qiime2
qPCR	quantitative polymerase chain reaction
RG	region
R	reverse primer
RWA	Russian wheat aphid
s	structure
SFL	sum of free living
SFL25	sum of free living from cp 2 to 5
SFW	soil food web
SG	soil group
SI	structure index
ST	soils
SU	suitable host
SUM	sum of all trophic groups
SYBR	Synergy Brands
TAF	triethanolamine formalin
US	unsuitable host

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Meloidogyne hapla distribution and significance of its parasitic variability (PV) in Michigan

The northern root-knot nematode (NRKN, *Meloidogyne hapla* Chitwood, 1949) is one of the most widely distributed plant-parasitic nematodes (PPN) across all continents, but Antarctica (CABI, 2002; Hunt and Handoo, 2010). Globally, *M. hapla* has broad range of dicotyledonous plants and weed hosts (Widmer et al., 1999). In Michigan and the United States Mid-West, *M. hapla* has particular economic significance in vegetable crops (Grabau et al., 2017; Melakeberhan and Wang, 2012; Melakeberhan et al., 2012; Melakeberhan et al., 2007). *Meloidogyne hapla* symptoms include galling, stunting, chlorosis, and wilting in all susceptible crops, and forking in carrots, leading to lower quantity and quality of crop yield (Anon, 2005; Mitkowski and Abawi, 2003). Crop losses associated with *M. hapla* infestation could be as high as 70% (Widmer et al., 1999).

In addition to its broad distribution and host range, there are four major challenges why *M. hapla* continues to be a problem and managing it is difficult. The first challenge is that there are no commercially available resistant crops (Melakeberhan and Wang, 2013), broad-spectrum nematicides like methyl bromide, dazomet, aldicarb, oxamyl and carbofuran are restricted because of human and environmental health reasons (Barry et al., 2012; Budnik et al., 2012; Desaeger et al., 2017; Oka, 2020; van der Putten et al., 2006; Xue et al., 2000), and its broad host range minimizes the benefits of crop rotation.

The second challenge is that *M. hapla* is one of the root-knot nematodes with PV where *M. hapla* populations have similar morphology and genetics (Liu and Williamson, 2006; Opperman et al., 2008), but reproduce at different rates and have different

pathogenicity on the same crop variety grown under standard growth conditions (Melakeberhan et al., 2007; Melakeberhan and Wang, 2013). The two main indicators of PV are the nematode's reproductive potential and galling. Reproductive potential as an indicator of PV, refers to the total numbers of nematodes recovered after a predetermined period following inoculation. For example, PV has been demonstrated on *M. incognita* in cotton (Kirkpatrick and Sasser, 1983) and pepper (Bucki et al., 2017), and *M. paranaensis* in coffee (Santos et al., 2018). In Michigan and elsewhere, *M. hapla* PV has been demonstrated in many crops including celery, carrot, potato, and tomato (Liu and Williamson, 2006; Melakeberhan et al., 2007; Melakeberhan and Wang, 2013; Murata and Uesugi, 2021; Stephan and Trudgill, 1982). While we know about *M. hapla* PV on several crops, the contributory or related soil factors are poorly understood. Managing nematodes without broad-spectrum options is hard, however the presence of PV makes it more difficult.

The third challenge for successful management is the complexity and variation of the production landscape and the connections between *M. hapla* PV and soil groups. For example, major vegetable production in the lower peninsula of Michigan alone spans twenty-eight (28) counties (**Figure 1.1**). The soils in these regions range from mineralized sandy to clay texture, low (mineral) to high (muck) organic matter, and 5.5 to 7.0 pH (Melakeberhan et al., 2007; Melakeberhan et al., 2010; Melakeberhan and Wang, 2012). Most of these vegetable production soils are under conventional tillage and receive agricultural inputs (chemical pesticides and fertilizers) multiple times per growing season to support large-scale monoculture (Castillo et al., 2017; Tsiafouli et al., 2015), and the presence of *M. hapla* PV in the western and southwestern vegetable production regions of Michigan is well documented (Melakeberhan et al., 2007; Melakeberhan and Wang, 2012). Furthermore, *M. hapla* populations from mineral soils showed more parasitism than the populations from muck soils (Melakeberhan et al., 2010; Norton, 1978), but the underlying mechanisms are unknown

(Melakeberhan et al., 2007). A considerable amount of studies on *M. hapla* biology (Vestergård, 2019) and its host suitability (Melakeberhan and Wang, 2012) so far suggest that the soils may have some influence on *M. hapla* PV.

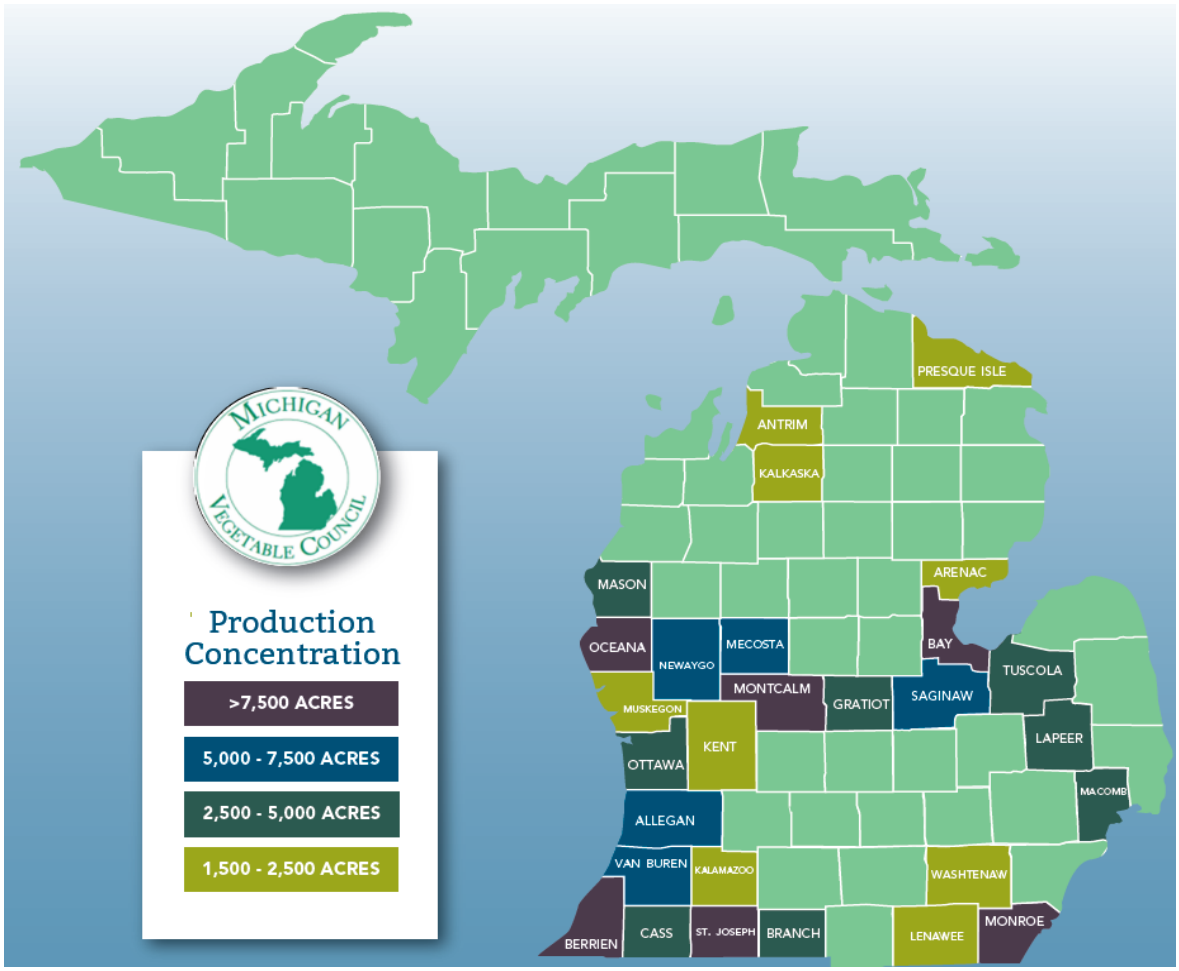

Figure 1.1 | Map of the State of Michigan showing vegetable production counties with total production areas of more than 7500 acres (http://www.michiganvegetablecouncil.org/industry-facts--stats.html).

The fourth challenge is the knowledge gaps on the relationship between *M. hapla* PV and the soil biophysicochemical changes that production practices generate in the highly variable soil types. Studies previously conducted on tillage and its effect on nematodes

produced mixed results. Some research findings showed tillage to be of less importance on nematode population dynamics (Grabau and Chen, 2016; Hu et al., 2017; Schmidt et al., 2017; Shuang et al., 2016), while other research had contrasting findings on nematode population dynamics after tillage or no-tillage practices (Grabau and Chen, 2016). It is, however, worth noting that tillage affects soil organic matter content (Ito et al., 2015) and nutrient cycling and water retention capacity (Robertson et al., 2014; Swinton et al., 2007). Regardless of the soils present in agricultural fields, all these interventions ultimately alter the soil health conditions in the soils where *M. hapla* exists (Tahat et al., 2020; Workie, 2017). Soil health is defined as the capacity of a soil to function as a medium that sufficiently provides the needed ecosystem services (Bennett et al., 2010; Lal, 2016; Robertson et al., 2014) and has the biological, physical and chemical components that need to be kept in balance at all times (Lal, 2011). Unfortunately, there is no information on the relationship between soil health conditions and *M. hapla* occurrence, much less its PV. Thus, understanding the soil health conditions of the fields where *M. hapla* occurs is central to developing a fundamental knowledge towards determining the mechanisms of its PV and designing potential management strategies. In this regard, changes in beneficial nematodes population dynamics could be a good indicator of soil health conditions (Ferris et al., 2001).

Role of beneficial nematodes as bioindicators of soil health conditions.

Understanding PV in *M. hapla* populations could be best achieved by considering soil health conditions and the role of the soil food web (SFW). The SFW is central to regulating the processes that drive the belowground biophysicochemical changes that are critical to soil health (Doran and Zeiss, 2000; Sánchez-Moreno, 2018) and nematodes are major players in nutrient cycling (DuPont et al., 2009; Melakeberhan et al., 2021a; Yeates, 2003). In addition, nematodes are excellent bioindicators of soil ecosystem changes because they are ubiquitous

and have a wide range of resistance to disturbance (Bongers, 1990; Desgarennes et al., 2018; Ferris et al., 2001). In order to measure ecosystem changes, it is essential to summarize the complexity of the various taxa into an index or value (Yeates and Bongers, 1999). The key to creating these indices is the classification of nematodes into functional guilds. When nematodes are classified into colonizer-persister groups (c-p) based on their life history and reproductive potential, each nematode functional guild is assigned a cp score between 1 to 5.

Based on the classification of the c-p groups, the soil health condition is illustrated using the Ferris et al., (2001) soil food web (SFW) model (**Figure 1.2**). The SFW model uses the relationship between changes in nematode population dynamics in response to resource and reproductive potential (Enrichment Index, EI) and resistance to disturbance (Structure Index, SI). The relationship between EI (x-axis) and SI (y-axis) categorizes soil conditions in terms of nutrient cycling potential and agroecosystem suitability in four quadrants from best to worst case scenarios (**Figure 1.2**). These are: enriched and unstructured (Quadrant A, disturbed), enriched and structured (Quadrant B, best case scenario), resource limited and structured (Quadrant C), or resource-limited and minimal structure (Quadrant D, degraded, worst case). Quadrants B are bacterial feeding nematode dominated and Quadrant D is biologically depleted and nutritionally degraded.

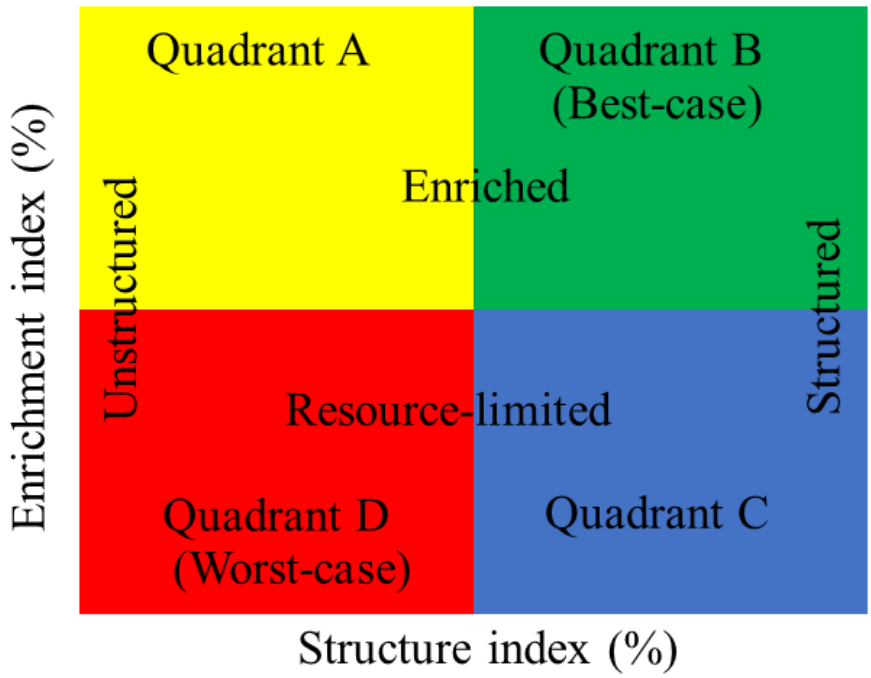

Figure 1.2 | Simplified soil food web model using nematode bioindicators to estimate food and reproduction (Enrichment Index (EI), vertical-axis) and resistance to disturbance (Structure Index (SI), horizontal-axis). Soil health conditions from the best- (Quadrant B) to worst- (Quadrant D) case-scenarios for nutrient cycling and agroecosystem sustainability (Ferris et al., 2001). Quadrant A has high EI and low SI indices indicating an nitrogen (N)-enriched (boom and bust manner) soil with low C:N ratio and dominated by bacterial feeding nematodes with fast reproduction and resistance to disturbance. Quadrant B has high EI and SI indices showing an N-enriched (stable and steady) soil with low C:N ratio and dominated by slow reproducing and disturbance sensitive nematodes. Quadrant C has a low EI and high SI indices indicating a moderately available N, with moderate C:N ratio dominated mostly by slow reproducing and disturbance sensitive fungal-feeding nematodes. Quadrant D has low EI and SI showing an N-depleted soil which is dominated by fast reproducing disturbance tolerant fungal feeding nematodes.

Meloidogyne hapla biology and soil microbiome associations

Whether the soil health is balanced or out-of-balance, soils are a dynamic environment and it is reasonable to assume that *M. hapla* and other organisms that exist therein have to adapt to those conditions (McSorley, 2003; Melakeberhan et al., 2004). In this context, considering

the biology of *M. hapla* is instructive (**Figure 1.3**; (Weerasinghe et al., 2003). The life cycle of *M. hapla* starts with an egg laid within an egg-mass that may be fully or partially embedded in a plant host root. The first molt occurs in the egg and the 2nd stage juvenile hatches, migrates through the soil to infect roots with its stylet, establishes a feeding site, and becomes sedentary. The 3rd and 4th stage juveniles and adults are sedentary. As the 2nd stage juvenile migrates through the soil and probes roots with its stylet, it interacts with a myriad of microbes (Davies, 2005; Topalović et al., 2019). The associations range from fungal spores and an assortment of bacteria attaching to and parasitizing *M. hapla* cuticle (Elhady et al., 2017; Viaene and Abawi, 1998) to mortality by *Pasteuria* spp. (Davies and Williamson, 2006), and diminishing (Topalović et al., 2019) or enhancing *M. hapla*'s ability to infect host-plant (Topalović et al., 2022).

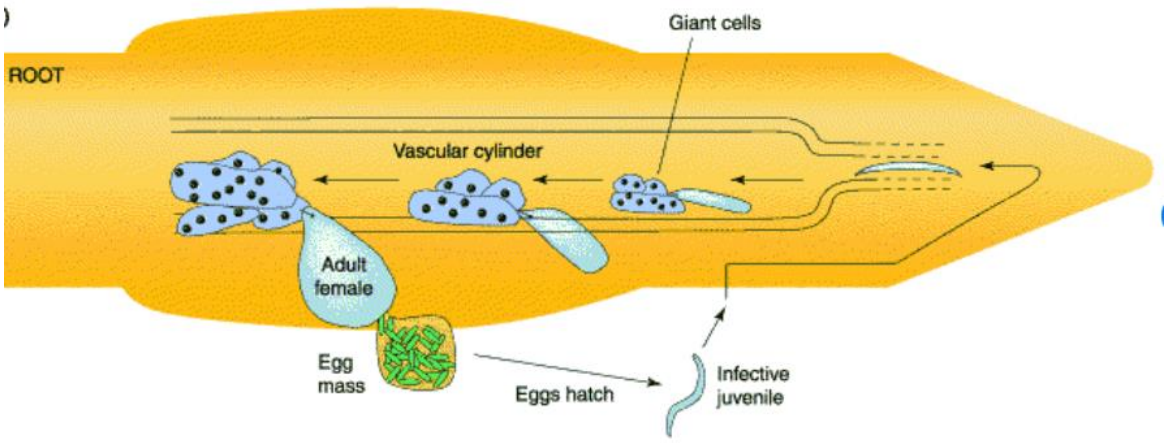

Figure 1.3 | An illustration of the life cycle of a root-knot nematode starting from the egg through the adult stage (Weerasinghe et al., 2003).

While there is little information on *M. hapla* PV and inhibitory, beneficial and/or mutual interactions with soil microbiome, it is worth looking at other organisms that have

stylets and with similar functions as that of *M. hapla*. For example, the Russian wheat aphid (RWA, *Diuraphis noxia*) uses a stylet to deliver salivary proteome, known as effectors, to suppress plant host defense responses (Miles, 1999; Mugford et al., 2016). Recent study by Luna et al., (2018) found that symbiotic bacteria (*Acinetobacter* spp., *Erwinia* spp., *Arthrobacter* spp., *Pantoea* spp., and *Enterobacter* spp.) isolated from the RWA enhanced virulence (wheat leaf chlorosis or necrosis) while the absence resulted in diminished virulence. In instances where only the bacteria were inoculated on the leaves, even at high doses, there were no leaf chlorosis or necrosis. Thus, showing the association with microbes enhanced virulence. On another hand, the entomopathogenic fungi like *Beauveria bassiana*, *Verticillium lecanii, Conidiobolus obscurus, Pandora neoaphidis, P. radicans* and *Neozygites fresenii* have been identified as lethal to the RWA (Feng et al., 1990; Ward et al., 2020). Whether or not *M. hapla* PV has or does not have any unidentified associations with soil microbiome that it may pick up during the migration through the soil to find the roots is yet to be determined. However, this will require a broad approach to quantifying biophysicochemical conditions in the soils where *M. hapla* exists. The biophysicochemocal conditions will be comprised of the microbial community, nematode trophic groups, sand, silt clay, pH, organic matter, nitrate and ammonium.

The project goal, objectives, and hypotheses

The broad occurrence of *M. hapla* in the Michigan vegetable production landscape and presence of more parasitism in populations isolated from mineral than from muck soils raises a question. i.e., Is this because *M. hapla* is adapted to broad soil conditions and/or are there specific conditions that favor or do not favor PV? This overarching question is best answered by understanding the soil conditions (**Figure 1.4**). Therefore, the goal of this project was to investigate the soil biophysicochemical conditions associated with *M. hapla* PV.

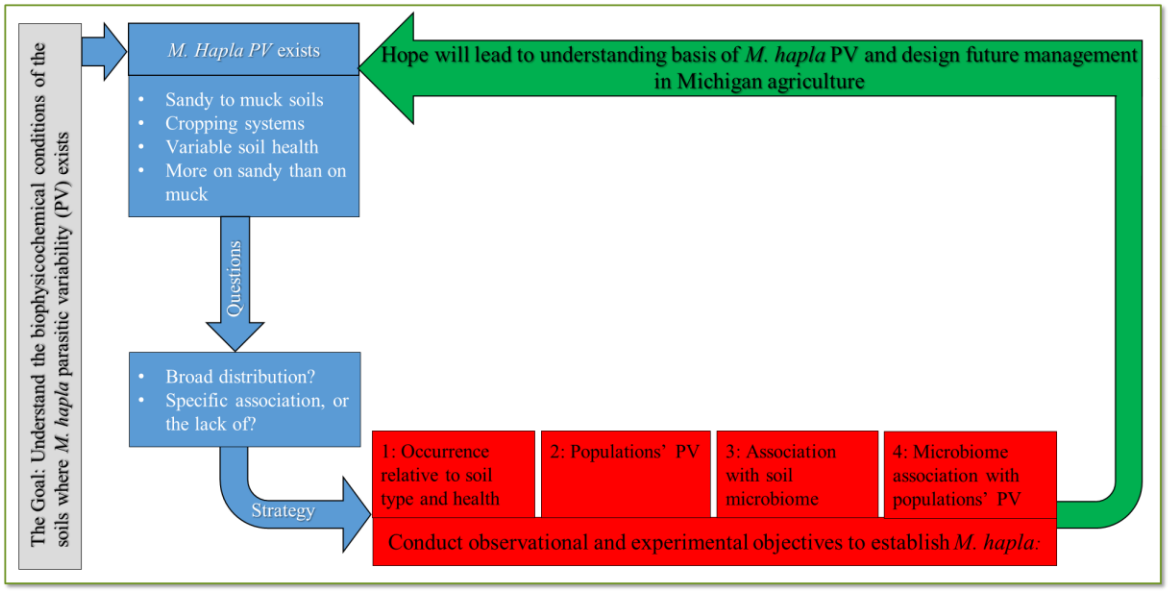

Figure 1.4 | A graphical abstract illustrating my project goal, the different soil conditions of sandy to muck soil, cropping systems, and the variable soil health conditions *M. hapla* PV exists, the sandy soil *M. hapla* populations with a higher parasitism than muck populations, research questions, observational and experimental objectives, and how their synthesis lays the basis for potential biophysicochemical associations with PV.

In order to address the goal, experimental and observational approaches were used to test four objectives (**Figure 1.4**). These were to establish *M. hapla*: 1) occurrence relative to soil type and health, 2) populations' PV, 3) association with soil microbiome, and 4) microbiome associated with populations' PV (**Figure 1.4**).

The first objective (Chapter 3) was to test the hypotheses that *M. hapla* populations in mineral and muck soils were a) present in degraded (Quadrant D, worst-case) soil health conditions, and b) associated with low trophic group abundance. The second objective (Chapter 4) was to test the hypothesis that PV in *M. hapla* populations is related to specific SFW conditions. If true, *M. hapla* populations from degraded and disturbed SFW conditions should exhibit distinct reproductive potential in both soil groups. The third objective (Chapter 5) was to test the hypotheses that a) the soil microbiome differed significantly between soil

groups and soil health conditions, b) the core-microbial members were present in the *M. hapla* infested and non-infested fields, and c) there were microbial indicators associated with *M. hapla* occurrence or SFW conditions. The fourth objective (Chapter 6) was to test the hypothesis that either presence and/or absence of specific bacterial composition and functional groups were associated with *M. hapla* PV. The synthesis objective (Chapter 7) would make connections across the four objectives in **Figure 1.4,** with the hope of identifying the basis of *M. hapla* PV and to design future management strategies in Michigan agriculture.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Study sites and sampling design

Michigan vegetables are produced in mineral and muck soils and *M. hapla* is present in the major vegetable production areas of the lower peninsula of Michigan (Melakeberhan and Wang, 2013; **Figure 2.1 A**). Using the sampling design in **Figure 2.1 B,** and a soil sampling datasheet to gather field information (**Appendix B**), a total of 15 geo-referenced vegetable fields representing muck and mineral soil groups in the east, southwest and northwest regions were selected for sampling in June 2018 (**Table 2.1**). With the variable distribution of mineral and muck agricultural sites across the east, southwest and the northwest regions, the number of representative fields were not the same per region and soil group. Three each of muck and mineral soils were in the east, three mineral and one muck in the southwest, and two muck and three mineral soils in the northwest regions. The between field distances in the eastern region was 1 km for the two closest fields, a maximum of 25 km for the furthest and an average of 10 km for all fields. In the southwestern region, the distance between the two fields was 3 km, the furthest apart was 71 km and a 37 km average for all fields. The northwestern region's closest fields were 3 km apart (Fields 14 and 15), the furthest being 85 km apart and an average distance of 47 km between all fields. In addition, a natural vegetation adjacent to each field was marked as an indicator of changes in soil health conditions between agricultural (disturbed) and undisturbed soils. This is important in developing soil health adjustment strategies should there be any relationship between *M. hapla* presence or absence and soil health conditions.

Table 2.1 | Field GPS coordinates (latitude and longitude) of 15 agricultural fields sampled from Michigan by regions (east, southwest and northwest) and soil groups (mineral and muck).

Region	Soil group	Field	Latitude	Longitude
East	Mineral	1	43.068871°	-83.341871°
		2	43.092822°	-83.053522°
		3	43.071761°	-83.032147°
	Muck	4	43.065446°	-83.067139°
		5	43.050561°	-83.068175°
		6	43.094628°	-83.064396°
Southwest	Mineral	7	42.664865°	-86.047077°
		8	42.108973°	-86.368160°
		9	42.083001°	-86.367671°
	Muck	10	42.660808°	-85.996822°
Northwest	Mineral	11	43.840772°	-86.348123°
		12	43.826522°	-86.378970°
		13	43.764887°	-86.137222°
	Muck	14	43.351260°	-85.726828°
		15	43.197608°	-85.782483°

Soil sampling and partitioning

In each of the selected vegetable fields and adjacent natural vegetation, five separate spots each with approximately 25 m^2 area were flagged for sampling. In all cases, one geo-referenced flag was randomly marked about 30 cm from the crop row or the natural vegetation. Approximately 1 L of a composite of ten random cores of soil from around each flag was collected using a custom-made steel cone with a diameter of 2.5 cm and a depth of 15 cm (Melakeberhan et al., 2018). After thoroughly mixing each of the five composite cores for every field, ~100 cm^3 of soil was used for total nematode community extraction to ascertain soil health conditions, 300 cm^3 of soil was used to screen for *M. hapla* presence, 50 cm^3 for chemical, 50 cm^3 for organic matter, 200 cm^3 for soil texture and 10 cm^3 for microbiome analyses (**Figure 2.1 C**). Approximately 300 cm^3 of the remaining soil of each

sample per field was designated as voucher specimen (**Figure 2.1 C5**) and assigned unique field codes as shown in **Appendix C**. All samples were kept at 4 ºC, but the microbiome soil samples were kept at -80 ºC.

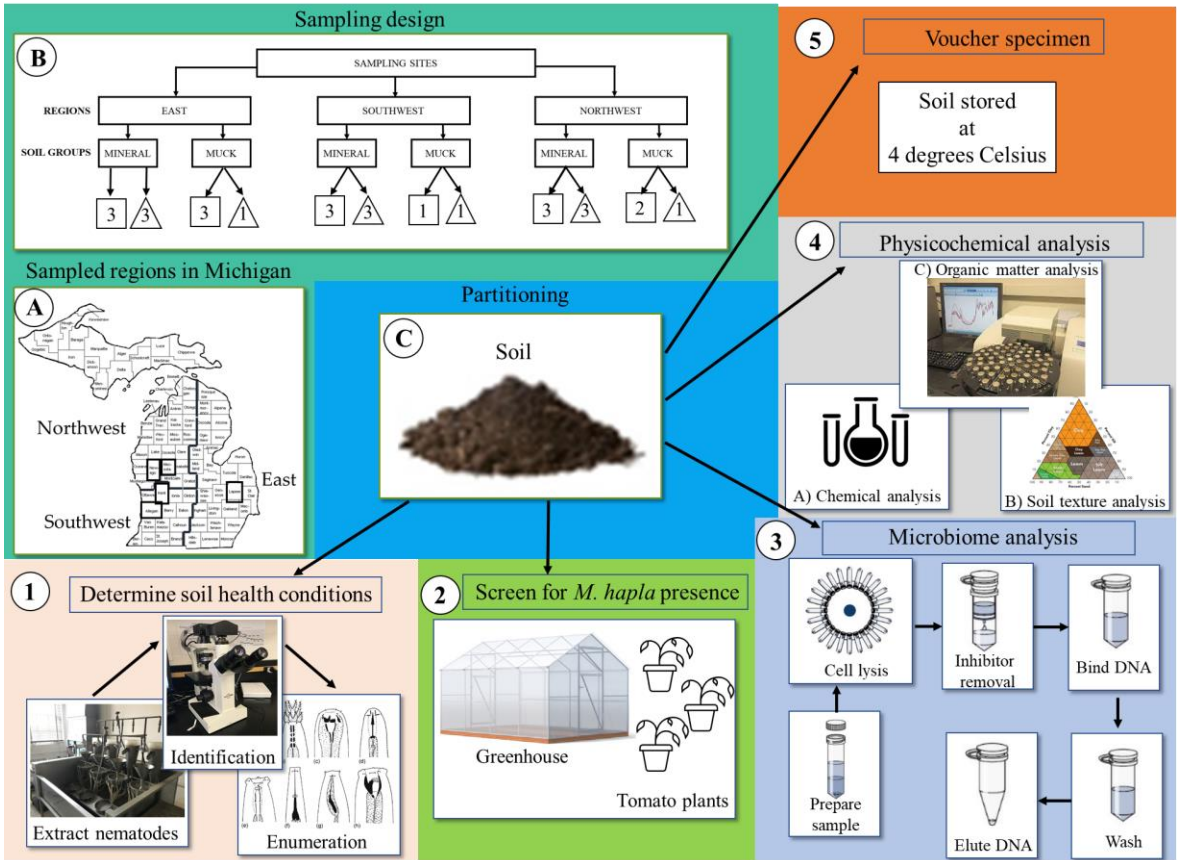

Figure 2.1 | Diagram of the three regions (east, southwestern and northwestern) in Michigan (A), the sampling design used (B), and how field soil was partitioned into five parts (C) to: determine soil health conditions (1), screen for *M. hapla* presence (2), perform microbiome analysis (3), conduct physicochemical analysis (4) and serve as soil voucher. In the sampling design, regions of the sampling sites had mineral and muck soils groups showing the numbers of sampled agricultural (disturbed) and natural vegetation (undisturbed) sites represented by square and triangle, respectively. A volume of 100 cm^3 of soil was used ascertain soil health conditions, 300 cm^3 of soil was used to screen for *M. hapla* presence, 300 cm^3 of soil was used for physicochemical analysis (50 cm^3 for chemical, 50 cm^3 for organic matter, 200 cm3 for soil texture), 10 cm^3 for microbiome analysis and 300 cm^3 as voucher specimen. Voucher soils are accessible in the Agricultural Nematology Laboratory of the MSU Department of Horticulture and each field was assigned unique field codes as shown in Appendix C.

Nematode extraction, fixing and enumeration

As illustrated in **Figure 2.1 C2**, vermiform nematodes were extracted from a 100 cm^3 volume of sub-sample of homogenized soil using a semi-automatic elutriator system as described in Avendaño et al., (2004) and Melakeberhan et al., (2018) and fixed in double TAF solution (14 ml 40% formalin: 4 ml tri- ethanolamine: 91 ml distilled water) (Hooper, 1986).

The total numbers of nematodes in each sub-sample were counted, and 100 individual nematodes were identified to genus level at 400× magnification using an inverted digital microscope (Accu-scope®) following identification keys of Bongers, (1994) and the University of Nebraska (Lincoln, NE, USA) nematode identification website (https://nematode.unl.edu/index.html). Nematodes were assigned to trophic groups of herbivore (HV), fungivore (FV), bacterivore (BV), omnivore (OV) and predator (PR) (Yeates et al., 1993; Okada and Kadota, 2003) and colonizer-persister groups (cp 1 resistant to disturbance and cp 5 sensitive to disturbance) (Bongers, 1990; Bongers et al., 1995). The total numbers of each genus in a sample were adjusted as described in Freckman and Ettema, (1993) and abundance of HV, BV, FV, OV and PR, all trophic groups (SUM), free-living nematodes (SFL), SFL of cp 2-cp 5 (SFL25), and trophic groups of cp 2-cp 5 (S25) expressed on a per 100 cm^3 basis.

Nematode community analysis for determining soil health conditions

The soil food web model utilizes nematode community dynamics in response to resource and reproductive potential and resistance to disturbance to establish soil health conditions. Weighted abundances of nematode guilds were processed to fit the enrichment and structure based on Ferris et al (2001) model as follows: structure ($s = \Sigma ks\ ns$), enrichment ($e = \Sigma ke\ ne$) and basal ($b = \Sigma kb\ nb$) conditions, with *k* representing specific weight of nematode guild and

n being the relative frequency of each functional guild for further calculations using formulae: structure index = 100(s/(s + b)) and enrichment index = 100(e/(e + b)). The soil health condition of each field was as either: disturbed (A), maturing (B), structured (C) or degraded (D).

Screening soils for *Meloidogyne hapla* presence

Screening for the presence of *M. hapla* was done using tomato (*Solanum lycopersicum*) 'Rutgers' (Melakeberhan et al., 2007; **Figure 2.1 C2**). Seedlings were mass-germinated in sterile soil in a glasshouse set at a diurnal cycle of 28 ± 3°C, 16 hr of light and 21 ± 3°C, 8 hr dark. Three 2-week-old seedlings were transplanted into a 300 cm^3 of each of the 135 soil sample potted in white styrofoam containers and maintained for 60 days under the same glasshouse conditions as the seedling germination. Plants were watered to saturation as needed and fertilized twice weekly with Scotts' Professional 20-20-20 (N-P-K) commercial mix (Scotts-Sierra Horticultural Products) (Melakeberhan and Wang, 2013). After 60 days, roots were washed free of soils and scored for presence or absence of *M. hapla* infection on a 0-5 modified index of Bridge and Page, (1980) where: 0 = no galls, 1 = few small galls, 3 = 50% of roots infested, main roots with galls, 4 = all main roots galled, few clean roots visible, and 5 = all roots severely galled, plant dying or dead. All samples were categorized as *M. hapla* positive and those without galling as negative. Sites with at least one of the five soil sub-samples testing positive for *M. hapla* were categorized as infested/positive sites.

Culturing *Meloidogyne hapla* populations

Among the sites testing positive for *M. hapla* (9 of the 15 agricultural fields), cultures of each of the 9 *M. hapla* positive populations were established from a single egg mass (Melakeberhan et al., 2007). Briefly, an individual egg mass from each of the positive populations was randomly selected and carefully removed with a clean tweezer from roots of the original culture and placed on a pencil-size hole of a two-week-old tomato (*Solanum lycopersicon*) cv. 'Rutgers' seedling contained in 500 cm^3 volume clay pots and allowed to multiply. Tomato seedlings were mass geminated and transplanted into steam-sterilized soil (a mix of topsoil, sphagnum peat, and sand supplied by Michigan State University Plant Science Greenhouses) two weeks after germination. Cultures were maintained in a greenhouse set to have a 28 ± 3 ^oC, 16-hour day, and 21 ± 3 ^oC, 8-hour dark diurnal cycle. The plants were watered daily as needed and fertilized twice every week with Scotts' Professional 20-20-20 (N-P-K) commercial mix. The 9 *M. hapla* cultures were allowed to build over several months in preparation for *M. hapla* egg extraction needed to characterize PV in Chapter 4.

Soil microbiome DNA extraction, library preparation, sequencing, and enumeration

As outlined in **Figure 2.2**, soil microbiome DNA was extracted from 2 g sub-sample of soil using the PowerSoil® DNA isolation kit (Qiagen, United States). All extractions included samples containing no sample (negative controls) and were stored at -80 ^oC (Longley et al., 2020).

Illumina MiSeq amplicon libraries targeting bacterial 16S rDNA with the primers 515F and 806R and fungal ITS rDNA with the primers ITS1f and ITS2 were constructed (White et al., 1990; Gardes and Bruns, 1993; Caporaso et al., 2011; Kozich et al., 2013). Libraries were prepared using Accuprime Pfx Super Mix. The polymerase chain reaction

(PCR cycles used for 16S and ITS are shown in **Appendix D** and in **Appendix E**, respectively. PCR products were normalized with the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, United States) and the final concentration of the library was determined using a SYBR green quantitative PCR (qPCR) assay with primers specific to the Illumina adapters (Kappa). Following normalization, samples were combined into one pool and concentrated with Amicon Ultra 0.5 mL 50K filters (EMD Millipore, Germany). Libraries were then cleaned with Agencourt AMPure XP magnetic beads to remove small fragments and primer dimers (Beckman Coulter, United States). Libraries were sequenced at the MSU Genomics Core with the Illumina MiSeq v2 500 cycles kit. Sequence data generated in this study have been deposited into the NCBI SRA archive under the following accession number: PRJNA833458.

