

Suppression of the Root-lesion Nematode Using Liquid Hog Manure

By

AMRO MAHRAN

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Plant Science

University of Manitoba

Winnipeg, Manitoba, Canada

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List of abbreviations

BLAST	Basic Local Alignment Search Tool
bp	base pair(s)
cm	centimeter
c-p scale	The Colonizer-Persister Scale for Nematodes
c-p 1	Enrichment Opportunist Nematodes
c-p 2	General Opportunist Nematodes
c-p 3, 4, and 5	Persister Nematodes
cv.	Cultivar
d	Day(s)
diam.	Diameter
DNA	Deoxyribonucleic Acid
e.g.	<i>Exempli Gratia</i>
ed.	Edition
Fig.	Figure
g	Gravity
g	Gram
h	Hieght
ha	Hectare(s)
hr	Hour(s)
i.d.	Inside Diameter
i.e.	<i>Id Est</i>
ITS	Internal Transcribed Spacer

IUPAC	International Union of Pure and Applied Chemistry
kb	Kilobase
kg	Kilogram
LHM	Liquid Hog Manure
m	Meter
min	Minute(s)
ml	Milliliter
mM	Millimolar
PCR	Ploymerase Chain Reaction
Pf	Final Nematode Population Density
Pi	Initial/Preplant Nematode Population Density
Rf	Reproduction Factor
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
sec	Second(s)
VFA	Short-chain Volatile Fatty Acids
wk	Week(s)

ABSTRACT

Mahran, Amro Mohamed. Ph.D., the University of Manitoba, March 2008. Suppression of the Root-lesion Nematode Using Liquid Hog Manure. Major Professors: Dr. Mario Tenuta (Advisor), and Dr. Fouad Daayf (co-Advisor).

Root-lesion nematodes, *Pratylenchus* spp., are serious pathogens of potato plants in temperate, tropical and subtropical regions of the world. Several management practices can control *Pratylenchus* spp. in soil; however, they all exhibit some limitations. Therefore, environmentally-safe, low-cost, and effective control strategies are needed as possible alternative to current strategies. This thesis was designed to assess if liquid hog manure (LHM) holds such potential. The objectives of this thesis were to determine: (i) the prevalence and identity of species of *Pratylenchus* spp. in Manitoba potato fields, (ii) if short-chain volatile fatty acids (VFA) in LHM are the constituents responsible for the manure's toxicity to *Pratylenchus* spp. using laboratory-based solution exposure experiments (iii) the effectiveness of LHM in killing *Pratylenchus* spp. in soil, and (iv) the impact of LHM on non-target nematode communities. *Pratylenchus* spp. were detected in 39% of 31 potato fields surveyed in Manitoba with population densities ranging, for positive fields, from 45 to 631 nematodes kg⁻¹ fresh soil. Morphometrics of female nematodes and molecular diagnosis (using species-specific PCR primers and species identification confirmation by molecular sequencing of PCR products and their comparison to the GenBank database) confirmed the species of *Pratylenchus* present in the potato fields to be *P. neglectus*. Potato, cv. Russet Burbank, was found to be a poor host to two populations of *Pratylenchus* spp. from Manitoba potato fields. From this, we conclude that *P. neglectus* does not seem to be a limitation to potato production in

Manitoba; thus, *P. penetrans*, the most wide spread and damaging species to potato, was used in successive studies to assess the use of LHM to control *Pratylenchus* spp. in potato fields. Under laboratory conditions, VFA (acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric, and *n*-caproic acids) accounted for the majority of the lethal effect of LHM exposure to *P. penetrans* under acidic conditions. The VFA in LHM killed *Pratylenchus* spp. in soil and acidification seemed to enhance this response when VFA concentration in the manure is low. LHM did not act as a soil fumigant eliminating soil trophic interactions, but increased bottom-up food web interactions. The VFA in LHM persisted in the soil for only four days with biological degradation being their mode of loss. In conclusion, LHM is potentially an effective and low-cost strategy to control *Pratylenchus* spp. and its efficacy improved by acidification when VFA concentration is low.

FOREWORD

This Thesis is presented in “Sandwich Style” as outlined by the Department of Plant Science and Faculty of Graduate Studies, University of Manitoba. A general introduction and review of literature precedes four manuscripts. Two manuscripts are published or in press, one is accepted after having been revised, and one is soon to be submitted to a journal. Each manuscript consists of an abstract, introduction, materials and methods, results and discussion with the later two either separate or combined depending on the preference of the journal. For consistency, the format of manuscripts is that of the Journal of Nematology. The manuscripts in order of presentation in the Thesis are as follows:

Mahran, A., Tenuta, M., Shinnars-Carnelley, T., and Daayf, F. Prevalence and species identification of *Pratylenchus* spp. in Manitoba potato fields and host suitability of Russet Burbank Potato. Intended for submission to the Canadian Journal of Plant Pathology.

Mahran, A., Tenuta, M., Hanson, M., and Daayf, F. 2008. Mortality of *Pratylenchus penetrans* by volatile fatty acids from liquid hog manure. Journal of Nematology 40:119-126.

Mahran, A., Conn, K., Tenuta, M., Lazarovits, G., and Daayf, F. 2008. Effectiveness of liquid hog manure and acidification to kill *Pratylenchus* spp. in soil. *Journal of Nematology* (*in press*).

Mahran, A., Tenuta, M., Lumactud, R. A., and Daayf, F. Liquid hog manure and its acidification alters a nematode community in soil. *Applied Soil Ecology* (accepted after receiving considerable revisions based on the journal review process; thus, published version is different than presented here).

A general discussion completes the Thesis. A united reference list is at the end of the thesis.

1.0 INTRODUCTION

Pratylenchus spp. Filipjev (1936) are serious parasites of potato (*Solanum tuberosum* L.) in temperate, tropical and subtropical regions of the world (Scurrah et al., 2005; Duncan and Moens, 2006; Castillo and Vovlas, 2007). *Pratylenchus* spp. attack potato plants, causing stunting, leaf yellowing, and severe necrotic lesions in the roots and tubers (Brodie et al., 1993) and cause yield losses as much as 25-73% (Olthof, 1986). The even greater economic damage occurs when some *Pratylenchus* spp. interact with the wilt fungus, *Verticillium dahliae* Kleb., forming the disease complex known as potato early dying when population levels of the two pathogens are too low to cause disease singly (Martin et al., 1982; Riedel et al., 1985; Rowe et al., 1985; MacGuidwin and Rouse, 1990; Rowe and Powelson, 2002). Potato early dying is considered a primary restraining factor to potato production in North America (Martin et al., 1982; Rowe et al., 1987).

The genus *Pratylenchus* is comprised of 68 valid species that parasitize and cause damage to a wide variety of plants (Duncan and Moens, 2006; Castillo and Vovlas, 2007). Only *P. alleni* Ferris, *P. brachyurus* (Godfrey) Filipjev and Schuurmans, *P. coffeae* (Zimmermann) Filipjev and S. Stekhoven, *P. crenatus* Loof, *P. neglectus* (Rensch) Filipjev and Schuurmans Stekhoven, *P. penetrans* (Cobb) Filipjev and Schuurmans Stekhoven, *P. scribneri* Steiner in Sherbakoff and Stanley, and *P. thornei* Sher and Allen are considered destructive to potato plants (Brodie et al., 1993; Ingham et al., 2005; Scurrah et al., 2005; Castillo and Vovlas, 2007). Moreover, *Pratylenchus* spp., that parasitize potato plants vary in their ability to interact with *V. dahliae* and consequently the induction of potato early dying. *Pratylenchus penetrans* and *P. thornei*

can interact synergistically with *V. dahliae* and induce potato early dying (Riedel et al., 1985; Wheeler et al., 1994; Wheeler and Riedel, 1994; Duncan and Moens, 2006), whereas *P. crenatus* cannot (Riedel et al., 1985; Duncan and Moens, 2006). *Pratylenchus scribneri* interacts with *V. dahliae* only at elevated temperatures (>32°C) (Riedel et al., 1985). Understanding of the interaction with *P. neglectus* is not clear. A population from Ontario, Canada, synergistically interacted with *V. dahliae* and one from Idaho, USA, did not interact with the fungus (Hafez et al., 1999). However, *P. alleni*, *P. brachyurus*, and *P. coffeae* interaction with *V. dahliae* has not been reported. Accordingly, it is crucial to determine the species and population levels of *Pratylenchus* spp. in potato soils to predict the potential risk of crop damage.

In recent years, potato producers in Manitoba believe that potato early dying to be a major constraint to achieving yield increase and, in many cases, is believed to be causing yield losses (Dr. Mario Tenuta, personal communication). Previous nematode surveys of potato fields conducted in Manitoba by Geisel (1991) and Manitoba Agriculture, Food and Rural Initiatives in collaboration with the Department of Soil Science, University of Manitoba, confirmed the presence of *Pratylenchus* spp. in 33% of 135 fields sampled with populations ranging from 4 to 5,300 nematodes kg⁻¹ soil (Geisel, 1991; Tenuta, et al., unpublished data). In addition, many potato producers in Manitoba have obtained commercial nematode pest analysis reports showing *Pratylenchus* spp. present in their fields. None of the surveys or the commercial analysis reports identified the species of *Pratylenchus* despite the clear importance of determining whether the *Pratylenchus* spp. in Manitoba potato fields can parasitize and damage potato plants as well as promote potato early dying disease complex.

Damage caused by *Pratylenchus* spp. to potato plants can be reduced by decreasing nematode populations in soil to non-injurious or sub-economic threshold populations (Viaene et al., 2006), which consequently contributes towards the management of potato early dying disease complex (Powelson and Rowe, 1993). Several management practices (e.g. crop rotation, resistant cultivars, biological control agents, and chemical nematicides) can reduce *Pratylenchus* spp. population in soil. However, many of these management practices have limitations (Castillo and Volvas, 2007). The wide host range of *Pratylenchus* spp. makes successful crop rotation difficult (Chen et al., 1995). The lack of resistant germplasm to incorporate into commercial cultivars limits the use of resistant crop cultivars (France and Brodie, 1995; Brodie, 1998). In addition, biological control agents have proven not to be effective in commercial fields (Hackenberg et al., 2000; Castillo and Volvas, 2007). The use of soil fumigation and nematicides is limited due to their environmental impact and high cost (Olthof, 1987; Olthof, 1989; Rich et al., 2004; Duncan and Moens, 2006). Therefore, there is an increasing need to develop low cost, environmentally-safe, and effective alternative strategies to control *Pratylenchus* spp. as possible alternative to currently used strategies. Organic amendments hold such potential, and application of a range of organic amendments has lead to considerable reduction in plant-parasitic nematode population densities through different mechanisms (Viaene et al., 2006).

Liquid hog manure (LHM), also known as liquid swine manure, is a product of commercial hog (*Sus scrofa domestica* L.) production. It is typically regarded as a waste rather than a resource and its disposal has become a considerable problem in modern agriculture (Shulte, 1977). Several studies have shown LHM to control plant pathogens

(Conn and Lazarovits, 1999; Conn and Lazarovits, 2000; Valocka et al., 2000; Tenuta et al., 2002; Conn et al., 2005; Xiao, et al., 2007 and 2008). Thus, we examined LHM as to whether it can be a low-cost, safe, and effective control strategy for *Pratylenchus* spp.

Various mechanisms and constituents contribute to how LHM controls plant pathogens. These mechanisms and constituents include the effects of ammonia, nitrous acid (Conn et al., 2005), and short-chain volatile fatty acids (VFA) (Tenuta et al., 2002; Conn et al., 2005). VFA have been found to be responsible for the mortality of plant-parasitic nematodes and soil-borne plant pathogens in several investigations (Conn and Lazarovits, 2000; Tenuta et al., 2002; Conn et al., 2005; McElderry et al., 2005; Xiao et al., 2007 and 2008). VFA (acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric, and *n*-caproic acids) in LHM are the products of bacterial anaerobic fermentation of carbohydrates and amino acids which takes place in the gastrointestinal tract of hogs and during LHM storage under anaerobic conditions (Zhu, 2000). The effect of VFA in LHM on *Pratylenchus* spp. is unknown though important in determining if they are lethal to the nematode.

For the LHM to compete as a *Pratylenchus* spp. management strategy with currently available ones, it should show high efficacy in killing *Pratylenchus* spp. in soil. Acidic conditions generating non-ionized forms of VFA in LHM are responsible for the mortality of *V. dahliae* under acidic conditions (Tenuta et al., 2005). Accordingly, investigating the efficacy of LHM in killing *Pratylenchus* spp. in soil is essential prior to its recommendation as a nematode management strategy. Moreover, testing the hypothesis that acidification of LHM would improve its efficacy by promoting the presence of lethal, non-ionized forms of VFA requires verification in soil.

An ideal amendment to control plant pathogens and pests should not be lethal to non-target beneficial organisms in the soil food web while being highly toxic to target organisms. The impact of VFA in LHM on nematode communities in soil is unknown though important in understanding how they affect the structure and function of the soil food web and in turn soil health. Nematode faunal analysis is an informative tool in assessing the structure and function of soil food webs and response to altered soil conditions (Freckman and Ettema, 1993; De Ruiter et al., 1994; Ferris et al., 1996; Bongers and Ferris, 1999; Bongers, 1999; Ferris et al., 2001). Nematode faunal analysis includes the characterization of a two-trajectory description of soil food web conditions. An enrichment trajectory, calculated as the enrichment index, based on the weighted abundance of opportunistic bacterivorous c-p 1 and fungivorous c-p 2 nematodes species that respond rapidly to prey resources. A structure trajectory, calculated as the structure index, based on the weighted abundance of larger, slower-reproducing bacterivore, fungivore, omnivore and carnivore c-p 3 to 5 value nematodes (Ferris et al., 2001).

Addition of readily available carbon from an external input (e.g. LHM), or dead soil organisms (e.g. due to application of broad spectrum pesticide to soil) will cause an increase in microbial activity in soil that can enhance opportunistic bacterial-feeding nematodes (Ferris et al., 2001). This is a “bottom-up” constraint on the size and activity of the food web and results in an enriched food web with high enrichment index value. The presence of high c-p nematodes that feed on opportunistic nematodes and competition among trophic levels provide “top-down” regulation of food web structure and function (Ferris et al., 2001) and over time leads to structured food web. In the case

that general mortality of the nematode community occurs, as following fumigation, dead organisms become a resource leading to a flush of microbial activity that enhance opportunistic bacterial-feeding nematodes (Ferris et al., 2001). However, the absence of high c-p nematodes, that are sensitive to disturbance, restrict the “top-down” population control which in turn result in an enriched food web and the structure index becomes very low (Wang et al., 2006). Accordingly, the impact of VFA in LHM on the structure and function of the soil food web and in turn soil health can be determined using the nematodes faunal analysis.

This thesis was designed to evaluate LHM as an effective control strategy for *Pratylenchus* spp. in potato fields. The objectives of the thesis were to determine: (i) the prevalence and species identification of *Pratylenchus* spp. in Manitoba potato fields, (ii) using a laboratory bioassay to determine if VFA in LHM can account for toxicity of the manure to *Pratylenchus* spp., (iii) the effectiveness of LHM in killing *Pratylenchus* spp. in soil, and (iv) using a microcosm experiment the impact of LHM on nematode communities in soil as indicators of soil health to evaluate its effect on soil food webs and soil health.

2.0 LITERATURE REVIEW

2.1 Potato (*Solanum tuberosum* L.), HOST PLANT

Potato is one of the most important sources of food for humans ranking fourth after rice (*Oryza sativa* L.), wheat (*Triticum* spp.), and corn (*Zea mays* L.) in terms of total production (Kaur and Mukerji, 2004), with global annual production reaching 325 million tons in 2007. China is the world's biggest potato producer, accounting for more than 20% of both the global potato-growing area and the world potato harvest, followed by the Russian Federation, India, and the United States. Europe, North America, and countries of the former Soviet Union were the major potato producers and consumers until the early 1990s. However, due to the increase in potato production and demand in Asia, Africa and Latin America, potato production in the world's developing countries exceeded that of the developed world, starting in 2005 (Food and Agriculture Organization of the United Nations, 2008).

Potato is very nutritious and it contains over 12 essential vitamins and minerals and a variety of essential amino acids (Thornton and Siczka, 1980). Moreover, it produces more energy and protein per unit area land than any other food crop (Kaur and Mukerji, 2004). For example, potato produces 54% and 78% more protein per unit of land area than wheat and rice, respectively (Stevenson et al., 2001). Potato is prepared and consumed in different ways, as a source of carbohydrates replacing rice or wheat, as a vegetable, or in the form of French fries, wafers, chips, mashed and boiled potatoes, and in soups. In addition, potato is used to produce alcoholic beverages such as vodka and akvavit in Eastern Europe and Scandinavia (Food and Agriculture Organization of the United Nations, 2008). Potato by-products are also given as feed to farm animals

(Radunz et al., 2003). Due to its importance as a staple food globally and the role it can play in providing food security and eliminating poverty, the United Nations declared 2008 the International Year of the Potato. Potato also has several industrial uses: its starch is widely used as an adhesive, binder, texture agent, and filler in the pharmaceutical, textile, wood, and paper industries. As well, potato peels and wastes from processing are fermented to fuel-grade ethanol (Food and Agriculture Organization of the United Nations, 2008).

Potato is grown in temperate, tropical, and subtropical regions of the world (Harris, 1992). It is an annual plant, about 30-100 cm tall and is vegetatively propagated through tubers. The tubers carry buds, known as eyes, which sprout and grow into plants. If tubers are exposed to light they turn green and become inedible because of the increased levels of solanine and chaconine that in turn may cause solanine poisoning if consumed; accordingly, they are grown in fields in ridges to maintain the developing tubers under soil. New tubers develop at flowering at the tip of the stolon as a lateral proliferation of storage tissue resulting from rapid cell division and enlargement. The stolon usually breaks off close to the tuber during harvest or dies with the plant on maturity (Hooker, 1983).

Thousands of potato varieties are grown worldwide (Food and Agriculture Organization of the United Nations, 2008). These varieties differ in time to maturity, yield, shape, color, texture, cooking characteristics, and taste. There are 156 potato varieties registered in Canada (Canadian Food Inspection Agency, 2008) with the variety Russet Burbank being the most widely spread. The leading ten varieties widely grown in

Canada and their characteristics are listed in Table 2.1 (Agriculture and Agri-food Canada, 2008; Canadian Food Inspection Agency, 2009).

Table 2.1 The leading ten potato varieties widely grown in Canada and their characteristics (modified from the Canadian Food Inspection Agency, 2009).

Variety*	Maturity	Yield	Tuber		Uses
			Shape	Skin	
Russet Burbank	Late	High	Long	Brown, strongly russeted	French frying
Shepody	Mid-season	Medium-high	Long	Smooth, buff	French frying
Ranger Russet	Late	High	Long	Brown, strongly russeted	French frying
Atlantic	Mid-season	High	Oval	Buff, rough (flaky)	Chipping
Kennebec	Mid-late season	High	Elliptical-oblong	Smooth creamy buff	Boiling, baking, chipping and French frying
Yukon Gold	Mid-season	Medium-high	Oval	Finely flaked yellowish white skin	Boiling, baking, and French frying
Chieftain	Mid-season	High	Oval-oblong	Smooth bright red skin	Boiling, chipping, and French frying
Russet Norkotah	Early- mid-season	Medium	Long-slightly oblong	Dark, russeted, and netted skin	Baking
Norland	Early	High	Oval-oblong	Red, smooth	Table market
Superior	Early-mid-season	Medium	Oval-oblong	Smooth to lightly flaked buff skin	Chipping

2.1.1 History of the potato

Potato is native to the Andean highlands of Peru and Bolivia in South America (Harris, 1992). Spaniards found potato in Peru at the time of their invasion beginning in

1524 and introduced it to Spain by 1580. From Spain, the plants went to other European countries. Potato was introduced to Ireland through trade with Spain in the 17th century. Soon after its introduction, potato became a very important staple food in Ireland.. In the 1840's, late blight disease (caused by the fungus *Phytophthora infestans* (Mont.) De Bary) destroyed the potato crop in Ireland which in part forced many Irish people to immigrate to the United States of America and Canada (Agrios, 2005). Oddly, potato came to the United States from Ireland. Acceptance of potato as a food in the United States was very slow in contrast to Ireland. The potato industry in Canada started in 1623, when a small patch of potato was grown at Annapolis Royal in the province of Nova Scotia. A barrel of potatoes was presented to the early Acadian settlers, who in turn used some of these for planting a small patch at Annapolis Royal. Since then, the potato industry has grown steadily over the years to become one of the most economically important crops in Canada (Thornton and Sieczka, 1980).

2.1.2 Economical importance of potato

Asia and Europe are the world's major potato producing regions, accounting for more than 80% of world production in 2007. However, in terms of potato yield per ha, North America leads with average yields being more than 36 tons ha⁻¹. In terms of potato consumption, Asia consumes almost half of the world's potato supply and that is due to its large population. However, Asia's potato consumption per person was a modest 25 kg per person in 2005. Europeans are the utmost potato consumers with consumption of 96 kg per capita. Per capita consumption is lowest in Africa and Latin America (Food and Agriculture Organization of the United Nations, 2008).

Canada ranks as the world's 13th largest potato grower (Food and Agriculture Organization of the United Nations, 2008). In 2008, the Canadian potato acreage stood at 151,069 ha and produced 4.7 million tons of potato. Potato production was concentrated in Prince Edward Island (24%), Manitoba (21%), Alberta (17%), and New Brunswick (15%) (Statistics Canada, 2008). Potato is one of Canada's most important horticultural crops as it accounts for one third of all vegetable farm cash receipts (approximately \$846 million) in the 2007 calendar year (Food and Agriculture Organization of the United Nations, 2008; Agriculture and Agri-food Canada, 2008). Canadian potato production has expanded to meet international demand for frozen potato products. In 2004, almost two million tons of potato were grown to meet export demand. Most exports are in the form of frozen French fries, destined mainly for market in the United States. Despite the potato's popularity, however, annual consumption in Canada declined from 89 kg per capita in 1994 to around 85 kg a decade later (Food and Agriculture Organization of the United Nations, 2008). This decline in potato consumption is a result of various diets as well as consumer awareness of the nutritional value of some of its preparation methods.

2.1.3 Potato in Manitoba

Manitoba is Canada's second largest potato producing province following Prince Edward Island. Commercial potato production in Manitoba is concentrated south and west of the city of Winnipeg in areas around the communities of Portage la Prairie, Winkler, Morden, Treherne, Holland, and Carberry. The potato industry is a vital and growing sector of Manitoba's agricultural economy. In 2008, 33,185 ha were planted to potato and produced 1.1 million tons of potato. There are about 1,500 jobs directly involved in production with many more involved in the processing and marketing

industry. The 2007 Manitoba potato crop alone was valued at \$205 million CAD, the value of processed and marketed potato being much more (Statistics Canada, 2008).

The marketing of all potatoes produced in Manitoba is regulated under the Manitoba Vegetable Producers' Marketing Plan that authorizes Peak of the Market, a growers owned non-profit corporation, to pass certain orders and regulations controlling the marketing of potatoes produced in Manitoba. Peak of the Market operates as a central selling desk. Producers deliver washed, graded table potatoes to Peak of the Market in bulk trailers. Peak of the Market pack the potatoes into 2.3, 4.5, and 9.0 kg bags and sells them under their label to wholesalers and retailers in Manitoba, other provinces, United States, and a few other countries (Manitoba Agriculture, Food Rural Initiatives, 2002; Geisel, 2003).

Potatoes grown in Manitoba under preplant contract for the processors are exempt from Manitoba Vegetable Producers' Marketing Plan regulations. Keystone Vegetable Producers' Association assists growers with price negotiations with the processing companies. The five major potato processors in Manitoba are Midwest Food Products, McCain Foods, J.R. Simplot, Old Dutch Foods, and Naleway Foods (Manitoba Agriculture, Food Rural Initiatives, 2002).

2.1.4 Diseases of potato

Potato plants are susceptible to many diseases caused by bacteria, fungi, viruses, mycoplasma, and nematodes (Agrios, 2005). Some of the most common diseases on potato are listed in Table 2.2.

Table 2.2 Common diseases of potato, their causal pathogens, and their geographical distribution (Harris, 1992).

Disease	Pathogen	Distribution
Fungal		
Early blight	<i>Alternaria solani</i> (Ellis and G. Martin) L.R. Jones and Grout	Worldwide
Fusarium wilt	<i>Fusarium</i> spp.	North America
Late blight	<i>Phytophthora infestans</i> (Mont.) de Bary	Worldwide
Rhizoctonia	<i>Rhizoctonia solani</i> Kühn	Worldwide
Canker	<i>Helminthosporium solani</i> Dur. and Mont.	Europe and North America
Silver scurf	<i>Verticillium albo-atrum</i> Reinke and Berthier	Worldwide
Verticillium wilt	<i>V. dahliae</i> Kleb.	Worldwide
Wart	<i>Synchytrium endobioticum</i> (Schilb.) Perc	
Bacterial		
Bacterial wilt	<i>Pseudomonas solanacearum</i> (Smith) Smith	Tropics
Blackleg	<i>E. carotovora</i> subsp. <i>carotovora</i> (Jones) Bergey et al.	Europe and North America
Pink eye	<i>P. fluorescens</i> Migula	Worldwide
Common scab	<i>Streptomyces scabies</i> (Thaxter) Lambert and Loria	Worldwide
Nematodes		
Golden cyst nematode	<i>Globodera rostochiensis</i> (Wollenweber) Mulvey and Stone	Worldwide
Pale cyst	<i>G. pallida</i> Stone	Worldwide
Northern root-knot nematode	<i>Meloidogyne hapla</i> Chitwood	North America and temperate areas
Columbia root-knot nematode	<i>M. chitwoodi</i> Golden, O'Bannon, Santo and Finley	North-west USA and the Netherlands
Root-lesion nematode	<i>Pratylenchus</i> spp.	Europe and North America
Tuber-rot nematode	<i>Ditylenchus destructor</i> Thorne	North America and Western Europe
Stem nematode	<i>D. dipsaci</i> (Kühn) Filipjev	Western Europe
Stubby root nematode	<i>Trichodorus</i> spp. Cobb / <i>Paratrichodorus</i> spp. Siddiqi	Europe and North America
Viruses		
Potato leafroll virus	Leafroll virus	Worldwide
Corky ringspot	Tobacco rattle virus	Worldwide
Mild mosaic	Virus A	Worldwide

2.2 The root-lesion nematode (*Pratylenchus* spp.)

2.2.1 Taxonomy

De Man described the first species of *Pratylenchus* in 1880 under the name *Tylenchus pratensis*. However, the genus *Pratylenchus* was not established until 1934 by Filipjev. The true generic diagnosis was published in 1936 as a group of nematodes possessing a tylenchoid pharynx overlapping the anterior portion of the intestine and a uniovarial gonad in adult females. The species that has been described by De Man in 1880 was then designated by Filipjev (1934) as the type of his new genus, *Pratylenchus*. The etymology of the genus was derived from the first three letters of the type species (*pratensis* = growing in a meadow) and *Tylenchus* (From the Greek: tylos = knob, *enchos* = spear). The type species was found in moist soil in a meadow near Leiden, The Netherlands (Castillo and Vovlas, 2007).

The classification of *Pratylenchus* spp. according to Siddiqi, 2000 is as follows:

Phylum Nematoda Rudolphi, 1808

Class Secernentea von Linstow, 1905

Subclass Tylenchia Inglis, 1983

Order Tylinchida Thorne, 1949

Suborder Tylenchina Thorne, 1949

Superfamily Hoplolaimoidea Filipjev, 1934

Family Pratylenchidae Thorne, 1949

Subfamily Pratylenchinae Thorne, 1949

Genus *Pratylenchus* Filipjev, 1936

2.2.2 *Pratylenchus* spp. life cycle

Pratylenchus spp. are migratory endoparasitic nematodes. All life stages (juveniles and adults) of *Pratylenchus* spp. can be found in both soil and roots. *Pratylenchus* spp. do not induce permanent feeding sites, but instead they feed and reproduce while migrating between or through plant cells. *Pratylenchus* spp. usually complete their life cycle in three to seven wk (Duncan and Moens, 2006; Castillo and Vovlas, 2007).

Egg laying: In *Pratylenchus* spp., parthenogenesis appears to be the most common mode of reproduction, which is implied from the absence of males and sperms in the female spermatheca (Duncan and Moens, 2006). After an egg passes the spermatheca, it is forced backward to the vulva through the movement of the tail and internal tissues. The tip of the egg then protrudes from the vulva. The eggshell is squeezed through the vulva opening with the egg's contents flowing from its unlaidd part through to its pole outside the vagina. When half of the egg passes through the vulva, its remaining part is expelled rapidly with the assistance of internal pressure and/or muscular contraction. The process of egg deposition is completed in approximately 2.5 min. At 23°C, adult females lay 1-2 eggs per day for a total of 16-35 eggs during a generation (Castillo and Vovlas, 2007). Adult females deposit eggs inside the cortical tissue, near the root surface, or outside infected plants along the length of the root (Zunke, 1990).