Bioinformatic analyses of 16S and ITS sequences were both performed using Qiime 2 version 2019.1 (Bolyen et al., 2019). First, sequences were analyzed for initial quality using FastQC. Due to lower quality of the reverse reads, only forward reads were analyzed further for ITS sequence while both forward and reverse reads were used for 16S sequences. The "join-pairs" method of the q2-vsearch plug-in was used to join the 16S sequence pairs. Afterward, both 16S and ITS library statistics were analyzed for quality distributions using the q2-quality-filter plugin. Additionally, error modeling, de-replication and denoising of sequences were performed with the default values of the q2-deblur plugin. The primers were trimmed and the read lengths for 16S and ITS truncated to 220 and 200 nucleotides, respectively. The taxonomies of 16S and ITS representative sequences were assigned using Greengenes 13.9, and CONSTAX2 (Gdanetz et al., 2017; Liber et al., 2021) against the UNITE database version 04.02.2020 (Abarenkov et al., 2020), respectively. All the analyses performed are accessible on github (https://github.com/larteyis/Scientific-Papers-R-Code/tree/main/Lartey_et_al_2021_Field_M.hapla_Associated_Microbiome).

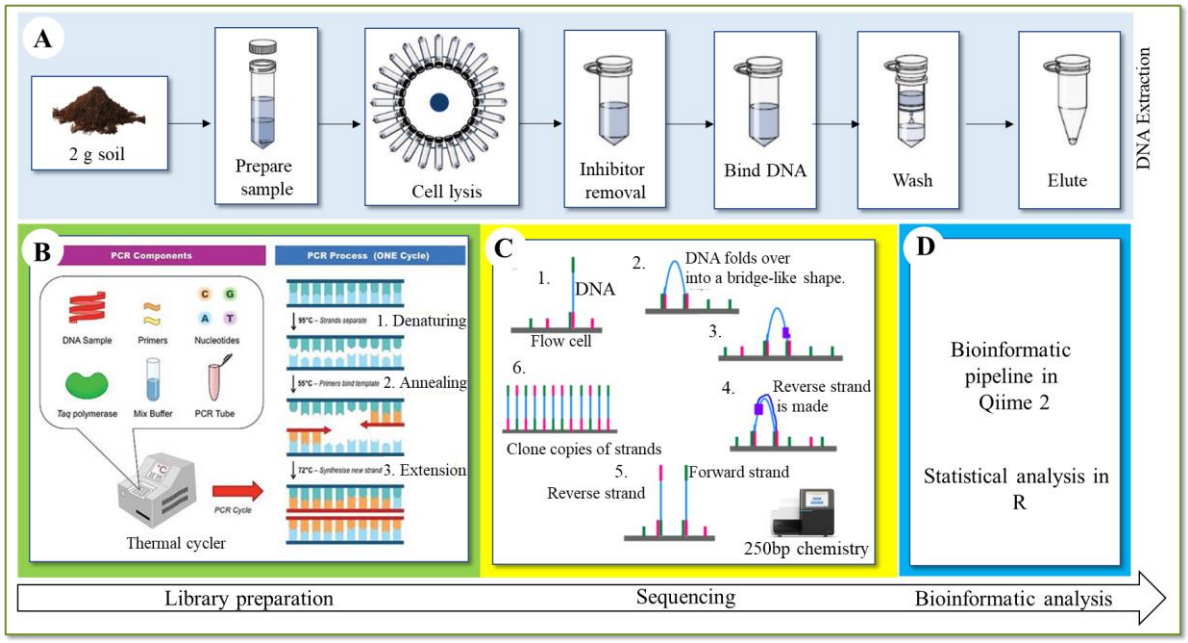

Figure 2.2 | Diagram of major steps followed for DNA extraction (A), library preparation (B), sequencing (C) and bioinformatic analysis (D) as part of the field soil microbiome analysis. DNA extraction was performed using the using the PowerSoil® DNA isolation kit with 2 g soil to prepare sample for cell lysis, removal of inhibitors, binding of DNA, washing and elution steps. The library preparation stage mixed DNA, primers (16S to target bacteria and ITS to target fungi), nucleotides, taq polymerase and buffer in a PCR tube, and placed the mixture in a thermocycler. The thermocycler repeated denaturing, annealing and extension steps to target bacteria and fungi. Sequencing procedure was done using the 250bp chemistry in a Miseq to clone copies of the bacterial and fungal DNA. Bioinformatic analysis was performed using the Qiime2 pipeline and statistical analysis was done in R (Bolyen et al., 2019; R Core Team, 2020).

Nematode microbiome DNA extraction, library preparation, sequencing, and enumeration

As illustrated in **Figure 2.3**, both field and greenhouse populations were extracted from a sub-sample of 100 cm^3 of soil with a semi-automatic elutriator using the method described by Avendaño et al., (2004) and Melakeberhan et al., (2018). Briefly, a 1:1:3 ratio of soil,

dish soap (non-phosphate) and tap-water was run through a semi-automatic elutriator, passed through sieves (850 µm, 250 µm and 20 µm, respectively), centrifuged (4000rpm in 456 sugar/L tap-water), carefully rinsed and collected in distilled water for storage (4 ^{o}C). For each of the field and greenhouse *M. hapla* populations, five second-stage juveniles were used. The *M. hapla* nematodes were picked with a clean needle (Genesse scientific, model 59-AWP-B) under a stereo microscope (Zeiss, model AX10) and dropped into a 30 µl worm lysis mixture (950 µl worm lysis solution (**Appendix F**) + 50 µl Proteinase K). In the next step, which was repeated five times, the nematode in the worm lysis mixture was frozen at -80 ^{o}C for 10 minutes and thawed at 25 ^{o}C. This was followed with an incubation step at 60 ^{o}C for 60 min and 95 ^{o}C for 15 min in a thermocycler. The final product was stored at -80 ^{o}C.

Illumina MiSeq amplicon libraries were constructed with the 515F and 806R primers to target the V4 region of the 16S bacterial rDNA. Libraries were prepared following a three step PCR protocol (Benucci et al., 2018; Caporaso et al., 2011) shown in **Appendix E.** First, unmodified primers were first used to enrich the target taxa. In the next step, frame shift incorporating primers were used (Chen et al., 2018; Lundberg et al., 2013). Third, a 10-nucleotide indexing set of barcodes and Illumina adapters were incorporated following the approaches used by Chen et al., (2018) and Lundberg et al., (2013). In order to minimize the amplification of mitochondria and chloroplast, PNA blocking clamps were used (Longley et al., 2020). The PCR products were run on a gel to confirm amplification. Following that, the concentrations of the PCR products were normalized (1-2 ng/µl) using the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, United States). After the normalization step, the normalized samples were pooled and concentrated using Amicon Ultra 0.5 mL 50K filters (EMD Millipore, Germany). The pooled libraries were cleaned with Agencourt AMPure XP magnetic beads (Beckman Coulter, United States) to remove primer dimers and

small fragments. The cleaned libraries created were submitted to the Michigan State University Genomics Core to be sequenced with an Illumina Miseq V3 600 cycles kit. Sequenced samples were demultiplexed using the Sabre software (Joshi, 2011). These sequences are available in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), BioProject PRJNA854890.

The bioinformatic analyses of 16S sequences were performed in Qiime 2 version 2019.1 (Bolyen et al., 2019). First, sequences were analyzed for initial quality using FastQC. After the initial quality testing, only the forward reads were considered for further analysis. Afterward, the 16S library statistics were analyzed for quality distributions using the q2-quality-filter plugin. Using the default values of q2-deblur plugin, error modeling, de-replication and denoising were performed. Additionally, primers were trimmed off and the sequence length truncated to at 200 nucleotides. The representative 16S sequences were assigned taxonomy using the ACT (alignment, classification, tree service; https://www.arb-silva.de/aligner/) tool of SILVA online database (Pruesse et al., 2012). In addition to the default settings used, the "minimum identity with query sequence" and the "reject sequences below identity" options were set to be 0.6 and 50%, respectively. All classification of sequences were based on the SILVA (Quast et al., 2013) database. All the analyses performed are accessible on github (https://github.com/larteyis/PAPER-Bacterial-composition-diversity-and-functional-groups-associated-with-Meloidogyne-hapla-popul).

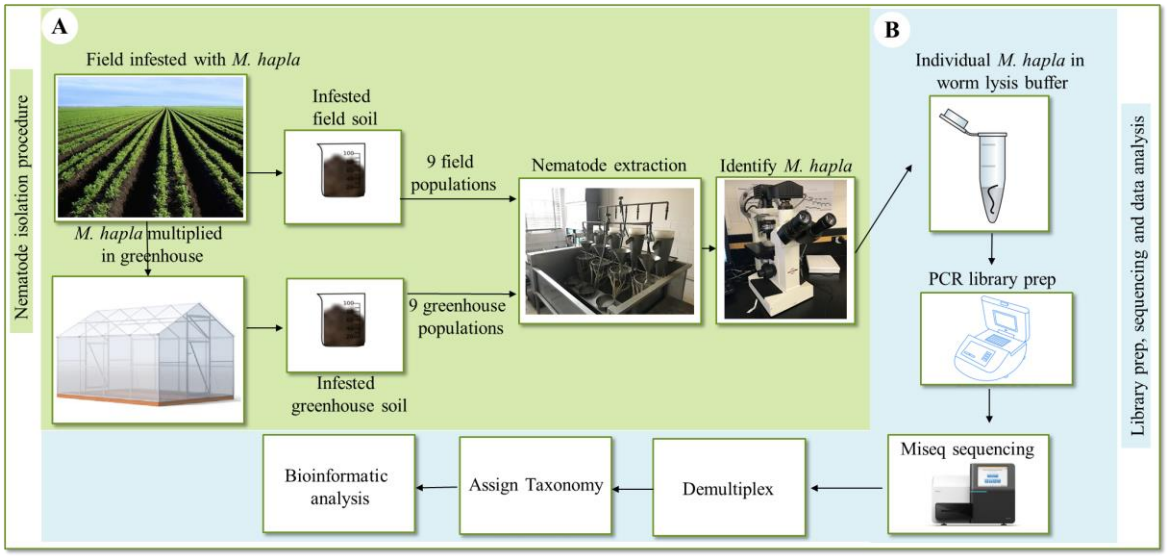

Figure 2.3 | Diagram of major steps followed for nematode isolation (A) and library preparation, sequencing and data analysis (B) procedures. Nematode isolation procedure involved 9 *M. hapla* populations sampled directly from infested field soil and the same populations from the field multiplied in the greenhouse over several generations. *Meloidogyne hapla* nematodes were extracted using a semi-automatic elutriator and identified using a microscope. For each population, 5 individual *M. hapla* nematodes were processed separately in a worm lysis buffer to extract DNA and a PCR library preparation was prepared to target bacteria 16S rDNA. The library was sequenced using a Miseq platform, demultiplexed, assigned taxonomy and bioinformatic analysis performed.

Analysis of soil physicochemical properties of field soils

All soil samples were taken to the Michigan State University Soil Testing Laboratory for analysis. A total of three samples were used for chemical analysis, whereas three samples were combined for soil texture or organic matter analysis. Before chemical analysis, the samples were forced air dried in an oven at 35 ^{o}C for 36hrs and was ground in a flail grinder. Subsequently, the ground sample was passed through a 2 mm mesh screen. The chemical analysis performed measured the available nitrate (NO_3^-), ammonium (NH_4^+) and pH (Huffman and Barbarick, 1981; Melakeberhan, 1999; Nelson, 1983). The percent organic

matter (OM%) content of samples was determined using the loss-on-ignition method (Heiri et al., 2001). For soil texture, the percentages of sand, silt, and clay were estimated following the particle size analysis (Bouyoucos, 1951).

Soil biophysicochemical data analysis of *M. hapla* infested field soils

Two sets of analyses were performed. First, a principal component analysis (PCA) in PAST v4.0 software to assess the physicochemical relationships of the fields from which *M. hapla* populations came from (Hammer et al., 2001). The composite relationships were assessed for NO_3^-, NH_4^+, pH, OM%, percent sand, silt, and clay across soil groups (mineral and muck) and soil health conditions (degraded and disturbed). Prior to generating the PCA with variance-covariance matrix, the values of dependent variables were standardized using the formula $z = (x-\mu/\sigma)$, where z was the standardized value, x was the observed value, μ. Variation was explained by principal component (PC) 1 on the x-axis and PC2 on the y-axis. Afterwards, several linear regression analyses were performed to assess the relationship between physicochemical parameters (NO_3^-, NH_4^+, pH, OM%, percent of sand, silt and clay) and parasitic variability (total number of nematodes in roots) using packages ggpubr and ggplot2 (Wickham, 2016) packages in R-studio (R Core Team, 2020).

Second, three separate redundancy analysis (RDA) were performed to assess the relationships of *M. hapla* populations with a) all soil bio-physicochemical parameters (ie trophic abundance (herbivore (HV), bacterivore (BV), fungivore (FV), predator (PR) and omnivore (OV) nematodes, sum of all trophic groups (SUM), sum of free-living nematodes (SFL), sum of free-living nematodes from cp 2-cp 5 (SFL25) and sum of cp 2-cp 5 nematodes (S25)), structure index (SI) and enrichment index (EI), physicochemistry (NO_3^-, NH_4^+, pH, OM, sand, silt and clay), bulk soil microbiome (bacterial and fungal genera abundance) and

isolated *M. hapla* field and greenhouse bacterial genera abundance, and parasitic variability (Gall, 2nd stage juvenile (J2), 3rd and 4th stage juveniles (J3/J4), adult , total, total (% infection)) at two inoculum levels (2000 and 4000 *M. hapla* eggs), b) bio-physicochemical parameters without EI, SI and nematode trophic group abundance and c) isolated field and greenhouse *M. hapla* microbial genera abundance, and parasitic variability. The decision to run an RDA over a CCA (canonical correspondence analysis) or PCA (principal component analysis) was determined by running a detrended correspondence analysis (DCA) to test assumptions that i) the eigen values were less than 50%, and ii) axis length was less than 3.5. The DCA was performed using the vegan package in R statistics (Dixon, 2003; R Core Team, 2020). Data of physicochemistry were transformed using logarithmic transformations, while all other variables were transformed using Hellinger, where needed. All relationships were plotted using the ggplot2 and ggrepel packages (Wickham, 2016).

CHAPTER 3

OCCURRENCE OF *MELOIDOGYNE HAPLA* RELATIVE TO NEMATODE ABUNDANCE AND SOIL FOOD WEB STRUCTURE IN SOIL GROUPS OF SELECTED MICHIGAN VEGETABLE PRODUCTION FIELD.

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Lartey, I., Kravchenko, A., Marsh, T., and Melakeberhan, H. (2021). Occurrence of *Meloidogyne hapla* relative to nematode abundance and soil food web structure in soil groups of selected Michigan vegetable production fields. Nematology, 23(9), 1011-1022.

Abstract

Despite considerable knowledge of distribution, biology and parasitic variability (PV) of *Meloidogyne hapla* in cropping systems, how its PV relates to soil health conditions remains unknown. This study investigated the relationship between the presence or absence of *M. hapla* with soil food web and the abundance of nematode community in muck (high organic matter) and mineral soils of 15 agricultural fields and adjacent natural vegetation across three vegetable production regions of Michigan, USA. *Meloidogyne hapla* was present in all regions and all muck soils, but only in some mineral soils. It was present in soils with degraded and disturbed soil food web conditions, but there was no pattern with nematode trophic group abundance. However, principal component analysis showed distinct relationships among *M. hapla* presence or absence, soils, nematode abundance parameters in agricultural and natural vegetation. The study lays down a foundation for more targeted investigations to understand any links between the PV of *M. hapla* and its soil environment.

Introduction

In the absence of broad spectrum nematicides and commercially available resistant cultivars, *Meloidogyne hapla* Chitwood (1949) continues to be a problematic nematode in many cropping systems globally (Kimpinski and Sanderson, 2004; Vestergård, 2019). It is particularly serious in temperate vegetable production systems where crop rotation has little value in controlling *M. hapla* because many of the crops are hosts (Melakeberhan and Wang, 2012; Melakeberhan et al., 2012; Grabau et al., 2017), and intensive agricultural and land use practices have resulted in degradation of soil health (Melakeberhan et al., 2007; Desaeger et al., 2017).

In addition to its broad distribution, *M. hapla* has parasitic variability (PV) where populations exhibit no detectable morphological or genetic differences (Liu and Williamson, 2006; Opperman et al., 2008) but infect and reproduce at different rates (Melakeberhan and Wang, 2013). Extensive studies in the biology (Vestergård, 2019) and host suitability (Melakeberhan et al., 2012) suggest that soil conditions may be contributing to *M. hapla* PV (Melakeberhan and Wang, 2013). For example, the incidence of parasitism was higher in mineral soils than in muck (high organic matter) soils (Melakeberhan et al., 2010). However, the exact mechanisms explaining these observations remain unknown.

Developing an integrated knowledge on how the environment where *M. hapla* thrives may influence its PV is critical to developing suitable management strategies; for example, soil types, production systems and practices, and soil health degradations, all of which, collectively, alter the biophysiochemical conditions in the soil (Melakeberhan et al., 2007; Gomiero et al., 2011; Lal, 2011; Chaparro et al., 2012; Turmel et al., 2015). In order for *M. hapla* and any organism to survive in such dynamic biophysiochemical environment, they must be adapting successfully to the conditions. For example, as the *M. hapla* second-stage juvenile hatches from the egg and migrates through the soil in search of the root system, it is

logical to expect that the juveniles are exposed to a range of harmful, harmless, and niche competitor biological organisms (Sánchez-Moreno and Ferris, 2007; Topalović et al., 2019, 2020; Zhou et al., 2019). If and how changes in the soil conditions directly or indirectly influence *M. hapla*'s PV remain unknown.

The goal of this project is to understand the basis of *M. hapla* PV in Michigan vegetable production soils with special emphasis on exploring any links between its PV and the ecosystem changes in its soil environment. Understanding the relationship between *M. hapla* distribution and soil health conditions as described by the Ferris et al., (2001) soil food web model could be a road map for identifying and connecting many of the missing links. The model uses changes in nematode community structure (x-axis) and enrichment (y-axis) to describe soil conditions in four quadrants: disturbed (A), maturing (B), structured (C) or degraded (D). Quadrant B would represent a best-case scenario and Quadrant D the worst case for agroecosystem. What factor(s) directly or indirectly influence *M. hapla* PV is unknown. Characterizing *M. hapla* presence or absence relative to the four quadrants is the first step towards clearly understanding its PV.

The objectives of this study were to determine how the presence and/or absence of *M. hapla* in selected mineral and muck soils relates to: *i*) the soil food web conditions; and *ii*) the nematode trophic group abundance. The hypothesis in relation to the soil food web was that *M. hapla* is present in degraded (Quadrant D) conditions. This is consistent with the notion that plant-parasitic nematodes are more prevalent in degraded than in non-degraded soil conditions (Wang and McSorley, 2005; Neher, 2010). The hypothesis in relation to abundance was that *M. hapla* presence is associated with low trophic group abundance. This is consistent with the notion of niche competition (Khan and Kim, 2007; Sánchez-Moreno and Ferris, 2007). A combination of both objectives will lead to a framework for targeted investigations to understand the basis of *M. hapla* PV in vegetable production systems.

Materials and methods

A greenhouse screening was used to determine presence or absence of *M. hapla* and a procedure to extract total nematode community for enumeration was used to determine the soil health conditions based on Ferris et al., (2001) model (see details in Chapter 2 as illustrated in **Figure 2.1 C1-2**).

Statistical analyses

Where applicable, residual diagnosis was applied to assess the assumptions of normality and homogeneity of the soil food web indices (EI and SI) and the data of nematode abundance parameters (HV, BV, FV, OV, and PR, SUM, SFL, SFL25 and S25). When the assumptions were not met, log transformation was performed. In order to determine how EI and SI were affected by soil, region, the presence or absence of *M. hapla*, land use, and their interactions in agricultural and adjacent natural vegetation, we used linear mixed effect model in PROC GLIMMIX (SAS-Institute, 2018). The fields were regarded as a random effect and were nested in soil, *M. hapla* presence/absence and region. Based on containment degree of freedom approximation, the statistical inference of the mixed ANOVA modelling was estimated by Restricted Maximum Likelihood. Differences of least square means of EI and SI by soil, region, the presence or absence of *M. hapla* and their interactions in the agricultural fields and natural vegetation are presented ($p<0.05$). In order to determine the soil food web conditions of individual agricultural fields and adjacent natural vegetation, we used PROC MIXED model where replicates (individual samples) were assigned as random effects and fields as fixed effects, and standard errors generated with Kenward-Roger method (SAS-Institute, 2018). Least squares means of EI and SI of the individual fields were plotted to describe the soil food conditions in four quadrants (Ferris et al., 2001).

Nematode abundance was analyzed in two ways. First, how abundance of HV, BV, FV, OV, and PR, SUM, SFL, SFL25 and S25 were separately affected by soil, region, the presence or absence of *M. hapla*, land use, and their interactions were statistically analyzed as described above. The results of back-transformed least square means of main, interacting and splicing effects were reported when they were found to be significantly different ($p<0.05$). Second, we used principal component analysis (PCA) to determine how abundance of HV, BV, FV, OV, and PR, SUM, SFL, SFL25 and S25 collectively change by soil, region, the presence or absence of *M. hapla*, and land use (R.Team, 2019). The first principal component (PC1) and PC2 were reported to explain the variation on the horizontal and vertical axis, respectively. In the PCA biplot, the relationship and location of nematode abundance (dependent) variables with regions and fields (independent) were shown by eigen value variation of each principal component. The PCA biplots were generated using the ggbiplot (Vu, 2011) and stats (R. Team, 2019) packages in RStudio. The eastern, northwestern and southwestern regions were represented by a square, a circle and a triangle, respectively.

Results

Screening for *M. hapla* presence

The glasshouse screening of the 15 fields showed that three mineral (Fields 2, 8 and 13) and six muck soil (Fields 4, 5, 6, 10, 14 and 15) in the agricultural fields were positive for *M. hapla* (**Table 3.1**). The *M. hapla* positive samples were in some mineral and all muck soils in the three regions. None of the adjacent natural vegetation sites tested positive for *M. hapla*. Host suitability at the time of sampling did not seem to be a major factor because soil samples from Fields 2, 4, 10, 14 and 15 (with suitable hosts) and Fields 5, 6, 8 and 13 (non-suitable hosts) tested positive for *M. hapla.* Fields 1 and 11 (with suitable hosts) and Fields

3, 7, 9 and 12 (non-suitable hosts) tested negative for *M. hapla* (**Table 3.1**). Voucher specimen of *M. hapla* from 9 fields prepared on slides are available in the Agricultural Nematology Laboratory of the Department of Horticulture (**Appendix G1**).

Table 3.1 | A regional (eastern, southwestern and northwestern of Michigan, USA), soil (mineral and muck) and soil type (loam, sandy loam, organic and loamy sand) categorization of the 15 agricultural fields sampled in June 2018 with their suitable host (SU: tomato, soybean, onion, vetch, carrot, parsnip and sugar beet) or unsuitable host (US: oats, corn, apple seedlings and wheat) crops and the presence or absence of *Meloidogyne hapla*.

Region	**Soil**	**Soil type**	**Field**	**Crops**	**SU or US**	**Presence/ absence of *M. hapla***
Eastern	Mineral	Loam	1	Tomato & pepper	SU	-
		Loam	2	Soybean	SU	+
		Sandy Loam	3	Oats	US	-
	Muck	Organic	4 *	Onion	SU	+
		Organic	5 *	Corn	US	+
		Organic	6 *	Corn	US	+
South-western	Mineral	Loamy Sand	7	Corn	US	-
		Loamy Sand	8	Apple seedlings	US	+
		Loamy Sand	9	Wheat & vetch	SU & US	-
	Muck	Organic	10	Onion	SU	+
North-western	Mineral	Sandy Loam	11	Carrot	SU	-
		Loamy sand	12	Corn	US	-
		Loamy sand	13	Wheat	US	+
	Muck	Organic	14 **	Parsnip	SU	+
		Organic	15 **	Sugar beet	SU	+

*Fields 4, 5 and 6 shared a common natural site based on their proximity.

**A shared natural site by Fields 14 and 15 was based on their proximity.

None of the adjacent natural vegetation tested positive for *M. hapla*.

Relationship of *M. hapla* presence with soil food web structure

The soil food web conditions were described based on the intersection of the enrichment and structure trajectories (**Figure 3.1**). None of the agricultural fields or adjacent vegetation had conditions that would be described as structured (data points in Quadrant C). The soil food web conditions of two mineral soil agricultural fields (11 and 12) in northwest that were negative for *M. hapla* and a muck soil in the natural vegetation adjacent to Field 4 in the east were the only locations described as maturing (Quadrant B, **Figure 3.1**). The soil food web conditions in the rest of the agricultural fields, irrespective of presence or absence of *M. hapla*, and the adjacent natural vegetation sites were either disturbed (Quadrant A) or boarderline between disturbed and degraded (Quadrant D).

There was no difference in structure index between agricultural and adjacent natural vegetation in the 15 sites. However, enrichment indices in mineral Fields 7 and 8 in the southwest and muck Fields 14 and 15 in the northwest were significantly lower than their corresponding natural vegetation (**Figure 3.1**). The enrichment indices in mineral soil Fields 11 and 12 in the northwest were higher than the corresponding natural vegetation. The structure index of Field 2, mineral and *M. hapla* positive, was significantly lower than *M. hapla* positive (Fields 4 and 8) and *M. hapla* negative agricultural fields (1, 3, 11 and 12) in both soils and three regions (**Figure 3.1**).

The enrichment index was similar while the structure index varied significantly by soil and *M. hapla*, region and soil, and region and *M. hapla* interactions in agricultural fields (**Table 3.2 A**). The structure index in muck soil with *M. hapla* present was lower than mineral soil with *M. hapla* either present or absent. The mineral soil in the eastern region had the lowest structure index compared to the rest of mineral and muck soils in eastern southwestern and northwestern regions. *Meloidogyne hapla* presence in the eastern region was associated with lower structure index value compared to the other region by *M. hapla*

interactions. In the adjacent natural vegetation (**Table 3.2 B**), structure index was similar, while enrichment index varied by region by soil interactions. Mineral soil in the eastern region showed a significantly lower enrichment compared to the other regions by soil interactions.

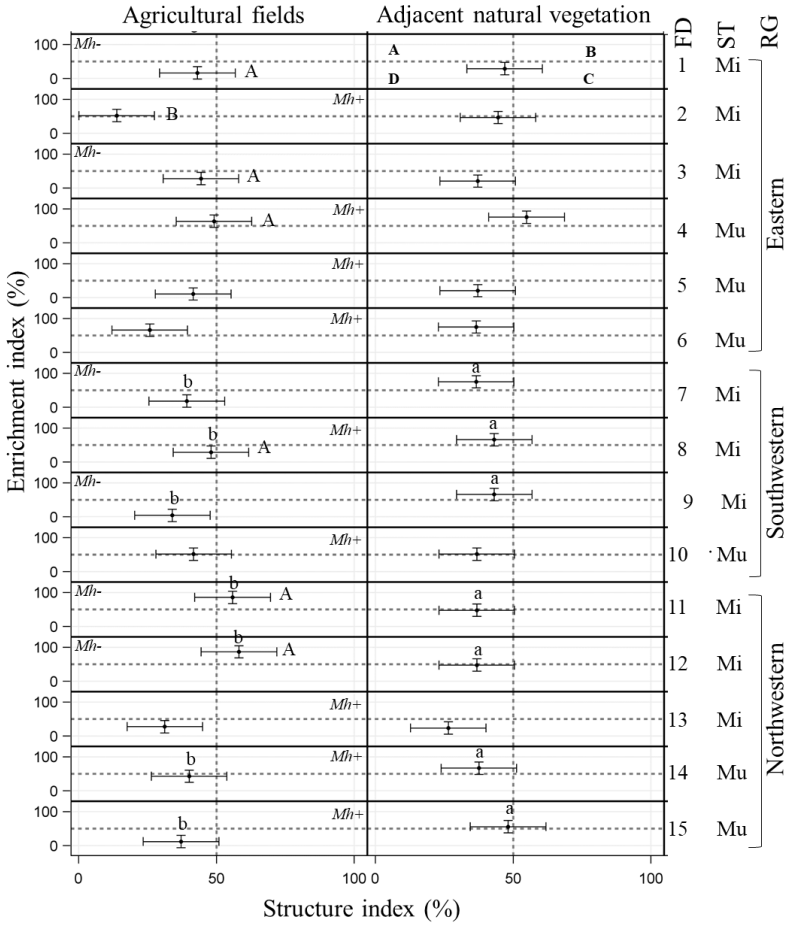

Figure 3.1 | The soil food web conditions of the 15 agricultural (left column) and adjacent natural (right column) vegetation sites (FD) in mineral (Mi) and muck (Mu) soils (ST) in the eastern, southwestern and northwestern regions (RG). Note: Quadrants A, B, C and D are based on the Ferris et al. (2001) model. *Meloidogyne hapla* was not detected in the natural vegetation. Its presence (Mh+) or absence (Mh-) in the agricultural fields are noted. Enrichment (vertical) standard error bars with no or same lower-case letters between agricultural and natural vegetation are not statistically different at $p<0.05$. Structure (horizontal) standard error bars with no or same upper-case letters within agricultural fields are not statistically different at $p<0.05$.

Table 3.2 | Mean enrichment (EI) and structure (SI) indices by region (RG: eastern, southwestern and northwestern of Michigan, USA), soil (ST: mineral and muck), and presence or absence of *Meloidogyne hapla* (Mh) in agricultural fields (A) and RG and ST in adjacent natural vegetation site (B).

Factors			A) Agricultural fields*	
RG	**ST**	**Mh**	**SI**	**EI**
-	Mineral	Present	37 ab	28
-		Absent	45 a	25
-	Muck	Present	25 b	32
-		Absent	-	-
Eastern	Mineral	-	23 b	25
	Muck	-	59 a	19
South western	Mineral	-	39 ab	10
	Muck	-	42 ab	33
North western	Mineral	-	43 a	52
	Muck	-	50 a	46
Eastern	-	Present	20 b	40
	-	Absent	64 a	10
South western	-	Present	42 a	30
	-	Absent	38 ab	12
North western	-	Present	34 ab	23
	-	Absent	60 a	85
			B) Adjacent natural vegetation *, **	
Eastern	Mineral	-	38	26 b
	Muck	-	40	51 ab
South western	Mineral	-	43	68 a
	Muck	-	34	49 ab
North western	Mineral	-	31	34 ab
	Muck	-	40	61 ab

*Least square means followed by the same or no letters within a category of RG × ST, RG × Mh, and ST × Mh and column not statistically different at $p<0.05$. RG × ST × Mh interaction was not significant.

** *M. hapla* not present.

Relationship of *M. hapla* presence with nematode trophic group abundance

A total of 59 nematode genera, belonging to 17 herbivores, 18 bacterivores, 7 fungivores, 8 predators and 9 omnivores, were identified in the sampled fields (**Table 3.3**). *Paratylenchus* and *Meloidogyne* in herbivores, *Cephalobus* and *Rhabditis* in bacterivores, and *Aphelenchus* and *Filenchus* in fungivores were the genera most observed.

Predators were not detected in the agricultural fields, and abundance of herbivore, bacterivore, fungivore and omnivore trophic groups, sum of all trophic groups, sum of free-living nematodes, sum of free-living nematodes from cp 2 to cp 5, and sum of cp 2 to cp 5 of all nematodes in the 15 agricultural fields and adjacent natural vegetation was low and highly variable (data not shown). Nematode abundance by region, soil group, presence and absence of *M. hapla*, and their interactions by landscape are shown in **Table 3.4**.

In the agricultural fields, abundance of bacterivores, sum of free-living nematodes from cp 2 to cp 5 and sum of cp 2 to cp 5 nematodes differed significantly ($p<0.05$) by region (**Table 3.4 A**). All abundance parameters, except for predators and omnivores, were significantly ($p<0.05$) higher in mineral than in muck soil in the eastern and southwestern regions, while the reverse was true in the northwestern region. The abundance of herbivores, sum of all trophic groups, sum of free-living nematodes, sum of free-living nematodes from cp 2 to cp 5, and sum of cp 2 to cp 5 of all nematodes was significantly ($p<0.05$) lower in the absence than in the presence of *M. hapla* in the eastern region, but the reverse was true in the northwestern region. There was no trend in southwestern region. Only the sum of free-living nematodes of cp 2 to cp 5, and the sum of cp 2 to cp 5 of all nematodes differed significantly ($p<0.05$) by region, soil and presence or absence of *M. hapla* (**Table 3.4 A**).

Because *M. hapla* was not present in the natural vegetation, abundance parameters were compared by soil and region (**Table 3.4 B**). The abundance of herbivores, bacterivores, fungivores, sum of all trophic groups, sum of free-living nematodes, and sum of cp 2 to cp 5

of all nematodes were significantly ($p<0.05$) higher in muck than in mineral soil. All abundance parameters differed significantly by soil group and region. In the eastern and northwestern regions, nematode abundance was significantly higher ($p<0.05$) in muck than in mineral soil. There was no clear trend in the southwestern region (**Table 3.4 B**).

The abundance of herbivores, omnivores and sum of all trophic groups were significantly ($p<0.05$) higher in the adjacent natural vegetation than in the agricultural fields. All but two nematode abundance parameters (herbivore and predator) differed significantly ($p<0.05$) by region and landscape. The abundance of herbivores, bacterivores, fungivores, of all trophic groups, sum of free-living nematodes, and sum of cp 2 to cp 5 of all nematodes were significantly different ($p<0.05$) by soil and landscape. Only omnivores, sum of free-living nematodes, sum of free-living nematodes of cp 2 to cp 5, and sum of cp 2 to cp 5 of all nematodes were significantly ($p<0.05$) different by soil, region and landscape (**Table 3.4 C**).

PCA relationships among abundance of nematode trophic (herbivore, bacterivore, fungivore, omnivore trophic groups, sum of all nematodes and free-living) and cp groups (free-living cp 2 to 5, and cp 2 to 5 of all nematodes) in agricultural fields and adjacent natural vegetation, *M. hapla* presence or absence, soils and regions showed distinct patterns (**Figure 3.2 A-F**). The pattern in the *M. hapla* negative mineral soil fields was generally influenced by PC1, whereas the pattern in the adjacent natural vegetation was strongly towards PC2 (**Figure 3.2 A, B**). The pattern of correlation in the *M. hapla* positive mineral and muck soils was strongly towards PC2 (**Figure 3.2 C, E**), while that of the corresponding natural vegetation was strongly influenced by PC1 (**Figure 3.2 D, F**).-

Table 3.3 | Genera of herbivore, bacterivore, fungivore, predator and omnivore nematodes and their colonizer-persister (cp) categories values observed in the soil samples from areas in Michigan, USA.

Herbivores*	cp **	**Bacterivores**	cp	**Fungivores**	cp	**Omnivores**	cp
Malenchus	2	*Bunonema*	1	*Aphelenchoides*	2	*Axonchiuma*	4
Paraphelenchus	2	*Diplogaster*	1	*Aphelenchus*	2	*Dorylaimus*	4
Paratylenchus	2	*Mesorhabditis*	1	*Aprutides*	2	*Ecumenicus*	4
Rotylenchus	2	*Pellioditis*	1	*Ditylenchus*	2	*Epidorylaimus*	4
Scutylenchus	2	*Pristionchus*	1	*Filenchus*	2	*Eudorylaimus*	4
Criconema	3	*Rhabditella*	1	*Nothotylenchus*	2	*Lamydorus*	4
Helicotylenchus	3	*Rhabditis*	1	*Paraphelenchus*	2	*Mesodorylaimus*	4
Heterodera	3	*Acrobeles*	2			*Microdorylaimus*	4
Hirschmaniella	3	*Acrobeloides*	2	**Predators**		*Prodorylaimus*	4
Hoplolaimus	3	*Anaplectus*	2	*Discolaimus*	3		
Loofia	3	*Cephalobus*	2	*Tripyla*	3		
Meloidogyne	3	*Cervidelus*	2	*Tripylina*	3		
Pratylenchus	3	*Chiloplacus*	2	*Ironus*	4		
Tylenchorhynchus	3	*Eucephalobus*	2	*Miconchus*	4		
Trichodorus	4	*Heterocephalobus*	2	*Mononchus*	4		
Axonchium	5	*Plectus*	2	*Mylonchus*	4		
Dorylaimellus	5	*Alaimus*	4	*Nygolaimus*	5		
		Bathyodonthus	4				

*Trophic groups were classified according to Okada and Kadota (2003) and Yeates et al., (1993).

**cp categories were based on Bongers (1990) and Bongers et al., (1995).

A list of nematode voucher specimen prepared on slides and available in the Agricultural Nematology Laboratory of the Department of Horticulture (**Appendix G2**).

Table 3.4 | Mean numbers of herbivore (HV), bacterivore (BV), fungivore (FV), predator (PR) and omnivore (OV) nematodes, sum of all trophic groups (SUM), sum of free-living nematodes (SFL), sum of free-living nematodes from cp 2 to cp 5 (SFL25), and sum of cp 2 to cp 5 nematodes (S25) per 100 cm^3 soil by region (RG: east, SW-southwestern and NW-northwestern of Michigan, USA), soil (ST: mineral and muck), and *Meloidogyne hapla* (Mh: present and absent) in agricultural fields (A), RG and ST in adjacent natural vegetation site (B), RG, ST and land use (C)(LU: agricultural and natural vegetations).