Embryogenesis, hatching, and molting: Eggs are laid at the one-cell stage, which in turn divides into two blastomeres 12 hr following egg laying. In the next 12 hr, a cleavage occurs in the anterior blastomere yielding the three-celled stage. Another cleavage occurs at the posterior blastomere 36 hr following egg laying which yields a four-celled stage. The pre-gastrula stage appears in the next 24 hr and remains for five days before it develops into gastrula. The gastrula develops into the J1 (first stage juveniles) after two days of its formation. The J1 are characterized by the absence of internal differentiation. The first molt occurs within two days to produce the J2 (second stage juveniles) (Castillo and Vovlas, 2007; Castillo et al., 1995). J2 hatches from the egg and hatching is influenced by the host type and age, and temperature (Pudasaini et al., 2008). *Pratylenchus* spp. J2 develop into the adult stage after passing through the J3 (third stage juveniles) and J4 (fourth stage juveniles). The cuticle is shed and replaced (molt) three times during the life cycle of *Pratylenchus* spp. The *Pratylenchus* spp. molting process involves three phases: (i) separation of the old cuticle from the epidermis; (ii) formation of a new cuticle from the epidermis; and (iii) the molted nematode expands in size and sheds the old cuticle (Zunke, 1990; Wright and Perry, 2006).

Host infection: Eggs hatch into J2 that in turn migrate and aggregate around the area where lateral roots emerge from the main root, the bases of root hairs, the root tip, and roots elongation zone. Invasion of plant tissue by *Pratylenchus* spp. involve both mechanical force from the stylet thrusting and pressure from the labial region and the secretion of cell wall degrading enzymes (e.g. cellulases and pectinases) through the stylet (Krusberg, 1960; Zunke, 1990; Castillo and Vovlas, 2007). When getting in contact

with the root surface, nematodes start abrasion by their lips along the root surface for a brief period presumably to recognize a suitable host. They then begin stylet thrusts for a period that varies according to the structure and thickness of the cell walls. In addition, *Pratylenchus* nematodes secrete cell wall degrading enzymes synthesized in their pharyngeal glands into plant cells via the stylet. These secretions participate in extracorporeal digestion to facilitate ingestion of cytoplasm components of the infected cell or may degrade the cell wall. Once a nematode punctures a cell wall it feeds on it for a short period before it moves through the epidermal cells. *Pratylenchus* spp. move through tissues by breaking down successive cell walls. Migration through the cortical tissue is accomplished by puncturing and penetrating neighboring cells with stylet thrusts at a corner of a cell followed by stylet thrusts at the opposite corner and then over the entire cell wall. This results in loss of cell turgor pressure and a gradual increase in the size of the nucleus followed by cell death along the nematode's route (Zunke, 1990; Castillo and Vovlas, 2007).

Feeding: The feeding process usually starts when an estimated 1-2 μm of the stylet tip has penetrated the cell wall and membrane and contacted the cytoplasm (Castillo and Vovlas, 2007). There are two types of feeding that take place by *Pratylenchus* spp. upon entering the plant roots. Brief feeding that extends for 5-10 min does not kill cells whereas extended feeding for several hr causes cell death (Zunke 1990). As the nematode starts the brief feeding, a small salivation zone appears around the stylet tip. The extended feeding is marked by a period of salivation of approximately two min, which result in a salivation zone around the stylet tip. After salivation, feeding commences and often continues for many hr. Defecation usually occurs every two to four

min without interrupted feeding. In response to feeding, the tonoplast of the cortical cells decline in size and the nucleus gradually hypertrophies during and after extended feeding periods and vacuole-like structures occasionally appear in the cytoplasm. When cell death occurs, discoloration of root surfaces called lesions can result. When levels of root damage are extremely high, leaves die followed by death of infected plants due to lack of a healthy root system to support the plant. After roots are damaged, *Pratylenchus* spp. leave the roots and search for another root to feed on (Duncan and Moens, 2006; Castillo and Vovlas, 2007).

2.2.3 *Pratylenchus* spp. in Canada

Pratylenchus spp. are widely distributed in Canada (Yu, 2008). *Pratylenchus* spp. have been found in the provinces of British Columbia, Alberta, Saskatchewan, Ontario, Quebec, Prince Edward Island, New Brunswick, Manitoba and Nova Scotia (Potter and Townshend, 1973; Johnson, 1977; Townshend et al., 1978; McKeown et al., 1994; Wang et al., 2000; Holzgang and Pears, 2007; Joshi and Jeffries, 2007; Yu, 2008). At least 11 species of *Pratylenchus* are associated with various crops in Canada (Table 2.3) (Yu, 2007 and 2008). *Pratylenchus penetrans* is the most widely spread and it is present in all provinces; however, *P. flakkensis* Seinhorst was detected only in Prince Edward Island and Quebec. In Ontario, all 10 *Pratylenchus* spp. were detected except for *P. flakkensis* (Yu, 2008).

Pratylenchus spp. are the dominant plant-parasitic nematode in potato fields in Canada. Only three species of *Pratylenchus*, *P. penetrans*, *P. crenatus*, and *P. neglectus*, were reported in potato fields with *P. penetrans* being the most dominant (Yu, 2008). In

Table 2.3 *Pratylenchus* spp. present in Canada, their host crops, and distribution according to Yu (2008).

<i>Pratylenchus</i> spp.	Crops	Distribution
<i>P. crenatus</i> Loof	Fruit tree, blueberry, tobacco, oat, strawberry, <u>potato</u> , turnip, carrot, hay, alfalfa, apple, prune, Douglas-fir, red clover, rhubarb, corn, timothy, sour cherry, roses, cedar, barely, nursery, and white clover	Nova Scotia, Prince Edward Island, Quebec, Ontario, Alberta, and British Columbia
<i>P. fallax</i> Seinhorst	Turf grass	Ontario and Saskatchewan
<i>P. flakkensis</i> Seinhorst	unknown	Prince Edward Island
<i>P. hexincius</i> Taylor and Jenkins	Fescue and Pea	Ontario and Alberta
<i>P. macrostylus</i> Wu	White Spruce, White birch, and Douglas fir	Ontario and British Columbia
<i>P. neglectus</i> (Rench) Filipjev and Schuurmans Stekhoven	Tobacco, asparagus, oat, strawberry, cherry, barely, peach, corn, tomato, pea, <u>potato</u> , pear, red clover, apple, alfalfa, timothy, African violet, rye, rose, prune, apricot, rhubarb, wheat, cedar, and begonia	Ontario, Manitoba, and British Columbia
<i>P. penetrans</i> (Cobb) Sher and Allen	Grape, tulip, onion, plum, bluegrass, bent grass rose, tulip, soybean, chrysanthemum, tobacco, asparagus, oat, strawberry, cherry, barely, peach, corn, tomato, pea, <u>potato</u> , pear, red clover, apple, alfalfa, timothy, African violet, rye, rose, prune, apricot, rhubarb, wheat, cedar, begonia, aspen, daffodil, and buckwheat	Nova Scotia, Prince Edward Island, New Brunswick, Quebec, Ontario, Manitoba, Saskatchewan, Alberta, and British Columbia
<i>P. pratensis</i> (De Man) Filipjev	English Holly, oat, corn, alfalfa, strawberry, and clover	New Brunswick, Manitoba, and Ontario
<i>P. sensilatus</i> Townshend and Anderson	Timothy	Ontario
<i>P. thornei</i> Sher and Allen	Wheat and turf grass	Ontario
<i>P. zea</i> Graham	Corn	Ontario

a survey of 50 potato fields representing over 5,600 ha in Simcoe County, Ontario, *P. penetrans* was present in 24% of the fields surveyed with median population density of 655 nematode kg⁻¹ soil; *P. crenatus* and *P. neglectus* were present in 24% and 14% of the fields, respectively, but with lower populations (Olthof et al., 1982). In another survey, *P. penetrans* was detected in 67% and 20% of soil samples collected from Prince Edward Island (30 sites) and New Brunswick (40 sites), respectively, with a population range of 70 to 4,730 nematodes kg⁻¹ soil in Prince Edward Island and 5,280 to 7,200 nematodes kg⁻¹ in New Brunswick (Kimpinski, 1987). In Québec, *P. penetrans* is the dominant plant-parasitic nematode present in potato fields (Olthof, 1987; Bélair et al., 2005).

In Manitoba, a survey was conducted in 1991 to determine the level of *V. dahliae* and *Pratylenchus* spp. in soils used for potato production in the main potato growing districts, i.e. Winkler, Portage la Prairie, Macgregor, and Carberry. Results of this survey show the presence of *Pratylenchus* spp. in all four districts with varying population levels (Geisel, 1991). In 2002, Tenuta et al. (in preparation) examined four potato fields with visual symptoms of early dying disease complex to identify the biological and environmental factors responsible for premature death and reduced yields. *Pratylenchus* spp. were detected in two of the four fields examined with population ranges from 40-5300 nematode kg⁻¹ soil. In 2003, another survey was conducted through the collaboration of the Department of Soil Science at the University of Manitoba and Manitoba Agriculture, Food Rural Initiatives covering 24 potato fields. *Pratylenchus* spp. were detected in 16 of the 24 fields sampled. The same group to determine the prevalence of *Pratylenchus* spp. in some potato fields conducted another survey in 2004. Phase I of the survey included six potato fields in Portage la Prairie and Winkler areas with

Pratylenchus spp. were detected in four of the six fields sampled with population ranges from 90-370 nematodes kg⁻¹ soil. In phase II of the survey, soil and root samples were collected from four potato fields in Portage la Prairie area with *Pratylenchus* spp. were detected in three of the four fields sampled with population ranges from 84-898 nematodes kg⁻¹ soil, while they were not detected in roots (Tenuta, unpublished data).

Yu (2008) identified preserved mounted *Pratylenchus* spp. specimens at the Canadian National Collection of Nematodes, Ottawa, Ontario, and reported that *Pratylenchus* species collected from Manitoba are *P. penetrans*, *P. pratensis*, and *P. neglectus*. *Pratylenchus penetrans* was present in a soil collected in 1956 from near the town of Darlingford on buckwheat (*Fagopyrum esculentum* Moench) and the Agriculture and Agri-food Canada, Morden Experimental Farm on plum (*Prunus* spp. L.). *Pratylenchus neglectus* was detected on corn in soil collected in 1971 from the Agriculture and Agri-food Canada, Brandon Experimental Farm. Moreover, *P. pratensis* was from an unknown locality and unknown host (Dr. Qing Yu, personal communication).

None of the surveys conducted in Manitoba identified the species of *Pratylenchus* in potato fields to the species level though important to predict risks of crop damage based on pre-plant nematode levels. In addition, the suitability of potato cv. Russet Burbank, the most widely grown potato variety in Manitoba, for *Pratylenchus* spp. in Manitoba has not been determined.

2.2.4 *Pratylenchus* spp. diagnosis

2.2.4.1 Morphological identification

Pratylenchus spp. diagnosis has relied on the morphology of adult females because they acquire more diagnostic characters than the males that are not common in most of *Pratylenchus* species (Loof, 1991; Castillo and Vovlas, 2007). However, females of different *Pratylenchus* spp. are very similar in gross morphology (Castillo and Vovlas, 2007); in addition, there is strong intra-specific variation with few species-specific diagnostic characteristics (Loof, 1978; Orui, 1996). Consequently, morphological identification of *Pratylenchus* species requires examination of several adult females by well-trained and experienced nematologists. Morphometric measurements (De Man Indices 1876 and 1880) and diagnostic data reported for females and the frequency of occurrence of males of the major *Pratylenchus* spp. present in Canada (Handoo and Golden, 1989) are listed in Table 2.4.

Although morphology is still considered the main means of *Pratylenchus* spp. identification, novel techniques based on biochemical and molecular analysis are being increasingly important for diagnostics, evaluation of genetic diversity of populations and the inference of phylogenetic relationships between taxa (Al-Banna et al., 1997; Duncan et al., 1999; Al-Banna et al., 2004; Subbotin and Moens, 2006).

2.2.4.2 Biochemical identification (Protein electrophoresis)

In this method, nematodes' soluble proteins are separated based on their different molecular masses using polyacrylamide or starch gels under an electric field. Thousands of soluble proteins can be extracted from *Pratylenchus* spp. or populations and after their

Table 2.4 Morphometric measurements (De Man Indices 1876 and 1880) and diagnostic data reported for females and the presence of males of the major *Pratylenchus* spp. present in Canada (Handoo and Golden, 1989).

<i>Pratylenchus</i> spp.	Character*								
	L	a	b	c	V%	Labial annuls	S	Lateral fields	Males
<i>P. crenatus</i>	0.32 – 0.59	19.7 – 29.9	4.9 – 7.9	16.4 – 26.8	78.2 – 86.3	3	14.0 – 18.0	4	Unknown
<i>P. fallax</i>	0.42 – 0.56	14.0 – 33.0	5.2 – 6.7	18.0 – 24.0	77.0 – 81.0	3	16.0 – 17.0	4	Present, common
<i>P. flakkensis</i>	0.42 – 0.57	20.0 – 27.0	5.2 – 7.1	12.0 – 18.0	73.0 – 77.0	2	17.0	4	Present
<i>P. hexincius</i>	0.34 – 0.54	18.2 – 28.8	5.9 – 8.4	16.0 – 22.7	75.3 – 82.2	2	14.5 – 15.4	6	Unknown
<i>P. macrostylus</i>	0.51 – 0.68	22.0 – 33.0	5.0 – 7.4	16.0 – 24.0	85.0 – 88.8	2	21.0 – 24.7	4	Present
<i>P. neglectus</i>	0.31 – 0.58	16.5 – 32.2	4.9 – 7.8	13.8 – 26.8	75.5 – 86.6	2	15.0 – 19.0	4	Present, very rare
<i>P. penetrans</i>	0.43 – 0.81	19.0 – 32.0	5.3 – 7.9	15.0 – 24.0	75.0 – 81.0	3	15.0 – 17.0	4	Present, common
<i>P. pratensis</i>	0.40 – 0.63	21.8 – 30.3	5.5 – 7.6	13.7 – 26.8	76.0 – 80.0	3	12.0 – 16.0	4	Present, common
<i>P. sensillatus</i>	0.57 – 0.69	28.0 – 42.0	7.1 – 8.3	20.0 – 31.0	77.0 – 81.0	3	15.0 – 17.0	4	Unknown
<i>P. thornei</i>	0.45 – 0.77	26.0 – 36.0	5.5 – 8.0	18.6 – 25.1	74.0 – 79.0	3	15.0 – 19.0	4	Present, very rare
<i>P. zea</i>	0.36 – 0.58	25.0 – 30.0	5.4 – 8.0	17.0 – 21.0	66.0 – 76.0	3	15.0 – 17.0	4	Unknown

* L = Mean length in millimeters; a (ratio) = Body Length/Greatest body width; b (ratio) = Body Length/Distance from anterior end to junction of Esophagus; c (ratio) = Body length/ Tail length (from the anus to the posterior end); V% = Vulva in relation to the head % of body length; S = Stylet length in μm .

staining, specific band patterns can be found for each species or population (Subbotin and Moens, 2006). Differences in banding patterns between species or populations can be used in studying protein polymorphism and genetic diversity among *Pratylenchus* populations (Payan and Dickson, 1990). In isoelectric focusing electrophoresis, extracted soluble proteins from *Pratylenchus* spp. populations are separated based on their charge in a pH gradient that yield more stable profiles and separate proteins into sharp bands. Extensive characterization of isozymes has been carried out for *Pratylenchus* spp. These studies revealed wide variation between populations of *Pratylenchus* spp. Payan and Dickson (1990) compared five populations of *P. brachyurus* from various geographical regions and hosts and one *P. scribneri* population by analyzing 18 enzyme systems (using 250 female nematodes) using isoelectric focusing electrophoresis. Only malate dehydrogenase, phosphoglucomutase, and phosphoglucose isomerase were detected from all five *P. brachyurus* populations and *P. scribneri*. Three distinct phenotypic groups were found in the malate dehydrogenase and phosphoglucomutase systems for *P. brachyurus* populations, but only a single electromorph was detected for phosphoglucose isomerase. Multiple electromorphs for malate dehydrogenase, phosphoglucomutase, and phosphoglucose isomerase were detected for *P. scribneri*; there was no similarity among these patterns with those from *P. brachyurus*. They did not observe phenotypic differences in phosphoglucose isomerase between females and mixed juveniles of a population of *P. brachyurus*.

A major limitation to using protein patterns and isozyme phenotypes in *Pratylenchus* spp. diagnosis is the large numbers of nematodes that are required for analysis. In addition, the protein examined may not be present in all life stages of the

nematode and that expression of a certain protein may be influenced by environmental factors (Jones et al., 1997; Castillo and Vovlas, 2007).

2.2.4.3 Molecular identification

Polymerase chain reaction (PCR)

In polymerase chain reaction (PCR), large number of copies of DNA molecules are produced by means of an enzyme catalyst (Subbotin and Moens, 2006). PCR is rapid, efficient, reliable, and does not require expertise in nematode taxonomy and morphology. PCR could be used as a rapid diagnostic tool for commercial and research applications for disease forecasting and management (Yan et al., 2008) and it is frequently used in diagnosis of *Pratylenchus* spp. (Castillo and Vovlas, 2007). Al-Banna et al., (2004) designed five forward species-specific primers from the internal variable portion of the D3 expansion region of the 26S rDNA for *P. penetrans*, *P. neglectus*, *P. thornei*, *P. vulnus* Allen and Jensen, and *P. scribeniri*. Each primer was used with a single, common reverse primer (D3B). The optimized species-specific primers produced unique amplicons from their respective target and did not amplify DNA from other *Pratylenchus* species. More recently, Yan et al., (2008) designed a set of PCR primers from 28S rRNA gene sequences of the D3 expansion domain that was capable of detecting and identifying *P. neglectus* and *P. thornei* from soil. PCR products of 144 bp for *P. neglectus* and 288 bp for *P. thornei* differentiated the two species.

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP)

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) includes amplification of DNA fragments from different species or populations of

Pratylenchus spp. using PCR. These DNA fragments are then digested using a set of restriction enzymes. Following digestion, the resulting fragments are separated by electrophoresis. If the digestion process yielded fragments with different lengths, the digestion of the PCR products yields restriction fragments length polymorphism profiles (Subbotin and Moens, 2006). PCR–RFLP of the internal transcribed spacer (ITS) region of the ribosomal RNA gene is a very reliable method for identification of *Pratylenchus* spp. (Powers and Harris, 1993; Orui, 1996). RFLP in ITS region of rDNA amplified by PCR was a reliable and precise method for differentiating *P. penetrans*, *P. coffeae*, *P. vulnus* and an unidentified species very similar to *P. coffeae* using the endonucleases *AluI*, *DdeII*, *HhaI*, *HinfI*, and *TaqI* (Orui, 1996).

The PCR–RFLP technique was used to establish a reliable diagnostic method for 18 *Pratylenchus* species: *Pratylenchus agilis* Thorne and Malek, *P. bolivianus* Crobet, *P. brachyurus*, *P. coffeae*, *P. crenatus*, *P. fallax*, *P. goodeyi* Sher and Allen, *P. loosi*, *P. mediterraneus* Crobett, *P. neglectus*, *P. penetrans*, *P. pratensis*, *P. pseudocoffeae* Mizukubo, *P. scribneri*, *P. subranjani* Mizukubo, Toida, Keereewan and Yoshida, *P. thornei*, *P. vulnus*, and *P. zaeae*. ITS regions from all species and populations were amplified using PCR and then were examined. These ITS regions revealed large differences in length ranging in size from 900 to 1250 bp. Five restriction enzymes (*CfoI*, *DdeI*, *HindIII*, *HpaII*, and *PstI*) were used to digest the rDNA fragments. All *Pratylenchus* species can be differentiated from each other by a combination of at least two enzymes. *CfoI* differentiated all nematode species with the exception of *P. fallax*, *P. penetrans*, and *P. pseudocoffeae*. *Pratylenchus fallax* was further separated by a *DdeI* restriction and *P. pseudocoffeae* by a *PstI* digestion. Upon *CfoI*, *DdeI*, *HindIII*, or *HpaII*

digestion, it was possible to separate the three *P. coffeae* populations studied from each other (Waeyenberge et al., 2000).

Multiplex polymerase chain reaction (Multiplex PCR)

Multiplex PCR enables the detection of one or several nematode species in a mixture of nematodes by a single PCR test using a number of primers simultaneously (Subbotin and Moens, 2006; Castillo and Vovlas, 2007). Multiplex PCR reduce the time and cost of diagnostics. In this method, different primer pairs in the same amplification reaction amplify two or more unique targets of DNA sequences in the same sample (Subbotin and Moens, 2006). Diagnostics using multiplex PCR with species-specific primers (Mi1-Mi2 and Pc1-Pc2), designed based on the ITS region of *M. incognita* (Kofoid and White) Chitwood and *P. coffeae*, enabled the detection of two specific bands derived from the two plant-parasitic nematodes even when the genome of other nematodes was mixed in the template (Saeki et al., 2003).

Qiu et al., (2005) designed four pairs of species-specific primers from ITS sequences of rDNA from *P. penetrans*, *P. vulnus*, *P. scribneri*, and *P. thornei* that amplified 470, 368, 617, and 603 bp DNA fragments, respectively. The same temperature was used in the PCR with all four pairs of primers and different size of DNA was amplified by different primers. This allowed the usage of multiplex PCR to amplify DNA from target species or separate species in a mixed population. The primers also amplified target DNA from soil extracts of field samples at levels as low as a single lesion nematode among other free-living or plant-parasitic nematodes.

Random amplified polymorphic DNA (RAPD)

In this method, a single random short primer (approximately ten nucleotides long) is used for creating genomic fingerprints. The short primer anneals to several similar sequences within the genome during the PCR cycle. If two complementary sequences are present on opposite strands of a genomic region in the correct orientation and within a close enough distance from each other, the DNA fragment between them can be amplified by PCR. Amplified DNA fragments obtained using different random primers from different samples are separated on gels and compared. The hypothesis behind this technique is that if primer hybridization site in a genome differs by even a single nucleotide, the change can lead to elimination of a specific amplification product (Subbotin and Moens, 2006). Random amplified polymorphic DNA was successful in discriminating seven isolates of *P. vulnus* from different geographical areas and different hosts and one isolate of *P. neglectus*. Random amplified polymorphic DNA patterns among the seven *P. vulnus* isolates were similar, although high intraspecific variability was present (Pinochet et al., 1994).

2.2.5 *Pratylenchus* spp. pathogenicity

2.2.5.1 Symptoms caused by *Pratylenchus* spp.

Aboveground symptoms of *Pratylenchus* spp. infection are not obvious and resemble those of other soil-borne diseases, nutrient deficiencies, insect damage, and/or environmentally induced stress (Duncan and Moens, 2006; Castillo and Vovlas, 2007). *Pratylenchus* spp. infection in the field occurs as patches of stunted and chlorotic plants, which give the field a ragged appearance. Damage is often most severe in the centre of

these areas, diminishing towards the edge to normal appearing plants (Castillo and Vovlas, 2007).

Belowground symptoms of *Pratylenchus* spp. infection occur as brown to reddish necrotic lesions parallel to the root axis and eventual secondary decomposition of roots. Heavily infected root systems are reduced in size and may show other symptoms such as ‘witch’s broom’, irregular root swelling and stunted rootlets. Symptoms in tubers, such as potato, range from scabby or sunken lesions (caused by *P. scribneri*) to warty outgrowth (caused by *P. penetrans*) (Duncan and Moens, 2006).

2.2.5.2 *Pratylenchus* spp. host range

Pratylenchus spp. are polyphagous plant-parasitic nematodes that feed on many different hosts (Castillo and Vovlas, 2007). Host suitability to *Pratylenchus* spp. is assessed by measuring their reproduction on plants after being artificially inoculated (Lewis, 1987). Several species of *Pratylenchus* have wide host range but their economic damage has been characterized for relatively few crops (Duncan and Moens, 2006). Host range studies on *P. penetrans* showed that it could parasitize nearly 400 plants including fruit, vegetable and cereal crops (Castillo and Vovlas, 2007).

2.2.5.3 *Pratylenchus* spp. damage thresholds

The degree of crop damage by *Pratylenchus* spp. is dependent on the nematode population densities in soil prior to planting or population densities in plants roots during the growing season (Castillo and Vovlas, 2007). Plants generally can tolerate low to moderate population levels of *Pratylenchus* spp. without occurrence of economic damage and the number of nematodes below which economic damage does not occur is called the

“damage threshold”. When nematode numbers increase above the damage threshold, economical damage occurs and crop yield decreases. Examples of damage threshold densities of *Pratylenchus* spp. most common in Canada on different host plants are listed in Table 2.5.

2.2.5.4 *Pratylenchus* spp. interaction with other plant pathogens

***Pratylenchus* spp. interaction with other plant-parasitic nematodes**

The population dynamics of plant-parasitic nematodes are influenced both by host plant identity and by the presence of other root-feeding nematodes (Brinkman et al., 2008). *Pratylenchus* spp. distribution and population densities in the field may reflect competitive interactions with other plant-parasitic nematodes (Castillo and Vovlas, 2007). When *Tylenchorhynchus ventralis* (Loof) Fortuner and Luc, and *P. penetrans* were added in different densities to natural dune grass (*Ammophila arenaria* L.); *P. penetrans* suppressed the development of *T. ventralis*, but only when it was added to the plant in relatively high densities that exceeded the typical field density of *P. penetrans* (Brinkman et al., 2004). Therefore, Brinkman et al., (2004) concluded that competition by *P. penetrans* is not a likely mechanism for the regulation of *T. ventralis* populations. Sequential inoculation of *P. penetrans* did not influence the development of *T. ventralis* more than inoculation at the same time. Despite their inhibiting effect on the development of *T. ventralis*, *P. penetrans* did not counteract the negative effect of *T. ventralis* on plant biomass.

Table 2.5 Damage threshold densities of three of the most common *Pratylenchus* spp. in Canada on different host plants (adapted from Castillo and Vovlas, 2007).

<i>Pratylenchus</i> spp.	Host plant	Damage threshold (nematodes kg ⁻¹ soil)	Reference
<i>P. neglectus</i>	Alfalfa (<i>Medicago sativa</i> L.)	2,000	Griffin and Gray (1990)
	Potato (<i>Solanum tuberosum</i> L.)	1,500	Umesh and Ferris (1994)
<i>P. penetrans</i>	Alfalfa (<i>Medicago sativa</i> L.)	1,000	Griffin (1993)
	Clover (<i>Trifolium</i> spp.)	1,700	Willis and Thompson (1969)
	Potato (<i>Solanum tuberosum</i> L.)	1,000-2,000	Olthof and Potter (1973); Olthof (1987)
	Tomato (<i>Solanum lycopersicum</i> L.)	450	Miller (1978)
<i>P. thornei</i>	Chickpea (<i>Cicer arietinum</i> L.)	30	Di Vito et al., (1992)
	Faba bean (<i>Vicia faba</i> L.)	2,200	Di Vito et al., (2000)

The distributions of *P. coffeae* and *Tylenchulus semipenetrans* Cobb in a central Florida citrus orchard were mutually exclusive. In an experiment conducted in the orchard, indigenous populations of either species did not prevent infection by the other species. Inoculation with either *T. semipenetrans* or *P. coffeae* tended to reduce the population size of the other nematode species. In greenhouse tests, one or the other of the two species parasitized individual feeder roots predominantly. Host response to parasitism in dual infections did not differ from response to single infection by either species (Kaplan and Timmer, 1982).

Under greenhouse conditions, population densities of *P. penetrans* and *M. incognita* were reduced when both nematodes inhabited the same tomato roots. Population density of *P. penetrans* alone after 50 days was about four times higher than when *M. incognita* was present. Conversely, *M. incognita* reproduced twice as fast alone as in combination with *P. penetrans*. No difference in the density of *P. penetrans* was observed when they were inoculated either 10 days prior to or after introduction of *M. incognita*. Root entry by *P. penetrans* was significantly inhibited by the presence of *M. incognita*. Split-root experiments showed that the inhibitory effects of *M. incognita* upon reproduction of *P. penetrans* involved factors other than the availability of feeding sites. On the other hand, the inhibitory effects of *P. penetrans* on *M. incognita* appeared to be primarily due to the quantity of available roots (Estores and Chen, 1972).

***Pratylenchus* spp. interaction with bacteria**

Pratylenchus spp. can interact synergistically or antagonistically with plant-pathogenic bacteria yielding disease complexes (Sitaramaiah and Pathak, 1993) or (Castillo and Vovlas, 2007). *Pratylenchus* spp. can interact synergistically with the bacteria by acting as a predisposing agent by providing wounds for the entry of the bacterial pathogen, or by breaking host resistance to bacteria (Castillo and Vovlas, 2007).

For example, *P. penetrans* interacted with *Pseudomonas viridiflava* (Burkholder) Dowson, *P. corrugate* Roberts and Scarlett, and *P. marginalis* (Brown) Stevens and lead to greater growth reductions than caused by a single pathogen, suggesting synergistic relationships. *Pratylenchus penetrans* produced root wounds that were used by the bacteria (Bookbinder et al., 1982).

Antagonism between *Pratylenchus* spp. and bacteria has also been reported (Castillo and Vovlas, 2007). As an example, *P. chlororaphis* (Guignard and Sauvageau) Bergey strain Sm3 reduced numbers of *P. penetrans* per gram of strawberry roots after six to 10 wk of strawberry roots inoculation with the bacteris in three separate greenhouse experiments with soils from southern British Columbia, Canada (Hackenberg et al., 2000).

***Pratylenchus* spp. interaction with pathogenic fungi**

Pratylenchus spp. are among the plant-parasitic nematodes genera most commonly reported to be involved in disease complexes with fungal pathogens (Back et al., 2002). Several *Pratylenchus* spp.-fungus interactions have been documented in literature with the interaction between *V. dahliae* and *Pratylenchus* spp. in potato early dying disease complex being one of the most notorious.

Potato early dying is a limiting factor to potato production in many areas of North America (Martin et al., 1982; Powelson and Rowe, 1993). Populations of *Pratylenchus* spp. aggravate early dying of potato caused by *V. dahliae* (Martin et al., 1981; Wheeler et al., 1992; Bowers et al., 1996; Hafez et al., 1999). *Pratylenchus* spp. vary in their ability to interact with *V. dahliae* and to induce potato early dying. *Pratylenchus penetrans* and *P. thornei* can interact synergistically with *V. dahliae* (Riedel et al., 1985; Wheeler et al., 1994; Wheeler and Riedel, 1994; Duncan and Moens, 2006), but *P. crenatus* does not (Riedel et al., 1985; Duncan and Moens, 2006). *Pratylenchus neglectus* has not been studied extensively with a population from Ontario, Canada, that interacted with *V. dahliae* and one from Idaho, United States, which did not interact with the fungus (Hafez

et al., 1999). *Pratylenchus scribneri* interacts with *V. dahliae* only at elevated temperatures (>32 °C) (Riedel et al., 1985). In this pathosystem, interactions were also dependent on fungal genotype. Botseas and Rowe (1994) in field micro-plots and greenhouse experiments found that *P. penetrans* interacts synergistically with *V. dahliae* VCG 4A to increase wilt severity, but not VCG 4B.

Potato early dying usually develops throughout infested potato fields typically halfway through the growing season with the disease becoming more severe toward the end of the growing season and the period of tuber bulking. This results in a decline in tuber size and total marketable yield (Rowe and Powelson, 2002). Tuber yield reduction can also take place without showing any symptoms on the infected plants (Rowe et al., 1985).

The two pathogens, *V. dahliae* and *Pratylenchus* spp., interact synergistically; together they cause severe vascular wilt and significantly higher yield reductions at population levels that have little or no effect when each of the two pathogens is present alone. The role that *Pratylenchus* spp. play in potato early dying is very crucial. The presence of the nematode with population equal or more than 1 g⁻¹ soil synergistically interacts with low populations of the fungus that in the absence of the nematodes would not cause disease (Saeed et al., 1998). The mechanism of the synergism between *Pratylenchus* spp. and *V. dahliae* is unclear. However, several hypotheses have been suggested to elucidate the mechanisms of their interaction. One of the hypotheses proposed is that the fungus utilizes the nematode-induced wounds to access the plant tissues. Bowers et al., (1996) infested soil in the greenhouse with known densities of *V. dahliae* and *P. penetrans* and then they harvested the plants three, five, seven, and nine

wk after planting. They observed that root infection by *V. dahliae* was through the root tip and the zone of elongation, regardless of the presence of *P. penetrans*. Five wk after planting, roots grown in soil infested with *V. dahliae* alone had a very low percentage of infected root tips (1.2%), which was significantly less than infected roots growing in soil infested with *V. dahliae* and *P. penetrans* (2.3%). They also found that roots were colonized by *V. dahliae* largely when grown in soil infested with *V. dahliae* and *P. penetrans* than in soil infested with *V. dahliae* alone. This trend continued with differences between treatments more clear after seven wk. Infection by *V. dahliae* was not observed to be associated with the site of nematode feeding.

Proposed another hypothesis to explain the mechanism of interaction between *Pratylenchus* spp. and *V. dahliae* is that the nematode affects host physiology in some way that results in altered or delayed host response to colonization by the fungus (Bowers et al., 1996). Rotenberg et al., (2004) separated *V. dahliae* and *P. penetrans* by inoculating roots with the nematode prior to injecting fungal conidia into the stem vasculature. Treatments were *P. penetrans* alone, *V. dahliae* alone, nematode and fungus together, and a no pathogen control. They observed that potato plants infected with the two pathogens contained more colony forming units of *V. dahliae* in stem sap than those infected by the fungus alone. They reported that infection of, cv. Russet Burbank, roots by *P. penetrans* systemically affects disease physiology associated with stem vascular infection by *V. dahliae*. They concluded that the role of the nematode in the fungus/host interaction is more than to facilitate extra-vascular and vascular entry of the fungus into potato roots. Accordingly, the role of *Pratylenchus* spp. in the potato early dying complex is systemic, suggesting that the nematode affects host physiology in some way that

promotes vascular colonization with *Verticillium* spp. However, the mechanism by which the nematodes suppress the defense mechanisms in potato plants against the fungus is not clear.

In another example of *Pratylenchus* –fungus interaction is the interaction between *P. neglectus* and the most frequently isolated fungi from wheat in Australia (*Pyrenochaeta terrestris* (Hansen) Gorenz, Walker and Larson, *Pythium irregulare* Buisman, *Fusarium oxysporum* Schltdl., and *Gaeumannomyces graminis* (Sacc.) Arx and Olivier plus *F. equiseti* (Corda) Sacc.). *Pratylenchus neglectus* synergistically interacted with these fungi and significantly increased severity of root lesions. Moreover, in chickpea (*Cicer arietinum* L.), *P. thornei* infections increased the severity of root necrosis by *F. oxysporum* f. sp. *ciceris* race 5 on both a wilt susceptible and wilt resistant chickpea cultivars irrespective of length of incubation time, densities of nematodes, fungal inoculation levels, and experimental conditions (Castillo et al., 1998).

2.2.6 *Pratylenchus* spp. management strategies

Once introduced into a field, *Pratylenchus* spp. are very hard to eradicate (Castillo and Vovlas, 2007). However, their ability to damage crops can be reduced by reducing the nematode numbers in soil through the application of several management strategies as: crop rotation, cultural practices, use of nematicidal plants, physical control, host plant resistance, biological control, and chemical control (Duncan and Moens, 2006; Castillo and Vovlas, 2007).

2.2.6.1 Cultural practices

Crop rotation

Crop rotation is one of the most important strategies used for the management of plant-parasitic nematodes (Duncan and Moens, 2006). Crop rotation reduce the population of *Pratylenchus* spp. in soil through the use of non-, poor or inhibitory hosts in rotation with susceptible crops to increase the duration before the next susceptible crop is grown (Viaene et al., 2006). The wide host range of *Pratylenchus* spp. makes designing a crop rotation that includes non-hosts to this nematode difficult. Accordingly, the use of crop rotation to manage *Pratylenchus* spp. is often considered a limited option (Castillo and Vovlas, 2007). However, some effective rotations have been developed to control *Pratylenchus* spp. For example, in Sonora, Mexico, *P. thornei* numbers, and subsequently damage to wheat, was reduced on a commercial scale when wheat was rotated with corn, cotton, and soybeans and wheat was not grown for two consecutive years (Van Gundy et al., 1974). In southeastern United States, multi-crop systems, corn-peanut-cotton-soybean and cotton-soybean-corn-peanut, were effective in suppressing *Pratylenchus* spp. population densities (Johnson et al., 1975). In Ontario, Canada, growing forage millet (var. CFPM 101) or grain millet (var. CGPMH-1) in rotation with tobacco rather than the crop commonly grown in rotation with tobacco, winter rye (*Secale cereale* L.), suppressed *P. penetrans* populations in soil and yielded 19 to 70% higher tobacco yield following forage millet and 18 to 65% higher following grain millet (Amankwa et al., 2006). *Pratylenchus penetrans* population in potato soil was reduced by one or two years of rotation to non-host or antagonistic plants such as Saia oat (*Avena strigosa* Schreb.),

Polynema marigold (*Tagetes erecta* L.), or Brown Daisy (*Rudbeckia hirta* L.) which in turn may reduce the severity of potato early dying (La Mondia, 2006).

Antagonistic plants

Plants antagonistic to nematodes are those that produce nematicidal compounds (e.g. dithioacetylenes, glycosides, glucosinolates) (Ferraz and De Freitas, 2004). The production and active release of toxic substances while the crop is growing or after incorporation into the soil is usually responsible for control. A large number of plants have been shown to contain nematicidal compounds when extracted from the tissue and tested *in vitro*. Species of marigold (*Tagetes* spp. L.), sunn hemp (*Crotalaria juncea* L.), castor Bean (*Ricinus communis* L.), partridge pea (*Chamaecrista fasciculata* L.), asparagus (*Asparagus officinalis* L.) and sesame (*Sesamum indicum* L.) are examples of such plants, which can successfully reduce populations of *Pratylenchus* spp. as well as other species. In a field trial in Prince Edward Island, Canada, marigold cultivars (*T. tenuifolia*, cv. NemaKill and cv. Nemanon) reduced *Pratylenchus* spp. population and increased potato tuber yield by up to 14% when potato followed marigolds (Kimpinski et al., 2000). In another field trial in Quebec, Canada, Pearl millet (*Pennisetum glaucum* L.) reduced *P. penetrans* populations after a one-year rotation. The following year, marketable potato cv. Superior yields were negatively correlated with initial *P. penetrans* densities in two experimental sites (Bélair et al., 2005).

Organic amendments and green manure

Incorporation of organic matter to the soil through soil amendments and green manures result in increasing diversity and activities of soil microbes, including beneficial ones, and reducing the numbers of plant-parasitic nematodes. Organic amendments and

green manure reduce plant-parasitic nematode populations by a number of mechanisms including: (a) nutrition-mediated increase in the resistance of the plant to the nematode, (b) release of compounds toxic to the nematodes, preformed like phenols, tannin, azadirachin, ricinin or derived from the decomposition process in the soil like ammonia, nitrous acid, and VFA, (c) alteration of the soil chemical and physical environment that reduces the persistence and activity of plant-parasitic nematodes, and (d) promotion of the activity of soil microorganisms antagonistic to the plant-parasitic nematodes (Widmer et al., 2002; Viaene, et al., 2006; Castillo and Vovlas, 2007).

The most common organic amendments that have been used in nematodes control include: oil cakes (e.g., *Azadirachta indica* L., *Arachis villosulicarpa* Hoehne, *Gossypium* spp. L., and *Glycine max* L. Merr.), agro-industrial wastes (e.g. sawdust and tree bark, cellulose waste, and rice and coffee husks), animal and urban waste (e.g. chicken manure, farmyard manure, liquid manures, fish remains, bone meal, and raw sewage), and plant residues (e.g. *Ascophyllum nodosum* L., *A. indica* L. leaves, *Brassica oleracea* L. leaves, and *Ananas comosus* L. (Merr.) leaves) (Sikora et al., 2005).

Several reports have shown that anaerobically digested or stored manures are capable of killing plant pathogens and pests such as plant-parasitic nematodes. In a field experiment, application of liquid dairy manure to forage crops reduced populations of *Pratylenchus* spp. and the stubby nematode, *Paratrichodorus* spp. (Timper et al., 2004). Also, anaerobically digested cattle manure added to soil in a pot study reduced damage severity to tomato by *M. incognita* as well as their egg mass numbers and soil populations (Jothi et al., 2003) and reduced survival of *P. penetrans* (Min et al., 2007).

Incorporating cover crops into the soil as green manure can significantly reduce the numbers of *Pratylenchus* spp. in soil through chemical or biological mechanisms (Widmer et al., 2002; Castillo and Vovlas, 2007). Chemical mechanisms include the release of compounds into the soil that are toxic or antagonistic to *Pratylenchus* spp. For example, oil radish (*Raphanus sativus* L.) incorporated as a green manure reduced soil population densities of *P. neglectus* due to the production of nematicidal compounds such as breakdown products of glucosinolates (Al-Rehiyani and Hafez, 1998). Biological mechanisms include providing an environment suitable for the increase of microorganisms antagonistic or parasitic to plant-parasitic nematodes, including those that may produce allelochemicals (Widmer et al., 2002).

Liquid hog manure (LHM)

Hog production in Canada

In the third quarter of 2008, Canadian hog producers reported their inventories at 12.8 million hogs (Statistics Canada, 2008). Quebec was the largest hog producer in Canada with 4 million hogs followed by Ontario with 3.2 million hogs. Manitoba comes in the third place with 2.75 million hogs (Statistics Canada, 2008).

Liquid hog manure production, storage, and usage

The 12.8 million hogs in Canada produce 25.6 million tons of manure annually. Following its production, LHM is collected and stored in different storage structures. These structures include earthen storage and concrete and steel tanks. LHM is regarded as a waste product and its disposal has become an important problem in modern agriculture (Shulte, 1977). LHM is an excellent source of plant nutrients and helps to improve soil structure, aeration, and water holding capacity. The use of LHM as a fertilizer for crop

production is a very beneficial way to recycle manure nutrients within an agricultural system (Manitoba Agriculture, Food Rural Initiatives, 2007). The average N, P, K content of LHM is 2.8, 2.4, and 1.7 kg m⁻³, respectively. Producers can often obtain LHM free of charge from neighboring hog rearing operations requiring its disposal. However, farmers often purchase it based on its content of plant nutrients. Due to the cost associated with transporting and applying liquid manure, there is a restriction on the distance that manure can economically be moved (Nagy et al., 2000). There are four main categories of hog manure land application techniques including broadcast (no incorporation), broadcast and incorporation, high disturbance injection, and low disturbance injection (Manitoba Agriculture, Food Rural Initiatives, 2007).

Liquid hog manure usage as a plant pathogens control strategy

LHM has been added to agricultural lands for centuries as a resource of essential plant nutrients and organic matter (Shulte, 1977). Several studies have also shown LHM to control plant pathogens. Application of LHM to potato fields reduced the incidence of verticillium wilt and potato scab, caused by *V. dahliae* and *S. scabies*, respectively, and reduced populations of *Pratylenchus* spp. (Conn and Lazarovits, 1999). It also reduced populations of plant-parasitic nematodes when surface-applied to grassland (Valocka et al., 2000). In a pot study, LHM added to soil inhibited egg production, hatch and survival of second stage juveniles (J2) of *Heterodera glycines* Ichinohe (Xiao et al., 2007 and 2008).

LHM was shown to control plant pathogens through various mechanisms including the toxic effects of ammonia and nitrous acid (Conn et al., 2005), and VFA (Conn et al., 2005; Tenuta et al., 2002).

Ammonia mechanism

When soil microorganisms degrade LHM, nitrogen excess of the microorganisms' needs is released into soil solution in the form of ammonia (NH_3). NH_3 forms only when soil pH rises above 8.5 (Tenuta and Lazarovits, 2002). Ammonia is quickly converted to ammonium (NH_4^+) causing the soil pH to increase. As the pH increases, some NH_4^+ is converted back into NH_3 . NH_4^+ is nontoxic to plant pathogens even at high concentrations, whereas NH_3 is very toxic to pathogens (Warren 1962; Michel and Mew, 1998; Tsao and Oster, 1981).

Nitrous acid mechanism

This mechanism involves the production of nitrous acid (HNO_2) which is toxic to plant pathogens (Loffler et al., 1986; Michel and Mew, 1998; Tenuta and Lazarovits, 2002). The conversion of NH_4^+ into nitrite (NO_2^-) and then nitrate (NO_3^-) through bacterial nitrification causes soil pH to drop. If it goes below pH 5.5, then NO_2^- takes the chemical form HNO_2 . Nitrite is nontoxic, whereas HNO_2 is very toxic to plant pathogens, including *V. dahliae*, *S. scabies*, *F. oxysporum* f. sp. *lycopersici*, *Sclerotinia sclerotiorum* (Lib.) de Bary, and also to crop and weed seeds (Tenuta and Lazarovits, 2002). Nitrous acid is 100–500 times more toxic than ammonia and requires three to five wk to reach maximum effect whereas ammonia toxicity occurs within a week. Soil pH is the crucial

driving factor in determining the relative amounts of toxic (NH_3 or HNO_2) and nontoxic (NH_4^+ or NO_2^-) compounds (Tenuta and Lazarovits, 2002).

Volatile fatty acids (VFA)

Five VFA (acetic, propionic, *n*-butyric, *n*-valeric, and *n*-caproic acids) and two isomers (isobutyric and isovaleric acids) are present in LHM (Table 3.2). Isobutyric and isovaleric acids are isomers of *n*-butyric and *n*-valeric acids, respectively. Both *n*-butyric, *n*-valeric acids, and their isomers share the same chemical formula in the same proportions but they differ in properties because of the differences in the arrangement of atoms. These VFA in LHM are the products of bacterial anaerobic fermentation of carbohydrates and amino acids that takes place in the gastrointestinal tract of hogs and during storage under anaerobic conditions (Zhu, 2000). VFA are present in LHM at differing individual concentrations. Acetic acid is the most dominant volatile fatty acid in LHM representing about 60% of the total amount of VFA. Propionic acid comes next representing about 25% of the total amount of VFA. Butyric, isobutyric, and *n*-valeric and isovaleric acids collectively represent about 3 to 10% of total VFA in LHM (Spoelstra, 1980). Acidic conditions generating non-ionized forms of VFA in LHM were shown to be the form responsible for the suppression of microsclerotia of *V. dahliae* (Conn and Lazarovits, 2000; Conn et al., 2005; Tenuta et al., 2002). In laboratory and pot studies, LHM, enriched in VFA, addition to soil inhibited egg production, hatch, and survival of J2 of soybean cyst nematode, *H. glycines* (Xiao et al., 2007 and 2008). In a laboratory experiment, butyric and propionic acids reduced the *Tylenchorhynchus* spp. population in unsaturated soil under both aerobic and anaerobic conditions (McElderry et al., 2005). Drenching strawberry plants infested with *P. penetrans* with butyric acid (0.1

and 1 M) reduced nematode densities by 98–100% (Browning et al., 2006). VFA inhibit the uptake of substrates such as amino acids, organic acids, and phosphate from surrounding media by inhibiting oxidative phosphorylation of active transport systems in cell membranes (Freese et al., 1973).

Table 2.6 Characteristics of short-chain volatile fatty acids (C2-C6) (VFA) in liquid hog manure (LHM).

VFA	IUPAC nomenclature ⁽¹⁾	Formula	Molecular weight	pK _a	Vapor pressure (mm Hg at 20 °C)
Acetic	Ethanoic acid	CH ₃ COOH	60.1	4.7	11.00
Propionic	Propanoic acid	CH ₃ CH ₂ COOH	74.1	4.8	2.90
Isobutyric	2-Methylpropanoic	(CH ₃) ₂ CHCOOH	88.1	4.8	1.50
<i>n</i> -Butyric	Butyric acid	CH ₃ (CH ₂) ₂ COOH	88.1	4.8	0.84
Isovaleric	3-Methylbutanoic	(CH ₃) ₂ CHCH ₂ COOH	102.1	4.8	0.44
<i>n</i> -Valeric	Pentanoic acid	CH ₃ (CH ₂) ₃ COOH	102.1	4.8	0.15
<i>n</i> -Caproic	Hexanoic acid	CH ₃ (CH ₂) ₄ COOH	116.2	5.0	0.20

⁽¹⁾ International Union of Pure and Applied Chemistry (IUPAC) nomenclature.

Production of volatile fatty acids by ruminants fed forage

Fermentation of carbohydrates to VFA

Ruminal fermentation of both dietary proteins and dietary carbohydrates contribute to VFA production; however, fermentation of dietary carbohydrates is the major contributor to VFA production in roughage-fed ruminants. Plant carbohydrates can be classified based on their structure to; the complex structural carbohydrates (e.g. pectin, hemicelluloses, and cellulose), the storage reserve carbohydrates (e.g. sucrose, fructose,

and starch), and the simple sugars (e.g. glucose, fructose, and galactose) (van Houtert, 1993).

Step 1: Fermentation of carbohydrates

Cellulose and hemicelluloses

Cellulose and hemicelluloses are the most abundant carbohydrates in the hogs feed and both are fermented gradually in the rumen. Cellulose are broken down by cellulase complexes that are excreted externally by ruminal bacteria (e.g. *Fibrobacter succinogenes* (Hungate) Montgomery, *Ruminococcus flavefaciens* Sijpesteijn, and *R. albus* Hungate), certain fungi (e.g. *Micromonospora ruminantium* Maluszynska and Janota-Bassalik) and protozoa (e.g. species of the genera *Diplodinium*, *Entodinium*, *Eudiplodinium* and *Polyplastron*) and through a number of biochemical steps glucose is produced. However, ruminal bacteria break down hemicelluloses to fructose and trioses (van Houtert, 1993; Leschine, 1995; Fondevila and Dehority, 1996).

Pectin

Plant cell wall pectin is fermented by extracellular pectolytic enzymes excreted by bacteria to pectic acid and galacturonic acid and then to fructoses and trioses (van Houtert, 1993).

Storage carbohydrates

Starches (i.e. amylose and amylopectin) are broken down by amylases and other carbohydrases produced from bacteria and protozoa to maltose and subsequently to glucose units in the rumen (van Houtert, 1993).

Step 2: Fermentation to pyruvate

All the carbohydrate monomers that are formed are then converted to pyruvate through glycolysis. Glucose and fructose monomers are phosphorylated and then split to yield glyceraldehyde- 3-phosphate, whereas triose-phosphate monomers are directly converted to glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate is then converted, again via a number of steps, to phosphoenolpyruvic acid and finally to pyruvate (van Houtert, 1993).

Step 3: Fermentation of pyruvate to volatile fatty acids

Pyruvate in the rumen is then converted enzymatically to VFA through a number of major pathways by a large number of microbes. Fermentation of feed to the various VFA is determined by conditions favoring or diminishing the activities of particular groups of ruminal microorganisms, the availability and nature of the substrate, the presence of electron donors and acceptors, and microbial interactions (van Houtert, 1993).

Fermentation to acetate

A major pathway for the conversion of pyruvate to acetate in the rumen is the cleavage of pyruvate to acetyl-phosphate and formate that is catalyzed by pyruvate-formate lyase (van Houtert, 1993).

Fermentation to propionate

The formation of propionate from carbohydrates takes place via two major pathways in the rumen of hogs depending on the feed of hogs. In the first pathway, pyruvate is condensed with carbon dioxide to form oxaloacetate or malate that in turn is

converted via fumarate to succinate. Succinate is then used as a substrate by other microbial species, which convert it to propionate through decarboxylation. This pathway dominates propionate production in the rumen of animals that are fed roughages. A second pathway of production involves the conversion of pyruvate to propionate via lactate and acrylate. This pathway takes prominence only in the rumen receiving high levels of grain (van Houtert, 1993).

Fermentation to butyrate

Butyrate is quantitatively the least important of the three major VFA in rumen fluid of animals. Reversal of the classical oxidation of fatty acids appears to be the main mechanism for condensation of two mol acetate into one mol butyrate. Two moles of acetate are activated to acetyl-CoA, which is then condensed to one mol acetoacetyl-CoA. This is then converted in a number of steps to butyrate. Carboxylation of acetyl-CoA to malonyl-CoA and the subsequent reaction of malonyl-CoA with acetyl-CoA to yield acetoacetyl-CoA is another potential pathway leading to butyrate synthesis (van Houtert, 1993).

2.2.6.2 Physical control

Physical control of *Pratylenchus* spp. includes soil solarization and thermotherapy. Soil solarization, heating soil under transparent plastic tarps, has been used effectively to disinfest soil of plant pathogens (Katan, 1981; McGovern et al., 2002). Moistened soil covered with polyethylene sheeting can provide significant reductions in nematode populations. The effect reduces with depth, but solarization for at least four to six wk will increase soil temperatures to about 35–50 °C to depths of up to about 30 cm and, depending on soil type and prior tillage, will reduce nematode infestations

significantly (Viaene et al., 2006). Solarization has been successfully used in controlling *Pratylenchus* spp. (Castillo and Vovlas, 2007). In Australia, solarization of artificially inoculated soils reduced inoculum levels to at least a depth of 10 cm and effectively controlled *P. penetrans* in celery (Porter and Merriman, 1985). In Syria, solarization for six to eight wk during the summer greatly reduced the population of *P. thornei* in soil and increased the yield of chickpea (Di Vito et al., 1991).

Thermotherapy, treatment of plant material with hot water, is also an option to control *Pratylenchus* spp. (Castillo and Vovlas, 2007). In east Africa, treating cooking bananas (*Musa* spp., AAA group, subgroup Matoke) with hot water (53 to 55 °C for 20 min.) slowed down the build-up of *P. goodeyi* (Elsen et al., 2004).

2.2.6.3 Host plant resistance

The phase out of many nematicides and fumigants (e.g. methyl bromide) from use due to increasing concern about environmental contamination has increased the importance of plant resistance and tolerance to plant parasitic-nematodes as a control strategy. Resistant cultivars are environmentally benign, durable, and cost-efficient control strategy for plant-parasitic nematodes (Cook and Starr, 2006). Resistance is typically defined as the ability of a plant to inhibit reproduction of a given nematode relative to a susceptible plant that supports high levels of reproduction. However, tolerance and intolerance is a measure of ‘host sensitivity’ and may be determined by assessing plant growth in the presence of the nematode, or in crop terms, measuring yield loss caused by nematodes (Cook and Starr, 2006). The ideal resistant cultivar should yield as much as cultivars with similar genetic yield potential treated with nematicides and should have no need for specialized equipment and growing techniques and therefore incur no additional costs (Cook and Starr, 2006; Castillo and Vovlas, 2007).

Resistance is currently available to *Pratylenchus* spp. in a number of crops, e.g. fruit trees, woody plants, roses (*Rosa* spp. L.), strawberries (*Fragaria* spp. L.), and raspberries (*Rubus* spp. L) (Castillo and Vovlas, 2007). The lack of resistant potato germplasm made the breeding for resistance unsuccessful so far (Brodie, 1998). Some host resistance has been identified in potato plants to *Pratylenchus* spp. (Castillo and Vovlas, 2007). Reproduction of four *P. penetrans* isolates from Cornell, Wisconsin, Long Island, and Adirondack was measured on three potato cultivars (cvs. Russet Burbank, Butte, and Hudson) and two breeding lines (NY85 and L118-2). Potato cv. Butte, Hudson and L118-2 were identified as resistant to *P. penetrans* from Cornell and susceptible to the isolate from Long Island (France and Brodie, 1995).

2.2.6.4 Biological control

Several antagonistic organisms are capable of reducing the ability of *Pratylenchus* spp. to survive in soil and reproduce (Castillo and Vovlas, 2007). *Pratylenchus* spp. antagonistic organisms include bacteria, fungi, entomopathogenic nematodes, and nematicidal plants (Castillo and Vovlas, 2007).

Bacteria

Bacteria antagonistic to plant-parasitic nematodes produce antibiotics, enzymes, or toxins that affect the nematode's behavior, feeding, or reproduction (Viaene et al., 2006). Several bacterial endophytes isolated from African (*T. erecta* L.) and French (*T. patula* L.) marigold, namely: *Microbacterium esteraromaticum* (Omelianski) Takeuchi and Hatano and *Kocuria varians* (Migula) Stackebrandt reduced the population density of *P. penetrans* in soils around the root zone of potato plants, without any yield reduction

(Sturz and Kimpinski, 2004). In greenhouse experiments, *P. putida* Trevisan recovered from wheat soil suppressive to *Pratylenchus* spp. suppressed apple replant disease caused by *P. penetrans* (Mazzola et al., 2002).

Nematophagus fungi

Several nematophagus fungi are capable of reducing *Pratylenchus* spp. populations (Castillo and Vovlas, 2007). In a greenhouse experiment, *Hirsutella rhossiliensis* Minter and Brady (Deuteromycotina: Hyphomycetes) reduced the population of *P. penetrans* entering roots of potato plants by 25%. In addition, the fungus suppressed the nematode population in roots (Timper and Brodie, 1994). *Monacrosporium lysipagum* (Drechsler) Subram killed 81% of *P. neglectus* under laboratory conditions using adhesive knobs within 20 hr (Khan et al., 2006). The nematode-parasitic fungus *Myrothecium verrucaria* (Albertini and Schweinitz) Ditmar produces a mixture of compounds (proteins, sugars, and lipids) that have nematicidal effect and are commercially registered under the name “DiTera”. DiTera is active against many plant-parasitic nematodes including *Pratylenchus* spp. (Chitwood, 2003). Treating *P. penetrans* with 1,380 mg liter⁻¹ DiTera for 72 h immobilized 90% of the nematode population. Moreover, DiTera at 1380 mg liter⁻¹ applied at planting of strawberries provided significant *P. penetrans* suppression (Pinkerton and Kitner, 2006).