Factors			A) Agricultural fields*								
RG	**ST**	**Mh**	**HV**	**BV**	**FV**	**PR**	**OV**	**SUM**	**SFL**	**SFL25**	**S25**
East	-	-	4	10 ab	7	0	4	19	16	17 a	20 a
SW	-	-	2	17 a	4	0	4	20	20	18 a	18 ab
NW	-	-	2	6 b	5	0	2	12	11	11 b	12 b
East	Mineral	-	9 a	9 ab	10a	0	10	20 ab	14 ab	16 ab	11 b
	Muck	-	0 b	7 ab	4 ab	0	1	10 b	14 ab	17 ab	22 a
SW	Mineral	-	4 ab	19 a	8 ab	0	4	24 a	23 a	21 a	10 b
	Muck	-	0 b	9 ab	0 b	0	2	11 ab	11 ab	10 b	17 ab
NW	Mineral	-	1 ab	5 b	6 ab	0	4	11 b	10 b	10 b	24 a
	Muck	-	6 ab	10 ab	6 ab	0	1	17 ab	15 ab	13 ab	11 b
East	-	Present	11a	14	9	0	5	28 a	21 a	18 a	26 a
	-	Absent	1 b	4	4	0	3	6 b	9 ab	15 ab	10 cd
SW	-	Present	2 ab	14	3	0	4	17 ab	16 ab	15 ab	16 bc
	-	Absent	0 b	14	2	0	1	17 ab	16 ab	16 ab	16 bcd
NW	-	Present	1 b	4	8	0	6	10 b	9 b	9 b	9 d
	-	Absent	5 ab	11	5	0	1	19 a	17 ab	15 ab	18 ab
RG x ST x Mh (*P* values)			0.08	0.118	0.391	0.860	0.245	0.062	0.109	0.049**	0.009**

Table 3.4 (cont'd)

			B) Adjacent natural vegetation*								
RG	**ST**	**Mh**	**HV**	**BV**	**FV**	**PR**	**OV**	**SUM**	**SFL**	**SFL25**	**S25**
-	Mineral	-	6 b	8 b	3 b	0	3	15 b	12 b	11	13 b
-	Muck	-	12 a	12 a	6 a	0	7	24 a	19 a	17	22 a
East	Mineral	-	7 b	7 c	2 b	0 b	3 b	13 c	10 d	10 c	12 c
	Muck	-	13 a	12 ab	4 b	1a	5 b	24 b	18 b	15 b	22 b
SW	Mineral	-	6 b	9 bc	4 b	0 b	5 b	17 c	15 bc	15 b	18 b
	Muck	-	8 ab	7 bc	2 b	0 b	1 b	13 c	9 d	7 c	10 c
NW	Mineral	-	5 b	7 bc	3 b	0 b	2 b	14 c	11 cd	9 c	10 c
	Muck	-	16 a	15 a	9 a	0 b	18 a	30 a	26 a	26 a	29 a

RG	**ST**	**LU**	**C) Agricultural and natural vegetation***								
-	-	Agricultural	2 b	10	5	0	3 b	16 b	15	14	16
-	-	Natural	9 a	9	4	0	5 a	18 a	14	13	16
RG x LU (*P* values)			0.7	0.001**	0.031**	0.549	<0.001**	0.001*	<0.001*	<0.001*	<0.001*
ST x LU (*P* values)			0.001**	0.037**	<0.001**	0.56	0.579	<0.001**	0.003**	<0.001**	<0.001**
LU x RG x ST (*P* values)			0.714	0.387	0.596	0.376	<0.001**	0.144	0.007**	<0.001**	0.006**

*Means followed by the same or no letters within a category of RG × ST, RG × Mh, and ST × Mh and column not statistically different at $p<0.05$.

**RG × ST × Mh, RG × LU, ST × LU and LU × RG × ST interaction effects are significant.

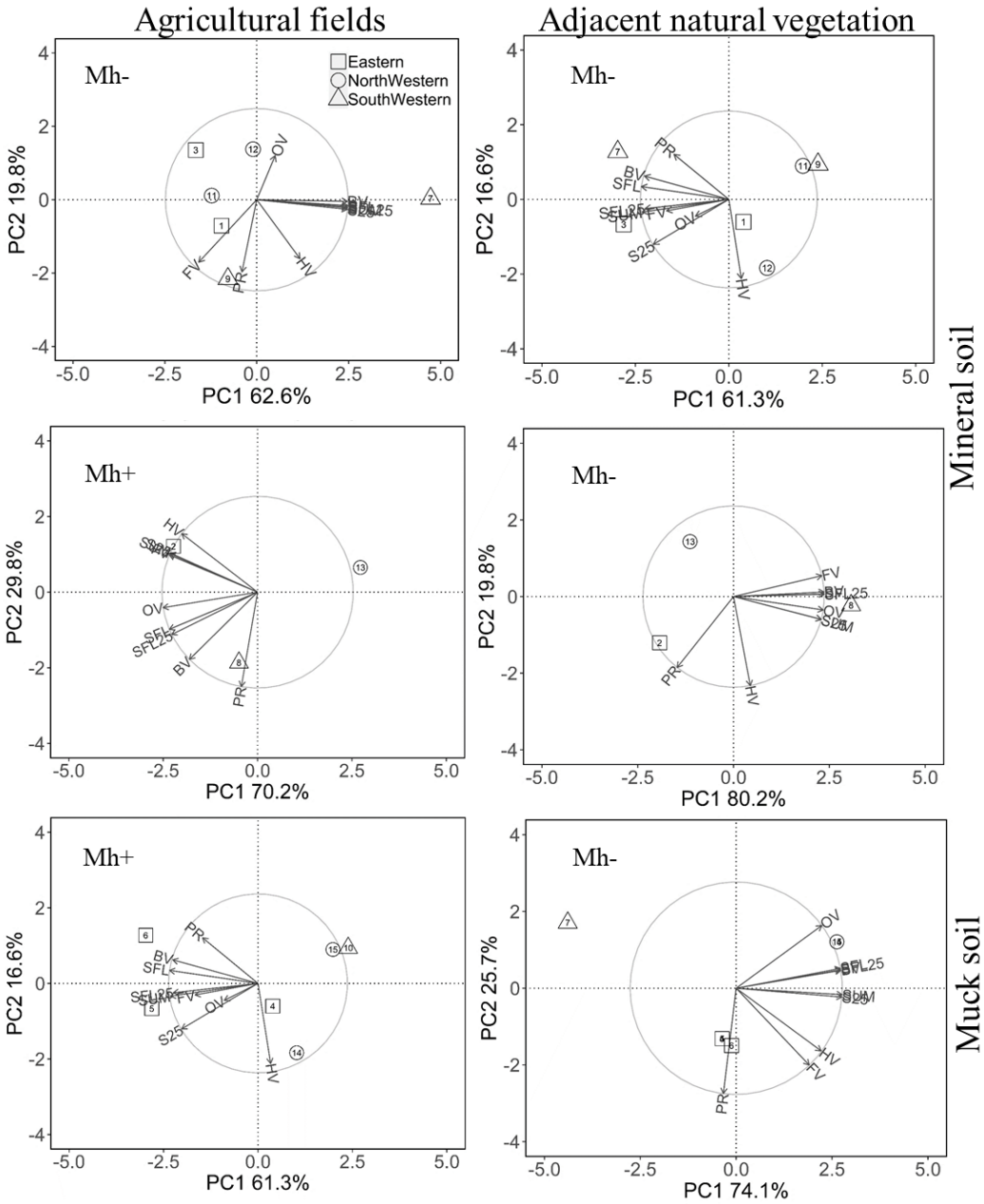

Figure 3.2 | Principal component analysis of the relationships among herbivore (HV), bacterivore (BV), fungivore (FV), predator (PR) and omnivore (OV) nematodes, sum of all trophic groups (SUM), sum of free living nematodes (SFL), sum of free living nematodes from cp 2 to cp 5 (SFL25), and sum of cp 2 to cp 5 nematodes (S25) the 15 sampling sites (agricultural fields and corresponding natural vegetation), *M. hapla* (presence, Mh+ or absence, Mh-), soils (mineral and muck), and regions (eastern-square, southwestern-triangle, and northwestern-circle).

Discussion

There is little published information about the presence or absence of *M. hapla* and soil health conditions as defined by nematode community structure. By broadly describing the relationship between *M. hapla* presence or absence and soil health conditions across selected regions and soils, this study advances our understanding of *M. hapla* distribution in Michigan vegetable production fields. Host suitability at the time of sampling had no relationship with presence or absence of *M. hapla*. Presence of *M. hapla* in all three regions and all of the agricultural fields with muck soil, but only in 3 of the 9 fields with mineral soils, suggests that its distribution is broad and variable (Melakeberhan et al., 2007; Melakeberhan and Wang, 2012; Melakeberhan et al., 2013).

Our first hypothesis was that *M. hapla* is present in degraded (Quadrant D) soil health conditions seems to be partially supported in mineral soils, but is inconclusive in muck soils. Based on the relationship between the enrichment index and structure index trajectories, only two agricultural fields with mineral soil in the northwestern region had what is considered maturing (Quadrant B) or best conditions for nutrient cycling. The rest of the agricultural fields in both soils had either disturbed (Quadrant A) depleted and/or degraded (Quadrant D) soil health conditions, suggesting that the soils have less than ideal conditions for nutrient cycling and overall soil health. The presence of *M. hapla* in disturbed (Quadrant A) and/or depleted and degraded (Quadrant D) and its absence in maturing (Quadrant B) soil health conditions in mineral appear partially to support the hypothesis. However, the absence of muck soils with maturing (Quadrant B) soil health conditions and *M. hapla* presence in degraded (Quadrant D) conditions makes the hypothesis less conclusive for muck soil.

A comparison of soil health conditions between the natural vegetation and agricultural fields was designed to assess how far the latter has been degraded and what adjustments may be considered if clear trends developed between soil health and presence or absence of *M.*

hapla. In this case, the natural vegetation soils were as stressed as the agricultural fields, but the variability of nematode abundance across sites was less than that of the agricultural fields. This suggests that the natural vegetation may have had some disturbance in the past (Neher, 2010).

Among other things, changes in either enrichment index or structure index have been used to identify soil conditions in relation to the ecosystem function (Wang and McSorley, 2005). In this study, how enrichment index and structure index varied by region, soil and *M. hapla* interactions seem to indicate differences. Without considering presence or absence of *M. hapla*, differences in structure index were observed by soil and region interaction. When considering presence or absence of *M. hapla*, differences in structure index by soil and region were observed. These results suggest that structure index as an indicator of soil conditions is likely to be location specific.

Our second hypothesis was that the presence of *M. hapla* is associated with low trophic group abundance. The expected outcomes were that there would consistently be a significantly ($p<0.05$) low trophic group abundance associated with *M. hapla* presence than in soils without *M. hapla*. Based on the numerical relationships of abundance parameters, this hypothesis was rejected. Abundance of herbivores, bacterivores, fungivores, omnivores, and sum of all trophic groups, sum of free-living nematodes and of their cp 2 to cp 5, and sum of cp 2 to cp 5 of all nematodes trophic groups were low and highly variable across the 15 agricultural fields. When the abundance parameters were compared by combinations of region, soil and presence or absence of *M. hapla* in the agricultural fields, variable patterns emerged for herbivores, bacterivores, fungivores, sum of all trophic groups, sum of free-living nematodes, sum of free-living from cp 2 to cp 5 and sum of cp 2 to 5 nematodes. As other authors have reported on other nematodes, variable abundance by trophic or across

trophic and cp groups is to be expected (Gavassoni et al., 2007; Donald et al., 2009; Cheng et al., 2018).

The Michigan vegetable production landscape where *M. hapla* exists has diverse soil conditions. A principal component analysis of the relationship among nematode abundance parameters, region, soil and presence or absence of *M. hapla* revealed distinct patterns explaining over 75% of the variations. The patterns of correlations in both muck and mineral soils where *M. hapla* was present were similar in the agricultural fields. While this suggests that similar changes may be taking place in both soils, what the exact changes are or how these patterns relate to *M. hapla* PV in each of the soils is yet to be determined. The opposite patterns of correlations between the adjacent natural vegetation and the *M. hapla* positive agricultural fields suggest differences between land use practices. The patterns of correlations in the *M. hapla* negative mineral soil had no clear trend and the adjacent natural vegetation soil had an opposite trend compared with those adjacent to *M. hapla* positive fields. Whether or not *M. hapla* absence in these fields is a matter of not being introduced, or detected, or the soil conditions influenced its presence remains to be determined.

Conclusions

In summary, this study advances our understanding of the broad distribution of *M. hapla* across Michigan vegetable growing regions. Its presence relative to abundance of nematode trophic groups does not seem to have a clear trend. The soil conditions associated with *M. hapla* presence in the sampled sites were mostly disturbed and/or degraded, partially supporting the working hypothesis for mineral soil, but not for muck soil. The distinct multifactor trophic group abundance patterns by soil, land use and/or *M. hapla* presence or absence lays down a foundation for more targeted investigations to understand any links between its PV and ecosystem to microbiome level changes in its soil environment.

CHAPTER 4

PARASITIC VARIABILITY OF *MELOIDOGYNE HAPLA* RELATIVE TO SOIL GROUPS AND SOIL HEALTH CONDITIONS

Abstract

While *Meloidogyne hapla* populations in the Michigan vegetable production landscape exist in degraded and disturbed soil health conditions of mineral and muck soils, the parasitic variability (PV) of these populations are not known. In this study, nine *M. hapla* populations from muck and mineral soils with degraded and disturbed SFW conditions from three regions were used to test the hypothesis that PV varies among the studied populations. In an experiment replicated three times, the populations were inoculated at 2000 and 4000 eggs/300 cm^3 of soil. Two populations,13 and 8, from degraded mineral soils had significantly higher reproductive potential from the rest of the populations in both SFW conditions but the root galls were variable and inconsistent. The *M. hapla* populations' reproductive potential varied by the interactions of SFW condition, soil group and region, indicating that the conditions where PV exists are likely to be variable within or across soil groups.

Introduction

Parasitic variability (PV) of plant parasitic nematodes (PPN) is considered one of the main problems associated with pest management in agriculture. It involves the variability in nematode virulence and pathogenicity, where PPN look the same but act different. Depending on the nematode species, factors linked with PPN PV include genetic variation (Plowright et al., 2013) and virulence phenotype (Mitchum et al., 2007) resulting in quantitative and/or qualitative crop yield losses (Kirkpatrick and Sasser, 1983; Bucki et al., 2017; Chen, 2020). In the case of *Meloidogyne hapla*, populations show no detectable morphological or genetic differences (Liu and Williamson, 2006) but differ in inducing galling and reproductive potential (Melakeberhan et al., 2007; Melakeberhan and Wang, 2013). Here, reproductive potential, an indicator of PV, refers to the total numbers of nematodes recovered after a predetermined period following inoculation on a susceptible host.

It has been established that the distribution of *M. hapla* spans multiple soil groups and landscapes receiving agricultural practices and inputs such fertilizers, insecticides, herbicides and nematicides multiple times per year (Melakeberhan et al., 2007) and that populations from mineral soils appear to have higher reproductive potential than those from muck soils (Melakeberhan et al., 2010). A combination of agricultural inputs and practices alter soil physicochemistry and may have direct and/or indirect effects on the resident organisms (Babin et al., 2019; Liu et al., 2019; Wendeborn, 2020; Xia et al., 2019). How *M. hapla* populations' PV relate to the soil conditions from where they came remains unknown.

As part of understanding the mechanisms of *M. hapla* PV in vegetable production, Lartey et al., (2021) investigated the relationship between *M. hapla* and the soil food web (SFW, Ferris et al., 2001) as an indicator of soil health conditions in 6 muck soil and 9 mineral soil fields in 3 regions of the lower peninsula of Michigan. The SFW model uses the

relationship between the structure index (SI, x-axis) and enrichment index (EI, y-axis) to categorize the agroecosystem fitness and nutrient cycling potential of the soil conditions as disturbed (Quadrant A), maturing (Quadrant B, best case), matured (Quadrant C) and degraded (Quadrant D, worse case) (Chapter 1, **Figure 1.2**). While measuring changes in either EI or SI and agronomic parameters provides linear relationship (Melakeberhan et al., 2021b), the quadrants that the SFW model generates using the intersection of EI and SI makes it possible to describe the soil conditions where an organism exists. The study revealed three points: first, *M. hapla* was present in all regions and muck soils and 3 mineral soils, confirming earlier studies (Melakeberhan et al., 2007; Melakeberhan and Wang, 2013). Second, principal component analysis of nematode community abundance in the *M. hapla* positive fields showed different patterns of correlations in muck and mineral soils. This suggested that the soil conditions that affect *M. hapla* (and other nematodes) are likely to have biological differences. Third, the SFW conditions of the fields where *M. hapla* was found were described as degraded (nutrient depleted and less structured) and disturbed (nutrient enriched and less structured). How the degraded and disturbed SFW conditions relate to *M. Hapla* populations' PV within or between soil groups is unknown.

The objective of the study reported was to determine if the *M. hapla* populations found in muck and mineral soils with disturbed and degraded SFW conditions of muck and mineral soils exhibit PV. The working hypothesis was that PV in *M. hapla* populations is related to specific SFW conditions. If true, *M. hapla* populations from degraded and disturbed SFW conditions should exhibit distinct reproductive potential in both soil groups. If the hypothesis is false, *M. hapla* populations from either degraded and/or disturbed SFW conditions should exhibit PV in one or none of the soil groups. This would mean that the conditions where PV exists are variable. The outcome of this research provides a foundation

for understanding the specific mechanisms of the interactions driving PV in *M. hapla* populations in Michigan vegetable production landscapes.

Materials and methods

Soils, SFW conditions and regions where *M. hapla* populations came from.

The *M. hapla* populations used in this study represent 9 of the 15 georeferenced fields and described in a previous study (Lartey et al., 2021). As used here the population numbers correspond to field numbers. Three of the populations (13, 8 and 2) were in mineral and six (4, 5, 6, 10, 14 and 15) were in muck soil groups. Populations 13, 8, 5, 14 and 15 came from degraded and populations 2, 4, 6 and 10 came from disturbed SFW conditions (Ferris et al., 2001). Populations 13, 14 and 15 were from the northwestern, 8 and 10 from the southwestern, and 2, 4, 5 and 6 from the eastern vegetable production regions of the lower peninsula of Michigan.

Experimental design, set up and measurements

A greenhouse experiment was conducted to test similarities and/or differences of the 9 *M. hapla* populations' reproductive potential and inducing galls on Rutgers tomato under the same greenhouse set up as the cultures. Each *M. hapla* population was inoculated at 2000 (low) and 4000 (high) eggs in 300 cm^3 of soil per pot. The inocula were designed to test if the level of inoculation makes a difference in expressing PV. Each inoculum treatment was replicated 6 times. Six water-treated plants served as controls (check). The experiment had a total of 114 experimental units (9 *M. hapla* populations x 2 inocula x 6 replicates + 1 control [6 replicates]). The experiment was repeated three times. Each experimental run was terminated at 30 days after inoculation, enough time to complete one generation (Inserra et

al., 1983; Melakeberhan et al., 2007). Experiments 1, 2 and 3 were conducted during November (2019), January (2020) and May (2020) months and accumulated 398, 412 and 667 degree-days (DD, base 10 ^{o}C), respectively.

Egg inocula were obtained from the individual *M. hapla* population root cultures (Chapter 2; **Figure 4.1**) using a modified protocol of Hussey and Boerma (1981). Briefly, roots were gently separated from the soil, gently washed under flowing tap water to prevent egg mass loss, cut into small pieces (~1 cm long), placed in 5% bleach solution, and shaken vigorously for 5 minutes in a flask. The eggs were sieved and cleaned as described in Hussey and Boerma (1981) and numbers estimated (Zuckerman, 1985). As part of accounting for any differences in nematode developmental stages in roots, stages of embryogenesis of each of the 9 *M. hapla* populations were determined by taking 1 ml aliquots and classifying the first eggs into undifferentiated (single-cell to 8-cell embryo) or differentiated (elongating to a 3-fold embryo) as illustrated by Calderón-Urrea et al., (2016). Three 1 ml aliquots were taken for each *M. hapla* population in each experiment, and the average number of differentiated and undifferentiated eggs estimated using an inverted digital microscope at 400× magnification (Accu-scope®). The proportion of undifferentiated and differentiated egg inoculum was similar across experiments ($P<0.05$). However, the proportion of undifferentiated (67.4% -76.7%) were significantly more than the differentiated (26.4% - 32.8%) stages.

At the end of each experiment, roots were gently separated from the soil and cleaned under running tap water, assessed for galling from 0 (no galling) to 5 (all roots severely galled, plant dying, or dead) using a modified Bridge and Page, (1980) scale. A 2 g sub-sample was stained and stored at 4 ^{o}C until nematodes were counted (Melakeberhan et al., 2004). Nematode developmental stages in roots were determined as second stage juvenile

(J2), third and fourth stage juvenile (J3/J4), and adults (Melakeberhan et al., 2004; Preston et al., 2003).

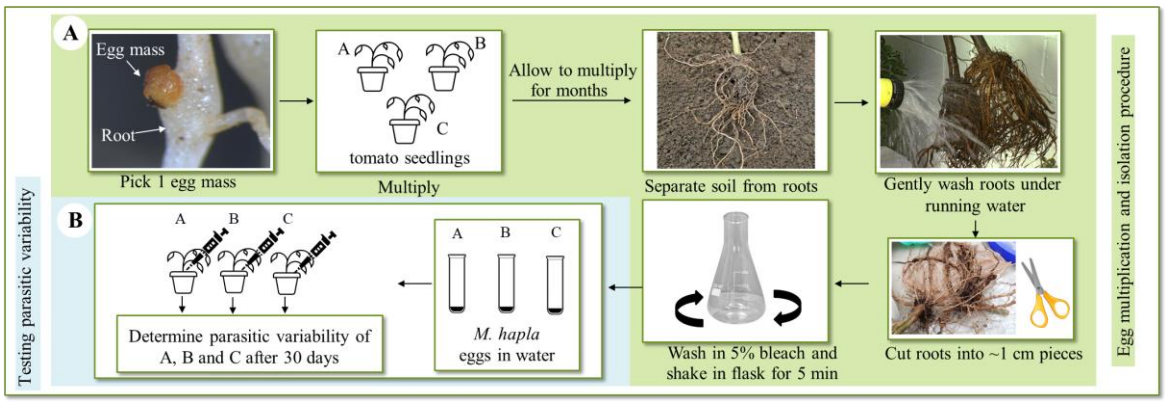

Figure 4.1 | Diagram outlining the procedures for *M. hapla* egg multiplication and isolation (A) and parasitic variability tests (B). The egg multiplication and isolation procedure had a single egg mass inoculated in soil with tomato seedlings and allowed to multiply for several months, roots were separated from soil, gently washed under running water, cut into ~1 cm pieces, washed in 5% bleach, shaken for 5 minutes and rinsed eggs in water. The parasitic variability test had an estimated number of eggs in tubes inoculated on 2-week-old tomato seedlings for a 30 day experiment.

Data analysis

Differences of nematode developmental stages (J2, J3/J4 and adult) and PV indicators (galling, total infection, and percent infection) of the 9 *M. hapla* populations (MH) within different inoculum (IN) levels (2000 and 4000 eggs) were analyzed using mixed model approach in PROC GLIMMIX (SAS-Institute, 2018). The statistical model consisted of fixed effects of MH and IN and the interaction between them, and random effects of experiment runs. If, after residual diagnostics, the assumptions of homogeneity of variances and normality of the residues were found to be violated, the log transformed (Log(x) +1) values of developmental stages and PV indicators were used. When either the MH by IN interaction

or simple effect (slicing) tests by MH and IN levels were found to be statistically significant, then multiple comparisons among the means of the nematode developmental stages and PV indicators for MH within each IN level was presented. A test of the high and low inoculum effect on each MH was conducted using t-tests ($p<0.05$).

To determine the effect of regions (RG), soil groups (SG), soil food web (SFW) conditions and IN on the *M. hapla* developmental stages and PV indicators, the statistical models were implemented in PROC GLIMMIX (SAS-Institute, 2018). Two separate three-way models were fitted. The first model included the main effects of SG, IN, and SFW and their interactions. Experiments were included in the model as a random effect. MH was also treated as a random effect nested within respective SFW and SG, thus it was used as an error term for testing the SFW and SG effects. The pots assigned to individual IN levels were also included in the model as random effects and used as an error term for testing the effects of IN and its interactions with SFW and SG. The second model included the main effects of SG, IN, and RG and their interactions, with experiments, MH nested in SG and RG, and the pots nested within IN, MH, SG and RG as random effects. The latter two terms were used for testing the SG and RG effects, and IN and its interactions with SG and RG effects, respectively.

Results

Meloidogyne hapla developmental stages in roots

The numbers of *M. hapla* J2, J3/J4 and adult stages found in the roots differed significantly ($p<0.05$) by population and inoculum, and the J3/J4 and adult stages by the interaction of population and inoculum (**Table 4.1**). Across populations, fewer J2s than J3/J4 and adults per 2 g of root were recovered. The numbers of J2s ranged from 2.8 to 5.7 in the low inoculum compared with 11.7 to 16.8 in the high inoculum. In the low inoculum, the numbers of J3/J4 were between 17.9 to 124.5 and 30.2 to 255.3 and those of the adult stages were between 10.4 to 98.4 and 37.8 to 230.1 in the low and in the high inoculum, respectively. In the high inoculum, the J3/J4s were between 30.2 to 255.3 and the adult nematodes between 37.8 to 230.1 (**Table 4.1**). All nematode developmental stages were significantly ($p<0.05$) higher in the high than in the low inoculum.

Within inoculum and across populations, the number of J2s recovered in roots of Population 6, 8, 13 and 15 of the high inoculum were significantly higher than those of Populations 5 and 10; whereas only those of Population 2 were significantly higher than those of Population 14 in the low inoculum (**Table 4.1**). The number of J3/J4s and adults recovered in roots of Population 13 in both inocula were significantly more than those of Population 8 and both populations were significantly more than the rest of the populations (**Table 4.1**).

Table 4.1 | Mean number of second stage (J2), third and fourth stage (J3/J4) juveniles and adult nematode developmental stages recovered from the low (2000) and high (4000) egg inocula (IN) treatments across 9 *M. hapla* (MH) populations and the two-way interaction (p-values) of MH and IN.

		Nematode developmental stages/ 2g of roots* **											
		J2				**J3/J4**				**Adult**			
	MH	**2000**		**4000**		**2000**		**4000**		**2000**		**4000**	
	13	3.8	ab*	15.1	ab	125.0	a*	255.0	a	98.4	a*	230.0	a
	8	4.8	ab*	15.3	ab	82.4	b*	187.0	b	71.1	b*	125.0	b
	2	5.7	a*	14.3	abc	51.8	c*	83.8	c	40.0	c*	60.2	d
	4	5.3	ab*	13.6	bc	39.4	cd	49.0	efg	33.6	cd*	65.5	cd
	5	4.5	ab*	11.7	c	21.9	de	37.1	fg	36.7	c*	79.5	c
	6	3.5	ab*	16.8	a	35.6	cde*	73.8	cd	44.0	c*	80.5	c
	10	3.7	ab*	11.7	c	17.9	e	30.2	g	29.7	cd*	60.6	d
	14	2.8	b*	12.9	bc	33.6	cde*	68.2	cde	18.4	de*	37.8	e
	15	4.1	ab*	14.6	ab	29.4	de*	53.0	def	10.4	e*	53.2	de
P-values	MH	0.0392**				<0.0001**				<0.0001**			
	IN	<0.0001**				<0.0001**				<0.0001**			
	MH*IN	0.0547				<0.0001**				<0.0001**			

**M. hapla* population mean significantly ($p<0.05$) higher in the 4000 than the 2000 inocula for a developmental stage.

**P-value of two-way ANOVA is statistically different at $p<0.05$

*** The number of nematodes across populations in each inoculum group represented with different letters were significantly different ($p<0.05$)

The numbers of all developmental stages in roots within and/or between inocula varied by soil group and the region from where they came (**Table 4.2**). Similar numbers of J2s were recovered in both soil groups and three regions within an inoculum, but significantly differed between inocula ($p<0.05$). The number of J3/J4 and adult nematodes recovered in high inoculum from mineral soil was significantly higher than other soil and inoculum groups. The J3/J4 in the low inoculum from muck soil were lower than both inocula from mineral soil. Across regions, more J2s were recovered in the high than in the low inoculum. The numbers of J3/J4 and adults in the southwest and northwest were significantly more than in the high inoculum the east and low inoculum in all regions. The numbers of J3/J4 and adult stages were significantly affected by two- and three-way interactions (**Table 4.2**).

Table 4.2 | Mean number of second stage (J2), third and fourth stage (J3/J4) juveniles and adult nematode developmental stages of *M. hapla* populations recovered across regions (RG: East, Southwest-SW and Northwest-NW), soil groups (SG: Muck and Mineral), from inocula (IN: 2000 and 4000) and the three-way interaction (p-values) of RG, SG and IN.

Factors***			Nematode developmental stages/ 2g of roots*		
RG	**SG**	**IN**	**J2**	**J3/J4**	**Adult**
-	Muck	2000	4.6 b	36.2 c	36.8 b
-	Mineral		4.0 b	58.4 bc	47.0 b
-	Muck	4000	14.1 a	59.2 b	66.7 b
-	Mineral		13.9 a	120.2 a	105.1 a
E	-	2000	4.8 b	37.2 d	38.6 d
SW	-		4.3 b	50.2 cd	50.4 cd
NW	-		3.6 b	62.5 bc	42.4 cd
E	-	4000	14.1 a	60.9 c	71.4 c
SW	-		13.5 a	108.9 b	92.3 b
NW	-		14.2 a	125.5 a	107.0 a
P-values	SG		0.225	0.001**	0.001**
	IN		0.002**	0.002**	0.001**
	RG		0.756	0.015**	0.006**
	SG*IN		0.673	0.006**	0.019**
	SG*RG		0.746	0.016**	0.001**
	RG*IN		0.694	0.038**	0.021**
	SG*RG*IN		0.750	0.047**	0.016**

*Means followed by same letters across SG x IN or RG x IN are not statistically different at $p<0.05$.

**P-values of the three-way ANOVA are statistically different at $p<0.05$.

***Factors not considered for mean comparisons are replaced by a dash (-).

Across disturbed and degraded SFW conditions, more J2s were recovered in the high than in the low inoculum (**Table 4.3**). Significantly more J3/J4 and adult stages were recovered in the high inoculum from degraded soil conditions than from disturbed conditions and low inoculum from disturbed and degraded conditions (**Table 4.3**). Within each inoculum, the numbers of J3/J4 and adult stage nematodes in roots from degraded SFW conditions were more than in roots from the disturbed conditions ($p<0.05$). There were more

two- and three-ways interaction effects of soil group, soil food web and inocula on J3/J4 than adult stages (**Table 4.3**).

Table 4.3 | Mean number of second stage (J2), third and fourth stage (J3/J4) juveniles and adult nematode developmental stages of *M. hapla* populations recovered from soil health (SFW: disturbed and degraded) conditions and inocula (IN: 2000 and 4000) and the three-way interaction (p-values) of soil group (SG), SFW and IN.

	Factors		Nematode developmental stages/ 2g of roots*		
	SFW	IN	J2	J3/J4	Adult
	Disturbed	2000	8.8 b	40.8 d	45.8 c
	Degraded		9.8 b	130.9 b	104.1 b
	Disturbed	4000	13.5 a	51.9 c	62.8 b
	Degraded		14.9 a	175.5 a	138.4 a
P-values	SG		0.2036	0.0049**	0.0525
	IN		<.0001**	0.0003**	0.0053**
	SFW		0.5600	0.0291**	0.1126
	SG*IN		0.9110	0.0041**	0.2955
	SG*SFW		0.8168	0.0281**	0.0451**
	SFW*IN		0.6126	0.0079**	0.1107
	SG*SFW*IN		0.3972	0.0119**	0.1253

*Means followed by same letters across SFW x IN not statistically different at $p<0.05$.

**P-values of the three-way ANOVA are statistically different at $p<0.05$.

Effect on galling, total and percent infection

The severity of galls, total number of nematodes recovered from roots and percent infection differed by population, inocula and/or the interactions of population and inocula (**Table 4.4**). The lowest (0.9) and highest (1.8) galling in the low inoculum and 1.4 and 3.1 in the high inoculum were observed in roots inoculated with Populations 5, 13, 14 and 13, respectively. Galling was significantly more severe in the high inoculum than in the low inoculum for all populations, but Population 10. Within the low inoculum, Populations 4 and 13 caused

significantly more galling than the rest of the populations. Within the high inoculum, Populations 2, 8 and 13 caused the most and Population 14 the least galling (**Table 4.4**).

Total infection was significantly higher in the high inoculum than in the low inoculum for all populations. The total number of nematodes observed were between 42.7 (lowest) and 226.0 (highest) in the low inoculum, and between 96.9 and 495.6 in the high inoculum, and were found in roots inoculated with Populations 15, 13, 10 and 13, respectively. In both inocula, total infection was the highest in Population 13 followed by Population 8 and both populations were significantly different from the rest of the populations. In the low inoculum, Population 2 had significantly higher total infection than Populations 5, 14 and 15. In the high inoculum, Population 2 and 6 had significantly higher total infection than Populations 10, 14 and 15, and Population 6 from Populations 4 and 5 (**Table 4.4**).

Percent infection was higher in high than in the low inoculum of Population 13, but similar between the inocula across the *M. hapla* populations (**Table 4.4**). In both inocula, the percent infection was the highest in Population 13 followed by population 8 and both significantly different from the rest of the populations. Within the low inoculum, Population 2 had higher percent infection than Populations 5, 6, 10, 14 and 15, and Populations 4 and 6 from Populations 10, 14 and 15. Within the high inoculum, percent infection of Populations 6 was higher than Populations 10, 14 and 15, Population 2 than Population 10 (**Table 4.4**).

Table 4.4 | Means of galls, total and total (% Infection) associated with the low (2000) and high (4000) egg inocula (IN) treatments across 9 *M. hapla* (MH) populations and the two-way interaction (p-values) of MH and IN.

		Gall*				Nematodes in 2g of roots***							
						Total				Total (% Infection)			
	MH	2000		4000		2000		4000		2000		4000	
	13	1.8	a*	3.1	a	226.0	a*	495.6	a	11.3	a*	12.4	a
	8	1.0	b*	2.9	a	157.0	b*	322.2	b	7.9	b	8.1	b
	2	1.1	b*	2.9	a	96.0	c*	155.6	cd	4.8	c	3.9	cd
	4	1.7	a*	2.3	b	76.7	cde*	123.6	de	3.9	cd	3.1	cde
	5	0.9	b*	2.4	b	61.5	def*	123.5	de	3.1	de	3.1	cde
	6	1.1	b*	2.3	b	82.2	cd*	166.1	c	4.2	cd	4.2	c
	10	1.0	b*	2.4	b	50.2	ef*	96.9	e	2.6	e	2.5	e
	14	1.1	b	1.4	c	53.9	def*	113.8	e	2.7	e	2.9	de
	15	1.1	b*	2.3	b	42.7	f*	115.6	e	2.2	e	2.9	de
P-values	MH	<0.0001**				<0.0001**				<0.0001**			
	IN	<0.0001**				<0.0001**				0.5912			
	MH*IN	<0.0001**				<0.0001**				0.2189			

* *M. hapla* population mean significantly ($p<0.05$) higher in the 4000 than the 2000 inocula for a developmental stage.

**P-values of two-way ANOVA is statistically different at $p<0.05$

*** The number of nematodes across populations in each inoculum group represented with different letters were significantly different ($p<0.05$)

Galling was significantly higher in high than in the low inoculum for both soil groups and all regions, and not affected by two- or three-way interactions of soil group, region and inocula (**Table 4.5**). Total number of nematodes recovered in the high inoculum from mineral soil was significantly more than the high inoculum and low inoculum in both soils. Total infection in the low inoculum from muck soil was lower than from the high in muck soil. Total infection of the high inoculum in northwest had the highest and the low inoculum from the east region the lowest total infection. The high inoculum from southwest had higher infection than the high inoculum from the east region and low inoculum from all three regions. Total infection was significantly affected by two- and three-way interactions soil group, region and inocula. Percent infection was higher in the high inoculum in mineral soil than in muck soil. The high inoculum in the northwest had the highest percent infection in mineral and the lowest in muck soil. The high inoculum in the northwest and southwest had higher percent infection than those from the east region and all regions at the low inoculum (**Table 4.5**).

Table 4.5 | Means of galls, total and total (% Infection) associated with *M. hapla* populations recovered across regions (RG: East, Southwest-SW and Northwest-NW), soil groups (SG: Muck and Mineral), from inocula (IN: 2000 and 4000) and the three-way interaction (p-values) of RG, SG and IN.