Muscodor albus Worapong, Strobel and Hess is a fungus that produces a mixture of volatile organic compounds capable of killing a broad range of plant fungi and bacteria. Exposure of *P. penetrans* to the fungus in sealed chambers caused 82% nematode mortality and 100% mortality in bean roots and soil in greenhouse (Riga et al.,

2008). Immersion of *P. penetrans* juveniles and adults in a nutrient broth culture of *Lysobacter enzymogenes* Christensen and Cook strain C3 led to death and disintegration of the nematodes. The death and disintegration of the nematodes was attributed to the toxins and enzymes produced by this fungus (Chen et al., 2006). However, biological control agents has not proven to be effective in production systems (Hackenberg et al., 2000; Castillo and Volvas, 2007).

Predaceous nematodes

Several predacious nematodes belonging to the groups Dorylaimid, Diplogastrid, and Mononchid have been reported to control *Pratylenchus* spp. The significance of these nematodes' predation on *Pratylenchus* spp. under field conditions has not been determined and their importance as control strategy is still under assessment (Castillo and Vovlas, 2007).

2.2.6.5 Chemical control

Pratylenchus spp. chemical control is usually an option when cultural, physical, and biological methods are not sufficient in reducing the damage caused to high value crops (Castillo and Vovlas, 2007). However, due to their impact on the environment and humans, there are increasing environmental and health concerns around their use (Chitwood, 2003). Chemical nematicides are classified based on their physical form into two main groups; fumigants and non-fumigants (Whitehead, 1998; Haydock et al., 2006).

Fumigants

Fumigants are chemicals that upon their application to the soil produce volatiles that spread through air in the soil pores to kill plant-parasitic nematodes. They are multi-

purpose or broad-spectrum, being non-specific to nematodes, but effective against several kinds of pests and pathogens including weeds (Whitehead, 1998; Haydock et al., 2006).

Fumigants are classified into two groups; one group consists of compounds based on halogenated hydrocarbons and the other group release methyl isothiocyanate as an active ingredient. The halogenated hydrocarbons group includes 1,3-Dichloropropene (1,3-D) and methyl bromide that affect biochemical pathways in protein synthesis and respiration. The other group includes sodium *N*-methyldithiocarbamate, metam sodium, and dazomet (3,5-dimethyl-1,3,5-thiadiazinane-2-thione) that degrade in soil to release methyl isothiocyanate. When cyanide enters the nematode, it prevents the utilization of oxygen that in turn prevents respiration (Haydock et al., 2006). The most common fumigants that are used to control *Pratylenchus* spp. are chloropicrin, dazomet, 1,3-D, Ethylene dibromide (EDB), metam sodium and methyl bromide (Castillo and Vovlas, 2007).

Non-fumigants

Non-fumigant nematicides are chemicals applied to the soil as granular or liquid formulations and depend heavily on initial mixing with soil and local redistribution in solution in soil water (Haydock et al., 2006). Non-fumigant nematicides are classified into two groups; organophosphates (e.g. fenamiphos, ethoprophos, and fosthiazate) and carbamates (e.g. aldicarb, carbofuran, oxamyl) (Haydock et al., 2006; Castillo and Vovlas, 2007). At low concentrations, non-fumigant nematicides disrupt chemoreception and the ability of the nematode to locate the host plant root; however, at higher concentrations they disrupt nematode hatch and movement. The active substance in non-

fumigant nematicides is absorbed through the nematode's cuticle where it disrupts functioning of the nervous system by binding with the enzyme acetylcholinesterase (AChE) (Haydock et al., 2006). Carbamates and organophosphates are often termed 'nematistats' or 'nematistics' because at field rates their effects are reversible. However, even if recovery does follow exposure to a sublethal dose, nematodes may not be sufficiently strong to locate the host plant (Haydock et al., 2006).

In a microplot study at Jordan Station, Ontario, Canada, oxamyl ((EZ)-N,N-dimethyl-2-methylcarbamoyloxyimino-2-(methylthio)acetamide) (32 mg ml^{-1}) applied to cut potato, cv. Russet Burbank tubers, grown in fine sandy loam infested with *P. penetrans* reduced nematode population densities in the soil at midseason and in the soil and roots at harvest. In another micropot, seed pieces treated with oxamyl at 96 mg ml^{-1} or at 32, 64, and 96 mg ml^{-1} followed by a polymer sticker: water (1:4) dip caused phytotoxicity. A pre-plant soil treatment with 1,3-D reduced numbers of *P. penetrans* at planting and increased tuber yields in for two years (Olthof and Townshend, 1991). Soaking potato seed pieces or whole tubers in various concentrations of oxamyl (in aqueous solutions of 1 to 32 mg ml^{-1}) reduced populations of *Pratylenchus* spp. in roots and soil and increased plant growth under glasshouse conditions in Ontario, Canada (Townshend and Olthof, 1988). In Ontario, Canada, oxamyl has been shown to provide potato with protection against *P. penetrans* damage and increased marketable yield of tubers by as much as 44% (Olthof et al., 1985). Applications of aldicarb (2-methyl-2-(methylthio)propanal O-(N-methylcarbamoyl)oxime) to potato cv. Superior have increased tuber yield by as much as 40% when the *P. penetrans* population was large (Olthof, 1986).

Novel nematicidal compounds

Some natural nematicidal compounds have been evaluated to control *Pratylenchus* spp. (Castillo and Vovlas, 2007). Thiarubrine C, a polyacetylenic 1,2-dithiin, isolated from the roots of *Rudbeckia hirta* L. showed strong nematicidal activity in vitro and growth chamber assays against *P. penetrans*. A 12 to 24 hr exposure to Thiarubrine C was sufficient for nematode mortality (Sanchez de Viala et al., 1998).

Table 2.7 Important nematicides registered in Canada.

Nematicide	Chemical group	Trade name	Formulation
Carbofuran	Carbamate	Furadan	Liquid
Dazomet	Methyl isothiocyanate liberator	Basamid	Microgranule
1, 3-Dichloropropene	Halogenated hydrocarbon	Telone	Liquid
Metam sodium	Methyl isothiocyanate liberator	Vapam	Liquid
Oxamyl	Oxime carbamate	Vydate	Microgranule, Liquid

ClandoSan, made from processed crab and crawfish exoskeletons, contains large amounts of chitin and urea. Its nematicidal activity is believed to result from the stimulation of populations of chitinolytic microflora that cause decreased nematode egg viability through degradation of eggshells (Chitwood, 2003; Chen et al., 2006).

A blend of extracts from the prickly pear (*Opuntia lindheimeri* (Engelm.) Parfitt & Pinkava), the oak (*Quercus falcata* Michx.), the sumac (*Rhus aromatic* Aiton), and the

mangrove (*Rhizophora mangle* L.) called “Sincocin” have shown to have nematicidal effect on a variety of plant-parasitic nematodes. However, its mode of action has not been fully elucidated (Chitwood, 2003).

2.3 Nematode community structure as bioindicator of soil health

Based on their life history, nematodes are ordered on a colonizer–persister scale (c-p scale) that ranges from one (colonizers) to five (persisters) (Bongers, 1990; Bongers and Ferris, 1999). Colonizer nematodes have short life-cycle and short generation time, are numerically dominant in samples, show high fluctuations in population densities, have high colonization ability, are tolerant to disturbance, have large gonads and produce numerous small eggs, and have survival structures to overcome unfavorable conditions. Examples of colonizers include nematodes of the families Rhabditidae, Panagrolaimidae, Diplogasteridae, and Monhysteridae (Bongers, 1990). These nematodes flourish under conditions of abundant bacterial growth that can occur following application of broad-spectrum pesticides, in soil, such as fumigants (Yeates et al., 1991; Ettema and Bongers, 1993; Wang et al., 2006) or following addition of readily decomposable materials to soil, such as animal manures (Griffiths et al., 1994; Bongers and Bongers, 1998; Bulluck et al., 2002; Ferris and Matute, 2003; Nahar et al., 2006). On the other hand, persister nematodes have a long life cycle and low reproduction rate, have small gonads and produce few offspring, and are sensitive to disturbance. These nematodes have been shown to be sensitive to different stressors such as metal (Zullini and Peretti, 1986; Korthals et al., 1996 and 2000; Georgieva et al., 2002), acidification (Dmowska, 1993; Ruess et al., 1996) nematicides (Smolik, 1983), and nitrogen (Tenuta and Ferris, 2004).

As well many species have intermediate characteristics and occupy intermediate positions on the c-p scale (Bongers, 1990).

Analysis of abundance of taxa comprising nematode communities in soil, sometimes referred to as nematode faunal analysis (Ferris et al., 2001), is an excellent tool in assessing the structure and function of soil food webs and response to altered soil conditions (Freckman and Ettema, 1993; De Ruiter et al., 1994; Ferris et al., 1996; Bongers and Ferris, 1999; Bongers, 1999; Ferris et al., 2001). This is due to the nematodes characteristics that include: (a) having high diversity and abundance in soil environments with complex food webs, with almost 20,000 nematode species having been described; (b) living in soil capillary water and having a permeable cuticle provides them with direct contact with their microenvironment; (c) being able to respond rapidly to disturbance and enrichment; (d) some are slow growing and reproduce slowly thus establishment following disturbance is slow ; (e) they do not migrate rapidly from stressful conditions and different genera vary in their sensitivity to different forms of stress; (f) they are relatively easy to isolate and identify to the Genus levels by well trained personnel; (g) they occupy key positions in soil food webs, they feed on nearly all soil organisms and are food for many others and (h) there is a clear relationship between nematode mouth structure and their food resource (Bongers and Ferris, 1999).

Ferris et al., (2001) developed the nematode faunal analysis that included the characterization of a two-trajectory description of soil food web conditions. The enrichment trajectory, calculated as the enrichment index, is based on the weighted abundance of opportunistic bacterivorous c-p 1 and fungivorous c-p 2 nematodes species that respond rapidly to prey resources. The structure trajectory, calculated as the structure

index, is weighted on the abundance of larger, slower-reproducing bacterivore, fungivore, omnivore and carnivore c-p 3 to 5 value nematodes. Both trajectories have cp-2 guilds as their origin.

Availability of organic resources through external input (e.g. addition of organic matter to soil), mortality of organisms (e.g. due to application of broad spectrum pesticide to soil), or favorable shifts in the environment (Odum, 1985) cause an increase in microbial activity in soil that enhance opportunistic bacterial-feeding nematodes (Ferris et al., 2001) which in turn result in enriched food web with high enrichment index. The introduction of organic resources to soil represents a “bottom-up” constraint on the size and activity of the food web. The presence of high c-p nematodes that feed on opportunistic nematodes and competition among trophic levels provide “top-down” regulation of food web structure and function (Ferris et al., 2001) and over time leads to structured food web with high structure index value. In the case that mortality of the general nematode community occurs as following fumigation, dead organisms become a resource leading to a flush of microbial activity that enhance opportunistic bacterial-feeding nematodes (Ferris et al., 2001). In the absence of high c-p nematodes, that are sensitive to fumigation, the food web becomes enriched and the structure index becomes very low (Wang et al., 2006). Accordingly, nematodes faunal analysis can be used as a tool to determine the effect of LHM on the structure and the function of the soil food web. In addition, it can be used to determine if LHM behave as a fumigant in soil possessing general toxicity to various nematodes groups and eliminating trophic interactions.

MANUSCRIPT 1

3.0 Prevalence and Species Identification of *Pratylenchus* spp. in Manitoba Potato Fields and Host Suitability of Russet Burbank Potato

3.1 Abstract

The objectives of this study were to determine the prevalence and the species of *Pratylenchus* spp. in Manitoba potato (*Solanum tuberosum*) fields with history of potato early dying disease using both morphometric and molecular techniques. In addition, determine the suitability of “Russet Burbank” potato as a host for *Pratylenchus* spp. present in Manitoba. During the summers of 2004 and 2005, 283 soil samples were collected from 31 potato fields in 11 rural municipalities in Manitoba with a history of potato early dying. *Pratylenchus* spp. were detected in 39% of the fields sampled and its population density in infested soil samples ranged from 45 to 631 nematodes kg⁻¹ fresh soil with median population density of 125 nematodes kg⁻¹ fresh soil. Morphometrics of female nematodes and molecular diagnosis (polymerase chain reaction followed by molecular sequencing of the products and comparison to the GenBank BLAST database) showed that the species of *Pratylenchus* prevalent in potato fields surveyed is *P. neglectus*. Potato, cv. Russet Burbank, was found to be a poor host to two populations of *Pratylenchus* spp. present in Manitoba potato fields. It is assumed that the presence of suitable host plants, cereals and canola, in rotation with potato in Manitoba and favorable soil texture favored the prevalence of *P. neglectus* rather than the most widespread species in soil regularly cropped to potato in eastern United States and Canada, *P. penetrans*. More studies are needed to determine whether *Pratylenchus* spp. in Manitoba

are causing damage to crops in rotation with potato and if they play a role in the early dying disease complex.

3.2 Introduction

Root-lesion nematodes, *Pratylenchus* spp. Filipjev 1936, are serious pathogens of potato (*Solanum tuberosum* L.) plants in temperate, tropical and subtropical regions of the world (Scurrah et al., 2005; Duncan and Moens, 2006; Castillo and Vovlas, 2007). Among *Pratylenchus* species, only *P. alleni*, *P. brachyurus*, *P. coffeae*, *P. crenatus*, *P. neglectus*, *P. penetrans*, *P. scribneri*, and *P. thornei* are considered detrimental to growth of potato plants (Brodie et al., 1993; Ingham et al., 2005; Scurrah et al., 2005; Castillo and Vovlas, 2007). Typically in eastern United States and Canada, *P. penetrans* is the most widespread species in soil regularly cropped to potato while *P. crenatus* and *P. scribneri* are less common and *P. neglectus*, *P. alleni* and *P. vulnus* are the least common (Dickerson et al., 1964; Kimpinski, 1979; Brown et al., 1980; Rowe et al., 1987). *Pratylenchus* spp. attack potato plants causing stunting and yellowing of the plants and necrotic lesions in the roots and tubers (Brodie et al., 1993) causing as much as 25-73% yield losses (Olthof, 1986). The greatest economic damage occurs when some *Pratylenchus* spp. interact with the wilt fungus, *Verticillium* spp., resulting in a disease complex known as potato early dying (Martin et al., 1982; MacGuidwin and Rouse, 1990; Rowe and Powelson, 2002). Potato early dying causes significant reduction in tuber size and total marketable yield that confines potato production in North America (Martin et al., 1982; Rowe et al., 1987). *Pratylenchus* spp. vary in their ability to interact with *V. dahliae* and to exasperate potato early dying. *Pratylenchus penetrans* and *P. thornei* interact synergistically with *V. dahliae* and induce potato early dying (Riedel et

al., 1985; Wheeler et al., 1994; Wheeler and Riedel, 1994; Duncan and Moens, 2006), whereas *P. crenatus* does not (Riedel et al., 1985; Duncan and Moens, 2006). *Pratylenchus neglectus* has not been studied extensively. A population from Ontario, Canada, was shown to interact with *V. dahliae* and to cause tuber yield reduction that exceeded that caused by the fungus alone. Another population from Idaho, USA, did not interact with the fungus (Hafez et al., 1999). *Pratylenchus scribneri* interacts with the fungus to increase potato early dying severity only at elevated temperatures (> 32 °C) (Riedel et al., 1985). Therefore, it is important to properly identify *Pratylenchus* spp. populations to species to properly assess the risk of potential crop damage.

Manitoba is Canada's second largest potato producing province (Statistics Canada, 2008). In recent years, producers and processors believe potato early dying to be a major confinement to achieving yield increases and in many cases it is believed to be causing yield decline (Dr. Mario Tenuta, personal communication). Surveys conducted in 1991 (Geisel, 1991), and 2002 to 2004 (Tenuta et al., unpublished) found *Pratylenchus* spp. to occur in 33% of 135 fields sampled with populations ranging from 4 to 5,300 nematodes kg⁻¹ soil. In the past several years, many potato farmers in Manitoba have obtained commercial nematode pest analysis reports showing *Pratylenchus* spp. to be present in their fields. However, none of the surveys or the commercial analysis reports identified the species of *Pratylenchus* present in Manitoba. However, this information is important in determining whether the species present in Manitoba can parasitize and damage potato, and also be involved in the potato early dying disease complex.

The objectives of this study were to determine the frequency of occurrence and population density of *Pratylenchus* spp. in Manitoba potato fields. Field populations were

identified to species of *Pratylenchus* using both morphometric characters and molecular techniques (PCR). The suitability of the dominantly grown potato variety in Manitoba, cv. Russet Burbank, to be a host to populations of *Pratylenchus* spp. present in Manitoba was characterized.

3.3 Materials and Methods

3.3.1 *Pratylenchus* spp. prevalence in Manitoba:

During the summers of 2004 and 2005, 283 soil samples were collected for nematodes analysis from 31 commercial fields having potato in the sampling year and known to have previously visual symptoms of potato early dying (communication between Dr. Tracy Shinnars-Carnelley and growers). The fields were present in 11 rural municipalities (Carberry, Carman, Dufferin, MacGregor, Manitou, Portage la Prairie, Rhineland, Rosebank, Stanley, Westbourne, and Winkler) representing the areas of main potato production in Manitoba. Between the beginning of July and the end of August 2004 (mid to late growing season), 179 soil samples were collected from nine fields. Soil samples were collected from healthy and non-healthy (showing symptoms of potato early dying) patches, approximately half an acre area, in each field. Each soil sample consisted of 20 subsamples collected within each section using a soil-sampling probe (2.5 cm diam.) between potato plants in a hill (15 to 20 cm depth). Because of the low frequency of occurrence of *Pratylenchus* spp. in potato fields sampled in 2004, we reduced the number of samples collected from each field in 2005 in order to sample more fields. Between mid June and end of August 2005, 104 soil samples were collected from 22 fields. Each field was divided into 10-acre sections and one soil sample was collected from each section. Each soil sample consisted of 20 subsamples as described above. Soil

samples were put into polyethylene bags, then placed on ice in an insulated chest and transported to the University of Manitoba. The soil samples were then stored at 5°C and determination of *Pratylenchus* spp. numbers were determined within three days of collection.

A subsample (100 g) of each soil sample was used for nematode extraction and analysis. Nematodes were extracted using Cobb's sieving and decanting (using USA Standard Test Sieve 100 then 400 mesh) followed by sugar flotation (using USA Standard Test Sieve 500 mesh) (Ingham, 1994). Nematodes were then placed in a gridded counting dish for total number determinations. The total number of nematodes in each dish was counted using a dissecting microscope at 80× magnification (Nikon SMZ645 with bottom oblique angle illumination; Nikon Canada, Mississauga, ON). Population densities of *Pratylenchus* spp. were determined by identifying the first 100 nematodes encountered in each sample to the genus level using an inverted compound microscope at x400 magnification (Motic AE31; Geneq, Montreal, PQ), and then, multiplying the fraction of *Pratylenchus* spp. in the identified 100 nematodes by the total count of nematodes in each sample (Freckman and Ettema, 1993; Ferris et al., 1996).

3.3.2 Morphological identification:

Individual female *Pratylenchus* spp. were handpicked using an eyelash probe from a nematode population extracted from some potato fields being positive for *Pratylenchus* spp. The nematodes were extracted as described above. Each female was transferred to a drop of glycerol (ACS grade; Fisher Scientific, Nepean, ON) on a glass slide and killed by mild heating using a hotplate. A cover slip was then placed on the slide. De Man Indices (a, b, c, L, V, s; De Man, 1876 and 1880) and number of lateral

fields were determined using an eyepiece micrometer and a brightfield light microscope (Olympus BH-2; Olympus Canada, Markham, ON). Most measurements were determined at x400 magnification except for the total body length (L) which was determined at x250 magnification and number of lateral incisures determined using oil immersion at x1,000 magnification. *Pratylenchus* spp. were identified to the species level following the key and the diagnostic compendium to the species of the Genus *Pratylenchus* by Handoo and Golden (1989).

3.3.3 Scanning electron microscope:

Pratylenchus spp. were imaged using a scanning electron microscope to determine the number of lip annuli in the head region. Thirty *Pratylenchus* spp. individuals of mixed juvenile and adult stages were handpicked from nematode extractions from each 13-05 and 20-05 soil. Nematodes were killed and fixed in hot (40 to 50 °C) 10% formalin w/v (4% formaldehyde solution; ACS grade, Fisher Scientific). These specimens were then sent by overnight courier to Dr. Manuel Mundo-Ocampo, (Department of Nematology, University of California, Riverside, CA) who performed the scanning electron microscope examination.

For scanning electron microscope observations, formalin fixed nematodes were rinsed in several changes of 0.1 M phosphate buffer and post-fixed in 4% aqueous osmium tetroxide for 4 hr. They were then rinsed again with several changes of cold 0.1 M phosphate buffer in a 15-min period and then dehydrated by exposure to increasing concentration of ethanol in solution (20% through to 100% ethanol). The dehydrated nematodes were then critical point dried (Tousimis Autosamdri-810; Tousimis, Rockville, MD). The specimens were then mounted on top of double sticking copper tape

attached to aluminum stabs. Stabs with mounted nematodes were then coated for three min with a 25 nm layer of gold palladium using an auto sputter coater (Cressington 108; Cressington Sci., Watford, England). Specimens were then imaged by scanning electron microscopy (XL-30/FEG Phillips 35 Scanning Electron Microscope; Philips, Eindhoven, Netherlands) operated at 10 kV.

3.3.4 Total DNA extraction:

DNA was extracted from *Pratylenchus* spp. specimens for molecular identification using the DNA extraction and PCR procedures according to Al-Banna et al., (1997, 2004) and sequencing of the D3 expansion region of amplified 26S rDNA.

Approximately 100 *Pratylenchus* spp. individuals of mixed juvenile and adult stages were obtained from nematode extractions of the three soils containing the highest *Pratylenchus* spp. population found (4-05, 13-05, and 20-05). The individuals for a soil were transferred using an eye-lash probe to a PCR reaction tube containing 300 μ l extraction buffer (0.1 mM Tris-HCl 0.05 mM EDTA, 0.2 mM NaCl, 1% SDS, and 0.5 mg proteinase K ml^{-1} extraction buffer) (Al-Banna et al., 2004). The nematodes in reaction tubes were then stored at $-80\text{ }^{\circ}\text{C}$ overnight. The following morning, the reaction tubes were placed in a water bath for 1 hr at $65\text{ }^{\circ}\text{C}$, and then heated at $95\text{ }^{\circ}\text{C}$ for 1 min and at $99\text{ }^{\circ}\text{C}$ for 3 min in a thermocycler (Techne Flexigene, Techne Inc., Burlington, NJ). The DNA was then extracted from the lysate using phenol/chloroform, precipitated with ethanol and subsequently dissolved in TE (10 mM Tris-HCl, and 1 mM EDTA, pH 8.0).

3.3.5 Amplification of the D3 region:

The D3 expansion region (345 bp) of the 26S rDNA was amplified by PCR from the extracted DNA using the forward primer D3A (5'-GAC CCG TCT TGA AAC ACG GA-3') and the reverse primer D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') following Al-Banna et al., (1994, 2004).

3.3.6 Amplification using species-specific primers:

A 1:500 dilution (vol. vol.⁻¹ 10x PCR reaction buffer) of the D3 region PCR product was used as a template in a species-specific PCR primer assay. Forward species-specific primers for *P. negelectus* (PNEG), *P. penetrans* (PPEN), *P. scribneri* (PSCR), *P. thornei* (PTHO) and *P. vulnus* (PVUL) and the universal reverse primer, D3B, were used and the PCR assay was conducted as described by Al-Banna et al., (2004) as follows: amplification was performed in a PCR reaction tube having a 25- μ l reaction mix containing the DNA template, 2 units of Taq polymerase, 200 μ M dNTPs, 0.8 μ M of each primer and 2.5 mM MgCl₂. Amplification cycles were conducted in a thermocycler (Techne Flexigene, Techne Inc., Burlington, NJ) as follows: 95 °C for 3 min; 35 cycles at 95 °C for 1 min; 62 °C for 1 min; 72 °C for 1 min; and a final extension step of 7 min at 72 °C. Aliquots of 10 μ l of the PCR products were separated by electrophoresis in 1.7% agarose gel, stained with ethidium bromide, and observed under UV trans-illumination.

3.3.7 DNA Sequencing:

The D3 region and the species-specific PCR product bands were excised from agarose gel using sterilized, scalpels. The DNA was extracted and purified using an

extraction kit (QIAquick, Qiagen Inc., Mississauga, ON). Extracted DNA was then analyzed for nucleotide sequences (Macrogen Corp., Rockville, MD).

3.3.8 Basic Local Alignment Search (BLAST):

The sequence of nucleotides of the DNA products was compared to nucleotide sequences in the GenBank database using the basic local alignment search (BLAST) to determine best match to *Pratylenchus* spp. in the database. The significance of the match was determined using the Expect value (E) parameter that describes the number of hits expected to find by chance when searching a database of a particular size.

3.3.9 Suitability of Russet Burbank potato as a host in commercial field conditions for a population of *Pratylenchus* spp. in Manitoba:

In the summer of 2006, an experiment was conducted at a commercial potato seed farm in southern Manitoba (field 20-05 in the survey). The experiment examined if a resident *Pratylenchus* spp. population are capable of parasitizing and reproducing on potato, cv. Russet Burbank, under commercial field conditions. The soil at that field had a pH of eight, organic matter content of 4.0%, and a loam texture. The field was previously cropped to spring wheat and corn in the years 2004 and 2005, respectively. Forty-five days after potato planting, 12 two-meter long sections on potato rows were randomly chosen over the field for total nematode and *Pratylenchus* spp. population monitoring over the growing season. Four of the 12 sections did not have detectable levels of *Pratylenchus* spp. and were thus excluded. Each section was marked and labeled using plastic flag markers. Planting of potato cv. Russet Burbank occurred May 1, 2006. Soil and root samples were collected from each section 6.5, 8.5, 10.5, 12.5, 14.5, and 16.5 wk after planting. At the 16.5 wk sampling date, the plants in the field were killed by the

producer by removing the vegetative part of the plants. Each soil sample consisted of 20 subsamples collected using a soil-sampling probe (2.5 cm diam.) within a hill and between potato plants (15-20 cm depth). Soil and root samples were put in polyethylene bags, then placed on ice in an insulated chest and transported to the University of Manitoba for nematode analysis. Soil samples were extracted and analyzed as previously explained in this chapter. Nematodes were extracted from 10 g of fresh roots using a Baermann funnel technique (Hooper et al., 2005). For each replicate a reproduction factor ($Rf = Pf/Pi$) was calculated, where Pf = total number of *Pratylenchus* spp. from soil at harvest and Pi = initial number of *Pratylenchus* spp. (in this experiment at 45 days following planting). The Rf values were used to categorize the potato plants into four groups according to Al-Rehiyani and Hafez (1998) as follows: good host leading to large increase in *Pratylenchus* spp. population ($Rf > 3$); maintenance host supporting the nematode population without large increase ($1 < Rf < 3$); poor host resulting in decline in nematode population ($0.1 < Rf < 1$); and non hosts resulting in elimination of the nematode population from soil ($Rf < 0.1$).

3.3.10 Host suitability of Russet Burbank potato in growth chamber conditions to two populations of *Pratylenchus* spp. in Manitoba:

This experiment was conducted in the fall of 2006 in a growth chamber (model PGW36, Conviron, Winnipeg, MB) at the Department of Soil Science, University of Manitoba. Soils from two commercial potato fields in Manitoba were used in this experiment. The first soil was collected from the field used in the field study (designated field 20-05 in the survey). The other soil was collected from a commercial processing potato farm south of Portage la Prairie (designated field 13-05 in the survey). The soil of

that field had a pH of 7.6, organic matter content of 1.8%, and a loamy sand texture. For each field, four replicate samples of soil were collected at random locations in each field (to 15 cm depth) and placed in polyethylene bags with soil from each replicate kept separate from others. Before setup of the experiment, each replicate soil was mixed thoroughly by hand and *Pratylenchus* spp. and total nematode populations were determined as previously described here. Then 25 kg soil was placed in a plastic pail (30 cm diam. × 40 cm height) with holes made at the bottom for drainage. The pails were arranged randomly in the growth chamber at 22 °C and 16 hr light per day. One seed tuber, cv. Russet Burbank, was planted in each pail and watered with deionized water every 2 days. Soil and root samples were collected from each pot 28, 42, and 56 days following tuber planting for nematode analysis. Soil samples were processed for nematode analysis as previously mentioned in this paper. Potato roots were washed carefully using tap water to remove any adhering soil particles to the root surface and then cut into 1 cm long pieces. Nematodes were extracted from roots by placing 1 g in 50 ml dH₂O in 250 ml Erlenmeyer flasks. The flasks were then agitated for 48 hr at 60 rpm at 22 °C using refrigerated incubator shaker (Model C24KC, New Brunswick Sci., Edison, NJ). Nematodes were collected after 48 hr on a 38µm opening mesh screen and then transferred to a plastic counting dish. Counts of total nematodes and *Pratylenchus* spp. were determined as described previously here and population level expressed on number per mass of fresh root. A reproduction factor was also calculated as previously explained here with initial *Pratylenchus* spp. population (Pi) is determined at experiment setup.

3.3.11 Statistical analysis:

Results for both the field and the growth chamber experiments were tested for normality using the Shapiro-Wilk normality test prior to analysis of variance (ANOVA) using the Proc GLM procedure of the statistical software program, SAS (SAS Institute Inc., Cary, NC). Variable used in the statistical analysis were the number of *Pratylenchus* spp. kg⁻¹ soil and numbers g⁻¹ fresh roots. Means of nematode population densities were compared using the Student-Newman-Keuls method ($\alpha < 0.05$) using the GLM procedure of the SAS computer software package (SAS Institute Inc., Cary, NC).

3.4 Results and Discussion

This study aimed to determine the species of *Pratylenchus* spp. in commercial potato fields in Manitoba. Previous surveys and potato pest analysis reports commissioned by farmers have shown *Pratylenchus* spp. to be present in potato fields of Manitoba. We set out also to determine if *Pratylenchus* spp. in Manitoba uses potato as a host as this would imply a needed change in management of potato in the province to limit production losses from these pests.

3.4.1 *Pratylenchus* spp. prevalence in Manitoba:

In 2004, six of the nine potato fields sampled had detectable levels of *Pratylenchus* spp. (Table 3.1). *Pratylenchus* spp. populations in infested soil samples were considered low and ranged from 90 to 160 nematodes kg⁻¹ fresh soil with median population density of 120 nematodes kg⁻¹ fresh soil (Table 3.1). *Pratylenchus* spp. frequencies of occurrence in infested fields indicate that the nematode occurs in patches in the fields (Table 3.1). In 2005, *Pratylenchus* spp. were detected in six of the 22 fields

sampled. In infested soil samples, *Pratylenchus* spp. populations ranged from 45 to 631 nematodes kg⁻¹ fresh soil with median population density of 164 nematodes kg⁻¹ fresh soil. *Pratylenchus* spp. frequency of occurrence in each field shows that its distribution in infested fields is also in patches (Table 3.1).

A total of 31 potato fields were sampled over 2004 and 2005 in Manitoba. *Pratylenchus* spp. were detected in 39% of the fields. *Pratylenchus* spp. population in infested soil samples ranged from 45 to 631 nematodes kg⁻¹ fresh soil with median population density of 125 nematodes kg⁻¹ fresh soil. The prevalence of *Pratylenchus* spp. in this survey was consistent with its prevalence in previous surveys conducted in Manitoba by Geisel, 1991, and 2002 to 2004 by Tenuta et al., (unpublished); however, its population density in infested soil samples in this survey was lower. *Pratylenchus* spp. in the potato fields we surveyed in Manitoba were less prevalent with lower population densities compared to the potato-growing regions in New Brunswick. Over the years 1990 and 1991, a survey of potato fields in New Brunswick showed the presence of *Pratylenchus* spp. in 93% of the 46 fields surveyed in 1990 and in 86% of the 43 fields surveyed in 1991. In 1990 and 1991, populations averaged 910 and 410 nematodes g⁻¹ dry root and 1030 and 720 nematodes kg⁻¹ dry soil, respectively (Kimpinski et al., 1998). However, *Pratylenchus* spp. were less prevalent in Simcoe County, Ontario, as it was detected in only 24% of 50 potato fields surveyed with median population density of 655 nematode kg⁻¹ soil.

3.4.2 *Pratylenchus* spp. identification:

The ranges of nearly all morphometric and derived variables (ratios) for female nematodes, from all sampled fields, overlapped with those reported for *P. neglectus*

(Table 3.2; Fig 3.1A). The stylet length of the nematodes from the field 7-04 was out of the range, slightly shorter, reported for *P. neglectus* by Handoo and Golden (1989). And the b ratio of the 9-05 field was less than the range reported for *P. neglectus* by Handoo

Table 3.1 Potato fields containing *Pratylenchus* spp. in the 2004 and 2005 surveys. Frequency of nematode occurrence in samples collected from a field, mean population of *Pratylenchus* spp. in soil samples containing the nematode and ± 1 standard deviation and percent coefficient of variation given for fields having three or more samples containing the nematode.

Field	Frequency of <i>Pratylenchus</i> spp. occurrence	<i>Pratylenchus</i> spp. kg ⁻¹ fresh soil	Standard Deviation (% CV of the mean)
2004 (9 fields sampled)			
2-04	1/18	90	
3-04	1/18	103	
4-04	3/18	115	28 (24)
5-04	2/25	129	
7-04	12/20	160	86 (54)
9-04	3/15	99	25 (25)
2005 (22 fields sampled)			
1-05	1/4	226	
4-05	4/4	238	184 (77)
9-05	1/4	45	
13-05	1/4	631	
14-05	2/5	93	
20-05	3/3	230	152 (66)

and Golden (1989). Only nematodes from the two soils 13-05 and 20-05 were examined using scanning electron microscope to determine the number of lips annuli at the head region. Scanning electron microscope micrographs of *Pratylenchus* spp. in these two soils show that the nematodes have two lips annuli at the head region (Fig. 3.1 B; Table 3.2) which is a characteristic of *P. neglectus* (Handoo and Golden, 1989; Castillo and Vovlas, 2007). In addition, scanning electron microscope micrographs observations were consistent with those made by the brightfield light microscope that the nematodes have

four lateral fields at mid body (Table 3.2; Fig. 3.1C) which is characteristic of *P. neglectus* (Handoo and Golden, 1989; Castillo and Vovlas, 2007). Scanning electron microscope micrographs also show that the lateral fields started at approximately the 9th body annul (Fig 3.1D) which is characteristic of *Pratylenchus* spp. in general (Handoo and Golden, 1989; Castillo and Vovlas, 2007). Only three male *Pratylenchus* spp. were found in the soil samples collected during 2004 and 2005. This is a characteristic of *P. neglectus* as its reproduction is usually parthenogenetic (Castillo and Vovlas, 2007). Accordingly, morphometrics of *Pratylenchus* spp. present in Manitoba potato fields surveyed indicate that the prevalent species of *Pratylenchus* is *P. neglectus*.

Amplification of the D3 expansion region of the large subunit 26S rRNA gene for *Pratylenchus* spp., from a section of each of the three fields harboring the highest nematode population, yielded a single PCR product with an approximate length of 345 bp. This indicates that the used primers amplified only the target DNA region. Application of the species-specific primers developed by Al-Banna et al., (2004) showed that only *P. vulnus* and *P. neglectus* primers amplified template DNA and no other primers were capable of producing an amplicon from the template DNA (Fig 3.2). However, the nematodes used were collected from soil and these nematodes could be contaminated with a wide range of diverse DNA residues that may sometimes lead to false positives (Volossiuk et al., 2003). DNA sequencing provides a check that eliminates false positives of this type (Volossiuk et al., 2003). Accordingly, the D3

Table 3.2 Morphometric measurements (DeMan Indices 1876 and 1880) for females of *Pratylenchus* spp. present in Manitoba potato fields surveyed in 2004 and 2005 and reported for *P. neglectus* (Handoo and Golden, 1989). Presented is the range followed by the mean (± 1 standard deviation of the mean). The number of females used in the determination for each field is given in parenthesis next to the field identifier.

Field	Character													
	a ¹		b		c		L (μm)		V (%)		s (μm)		Labial annuls (n)	Lateral incisures (n)
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean		
7-04 (n=15)	18.6 – 25.5	21.8 (0.6)	4.3 – 5.6	5.0 (0.1)	16.7 – 35.6	21.9 (1.2)	398 – 498	440 (0.0)	68.5 – 86.2	80.7 (1.2)	12.0 – 16.0	14.0 (0.3)	nd	4
1-05 (n=10)	19.0 – 24.1	21.6 (0.5)	5.1 – 5.5	5.3 (0.1)	19.2 – 23.3	21.2 (0.5)	408 – 517	433 (8.2)	77.8 – 86.0	81.6 (0.8)	12.0 – 17.0	15.0 (0.4)	nd	4
4-05 (n=15)	20.9 – 23.6	22.0 (0.2)	4.9 – 5.8	5.2 (0.1)	19.2 – 23.8	21.0 (0.4)	384 – 521	424 (8.9)	74.5 – 84.4	81.0 (0.6)	13.0 – 18.0	16.0 (0.4)	nd	4
9-05 (n=2)	19.6 – 23.3	21.4 (1.8)	4.6 – 4.8	4.7 (0.1)	22.0 – 32.3	22.7 (0.6)	408 – 441	424 (16.6)	82.8 – 84.9	83.8 (1.0)	15.0 – 17.0	16.0 (1.3)	nd	4
13-05 (n=4)	20.2 – 22.0	21.2 (0.5)	5.0 – 5.5	5.3 (0.1)	21.9 – 23.5	22.6 (0.4)	384 – 496	424 (19.1)	81.2 – 84.9	83.2 (0.8)	15.0 – 19.0	16.0 (0.6)	2	4
14-05 (n=15)	18.8 – 24.1	21.9 (0.4)	4.9 – 5.7	5.2 (0.1)	20.0 – 24.1	21.9 (0.3)	389 – 502	435 (7.2)	78.1 – 84.8	81.5 (0.5)	12.0 – 17.0	15.0 (0.4)	nd	4
20-05 (n=15)	19.2 – 25.2	21.6 (0.5)	4.5 – 5.9	5.1 (0.1)	17.1 – 24.6	21.2 (0.5)	436 – 498	433 (8.2)	77.1 – 88.2	81.6 (0.8)	12.0 – 17.0	15.5 (0.4)	2	4
<i>Pratylenchus neglectus</i> ²	16.5-32.2	-	4.9 – 7.8	-	13.8 – 26.8	-	310 – 580	-	75.5 – 86.6	-	15.0 – 19.0	-	2	4

nd = not determined

¹ a = Total body length divided by maximum body diameter; b = Total body length divided by pharyngeal length; c = Total body length divided by tail length; L = Total body length (head to tail tip); V% = Position of vulva from anterior end expressed as percentage of body length; and s = stylet length.

² *Pratylenchus neglectus* range according to Handoo and Golden, (1989).

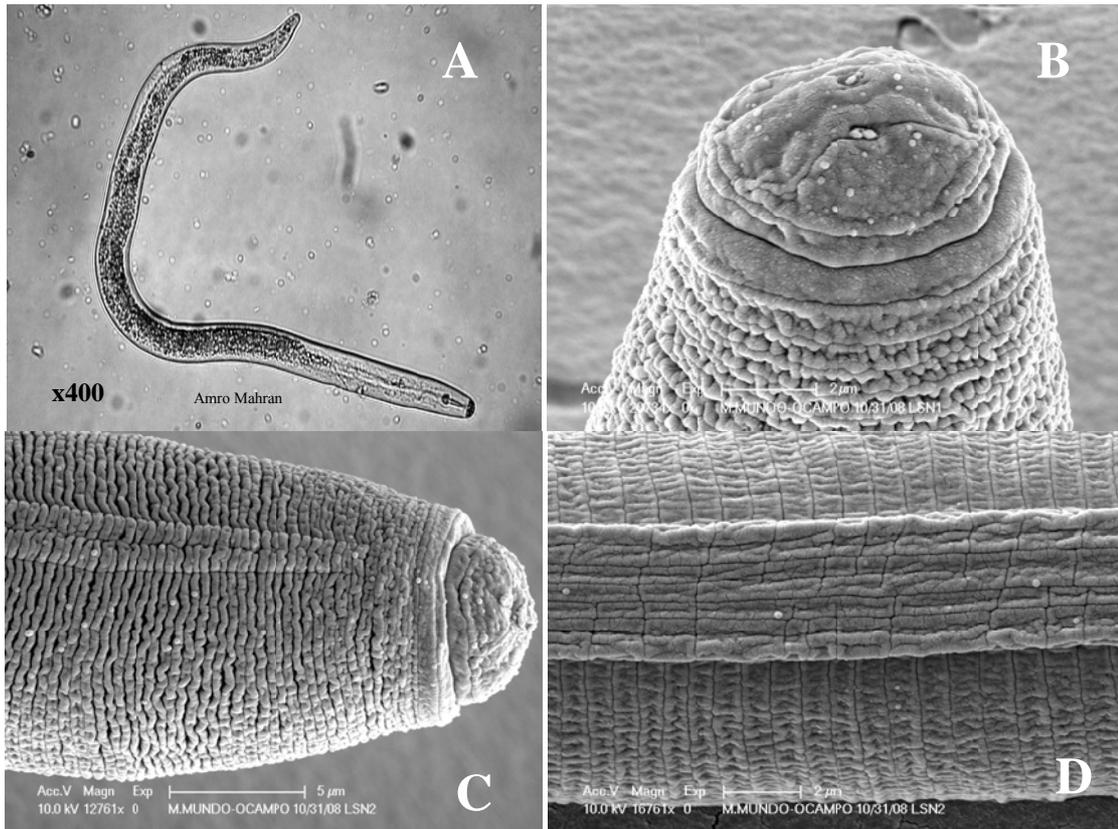


Figure 3.1 *Pratylenchus* spp. from Manitoba potato fields (13-05 and 20-05). A: light microscope image of a whole nematode; and scanning electron micrographs of; B: anterior region showing two lip annuli; C: lateral field at mid-body showing four lateral fields; and D: origin of lateral field showing lateral fields starting at approximately 9th body annual. The scanning electron micrographs were produced by Dr. Manuel Mundo-Ocampo (Department of Nematology, University of California, Riverside).

region and species-specific primer products from *P. vulnus* and *P. neglectus* were sequenced and compared to the sequences in GenBank, using BLAST search. The D3 region and the species-specific primers' amplicons matched with sequences from *P. neglectus* ($E= 2e^{-92}$ to $3e^{-93}$). Therefore, the results of the morphometrics agreed with

those obtained from the molecular methods support that the species of *Pratylenchus* spp. prevalent in the surveyed Manitoba potato fields is *P. neglectus*.

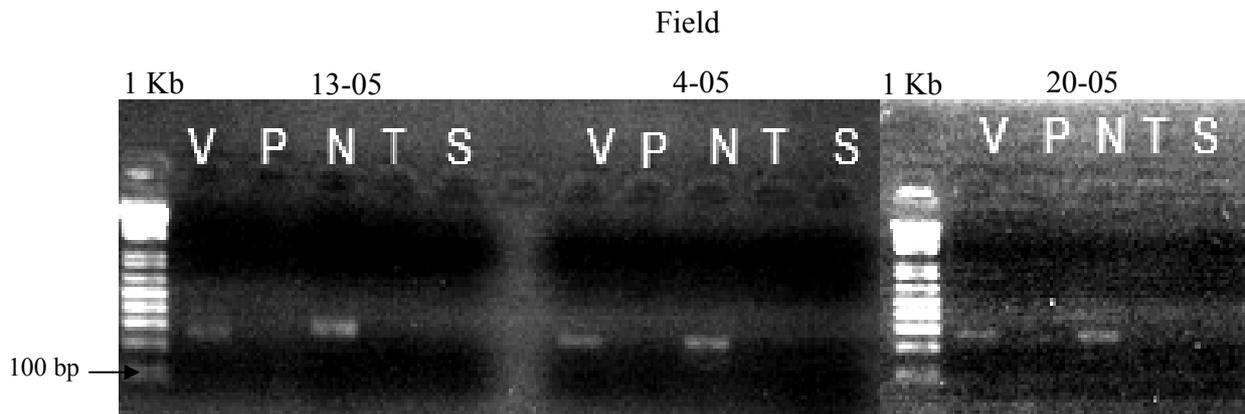


Figure 3.2 Agarose gels (1.7%) follow electrophoresis and stained with ethidium bromide for PCR products of *Pratylenchus* spp. from three potato fields in Manitoba (13-05, 4-05 and 20-05) using species-specific primers (Al-Banna et al., 2004). A common D3B primer and the following species-specific primers were used: *P. vulnus* (V); *P. penetrans* (P); *P. neglectus* (N); *P. thornei* PTHO (T); *P. scribneri* PSCR (S). Image of the electrophoresis products for field 20-05 was digitally added to that for fields 13-05 and 4-05.

Pratylenchus neglectus is geographically distributed in temperate and subtropical regions of most continents parasitizing several hosts including potato (Duncan and Moens, 2006; Castillo and Vovlas, 2007). In North America, *P. neglectus* was detected in potato fields in Ontario (Olthof et al., 1982); Alberta (Merrifield, 2007); Idaho and Eastern Oregon (Hafez et al., 1992); New York (Timper and Brodie, 1997); and Ohio (Brown et al., 1980). However, it was not detected in potato fields surveyed by Kimpinski et al., 1998, in New Brunswick.

Pratylenchus penetrans is the most widespread species of *Pratylenchus* in soil regularly cropped to potato in eastern United States and Canada (Rowe et al., 1987). The non-prevalence of *P. penetrans* in the potato fields surveyed in Manitoba was surprising. *Pratylenchus penetrans* was also not prevalent in potato growing regions of Australia where it was only detected in two of 77 potato fields surveyed; whereas, other species of *Pratylenchus* were detected in 87% of the fields and on roots in 92% of the fields (Harding and Wicks, 2007).

The distribution and population development of *Pratylenchus* spp. within a region are affected by hosts suitability (Duncan and Moens, 2006). *Pratylenchus neglectus* was the most predominant *Pratylenchus* spp. present in cereal and forage crops in Ontario and thus concluded that it is a cereal and forage parasite (Potter and Townshend, 1973). Canola as well has been shown to be a good host for *P. neglectus* (Taylor et al., 2000; Fatemy et al., 2006). On the other hand, *P. penetrans* occurred more frequently in fruit and tobacco growing areas and has thus been concluded to be a fruit and tobacco parasite (Potter and Townshend, 1973). In the potato fields sampled in this survey, cereals and canola are normally rotated with potato and thus favoring the presence of *P. neglectus* rather than *P. penetrans*. Which may explain the presence of *P. neglectus* rather than *P. penetrans*.

Soil texture also influences the distribution of *Pratylenchus* spp. within a region (Duncan and Moens, 2006; Castillo and Vovlas, 2007). *Pratylenchus neglectus* is found more frequently in clay or loamy soil (Loof, 1978) and its pathogenicity and reproduction is greatest in sandy loam and loamy sand soil (Griffin, 1996; Townshend, 1972; Olthof, 1990) rather than clay soils (Scholte and s'Jacob, 1989). However, *P. penetrans* is found

more frequently in sandy soils (Loof, 1978; Florini et al., 1987). The soil texture in potato fields that harbored high *Pratylenchus* spp. population density in this study were loam and loamy sand; thus, favoring *P. neglectus* rather than *P. penetrans*.

3.4.3 Suitability of Russet Burbank:

The suitability of any crop to *Pratylenchus* spp. can be assessed by measuring the ability of nematodes to reproduce on the crop (Lewis, 1987). The reproduction factor (*Rf*) has been widely used to define a crop and cultivar to plant-parasitic nematodes including *Pratylenchus* spp. (Marull and Pinochet, 1991; Al-Rehiyani and Hafez, 1998; Taylor et al., 2000; Bélair, 2007). In the field experiment, *Pratylenchus* spp. population declined from 1,034 nematodes kg⁻¹ soil by mid June to 248 nematodes kg⁻¹ soil at harvest with a reproduction factor of 0.23 ± 0.05 (Fig. 3.3). Moreover, no *Pratylenchus* spp. were detected in potato roots (data not shown). However, total number of nematodes did not decline over the same period of time (Fig. 3.3). Accordingly, potato cv. Russet Burbank is designated poor host to *Pratylenchus* spp. present in Manitoba fields under commercial field conditions.

The results obtained from the field experiment were similar to those from the growth chamber trials. In the soil collected from field 13-05, the population of *Pratylenchus* spp. declined from 837 nematodes kg⁻¹ soil at experiment setup to 116 nematodes kg⁻¹ soil eight wk later yielding a reproduction factor of 0.14 ± 0.03 (Fig. 3.4). In soil 20-05, *Pratylenchus* spp. population declined from 2,324 to 2,115 nematodes kg⁻¹ soil over the same period yielding a reproduction factor of 0.75 ± 0.14 (Fig. 3.4). Total nematode population did not decline over the same period in the two soils. *Pratylenchus*

spp. population in roots 28 days after tuber planting were not significantly different ($P \leq 0.05$) from those present in roots 56 days after tubers planting (Table 3.3). Accordingly, potato cv. Russet Burbank is a poor host for *Pratylenchus* spp. present in Manitoba potato fields under growth chamber conditions. These results contradict those of Olthof (1990),

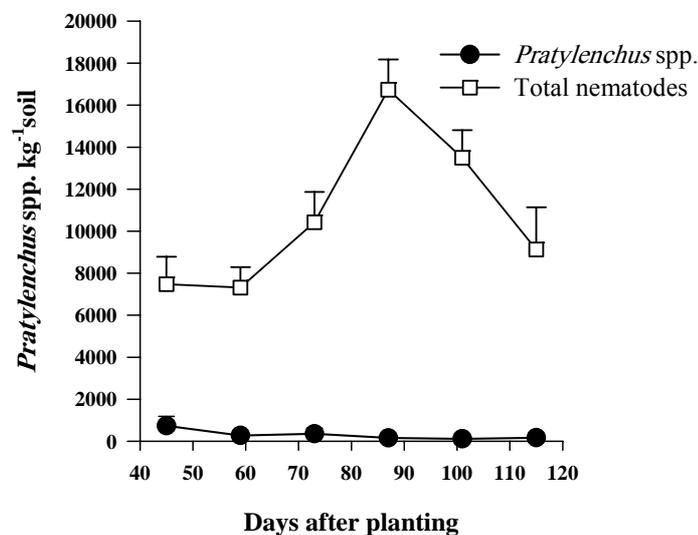


Figure 3.3 Abundances of total nematodes and *Pratylenchus* spp. in soil over the growing season on cv. Russet Burbank, potato in a commercial potato field in Manitoba in 2006. Data shown are the mean of eight independent replicates (+ 1 standard deviation of the mean). Planting date was June 14, 2006.

that an Ontario population of *P. neglectus* is an economically important parasite of potato as it reproduces well on potato and can cause yield losses up to 22%. Hafez et al., (1999) found a *P. neglectus* population from Ontario, Canada, infected and reproduced on Russet Burbank and Butte potato cultivars and Umesh and Ferris, (1994) showed that a *P. neglectus* population from potato fields near Tulelake in the Klamath basin,

California, lowered potato root weight in pot studies. Our results agree with Davis et al., (1992) that showed potato cv. Butte grown in Idaho for five years reduced the population of *P. neglectus* in soil and roots of Russet Burbank potato grown in year six.

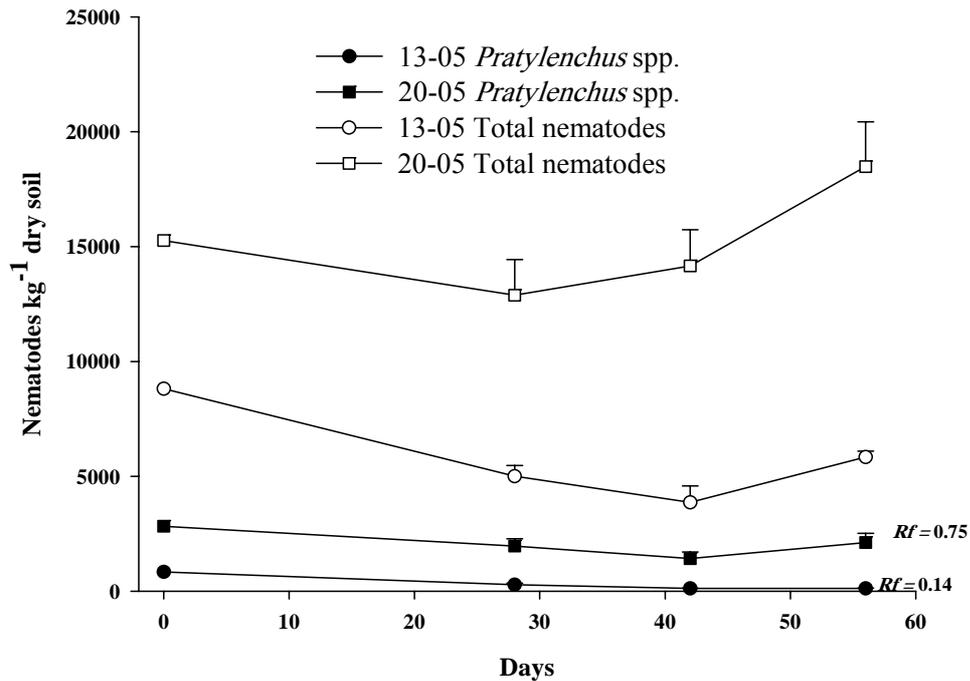


Figure 3.4 Abundance of total nematodes and *Pratylenchus* spp. in soil from two commercial potato field soils from Manitoba (13-05 and 20-05) planted to, cv. Russet Burbank, potato and grown over an eight-week period in a growth chamber. Data shown are the mean of four independent replicates (+ 1 standard deviation of the mean). Reproduction factor of *Pratylenchus* spp. (*Rf*) is shown and calculated as (final nematode populations / initial population).

Pratylenchus neglectus populations showed variability in their pathogenicity to several crops (Griffin, 1991; Griffin, 1993; Griffin and Jensen, 1997) including potato (Hafez et al., 1999). The variability in pathogenicity of *P. neglectus* populations may

indicate the presence of physiological races. Hafez et al., (1999) showed in greenhouse studies that a population of *P. neglectus* from southern Ontario added to Russet Burbank potato plants in soil and sand mix (1:3) reproduced faster and caused more tuber yield loss in potato compared to another population from Parma, Idaho. More studies are needed to determine if there are physiological races of *P. neglectus*.

Table 3.3 Abundance of *Pratylenchus* spp. extracted from roots of Russet Burbank potato grown in field soil from two potato fields (13-05 and 20-05) from Manitoba. Plants were grown in a growth chamber over a 56-day period. Data shown are the mean of four independent replicates (± 1 standard deviation of the mean).

Soil	Days		
	28	42	56
	<i>Pratylenchus</i> spp. g ⁻¹ fresh root		
13-05	16 \pm 6	45 \pm 11	30 \pm 13
20-05	26 \pm 9	28 \pm 10	31 \pm 4

Pratylenchus neglectus populations have also shown variability in their interaction with the wilt fungus *V. dahliae* and the induction of early dying of potato. Hafez et al., (1999) showed that the *P. neglectus* population from Ontario, Canada, interacted synergistically with *V. dahliae* on potato but another population from Idaho did not. Although we showed in this experiment that *P. neglectus* in Manitoba potato fields did not reproduce on potato cv. Russet Burbank; however, its ability to interact synergistically with *V. dahliae* is unknown. This information is important for management decisions of Potato early dying disease in Manitoba.

The two populations of *P. neglectus* studied here did not reproduce on, cv. Russet Burbank, which may indicate that the nematodes were using crops other than potato as a primary host. Cereals and canola are commonly rotated with potato in Manitoba. Cereals are good hosts for *P. neglectus* and severely damaged by this nematode (Castillo and Vovlas, 2007). Wheat is a good host of *P. neglectus* (Taylor et al., 2000) and the nematode caused approximately 20% reduction in yield in Australia (Taylor et al., 1999). *Pratylenchus neglectus* was present in 96% of 81 wheat and 52 barley fields in 13 southeastern Idaho counties surveyed (Strausbaugh et al., 2004). In the Pacific Northwest of the USA, *P. neglectus* reduced yield of spring wheat cultivars by approximately 8 to 36% (Smiley et al., 2005). Canola is also a good host for *P. neglectus* as shown in field studies (Fatemy et al., 2006; Taylor et al., 2000). More studies are needed to determine if *Pratylenchus* spp. in Manitoba is causing damage to cereals and canola in rotation with potato crop.

In conclusion, *Pratylenchus* spp. were present in 39% of the potato fields sampled from the major potato growing areas in Manitoba with populations in infested soil samples ranging from 45 to 631 nematodes kg⁻¹ fresh soil and median population density of 125 nematodes kg⁻¹ fresh soil. Results of morphometrics and molecular diagnosis of *Pratylenchus* spp. are consistent with the species of *Pratylenchus* spp. found to be *P. neglectus*. Potato, cv. Russet Burbank, was found to be a poor host to two populations of *Pratylenchus* spp. present in Manitoba potato fields. We assume that the presence of suitable host plant, cereals and canola, in rotation with potato in Manitoba and favorable soil texture favored the prevalence of *P. neglectus* rather than *P. penetrans*.