Factors***			Gall*	Nematodes in 2g of roots*	
RG	**SG**	**IN**		**Total**	**Total (% Infection)**
-	Muck	2000	1.2 b	77.6 c	3.9 ab
-	Mineral		1.2 b	109.4 bc	5.5 ab
-	Muck	4000	2.5 a	140.0 b	3.5 b
-	Mineral		2.4 a	239.2 a	6.0 a
E	-	2000	1.2 b	80.5 d	4.0 cd
SW	-		1.0 b	104.8 cd	5.2 cd
NW	-		1.4 b	108.5 c	5.4 ab
E	-	4000	2.5 a	146.4 c	7.2 d
SW	-		2.6 a	215.0 b	10.8 bc
NW	-		2.3 a	246.7 a	12.4 a
P-values	SG		0.080**	0.001**	0.001**
	IN		0.005**	0.001**	0.572
	RG		0.812	0.006**	0.007**
	SG*IN		0.183	0.003**	0.883
	SG*RG		0.315	0.003**	0.003**
	RG*IN		0.486	0.010**	0.129
	SG*RG*IN		0.970	0.010**	0.426

*Means followed by same letters in RG x IN or SG x IN not statistically different at $p<0.05$.

**P-values of the three-way ANOVA are statistically different at $p<0.05$.

***Factors not considered for mean comparisons are replaced by a dash (-).

Galling was significantly higher in the high than in the low inoculum in both SFW groups and not affected by two- or three-way interactions of soil group, soil food web and inocula (**Table 4.6**). Total infection significantly differed by soil conditions and inocula as well as the two- and three-way interaction of soil group, soil conditions, and inocula. The high inoculum from degraded soil had the highest and the low inoculum from disturbed the lowest infection. Percent infection was higher in the high inoculum than in the low inoculum

in degraded and disturbed soil conditions. The effect of soil groups, and the interactions of soil groups and soil conditions and inoculum on percent infection was significant (**Table 4.6**).

Table 4.6 | Means of gall, total and % Infection associated with *M. hapla* populations recovered from soil health (SFW: disturbed and degraded) conditions and inocula (IN: 2000 and 4000) and the three-way interaction (p-values) of soil group (SG), SFW and IN.

	Factors		Gall*	Nematodes in 2g of roots*	
	SFW	IN		Total	% Infection
	Disturbed	2000	1.7 B	92.2 D	4.6 B
	Degraded		2.1 B	242 B	12.1 B
	Disturbed	4000	2.2 A	128.3 C	6.4 A
	Degraded		3.0 A	328.7 A	16.4 A
P-values	SG		0.0635	0.0110**	0.0095**
	IN		0.0007**	0.0006**	0.9260
	SFW		0.8528	0.0462**	0.0531
	SG*IN		0.1090	0.0365**	0.6818
	SG*SFW		0.3057	0.0270**	0.0269**
	SFW*IN		0.6564	0.0259**	0.0447**
	SG*SFW*IN		0.8156	0.0331**	0.2488

*Means followed by same letters across SFW x IN not statistically different at $p<0.05$.

**P-values of the three-way ANOVA are statistically different at $p<0.05$.

Discussion

The nine *M. hapla* populations from muck and mineral soils with degraded and disturbed SFW conditions from three regions of the lower peninsula of Michigan overall separated into highest (Population 13), medium (Population 8) and lowest (Populations 2, 4, 5, 6, 10, 14 and 15) PV categories based on the reproductive potential. Populations 8 and 13 are from mineral soils and Populations 5, 14 and 15 are from muck soils with degraded SFW conditions. Population 2 from mineral soil and Populations 4, 6 and 10 from muck soils are from disturbed SFW conditions. Consequently, the hypothesis that PV in *M. hapla* populations was related to specific SFW conditions is not supported. The *M. hapla* populations' developmental stages recovered in roots, total, percent infection and galling varying by SFW condition, soil group and region where they came from alone and/or by their interactions support an alternative hypothesis that the conditions where PV exists are likely to be variable within or across soil groups.

The use of 2000 and 4000 eggs per 300 cm^3 soil inoculum levels was designed to see if the same PV trends exist across inocula. As expected and observed in previous studies (Park *et al*., 2005; Viaene & Abawi, 1996), galling, the relative proportion of nematode developmental stages in roots and total infection were higher when inoculated with 4000 eggs than with 2000 eggs. The similar trends in the PV indicators across the *M. hapla* populations within an inoculum level suggests that the same information may be deciphered from a single inoculum level (Mennan et al., 2006).

The higher proportion of undifferentiated than differentiated eggs of the inoculum cohort seems to be reflected in the proportion of J2s, J3/J4 and adults recovered in roots. More J3/J4s and adults than J2s were recovered across the *M. hapla* populations. Given the number of DD (between 398 and 667) accumulated during the studies, the J3/J4s and adults

are likely to be from the inoculum cohort (group of nematodes the penetrated roots around the same time) and the J2s an initiation of a second generation (East et al., 2019; Inserra et al., 1983; Vrain et al., 1978). Thus, any differences in reproductive potential among the populations are likely to be independent of the inoculum cohort.

Differences in reproductive potential among *M. hapla* populations have been previously reported (Melakeberhan and Wang, 2013; Stephan and Trudgill, 1982). Regardless of the differences in galling and in the proportion of the nematode developmental stages recovered in the roots within or across the *M. hapla* populations in this study, the total and percent infection revealed that Populations 13 and 8 had the highest reproductive potential. Why Populations 13 and 8 differed in reproductive potential between each and from the rest of the populations remains to be determined.

The higher reproductive potential of nematode populations originating from mineral compared to muck soil in this study are consistent with earlier reports (Melakeberhan et al., 2007; Norton, 1978). What is significant and new here is that the SFW conditions from where Populations 13 and 8 originated degraded (Ferris *et al.*, 2001). However, populations in muck soils with the same level of SFW degradation scale were among those with the lowest reproductive potential. If the degraded SFW condition is a contributing factor to differences in *M. hapla* populations' reproductive potential, this study suggests that the effect is not the same in mineral and muck soil groups. What factors in the degraded SFW conditions in mineral and muck soils are contributing to increase or decrease in *M. hapla* populations' reproductive potential is unknown.

The locations from where these *M hapla* populations came have highly variable crop production systems (Lartey et al., 2021), and are similar to studies that reported PV in other nematodes (Melakeberhan et al., 2010; Riggs and Schmitt, 1988; Stephan and Trudgill,

1982). It also is known that production systems alter the soil biophysicochemistry that may directly or indirectly influence nematodes (Babin et al., 2019; Liu et al., 2019; Mateille et al., 2020; Melakeberhan et al., 2007). In Field 15 for instance, the use vydate as a plant parasitic nematode control (**Appendix B**) likely influenced characteristics of nematode populations sampled. The two- to four-way interaction effects of inoculum, soils group, SFW conditions, and region on PV indicators in this study suggest that the factors contributing to *M. hapla* populations' reproduction potential are likely to be many and without a clear trend in both soil groups.

Conclusion

In conclusion, this study for the first-time establishes connections between *M. hapla* populations' reproductive potential and SFW conditions. This, in turn, lays down a foundation for exploring the contributing biophysicochemical factors to parasitic variability.

CHAPTER 5

CHARACTERIZING MICROBIAL COMMUNITIES ASSOCIATED WITH THE NORTHERN ROOT-KNOT NEMATODE (*MELOIDOGYNE HAPLA*) OCCURRENCE AND SOIL HEALTH

Abstract

While PV of *Meloidogyne hapla* populations from Michigan vegetable production fields seem to have a relationship with the soil health conditions they exist in, it is not known how *M. hapla* occurrence relates to microbial communities. In order to improve understanding of soil biophysicochemical conditions of the environment where *M. hapla* populations exhibited PV, this study characterized the soil microbial community structure associated with *M. hapla* occurrence and soil health conditions in 15 Michigan mineral and muck vegetable production fields, and identified core microbiome and indicator species related to PV. Soils were collected, their nematode communities were characterized and assessed for PV, and high throughput sequencing of 16S and Internal Transcribed Spacer (ITS) rDNA was used to characterize bacterial and fungal communities in soil samples. Results showed that, *M. hapla*-infested, as well as disturbed and degraded muck fields, had lower bacterial diversity (observed richness and Shannon) than corresponding mineral soil fields or non-infested mineral fields. Bacterial and fungal community abundance varied by soil group, soil health conditions and/or *M. hapla* occurrence. A core of 39 bacteria and 44 fungi OTUs occurred *M. hapla* infested and non-infested fields. An indicator of 25 bacteria OTUs were associated with *M. hapla* presence or absence. Collectively, the results provide a foundation for in-depth understanding of the environment where *M. hapla* exists and conditions associated with PV.

Introduction

The occurrence of *Meloidogyne hapla* populations with parasitic variability (PV) across Michigan vegetable production fields are associated with different soil health conditions (Lartey et al., 2021; Chapter 4). Moreover, these populations show no genetic or morphological variation (Liu and Williamson, 2006; Opperman et al., 2008), but differ in how they elicit a plant host reaction such as galling and reproductive potential (Melakeberhan et al., 2007; Melakeberhan and Wang, 2013; Chapter 4). The soils where *M. hapla* occurs range from sandy and low in organic matter to muck soils with high organic matter, and it has been established that populations from mineral soils have higher parasitism than those from muck soils (Melakeberhan et al., 2010). However, the mechanisms by which *M. hapla* PV relates to soil type remains unknown.

In order to understand how *M. hapla* PV relates to soil types in the landscapes, it is important to consider soil health and the soil environment in relation to the biology of *M. hapla*. Soil health, defined as a given soil's ability to function and deliver desired ecosystem services, has biological, physicochemical, nutritional, structural and hydrological integrity components (Lal, 2011). Intensive cultivations and agricultural inputs in the landscapes where *M. hapla* exists have resulted in the varying degrees of degradation of the soil health conditions (Lartey et al., 2021).

Soil is a dynamic environment and it is reasonable to assume that an organism that exists therein has to adapt to soil health conditions (McSorley, 2003; Melakeberhan et al., 2004). Within this context, it is worth considering the biology of *M. hapla*, which has an egg, 2nd, 3rd and 4th stage juveniles and adult stage (East et al., 2019). Eggs are laid in a gelatinous matrix completely inside a root or exposed to the soil. The 2nd stage juvenile, infective stage, hatches from the egg, migrates through the soil, pierces the root with its stylet, establishes a feeding site and draws nutrient from the host. The 3rd and 4th stages are completely inside the

root. The 2nd stage juvenile has the most exposure to all of elements in the soil environment. Hence, quantifying soil health is necessary in order to determine if any relationship between *M. hapla* presence and/or PV and soil conditions exist.

Recently, Lartey et al. (2021) used the beneficial nematode community analysis-based Ferris et al. (2001) soil food web (SFW) model to map out *M. hapla* distribution in 15 mineral and muck fields in three vegetable production regions of Michigan. The SFW model uses the relationship between changes in nematode population dynamics in response to resource and reproductive potential (Enrichment Index, EI) and resistance to disturbance (Structure Index, SI). The relationship between EI (x-axis) and SI (y-axis) categorizes soil conditions in terms of nutrient cycling potential and agroecosystem suitability in four quadrants from best to worst case scenarios. These are: enriched and unstructured (Quadrant A, disturbed), enriched and structured (Quadrant B, best case scenario), resource limited and structured (Quadrant C), or resource-limited and minimal structure (Quadrant D, degraded, worst case). Quadrants B are bacterial feeding nematode dominated and Quadrant D is biologically depleted and nutritionally degraded. *Meloidogyne hapla* was found in soil conditions fitting the disturbed (Quadrant A) and degraded (Quadrant D) in both soil groups (Lartey et al. (2021). In a follow up study, two of the *M. hapla* populations (8 and 13) from mineral and degraded soils had significantly higher reproductive potential than the rest of the populations (Chapter 4). While confirming earlier reports that *M. hapla* populations from mineral soils have higher reproductive potential than populations from muck soils (Melakeberhan et al., 2010; Melakeberhan and Wang, 2012), this study for the first time established a soil health component to *M. hapla* PV.

The field observation and experimental studies raises the overarching question: Could there be general and/or specific connections between *M. hapla* PV and the soil health and biophysicochemical components therein? The role of soil microbiome in regulating nutrient

cycling and soil health (Chaparro et al., 2012; Pajares and Bohannan, 2016; Saleem et al., 2019; Sanjuan et al., 2020) and nematode-microbiome antagonistic (Chen and Dickson, 1998; Topalović et al., 2020) and mutual (Cao et al., 2015; Colagiero et al., 2020; Tian et al., 2015; Yergaliyev et al., 2021) interactions are well documented. At this stage, it is difficult to tell what direct and/or indirect relationship, if any, may exist between the presence or absence of *M hapla* with the soil microbiome and soil conditions. However, it is worth noting the occurrence of *M. hapla* in disturbed and resource-enriched (Quadrant A) and degraded and resource-depleted (Quadrant D) in mineral and muck soils, and that populations with the highest reproductive potential were from mineral soil with degraded soil health conditions (Chapter 4). If there is any attributable relationship between soil health conditions and *M. hapla* PV, microbial community composition and/or structure will be a likely indicator.

Our objectives in this study were three-fold. First, we aimed to characterize the soil microbiome structure and diversity in mineral and muck soil fields with varying soil health conditions and presence or absence of *M. hapla*. We hypothesize that soil microbiome would differ significantly between soil groups and soil health conditions. Our second aim was to determine the core-microbiome associated with *M. hapla* occurrence across fields using the abundance-occupancy distributions as suggested by Shade and Stopnisek, (2019). Here, we define the core-microbiome as the most abundant and prevalent taxa shared across most fields. We hypothesize that the core-microbial members were present in at least most of the *M. hapla* infested and non-infested fields. Our third aim was to identify indicator-microbes associated with *M. hapla* occurrence and soil food web (SFW) conditions. Based on *M. hapla* occurrence or SFW conditions, indicator-microbes are distinguishable across the different soil health conditions. We hypothesize that there are microbial indicators associated with *M. hapla* occurrence or SFW conditions. Collectively, the outcomes of this research will help to establish foundations towards understanding the mechanism of *M. hapla* PV.

Materials and methods

Sample Sites with *Meloidogyne hapla* Occurrence

Samples were collected from 6 muck and 9 mineral soils in the eastern, southwestern and northwestern vegetable production areas in the lower peninsula of Michigan, USA**.** The distribution of sampled fields in the three regions, and their soil health conditions as described by the SFW model, soil groups, and presence or absence of *M. hapla* are graphically depicted in **Figure 5.1** (Lartey et al., 2021). Three of the muck soil fields are located in the east, 1 in the southwest and 2 northwest, and the 9 mineral soil fields were equally distributed across the 3 regions. Three muck and 1 mineral soil fields were characterized as having disturbed (Quadrant A), 3 muck and 6 mineral fields as degraded (Quadrant D), and 2 mineral fields matured (Quadrant B) SFW conditions. *Meloidogyne hapla* was present in all muck and 3 mineral soils (**Figure 5.1**; Lartey et al., 2021).

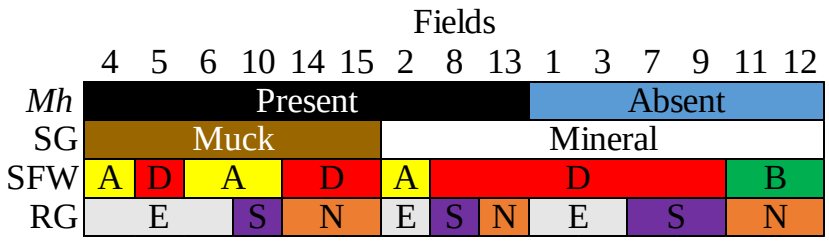

Figure 5.1 | Description of the 15 agricultural fields showing *M. hapla* occurrence (*Mh*: Present [black] and Absent [blue]), soil group (SG: Mineral [white] and Muck [brown]), soil food web conditions (SFW: D-Degraded [red], A-Disturbed [yellow], B-Maturing [green]), and regions (E-Eastern [grey], S-Southwestern [purple] and N-Northwestern [orange]).

Statistical Analyses

Data files containing OTU tables, taxonomy, mapping, and OTU sequences were loaded into the R (version 4.0.2) statistical environment (R Core Team, 2020) and used to create a phyloseq object for further analysis in the phyloseq package (McMurdie and Holmes, 2013). Sequences belonging to non-target organisms including Archaea, chloroplast and mitochondria were removed from 16S data prior to performing analysis (Zhang et al., 2019). OTUs determined to be contaminants in negative controls were removed with the decontam package (Davis et al., 2018). Alpha diversity (within sample diversity) was estimated for each sample following recommendations in McMurdie and Holmes, (2014). The alpha diversity was estimated using observed richness (Simpson, 1949) and Shannon diversity (Hill, 1973) within the BiodiversityR and vegan packages (Dixon, 2003; Kindt and Coe, 2005). OTU richness and Shannon diversity were visualized for each field with boxplots in ggplot2 (Wickham, 2016). Differences in alpha diversity means across fields were tested for statistical significance using Kruskal Wallis tests in the stats package (R Core Team, 2020). Afterward, a pairwise Wilcox test with an FDR (false discovery rate) p value correction was performed on fields according to *M. hapla* occurrence in soil groups and soil health conditions. Following alpha diversity analyses, OTUs with less than five reads in a single sample were placed to zero to account for tag switching and to account for PCR errors (Lindahl et al., 2013; Oliver et al., 2015). Stacked-barplots for bacterial communities were created in ggplot2 to show phyla and taxonomic classes with >2% relative abundance while classes with <2% abundance grouped as other (Wickham, 2016). Fungal stacked-barplots were created to show all phyla and families with >1.5% relative abundance while families with <1.5% abundance grouped as other. Next, data were normalized by cumulative sum scaling in the metagenomeseq package (Paulson et al., 2013). Following normalization, beta diversity was analyzed in the phyloseq and vegan packages by creating Principal Coordinate

Analysis (PCoA) plots with the "ordinate" and "plot_ordination" functions. Here, *M. hapla* occurrence relative to soil group and region community patterns were reported. An ellipse covering 70% of data points was drawn to show clusters of *M. hapla* occurrence in soil groups. Community patterns identified in PCoA plots were tested for statistical significance using PERMANOVA as implemented by the "adonis" function in vegan. Homogeneity of variance between modeled groups was analyzed with the "betadisper" function in vegan.

The most prevalent (core) microbes were identified following the "increase-method" described by Shade and Stopnisek (2019). Briefly, microbial OTUs were ranked by occupancy across fields, the proportion of total community explained by core subset taxa estimated using the Bray-Curtis method for beta-diversity, and core-taxa identified at 2% as the threshold for a marginal return in the explanatory value. The taxonomic genera of the identified core-taxa were assigned using NCBI nucleotide BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and then visualized using relative abundance stacked-plots.

Taxa closely associated with (a) *M. hapla* occurrence across the fields and (b) soil health conditions were determined with the indicspecies package (Cáceres and Legendre, 2009). Following identification of indicator OTUs, p-values were FDR adjusted, and only taxa with adjusted $p<0.05$ were considered to be indicators. The top 25 most abundant identified indicator taxa associated with *M. hapla* occurrence were used to create heatmaps displaying the relative abundance distributions across fields in the ComplexHeatmap package in R (Gu et al., 2016). The taxonomic genera of the top 25 indicators were assigned using NCBI nucleotide BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Indicators associated with soil health conditions were visualized with a Venn diagram. All R code and files for

producing figures and tables including metadata and OTU tables are available at: https://github.com/larteyis/Scientific-Papers-R-Code/tree/main/Lartey_et_al_2021_Field_M.hapla_Associated_Microbiome.

Results

To study bacteria and fungi communities associated with the northern root-knot nematode (*Meloidogyne hapla*), a total of 75 soil samples were collected for high-throughput amplicon sequencing. These included the 6 muck (4, 5, 6, 10, 14 and 15) and 3 mineral soils (2, 8 and 13) from fields infested with *M. hapla*, and 6 non-infested mineral soil fields (1, 3, 7, 9, 11 and 12) with either degraded, disturbed or maturing soil health conditions in three regions. Each of these 75 samples were analyzed for 16S, as well as for ITS rDNA fungal diversity. The total raw sequence reads obtained for 16S and ITS libraries were 3,443,432 and 1,283,828, respectively. The quality filtering procedure obtained 12,906 OTUs for 16S and 2,067 OTUs for ITS.

Bacterial community composition

Only 10 phyla had greater than 2% relative abundance. Phyla with less than 2% are presented as other (**Figure 5.2 A**). The detected phyla were present at varying proportions and occurring in all fields or varying by presence or absence of *M. hapla*, soil health conditions and/or region. Acidobacteria (9.3% to 18.3%), Actinobacteria (8.7% to 19.8%), Bacterioidetes (3.4% to 15.8%), Chloroflexi (2.9% to 9.8%), Planctomycetes, (2.2% to 6.0%), Proteobacteria (32.3% to 52.3%), and other (2.9% to 5.5%) were present in soils from all fields. Verrucomicrobia and Gemmatimonadetes were absent in disturbed SFW and *M. hapla*-infested muck Field 4 (east region) and non-infested mineral Field 7 (southwest

region), respectively. Nitrospirae was present only in *M. hapla*-infested and disturbed Field 4 (east) and degraded Fields 14 and 15 (northwest) muck soils. Firmicutes were present in *M. hapla*-infested and disturbed SFW muck Field 10 (southwest) and degraded mineral Fields 8 (southwest) and 13 (northwest), as well as in non-infested mineral Fields 1 and 3 (east) and 7 (southwest) with degraded and 11 and 12 (northwest) with maturing SFW conditions (**Figure 5.2 A**).

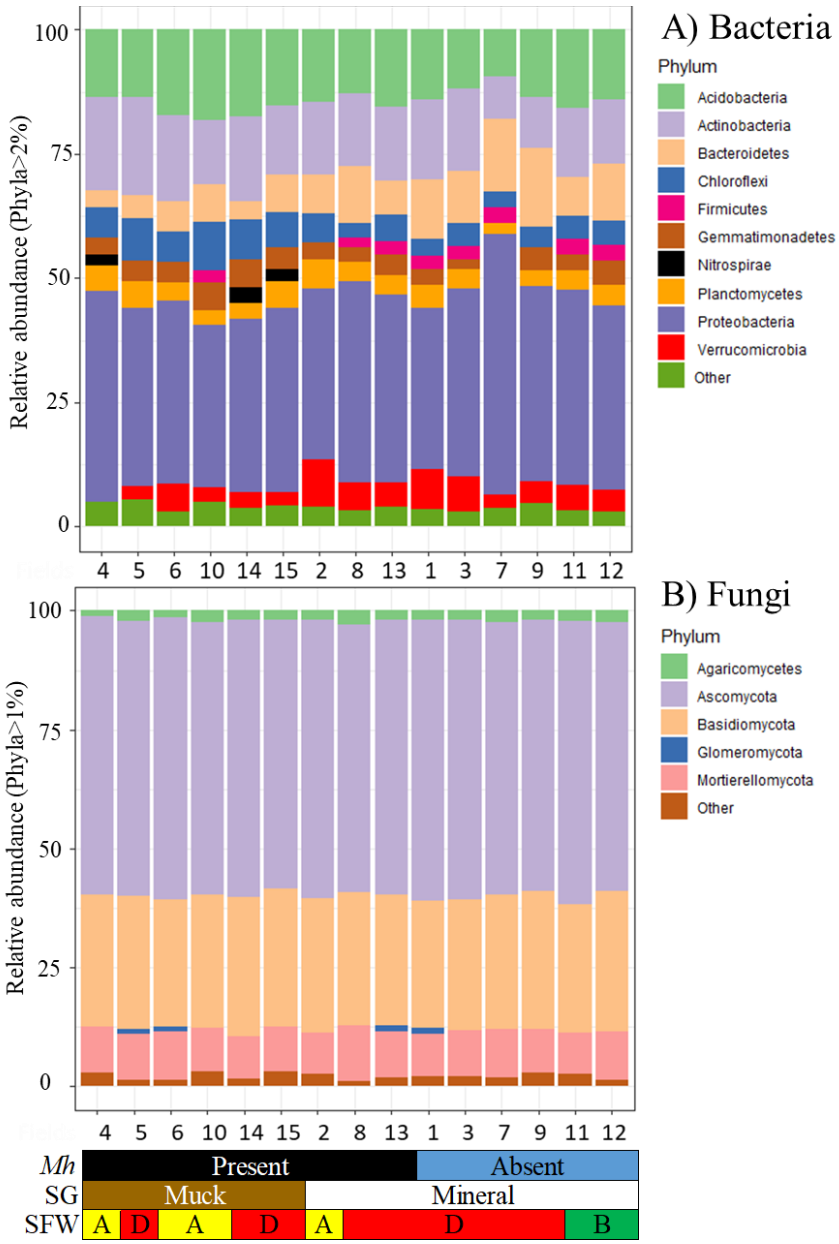

Figure 5.2 | Stacked bar plots of 15 agricultural fields by *M. hapla* occurrence (*Mh*: Present [black] and Absent [blue]), soil group (SG: Mineral [white] and Muck [brown]) and soil food web conditions (SFW: D-Degraded [red], A-Disturbed [yellow], B-Maturing [green]) of (A) bacteria phyla, and (B) fungi phyla. Colors of bacteria and fungi correspond with colors in the stacked bar plots and each bar represents a field. Other unassigned bacterial and fungal phyla were classified as other. Relative abundance of bacterial and fungal phyla variable across the 15 sampled fields.

Of the 27 classes of bacteria with more than 2% relative abundance, Alphaproteobacteria (12.7% to 21.7%), Acidobacteria-6 (2.5% to 8.6%), Actinobacteria (6.2% to 9.8%), Betaproteobacteria (5.0% to 8.0%), Deltaproteobacteria (4.3% to 11.4%), Gammaproteobacteria (3.9% to 14.3%) and those with less than 2% relative abundance (15.4% to 24.7%) occurred in all fields (**Figure 5.3 A**). The less prevalent classes had variable absence or presence mostly relative to soil group and *M. hapla* occurrence. These included absence of Saprospirae and Spartobacteria in one or more of the muck fields with varying soil health conditions, Chloracidobacteria in disturbed Field 6 (muck) and in Field 2 (mineral), and Thermoleophilia in degraded Field 7 (mineral). Presence of Acidimicrobiia, Anaerolineae, DA052, Ellin6529, Gemm-1, and Nitrospira was limited to one or more of the muck fields while Ktedonobacteria and Phycisphaerae were limited to mineral soil Field 2. Acidobacteriia and Solibacteres were present one or more disturbed and/or degraded *M. hapla* infested muck and mineral soils. Cytophagia, Gemmatimonadetes and Pedosphaerae were present in infested and/or non-infested mineral and muck soils; whereas, Sphingobacteriia and Sva0725 were present in infested and/or non-infested mineral soils.

Fungal community composition

Only 5 fungal phyla had greater than 1% relative abundance. The phyla with less than 1% relative abundance were represented as other (**Figure 5.3 B**). Agaricomycetes (1.3% to 3.0%), Ascomycota (56.3% to 59.6%), Basidiomycota (26.8% to 29.4%), and Mortierellomycota (8.6% to 11.6%) and other phyla (1.2% to 3.0%) were present in all of the fields. Glomeromycota (1.1% to 1.2%) were present only in *M. hapla*-infested muck fields with degraded (Fields 5) and disturbed (Field 6) in the eastern and degraded mineral (Fields 13) in the northwest and non-infested (Field 1) in the eastern regions.

Twenty-nine families with at least 1.5% relative abundance were detected (**Figure 5.3 B**). Cantharellales_fam_incertae_sedis (1.5% to 2.8%), Herpotrichiellaceae (2.3% to 3.1%), Lasiosphaeriaceae (1.7% to 5.6%), Mortierellaceae (10.9% to 14.7%), Mrakiaceae (2.3% to 4.9%), Nectriaceae (15.0% to 20.3%), Pleosporaceae (1.6% to 2.9%) and Strophariaceae (2.5% to 4.4%) were found in all of the fields. The less prevalent families had variable with no particular trend to soil group and/or *M. hapla* occurrence. For example, Chaetosphaeriaceae was absent in a non-infested and mineral (Field 12) with maturing soil health conditions while Lectera and Russulaceae were absent in infested mineral and muck soils and/non-infested mineral soils with varying soil health conditions. Aspergillaceae, Clavulinaceae and Sebacina were present in one or more muck fields with degraded and/or disturbed soil health while Ceratobasidiaceae and Trimorphomycetaceae were present in degraded and *M. hapla* infested mineral soil (Field 8). Hypocreaceae and Plectospaerellaceae were present in degraded and *M. hapla* infested (Filed 8) and non-infested degraded (Field 7) and maturing (Field 11) mineral soils. Archaeorhizomycetaceae, Cladosporiaceae, Clavariaceae, Clavicipitaceae, Didymellaceae, Hydnodontaceae, Inocybaceae, Marasmiaceae, Sebacinaceae Tricholomataceae and Vibrisseaceae had broad distribution across fields and *M. hapla* infestations (**Figure 5.3 B**).

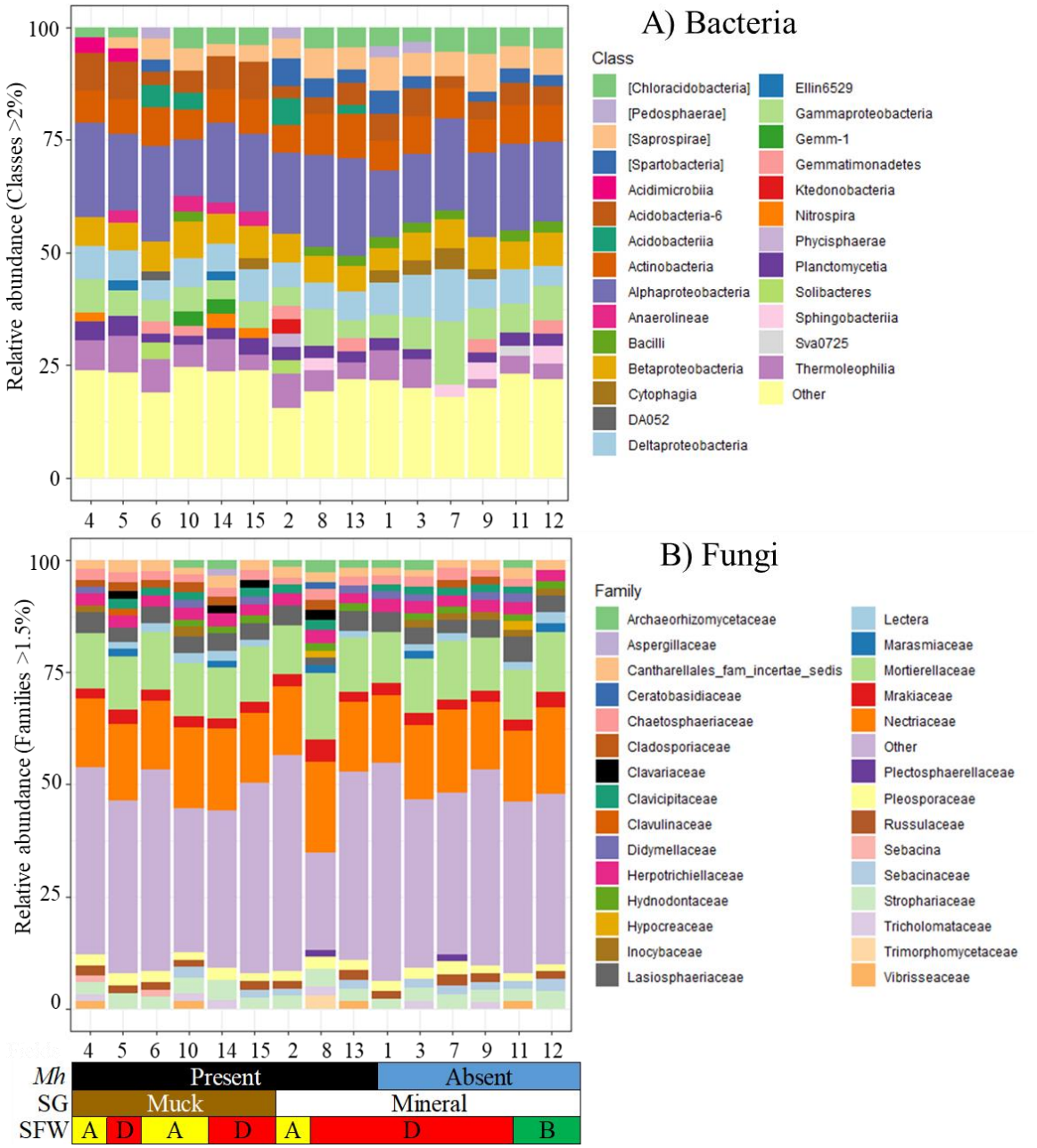

Figure 5.3 | Stacked bar plots of 15 agricultural fields showing the relative abundance by *M. hapla* occurrence (*Mh*: Present [black] and Absent [blue]), soil group (SG: Mineral [white] and Muck [brown]) and soil food web conditions (SFW: D-Degraded [red], A-Disturbed [yellow], B-Maturing [green]) for (A) bacterial classes (>2%), and (B) fungal families (>1.5%). Colors of bacteria and fungi correspond with colors in the stacked bar plots and each bar represents a field. Other unassigned bacterial and fungal groups were classified as other. Relative abundance of bacterial (classes) and fungal (families) groups were variable across the 15 sampled fields.

Bacterial alpha diversity

Alpha diversity of each field was measured using the observed richness and Shannon diversity. Bacterial richness and diversity differed significantly ($p<0.05$) across all muck and mineral fields (**Figure 5.4 A, B**). Observed richness in muck fields (1 to 2,825 [average 1,193]) was generally lower than in mineral fields (1,187 to 3,403 [average 2,204]). Observed richness in *M. hapla*-infested and disturbed SFW muck Fields 4, 6 and 10, and degraded Fields 5, 14 and 15 was lower than in disturbed (Field 2) and degraded (Fields 8 and 13) mineral soil fields (**Figure 5.4 A**). Shannon diversity in muck (0.0 to 7.2 [average 5.7]) and mineral (6.1 to 7.5 [average 6.9]) fields as well as *M. hapla* infestation and SFW conditions was similar to the observed richness (**Figure 5.4 B**).

Fungal alpha diversity

The observed richness of mineral (1 to 758 [average 185]) and muck (1 to 485 [average 138]), and the Shannon fungal diversity of mineral (0.0 to 6.0 [average 4.3]) and muck (0.0 to 5.6 [average 4.2]) were similar across soil groups (**Figure 5.4 C, D)**. In fields, the observed richness of fungi was similar regardless of soil groups, SFW conditions or *M. hapla* infestation (**Figure 5.4 C**). Similarly, the Shannon diversity of fungi was similar across the fields of different soil groups, SFW conditions or *M. hapla* infestation. (**Figure 5.4 D**).

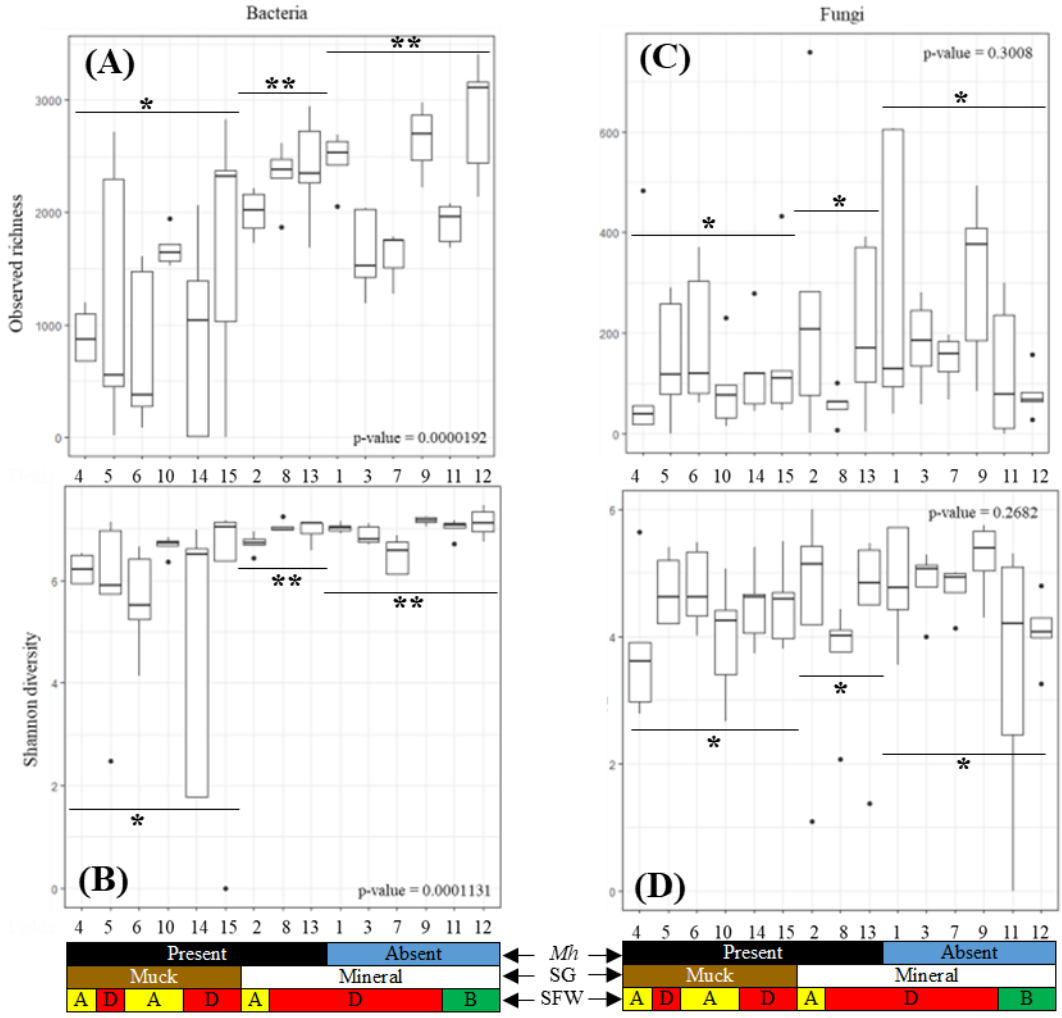

Figure 5.4 | Alpha diversity boxplots of 15 agricultural fields by *M. hapla* occurrence (*Mh*: Present [black] and Absent [blue]), soil group (SG: Mineral [white] and Muck [brown]) and soil food web conditions (SFW: D-Degraded [red], A-Disturbed [yellow], B-Maturing [green]) of (A) bacterial observed richness, (B) bacterial Shannon diversity, (C) fungal observed richness, and (D) fungal Shannon diversity. Outliers on boxplots are displayed as dots. Kruskal Wallis tests were performed to determine significant differences across fields and p-values shown. A pairwise Wilcox test with an FDR p-value correction compared alpha diversity by soil groups based on *M. hapla* occurrence (muck with *M. hapla* present, mineral with *M. hapla* present and mineral with *M. hapla* absent). Each boxplot represents a field. Lines show groups and the asterisk (*) symbol shows differences/similarities of groups. Different asterisks were used to note significant difference ($p<0.05$).