MANUSCRIPT 2

4.0 Mortality of *Pratylenchus penetrans* by Volatile Fatty Acids from Liquid Hog Manure

4.1 Abstract

As part of our research program assessing the use of liquid hog manure (LHM) to control root-lesion nematodes, *Pratylenchus penetrans*, a series of acute toxicity tests was conducted to: (i) examine if non-ionized forms of volatile fatty acids (VFA) are responsible for the mortality of *P. penetrans* exposed to LHM under acidic conditions, (ii) determine if *Caenorhabditis elegans* can be a surrogate for *P. penetrans* in screening tests by comparing their sensitivities to VFA, (iii) characterize the nematicidal effect of individual VFA in LHM to *P. penetrans*, and (iv) determine whether individual VFA in LHM interact in their toxicity to *P. penetrans*. LHM was significantly ($P \leq 0.05$) more toxic to *P. penetrans* than a mixture of its main VFA components at concentrations of 5% and 10% (vol. VFA or LHM vol.⁻¹ in buffer). *Pratylenchus penetrans* was more sensitive to acetic acid than *C. elegans*, whereas the sensitivity of both nematode species to *n*-caproic acid was similar. Individual VFA vary in their lethality to *P. penetrans*. *n*-valeric acid was the most toxic (LC₉₅= 6.8 mM), while isobutyric acid was the least toxic (LC₉₅= 45.7 mM). Individual VFA did not interact in their toxicity to *P. penetrans*, and their effects were considered additive. VFA account for the majority of the lethal effect of LHM to *P. penetrans* under acidic conditions. *C. elegans* cannot be used as a surrogate to *P. penetrans* in toxicity studies using VFA. The efficacy of LHM to control *P. penetrans* can be evaluated by assessing its VFA content prior to application, and this evaluation is

facilitated by the fact that the interaction of individual VFA appears to be simply additive.

4.2 Introduction

Pratylenchus penetrans, (Cobb, 1917) Filipjev and Schuurmans Stekhoven (1941), is considered one of the most economically important plant-parasitic nematodes (Castillo and Vovlas, 2007). This is due to its wide host range, as it has been reported from over 350 hosts (Corbett, 1973) and on every continent except for Antarctica (Castillo and Vovlas, 2007). It is a serious nematode pest of potato throughout several potato growing regions in the world (Castillo and Vovlas, 2007), with tuber yield losses estimated to be as much as 20-50% in infested fields (Oostenbrink, 1954). *Pratylenchus penetrans* attacks potato plants causing poor growth and yellowing of the foliage and severe necrosis in roots and tubers (Brodie et al., 1993). The greatest economic damage occurs when *P. penetrans* interacts with the wilt-causing fungus *Verticillium* spp., forming a disease complex known as early dying of potatoes (Martin et al., 1982; MacGuidwin and Rouse, 1990; Rowe and Powelson, 2002). Early dying of potatoes is a significant limiting factor to potato production in various areas in North America (Martin et al., 1982; Rowe et al., 1987).

There is an increasing need to develop environmentally safe and effective alternative strategies to control plant-parasitic nematodes to replace many of the nematicides and fumigants that have been abandoned or restricted due to environmental concerns (Rich et al., 2004). Organic amendments hold such potential, and Linford et al., (1938) were the first to report the nematicidal effects of such additions. Since then, application of a range of organic amendments has been found to lead to considerable

reduction in plant-parasitic nematode population densities through different mechanisms (Viaene et al., 2006). Application of acidified liquid hog manure (LHM), also known as liquid swine manure (LSM), in microplots and field studies reduced the population of plant-parasitic nematodes including *P. penetrans* below densities that would directly reduce potato yield (Mahran et al., (in preparation)). We were encouraged to determine the nature of suppression of root lesion nematodes by LHM. Short-chain volatile fatty acids (VFA) in LHM have been the focus of several investigations as to how plant-parasitic nematodes and soil-borne plant pathogens have been suppressed. VFA accumulate in LHM after being produced through bacterial fermentation of amino acids which are produced during protein degradation and carbohydrate breakdown either in the gastrointestinal tract of hogs or during their storage under anaerobic conditions (Zhu, 2000).

Non-ionized forms of VFA (acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acids) are generated when typically pH-neutral LHM is either acidified or added to acid soil, and it is these forms that were responsible for the death of the microsclerotia of *V. dahliae* (Tenuta et al., 2002; Conn et al., 2005). In addition, VFA in LHM were associated with the suppression of the soybean cyst nematode in greenhouse studies (Xiao et al., 2007). Also, *n*-butyric acid is capable of killing various soil-borne fungi (Browning et al., 2006), both ecto-parasitic and endo-parasitic plant-parasitic nematodes (Browning et al., 2004, 2006) and nematodes from various trophic groups (Browning et al., 2004).

Pre-parasitic juveniles of *P. penetrans* and the standard model nematode, *Caenorhabditis elegans* Maupas 1900, share many similar traits. Thus, much of the

knowledge that has been gained from *C. elegans* could be transferable to plant-parasitic nematodes (Costa et al., 2007). *Caenorhabditis elegans* has been successively used as a model nematode for plant parasites, especially in elucidating gene functions (McCarter et al., 2003). Advancements that have been achieved in *C. elegans*' neurobiology can be used in studying plant-parasitic nematodes' behavior, especially in neuronal function, which is largely conserved among nematodes (Schafer, 2005). *Caenorhabditis elegans* is a bacterial feeding nematode, 1.0-1.5 mm long, and can be easily maintained in the laboratory in large numbers (Costa et al., 2007). It completes its life cycle in less than three days at 25°C, with an average life-span of two to three wk, during which it can produce 300 to 350 offspring (Hope, 1999). These traits make it easier to study than *P. penetrans*, which is more difficult to maintain in large numbers in the laboratory because of its obligate plant parasitism and its longer life cycle (Costa et al., 2007). If the sensitivity of *P. penetrans* and *C. elegans* to the VFA in LHM is similar, then *C. elegans* could be used as a simple surrogate to *P. penetrans* in toxicity tests.

As part of our research program of developing LHM to control *P. penetrans*, a series of acute toxicity tests (bioassays) were conducted to: (i) examine if VFA are the compounds responsible for the suppression of *P. penetrans* juveniles exposed to LHM under acidic conditions, (ii) determine if *C. elegans* can be a surrogate for *P. penetrans* in screening tests by comparing their sensitivities to VFA, (iii) determine if the nematicidal effect of individual VFA in LHM varies to the root-lesion nematode and (iv) determine the nature of interaction (additive, synergistic or antagonistic) of individual VFA in their toxicity to *P. penetrans*.

4.3 Materials and Methods

4.3.1 Manure collection and analysis:

In June 2006, LHM was collected from an earthen storage lagoon at a commercial hog farm in south eastern Manitoba, Canada. The manure had a pH of 7.2, 2.7% dry mass, 0.6% total N, 0.01% total P, 0.25% total K, and 2.5 mg NO_3^- and NO_2^- – N/liter fresh manure. The manure was centrifuged (10 min at 3,400 x g) to remove particulates. An aliquot of the supernatant was analyzed for its main VFA constituents (C2 to C6 including isomers), while the rest was frozen at -20°C until use in the toxicity tests. Concentrations of individual VFA were determined using chemical suppression ion exclusion chromatography and conductivity detection (Tenuta et al., 2002) using a Dionex ion chromatography system, ICS-1000 (Dionex Corp., Sunnyvale, CA). The chromatograph was equipped with an IonPac ICE-AS1 (9 x 250 mm) analytical column and Anion Micromembrane Suppressor (AMMS-ICE II). Diluted LHM (25x in dH_2O) contained in 1 ml vials with filter caps (Dionex Corp.) were injected (25 μl) using an automated sampler (AS40, Dionex Corp.). For analysis heptafluorobutyric acid 1 mM (Acros Organics Inc.) with 5% acetonitrile (vol./vol.) was used as the eluent. Commercially available individual VFA (Table 4.1) were used to calibrate and quantify the samples using an 8 point standard curve.

4.3.2 Nematode cultures:

Pratylenchus penetrans were supplied by Agriculture and Agri-Food Canada (Agassi, BC) and maintained in the laboratory on carrot (*Daucus carota* L.) disk cultures (O'Bannon and Taylor, 1968). After about 8 wk, nematodes were recovered by washing

the plates with 10 ml dH₂O and were collected on a 25- μ m pore diam. mesh screen (USA Standard Test Sieve, 500-mesh) for use in the bioassays.

Table 4.1 Individual volatile fatty acids (VFA) and their commercial product grade, their IUPAC nomenclature and concentration range used in Bioassays 2 and 3.

VFA	IUPAC nomenclature ⁽¹⁾	Concentration range (mM)
Acetic acid ⁽²⁾	Ethanoic acid	0, 16, 32, 48, 64, 80, 96 and 112
Propionic acid ⁽³⁾ , 99%	Propanoic acid	0, 4, 8, 12, 16, 20, 24, 28 and 32
Isobutyric acid ⁽³⁾ , 99%	2-Methylpropanoic acid	0, 8, 16, 24, 32, 40, 48, 56 and 64
<i>n</i> -Butyric acid ⁽³⁾ , 99+%	Butyric acid	0, 7, 14, 21, 28, 35, 42, 49 and 56
Isovaleric acid ⁽³⁾ , 99%	3-Methylbutanoic acid	0, 8, 16, 24, 32, 40, 48, 56 and 64
<i>n</i> -Valeric acid ⁽³⁾ , 99%	Pentanoic acid	0, 2, 4, 6, 8, 10, 12, 14 and 16
<i>n</i> -Caproic acid ⁽⁴⁾ , 99+%	Hexanoic acid	0, 1.5, 3, 4.5, 6, 7.5 and 9

⁽¹⁾ International Union of Pure and Applied Chemistry (IUPAC) nomenclature.

⁽²⁾ Sodium Acetate Anhydrous (Fused Crystals/Certified ACS), Fisher Chemical.

⁽³⁾ Source: Sigma-Aldrich.

⁽⁴⁾ Source: Acros Organics.

Caenorhabditis elegans var. Bristol (wild type strain N2) were provided by the Caenorhabditis Genetics Centre. *Caenorhabditis elegans* nematodes were maintained in 100 x 15 mm sterilized plastic petri plates (Fisher Scientific, Inc.) containing rich nematode growth medium (RNGM) (Tenuta and Ferris, 2004) with a lawn of *Escherichia coli* strain OP50 as a food source. Prior to each bioassay, 200 μ l of *E. coli* (OP50) L-Broth grown for 24 hr was spread to the surface of a new RNGM plate and incubated at 22°C to grow overnight to form a bacterial lawn. Several hermaphrodites were then transferred to the petri plates and incubated at 22°C for five d. Juvenile nematodes then were harvested by washing the plates with de-ionized water and passing through a 25- μ m pore diam. mesh screen.

4.3.3 The bioassay:

To conduct the bioassay, 300 nematodes in 0.5 ml dH₂O were added to 9.5 ml test solution in citric acid-NaOH buffer solution (final concentration of citric acid 23 mM) at pH 4.5 in 15 ml polyethylene test tubes (Fisher Scientific, Inc.). The pH of the test solution was adjusted using 1 M solutions of citric acid and NaOH as needed. A pH of 4.5 was used because at this pH the majority of the VFA will be in their toxic, non-ionized VFA forms (e.g., acetic acid instead of acetate). Preliminary studies showed 300 nematodes yielded a high percentage of recovery (> 90%) in the buffered solution at pH 4.5. Nematodes in the test solutions were incubated for 24 hr at 22°C in the dark. To recover the nematodes that survived the test exposure, nematodes were rinsed in tap water three times with centrifugation between each step (5 min at 1,300xg) and decanting to remove any residual test solutions. Afterwards, live nematodes were obtained using a micro-Baermann tube system (Tenuta and Ferris, 2004). Briefly, nematodes were washed into PVC tubes (2.1 cm diam. × 1.4 cm h) opened at one end, with the other end covered with a double layer of Kimwipe laboratory tissue (Kimberly-Clark, Inc.) using a rubber band. The PVC tubes were placed over plastic paper clips to allow some space for the nematodes to move downwards into a gridded counting dish filled with tap water. The dishes with the PVC tubes in them were placed on a tray and covered to reduce water loss and stored at 22°C for 24 hr. The exteriors of tubes and plastic paper clips were rinsed into the gridded counting dishes to dislodge any nematodes adhering on the outside of the extraction tube into the dish. The nematodes in each dish were counted using a dissecting microscope at x200 magnification. The numbers of nematodes recovered were indexed to the control mean using the following equation: (Numbers of nematodes recovered from

each treatment replicate x 100)/ (Average number of nematodes recovered in control buffer solution alone). Each treatment had three replicates, and each bioassay was repeated twice. The pH of the test solution was measured using an Accumet pencil-thin epoxy body gel-filled combination electrode with Ag/AgCl reference (Fisher Scientific), and the concentration of non-ionized plus ionized VFA was measured, using the method described previously, before and after the exposure of nematodes to confirm that exposure levels to VFA remained constant over the test period.

4.3.4 Bioassay 1. Comparing toxicity of LHM and mixture of its VFA to *P. penetrans*:

To determine if the VFA in the LHM are responsible for the suppression of *P. penetrans*, a mixture of VFA equivalent in concentration to those present in the LHM was prepared using commercially available VFA (Table 4.1) and compared to LHM in a bioassay. The VFA mixture and the LHM were prepared in a buffered solution of citric acid-NaOH at pH 4.5. The mixture of VFA and the LHM were compared at different concentrations: 0, 5, 10, and 15% (vol. VFA or LHM/vol. in buffer solution). These concentrations are equivalent to the rate of application of LHM to field soil of 0, 23,400, 46,800, and 70,200 liters/ha (equal to 0, 2,500, 5,000 and 7,500 US gal/acre) based on a soil moisture content of 30% and a depth of incorporation of LHM in soil to 15 cm.

4.3.5 Bioassay 2. Relative sensitivity of *C. elegans* and *P. penetrans* to VFA:

This bioassay compared the sensitivities of *C. elegans* and *P. penetrans* to increasing concentrations of acetic acid and *n*-caproic acid (Table 4.1) in a citric acid-

NaOH buffer solution at pH 4.5. The final test concentrations used were chosen based on preliminary range-finding toxicity tests (Table 4.1). The concentration of non-ionized forms in the exposure tests was estimated using the Henderson-Hasselbalch equation (Hasselbalch, 1916), pH of the test solution and VFA (non-ionized plus ionized) concentration. The bioassay was intended to allow the calculation and comparison of LC₅₀ for both acids to the two nematodes.

4.3.6 Bioassay 3. Characterization of individual VFA lethality to *P. penetrans*:

Bioassays to determine the sensitivity of *P. penetrans* to individual VFA present in the LHM were conducted. In these bioassays, *P. penetrans* was exposed to increasing concentrations of acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric and *n*-caproic (Table 4.1) acids in the citric acid-NaOH buffer solution at pH 4.5. The range of concentrations used was chosen based on preliminary toxicity tests using a wide range of concentrations. The concentration of non-ionized forms in the exposure tests was determined as previously described. The concentration of interest was LC₉₅ (the concentration of individual VFA that killed 95% of the nematodes under study in 24 hr.).

4.3.7 Bioassay 4. Interaction of individual VFA in the LHM in their toxicity to *P. penetrans*:

Tests were conducted to assess the role of individual VFA in a mixture scenario to try to understand their potential to interact additively, synergistically or antagonistically in their toxicity to *P. penetrans* when found in LHM. The recovery of live *P. penetrans* in

individual test solutions of VFA of concentration equal to an LC₁₀ was compared to the recovery in a mixture solution of individual VFA with each of them at LC₁₀ strength.

If the lethality of the mixture of the VFA to the nematodes exceeded the cumulative effect of individual VFA, this was deemed to be an indicator that the interaction between individual VFA was synergistic. However, if the lethality of the mixture to *P. penetrans* was less than that of the cumulative of individual VFA, this was deemed to be an indicator that the interaction between individual VFA was antagonistic. If the toxicity of the mixture of the VFA was equal to that of the cumulative toxicity of individual VFA, this was deemed to be an indicator that the effect of individual VFA was additive.

4.3.8 Statistical analysis:

Percent nematode survivorship relative to the mean of the control solution (buffer solution alone) is reported. To calculate the lethal concentrations (LC₁₀, LC₅₀ or LC₉₅) and 95% confidence intervals, the data for each replicate from the two experiments were fit to a logistic model using the computer software SAS (Stephenson et al., 2000) using re-parameterized models by incorporation of the LC₁₀, LC₅₀ and LC₉₅ into the equation. The model for estimating LC₁₀ was: $y = t/(1 + (0.1/0.9)*(dose/x)^b)$; for LC₅₀: $y = t/(1 + (dose/x)^b)$; and for LC₉₅: $y = t/(1 + (0.95/0.05)*(dose/x)^b)$, where t = survivorship of nematodes in control solution, dose = experimental concentration of non-ionized VFA (mM), x = desired LC value and b = slope of the curve. The results presented are the average LC₁₀, LC₅₀ or LC₉₅ of the combined means from the two experiments. Analysis of variance (ANOVA) ($P \leq 0.05$) followed by Tukey's multiple comparisons ($P \leq 0.05$)

of the arcsine transformed data was used to compare LHM and a mixture of its VFA in their lethality to *P. penetrans* in Bioassay 1. In Bioassay 2, the sensitivity of *P. penetrans* and *C. elegans* to acetic and *n*-caproic acids was compared using ANOVA ($P \leq 0.05$) followed by Tukey's multiple comparisons ($P \leq 0.05$) of the LC₅₀ estimated value of individual tested acids. In Bioassay 4, the cumulative effect of individual VFA was compared with a mixture of them at the LC₁₀ strength using ANOVA ($P \leq 0.05$).

4.4 Results

4.4.1 LHM analysis:

Five short-chain VFA (from C2-C6) and two isomers were present in the LHM at differing concentrations. Acetic acid was the most dominant VFA in the LHM, having a concentration of 190.3 mM representing more than half the total amount of VFA in the LHM. The LHM used contained nearly equal concentrations of both *n*-butyric and propionic acids, 53.9 and 50.7 mM, respectively, with each one constituting approximately 15% of the total VFA in the LHM. The LHM contained lower concentrations of isobutyric and isovaleric acids, 22.8 and 12.5 mM respectively, representing 3.6 and 6.6% of the total VFA present in the LHM, respectively. *n*-caproic and *n*-valeric acids were present at lower concentrations, 8.7 and 8.5 mM, respectively, each composing about 2.5% of the total VFA in the LHM.

4.4.2 Bioassay 1. Comparing LHM and a mixture of its VFA in their lethality to *P. penetrans*

The bioassay results showed that LHM was significantly more lethal to *P. penetrans* than the mixture of its primary VFA (Fig. 4.1). At the 5% concentration, LHM

was significantly ($P \leq 0.05$) more lethal to *P. penetrans* than the mixture of its VFA. LHM killed 60% of the exposed nematodes, and the mixture of its VFA caused 40% mortality (Fig. 4.1). With the 10% concentration, LHM was still significantly ($P \leq 0.05$) more lethal than the VFA mixture killing 99% of the nematode population, while the VFA mixture was slightly less lethal, killing 88% of the nematode population relative to the control (Fig. 4.1). Total mortality of the nematode test population was achieved at 15% (vol/vol buffer) for both the LHM and the VFA mixture (Fig. 4.1).

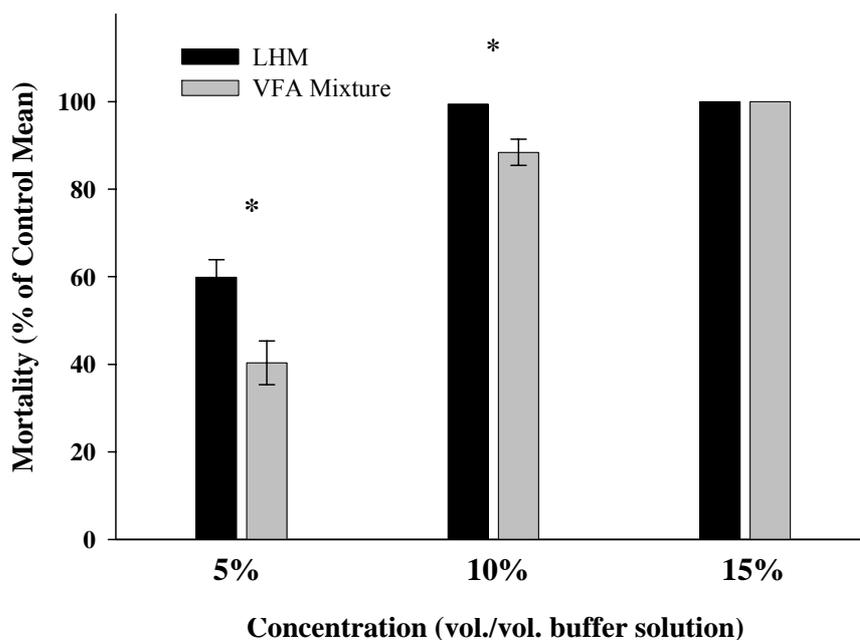


Figure 4.1 Results of Bioassay 1 for the comparison of liquid hog manure (LHM) and a mixture of its volatile fatty acids (VFA mixture) in citric acid-NaOH buffered solution (pH = 4.5) in their lethality to *Pratylenchus penetrans*. Shown are the average of two trials with three replicates each ($n = 6$) \pm 95% confidence interval. * indicates statistically significant ($P \leq 0.05$) differences according to Tukey's multiple comparison test using arcsine transformed data.

4.4.3 Bioassay 2. Comparison of *C. elegans* and *P. penetrans* sensitivity to VFA:

Pratylenchus penetrans and *C. elegans* showed different levels of sensitivity to acetic acid (Fig. 4.2, Table 4.2). *Pratylenchus penetrans* was more sensitive ($P \leq 0.05$) to acetic acid than *C. elegans*, with LC₅₀ values of 8.5 (± 1.0) and 23.3 (± 1.4) mM, respectively. The sensitivity of the two nematode species to the larger chain VFA, *n*-caproic acid, was the same ($P \leq 0.05$), with an LC₅₀ approximate value of 2.3 mM for the two nematodes.

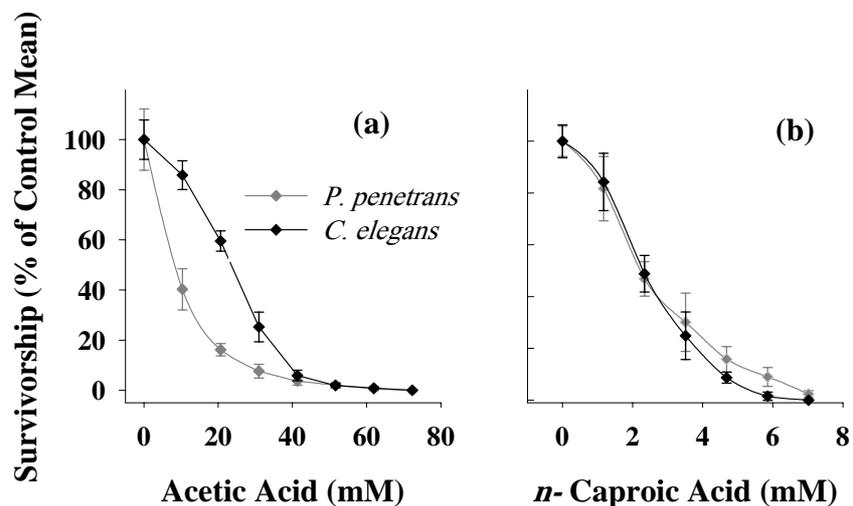


Figure 4.2 Results of Bioassay 2 for comparison of the sensitivity of *Pratylenchus penetrans* and *Caenorhabditis elegans* to non-ionized concentration of (a) Acetic and (b) *n*-Caproic acids in citric acid-NaOH buffer solution at pH 4.5. Shown are the average of two trials with three replicates each ($n = 6$) \pm 95% confidence intervals.

4.4.4 Bioassay 3. Screening of individual VFA based on their lethality to *P. penetrans*:

Individual VFA were found to vary in their lethality to *P. penetrans* (Table 4.3, Fig. 4.3). VFA based on determined LC₉₅ values to *P. penetrans*, from the most to least

lethal, were as follows: *n*-valeric, *n*-caproic, propionic, *n*-butyric, acetic, isovaleric and isobutyric acid (Table 4.3, Fig. 4.3).

Table 4.2 The lethal concentration to 50% of the tested population (LC₅₀) ± 95% confidence interval of non-ionized forms of acetic and *n*-caproic acids in citric acid-NaOH buffer solution (pH = 4.5) for *Pratylenchus penetrans* and *Caenorhabditis elegans* in Bioassay 2.

		LC ₅₀ (mM)
Acetic acid	<i>C. elegans</i>	23.3 ^a (± 1.4) a
	<i>P. penetrans</i>	8.5 (± 1.0) b
<i>n</i> -Caproic acid	<i>C. elegans</i>	2.3 (± 0.3) a
	<i>P. penetrans</i>	2.3 (± 0.2) a

^a LC₅₀ values of each acid for the two nematodes followed by the same letter are not significantly different from another ($P \leq 0.05$) as determined by Tukey's multiple comparison test.

4.4.5 Bioassay 4. Interaction of individual VFA in the LHM in their lethality to *P. penetrans*:

The calculated LC₁₀ concentrations of the individual VFA, acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acids, are given in Tables 4.3 and 4.4. The sum mortality from individual VFA to *P. penetrans* was not significantly different ($P \leq 0.05$) from the mixture of individual VFA with their concentration set to the calculated LC₁₀ under our test conditions (Table 4.4). These results indicate that individual VFA do not appear to be interacting synergistically or antagonistically in their toxicity to *P. penetrans* and that their effect is likely additive.

Table 4.3 Volatile fatty acids (VFA) used in this study arranged according to the lethal concentration of their non-ionized forms to 95% of the tested population ($LC_{95} \pm 95\%$ confidence interval, their $LC_{50} \pm 95\%$ c.i., their $LC_{10} \pm 95\%$ c.i., and model parameters b and t.

VFA	LC_{95} (mM)	LC_{50} (mM)	LC_{10} (mM)	b ^a	t ^a
<i>n</i> -Valeric	6.8 (\pm 0.6)	3.7 (\pm 0.2)	2.3 (\pm 0.2)	4.8	100
<i>n</i> -Caproic	8.0 (\pm 1.3)	2.3 (\pm 0.2)	0.9 (\pm 0.2)	2.3	100
Propionic	14.6 (\pm 1.2)	9.9 (\pm 0.3)	7.3 (\pm 0.7)	7.5	100
<i>n</i> -Butyric	25.3 (\pm 2.6)	8.6 (\pm 0.5)	3.8 (\pm 0.5)	2.7	100
Acetic	37.8 (\pm 6.8)	8.5 (\pm 0.9)	2.8 (\pm 0.7)	2.0	100
Isovaleric	39.8 (\pm 4.1)	16.6 (\pm 0.9)	8.6 (\pm 3.0)	3.4	100
Isobutyric	45.7 (\pm 4.7)	14.6 (\pm 0.9)	6.2 (\pm 0.8)	2.6	100

^a See text for models used and explanation of model parameters.