Beta diversity of bacterial communities

A principal coordinate analysis (PCoA) of all field samples was used to show patterns in bacterial communities (**Figure 5.5 A**). Regions were represented by circle (east), triangle (northwest) and square (southwest), and *M. hapla* presence in muck (green) and in mineral (red), and absence (blue). Across soil groups, bacterial community patterns distinctly separated between muck and mineral soils samples along the x- (11.8%) and y-axes (9.3%). In mineral soil, *M. hapla*-infested samples shared bacterial communities with non-infested samples. The ellipse of *M. hapla* infested mineral soil samples slightly overlapped with that of infested muck soil samples. Bacterial communities showed little graphical separations by region.

Results of PERMANOVA showed that bacterial diversity was significantly ($p<0.05$; perm. = 9999) affected by soil groups (SG), regions (RG), SFW conditions, *M. hapla* (MH) occurrence and their interactions (**Table 5.1**). Between 38.0% and 6.9% variation (R^2) could be explained by all the variables. The test of homogeneity of variance showed a significant ($p<0.05$) within group dispersion of regions.

Beta diversity of fungal communities

The PCoA of fungal communities did not reveal any patterns based on *M. hapla* occurrence and soil groups (**Figure 5.5 B**). Approximately 30% (15.9% on the x-axis and 14.3% on the y-axis) of the total variation were accounted for. No observable separations along the x- and y-axes were noted in soil groups, regions by *M. hapla* occurrence.

The PERMANOVA showed that fungal diversity was significant ($p<0.05$; perm = 9999) by SG, RG*MH, and SG*RG*SFW*MH interactions, accounting for 2.4%, 10% and 15% of the variation (R^2), respectively (**Table 5.1**). The homogeneity of variance test did

not reveal any significantly different ($p<0.05$) within group sample dispersion of any variable.

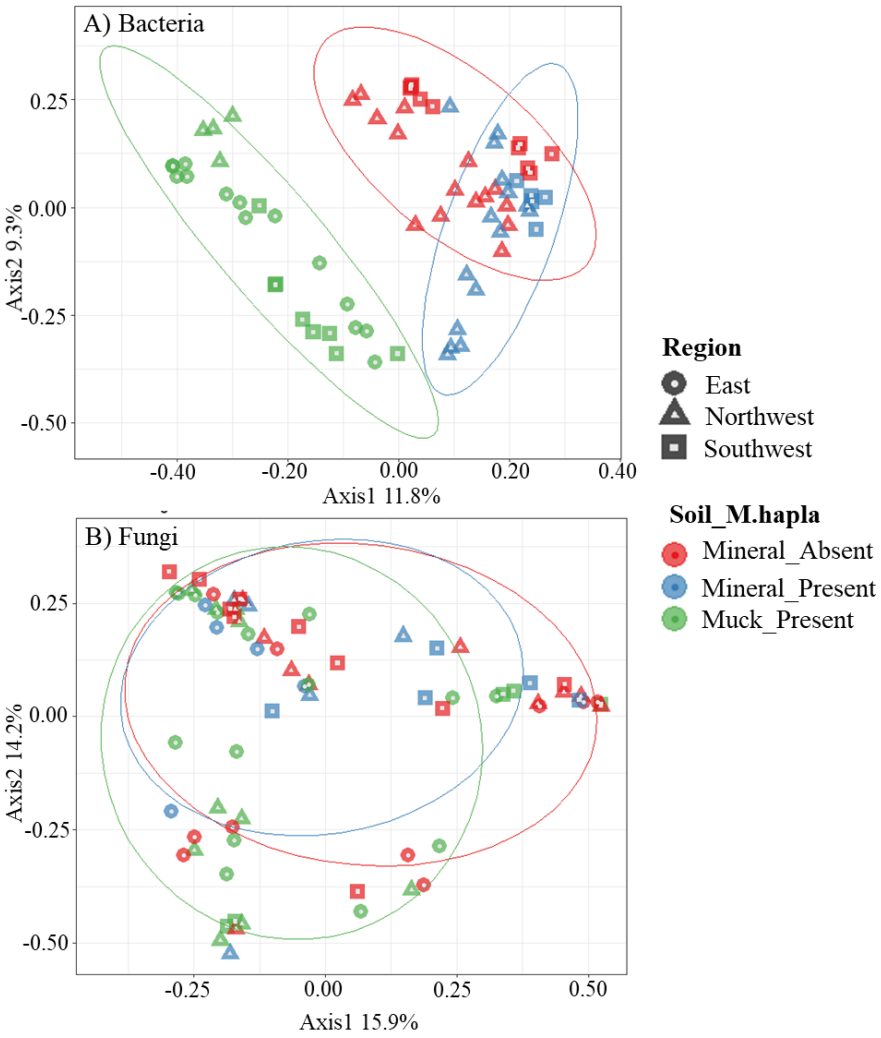

Figure 5.5 | Principal coordinates analysis plots, based on Bray-Curtis dissimilarity, of (A) bacterial communities, and (B) fungal communities. Colors represent *Meloidogyne hapla* occurrence in soil groups (Mineral_Absent: red, Mineral_Present: blue, Muck_Present: green), while the shapes represent regions (east: circle, northwest: triangle, southwest: square) of the lower peninsula of Michigan. Categories were separated with a 70% ellipse.

Table 5.1 | Permutational multivariate analysis of variance *adonis* and multivariate homogeneity of groups dispersions analysis (betadisper) results for bacteria and fungi communities associated with *M. hapla* occurrence (MH), soil group (SG), region (RG), soil health (SFW) conditions, and interactions 15 agricultural fields.

	BACTERIA					**FUNGI**				
	PERMANOVA			**DISPERSION**		**PERMANOVA**			**DISPERSION**	
Variable	***F-value***	***R^2***	***P-value***	***F-value***	***P-value***	***F-value***	***R^2***	***P-value***	***F-value***	***P-value***
MH	5.353	0.069	**0.0001**	43.036	**0.001**	1.495	0.020	0.0966	0.702	0.427
SG	8.368	0.104	**0.0001**	12.644	**0.001**	1.788	0.024	**0.0383**	1.045	0.349
RG	3.685	0.093	**0.0001**	0.764	0.467	1.141	0.031	0.2544	0.478	0.608
SFW	3.867	0.098	**0.0001**	14.110	**0.001**	1.198	0.032	0.1985	0.417	0.676
SG:MH	5.928	0.142	**0.0001**	12.258	**0.001**	1.241	0.033	0.1623	0.631	0.551
RG:MH	3.647	0.173	**0.0001**	17.803	**0.001**	1.524	0.100	**0.0065**	0.303	0.918
SFW:MH	3.881	0.142	**0.0001**	14.547	**0.002**	1.172	0.047	0.1991	0.333	0.797
SG:SFW:MH	4.608	0.252	**0.0001**	8.154	**0.001**	1.241	0.083	0.0884	0.560	0.749
SG:RG:SFW:MH	3.914	0.380	**0.0001**	2.591	**0.015**	1.269	0.150	**0.0330**	0.215	0.989

Significant P-values are indicated in bold

Core bacterial communities

Across all of the muck and mineral fields, 39 core bacterial OTUs were detected. These were classified into 11 genera, and unassigned core OTUs labeled as unclassified (**Figure 5.6 A**). *Arthrobacter* (17.8% to 67.8%), *Devosia* (1.1% to 10.0%), *Kaistobacter* (0.9% to 53.3%) and the unclassified taxa were the most common genera across fields regardless of soil groups, SFW conditions and *M. hapla* occurrence. Other less prevalent core genera were present in both soil groups with either disturbed (Fields 2, 4, 6 and 10), degraded (Fields 1, 3, 7, 8, 9 and 13) or maturing (Fields 11 and 12) and *M. hapla*-infested muck (Fields 4, 5, 6, 10, 14 and 15) and mineral (Fields 2, 8 and 13) or non-infested (Fields 1, 3, 7, 9, 11 and 12) fields. *Adhaeribacter, Balneimonas, Dactylosporangium, Paenibacillus, Reyranella, Rhodoplanes, Sphingobium* and *Turicibacter* were variable across fields. The core bacteria were made up of the suppressive soil (*Arthrobacter* and *Dactylosporangium*), nematicidal (*Devosia*), enhanced nematode parasitism (*Kaistobacter*), plant growth promoter (*Paenibacillus*), soybean cyst associated (*Reyranella*), root knot nematode associated (*Rhodoplanes*), polysaccharide_degrader (*Sphingobium*) functional groups. On the other hand, little information on *Adhaeribacter, Balneimonas,* and *Turicibacter* interaction with nematode exist in literature.

Core fungal communities

Forty-four OTUs were detected as the core fungal communities across muck and mineral fields and classified into 6 genera and the unassigned OTUs grouped as unclassified (**Figure 5.6 B**). *Fusarium* (20.0% to 33.4%) was common in all fields regardless of soil groups, SFW conditions and *M. hapla* presence. As part of the core bacteria, *Alternaria, Bahusutrabeeja , Lectera* and *Saitozyma* varied in soil groups, SFW conditions and *M. hapla* infestation. The core fungi were plant pathogenic (*Fusarium, Alternaria* and *Lectera*)

and polysaccharide degrader (*Saitozyma*) functional groups. However, little is known about the function of *Bahusutrabeeja.*

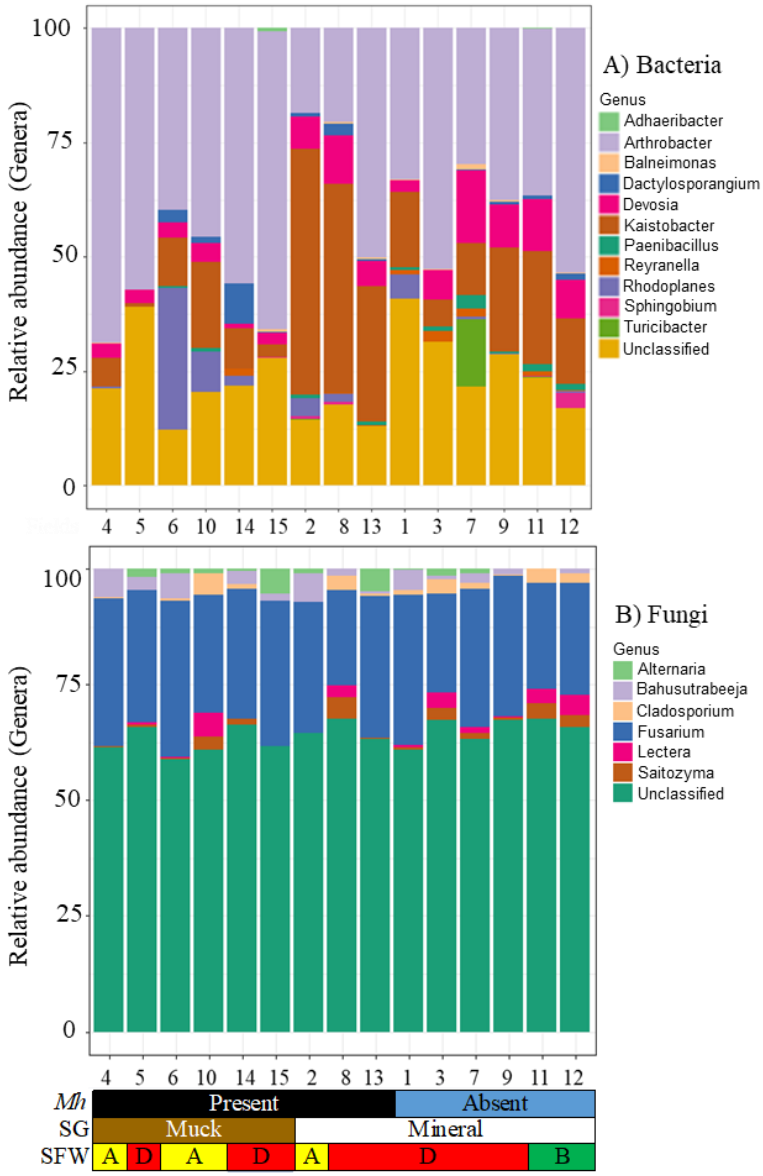

Figure 5.6 | Stacked bar plots of 15 agricultural fields by *M. hapla* occurrence (*Mh*: Present [black] and Absent [blue]), soil group (SG: Mineral [white] and Muck [brown]) and soil food web conditions (SFW: D-Degraded [red], A-Disturbed [yellow], B-Maturing [green] for core (A) bacterial, and (B) fungal communities. Colors of bacteria and fungi correspond with colors in the stacked bar plots and each bar represents a field. Other unassigned bacterial and fungal genera assigned as unclassified. Relative abundance of bacterial and fungal genera was variable across the 15 sampled fields.

Indicators of *M. hapla* occurrence and soil health conditions

A heatmap of the top 25 most relatively abundant indicator bacterial OTUs associated with the occurrence of *M. hapla* in all muck and mineral fields are shown in **Figure 5.7**. The indicator OTUs associated with *M. hapla* occurrence were clustered by hierarchical clustering on the y-axis, whereas fields were clustered by Bray Curtis dissimilarity on the x-axis. All mineral soil Fields (8 and 13 and 1, 3, 7, 9, 11 and 12), but Field 2, clustered separately from all the of the muck soil fields. Accordingly, the relatively abundant OTUs in mineral fields were OTU1 to OTU16. The relatively abundant OTUs in the muck Fields 4, 5, 6, 10, 14 and 15 and mineral Field 2, accordingly, were OTU17 to OTU25. On the y-axis, the relatively more abundant OTUs associated with *M. hapla* presence formed a separate cluster from the OTUs associated with *M. hapla* absence.

The indicator bacteria with their functional groups were : *Kaistobacter* (enhanced nematode parasitism), *Chloracidobacteria* (nematicidal), *Rhizobium* (nitrogen fixer), *Brevundimonas* and *Solibacterales* (plant growth promoter), *Flavitalia populi* (plant pathogenic), *Sorangium* (polysaccharide degrader), *Sphingobacteriales* (root knot nematode associated), *Balneimonas* and *Gemmatinomonas* (suppressive soil), *Oxalicibacterium*, *Skermanella*, *Thauera*, *Actinobacteria*, *Syntrophobacteraceae* and *Pedosphaerales* (other). The relatively abundant OTUs in the muck Fields 4, 5, 6, 10, 14 and 15 and mineral Field 2 were: *Actinobacteria*, *Chloroflexi* (OTU17 and 24), and *Solirubrobacterales* (nematicidal), *Afifella* (root knot nematode associated), *Actinoplanes* (suppressive soil), *Phycicoccus*, *Pirellulaceae* and *Gaiellaceae* (other).

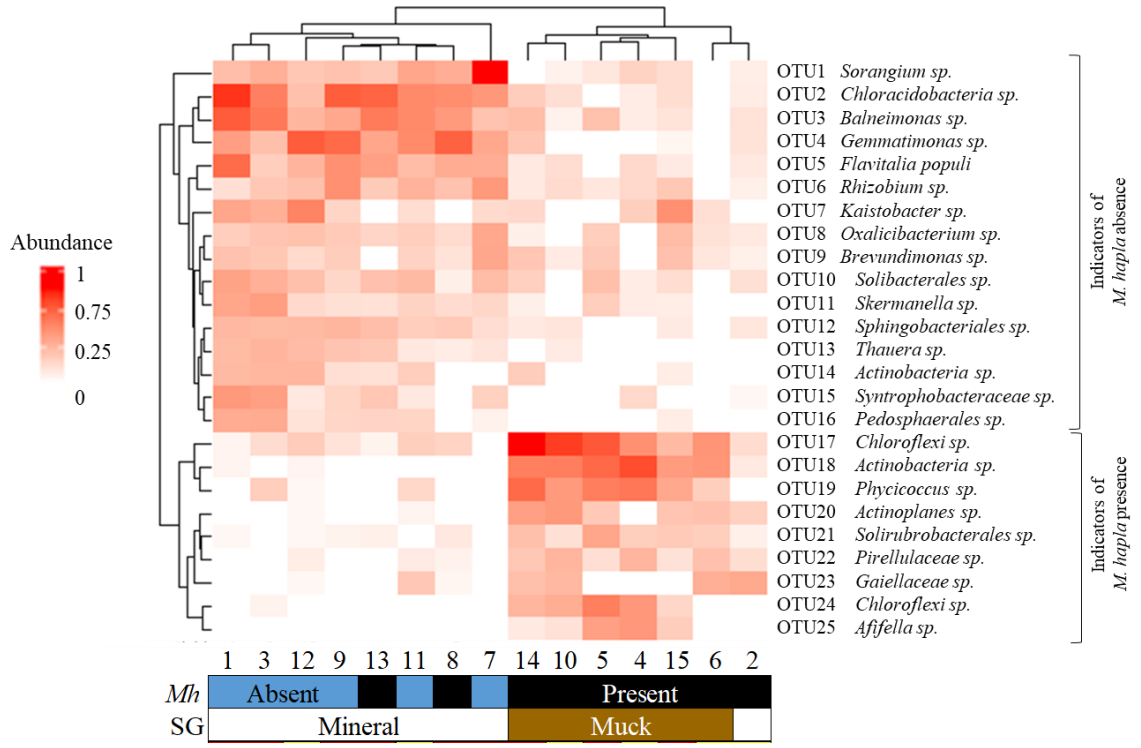

Figure 5.7 | Bacterial indicator heatmap of the top 25 most abundant of OTUs associated with *M. hapla* occurrence (*Mh*: Present [black] and Absent [blue]) across soil groups (SG: Mineral [white] and Muck [brown]). Deeper red color corresponds with a higher bacteria abundance. On the y-axis (hierarchical clustering) is the lower cluster showing indicators associated with *M. hapla* presence (OTU17 to OTU25) and the upper cluster (bray Curtis dissimilarity) showing indicators of *M. hapla* absence (OTU1 to OTU16). The lower cluster with taxa like *Chloroflexi* and *Actinobacteria* was a strong indicator of *M. hapla* presence in muck soils and the mineral Field 2. The top cluster with taxa like *Sorangium* and *Chloracidobacteria* was a strong indicator of mineral fields with and without *M. hapla*. Fields 13 and 8 which were associated with the high and medium PV in Chapter 4 were separated from other low PV populations.

The SFW conditions were disturbed (Fields 2, 4, 6 and 10), degraded or worst case (Fields 1, 3, 7, 8, 9 and 13) and maturing or best case (Fields 11 and 12). A total of 1065 indicator OTUs were associated with the disturbed, degraded and maturing categories of soil health conditions (**Figure 5.8**). Of these, five OTUs were specific to degraded, 89 OTUs to

disturbed, and 787 OTUs to maturing soil health conditions. Soils with degraded SFW conditions had 135 OTUs and those with disturbed conditions shared 49 OTUs with soils that had the maturing conditions. There were no OTUs shared between disturbed, degraded and maturing soil health conditions.

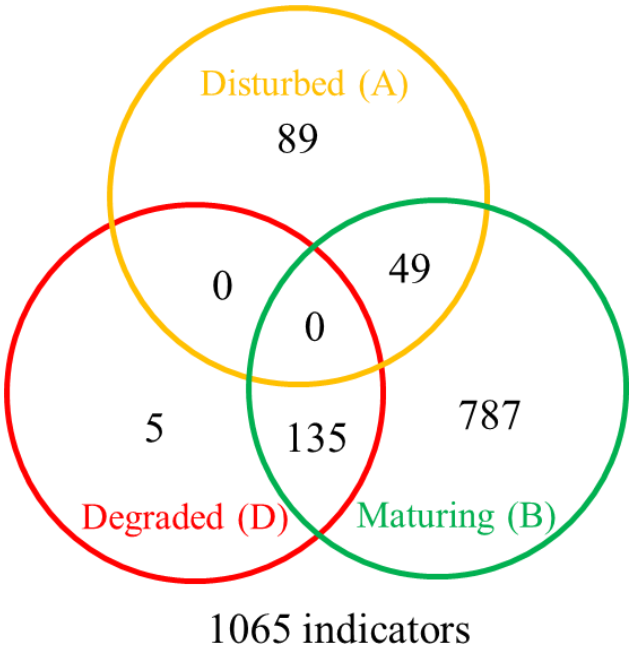

Figure 5.8 | Venn diagram showing the distribution of 1065 bacterial indicators associated with disturbed (A-yellow), degraded (D-red) and maturing (B-green) soil health conditions. The degraded had 5 unique indicators, the disturbed with 89 and the maturing conditions with 787 indicators. The maturing conditions shared 135 indicators with degraded and 49 indicators with the disturbed conditions. No indicators were shared by degraded and disturbed conditions or by all three soil health conditions. A complete list of soil health condition indicators is available using this link (https://docs.google.com/spreadsheets/d/1FaYYF25SXTXSKVtTWaJg-TtPEzcwvVme/edit?usp=sharing&ouid=101736667728877041455&rtpof=true&sd=true).

Discussion

A growing body of research indicates that *M. hapla* populations from different soil conditions exhibit parasitic variability (PV, Chapter 4; Melakeberhan et al., 2007; Melakeberhan and Wang, 2013), but little is known about the biophysicochemical basis or mechanisms leading to PV in general and any role of soil microbiome in particular. This study was designed to develop a baseline information towards understanding associations among soil microbiome, soil health and *M. hapla* occurrence.

Microbial diversity and community composition

The first aim was to characterize the soil microbiome structure and diversity in mineral and muck fields that varied in soil health conditions and *M. hapla* occurrence. Our hypothesis that soil microbiome would differ significantly between soil groups (SG) and soil health conditions (SFW) was partially supported by bacterial community composition varying by SG, region (RG), SFW and *M. hapla* occurrence (MH) and that of fungi by SG*RG*SFW*MH. The lower bacterial diversity (observed richness and Shannon) in muck fields with disturbed and degraded soil health conditions than in *M. hapla* infested or non-infested mineral soil with similar degradations also support our hypothesis. In contrast, the lack of difference in diversity and richness of fungi by RG, SFW or MH, but SG does not support the hypothesis. This suggests that, while bacterial composition had several drivers influencing the community structure in the soil only a limited set of factors influence fungal communities. Perhaps, this explains the differences observed in bacterial diversity and lack of difference in fungal diversity.

The variable effects of soil type and/or region on bacterial and fungal community composition and/or diversity are consistent with established facts (Fierer and Jackson, 2006; Zhou and Fong, 2021; Lupatini et al., 2012). These results combined with soil health

conditions and the presence or absence of *M. hapla* reveal the relative abundance of the bacterial and fungal communities commonly occurring in all fields and those varying by SG, RG, SFW and/or MH. The commonly occurring bacterial and fungal phyla like Proteobacteria and Ascomycota, respectively, were the most abundant (**Figure 5.2**). Similarly, bacterial classes like Alphaproteobacteria, and fungal families like Basidiomycota were present in high proportions and in all fields (**Figure 5.3**). On the other hand, other bacterial and fungal taxa had low relative abundance as well as absence or presence that varied by SG, RG, SFW and/or *M. hapla* occurrence. The bacterial phylum like Verrucomicrobia and 19 out of the 27 classes, and the fungal phylum like Glomeromycota and 22 of the 29 families had low relative abundance as well as absence or presence that varied by SG, RG, SFW and/or *M. hapla* occurrence (**Figure 5.2; Figure 5.3**). As shown in Chapter 3, *M. hapla* was present in 9 out of the 15 fields in the three regions and soil groups with varying soil health conditions. Population 13 had the highest, Population 8 medium, and the rest of the populations (2, 4, 5, 6, 10, 14 and 15) low parasitism, and Populations 5, 8, and 13 came from degraded and Populations 2, 4, 6 and 10 from disturbed soil health conditions (Chapter 4). In this regard, it is more informative to look at the absence or presence of the bacterial and fungal communities relative to the PV of the *M. hapla* populations and the soil health conditions. For example, the bacterial phyla Verrucomicrobia and Gemmatimonadetes and classes Saprospirae, Spartobacteria and Chloracidobacteria were absent in one or more of the low PV category populations with either disturbed and/or degraded soil health conditions (**Figure 5.2; Figure 5.3**). Nonetheless, different taxa had different roles in the soil. Verrucomicrobia in the *Xiphinema* spp nematode is associated with pathogenesis (Lazarova et al., 2016; Vandekerckhove et al., 2000). Gemmatimonadetes are known to be abundant in root knot nematode suppressive soils, but Saprospirae and Chloracidobacteria are present in root knot nematode infested and non-infested soils (Zhou

et al., 2019). In tomato, Spartobacteria is known to be associated with the corcky-root disease complex (Lamelas et al., 2020).

The presence of the bacterial phyla Nitrospirae and classes like Acidimicrobiia, limited to one or more of the muck fields, and Ktedonobacteria and Phycisphaerae to mineral soil Field 2 is an example of the association of these communities with the soil where the low PV category populations came from (**Figure 3 A**). While present in *M. hapla*-infested soils, the fungi families Ceratobasidiaceae and Trimorphomycetaceae were limited to mineral soils and Aspergillaceae, Clavulinaceae and Sebacina the bacterial classes Acidobacteriia and Solibacteres were present in both soil groups (**Figure 3 A**). The presence of Hypocreaceae and Plectospaerellaceae in *M. hapla*-infested (Field 8) and non-infested degraded (Field 7) and maturing (Field 11) mineral soils, while the bacterial phylum Firmicutes were present in disturbed muck (Field 10) and degraded (Fields 8 and 13) and non-infested mineral (Fields 1, 3, 7, 11 and 12) soil shows their broad distribution across soil degradations with varying soil health degradations (northwest) and maturing SFW conditions. Solibacteres has a negative abundance relationship with *Meloidogyne* spp (Castillo et al., 2017), Nitrospirae suppresses root knot nematode infection, Acidimicrobiia are in root knot nematode infested and non-infested rhizosphere, Phycisphaerae is associated with the root knot nematode infection, and Anaerolineae is enriched in root knot nematode non-infested plants (Zhou et al., 2019). Ktedonobacteria, Nitrospira and Trimorphomycetaceae have nematicidal properties against the root knot nematode affecting soybeans (Toju and Tanaka, 2019). Ceratobasidiaceae, Hypocreaceae and Aspergillaceae are involved in the pine wilt disease vectored by *Bursaphelenchus xilophilus* (Vicente et al., 2021) and Sebacina is an indicator of the absence of the pine wilt disease (Liu et al., 2021). However not much is known about DA052, Ellin6529, Gemm-1, Clavulinaceae and Acidobacteriia. Finally, the bacterial classes like Cytophagia and Sphingobacteriia (**Figure 3**

A) and fungal families like Sebacinaceae and Tricholomataceae (**Figure 3 B)** had broad but variable presence across SG, RG, SFW and MH, suggesting that these groups were widely distributed regardless of soil conditions, *M. hapla* occurrence or geography. Cytophagia is known to occur in both infested and non-infested root-knot nematode soils (Zhou et al., 2019) and Sphingobacteriia negatively impacts *Caenorhabditis elegans* population numbers (Dirksen et al., 2016). Sebacinaceae , however, antagonizes cyst nematode infection and development in *Arabidopsis* roots (Daneshkhah et al., 2013), and Tricholomataceae negatively affects the activity of the pinewood nematode (*Bursaphelenchus xilophilus*) (Ishizaki et al., 2015).

Core microbial communities

Our second aim was to identify core soil microbiome associated with the occurrence of *M. hapla* across fields using the abundance-occupancy distributions as suggested by Shade and Stopnisek, (2019). We identified 39 bacterial and 44 fungal as core-microbes present in at least most of the *M. hapla* infested and non-infested fields, supporting our hypothesis. *Fusarium,* a ubiquitous and diverse fungal genus, and the bacterial genera *Arthrobacter*, *Devosia*, *Kaistobacter* (as well as unclassified taxa) were present in all of the fields. *Arthrobacter* includes decomposers (Cacciari and Lippi, 2009), *Kaistobacter* appears to suppress bacterial wilt (Liu et al., 2016), and *Devosia* has a soil toxin degrading (Talwar et al., 2020), as well as antagonistic traits against the plant-parasitic nematodes *Pratylenchus neglectus, M. chitwoodi* and *Globodera pallida* (Castillo et al., 2017; Eberlein et al., 2016). If the core microbiome is related to PV, it is reasonable to assume that the variable groups may be involved.

The presence of the rest of the 39 bacteria and 44 fungi core OTUs in the fields across the three regions varied by soil group and *M. hapla* occurrence. The *M. hapla*

Populations 2, 8 and 13 were from mineral soil and Populations 4, 5, 6, 10, 14 and 15 were from muck soil. Population 13 had significantly higher reproductive potential from Population 8, and both from the rest of the populations (Chapter 4). Whether or not the core microbial populations contribute to *M. hapla* PV is yet to be determined, but their presence or absence is worth noting. The higher reproductive potential of Population 13 lacked the fungus *Alternaria* and the bacteria *Reyranella* and *Rhodoplanes.* On the other hand, Population 8 with a medium PV lacked bacteria *Paenibacillus* and *Reyranella* and the fungus *Lectera. Paenibacillus* is a beneficial bacteria that enhances plant growth through nitrogen fixation, phosphate and potassium solubilization (Patowary and Deka, 2020). *Reyranella* is found to be associated with *Heterodera glycines* (Hu et al., 2017) and *Rhodoplanes* has a positive relationship with *M. incognita* (Castillo et al., 2017). *Alternaria* is a pathogen of the citrus black rot disease (Umer et al., 2021) and *Lectera* is a legume pathogen (Cannon et al., 2012). Otherwise, the detection of the other core bacteria and fungi in mineral and muck soils infected with one or more of the respective populations suggests that the *M. hapla* populations were exposed to common microbiome.

With regards to soil health conditions, *Balneimonas, Dactylosporangium, Rhodoplanes* and *Sphigobium* and the fungi *Bahusutrabeeja, Lectera* and *Saitozyma* were common to the disturbed and degraded muck and mineral soils. The bacteria *Paenibacillus* was detected in disturbed and degraded mineral and disturbed muck and the fungus *Cladosporium* in disturbed mineral and disturbed and degraded muck soil. Although there is not much information about *Bahusutrabeeja* and *Balneimonas* in the literature, other core microbes associated with soil health condition had different roles. *Dactylosporangium* was found in *Heterodera glycines* suppressive soils (Topalović et al., 2020). *Saitozyma* is a yeast (Li et al., 2020) and *Sphingobium* produces enzymes which allow sugars to be degraded (Balows et al., 1992; Wu et al., 2017). *Cladosporium* is involved in the increase of systemic

defense in pine to reduce the incidence of *Bursaphelenchus xylophilus* infectivity (Chu et al., 2019). While the role of the core microbes reported herein relative to *M. hapla* PV is unknown, documenting their presence or absence is helpful towards understanding the SFW conditions in which *M. hapla* exists.

Indicator bacterial communities relative to *M. hapla* occurrence

The third aim of this study was to identify indicator-microbes associated with *M. hapla* occurrence and soil food web (SFW) conditions. Twenty-five bacterial OTUs were indicators for *M. hapla* presence or absence, and none of the 1065 soil health indicator OTUs were common to the three categories - disturbed, degraded and matured. The clustering of the bacterial communities by *M. hapla* and soil health conditions partially support the hypothesis that there are indicator-microbes associated with *M. hapla* occurrence or SFW conditions (**Figure 5.7**). While the bacterial indicator species enrichment in the fields was variable, all of the mineral soil fields, but Field 2, clustered separately from the muck fields, all of which are *M. hapla* infested. This suggests that there may be soil-specific factors driving the indicator species. The infested muck soils regardless of SFW conditions were clustered and shared *Chloroflexi* sp. (OTU17), *Actinobacteria* sp., *Phycicoccus* sp., *Solirubrobacterales* sp., and *Pirellulaceae* sp. *Chloroflexi* and *Solirubrobacterales* are part of a consortia of anti-nematode bacteria in the rhizosphere of soybean plants attacked by root-knot nematodes (Toju and Tanaka, 2019) and *Actinobacteria* introduced to strawberry root by *Pratylenchus penetrans* cause a decline in strawberry yield. However, little is known about the role of *Phycicoccus* and *Pirellulaceae.*

Although *M. hapla* populations in Field 2 had similar PV category as those from muck soil (Chapter 4), why it separated with the muck soils is unknown. The fields where *M. hapla* populations 13 and 8, with highest PV, and Population 2 came from had

Sorangium sp., *Chloracidobacteria* sp., *Balneimonas* sp., *Gemmatinomonas* sp., *Flavitalia populi, Rhizobium* sp., *Oxalicibacterium* sp., *Solibacterales* sp., *Sphingobacteriales* sp., *Chloroflexi* sp. (OTU17), and *Solirubrobacterales* sp. in common. Yet, Populations 8 and 13 were separate from Population 2 and all of the populations from muck soil. It is unknown if the enrichment of *Actinoplanes* sp, and *Actinobacteria* sp. (OTU18) in Field 2, none in Field 8, and that of *Actinobacteria* sp. (OTU14) and *Pedosphaerales* sp. in Field 13, and that, the latter two fields were degraded and the former field disturbed soil health conditions contributed the similarity and differences in clustering of the these populations. *Sorangium* has enzymes which breaks down plant cell walls (Li et al., 2013) and *Chloracidobacteria* is part of the microbiome in the rhizosphere known to suppress root knot nematode infection (Zhou et al., 2019). *Balneimonas* is associated with amendment treated soils that suppresses verticillium wilt (Inderbitzin et al., 2018), *Gemmatinomonas* and *Actinoplanes* are present in plant parasitic nematode suppressive soils (Topalović et al., 2020) and *Flavitalia* is associated with apple replant disease (Kanfra et al., 2022). *Rhizobium* fixes nitrogen in plant to enhance growth (Maróti and Kondorosi, 2014), *Solibacterales* enhances plant growth by mobilizing phosphorus in the soil (Bergkemper et al., 2016) and *Sphingobacteriales* is associated with *M. incognita* infection in tomato roots (Tian et al., 2015). The function of *Oxalicibacterium* and *Pedosphaerales* were not known. Whether or not the shared indicators have a relationship with PV, is not fully understood.

Meloidogyne hapla was isolated in mineral and muck soils with disturbed, degraded and maturing soil health conditions (Lartey et al., 2021), and Populations 13 and 8 from degraded mineral soil had significantly higher reproductive potential than the rest of the populations in both soil health categories (Chapter 4). Out of the 1,065 indicator bacterial species that were found across the soil health categories, 73.9% in the maturing, 8.4% in the disturbed, 0.4% in the degraded, supporting generally known facts about soil degradation

relative to microbial communities (Chapter 3). The soils with maturing shared 8.4% with disturbed and 12.7% with the degraded soil health conditions, suggesting that some commonality between the overlapping soil health conditions. The lack of common indicator OTUs between disturbed and degraded soil health suggests that the conditions in these soil health groups select for different microbes. Whether or not these differences contribute to differences in *M. hapla* populations PV has yet to be determined.

Characterizing the microbial communities in soils where *M. ha*pla occurs relative to PV is an important first step of knowing the soil environments populations survive and interact with. However, whether all or some of the soil microbes are directly, indirectly or of no consequence relative to PV demands further investigation. By isolating *M. hapla* nematodes and characterizing the associated microbes just like the Russian wheat aphid (Luna et al., 2018) this will answer question: What microbial composition and functional groups could be associated with *M. hapla* PV?

Conclusions

This study provides insights into the microbial community structure, the core- and indicator-microbiome associated with *M. hapla* occurrence in mineral and muck soil groups across disturbed and degraded soil food web conditions. We established that *M. hapla*-infested and disturbed and degraded muck fields had lower bacterial diversity (observed richness and Shannon) than corresponding mineral soil fields or non-infested mineral fields. Bacterial and fungal abundance varied by soil group, soil health conditions and/or *M. hapla* occurrence. A core of 39 bacteria and 44 fungi OTUs occurred either across all fields or varying by soil group, soil health conditions and/or *M. hapla* occurrence. We found 25 indicator bacteria OTUs associated with *M. hapla* presence or absence. Taken together, these findings reveal the microbial communities which exist with *M. hapla* populations in different soil groups and

soil health conditions and how it may be related to PV.

CHAPTER 6

COMPOSITION AND FUNCTION OF BACTERIAL COMMUNITIES ASSOCIATED WITH THE NORTHERN ROOT-KNOT NEMATODE (*MELOIDOGYNE HAPLA*) POPULATIONS SHOWING PARASITIC VARIABILITY

Abstract

A diverse soil microbial community structure is known to co-exist with *Meloidogyne hapla* occurrence across different soil health conditions in mineral and muck Michigan vegetable production fields. However, it is not known what microbial relationships or the lack of, are associated with *M. hapla* populations' PV. Using *M. hapla* populations isolated from mineral and muck soil fields with disturbed and degraded soil health conditions, and their greenhouse cultures showing PV, the populations were studied for the presence and/or absence of specific bacterial composition and functional groups. The 16S DNA of field and greenhouse isolated *M. hapla* populations were sequenced to characterize bacteria. The results showed 65 genera in the field and 61 genera in the greenhouse had a variable composition with 14 known functional groups (in brackets) and unknown or other groups. Population 13 with the highest PV was associated with the unique absence of *Mesorhizobium* (nitrogen fixer) and *Ohtaekwangia* (suppressive soils) in the field and *Devosia* (nematicidal), *Kibdelosporangium* (anti bacteria), *Mycobacterium* (animal pathogenic) and *Steroidobacter* (suppressive soil) in the greenhouse. On the other hand, the medium PV of Population 8 was uniquely associated with the presence of *Brevundimonas* (plant growth promoter), *Candidatus Udaeobacter* (antibiotic resistant), *Hyphomicrobium* (other), *Mucilaginibacter* (plant growth promoter), *Nocardioides* (nematicidal), *Roseiarcus* (other) and *Solirubrobacter* (nematicidal) in the field and *Hyphomicrobium* (other) in the greenhouse. The low reproductive potential was found to be associated with several bacteria. The findings of this study lay the foundation in establishing potential cause-and-effect relationships associated with *M. hapla* PV.

Introduction

Meloidogyne hapla occurrence across mineral and muck fields with different soil health conditions have been found to co-exist with a diversity of soil microbial communities (Chapter 5). However, information on *M. hapla* distribution and PV relative to soil biophysicochemical and soil health conditions is limited. Recent studies designed to understand the soil environment in which *M. hapla* exists in the lower peninsula of Michigan (USA) revealed three points. First, *M. hapla* distribution in mineral and muck soils was related to soil food web (SFW) conditions described in the Ferris et al., (2001) model as disturbed (enriched and unstructured), degraded (resource-limited and minimal structure) or maturing (enriched and structured) (Lartey et al., 2021). Degraded SFW conditions have been correlated with poor soil health conditions (Melakeberhan et al., 2021a). Second, *M. hapla* populations isolated from mineral soils with degraded soil health conditions had higher PV (based on the reproductive potential) than populations from mineral and muck soils with similar conditions (Chapter 4). Third, analysis of the soil microbiome in the soils where *M. hapla* was isolated from identified 39 bacterial and 44 fungal core-microbiome, and 1065 indicator bacteria were associated with soil health conditions and/or *M. hapla* occurrence (Chapter 5). Core-microbiome are defined as the most abundant and prevalent taxa across fields (Shade and Stopnisek, 2019). Indicator species on the other hand are the microbes with an increased occurrence or abundance associated with a group of sites of a similar characteristic(s) (Cáceres and Legendre, 2009).

In general, a myriad of soil microbes have varying association with *M. hapla* (Davies and Williamson, 2006; Topalović et al., 2019). The associations range from fungal spores and an assortment of bacteria attaching to and parasitizing *M. hapla* cuticle (Elhady et al., 2017; Viaene and Abawi, 1998) to lethal-parasitism of *Pasteuria* spp. (Davies and Williamson,

2006), and diminishing (Topalović et al., 2019) or enhancing *M. hapla*'s ability to infect host-plant (Topalović et al., 2022). However, if and how the core bacteria and fungi and/or the indicator bacteria identified in the soils where *M. hapla* occurred may or may not relate to *M. hapla* PV and/or the populations isolated from the fields is unknown.

From the *M. hapla* biology point of view, the 3rd, 4th and adult stages inside the root system and eggs are laid in gelatinous matrix inside the root or protruding out of the root (Desaeger, 2019). The second-stage juvenile, the infective stage, hatches from the egg and migrates through the soil to find the host roots and is most likely to have exposure to soil microbes and the environment therein. What, if any, associations between *M. hapla* and soil microbes that relate to PV may or may not exist is unknown. In this context, we define association as any microbes detected with DNA extract of an *M. hapla* population.

This study is part of a project whose goal is to identify the mechanisms of *M. hapla* PV through understanding the soil environment in which *M. hapla* exists. The study presented herein expands the base line biophysicochemical information of the soil environment in which *M. hapla* PV exists (Lartey et al., 2021) by focusing on associations, or the lack of, between *M. hapla* and bacteria. The objective was to characterize composition and function of bacterial communities associated with *M. hapla* populations isolated from mineral and muck soil fields with disturbed and degraded SFW conditions and their greenhouse cultures showing PV (Lartey et al., 2021). Other studies have shown associations of bacteria (for example *Paraburkolderia*, *Lectera* and *Penicillium*) with cuticles of *M. hapla*, *M. incognita* and *Pratylenchus penetrans* (Elhady et al., 2017; Topalović et al., 2019). We know that these *M. hapla* populations have PV (Chapter 4) and that there are core bacterial- and fungal-microbiome as well as indicator bacteria associated with soil health conditions and/or *M. hapla* occurrence (Chapter 5). Our hypothesis is that either presence and/or

absence of specific bacterial composition and functional groups are associated with *M. hapla* PV. Knowing whether or not there is an association between specific bacteria and *M. hapla* populations will advance of our understanding of the environment in which *M. hapla* exists and potentially lead towards identifying cause-and-effect relationships of its PV.

Materials and methods

Meloidogyne hapla Populations

Three *M. hapla* populations (2, 8 and 13) from mineral soil and six (4, 5, 6, 10, 14 and 15) from muck soil were collected from vegetable production fields in the eastern, southwestern and northwestern regions in the lower peninsula of Michigan, USA (Lartey et al., 2021). Populations 13, 14 and 15 were from the northwest, Populations 8 and 10 from the southwest, and Populations 2, 4, 5 and 6 were from the eastern regions. Populations 5, 8, 13, 14 and 15 came from degraded and Populations 2, 3, 6 and 10 from disturbed soil health conditions (**Figure 6.1**; Chapter 4). Population 13 had the highest reproductive potential than all of the populations, and Population 8 from Populations 2, 4, 5, 6, 10, 14 and 15 (Chapter 4). In this study, field and greenhouse populations were used. The field populations were isolated from the original soil samples. The greenhouse populations were isolated from greenhouse cultures maintained in a steam-sterilized (100 ^{o}C for 8 hrs) mix of top soil, sphagnum peat and sand (supplied by Michigan State University Plant Science Greenhouses [MSUPSG]) on tomato (*Solanum lycopersicon*) cv. ‘Rutgers’ a standard for testing PV at the MSUPSG over three years (Chapter 4).

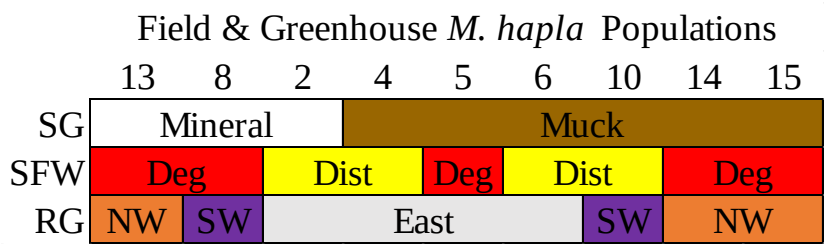

Figure 6.1 | Description of the 9 *M. hapla* populations isolated from field and greenhouse populations of different soil groups (SG: Mineral [white] and Muck [brown]), soil food web conditions (SFW: Deg-Degraded [red], Dist-Disturbed [yellow]) and regions (RG: East [grey], SW-Southwest [purple] and NW-Northwest [orange]). Sequence of *M. hapla* populations from mineral soils arranged from high to low parasitic variability (PV) but muck populations with low PV (reproductive potential) are arranged numerically.

Statistical and Data Analyses

Data files containing OTU tables, taxonomy, mapping, and OTU sequences were loaded into the R (version 4.0.2) statistical environment (R Core Team, 2020) and used to create a phyloseq object for further analysis, using the phyloseq package (McMurdie and Holmes, 2013). OTU contaminants were removed with a negative control using the decontam package (Davis et al., 2018). Afterwards, three sets of analyses were performed.

First, bacterial phyla and genera composition, and relative abundance were visualized using stacked bar plots for field and greenhouse populations. Afterward, the alpha diversity was estimated for observed richness (Simpson, 1949) and Shannon diversity (Hill, 1973) using the microbiome and vegan packages (Dixon, 2003), and boxplots of the alpha diversity data created with the ggplot2 package (Wickham, 2016). In order to determine statistical differences, Kruskal Wallis test in the stats package was conducted across populations (R Core Team, 2020).

Second, in order to visualize and compare microbial communities patterns by soil group and SFW conditions; first, OTUs of less than 5 reads in a sample were filtered out to account for PCR errors (Oliver et al., 2015). Next, the metagenomeseq package (Paulson et al., 2013) was used to normalize the data by cumulative sum scaling. The soil group and SFW microbial community patterns associated with *M. hapla* populations were investigated by creating principal coordinates analysis (PCoA) plots with the ordinate and plot_ordination functions. The statistical significance of microbial community patterns was tested with PERMANOVA as implemented by the adonis function of the vegan package. Afterwards, the within sample variance (homogeneity of variance) were statistically tested with the "betadisper" function found in vegan.

Third, the bacterial composition and relative abundance of assigned functional groups was created. The bacterial genera from the first set of analyses were assigned functional groups based on literature. Across field and greenhouse *M. hapla* populations the functional groups were visualized using stacked bar plots. All the analyses performed are accessible on github (https://github.com/larteyis/PAPER-Bacterial-composition-diversity-and-functional-groups-associated-with-Meloidogyne-hapla-popul).

Results

Community composition

Nine bacterial phyla with 65 genera in the field and 61 genera in the greenhouse *M. hapla* populations were detected at varying proportions (**Figure 6.2**; **Figure 6.3**). The absence or presence and relative abundance of the bacterial communities associated with the populations from mineral (Populations 2, 8 and 13) or muck (Populations 4, 5, 6, 10, 14 and

15) soils with either disturbed (Populations 2, 4, 6 and 10) or degraded (Populations 8, 13, 14 and 15) SFW conditions, or high (Population 13), medium (Population 8) or low (Populations 2, 4, 5, 6,10, 14 15) PV category was highly variable (**Figure 6.3**). Thus, the phyla and genera are described as common to all *M. hapla* populations, variable among, specific to or lacking in certain populations from the field and greenhouse.

The phyla Actinobacteria, Bacteroidetes, Firmicutes, Myxococcota and Proteobacteria were present in all of the field and greenhouse populations; whereas, the presence of Acidobacteria Chloroflexi, Planctomycetes and Verrucomicrobia had a low and variable relative abundance in both sets of populations (**Figure 6.2 A, B**). At the genera level, *Acidibacter, Actinophytocola, Amycolatopsis, Bradyrhizobium, Candidatus Phytoplasma, Cellvibrio, Chitinophaga, Clostridium sensu stricto, Duganella, Flavobacterium, Frankia, Lechevalieria, Massilia, Paenibacillus, Pseudomonas, Pseudonocardia, Rheinheimera, Rhizobacter* and the unclassified genera were common to all field and greenhouse populations (**Figure 6.3 A, B**). *Haliangium*, *Kibdelosporangium*, *Mycobacterium*, *Novosphingobium*, *Rhizobium*, *Rhodoplanes*, *Sphingomonas* and *Streptomyces* were common to all field populations but variable among the greenhouse populations. Presence or absence of the rest of the genera varied in both the field and greenhouse populations.

Population 13 had *Inquilinus* in common with Populations 5 and 8 and *Caulobacter* with Populations 5 and 15 in the field, and *Halomonas* with Populations 8 and 10 and *Limnohabitans* with Populations 2, 4, 5 and 15 in the greenhouse. *Mesorhizobium* and *Ohtaekwangia* in the field and *Devosia, Kibdelosporangium, Mycobacterium* and *Steroidobacter* in the greenhouse were absent in Population 13. *Streptomyces* in the greenhouse and *Acidothermus, Devosia, Limnohabitans* and *Mycoplasma* in the field were

absent in Population 13 and one or more of the low PV category populations (**Figure 6.3 A, B**).

Presence of *Brevundimonas, Candidatus Udaeobacter, Hyphomicrobium, Mucilaginibacter, Nocardioides, Roseiarcus* and *Solirubrobacter* in the field and *Hyphomicrobium* in the greenhouse was limited to Population 8. Population 8 had the presence of *Catenulispora*, *Gemmata,Pedomicrobium, Rhodomicrobium, Sphingobium, Phenylobacterium, Variovorax* and *Xanthomonas* in the field, *Brevundimonas, Chryseolinea, Kribbella, Mucilaginibacter, Mycoplasma, Niastella, Novosphingobium, Ohtaekwangia, Paraburkholderia, Rhodoplanes* in the greenhouse, and *Dokdonella*, *Labrys*, *Nocardia* and *Polaromonas* in both field and greenhouse populations in common with one or more of the low PV category populations (**Figure 6.3 A, B**).

The low PV category populations had some unique presence and absence as well. Presence of *Chryseolinea* and *Fluviicola* in the field, *Holdemanella, Nocardioides, Pedomicrobium, Sphingobium, Solirubrobacter*, *SM1A02* and *Xanthomonas* in the greenhouse, *Bryobacter* in both field and greenhouse populations were limited to Populations 2. Similarly, and *Holdemanella* and *SM1A02* in the field and *Roseiarcus* in the greenhouse were specific to Population 15 and 6, respectively. *Actinospica* in Populations 2, 4 and 14, and *Kribbella* in Populations 5 and 15 were absent in the field (**Figure 6.3 A**).

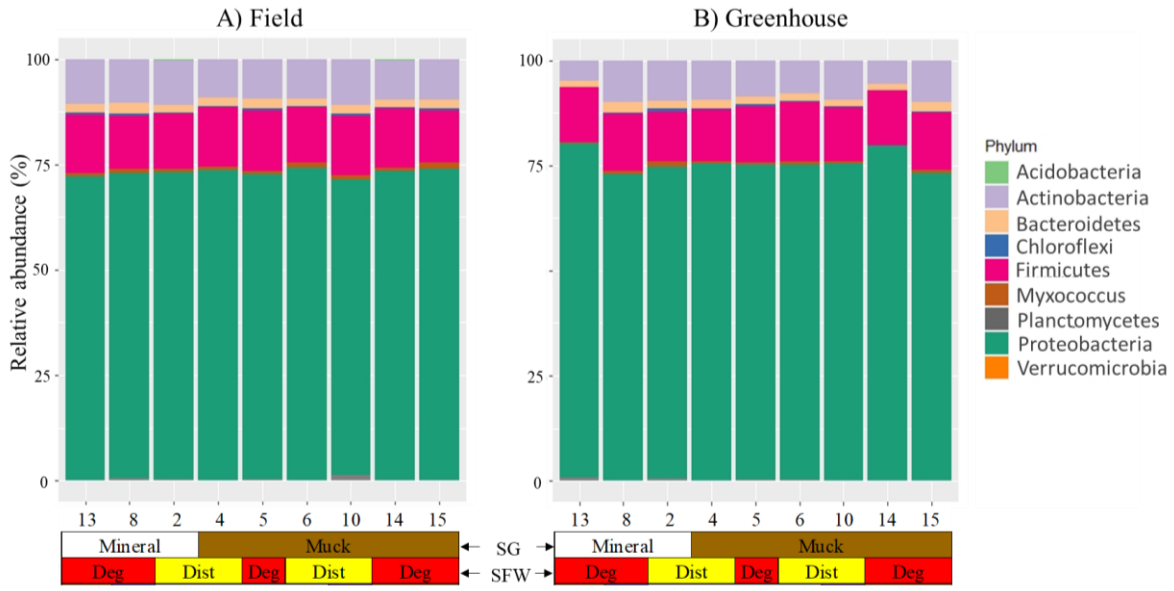

Figure 6.2 | Stacked bar plots showing the relative abundance of bacteria genera associated with field (A) and greenhouse (B) *M. hapla* populations originating from different soil groups (SG: Mineral [white] and Muck [brown]) and soil food web conditions (SFW: Deg-Degraded [red], Dist-Disturbed [yellow]). Colors of bacterial phyla correspond with colors in the stacked bar plots and each bar represents a population in either field or greenhouse. Relative abundance of phyla was variable across the field and greenhouse populations. Sequences were assigned to taxonomic groups using the ACT (alignment, classification, tree service; https://www.arb-silva.de/aligner/) tool of SILVA online database.

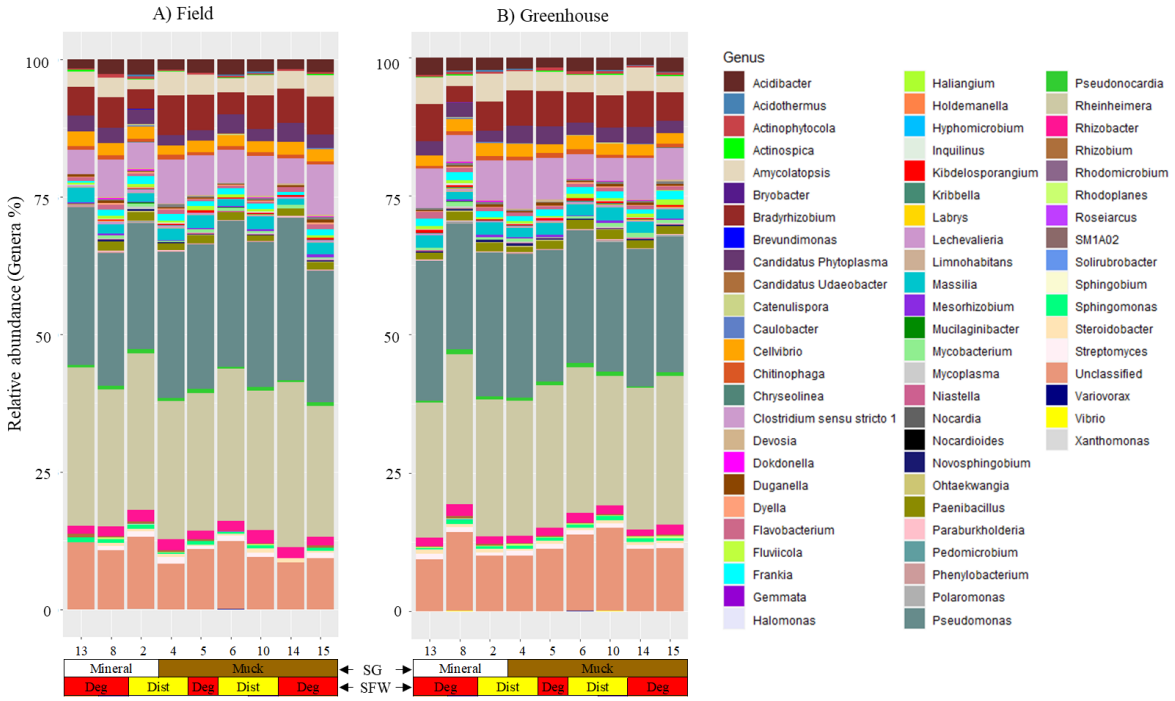

Figure 6.3 | Stacked bar plots showing the relative abundance of bacterial genera associated with (A) field and (B) greenhouse *M. hapla* populations originating from different soil groups (SG: muck [brown] and mineral [white]) and soil food web conditions (SFW: Deg: Degraded [red] and Dist: Disturbed [yellow]). Sixty genera were detected in both field and greenhouse populations, five (*Norcardia, Rhodomicrobium, Gemmata, Fluviicola and Vibrio*) in only field populations, and one (*Steroidobacter*) in only greenhouse populations. Other OTUs which were not assigned genera were labelled as unclassified. Colors of bacteria and fungi correspond with colors in the stacked bar plots and each bar represents a field. Relative abundance of bacterial genera was variable across the field and the greenhouse populations. Sequences were assigned to taxonomic groups using the ACT (alignment, classification, tree service; https://www.arb-silva.de/aligner/) tool of SILVA online database. Each numbered vertical bar of the plot represents an *M. hapla* population in the field or the greenhouse.

Functional groups

Sixty-five of the bacterial genera detected in the field and greenhouse *M. hapla* populations belonged to 14 functional bacterial groups (**Figure 6.4**; **Table 6.1**). The functional groups and the numbers of genera (in brackets) were: animal-pathogenic (2), anti_bacteria (3), anti-fungi (4), antibiotic-resistant (1), enhanced-nematode-parasitism (1), iron reducing (1), nematicidal (12), nitrogen_fixer (5), others (12), plant_growth_promoter (8), plant_pathogenic (5), polysaccharide-degrader (1), root_knot_nematode_associated (2), soybean-cyst-associated (1) and suppressive_soil bacteria (8) (**Table 6.1**). Another 12 identified genera (*Bryobacter, Catenulispora, Dokdonella, Fluviicola, Holdemanella, Hyphomicrobium, Inquilinus, Labrys, Limnohabitans, Pseudonocardia, Roseiarcus* and *SM1A02*) with little known functions were classified as other. The numbers of bacterial functional groups and genera had varying relative abundance with no clear trends between the field and greenhouse *M. hapla* populations (**Figure 6.4**; **Table 6.1**). The functional groups and genera are described as common to both sets of populations, varying, or specific presence or absence in certain field and greenhouse populations.

Table 6.1 | Functional groups microbial genera found in field and greenhouse *M. hapla* populations.

Functional groups	**Phyla**	**Genera**
Animal_pathogenic	Actinobacteria	*Mycobacterium*
	Bacteroidetes	*Flavobacterium*
Anti_bacteria	Actinobacteria	*Kibdelosporangium*
	Actinobacteria	*Lechevalieria*
	Proteobacteria	*Rheinheimera*
Anti_fungi	Actinobacteria	*Acidothermus*
	Proteobacteria	*Paraburkholderia*
	Bacteroidetes	*Chryseolinea*
	Proteobacteria	*Haliangium*
Antibiotic_resistant	Verrucomicrobia	*Candidatus Udaeobacter*
Enhance_nematode_parasitism	Proteobacteria	*Novosphingobium*
Iron_reducing	Proteobacteria	*Acidibacter*
Nematicidal	Proteobacteria	*Cellvibrio*
	Bacteroidetes	*Chitinophaga*
	Proteobacteria	*Devosia*
	Proteobacteria	*Duganella*
	Planctomycetes	**Gemmata*
	Actinobacteria	*Nocardioides*
	Proteobacteria	*Pedomicrobium*
	Proteobacteria	*Phenylobacterium*
	Actinobacteria	*Solirubrobacter*
	Actinobacteria	*Streptomyces*
	Proteobacteria	**Vibrio*
	Proteobacteria	*Xanthomonas*
Nitrogen_fixer	Proteobacteria	*Rhizobium*
	Proteobacteria	*Bradyrhizobium*
	Actinobacteria	*Frankia*
	Proteobacteria	*Mesorhizobium*
	Proteobacteria	**Rhodomicrobium*

*Microbial genera in field but not in greenhouse populations.

**Microbial genera in greenhouse but not in field populations.

All untagged genera were found in both field and greenhouse populations.

Table 6.1 (cont'd)

Functional groups	Phyla	Genera
Other	Acidobacteria	*Bryobacter*
	Actinobacteria	*Catenulispora*
	Proteobacteria	*Dokdonella*
	Bacteroidetes	**Fluviicola*
	Firmicutes	*Holdemanella*
	Proteobacteria	*Hyphomicrobium*
	Proteobacteria	*Inquilinus*
	Proteobacteria	*Labrys*
	Proteobacteria	*Limnohabitans*
	Actinobacteria	*Pseudonocardia*
	Proteobacteria	*Roseiarcus*
	Planctomycetes	*SM1A02*
Plant_growth_promoter	Actinobacteria	*Amycolatopsis*
	Proteobacteria	*Brevundimonas*
	Proteobacteria	*Caulobacter*
	Proteobacteria	*Halomonas*
	Bacteroidetes	*Mucilaginibacter*
	Actinobacteria	**Nocardia*
	Firmicutes	*Paenibacillus*
	Proteobacteria	*Sphingomonas*
Plant_Pathogenic	Firmicutes	*Candidatus Phytoplasma*
	Firmicutes	*Clostridium sensu stricto 1*
	Proteobacteria	*Dyella*
	Firmicutes	*Mycoplasma*
	Proteobacteria	*Rhizobacter*
Polysaccharide_degrader	Proteobacteria	*Sphingobium*
Root_knot_nematode_associated	Proteobacteria	*Pseudomonas*
	Proteobacteria	*Rhodoplanes*
Soybean_cyst_associated	Proteobacteria	*Polaromonas*
Suppressive_soils	Actinobacteria	*Actinophytocola*
	Actinobacteria	*Actinospica*
	Actinobacteria	*Kribbella*
	Proteobacteria	*Massilia*
	Bacteroidetes	*Niastella*
	Bacteroidetes	*Ohtaekwangia*
	Proteobacteria	***Steroidobacter*
	Proteobacteria	*Variovorax*

Animal_pathogenic (*Flavobacterium*)), anti-bacteria (*Lechevalieria* and *Rheinheimera*), nematicidal (*Cellvibrio, Chitinophaga* and *Duganella*), iron_reducing (*Acidibacter*), nitrogen-fixer (*Bradyrhizobium* and *Frankia*), other (*Pseudonocardia*), plant-growth-promoter (*Amycolatopsis* and *Paenibacillus*), plant-pathogenic (*Candidatus Phytoplasma, Clostridium sensu stricto 1* and *Rhizobacter*), root-knot nematode associated (*Pseudomonas*) and suppressive soil (*Actinophytocola* and *Massilia*) functional groups were present in the field and greenhouse populations (**Figure 6.4 A, B**). Animal-pathogenic (*Mycobacterium*), anti bacteria (*Kibdelosporangium*), anti_fungi (*Acidothermus, Chryseolinea, Haliangium* and *Paraburkholderia*) and enhanced_nematode_parasitism (*Novosphingobium*), nematicidal (*Streptomyces*), nitrogen_fixer (*Rhizobium*), plant_growth_promoter (*Sphingomonas*), and root_knot_nematode_associated (*Rhodoplanes*) were present in all fields but variablly in the greenhouse populations. Presence or absence of the antibiotic-resistant (*Candidatus Udaeobacter*), polysaccharide-degrader (*Sphingobium*) and the rest of the genera in the other 12 functional groups varied in both the field and greenhouse populations (**Figure 6.4 A, B**).

Population 13 had in common the presence of *Inquilinus* (other) in Populations 5 and 8 and plant_growth_promoters *Caulobacter* with Populations 5 and 15 in the field, and *Halomonas* with Populations 8 and 10 and *Limnohabitans* (other) with Populations 2, 4, 5 and 15 in the greenhouse. Population 13 had the absence of *Acidothermus* (anti-fungi), and *Mycoplasma* (plant_pathogenic) in the field and *Streptomyces* (nematicidal) in the greenhouse and *Devosia* (nematicidal) in the field and greenhouse in common with one or more of low PV category populations. The absence of *Mesorhizobium* (nitrogen_fixer) and *Ohtaekwangia* (suppressive soil bacteria) in the field, and *Devosia* (nematicidal) *Kibdelosporangium* (anti bacteria), *Mycobacterium* (animal pathogenic) and *Steroidobacter* (suppressive_soil bacteria) in the greenhouse was limited only to Population 13 (**Figure 6.4**

A, B).Presence of *Candidatus Udaeobacter* (antibiotic-resistant) and *Brevundimonas* and *Mucilaginibacter* (plant growth promoters), *Roseiarcus* (other), and *Nocardioides* and *Solirubrobacter* (nematicidal) in the field and *Hyphomycrobium* (other) in both field and greenhouse was limited to Population 8. Population 8 had plant growth promoters *Brevundimonas* and *Mucilaginibacter Niastella, Ohtaekwangia* and *Kribbella* (suppressive_soil bacteria), *Paraburkholderia* (anti fungi), *Mycoplasma* (plant pathogenic), *Chryseolinea* (anti-fungi), *Novosphingobium* (enhanced-nematode-parasitism), and *Rhodoplanes* (root_knot_nematode_associated) in the greenhouse, and , *Catenulispora* (other), *Gemmata, Pedomicrobium, Phenylobacterium, Xanthomonas* (nematicidal), *Rhodomicrobium* (nitrogen_fixer), *Sphingobium* (polysaccharide-degrader) and *Variovorax* (suppressive_soil bacteria) in the field, and *Dokdonella* and *Labrys* (others), *Nocardia* (plant_growth_promoters), *Polaromonas* (soybean-cyst-associated) in the greenhouse and field in common with one or more of the low PV category *M. hapla* populations (**Figure 6.4 A, B**).

Presence of *Chryseolinea* (anti-fungi) and *Fluviicola* (others) in the field, *Holdemanella* and *SM1A02* (others), *Nocardioides, Pedomicrobium, Solirubrobacter* and *Xanthomonas* (nematicidal), and *Sphingobium* (polysaccharide-degrader) in the greenhouse, *Bryobacter* (others) in both field and greenhouse populations were limited to Populations 2. Similarly, *Holdemanella* and *SM1A02* (others) in the field and *Roseiarcus* (other) in the greenhouse were specific to Population 15 and 6, respectively. Suppressive soil bacteria *Actinospica* in Populations 2, 4 and 14, and *Kribbella* in Populations 5 and 15 were absent in the field (**Figure 6.4 A, B**).

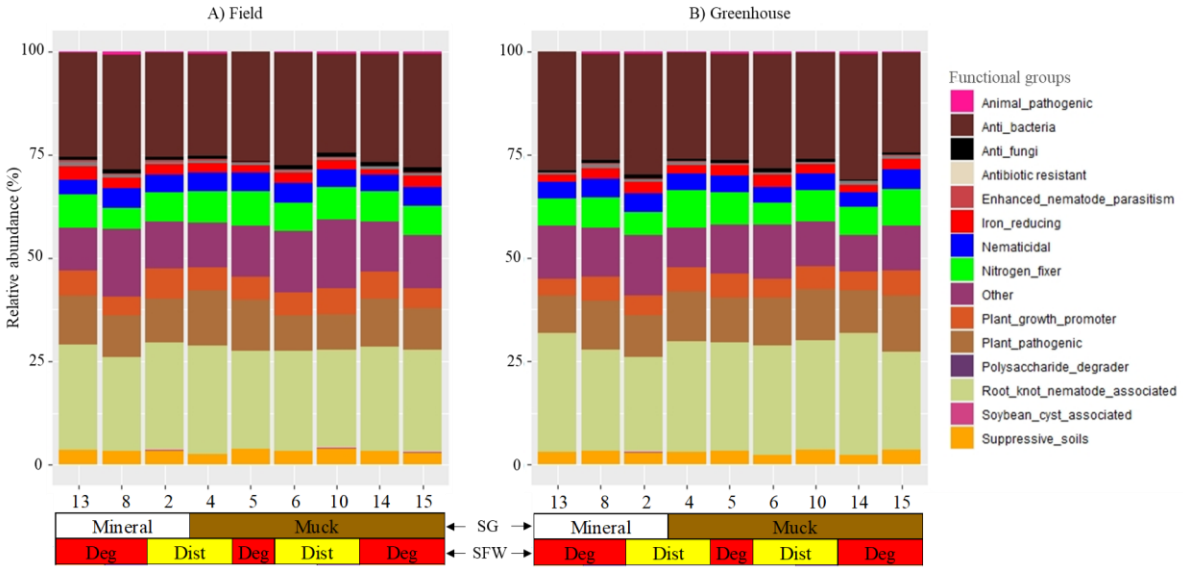

Figure 6.4 | Stacked bar plots showing the relative abundance of bacterial functional groups associated with field (A) and greenhouse (B) *M. hapla* populations originating from different soil groups (SG: Mineral [white] and Muck [brown]) and soil food web conditions (SFW: Deg-Degraded [red], Dist-Disturbed [yellow]). Greenhouse samples were isolated from tomato roots growing in a sterilized soil media for multiple generations. A list of bacterial genera assigned to each of the 15 functional groups are presented in Table 6.1. All genera which did not have a known function were grouped as "Other". Each numbered vertical bar of the plot represents an *M. hapla* population in the field or the greenhouse.

Alpha diversity

Alpha diversity was measured using the observed richness and Shannon diversity of bacterial communities associated with the field and greenhouse *M. hapla* populations (**Figure 6.5**). The observed richness and Shannon diversity were similar across the field populations (**Figure 6.5 A**). Neither soil group nor SFW conditions associated with the populations significantly influenced the richness and Shannon diversity. Similarly, the greenhouse populations had similar observed richness and Shannon diversity regardless of soil groups or SFW conditions previously associated with populations (**Figure 6.5 B**).

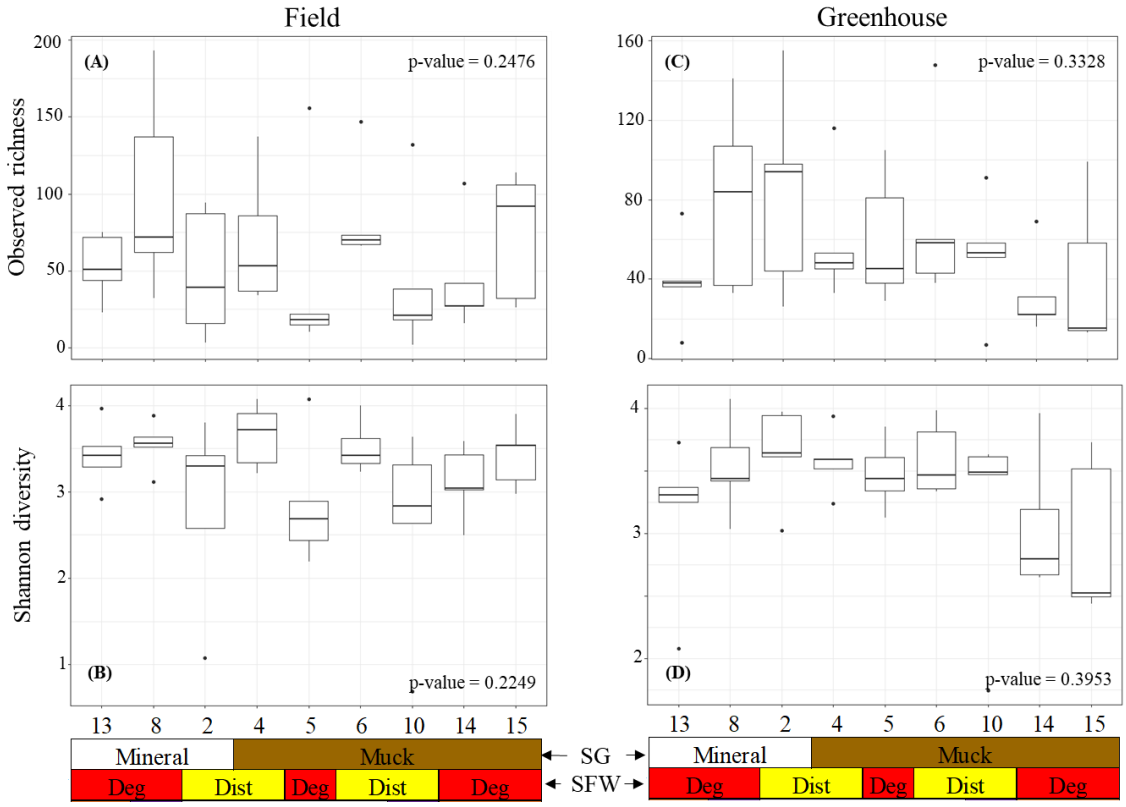

Figure 6.5 | Alpha diversity boxplots showing the bacterial (A) field observed richness, (B) field Shannon diversity, (C) greenhouse observed diversity, and (D) greenhouse Shannon diversity of *M. hapla* populations originating from different soil groups (SG: Mineral [white] and Muck [brown]) and soil food web conditions (SFW: Deg-Degraded [red], Dist-Disturbed [yellow]). Outliers on boxplots are displayed as dots. Kruskal Wallis tests were performed to determine significant differences across fields and p-values shown. Each numbered box plot represents an *M. hapla* population in the field or the greenhouse.