4.5 Discussion

The primary VFA present in the LHM were acetic, propionic, *n*-butyric, isobutyric *n*-valeric, isovaleric, and *n*-caproic acids. The concentrations of individual VFA can vary from one lagoon to another, according to the pigs' diet (Shriver et al., 2003; Lynch et al., 2007), storage conditions (aerobic or anaerobic), and the number and the age of animals in the barn. However, the relative concentration of individual VFA is generally consistent, with acetic and propionic acids dominating, followed by *n*-butyric, isobutyric, *n*-valeric and isovaleric acids at intermediate concentrations and *n*-caproic acid at lower concentrations (Spoelstra, 1980; Tenuta et al., 2002). Our results demonstrated that acetic and propionic acids are the most dominant VFA in the manure

(with concentrations of 190.3 and 53.9 mM, respectively) representing more than half the total amount of VFA present in LHM, and these results agreed with those of Spoelstra

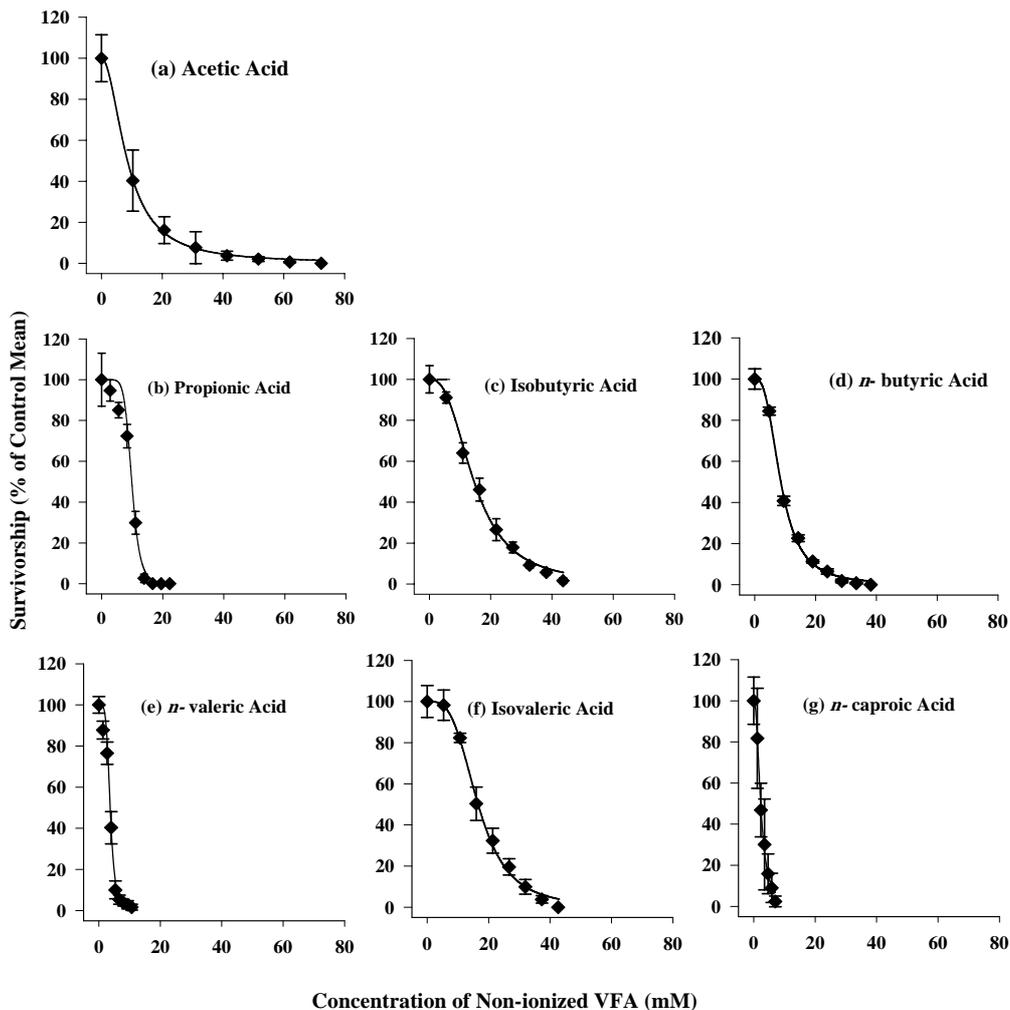


Figure 4.3 Results of Bioassay 3 for the mortality of *Pratylenchus penetrans* as a function of the non-ionized concentration of individual volatile fatty acids (VFA) present in the liquid hog manure (LHM), (a) Acetic acid, (b) Propionic acid, (c) Isobutyric acid, (d) *n*-Butyric acid, (e) Isovaleric acid, (f) *n*-Valeric acid and (g) *n*-Caproic acid in citric acid-NaOH buffer solution at pH 4.5. Data shown are the average of two trials with three replicates each ($n = 6$) \pm 95% confidence intervals. Fitted curves are the logistic models describing mortality of *P. penetrans* as a function of non-ionized concentration of individual VFA.

Table 4.4 The lethal concentration to 10% of the tested population (LC₁₀) ± 95% confidence interval of individual non-ionized forms of volatile fatty acids (VFA) in citric acid-NaOH buffer solution (pH=4.5) for *Pratylenchus penetrans* and the percentage nematode mortality ± 95% confidence interval and their cumulative mortality compared to that of the volatile fatty acid (VFA) mixture ± 95% c.i. in Bioassay 4.

Treatment	LC₁₀ (mM)^a	% Nematodes mortality^b
Acetic	2.8	9.5 ± 5.0
Propionic	7.4	14.1 ± 2.7
<i>n</i> - Butyric	3.8	15.3 ± 2.3
Isobutyric	6.2	8.4 ± 4.4
<i>n</i> -Valeric	2.3	15.6 ± 3.9
Isovaleric	8.6	13.0 ± 3.3
<i>n</i> -Caproic	0.9	7.1 ± 4.0
Cumulative for individual VFA		83.1 ^{c,d} ± 4.8
VFA mixture		84.7 ± 2.2

^a LC₁₀ values determined from previous bioassay.

^b Percentages are indexed to the control mean.

^c Cumulative percentage for individual VFA of nematode mortality does not equal sum of individual mortality as shown due to rounding of data.

^d The cumulative effect for individual VFA is not significantly different ($P \leq 0.05$) from the effect of the VFA mixture according to the ANOVA.

(1980) and Tenuta et al., (2002). LHM contained lower concentrations of *n*-butyric, isobutyric, *n*-caproic, *n*-valeric and isovaleric acids, 50.7, 22.8, 8.7, 8.5 and 12.5 mM, respectively, which generally agreed with Spoelstra (1980) and Tenuta et al. (2002). The manure used in the present study had 3-fold more *n*-butyric acid concentration than what Tenuta et al. (2002) used (53.9 mM and 16.3 mM, respectively).

Our results demonstrate that non-ionized forms of VFA are the dominant lethal agent in the LHM to the lesion nematode, *P. penetrans*, under acidic conditions. Other factors in LHM seem to contribute to the toxicity observed in *P. penetrans* because the mixture of VFA was slightly less lethal than the LHM itself at 5% and 10%. VFA greater than C6 could be present in LHM, as well as indoles, phenols, volatile amines and sulfur-containing compounds (Zhu, 2000). However, the effect of these compounds on *Pratylenchus* spp. or other pathogens and pests has not been studied. VFA have also been found to be responsible for the control of the microsclerotia of *V. dahliae* (Tenuta et al., 2002) that interact synergistically with *P. penetrans*, causing early dying of potatoes. However, individual VFA varied in their toxicity to *P. penetrans* and *V. dahliae*. While here *n*-valeric acid was found to be the most lethal to *P. penetrans* ($LC_{95} = 6.8$ mM), *n*-caproic acid was the most lethal to *V. dahliae* ($LC_{95} = 4.1$ mM) (Tenuta et al., 2002). In addition, individual VFA vary in their lethality to the two organisms. For example, acetic acid, which is the most dominant VFA in the LHM, was more lethal to *V. dahliae* ($LC_{95} = 26.2$ mM) (Tenuta et al., 2002) than to *P. penetrans* ($LC_{95} = 37.8$ mM), while propionic acid, the second-most dominant VFA in the LHM, was more lethal to *P. penetrans* ($LC_{95} = 14.6$ mM) than to *V. dahliae* ($LC_{95} = 27$ mM) (Tenuta et al., 2002). Accordingly, the effectiveness of the LHM in controlling plant pathogens will vary according to the target organism and the VFA profile in the LHM. Information regarding the sensitivity of the target plant pathogen to non-ionized forms of VFA and analysis of the VFA composition of the LHM is essential to predict the effectiveness of the LHM in controlling the target pathogen prior to manure application under field conditions.

Pratylenchus penetrans and *C. elegans* have similar sensitivities to *n*-caproic acid. However, *C. elegans* showed more tolerance to acetic acid than did *P. penetrans*. The *C. elegans* N2 strain is believed to have been originally isolated from mushroom compost. Like LHM, composts contain VFA, with acetic acid being dominant and > C4 VFA generally found in very low concentrations (Ozores-Hampton et al., 2001; Bog et al., 2002). Aquino and Santiago-Silva (2006) showed acetic acid persists longer in mushroom compost than propionic, *n*-butyric and *n*-valeric acids (*n*-caproic acid was not detected in the compost). Thus, *C. elegans* may be less sensitive to acetic acid because of its presence in the habitat the culture organism was isolated from originally. The difference in the sensitivity of the two nematodes to VFA indicates that the use of *C. elegans* as a model nematode for the plant-parasitic nematodes in toxicological studies with these compounds is not possible.

In the application of the LHM to a field, VFA are present in the LHM as a complex mixture, and, when applied to the field, the *P. penetrans* are exposed to these combinations. Our results show that individual VFA present in the LHM do not appear to have synergistic interactions between them and that their effect is likely additive. This additive effect could be explained by their shared common mode of action, which is the uncoupling of the substrate transport and oxidative phosphorylation from the electron transport system leading to the inhibition of the uptake of essential substrates like amino acids, organic acids and phosphate (Freese et al., 1973). Therefore, it should be possible to predict the overall effectiveness of the LHM to control *P. penetrans* based on the sum of lethal effects of individual VFA. VFA in LHM was shown to be lethal to *P. penetrans* here and also previously to *V. dahliae* (Tenuta et al., 2002) under acidic conditions. These

two pathogens interact synergistically in their damage to the potato plants, leading to the early dying disease complex (Rowe et al., 1987). That both are killed by VFA indicates great potential of VFA in LHM in controlling the early dying disease complex in acid soils.

In conclusion, our results demonstrate that the VFA in the LHM can account for the bulk of the toxicity observed in *P. penetrans* exposed to LHM under acidic conditions. *n*-valeric acid was the most toxic ($LC_{95}= 6.8$ mM), while isobutyric acid was the least ($LC_{95}= 45.7$ mM). Individual VFA did not appear to interact in their toxicity to *P. penetrans*, and their effects were deemed additive. Our results indicate that *C. elegans* cannot be used as a complete surrogate to *P. penetrans* in toxicity studies using VFA. The efficacy of LHM to control *P. penetrans* can be evaluated by assessing the VFA concentration in the manure prior to application and determination of non-ionized concentration of VFA nematodes are exposed to. This evaluation is facilitated by the fact that the interaction of individual VFA is additive.

MANUSCRIPT 3

5.0 Effectiveness of Liquid Hog Manure and Acidification to Kill *Pratylenchus* spp. in Soil

5.1 Abstract

The effectiveness of liquid hog manure (LHM) and LHM acidified to kill *Pratylenchus* spp. in potato soils was examined using two micro-plot and one field experiments. Micro-plot experiments were conducted in 2004 and 2005 using two slightly acid soils (designated BPF and MS) harbouring *Pratylenchus* spp. Treatments applied to the two soils in 2004 were: Control, Sulfuric acid (equivalent to 1,800 liters ha⁻¹), LHM (equivalent to 56,000 liters ha⁻¹), and Acidified LHM (equivalent to 2,400 liters sulfuric acid plus 56,000 liters LHM ha⁻¹). Treatments were repeated in 2005. Sulfuric acid was used to reduce soil pH to 4.3 and 5.5 in 2004 and 2005, respectively. By harvest in 2004, only Acidified LHM reduced *Pratylenchus* spp. population in BPF and MS soils by 94% and 91%, respectively. By harvest in 2005, LHM and Acidified LHM reduced populations by 56% and 61%, respectively, for BPF, and by 60% and 93%, respectively, for MS soil. The field experiment was conducted in 2005 on a commercial potato field. Different combinations of LHM (equivalent to 56,000 liters ha⁻¹) and sulfuric acid were added to field plots. At post-application, LHM acidified at pH 6.2 and 5.5 reduced *Pratylenchus* spp. populations by 51% and 89%, respectively. However, Acid (pH 4.6) and LHM treatments reduced populations by 43% and 74%, respectively. By harvest, no treatments had reduced populations compared to Control treatment. We conclude LHM and acidified LHM killed *Pratylenchus* spp. in the slightly acid soils examined consistent

with VFA toxicity being the mechanism; acidification seemed to enhance LHM to kill *Pratylenchus* spp. only when VFA concentration of LHM was relatively low.

5.2 Introduction

Pratylenchus spp., Filipjev (1936), have the widest host range among plant-parasitic nematodes and rank only next to root-knot and cyst nematodes in terms of their worldwide economic impact on crops (Sasser and Freckman, 1987; Duncan and Moens, 2006). Of the 68 *Pratylenchus* species, eight (*P. alleni*, *P. crenatus*, *P. neglectus*, *P. thornei*, *P. scribneri*, *P. coffeae*, *P. brachyurus*, and *P. penetrans*) are associated with poor growth of potato (*Solanum tuberosum*) in many production areas worldwide (Brodie et al., 1993; Ingham et al., 2005; Scurrah et al., 2005; Castillo and Vovlas, 2007). *Pratylenchus* spp. cause stunting and yellowing of potato plants and severe necrotic lesions in the roots and tubers (Brodie et al., 1993), resulting in yield loss as much as 25-73% (Olthof, 1986). In addition, three species, *P. crenatus*, *P. penetrans*, and *P. scribneri*, are capable of interacting synergistically with the wilt fungus, *Verticillium dahliae*, forming the disease complex known as early dying of potatoes when population levels of the two pathogens are too low to cause disease alone (Riedel et al., 1985; Rowe et al., 1985; Rowe and Powelson, 2002). *Pratylenchus penetrans* is the most important *Pratylenchus* spp. that enhances the development of early dying of potatoes (Castillo and Vovlas, 2007). Early dying of potatoes is a limiting factor in potato production in various areas in North America (Martin et al., 1982; Rowe et al., 1987).

Damage to potato caused by *Pratylenchus* spp. can be reduced by lowering pre-plant soil population levels to below an economic threshold population level (Viaene et al., 2006). Several management practices have been used to achieve this; however, some

of these practices have limitations (Castillo and Volvas, 2007). Crop rotation can be used to minimize damage by *Pratylenchus* spp.; however, the wide host range of *Pratylenchus* spp. makes successful crop rotation difficult (Chen et al., 1995). Use of resistant crop cultivars is limited by the lack of resistant germplasm to incorporate into commercial cultivars (France and Brodie, 1995; Brodie, 1998). In addition, biological control agents has not proven to be effective in production systems (Hackenberg et al., 2000; Castillo and Volvas, 2007). Soil fumigation and nematicides are restricted to high-value crops due to the high cost (Olthof, 1987; Olthof, 1989; Rich et al., 2004; Duncan and Moens, 2006).

Manures have been added to agricultural lands for centuries as a source of essential plant nutrients and organic matter (Shulte, 1977). Some studies have shown that anaerobically digested or stored organic wastes kill plant-parasitic nematodes. Liquid hog manure (LHM), also called liquid swine manure (LSM), reduced populations of *Pratylenchus* spp. when incorporated into potato fields (Conn and Lazarovits, 1999), and reduced populations of plant-parasitic nematodes when surfaced applied to grassland (Valocka et al., 2000). In a pot study, LHM added to soil inhibited egg production, hatch and survival of J2 stage juveniles of *Heterodera glycines* (Xiao et al., 2007 and 2008). Anaerobically digested cattle manure reduced damage severity to tomato as well as egg mass and population of *Meloidogyne incognita* in a pot study (Jothi et al., 2003). Also using a pot study, Min et al. (2007) found anaerobically digested cattle and swine manures to reduce populations of *P. penetrans* in an acid soil. Sequential sprinkler irrigation of liquid dairy manure to forage crops reduced populations of *Pratylenchus* spp. and *Paratrichodorus* spp. (Timper et al., 2004). In contrast, Forge et al. (2005) found

populations of *P. penetrans* increased with annual application of LHM to forage in neutral pH soil.

Several studies have shown LHM to control plant pathogens (Tenuta et al., 2002; Conn et al., 2005). Application of LHM to potato fields reduced the incidence of verticillium wilt and potato scab, caused by *V. dahliae* and *Streptomyces scabies*, respectively (Conn and Lazarovits, 1999). LHM was shown to control plant pathogens through various mechanisms including the effects of ammonia, nitrous acid (Conn et al., 2005), and short-chain volatile fatty acids (VFA) (Tenuta et al., 2002; Conn et al., 2005). VFA in LHM are the products of bacterial anaerobic fermentation of carbohydrates and amino acids which takes place in the gastrointestinal tract of hogs and during LHM storage under anaerobic conditions (Zhu, 2000). In a survey of hog farms in southwestern Ontario, Canada, it was found that LHM from finishing pig operations had sufficient VFA to be used as an effective control product to *V. dahliae* while LHM from sow operations did not (Conn et al., 2007). This was due to the manure being more diluted with wash water in sow operations compared to finishing pig operations. Acidic conditions generating non-ionized forms of short-chain VFA (acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acids) in LHM were shown to be responsible for the suppression of microsclerotia of *V. dahliae* (Conn and Lazarovits, 2000; Conn et al., 2005; Tenuta et al., 2002). More recently, using solution exposure studies, Mahran et al. (2008), concluded that VFA can account for the majority of the lethal effect of LHM to *P. penetrans* under acidic conditions. Also, LHM enriched in VFA was more effective in inhibiting egg production by *H. glycines* than the manure un-enriched in VFA (Xiao et al., 2007). In a laboratory experiment, short-chain fatty acids,

butyric and propionic, reduced *Tylenchorhynchus* spp. population in unsaturated soil under both aerobic and anaerobic conditions (McElderry et al., 2005).

As part of our research program to evaluate LHM as a strategy to manage *Pratylenchus* spp., the ability of LHM and acidified LHM to kill the nematodes in potato soils was examined using two micro-plot and one field experiment. LHM, either as is or acidified, was used to test whether the nematicidal effect of LHM occurs under field conditions and if acidification improves its effectiveness.

5.3 Materials and Methods

5.3.1 Micro-plot Experiment 2004:

The experiment was conducted at the Southern Crop Protection and Food Research Centre (Agriculture and Agri-Food Canada, London, Ontario, Canada) in the spring of 2004. Forty-eight micro-plots made from pieces (25 cm i.d. × 25 cm height) of perforated drainage tile buried in soil vertically 20 cm were used. There were six replicate tiles per treatment arranged in a randomized block design. Soil from two commercial potato fields (soil designated as BPF and MS) in the province of Prince Edward Island, Canada, harboring *Pratylenchus* spp. was used. Soil was collected the second week of May, 2004 to a 15cm depth from each field and couriered to London, Ontario for use.

The soils had a mixed population of plant-parasitic nematodes including *Criconea* spp., *P. crenatus*, *P. penetrans*, and *Tylenchorhynchus* spp. In both soils, over 70% of plant-parasitic nematodes were *P. crenatus* and *P. penetrans*. We identified at the University of Manitoba Potato Pathology Laboratory, the two *Pratylenchus* spp. using

PCR according to Al-Banna et al. (1997, 2004) and sequencing of the D3 expansion region of the 26S rDNA. Briefly, 20 individuals of mixed stages were handpicked and each placed into separate PCR reaction tubes containing 5 μ l extraction buffer (0.1 mM Tris-HCl, 0.05 mM EDTA, 0.2 mM NaCl, 1% SDS, and 0.5 mg proteinase K ml⁻¹). The tubes were placed at -80 °C overnight. The tubes were then heated in a water bath for 1 hr at 65 °C, then heated at 95 °C for 1 min and at 99 °C for 3 min in a PCR thermocycler (Techne Flexigene, Techne Inc., Burlington, NJ). The D3 expansion region of the 26S rDNA was amplified by PCR using the forward primer D3A (5'-GAC CCG TCT TGA AAC ACG GA-3') and the reverse primer D3B (5'-TCG GAA GGA ACC AGC TAC TA-3'). PCR products were separated by gel electrophoresis (1.7% agarose gel and ethidium bormide staining) and the resulting band excised from the gel, with the DNA extracted and purified using an extraction kit (QIAquick, Qiagen Inc., Mississauga, ON). The DNA products were then sequenced (Macrogen Corp., Rockville, MD). A Nucleotide-nucleotide BLAST search in the Genebank for the PCR product sequence of each of the 20 nematodes from each soil indicated excellent match of 8 to *P. penetrans* and 7 to *P. crenatus* ($E= 2^{-92}$ to $E= 3^{-93}$), five did not yield a match to a sequence in the database.

Before setup of the experiment, each soil was thoroughly mixed and then slightly dried to bring soil moisture to about 12% (w H₂O w⁻¹ dry soil), and 13 kg placed in polyethylene bags, one bag for each tile. The soil had been partially dried to allow addition of LHM and/or acid and mixing within bags. Drying was done by spreading soil onto a table for three hr at room temperature. Each soil was thoroughly mixed prior to drying and after drying to insure similar population of nematodes in 13 kg subsamples.

Soil amendments were added to each bag, mixed with the soil, and the soil placed in a tile. The pH of the soils was 6.6 and 6.1 for BPF and MS, respectively, organic matter content was 3.5 and 5.7% for BPF and MS, respectively, and the texture of the soils was sandy loam. The LHM used in this experiment was collected in spring 2004 from a below ground covered storage pit at a finishing pig barn in southwestern Ontario, Canada. The manure had 10.0 % dry matter, 0.95% total N, 0.54% NH_4^+ -N, 0.21% total P, and 0.47% total K concentration on an as-is basis. The total VFA concentration (ionized plus non-ionized forms) of the LHM was 149 mM (acetic, 81 mM; propionic, 29 mM; isobutyric, 6.4 mM; *n*-valeric, 8.1 mM; isovaleric, 7.9 mM; and *n*-caproic, 17 mM). The pH of each soil was reduced using sulfuric acid to a pH of 4.3 which was chosen to assure that there were more than sufficient concentration of non-ionized forms of the VFA to suppress *P. penetrans* based on previous 24 hour solution exposure studies (Mahran et al., 2008). The amounts of sulfuric acid needed to bring the pH of the soils and LHM down to pH 4.3 was determined by titrating samples of the soils and LHM with acid before setting up the experiment. Different combinations of sulfuric acid (98% concentrate) (Anchem Sales, London, ON) and LHM were added to soil on June 9, 2004 to provide the following treatments: Control (no sulfuric acid or LHM added), Sulfuric acid (12 ml per tile equivalent to 1,800 liters ha^{-1}), LHM alone (390 g per tile or 3% of soil mass, equivalent to 58,500 liters ha^{-1}), and Acidified LHM (16.3 ml sulfuric acid equivalent to 2,400 liters ha^{-1} sulfuric acid plus 390 g LHM). The Acidified LHM treatment required more sulfuric acid (4.3 ml per tile) than the Sulfuric acid alone treatment to prevent the LHM from raising soil pH above 4.3. The density of LHM was assumed to be 1 g ml^{-1} with the addition rate used estimated being 58,500 L ha^{-1}

assuming a soil bulk density of $1,300 \text{ kg m}^{-3}$, which is typical for sandy loam soil and incorporation depth of 15 cm, also typical for surface applied followed by discing incorporation of LHM. The same soil bulk density and incorporation depth were assumed for estimates of equivalent application rates of acid ha^{-1} . Sulfuric acid was added to soil first followed by LHM to overcome foaming when the acid is mixed with LHM. One potato tuber, cv. Snowden, was planted in each tile one week after addition of amendments. The soil was fertilized one week after planting with an N/P/K granular fertilizer blend at a rate of 260/84/79 kg ha^{-1} , respectively. For the LHM treatments, the amount of N/P/K obtained from the manure was taken into account and the amount of inorganic fertilizer reduced accordingly so that the total N/P/K added was equal for all treatments. The crop was irrigated using a sprinkler system as needed during the growing season. In the fall, first week of October 2004, soil samples were collected from each tile for nematode analysis as described later in this paper, and tubers were weighed. All tubers within a tile were collected at harvest. Plants senesced and died naturally two wk prior to soil collection and harvesting in this experiment and subsequent experiments presented here. Thus at time of soil collection plants roots were partially decomposed and likely endo-parasitic plant nematodes would have begun or completed migration to soil.

The soil pH and VFA concentration for each tile was determined immediately after treatment application (0 hr) in June and pH again determined in October at harvest using 1:2 soil:water (w:w) extracts. The effect of treatments on total nematode numbers, *Pratylenchus* spp. numbers, and yield of tubers ($> 3.17 \text{ cm}$ width) at harvest are also reported.

5.3.2 Micro-plot Experiment 2005:

The experimental set up was identical to 2004 except the initial soil pH after acid treatments was set to a higher pH being 5.5. The pH was set to 5.5 in 2005 for two reasons; (i) large amounts of sulfuric acid had been used in 2004 to lower the pH of the soil to 4.3, and (ii) in the acid only treatment in 2004, the soil pH did not return to control levels. Based on the pK_a values of individual VFA, the VFA concentration of the manure, and results of 24 hour solution exposure studies (Mahran et al., 2008), we expected there was still more than sufficient non-ionized VFA concentration in the Acidified LHM treatment to kill *Pratylenchus* spp. Different combinations of sulfuric acid (98% concentrate) and LHM were applied on June 9, 2005 to freshly collected BPF and MS soils to provide the following treatments: Control, Sulfuric acid (3.2 and 0.8 ml per tile equivalent to 500 and 112 liters ha^{-1} for BPF and MS, respectively), LHM alone (390 g per tile), and Acidified LHM (4.5 and 2.1 ml sulfuric acid equivalent to 670 and 300 liters ha^{-1} for BPF and MS, respectively plus 390 g of LHM per tile). The LHM was obtained in spring 2005 from the same storage pit as in 2004. The manure was more dilute than that used in 2004 having 2.2% dry matter, 0.29% total N, 0.22% NH_4^+ -N, 0.03% total P, and 0.29% total K concentration on an as-is basis. Yet the total VFA concentration (ionized plus non-ionized forms) of the LHM used in 2005 micro-plot study was greater than that in 2004 being 261 mM (acetic, 162 mM; propionic, 54 mM; isobutyric, 15 mM; *n*-valeric, 6.2 mM; isovaleric, 16 mM; and *n*-caproic, 7.8 mM). In the fall, last week of September 2005, and after natural senescence of the plants for two wk, soil samples were collected from each tile for nematode analysis as described in this paper, and tubers were weighed. The soil pH and VFA concentration for each tile was

determined immediately after treatment application (0 hr) in June and pH again determined in September at harvest using 1:2 soil:water (w:w) extracts. The effect of treatments on total nematode numbers, *Pratylenchus* spp. numbers, and tuber yield at harvest are also reported.

5.3.3 Field Experiment:

During the last week of April 2005, an experiment was set up at a commercial potato field with loamy sand soil (pH 6.9, 3.2% organic matter) near Aylmer, Ontario, Canada. Different sulfuric acid (50% concentrate) and LHM combinations were used to provide the following treatments: Control (no acid or LHM added), Sulfuric acid Treatment A (soil pH reduced to 5.8 using sulfuric acid equivalent to 3,400 liters ha⁻¹), Sulfuric acid Treatment B (soil pH reduced to 4.6 using sulfuric acid equivalent to 6,500 liters ha⁻¹), LHM (equivalent to 58,500 liters ha⁻¹), Acidified LHM treatment A (soil pH reduced to 6.2 using sulfuric acid equivalent to 4,000 liters ha⁻¹ plus LHM equivalent to 58,500 liters ha⁻¹), and Acidified LHM treatment B (soil pH reduced to 5.5 using sulfuric acid equivalent to 4,800 liters ha⁻¹ acid plus LHM equivalent to 56,000 liters ha⁻¹). The LHM was obtained in spring 2005 from the same storage pit as for the micro-plot experiments. The manure was more similar to that used in the 2005 micro-plot study having 1.6% dry matter, 0.26% total N, 0.21% NH₄⁺-N, 0.03% total P, and 0.26% total K concentration on an as-is basis. The VFA concentration (ionized plus non-ionized forms) in the LHM used in the field experiment was even greater than previously used in the micro-plot experiment being 367 mM (acetic, 233 mM; propionic, 70 mM; isobutyric, 20 mM; *n*-valeric, 9.4 mM; isovaleric, 25 mM; and *n*-caproic, 9.7 mM). Sulfuric acid (diluted 10-fold in water) and LHM were directly injected into the soil to a 15 cm depth

using a liquid manure tanker (20,000 liter capacity) fitted with an injector/cultivator tool bar (38 cm spacing between injectors and depth of cultivation 15 cm). In the treatments receiving both sulfuric acid and LHM, the acid was applied first followed by LHM.

The experimental design was a randomized block design with four replicate plots (15 x 5.5 meters) per treatment. There was a 9 m border between each replicate block. The field was clean of weeds prior to setup of the experiment. From each treatment plot, ten soil subsamples were collected pre-application, post-application (Day 0) and at harvest using a soil core sampler (2.5 cm diam.) to 15 cm and then combined together for a sample occasion. The post-application collection of soil occurred between 3 and 7 days following application because of inability to complete sampling on one day and arrangement of crew to sample. Previous microcosm experiments showed maximum mortality of *P. penetrans* occurred by 3 days of adding acidified LHM (Mahran et al., *submitted*) and thus the post-application sampling was expected to capture mortality resulting from treatment addition. Two wk following treatment, six rows of potato cv. Snowden were planted in each plot (90 cm between rows and seed potato planted 30 cm apart within row) at a depth of 15 cm. The plots were hilled once after planting. No extra fertilizer was applied to the plots receiving LHM; however, the other plots received a typical amount of fertilizer (N/P/K at a rate of 224/84/79 kg ha⁻¹). The site was irrigated throughout the potato growing period as needed using a center-pivot system. In the fall, second week of October, 2005, the middle two rows of each plot were harvested and the tuber yield determined. Soil pH was determined immediately post-application (Day 0) and post-harvest as previously described. The effect of the treatments on total nematode

and *Pratylenchus* spp. numbers at pre-application, post-application (Days 3 to 7) and harvest are reported. In addition tuber yield (> 3.17 cm width) at harvest is reported.