Beta diversity

The principal coordinate analysis (PCoA) of bacterial communities associated with either field or greenhouse *M. hapla* populations did not reveal any distinct patterns relative to soil groups and SFW conditions from where the soil samples were collected (**Figure 6.6 A, B**). Similarly, neither soil groups, SFW conditions or their interactions had a significantly different PERMANOVA (P>0.05; perm. = 9999) and homogeneity of variance results were

not significantly different (**Table 6.2**). However, a total of 45.4% variance (axis1: 25.2%, and axis2: 20.2%) and 42.7% variance (axis1: 22.7%, and axis2: 20.0%) could be explained by the graphs of field and greenhouse microbial communities, respectively (**Figure 6.6 A, B**).

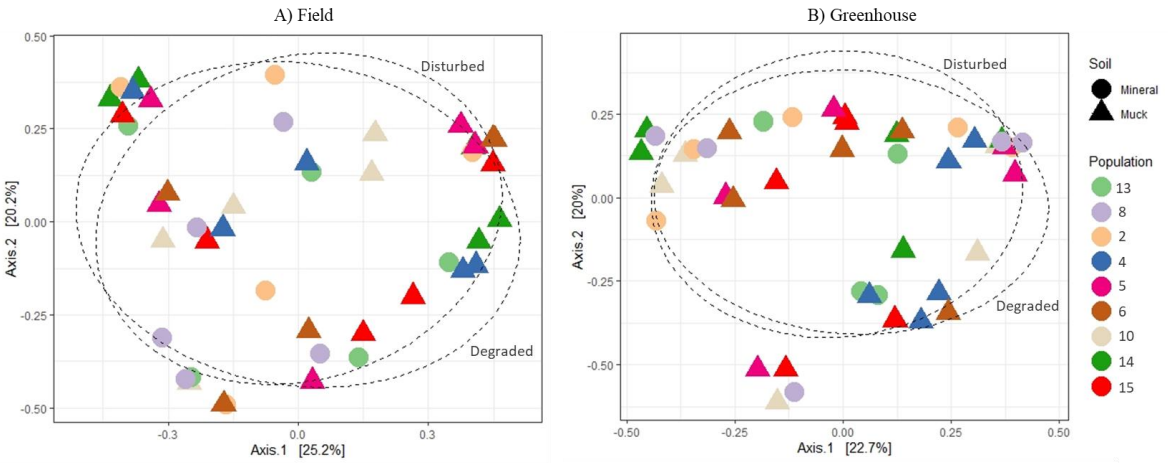

Figure 6.6 | Principal coordinates analysis plots, based on Bray-Curtis dissimilarity, of bacterial communities associated with, field (A) and greenhouse (B) *M. hapla* populations originating from different soil groups (muck - triangle and mineral - circle) and soil food web conditions (Degraded and Disturbed). Soil food web categories were separated with a 70% ellipse.

Table 6.2 | Permutational multivariate analysis of variance and multivariate homogeneity of groups dispersions analysis results microbial communities associated with field and greenhouse *M. hapla* based on soil group (SG), region (RG), soil health (SFW) conditions, and interactions.

	Field *M. hapla*					**Greenhouse *M. hapla***				
	PERMANOVA			**DISPERSION**		**PERMANOVA**			**DISPERSION**	
Variable	***F-value***	***R^2***	***P-value***	***F-value***	***P-value***	***F-value***	***R^2***	***P-value***	***F-value***	***P-value***
SG	1.101	0.025	0.347	2.23	0.627	0.472	0.011	0.879	0.008	0.922
RG	1.134	0.052	0.319	0.52	0.577	0.759	0.036	0.722	1.211	0.288
SFW	0.224	0.005	0.993	0.073	0.796	0.262	0.006	0.988	1.628	0.206
SG:SFW	0.775	0.054	0.755	0.095	0.963	0.662	0.046	0.885	0.727	0.529
SG:RG	0.859	0.099	0.697	0.221	0.942	0.517	0.062	0.994	0.497	0.78
SFW:RG	0.807	0.075	0.765	0.118	0.975	0.523	0.05	0.989	0.584	0.679
SG:RG:SFW	0.788	0.111	0.837	0.222	0.967	0.499	0.073	0.999	0.568	0.743

Significant P-values are indicated in bold

Discussion

This study aims to enhance our understanding of the relationships, or the lack, between *M. hapla* populations' PV and the biophysicochemical conditions of the belowground environment where the nematode thrives. If there is going to be any relationship between *M. hapla* PV and soil physicochemical conditions, it is likely to be from presence of conditions that directly or indirectly either enhance, hinder or their absence favors PV. From the PV standpoint, the *M. hapla* populations in this study fall into: a) stand alone or the highest, Population 13, b) medium, Population 8, and c) those with least reproductive potential, Populations 2, 4, 5, 6, 10, 14 and 15 (Chapter 4). Populations 2, 8 and 13 are from mineral soil and the rest are from muck soil. After establishing bacterial and fungal core-microbiomes and bacterial OTUs associated with either soil health conditions and/or *M. hapla* occurrence in the field (Chapter 5), we examined the composition and function of bacteria associated with field and greenhouse *M. hapla* populations. The results improve our understanding of the soil conditions in which *M. hapla* PV exists by describing the presence or absence of bacterial communities and functional groups common to both the field and the greenhouse populations, specific to either set of population, or variations within either population relative to the high, medium and low levels of reproductive potential.

Community diversity and composition

As indicated in the richness- and Shannon-based analyses, the bacterial communities showed no difference in their diversity in either the field or greenhouse, suggesting that the number of different species and the structure of bacteria were similar (Elhady et al., 2017). Similarly, the PCoA showed no pattern by population or soil group or conditions between the two sets of populations, suggesting that the bacterial diversity is independent of all soil group and SFW conditions (Schuelke et al., 2018). Although the diversity of the bacterial communities was

similar, the abundance and composition of the 65 genera in the field and 61 genera in the greenhouse populations belonging to nine phyla varied between and within the two sets of populations and more so at the genera than at the phyla levels.

The bacterial phyla and genera had varying proportions with no clear trend between the field and greenhouse populations. The phyla Actinobacteria, Bacteroidetes, Firmicutes, Myxococcota and Proteobacteria and the genera *Acidibacter, Actinophytocola, Amycolatopsis, Bradyrhizobium, Candidatus Phytoplasma, Cellvibrio, Chitinophaga, Clostridium sensu stricto, Duganella, Flavobacterium, Frankia, Lechevalieria, Massilia, Paenibacillus, Pseudomonas, Pseudonocardia, Rheinheimera, Rhizobacter* and the unclassified genera were common to all field and greenhouse *M. hapla* populations and at high proportions. Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Chloroflexi are known to be associated with *M. hapla* suppression (Topalović et al., 2022) and Verrucomicrobium synthesizing amino acids in the nematode *Xiphenema americanum* as a nutritional mutualist (Brown et al., 2015). Little is known about the role of Myxococcota and Planctomycetes relative to nematodes. While there is little published association of these phyla and/or genera and their varying proportions on *M. hapla* PV, it can be assumed that these bacterial communities may have the same effect on both sets of the populations (Topalović et al., 2019). On the other hand, the presence of the phyla Acidobacteria, Chloroflexi, Planctomycetes and Verrucomicrobia and the genera *Haliangium*, *Kibdelosporangium*, *Mycobacterium, Novosphingobium*, *Rhizobium*, *Rhodoplanes*, *Sphingomonas* and *Streptomyces* in all of the field, but variably in the greenhouse populations shows differences between the two sets of *M. hapla* populations. Whether or not the variable presence of these bacterial communities may be related to the observed PV differences among the populations (Chapter 4), remains to be determined.

However, presence or absence of the variable bacterial communities between the two sets of *M. hapla* populations appear to differ among the high (Population 13), medium (Population 8) and low PV category (Populations 2, 4, 5, 6, 10, 14 and 15) populations. For example, Population 13 only had the presence of *Inquilinus* in the field and *Halomonas* in the greenhouse in common with Population 8 and/or Populations 5 and 10, and *Caulobacter* and *Limnohabitans* in the greenhouse with two or more of the low PV category populations. Population 8, on the other hand, had *Catenulispora, Gemmata,Pedomicrobium, Rhodomicrobium, Sphingobium, Phenylobacterium, Variovorax* and *Xanthomonas* in the field, *Brevundimonas, Chryseolinea, Kribbella, Mucilaginibacter, Mycoplasma, Niastella, Novosphingobium, Ohtaekwangia, Paraburkholderia, Rhodoplanes* in the greenhouse, and *Dokdonella, Labrys, Nocardia* and *Polaromonas* in both field and greenhouse populations in common with one or more of the low PV category populations. This shows the medium and low PV category *M. hapla* populations have bacterial composition associated with them in common than with Population 13.

Population 13 did not have bacteria uniquely present with it, but Populations 8 and several of the low PV category populations did. These included the presence of *Brevundimonas, Candidatus Udaeobacter, Hyphomicrobium, Mucilaginibacter, Nocardioides, Roseiarcus* and *Solirubrobacter* in the field and *Hyphomicrobium* in the greenhouse was limited to Population 8, and *Chryseolinea* and *Fluviicola* in the field, *Holdemanella, Nocardioides, Pedomicrobium, Sphingobium, Solirubrobacter, SM1A02* and *Xanthomonas* in the greenhouse, *Bryobacter* to Population 2. While absence of *Streptomyces* in the greenhouse and *Acidothermus, Devosia, Limnohabitans* and *Mycoplasma* in the field were common to Population 13 and on or more of the low PV category populations, the absence of *Mesorhizobium* and *Ohtaekwangia* in the field and *Devosia, Kibdelosporangium, Mycobacterium* and *Steroidobacter* in the greenhouse were

unique to Population 13. Whether or not the absence of these genera in Population 13 have anything to do with its PV is yet to be determined. To better understand the associations of bacterial communities with *M. hapla* populations, however, it is worth considering the functions of all of the bacterial communities associated with all of the *M. hapla* populations.

Functional groups and their habitats.

Sixty-five of identified bacterial genera in the nine phyla represented 14 known functional groups and 12 genera were of unknown (other) functions. The 18 genera commonly associated with all of the field and greenhouse *M. hapla* populations belonged to nine known and one unknown functional groups. These were animal_pathogenic (*Flavobacterium*), anti-bacteria (*Lechevalieria* and *Rheinheimera*), nematicidal (*Cellvibrio, Chitinophaga* and *Duganella*), iron_reducing (*Acidibacter*), nitrogen-fixer (*Bradyrhizobium* and *Frankia*), other (*Pseudonocardia*), plant-growth-promoter (*Amycolatopsis* and *Paenibacillus*), plant-pathogenic (*Candidatus Phytoplasma, Clostridium sensu stricto 1* and *Rhizobacter*), root-knot nematode associated (*Pseudomonas*) and suppressive soil (*Actinophytocola* and *Massilia*) functional groups. *Flavobacterium* is a pathogen of the oriental beetle (*Blitopertha orientalis*), *Lechevalieria* produces rebeccamycin antibiotic and *Rheinheimera* toxins that kill *Euplotes aediculatus* (Schmidt et al., 2012; Chiellini et al., 2019). *Cellvibrio, Chitinophaga, Duganella, Pseudomonas, Actinophytocola* and *Massilia* are part of a bacterial consortium that negatively impact root-knot and cyst nematodes (Khan et al., 2016; Topalović et al., 2020; Toju and Tanaka, 2019). *Candidatus Phytoplasma* causes stunting and witches broom in several vegetable crops (Kumari et al., 2019), *Clostridium sensu stricto 1* causes soft rot disease of sweet potato (da Silva et al., 2019), and *Rhizobacter* gall disease of carrot (Goto and Kuwata, 1988). *Amycolatopsis* enhances plant growth by inhibiting charcoal rot disease caused by *Macrophomina phaseolina* (Gopalakrishnan et al., 2019) and *Paenibacillus* tomato

growth and root-mass production infested with *M. incognita* (Khan et al., 2008). *Acidibacter* is a mesophile that reduces iron (Falagán and Johnson, 2014) and *Bradyrhizobium* and *Frankia* are involved in plant root nodulation and nitrogen fixation (Elhady et al., 2020; Ghodhbane-Gtari et al., 2019). However, little is known about the role of *Pseudonocardia* in soil.

The genera that were common to the field populations, but variable in the greenhouse populations belonged to animal-pathogenic (*Mycobacterium*), anti bacteria (*Kibdelosporangium*), anti_fungi (*Acidothermus, Chryseolinea, Haliangium* and *Paraburkholderia*), enhanced_nematode_parasitism (*Novosphingobium*), nematicidal (*Streptomyces*), nitrogen_fixer (*Rhizobium*), plant_growth_promoter (*Sphingomonas*), and root_knot_nematode_associated (*Rhodoplanes*) functional groups. Given the diversity of bacteria, variations within functional groups are to be expected (Tang et al., 2021). *Mycobacterium* causes tuberculosis in cattle and *Kibdelosporangium* produces antibiotic substances like cycloviracins, aricidins and kibdelins (Grappel et al., 1985; Hlokwe et al., 2017; Shearer et al., 1986; Tomita et al., 1993). *Acidothermus* suppresses the activity of arbuscular mycorrhizal fungi, *Chryseolinea* suppresses *Fusarium* wilt of banana, *Haliangium* produces bioactive products against fungi and *Paraburkholderia* suppresses the root rot fungal pathogen *Cylindrocarpon destructans* (Colegate and Molyneux, 2007; Farh et al., 2015; Svenningsen et al., 2018). *Novosphingobium* synthesizes vitamin B12 which enhances *Pristionchus pacificus* parasitism against *Caenorhabditis elegans* (Akduman et al., 2020) and *Streptomyces* with biofumigation is lethal to *M. incognita* (Jin et al., 2019). *Rhizobium* fixes nitrogen in legumes for plant growth, *Sphingomonas* increases lateral roots and root hairs of *Arabidopsis thaliana* (Costa et al., 2021; Y. Luo et al., 2019) and *Rhodoplanes* was associated with *Meloigogyne* spp. (Engelbrecht et al., 2021). However, it is unknown if the variable presence of these genera in the greenhouse *M. hapla* populations is because of the

difference between the field and greenhouse soils or other undescribed biological associations or the lack of (Forero et al., 2019).

Out of the three levels of PV, the medium (Population 8) and low (Populations 2, 4, 5, 6, 10, 14 and 15) PV category populations had far more bacterial genera in common than the high PV category populations. For example, Population 8 alone had *Candidatus Udaeobacter* (antibiotic-resistant), *Nocardioides* and *Solirubrobacter* (nematicidal), *Brevundimonas* and *Mucilaginibacter* (plant growth promoters), and *Roseiarcus* and *Hyphomycrobium* (other functions). Moreover, the diversity of bacteria that Population 8 shared with the low PV category populations is particularly worth noting. These included *Chryseolinea* and *Paraburkholderia* (anti fungi), *Rhodoplanes* (root_knot_nematode_associated), *Novosphingobium* (enhanced-nematode-parasitism), *Kribbella* and *Variovorax* (suppressive_soil bacteria), *Polaromonas* (soybean-cyst-associated), *Brevundimonas* and *Mucilaginibacter Niastella, Nocardia* and *Ohtaekwangia* (plant_growth_promoters), *Gemmata, Pedomicrobium, Phenylobacterium, Xanthomonas* and, *Rhodomicrobium* (nitrogen_fixers), *Sphingobium* (polysaccharide-degrader) and *Catenulispora, Dokdonella* and *Labrys* (other). *Candidatus Udaeobacter* thrives in environments concentrated with antibiotics (Willms et al., 2020). *Nocardioides* has a negative relationship with *M. hapla* and *P. neglectus* numbers, and *Solirubrobacter* together with *Gemmata, Pedomicrobium, Phenylobacterium* and *Xanthomonas* have a negative impact on *Meloidogyne* spp (Sikder et al., 2021; Toju and Tanaka, 2019). *Brevundimonas* increases nitrogen intake to enhance potato growth, *Mucilaginibacter* promotes plant growth by enhancing rhizobacteria, *Rhodomicrobium* synthesizes enzymes that enable nitrogen fixation in plants, and *Nocardia* produces auxins that induce nodule-like structures to enhance the growth of *Casuarina glauca* (Fan et al., 2020; Ghodhbane-Gtari et al., 2019; Madigan et al., 1984; Naqqash et al., 2020). *Kribbella, Niastella, Ohtaekwangia* and *Variovorax* co-

operatively suppress the plant parasitic nematode *Heterodera glycines* (Topalović et al., 2020) and *Polaromonas* was detected in *Heterodera glycines* cyst (Nour et al., 2003). *Sphingobium* produces enzymes which allows sugars to be degraded (Balows et al., 1992; Wu et al., 2017). Given the amount of information available on the functional roles of different bacteria, not much was known about *Roseiarcus, Hyphomycrobium, Catenulispora, Dokdonella* and *Labrys*. While how the presence of these genera may or may not be related to the populations with medium and low PV categories is yet to be determined, these data provide basis for more targeted analyses of associations, or the lack of, among bacteria and *M. hapla* populations.

On the other hand, what Population 13 had in common with Population 8 and/or one or more of the low PV category populations were the presence of *Caulobacter* (plant_growth_promoter) and *Halomonas, Inquilinus* and *Limnohabitans* (other or unknown functions) and the absence of *Acidothermus* (anti-fungi), *Mycoplasma* (plant_pathogenic), and *Devosia* and *Streptomyces* (nematicidal) in the populations from the field and/or greenhouse. Without excluding how the presence or absence of these small number of bacterial genera may or may not interact with other factors to influence PV, we can assume that their effect on the high, medium, and low PV category populations could be similar. What separates Population 13 from the medium and low PV category populations is the unique absence of *Mesorhizobium* (nitrogen_fixer) and *Ohtaekwangia* (suppressive soil bacteria) in the field, and *Kibdelosporangium* (anti bacteria), *Steroidobacter* (suppressive_soil bacteria), *Devosia* (nematicidal) and *Mycobacterium* (animal pathogenic) in the greenhouse. *Caulobacter* increases root, leaf number and leaf size in *Arabidopsis thaliana* (D. Luo et al., 2019)mand *Mycoplasma* is a pathogen of plants like corn and citrus (Garnier et al., 2001). *Steroidobacter* in *Heterodera glycines* suppressive soils thrives in conditions with a relatively neutral pH and *Devosia* in *M. hapla* suppressive soils is

associated with fewer galls, egg masses, eggs, and a reduced rate of fecundity (eggs per egg mass) (Adam et al., 2014; Fahrbach et al., 2008; Topalović et al., 2020). *Mesorhizobium* is commonly associated with nodulation of legumes (Redding et al., 2018) and *Ohtaekwangia* is broadly adapted to aerobic, acidic and alkaline conditions (Yoon et al., 2011).

Connections with core and indicator groups

Analyses of microbiome in soils collected from the fields where the *M. hapla* populations were isolated from identified 39 core bacteria OTUs, defined as the most abundant and prevalent taxa across fields (Shade and Stopnisek, 2019), and 25 indicator OTUs, characterized by an increased occurrence or abundance associated with a group of sites of similar conditions (Cáceres and Legendre, 2009), associated with soil health conditions and/or *M. hapla* occurrence (Chapter 5).

Across all the field and greenhouse populations, one of the 11 core bacteria genera and none of the of 25 indicator bacterial genera were present in *M. hapla* populations. The complete absence of core bacteria *Kaistobacter* (enhanced nematode parasitism), *Reyranella* (soybean cyst associated), *Arthrobacter* and *Dactylosporangium* (suppressive soil), *Adhaeribacter, Turicibacter* and *Balneimonas* (other), and a similar absence of the indicator bacteria *Actinobacteria, Chloroflexi, Chloracidobacteria* and *Solirubrobacterales* (nematicidal), *Solibacterales* (plant growth promoter), *Balneimonas*, *Gemmatimonas* and *Actinoplanes* (suppressive soils), *Flavitalea populi* (plant pathogenic), *Afifella* and *Sphingobacteriales* (root knot nematode associated), *Sorangium* (polysaccharide degrader), and *Oxalicibacterium, Skermanella, Thauera, Syntrophobacteraceae, Gaiellaceae, Phycicoccus, Pirellulaceae* and *Pedosphaerales* (other) were noted. However, only the core bacteria *Paenibacillus* (plant growth promoter) was present in all the field and greenhouse populations (**Table 6.3; Table 6.4**). The core bacteria *Rhodoplanes* (root knot nematode

associated) and the indicator bacteria *Rhizobium* (nitrogen fixer) were common in all the field populations but variable in the greenhouse populations (**Table 6.3; Table 6.4**).

Population 13 with high PV had the unique absence of *Devosia* (nematicidal) core bacteria in the greenhouse, while no other core or indicator bacteria in the field and/or greenhouse were detected to be present or absent only in Population 13 (**Table 6.3; Table 6.4**).

Population 8 with medium PV had a limited presence of *Brevundimonas* (plant growth promoter) indicator bacteria in the field but other core or indicator bacteria in the field and/or greenhouse were not present or absent in only Population 8 (**Table 6.3; Table 6.4**).

Low PV category populations had *Sphingobium* (polysaccharide degrader) core bacteria present in the greenhouse in only Population 8, while the combination of one or more low PV category populations with Population 8 had *Sphingobium* core bacteria in the field and *Brevundimonas* (plant growth promoter) indicator bacteria in the greenhouse being present. Regardless of the presence or absence of the core or indicator bacteria, a variety of interactions between microbes initially detected in the soils and the *M. hapla* populations (**Table 6.3; Table 6.4**).

Table 6.3 | Taxonomy (Phylum, Class, Order, Family and Genera) and function of 25 bacterial indicators found in locations with *M. hapla* presence or absence.

Phylum	Class	Order	Family	Genera	Functional group
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Chloracidobacteria sp. (OTU2)*	Nematicidal
	Solibacteres	Solibacterales		*Solibacterales sp. (OTU10)*	Plant growth promoter
Actinobacteria				*Actinobacteria sp. (OTU14)*	Nematicidal
				Actinobacteria sp. (OTU18)	Nematicidal
	Actinobacteria	Micrococcales	Intrasporangiaceae	*Phycicoccus sp. (OTU19)*	Other
	Actinobacteria	Micromonosporales	Micromonosporaceae	*Actinoplanes sp. (OTU20)*	Suppressive soils
	Thermoleophilia	Solirubrobacterales		*Solirubrobacterales sp. (OTU21)*	Nematicidal
	Thermoleophilia	Gaiellales	Gaiellaceae	*Gaiellaceae sp. (OTU23)*	Other
Bacteroidetes	Chitinophagia	Chitinophagales	Chitinophagaceae	*Flavitalea populi (OTU5)*	Plant pathogenic
	Sphingobacteriia	Sphingobacteriales		*Sphingobacteriales sp. (OTU12)*	Root knot nematode associated
Chloroflexi				*Chloroflexi sp. (OTU17)*	Nematicidal
				Chloroflexi sp. (OTU24)	Nematicidal
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonales	Gemmatimonaceae	*Gemmatimonas sp. (OTU4)*	Suppressive soils
Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae	*Pirellulaceae sp. (OTU22)*	Other

Table 6.3 (cont'd)

Phylum	Class	Order	Family	Genera	Functional group
Proteobacteria	Deltaproteobacteria	Myxococcales	Sorangium	*Sorangium sp. (OTU1)*	Polysaccharide degrader
	Alphaproteobacteria	Hyphomicrobiales	Methylobacteriaceae	*Balneimonas sp. (OTU3)*	Suppressive soils
	Alphaproteobacteria	Hyphomicrobiales	Rhizobiaceae	[1]*Rhizobium sp. (OTU6)*	Nitrogen fixer
	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Kaistobacter sp. (OTU7)*	Enhanced nematode parasitism
	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Oxalicibacterium sp. (OTU8)*	Other
	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	[2]*Brevundimonas sp. (OTU9)*	Plant_growth_promoter
	Alphaproteobacteria	Rhodospirillales	Azospirillaceae	*Skermanella sp. (OTU11)*	Other
	Betaproteobacteria	Rhodocyclales	Zoogloeaceae	*Thauera sp. (OTU13)*	Other
	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	*Syntrophobacteraceae sp. (OTU15)*	Other
	Alphaproteobacteria	Hyphomicrobiales	Afifellaceae	*Afifella sp. (OTU25)*	Root knot nematode associated
Verrucomicrobiota	Verrucomicrobiae	Pedosphaerales		*Pedosphaerales sp. (OTU16)*	Other

[1]*M. hapla* microbe from all field Populations, and all but greenhouse Population 14.

[2]*M. hapla* microbe from field Populations 8, and greenhouse Population 8 and 2.

List of indicators adopted from a previous study by Chapter 5.

Table 6.4 | Taxonomy (Phylum, Class, Order, Family and Genera) and function of 11 core-bacteria found in locations with *M. hapla* presence or absence.

Phyla	Class	Order	Family	Genera	Functional group
Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	*Arthrobacter*	Nematicidal
	Actinobacteria	Micromonosporales	Micromonosporaceae	*Dactylosporangium*	Nematicidal
Bacteroidetes	Cytophagia	Cytophagales	Hymenobacteraceae	*Adhaeribacter*	Other
Firmicutes	Bacilli	Paenibacillales	Paenibacillaceae	[1] Paenibacillus	Plant_growth_promoter
	Erysipelotrichia	Erysipelotrichales	Turicibacteraceae	*Turicibacter*	Other
Proteobacteria	Alphaproteobacteria	Hyphomicrobiales	Methylobacteriaceae	*Balneimonas*	Other
	Alphaproteobacteria	Rhizobiales	Devosiaceae	[2] Devosia	Nematicidal
	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Kaistobacter*	Enhanced nematode parasitism
	Alphaproteobacteria	Rhodospirillales	Reyranellaceae	*Reyranella*	Soybean cyst associated
	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	[3] Rhodoplanes	Root_knot_nematode_associated
	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	[4] Sphingobium	Polysaccharide-degrader

[1] *M. hapla* microbe in all field and greenhouse Populations

[2] *M. hapla* microbe in field Populations 10 and 13, and all greenhouse Populations except in 13

[3] *M. hapla* microbe in all field Populations, and greenhouse Populations 2, 5, 10, 4 and 8

[4] *M. hapla* microbe in field Population 2

List of indicators adopted from a previous study by Chapter 5.

Conclusions

By determining the relationships, or the lack, between *M. hapla* populations' PV and associated bacteria this study established that the bacterial richness and Shannon diversity did not significantly differ across field and greenhouse isolated *M. hapla* populations. However, the variable bacteria genera composition and functional groups showed presence or absence relationships with *M. hapla* PV. Particularly, the highest PV of Population 13, the medium PV of Population 8 and the low PV of other populations did not all have the same bacteria composition and functional groups. These patterns seem to suggest that there may be differences in bacteria composition and could have a relationship with *M. hapla* PV. Therefore, this study lays the foundation in establishing potential cause-and-effect relationship of *M. hapla* PV.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Background

While it has been known that *M. hapla* is broadly distributed in Michigan vegetable production landscape and that populations from mineral soils had higher parasitic variability (PV) than those from muck soil (Melakeberhan et al., 2010), how all of these relate to the biophysiochemical conditions across time and space is unknown. These knowledge gaps made it difficult to know where to start to unravel the mechanisms of *M. hapla* PV. In order to address the goal of my dissertation, of understanding the biophysicochemical conditions of the soils where *M. hapla* PV exists, I laid out a research plan that was broad enough to account for the missing links and the complexities of the soils and soil conditions, and narrow enough to identify links that will lead to identifying potential routes towards understanding the mechanisms of *M. hapla* PV in Michigan agriculture (**Figure 7.1**). The emphasis on biophysicochemical conditions is based on the biology of *M. hapla*, which includes migration through the soil, and its eggs and infective second-stage juveniles coming in contact with all of the diversity and abundance of the soil macro- and micro-biome and the environment that they co-exist with. In addition, an understanding of *M. hapla* PV based on measuring the changes in biophysicochemical conditions will be a good platform to integrate the potential role of soil health. The totality of my observational and experimental research results have yielded two roadmaps answering the overarching question: Is *M. hapla*'s PV and broad distribution a function of adaptation and/or the association with, or the lack of, specific biophysicochemical factors? One roadmap will lead towards understanding potential role of biophysicochemical conditions on *M. hapla* PV and the other leads to developing soil health-based *M. hapla* management.

The roadmap towards understanding the biophysicochemical basis of *M. hapla* PV.

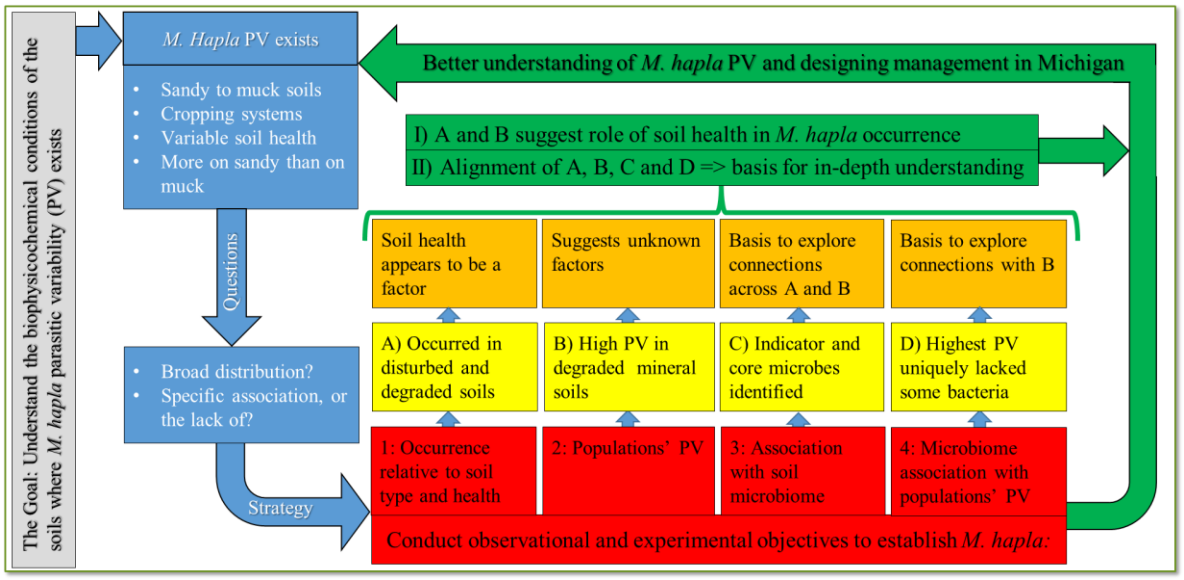

Figure 7.1 | A graphical abstract illustrating my project goal (grey box), the different soil conditions of sandy to muck soil, cropping systems, and the variable soil health conditions *M. hapla* PV exists, the sandy soil *M. hapla* populations with a higher parasitism than muck populations, research questions, and strategy (blue boxes and arrows), observational and experimental objectives (red boxes), key findings (yellow boxes), and conclusions (orange boxes) and how the findings (green box and arrows) collectively lead to understanding the potential biophysicochemical basis of PV and designing soil health management strategies.

The roadmap towards understanding the role of biophysicochemical conditions in *M. hapla* has four branches that converge into a complex web (**Figure 7.1**). The first two branches are the relationship between *M. hapla* distribution and PV of the populations collected from the mineral and muck soil fields. Describing that *M. hapla* populations from a number of mineral soil fields had highest PV (Chapter 3; Chapter 4) would be a confirmation of what has been well documented (Melakeberhan et al., 2010; Norton, 1978). However, describing the same PV information within the context of *M. hapla* occurrence in soils with disturbed and/or degraded soil health conditions (Chapter 3; Chapter 4) for the first time

opens a window into the environment where *M. hapla* exists. When soil health is out of balance, there are many cascading changes in the soil's biophysicochemical processes whose effect on the macro- and micro-biome (Díaz-Vallejo et al., 2021; Lammel et al., 2018; Pertile et al., 2021) and on nematodes in general is less understood, much less on *M. hapla* PV. At the very least, my research findings suggest that soil health may be a factor on *M. hapla* PV and distribution.

The third branch is identifying an association of core-bacteria and -fungi and indicator bacteria with *M. hapla* distribution and/or PV and soil health. While what these associations mean or how they individually and/or collectively may or may not relate to the mechanisms of *M. hapla* PV is yet to be determined, the data provide a starting point for developing a focused research in the future. For example, work by Topalović et al., (2022) suggest that the collective microbiome present in soil could be conducive or have a suppressive relationship on root-knot nematodes. In addition, studies by Adam et al., (2014) and Watson et al., (2020) have suggested a negative relationship between a plant parasitic nematodes ability to parasitize plants and the soil microbiome. In all these studies by other authors, the exact underlying mechanisms were not fully understood.

The fourth branch is how the *M. hapla* populations PV relate to bacteria isolated from the nematodes. As shown in Chapter 4, the *M. hapla* populations fall into three PV categories: high (Population 13), medium (Population 8) and low (Populations 2, 4, 5, 6, 10, 14 and 15). The medium and low PV category populations shared many bacteria in common while Population 13 specifically lacked *Mesorhizobium* and *Ohtaekwangia* in the field and *Devosia, Kibdelosporangium, Mycobacterium* and *Steroidobacter* in the greenhouse (Chapter 7). The fact that Populations 8 and 13 came from degraded mineral soil conditions seems to suggest a direct or indirect relationship of soil health condition on PV. Bringing together the

three branches will provide further insights as part of the roadmap towards understanding the biophysicochemical basis of *M. hapla* PV.

The four branches converge into a complex web that describes the alignment of the soil groups, soil health and biophysicochemical conditions and *M. hapla* PV reveal towards understanding potential mechanisms of the interactions. There are several layers to the alignment of the different factors.

First, a principal component analysis of physicochemical (sand, silt clay, pH, %OM, NO_3^-, NH_4^+) properties and soil health (degraded and disturbed) conditions of the *M. hapla* populations in three mineral and six muck soil fields revealed an important point. i.e. The two soil groups clustered separately with the low PV category populations being aligned with silt, clay, pH, %OM, NO_3^- and NH_4^+ in muck soils with disturbed and degraded soil health conditions; whereas, the high (Population 13) and medium (Population 8) PV groups favoring two of the three mineral soils, sand and degraded soil health conditions (**Figure 7.2**). Soil physical and chemical properties can either have a direct or indirect effect on nematodes (Melakeberhan, 1999; Melakeberhan et al., 2004; Norton, 1978). For example, *M. hapla* is known to be more virulent in sandy soils than in soils with high organic matter such as muck soils (Norton, 1978). Furthermore, soils with high nitrogen are associated with a lower reproductive potential of *M. hapla* (Melakeberhan et al., 2007).

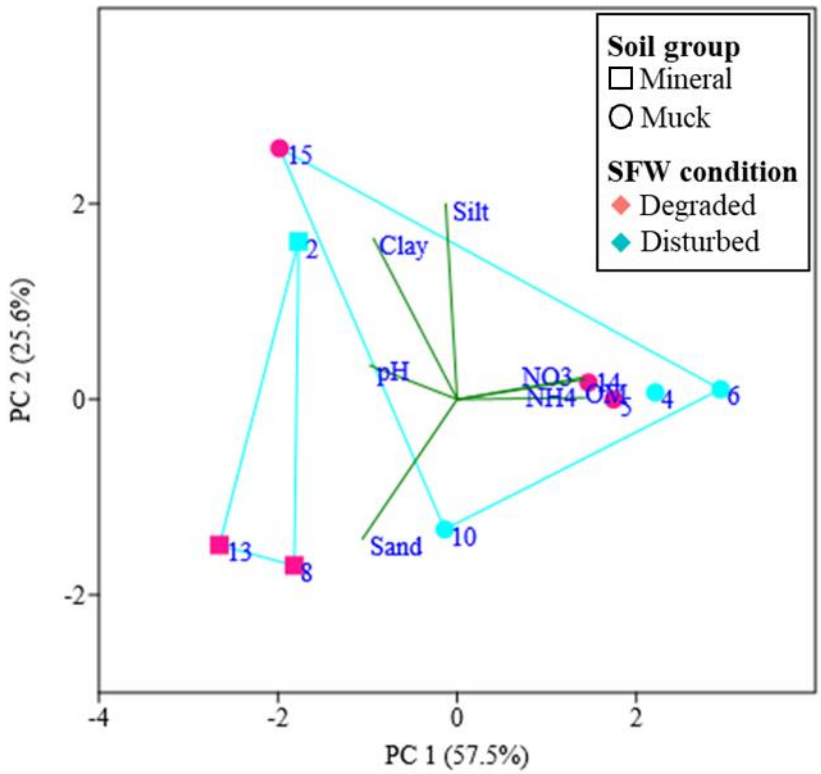

Figure 7.2 | Physicochemical relationships showing the analysis of the principal components of chemical (organic matter [OM], NO_3^-, NH_4^+ and pH) and physical (sand, silt and clay) properties of soil groups (mineral and muck) and soil health conditions (degraded and disturbed) in 9 agricultural fields with populations of *M. hapla.*

The second layer is the alignment of the biophysicochemical (microbial community, sand, silt clay, pH, %OM, NO_3^-, NH_4^+) and soil health (degraded and disturbed) conditions and the PV parameters (J2, J3.J4, adult, total, percent infectivity, galling) and inocula (2000 and 4000) of the *M. hapla* populations within a soil group (**Figure 7.3**). Because the soils had clustered separately, it is important to know how the different factors align relative to the high (Population 13), medium (Population 8) and/or low (Populations 2, 4, 5,6, 10, 14 and 15) PV category populations. In the muck soil where all of the populations had low PV, Population 4 was aligned with *Mycoplana, Pseudomonas* and silt, Populations 6 and 14 with *Saitozyma, Candidatus Koribacter, Bacteroides* and %OM, Population 10 with sand, *Kaistobacter, Geobacter* and *Alicyclobacillus,* and Populations 15 and 5 with *Bacillus,*

Nitrospirae, Afifella and clay (**Figure 7.3**). The biophysicochemical alignments of muck soil populations and their low PV seem to suggests they were all affected in a similar way.

In the mineral soil, each population was aligned with different biophysicochemical parameters (**Figure 7.3**). Population 13 was aligned with *Fusarium, Mortierella, Phythium, Phormidium* and *Arthrobacter*, Population 8 with *Rheinhimera* and Population 2 with silt, *Dactylosporangium, Flavobacterium and Candidatus Koribacter*. The distinct biophysicochemical alignments seem to suggest that the differences in PV were related to different soil parameters. On the other hand, Population 13 and 8 with the high and medium PV, respectively, had alignments with only microbes while the low PV of Population 2 had alignments with both microbes and physicochemical (i.e. silt) condition. This suggests that the silt content seem to favor the microbes closely aligned with Population 2.

Between the mineral and the muck *M. hapla* populations, silt as an individual parameter seem to be associated with low PV between Population 2 from mineral soil and Population 4 from muck. In both scenarios, silt seem to have a negative relationship with PV (**Figure 7.3**).

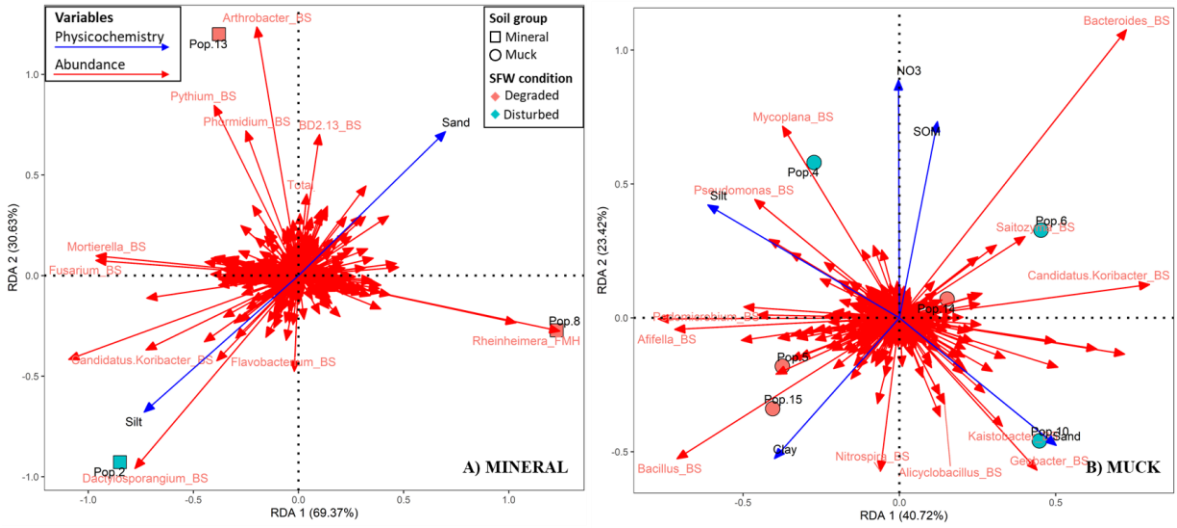

Figure 7.3 | Redundancy analysis ordinations showing 9 *M. hapla* populations [(A) mineral = 13, 8, 2, and (B) muck 4, 5, 6, 10, 14, 15)] relative to physicochemical parameters (blue arrow: NH_4^+, NH_3^-, pH, soil organic matter - som, sand, silt, and clay) and the abundance (red arrow) of the bacterial phyla of isolated field *M. hapla* nematodes and *M. hapla* parasitic variability parameters of *M. hapla* (J2, J3.J4, adult, total, percent infectivity, galling). The ending of each bacterial and fungal label for bulk soil (_BS), isolated field *M. hapla* (_FMH), and isolated greenhouse *M. hapla* (_GMH) is used to identify the source of the microbe.

The third layer is the alignment of the microbial community isolated from individual field and greenhouse cultures, soil health (degraded and disturbed) conditions and the PV parameters (J2, J3.J4, adult, total, percent infectivity, galling) and inocula (2000 and 4000) within a soil group (**Figure 7.4**). In the muck soil, Population 15 was aligned with J2 and total nematodes, Population 10 and 5 with *Acidothermus*, *Actinophytocola*, *Acidibacter*, *Actinospica*, adult, total infection, gall and J2 nematodes, Populations 4 and 6 with adult, total nematodes and total infection and *Bradyrhizobium*, and Populations 6 and 14 with J3/J4 nematodes and gall (**Figure 7.4**). This suggests that whether both microbes and PV parameters or only PV parameters were aligned with muck soil populations the PV was low.

In the mineral soil, the three populations were clustered apart from each other (**Figure 7.4**). Population 2 was associated with total, J3.J4, total infection and gall, Population 13 with adult nematodes, and Population 8 with total infection, total, J3.J4 nematodes and *Acidibacter*. *Acidibacter* is a mesophile that reduces iron (Falagán and Johnson, 2014) was common to all fields and greenhouse isolated *M. hapla* populations (Chapter 7), however, it's association with Population 8 in **Figure 7.4** seem to suggest it was less important to Population 13 and 2. The difference in abundance seems to be somehow related with the medium PV of Population 2. Additionally, the mineral population separation by between degraded and disturbed conditions along the RDA 1 axis suggests there could be some relationship between the PV outcomes of the high PV populations (13 and 8) and the low PV Population 2.

The patterns of microbial and PV alignments between the mineral and the muck soil *M. hapla* populations seem to be distinct(**Figure 7.4**). Across the muck and mineral soil, Not all the muck and mineral *M. hapla* populations had both PV parameter and microbial alignments. For example, mineral Population 8 was aligned with total infection, total, J3.J4 nematodes and *Acidibacter* but muck Populations 6 and 14 with J3/J4 nematodes and gall (**Figure 7.4**). This seem to suggest that different PV parameters relating to *M. hapla* populations were not affected equally by microbes and likely associated with PV differences.

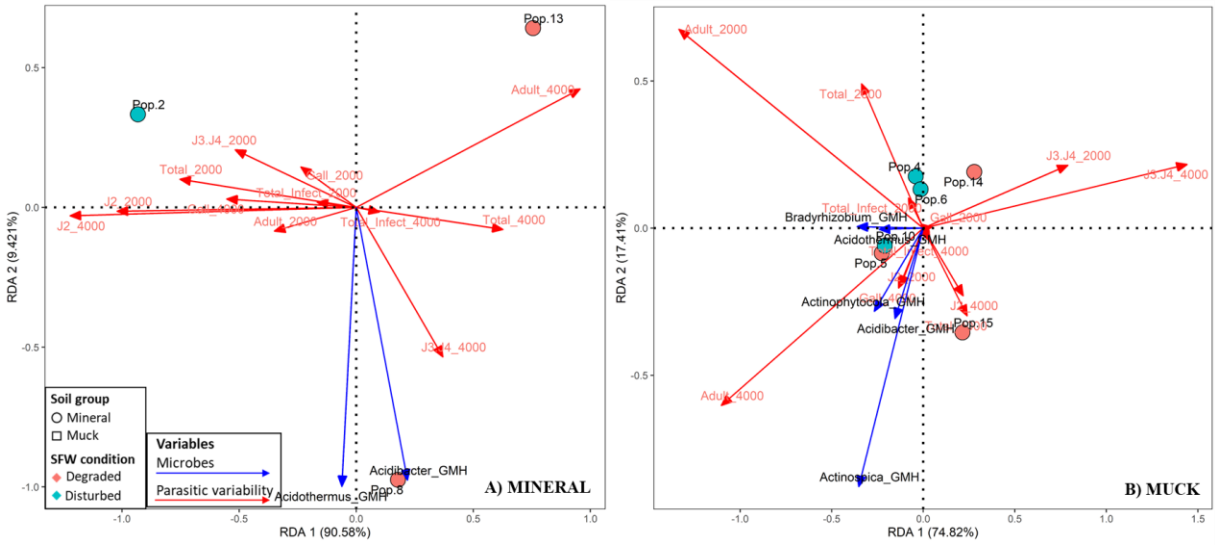

Figure 7.4 | Redundancy analysis ordinations showing 9 *M. hapla* populations [(A) mineral = 13, 8, 2, and (B) muck 4, 5, 6, 10, 14, 15)] relative to physicochemical parameters (blue arrow: NH4, NH3, pH, Organic matter - SOM, Sand, Silt and clay) and the abundance (red arrow) of bacterial and fungal genera from bulk soil, bacterial genera of isolated field and greenhouse *M. hapla* nematodes, and parasitic variability parameters (J2, J3/J4, Adult, Total, Percent infectivity, Galling) at two levels of inoculum levels (2000 and 4000). The ending of each bacterial and fungal label for bulk soil (_BS), isolated field *M. hapla* (_FMH), and isolated greenhouse *M. hapla* (_GMH) is used to identify the source of the microbe.

The fourth layer is an alignment of all of the biophysicochemical (microbial community, nematode trophic groups, sand, silt clay, pH, %OM, NO_3^-, NH_4^+) and soil health (degraded and disturbed) conditions and the PV parameters (J2, J3.J4, adult, total, percent infectivity, galling) and inocula (2000 and 4000) of the *M. hapla* populations across the muck and mineral soil groups included in the previous three layers (**Figure 7.5**). The muck soil populations were clustered separately from the mineral populations, suggesting that both soil groups were biophysicochemically different. Within the muck soil, Populations 6, 10 and 14 clustered with NH_4^+, NO_3^-, OM, *Bacteroides, Pseudomonas, Rheinheim*era and *Myrmecodia.* and Populations 4, 5 and 15 with silt, *Mycoplana, Afifella, Pedomicrobium* and *BD2.13*,

suggesting that these factors maybe directly or indirectly contributing to the low PV in muck soil.

A higher soil nitrogen, was previously found to be associated with a lower reproductive potential in *M. ha*pla (Melakeberhan et al., 2007) just like a high soil OM content is associated with low PV (Melakeberhan et al., 2010). *Bacteroides* was associated with *Heterodera glycines cyst* whereas, *Afifella and Pseudomonas* were root-knot nematode associated (Khan et al., 2016; Topalović et al., 2020). *Rheinheim*era had anti-bacterial properties and *Pedomicrobium* has nematicidal effect on nematodes (Chiellini et al., 2019; Toju and Tanaka, 2019). However, little is known about *Myrmecodia, Mycoplana* and *BD2.13.*

Within the mineral soil populations of *M. hapla*, Populations 2 and 13 aligned with pH, clay and *Bacillus* and Population 8 with sand, *Kaistobacter, Burkholderia, Dactylosporangium* and *Actinoallomurus.* Although Populations 2 (disturbed) and 13 (degraded) degraded soils have similar alignment with biophysicochemical conditions, they have extreme difference in PV. How these similarities and differences of alignments may affect PV remains unknown.

When microbes and physicochemical parameters are considered individually, *Bacillus, Burkholderia, Dactylosporangium* and *Actinoallomurus* are known to have antagonistic effect on PPNs (Farh et al., 2015; Ghahremani et al., 2020; Takeuchi et al., 1988; Topalović et al., 2020) while *Kaistobacter* enhance parasitism of PPN (Castillo et al., 2017). Acidic soil pH is known to negatively impact nematode population dynamics (Melakeberhan et al., 2004). Similarly, the fine soil texture of clay and silt with smaller air pores are less conducive environments for nematodes (Melakeberhan et al., 2010; Norton, 1978).

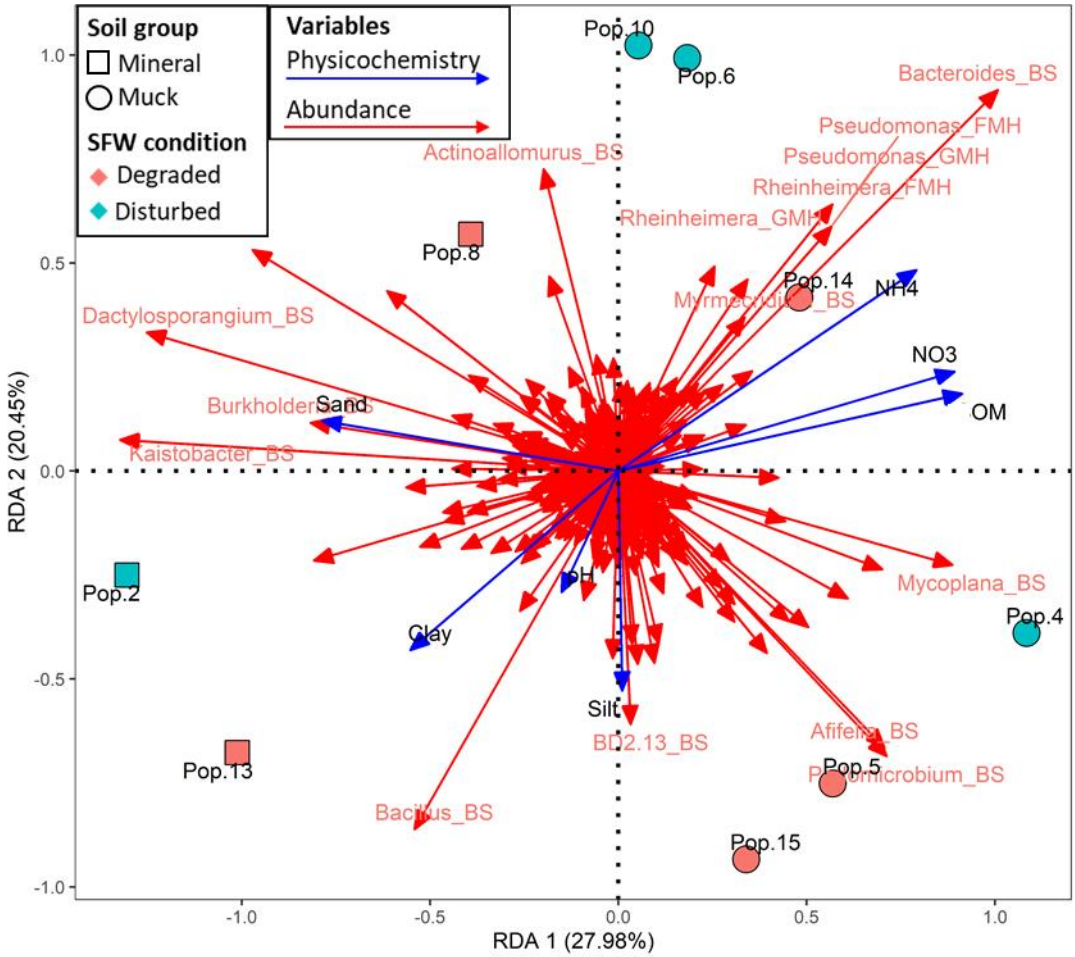

Figure 7.5 | RDA ordinations showing 9 *M. hapla* populations (mineral = 13, 8, 2, and muck 4, 5, 6, 10, 14, 15) relative to physicochemical parameters (blue arrow: NH_4^+, NO_3^-, pH, Organic matter - OM, Sand, Silt and Clay) and the abundance (red arrow) of bacterial and fungal genera from bulk soil, bacterial genera of isolated field and greenhouse *M. hapla* nematodes, trophic groups of the nematode community (Herbivore - HV, Bacterivore - BV, Fungivore - FV, Omnivore - OV, Predator – PR, Sum of all trophic groups - SUM, Free-living – SFL, Free-living from cp2 to cp5 – SFL25, sum of cp2 to cp5 – S25), parasitic variability parameters (J2, J3.J4, Adult, Total, Percent infectivity, Galling) at two inoculum levels (2000 and 4000 and enrichment (EI) and structural (SI) indices. The ending of each bacterial and fungal label for bulk soil (_BS), isolated field *M. hapla* (_FMH), and isolated greenhouse *M. hapla* (_GMH) is used to identify the source of the microbe.

From the three layers and the four branches that converge into a complex web, soil groups seem to be a major distinguishing factor in the broad perspective (**Figure 7.5**). However, when specific soil factors within each soil group are considered, it becomes evident that there are specific soil biophysicochemical factors which are aligned with the three levels of PV. Also, when the focus begins to shift from broad (**Figure 7.5**) to narrow (**Figure 7.3; Figure 7.4**) soil biophysicochemical relationships, the role of soil health in distinguishing high, medium, and low PV becomes apparent in the mineral soil. On the other hand, the lack of soil health related separation in **Figure 7.5** seems to suggest that considering nematode trophic group abundance as a biophysicochemical factor could complicate PV relationships. Thus, formulating future follow-up studies to determine the relationship between PV and the soil environment may not require trophic group abundance as a soil factor of interest.

The roadmap towards designing soil health-based *M. hapla* management.

Knowing the environment where an organism exists is an important element in designing conditions that favor or do not favor the organism's success in that environment. The second roadmap relates to potential role of soil health conditions in *M. hapla* distribution and/or its PV and what the data suggest in terms of the management strategies. As discussed in Chapter 1, soil health has physical, biological and chemical components. With regards to the chemical component, an investigation of the relationship between soil nitrogen (NO_3^- and NH_4^+) relative to soil groups revealed more nitrogen in the infested muck than in the mineral soils (**Table 7.1**). The *M. hapla* infested muck soils had an average NO_3^- content of between 57 to 356 mg/L, and NH_4^+ from 5 to 11 mg/L, while infested mineral soils had NO_3^- content between 9 and 40 mg/L, and NH_4^+ between 3 and 5 mg/L. These differences seem to be related to the high organic matter content of muck soils and the capacity to retain more soil nutrients than the mineral soils (Murphy, 2015).

A further look at the relationship between the soil nitrogen content and the assigned soil health condition of *M. hapla* infested mineral soils showed inconsistent trends (**Table 7.1**). In the degraded mineral soils, Population 13 had NO_3^- of 9 mg/L and NH_4^+ of 3 mg/L, and Population 8 with 40 mg/L and 5 mg/L, respectively. On the other hand, the disturbed condition of Population 2 had an NO_3^- content of 19 mg/L and an NH_4^+ of 4 mg/L. The low nitrogen content associated Population 13 was consistent with the nitrogen-depleted status of degraded soil conditions. Although more nitrogen is expected in the disturbed conditions of Population 2, there was more soil nitrogen in the degraded conditions of Population 8. This seems to be inconsistent with the nitrogen-enriched soil status expected of disturbed conditions versus the nitrogen depleted condition of degraded soils. While the NO_3^- and the NH_4^+ soil content were inconsistent for Population 8 and 2, other unaccounted factors may explain this trend. Both NO_3^- and the NH_4^+ only accounted for the inorganic nitrogen. However, the absence of the soil organic nitrogen values seems to explain the disparity.

Meloidogyne hapla infested muck soils with degraded conditions had an average NO_3^- content of 71 mg/L to 192 mg/L and the NH_4^+ of 5 mg/L to 9 mg/L, whereas the disturbed conditions were associated with 57 mg/L to 356 mg/L and 7 mg/L to 11 mg/L, respectively. Overall, the low nitrogen-enrichment in degraded soils is consistent with the soil nutrient availabilty description of Ferris et al., (2001).

Table 7.1 | Means and standard deviation (±) concentration of nitrate (NO_3^-) and ammonium (NH_4^+) present in *Meloidogyne hapla* infested fields across regions (east, southwest and northwest), soils groups (mineral and muck) and soil foodweb conditions (SFW: degraded and disturbed).

Region	Soil group	SFW	Field	NO_3^- (mg/L)	NH_4^+ (mg/L)
East	Mineral	Disturbed	2	19 ± 6	4 ± 1
	Muck		4	289 ± 38	8 ± 2
		Degraded	5	192 ± 23	8 ± 1
		Disturbed	6	356 ± 16	11 ± 1
Southwest		Degraded	8	40 ± 0	5 ± 0
		Disturbed	10	57 ± 11	7 ± 1
Northwest	Mineral	Degraded	13	9 ± 2	3 ± 0
	Muck		14	156 ± 34	9 ± 1
			15	71 ± 6	5 ± 1

Based on the PV characteristics of the 9 *M. hapla* populations mentioned in Chapter 4 and the soil health conditions they existed in allows creation of a framework for developing a management strategy. Since the *M. hapla* populations could be grouped into high (Population 13), medium (Population 8) and low (Populations 2, 4, 5, 6, 10, 14, 15) PV categories, the management strategy would encompass using the soil health conditions and biophysicochemical relationships identified to manage *M. hapla* PV in Michigan agriculture.

Manipulating the soil biology to reduce PV is one approach that can be used to manage *M. hapla* nematodes. As mentioned in Chapter 4, both the high PV of Population 13 and the medium PV of Population 8 were associated with degraded soils, while the low PV of Population 2 was related to disturbed conditions. This seems to suggest that the disturbed mineral soil condition was a less conducive environment. Contrary to what was observed for the mineral populations, no such relationship existed for *M. hapla* populations from muck soil. Muck populations from degraded conditions had a similar PV as those from disturbed

conditions. This suggests that the management of *M. hapla* PV should be the tailored for each soil group.

As soil health becomes out of balance, it becomes difficult to generate vital ecosystem services like *M. hapla* suppression. While several studies attest to the role of soil micro- (eg. bacteria) and macro-biome (eg. nematodes) in root-knot nematode suppression (Pyrowolakis et al., 2002; Sánchez-Moreno and Ferris, 2007; Topalović et al., 2020), similar conditions may be required to manage *M. hapla* PV. For example, a balanced soil health condition (Quadrant B) has an adequate representation of predators which can feed on other nematodes like *M. hapla* to control their numbers (Sánchez-Moreno and Ferris, 2007). Moreso, the presence of beneficial microbes like *Pasteuria penetrans* in the soil can control *M. hapla* numbers (Davies and Williamson, 2006). Similarly, in Chapter 7, I identified several microbes associated with nematode suppression like *Actinophytocola, Actinospica, Kribbella, Massilia, Niastella* and *Ohtaekwangia*. Since these were associated with isolated *M. hapla* populations, the management strategy would focus on creating conducive conditions for these potentially beneficial microbes. Some strategies reported to encourage the growth of beneficial microbes include introducing microbes as inoculants (Elnahal et al., 2022), and encouraging mixed cropping instead of monoculture (Eisenhauer et al., 2013, 2012).

In addition to managing soil health conditions in soils infested with *M. hapla*, it is equally important to consider what crops are chosen as part of a crop rotation. Overall, the biophysicochemical changes in this study could be the basis for formulating soil health-based management of *M. hapla* in the regions where it exists. However, one has to be mindful that biophysicochemical changes observed here in are heavily influenced by dicot and monocotyledon crops (Chapter 3) as well as their use and sequence in cropping systems. With dicotyledons being excellent host crops for *M. hapla* than monocotyledons (Widmer et al., 1999), the vegetable production industry of Michigan in particular where dicotyledenous

crops dominate is continually threatened. Thus, it makes sense that the right choice of crops in *M. hapla* infested fields could positively contribute to the management effort.

Conclusions

In conclusion, this project's aim was to know if *M. hapla*'s PV and broad distribution was a function of broad adaptation and/or the association with, or the lack of, specific biophysicochemical factors. Based on the synthesis of the soil health conditions *M. hapla* populations existed and the biophysicochemical relationships connected with PV, several points were deduced from the two roadmaps. The first roadmap found a) the broad distribution of *M. hapla* PV in disturbed and degraded conditions of mineral and muck soils being aligned with several distinct biophysicochemical factors, b) that different biophysicochemical alignments were related to high, medium and low *M. hapla* reproductive potential in the mineral soil, but different alignments in the muck soil were related to low reproductive potential, and c) the soil groups and soil health conditions populations existed had a relationship with *M. hapla* PV. The second roadmap showed d) the combination of soil group and the soil health conditions could form the basis of any *M. hapla* PV management strategy, e) that although the same soil health conditions were associated with *M. hapla* populations between the muck and mineral soil groups the management outcomes were different. Thus, this project established the basis for an integrated soil group and soil health strategy needed to design management strategies that may address *M. hapla* PV.

APPENDICES

MICHIGAN STATE
UNIVERSITY

DETERMINED NOT "HUMAN SUBJECTS"

November 30, 2018

To: Haddish Melakeberhan

Re: **MSU Study ID:** STUDY00001796
Principal Investigator: Haddish Melakeberhan
Determination Date: 11/30/2018

Title: Understanding Northern Root-Knot Nematode (Meloidogyne hapla) Parasitic Variability through the Multidisciplinary Approaches of Soil Biome and Environment.

The activity described in this submission was determined not to involve "human subjects" as defined by the U.S. Department of Health and Human Services (DHHS) regulations for the protection of human research subjects.

Definition of Human Subject
For DHHS, "human subject" means "a living individual about whom an investigator (whether professional or student) conducting research obtains: (1) Data through intervention or interaction with the individual, or (2) Identifiable private information." [45 CFR 46.102(f)].

Determination
This project will collect soil samples and environmental data in order to study the plant parasitic nematode Meloidogyne hapla parasitic variabilty. This activity is not about living individuals and no research data will be collected through any interactions or interventions with human subjects.

Hence, the activity does not involve human subjects.

Therefore, the federal regulations for the protection of human subjects would not apply to this activity and Michigan State University (MSU) Institutional Review Board (IRB) approval is not needed to proceed. However, please note that while MSU IRB approval is not required, other federal, state, or local regulations or requirements or ethical or professional standards may still be applicable based on the activity.

Modifications: If any of the activities described in this submission change, please contact the IRB office as the activity may involve human subject research and require IRB approval. For example, this determination is not applicable to activities that may be regulated by U.S. Food & Drug Administration (FDA), such as those involving drugs, medical devices, human food additives, color additives, electronic products, or any other test articles regulated by the FDA.

Modifications to Funding: Changes in funding may alter this determination. For example, MSU IRB review and approval is required if MSU receives an award through a grant, contract, or cooperative agreement directly from a federal agency,

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even where all non-exempt research involving human subjects are carried out by employees or agents of another institution.

For More Information: See HRPP Manual Section 4-3, Determination of Human Subject Research (available at hrpp.msu.edu).

Contact Information: If we can be of further assistance or if you have questions, please contact us at 517-355-2180 or via email at IRB@msu.edu. Please visit hrpp.msu.edu to access the HRPP Manual, templates, etc.

APPENDIX B | Soil sampling data sheet/questionnaire responses for 15 agricultural fields

Table B1 | General field data of the 15 agricultural fields collected from regions (east, southwestern and northwestern) and soil groups (mineral and muck) of Michigan showing the latitude, longitude, crop-rotation status, nutrient source, tillage, soil conditions and comments.

Region	Soil group	Field	Latitude	Longitude	Crop Rotation					Nutrient Source
					Yes/ No	Cereal	Legume	Cover Crops	Vegetables	
East	Mineral	1	43.068871°	-83.341871°	Yes	Barley	NA	NA	Several*	NA
		2	43.092822°	-83.053522°	Yes	NA	Soybean	NA	NA	Inorganic
		3	43.071761°	-83.032147°	Yes	Oats	NA	NA	NA	Inorganic
	Muck	4	43.065446°	-83.067139°	Yes	NA	NA	NA	Onion	NA
		5	43.050561°	-83.068175°	Yes	Corn	NA	NA	NA	NA
		6	43.094628°	-83.064396°	Yes	Corn	NA	NA	NA	NA
Southwest	Mineral	7	42.664865°	-86.047077°	Yes	Corn	NA	NA	NA	NA
		8	42.108973°	-86.368160°	NA	NA	NA	NA	NA	NA
		9	42.083001°	-86.367671°	Yes	Wheat	NA	Hairy vetch	NA	NA
	Muck	10	42.660808°	-85.996822°	Yes	Barley	NA	Sorghum	Several**	Inorganic
Northwest	Mineral	11	43.840772°	-86.348123°	Yes	NA	NA	NA	Carrot	Inorganic
		12	43.826522°	-86.378970°	Yes	Corn	NA	NA	NA	Organic
		13	43.764887°	-86.137222°	Yes	Wheat	NA	NA	NA	Inorganic
	Muck	14	43.351260°	-85.726828°	Yes	NA	NA	NA	Several***	Inorganic
		15	43.197608°	-85.782483°	Yes	NA	Soybean	NA	Several****	Inorganic

Table B1 (cont'd)

Region	Soil group	Field	Tillage		Soil condition	Comments
			[a]Conventional/ [b]Conservation	Frequency in a year		
East	Mineral	1	Conventional	1	Dry	Plastic mulch, drip irrigation & 45 to 50 years landuse
		2	Conventional	NA	Dry	
		3	Conventional	NA	Dry	Oats mixed with weeds
	Muck	4	Conventional	NA	Dry	Soil surface dry but wet underneath
		5	Conventional	NA	Moist	
		6	Conventional	NA	Moist	
Southwest	Mineral	7	Conventional	NA	Dry	
		8	Conventional	NA	Dry	Field converted to an orchard
		9	Conventional	NA	Dry	
	Muck	10	Conventional	NA	Dry	NPK 5-12-42, fungicide & herbicide applied
Northwest	Mineral	11	Conventional	NA	Dry	Field has corn plowed into soil
		12	Conventional	1	Moist	Green manure applied
		13	Conventional	NA	Dry	
	Muck	14	Conventional	NA	Dry	
		15	Conventional	1 or 2	Dry	Lorax, vydate, potash and urea applied to field

* Onion, parsnips, turnip, tomato, pepper, sugar beet, ** Celery, onion, radish, *** Parsnip, sugar beet, **** Carrot, parsnip, sugar beet,

[a]Conventional tillage has 100% disturbance, [b]Conservation tillage is either strip till, ridge till or mulch till

NA (Not available)

Questionnaire with responses filed at the Agricultural Nematology Lab of the Department of Horticulture

APPENDIX C | Assigned field codes for voucher specimen of 15 agricultural fields

Table C1 | Field codes of the 15 agricultural fields sampled from regions (east, southwestern and northwestern) and soil groups (mineral and muck) of Michigan, used as cross-reference labels for data and the voucher specimen.

Region	Soil group	Field	Field code
East	Mineral	1	JW
		2	PZ1
		3	PZ2
	Muck	4	EBR1
		5	EBR2
		6	EBR3
SW	Mineral	7	PN
		8	L
		9	M
	Muck	10	ED
NW	Mineral	11	OF2
		12	OB2
		13	OF
	Muck	14	JWA
		15	VS

APPENDIX D | PCR mix and thermocycle used for PCR in Chapter 2

Table D1 | Polymerase chain reaction (PCR) mix with volumes and thermocycle settings (temperature, time and cycles) used to amplify the 16 ribosomal region of bacteria.

PCR MIX			**THERMOCYCLE**		
PCR Mix	**µL**	**Total (µL)**	**Temp (°C)**	**Time(min)**	**Cycles**
Dream Taq	6.25	937.5	95	2:00	
ITS1F	0.375	60	95	0:20	30x
ITS2R	0.375	60	55	0:15	
H_20	2	320	72	5:00	
BSA	2	320	72	10:00	
DNA	1		4	inf	

APPENDIX E | PCR mix and thermocycle used for 3-step PCR in Chapter 2

Table E1 | Polymerase chain reaction (PCR) mix with volumes and thermocycle settings (temperature, time and cycles) used to amplify the internal transcribed spacer (ITS) region of fungal ribosome.

PCR MIX

Step 1		
PCR Mix	**µL**	**Total (µL)**
Dream Taq	6.25	937.5
ITS1F	0.375	60
ITS2R	0.375	60
H_20	2	320
BSA	2	320
DNA	1	

Step 2		
PCR Mix	**µL**	**Total (µL)**
Dream Taq	6.25	1000
ITS1F (with frameshift)	0.375	60
ITS2R (with frameshift)	0.375	60
H_20	1	160
BSA	2	320
DNA	2 from step1	

THERMOCYCLE

Step 1		
Temp (°C)	**Time(min)**	**Cycles**
95	5:00	
95	0:30	10x
50	0:30	
72	1:00	
72	7:00	
12	inf	

Step 2		
Temp (°C)	**Time(min)**	**Cycles**
95	5:00	
95	0:30	10x
50	0:35	
72	1:05	
72	7:00	
12	inf	

Table E1 (cont'd)

Step 3			**Step 3**		
PCR Mix	**µL**	**Total (µL)**	**Temp (°C)**	**Time(min)**	**Cycles**
Dream Taq	8	1280	95	5:00	
PCR F	0.5	80	95	0:40	
H_20	0.5	80	63	0:50	10x
Barcode	1		72	7:00	
DNA	4 from step2		72	7:00	
			12	inf	

APPENDIX F | Worm lysis buffer (WLB) mix used in Chapter 2

Table F1 | Worm lysis buffer (WLB) mix used to extract bacterial DNA associated with *Meloidogyne hapla* populations isolated directly from field soils and greenhouse cultures.

WLB Mix	**µL**
1M KCL	500
1M Tris pH 8.3	100
1M $MgCl_2$	25
NP40	45
Tween 20	45
2% Gelatin	50
dd H_20	9,235
Total	**10,000**

APPENDIX G | Voucher specimens

Table G1 | Voucher specimens of *Meloidogyne hapla* nematodes identified from the sampled fields and mounted on glass slides. The specimens were from the infested fields identified after the greenhouse screening of *M. hapla* in Chapter 2. The samples are stored in the Agricultural Nematology Laboratory, Department of Horticulture at MSU.

Region	Soil group	Field number	Nematode species	Number of individuals	Developmental stage
East	Mineral	2	*Meloidogyne hapla*	1	Female
	Muck	4	*Meloidogyne hapla*	1	Female
		5	*Meloidogyne hapla*	1	Female
		6	*Meloidogyne hapla*	1	Female
Southwest	Mineral	8	*Meloidogyne hapla*	1	Female
	Muck	10	*Meloidogyne hapla*	1	Female
Northwest	Mineral	13	*Meloidogyne hapla*	1	Female
	Muck	14	*Meloidogyne hapla*	1	Female
		15	*Meloidogyne hapla*	1	Female

Table G2 | Voucher specimens of nematode genera identified from the sampled fields and mounted on glass slides. The specimens were from the 15 fields across the lower peninsula of Michigan in Table 2.1. The samples are stored in the Agricultural Nematology Laboratory, Department of Horticulture at MSU.

Slide number	Nematode genera	Number of individuals	Developmental stage
1	*Ditylenchus*	1	Female
2	*Acrobeles*	1	Female
3	*Criconema*	1	Female
4	*Aphelenchus*	1	Female
5	*Eucephalobus*	1	Female
6	*Nygolaimus*	1	Female
7	*Cephalobus*	1	Female
8	*Filenchus*	1	Female
9	*Rhabditis*	1	Female
10	*Epidorylaimus*	1	Female
11	*Eucephalobus*	1	Female
12	*Pellioditis*	1	Female
13	*Malenchus*	1	Female
14	*Psilenchus*	1	Female
15	*Aphelenchoides*	1	Female
16	*Eucephalobus*	1	Female
17	*Pratylenchus*	1	Female
18	*Tripyla*	1	Female
19	*Anaplectus*	1	Female
20	*Eucephalobus*	1	Female
21	*Helicotylechus*	1	Female
22	*Acrobeloides*	1	Female
23	*Chiloplacus*	1	Female
24	*Mesorhabditis*	1	Female
25	*Paraphelenchus*	1	Female
26	*Meloidogyne*	1	Female
27	*Plectus*	1	Female
28	*Scutylenchus*	1	Female
29	*Heterocephalobus*	1	Female
30	*Paratylenchus*	1	Female

Table G2 (cont'd)

Slide number	Nematode genera	Number of individuals	Developmental stage
31	*Alaimus*	1	Female
32	*Rhabditella*	1	Female
33	*Nothotylenchus*	1	Female
34	*Rotylenchus*	1	Female
35	*Aprutides*	1	Female
36	*Loofia*	1	Female
37	*Lamydorus*	1	Female
38	*Prodorylaimus*	1	Female
39	*Heterodera*	1	Female
40	*Pristionchus*	1	Female
41	*Diploscapter*	1	Female
42	*Mesodorylaimus*	1	Female

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