5.3.4 Nematode Analysis:

Soil samples were placed on ice immediately upon collection and sent by overnight courier to the Soil Ecology Laboratory at the University of Manitoba for nematode extraction and analysis. The samples were then stored at 5 °C and processed within 1 week of being received. One hundred gram fresh weight subsample of each soil was used for nematode extraction and analysis. Nematodes were extracted using Cobb's sieving and decanting (using USA Standard Test Sieve 100 then 400 mesh) followed by sugar flotation (using USA Standard Test Sieve 500 mesh) (Ingham, 1994). Nematodes were then placed in a gridded plastic petri dish for nematode total number determination. The total number of nematodes in each dish was counted using a dissecting microscope at 80× magnification. Numbers of *Pratylenchus* spp. were determined by identifying 100 nematodes to the genus level using an inverted compound microscope at 400× magnification.

5.3.5 pH and Volatile Fatty Acid Analysis:

The entire contents of replicate tiles for the micro-plot experiments were placed in polyethylene bags after harvest and the soil thoroughly mixed. The composite of core samples for a field plot were also mixed in a polyethylene bag. A subsample (8 g) of soil was then added to cold water (40 ml) in polyethylene bags (Seward Stomacher Blending Bags; VWR International, Edmonton, AB). The bags were heat sealed and the slurry was mechanically disrupted (30 s) using a Stomacher homogenizer (Seward Medical, Worthing, UK). The bags were placed on an orbital shaker (200 rpm) for 1 h at 4°C. The

bags were removed from the cold, mechanically disrupted again, and allowed to sit at room temperature for 1 h. The pH of the standing solution was determined using a polymer body pH electrode (Cole-Parmer Canada Inc., Montreal, PQ).

The concentrations of ionized plus non-ionized forms of individual VFA in LHM and soil extracts were determined by ion exclusion chromatography using chemical suppression and conductivity detection (Dionex model 100, Dionex Corp., Sunnyvale CA). Following pH determination, a portion of the standing solution (1.5 ml) was transferred to microcentrifuge tubes and particulates in the solution were removed by centrifugation (10 min at 10,600 g). The analytical column used was an IonPac ICE-AS1 along with an AMMS ICE II chemical suppressor (Dionex Corp.). Extract solution contained in vials were introduced to the ion chromatograph using an autosampler equipped with a refrigerated chamber housing the vials (Waters 717 plus, Waters Associates, Milford, MA). The concentrations (in millimolar, mM) of non-ionized VFA were estimated using the concentration of ionized and non-ionized individual VFA, soil pH, soil moisture and the Henderson-Hasselbalch equation as previously described (Conn et al., 2005).

5.3.6 Statistical Analysis:

For the micro-plot experiments, the values presented for total nematode population, *Pratylenchus* spp. population, tuber yield, pH, and VFA concentration are the means of six independent replicate tiles. The data were tested for normality and subjected to ANOVA. The ANOVA showed no block but treatment effects. Means of treatments were compared using Student-Newman-Keuls method ($P < 0.05$) using the GLM procedure of the SAS computer software package (SAS Institute Inc., Cary, NC). For the

field study, the values presented for total nematode population, *Pratylenchus* spp. population, tuber yield, and pH are the means of four independent replicate plots. The data were tested for normality and subjected to ANOVA. The ANOVA revealed that there was no block effect. The means of treatments were compared as described for the micro-plot experiment.

5.4 Results and Discussion

This study examined the effectiveness of LHM to kill *Pratylenchus* spp. in soil. It further tested the hypothesis that acidification of LHM would improve efficacy by promoting the presence of lethal, non-ionized forms of VFA.

In the 2004 micro-plot experiment, Acidified LHM significantly reduced the *Pratylenchus* spp. population ($P < 0.05$) by harvest, being 94% and 91% of the Control treatment in BPF and MS soils, respectively (Table 5.1). In addition, it reduced the total nematode population ($P < 0.05$) by 50% and 38% in BPF and MS soils, respectively (Table 5.1). In contrast, application of LHM without acidification affected neither *Pratylenchus* spp. nor total nematode populations in the two soils (Table 5.1). Sulfuric acid treatment alone had no effect on total nematode population but increased *Pratylenchus* spp. population ($P < 0.05$) in BPF soil (Table 5.1). The increase in *Pratylenchus* spp. population in soil receiving the Sulfuric acid treatment alone is possibly related to the optimum pH for *P. penetrans* reproduction being 5.2 to 6.4 (Morgan and Maclean, 1968; Willis, 1972). The soil pH by the fall in the Sulfuric acid treatment was 5.7 and 5.6 for BPF and MS soils, respectively, lying within the optimum soil pH range for *P. penetrans* reproduction compared to the Control treatments being at the upper end of the range.

Table 5.1 Combinations of sulfuric acid and liquid hog manure (LHM) applied to BPF and MS soils in the 2004 micro-plot experiment. Shown are the mean total number of nematodes, root-lesion nematodes (*Pratylenchus* spp.) at harvest, and soil pH immediately after post-application (Day 0) and harvest, and also yield of tubers (> 3.17 cm width) at harvest.

Soil	Treatment ¹	Total nematodes (# kg ⁻¹ fresh soil)	<i>Pratylenchus</i> spp. (# kg ⁻¹ fresh soil)	Soil pH		Yield (g plot ⁻¹)
				Day 0	Harvest	
BPF	Control	15,500 a ²	2,660 b	6.6 a	6.3 a	319 b
	Sulfuric acid	13,300 ab	4,160 a	4.3 b	5.7 b	355 b
	LHM	14,000 ab	1,600 b	6.5 a	6.6 a	419 b
	Acidified LHM	7,500 b	154 c	4.5 b	5.4 b	551 a
MS	Control	16,800 a	3,660 a	6.1 a	6.2 a	301 b
	Sulfuric acid	16,100 a	4,050 a	4.3 b	5.6 b	303 b
	LHM	15,700 ab	2,480 a	6.1 a	6.2 a	433 b
	Acidified LHM	10,300 b	320 b	4.4 b	5.1 c	522 a

¹See text for details of treatments and their application rates for the 2004 micro-plot experiment.

²Values shown are the mean of six replicates. Values in a column for each soil followed by different letters are significantly different from one another ($P < 0.05$) as determined by the Student-Newman-Keuls test.

By harvest in the 2005 micro-plot study, Acidified LHM reduced ($P < 0.05$) the *Pratylenchus* spp. population compared to Control treatments by 61% and 93% in BPF and MS soils, respectively (Table 5.2). Total nematode population increased ($P < 0.05$) with Acidified LHM in BPF soil only. LHM addition alone also decreased ($P < 0.05$) *Pratylenchus* spp. populations by 56% and 60% for BPF and MS soils, respectively, but did not affect total nematode populations. Sulfuric acid alone treatment increased the *Pratylenchus* spp. population ($P < 0.05$) by 28% for the BPF soil but decreased ($P < 0.05$) total nematode population by 24%. The effect of LHM alone on *Pratylenchus* spp.

population was not different ($P > 0.05$) from that of Acidified LHM in BPF soil; however, Acidified LHM was more effective in reducing ($P < 0.05$) *Pratylenchus* spp. population in MS soil than LHM alone.

Table 5.2 Combinations of sulfuric acid and liquid hog manure (LHM) applied to BPF and MS soils in the 2005 micro-plot experiment. Shown are the mean total number of nematodes, root-lesion nematodes (*Pratylenchus* spp.) at harvest, and soil pH post-application and harvest, and yield of tubers (> 3.17 cm width) harvest.

Soil	Treatment ¹	Total nematodes (# kg ⁻¹ fresh soil)	<i>Pratylenchus</i> spp. (# kg ⁻¹ fresh soil)	Soil pH		Yield (g plot ⁻¹)
				Day 0	Harvest	
BPF	Control	7,620 b ²	1,450 a	6.7 b	6.7 b	509 b
	Sulfuric acid	5,780 c	1,160 a	6.2 c	6.2 c	576 ab
	LHM	9,110 ab	640 b	7.3 a	7.4 a	760 a
	Acidified LHM	9,850 a	560 b	6.1 c	6.6 b	776 a
MS	Control	6,270 ab	800 b	6.0 a	5.9	499
	Sulfuric acid	5,750 b	1,020 a	5.7 b	6.0	666
	LHM	6,620 a	320 c	6.2 a	6.3	732
	Acidified LHM	6,860 a	60 d	5.6 b	5.9	772

¹See text for details of treatments and their application rates for the 2005 micro-plot experiment.

²Values shown are the mean of six replicates. Values in a column for each soil followed by different letters are significantly different from one another ($P < 0.05$) as determined by the Student-Newman-Keuls test.

The population of *Pratylenchus* spp. was not different ($P > 0.05$) between treatments prior to the start of the field experiment (Table 5.3). Within a week of application to soil in the field study, Sulfuric acid B (soil pH reduced to 4.6), LHM, and the two acidified LHM treatments reduced ($P < 0.05$) *Pratylenchus* spp. populations to below that of the Control treatment (Table 5.3). The Acidified LHM B treatment reduced *Pratylenchus* spp. population by 89% and LHM, Acidified LHM A, and Sulfuric acid B treatments were reduced compared to Control populations by 74%, 51%, and 43%,

respectively. However, by harvest, the population of *Pratylenchus* spp. between treatments was not different ($P > 0.05$). Similarly, total nematode populations were different ($P < 0.05$) between treatments for only the post-application sampling occasion (Table 5.3). At this time, Acidified LHM increased ($P < 0.05$) total nematode population by 314% compared to the Control treatment.

The yield of tubers in the 2004 micro-plot study increased ($P < 0.05$) by 73% for each soil with Acidified LHM treatment compared to the Control treatment (Table 5.1). The yield increase was consistent with a decline in *Pratylenchus* spp. population with Acidified LHM indicating that soil levels of this pest or other pathogen/pest not studied here, could have been yield limiting. Tuber yield increased in the 2005 micro-plot study ($P < 0.05$) 52% and 49% with LHM and Acidified LHM treatment to BPF soil compared to the Control treatment (Table 5.2). For MS soil, tuber yield was not statistically different ($P > 0.05$) between treatments though a numerical increase of 55%, 47%, and 33% was observed for the Acidified LHM, LHM, and Sulfuric acid treatments, respectively. In the field study, there was no difference ($P > 0.05$) in tuber yield of treatments (Table 5.3).

That LHM alone was effective in reducing *Pratylenchus* spp. population in soil in the 2005 micro-plot and field experiment indicated the manure can be active in killing the pest. Acid alone treatment did not lower populations of the pest except for an addition rate bringing soil pH very low, to 4.6 (Sulfuric acid B treatment). This indicates lowering soil pH, unless drastic, does not reduce populations of *Pratylenchus* spp. However, acid supplementation to reduce soil pH below 6, increased the effectiveness of LHM to kill *Pratylenchus* spp. as evident in population reduction in the 2004 micro-plot experiment

Table 5.3 Combinations of sulfuric acid and liquid hog manure (LHM) applied to a field experiment at a commercial potato field near Aylmer, Ontario, Canada. Shown are; the mean total number of nematodes and root-lesion nematodes (*Pratylenchus* spp.) pre-application, post-application (3 to 7 days), and harvest; soil pH immediately post-application (Day 0) and harvest; and also the yield of tubers (> 3.17 cm width).

Treatment ¹	Total nematodes (# kg ⁻¹ fresh soil)			<i>Pratylenchus</i> spp. (# kg ⁻¹ fresh soil)			Soil pH		Yield (kg 30 m ⁻¹)
	Pre-application	Post-application	Harvest	Pre-application	Post-application	Harvest	Day 0	Harvest	
Control	7,970	5,440 b ²	5,200	1,750	1,220 a	350	6.9 a	5.7 ab	60
Sulfuric acid A	6,640	6,000 b	6,600	1,310	1,050 a	540	5.9 b	5.6 ab	60
Sulfuric acid B	8,120	9,430 b	7,050	1,120	690 b	70	4.6 d	4.9 b	44
LHM	6,640	3,920 b	7,160	1,040	320 bc	70	7.2 a	6.2 a	72
Acidified LHM A	8,240	4,420 b	3,310	1,420	600 b	50	6.2 b	5.6 ab	53
Acidified LHM B	5,400	24,000 a	6,620	1,340	140 c	210	5.5 c	5.3 ab	59

¹See text for details of treatments and their application rates for the field experiment.

²Values shown are the mean of four replicates. Values in a column followed by different letters are significantly different from one another ($P < 0.05$) as determined by the Student-Newman-Keuls test.

and further lower population for acidified LHM than LHM alone in the 2005 micro-plot experiment in MS soil. This indicates an interaction of acidification in somewhat improving the effectiveness of LHM to kill the pest.

Few studies have examined the effectiveness of LHM to kill plant-parasitic nematodes. Conn and Lazarovits (1999) showed LHM to reduce populations of *Pratylenchus* spp. in slightly acidic field soil. Xiao et al. (2007) showed VFA enriched LHM to be more effective in inhibiting egg production by *Heterodera glycines* than the manure un-enriched in VFA. They further demonstrated LHM inhibited *H. glycines* egg hatch and killed J2 stage of the pest in laboratory and greenhouse experiments (Xiao et al., 2008). LHM has been shown to control plant pathogens through various mechanisms including ammonia at soil pH > 8, nitrous acid (HNO₂) and non-ionized forms of VFA in soil of pH < 6 (Tenuta et al., 2002; Conn et al., 2005). The pH of soil used in these experiments were less than 8, thus a mechanism of ammonia toxicity was unlikely.

The findings of this study are consistent with VFA being a mechanism for killing *Pratylenchus* spp. in soil. Acidification of LHM resulted in greater concentration of non-ionized VFA in soil (Table 5.4) and increased efficacy of the manure to kill the pest for both soils in the 2004 micro-plot experiment and for MS soil in the 2005 micro-plot experiment. Further, LHM used in the 2005 micro-plot and field experiment had 75% and 146% greater total VFA concentration than used in the 2004 micro-plot experiment. The LHM alone treatment in the 2005 micro-plot and the field study were effective in killing *Pratylenchus* spp. compared to Control treatments.

Table 5.4 Combinations of sulfuric acid and liquid hog manure (LHM) applied to BPF and MS soils in micro-plot experiments in 2004 and 2005. Shown are the Total Volatile Fatty Acid (VFA; ionized plus non-ionized forms) in soil, Total VFA concentration in soil water, and non-ionized VFA in soil water immediately post-application (Day 0).

Soil	Treatment ¹	Total VFA (mg kg ⁻¹ dry soil)		Total VFA (mM in soil water)		Non-ionized VFA (mM in soil water) ²	
		2004	2005	2004	2005	2004	2005
BPF	Control	0 a ³	0 a	0 a	0 a	0 a	0 a
	Sulfuric acid	0 a	0 a	0 a	0 a	0 a	0 a
	LHM	59 b	50 ab	25 b	19 b	1 b	0.1 b
	Acidified LHM	84 b	124 b	38 c	61 c	24 c	3 c
MS	Control	0 a	0 a	0 a	0 a	0 a	0 a
	Sulfuric acid	0 a	0 a	0 a	0 a	0 a	0 a
	LHM	54 b	86 b	22 b	41 b	1 b	2 b
	Acidified LHM	93 c	119 b	47 c	56 b	33 c	8 c

¹See text for details of treatments and their application rates for the 2004 and 2005 micro-plot experiments.

²Concentration of total non-ionized VFA in soil taking into account soil moisture and pH.

³Values shown are the mean of six replicates. Values in a column for each soil followed by different letters are significantly different from one another ($P < 0.05$) as determined by the Student-Newman-Keuls test.

Non-ionized VFA were present in LHM and Acidified LHM soil treatments post-application in the micro-plot studies (Table 5.4). In 2005, the recovery of non-ionized VFA was lower in BPF than MS soil, and lower than for either soil in the 2004 microplot study. The *Pratylenchus* spp. population declined at the post-application sampling in BPF soil for the 2005 micro-plot study (Table 5.2) despite low concentration of non-ionized total VFA (0.1 mM). The low concentration of non-ionized VFA post-application was partly attributable to lower recovery of acetic acid plus acetate for the LHM alone treatment in BPF soil compared to Acidified LHM (5.5 compared to 40 mM,

respectively) and compared to LHM (26 mM) and Acidified LHM (36 mM) in MS soil for the 2004 micro-plot study. There seems to have been either degradation of acetic acid plus acetate (Sorensen, 1998) or sorption to soil in BPF soil. The later has been demonstrated in marine sediments (Shiba et al., 2001) and thus likely possible in soil.

Consistent with a decline in acetic acid plus acetate at post-application was a rise in soil pH from 6.7 to 7.4 in BPF soil receiving LHM alone (Table 5.2). pH of LHM is controlled by relative amounts of total VFA:(NH₃+NH₄⁺) (Paul and Beauchamp, 1989) where a decrease in total VFA results in a pH increase. Sorensen (1998) found a pH increase of 1 to 2 units for coarse-textured soil receiving VFA. Thus unlike solution exposure studies in which VFA concentrations can be kept constant, in the 2004 micro-plot study, rapid loss of acetic acid plus acetate and subsequent rise in pH resulted in low concentration of acetic acid. Thus is it possible determined non-ionized VFA concentration post-application was lower than for that in soil at time of sampling, thus, underestimating exposure concentration of non-ionized VFA to *Pratylenchus* spp.

The results indicate that variation in the concentration of VFA in LHM from even a single source has implications if application without acidification will be effective in killing *Pratylenchus* spp. Variation in VFA concentration from different operations was documented in southwestern Ontario, Canada, where it was found that LHM from finishing pig operations had sufficient VFA to kill microsclerotia of *V. dahliae* while LHM from sow operations did not (Conn et al., 2007). This was attributed to the use of greater amounts of wash water in sow compared to finishing pig operations. The results here indicate the need for determining the VFA concentration in stored LHM within operations if to be used as a pest management strategy.

We cannot rule out that mechanisms other than VFA contributed to the effectiveness of LHM to kill *Pratylenchus* spp. in this present study. The soils used were slightly acidic and thus could have allowed nitrous acid accumulation (Tenuta and Lazarovits, 2002; Tenuta and Lazarovits, 2004) as ammoniac nitrogen nitrified. Recently we demonstrated that acidified LHM was slightly more lethal to *P. penetrans* than a mixture of its individual acidified VFA components (Mahran et al., 2008). LHM can contain VFA > C6, indoles, phenols, volatile amines, and sulfur-containing compounds (Zhu, 2000) that may be lethal to plant-parasitic nematodes, though, untested. The application rates of LHM used in these experiments are typical of that applied to cropped soil. Determining if these other compounds promote the effectiveness of LHM as-is to kill nematode pests and pathogens is interesting because foregoing the addition of acid to LHM would be more practical than acidification.

Although acidification somewhat improved the efficacy of LHM to kill *Pratylenchus* spp., its practical use by farmers is perhaps limited. Addition rates of sulfuric acid will need to be monitored carefully to prevent excessive acidification of soil. For example, lowering of pH of BPF and MS soils in the 2004 micro-plot experiment resulted in pH remaining lower than that of the Control treatment by harvest (Table 5.1). Such acidic soil pH may lead to increasing the availability of some elements (e.g. Fe and Mn) to toxic levels to the crops. At the same time, acidity may cause reduction in the availability of some nutrients (e.g., Ca, Mg, and P) (Martini and Mutters, 1985a; Martini and Mutters, 1985b).

In the 2004 micro-plot experiment, the acidification treatments decreased ($P < 0.05$) soil pH at post-application (Day 0) and by harvest sampling occasions. In the 2005

micro-plot experiment, acidification treatments did result in lowering ($P < 0.05$) soil pH immediately post-application (Day 0) (Table 5.2). However by harvest, soil pH was only lower ($P < 0.05$) for the Sulfuric acid treatment in BPF soil whereas it was higher with LHM alone treatment. In the field experiment, acidification treatments did result in lowering soil pH with the order of effectiveness of treatments being Sulfuric acid B, Acidified LHM B, Sulfuric acid A, and Acidified LHM A (Table 5.3). By harvest, soil pH was lower for the Control treatment than at post-application (Day 0) by almost one pH unit. A decrease in soil pH following nitrogen source addition for soil having low pH buffering ability is common as nitrogen addition as fertilizer or organic sources results in pH reduction through nitrification of added nitrogen (Bolan and Hedley, 2003; Chien et al., 2008) and soil pH returning to former levels in subsequent spring presumably following leaching over winter of acidity. At harvest, only the Sulfuric acid B treatment had lower ($P < 0.05$) soil pH than that of the Control.

In the field experiment, we found addition of acid directly to LHM not possible because it resulted in foaming of the mixture, likely because of generation of non-ionized VFA gases. Instead, we applied concentrated sulfuric acid diluted 10 times in water to soil separate from LHM. Dilution was done to have enough volume to deliver the solution reliably to soil, enough volume of solution to mix within the top 15 cm of soil and to reduce the corrosiveness of the solution to the tank and application system. Clearly, a means of preventing foaming of acidified LHM is required to increase the practicality of the mixture addition to soil.

The nematode community in soil receiving acidified LHM treatments at harvest was by observation dominated by bacterial scavengers in the Cephalobidae and

Rhabditidae and fungal-feeders in the Aphelenchidae and Aphelenchoididae. These nematodes are adapted to grow rapidly in response to growth of bacterial and fungal decomposers in soil (Bongers and Ferris, 1999). Their seeming dominance indicates either acidified LHM may have been toxic to the soil food web with nematodes in those families subsequently flourishing in response to microbial decomposers proliferating on dead microbial biomass or microbial biomass proliferating on readily available C in LHM. The impact of acidification of LHM on soil food webs needs to be determined to understand its effect on soil health.

LHM and acidified LHM seem to be economically competitive to currently available fumigants used for *Pratylenchus* spp. control. Farmers can often obtain LHM at no cost from neighboring hog rearing operations requiring disposing of the material. Where disposal of LHM is not a concern, farmers receiving LHM often purchase it based on its value of plant nutrients. Estimated nutrient value of 58,500 L of LHM averaged for the three LHM products used here and based on average North American fertilizer market value from January 2007 to January 2008 is \$412 USD (N=\$0.88, P=\$1.06 and K=\$0.54 USD kg⁻¹; Oehmke et al. 2008). At the application rate, 58,500 liters ha⁻¹, and application cost of \$0.25 CDND L⁻¹ (Saskatchewan Agriculture and Food, 2006) being about \$0.20 USD L⁻¹, the nutrient equivalent value and cost of LHM application is estimated at \$533 USD ha⁻¹.

For soil acidification of LHM to be used by farmers to kill *Pratylenchus* spp., it must be more effective than LHM added alone. Clearly more studies are required to determine the benefit of acidification of LHM. In this study, there was improvement in the efficacy of LHM when acidified, being apparent in the 2004 micro-plot study using

an LHM of lower VFA concentration than used in the 2005 micro-plot and the field study. If following further scrutiny, acidification of LHM holds up to improve the effectiveness of LHM in killing nematode pests, a low-cost source of acid would be required. Such a source is currently available as a waste product from metal cleaning and metal pickling industries (Kobe and Fredrickson, 1956). Currently, these waste solutions are used as a source of ferrous sulfate. The estimated cost of sulfuric acid solution based on cost of ferrous sulfate solution (source: <http://www.theinnovationgroup.com>) and rate of application of 1,800 L ha⁻¹ is \$420 USD ha⁻¹. Accordingly, the total cost of acidified LHM application (LHM to 58,500 L ha⁻¹) would be \$953 USD ha⁻¹ if the LHM was only available for a cost. In comparison, the cost of a fumigant (e.g. Vapam (metam sodium)) application to control *Pratylenchus* spp. is approximately \$1,400 USD ha⁻¹ (based on 2008 prices and rate of application of 700 L ha⁻¹) (Dr. Robert Wick, personal communication). Accordingly, LHM application alone or acidified is a competitive option for *Pratylenchus* spp. control compared to fumigants. However, further testing of the effectiveness of LHM and necessity of acidification is needed.

In conclusion, micro-plot and field experiments showed that LHM is effective in reducing *Pratylenchus* spp. populations in at least the slightly acid soils examined here. The effectiveness of LHM was somewhat improved with acidification where the LHM was low in VFA concentration. The results of this study are consistent with VFA as the constituents responsible for toxicity of LHM to *Pratylenchus* spp. Further, application of LHM alone or acidified for *Pratylenchus* spp. control is potentially economically competitive to fumigants, however further studies are required to compare the effectiveness of LHM and acidification of LHM to fumigants.

MANUSCRIPT 4

6.0 Liquid Hog Manure and its Acidification Alters a Nematode Community in Soil

6.1 Abstract

Soil microcosm experiments were conducted to determine why opportunistic bacterial-feeding nematodes increase following addition of liquid hog manure (LHM) and acidified mixtures of the manure. A sandy loam soil harboring a wide range of nematode taxa, representing various trophic and colonizer-persister groups (c-p 1 through 5) and augmented with the plant-parasitic nematode, *Pratylenchus* spp., was used. Treatments were additions of LHM (0.15 v v⁻¹ soil water), mixtures of the manure (0.05, 0.10, and 0.15 v v⁻¹ soil water) and sulphuric acid to reduce soil pH to 5.5, as well as a sulphuric acid alone and a non-treated control. Nematode community analysis was conducted over a 28-day period. Four days post-treatment, populations of plant-parasitic nematodes, including *Pratylenchus* spp., decreased by at least 50% for all manure treatments compared to non-treated control and acid alone treatments. Thereafter, for all manure treatments, c-p 1 and c-p 2 nematode populations increased compared to non-treated and acid alone treatments. At day 28, c-p 1 and c-p 2 nematode populations, were at least greater, by 6,000 and 5,000 kg⁻¹, respectively, compared to non-treated control and acid alone treatments. In contrast, populations of c-p 3, 4, and 5 nematodes were not affected by the treatments. At day 28, the structure index, an assessment of the abundance of high c-p (c-p 3, 4 and 5) nematodes to c-p 2 nematodes, was 53 for all manure treatments and higher, being, 68 and 80 for acid alone and non-treated control treatments, respectively. The enrichment index, an assessment of the abundance of opportunistic c-p 1 and 2 nematodes compared to all c-p 2 nematodes, was about 70 for all manure treatments and

lower, being, 32 and 29 for acid alone and non-treated control treatments, respectively. The increase in opportunistic nematodes observed following manure treatment was primarily due to enrichment of the soil environment with readily degradable compounds. Volatile fatty acids (VFA) present in the manure persisted in the soil for only four days with biological degradation being their mode of loss. The results indicate LHM is effective in killing plant-parasitic nematodes without having the characteristic of a soil fumigant curtailing soil trophic interactions but rather increased bottom-up food web interactions.

6.2 Introduction

Animal manures and wastes are sources of plant nutrients and organic matter to soil in many agricultural production systems (Shulte, 1977). Anaerobically digested or stored animal manures and wastes are capable of killing plant pathogens (Conn and Lazarovits, 1999) and pests such as plant-parasitic nematodes (Valocka et al., 2000; Jothi et al., 2003; Timper et al., 2004; Min et al., 2007; Xiao et al., 2007 and 2008; Mahran et al., *in press*). Liquid hog manure (LHM) was shown to control plant pathogens through various mechanisms including short-chain volatile fatty acids (VFA) (Tenuta et al., 2002; Conn et al., 2005; Mahran et al., 2008). Acidic conditions generating non-ionized forms of short-chain VFA (acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acids) in LHM were shown to be responsible for the suppression of the wilt fungus, *Verticillium dahliae* Kleb. (Conn and Lazarovits, 2000; Tenuta et al., 2002; Conn et al., 2005). More recently, using laboratory solution exposure studies, Mahran et al. (2008), concluded that VFA can account for the majority of the lethal effect of LHM to *P. penetrans* (Cobb) Chitwood and Oteifa under acidic conditions. VFA added to soil in

pot studies (McElderry et al., 2005; Xiao et al., 2007) and those present in LHM added to field and micro-plots (Mahran et al., *in press*), reduced populations of plant-parasitic nematodes.

While examining extractions of soil cropped to potato having received acidified LHM, we observed large populations of opportunistic bacterial feeding nematodes of the Rhabditidae (unpublished data). These nematodes flourish under conditions of abundant bacterial growth that can occur following application of broad-spectrum pesticides, in soil, such as fumigants (Yeates et al., 1991; Ettema and Bongers, 1993; Wang et al., 2006) or following addition of readily decomposable materials to soil (Griffiths et al., 1994; Bongers and Bongers, 1998; Bulluck et al., 2002; Ferris and Matute, 2003; Nahar et al., 2006).

Anaerobically stored animal manures contain large amounts of easily decomposable organic C such as VFA (Kirchmann and Lunvall, 1993; Sorensen, 1998; Zhu, 2000), amino acids (Dewes and Hunsche, 1998; Zhu, 2000), and dissolved organic carbon (Royer et al., 2007). Following manure application to soil, these materials are rapidly utilized by soil microorganisms as a source of energy (Sorensen, 1998; Tenuta et al., 2000). Kirchmann and Lundvall, (1993) showed that VFA in hog and cattle slurries decomposed in soil within one to two days of application at 25 °C. They observed good correlation between amount of N immobilized and amount of VFA in soil and concluded that they are an easily decomposable source of energy for soil microorganisms.

The efficacy of any compound in killing soil organisms, whether beneficial or detrimental to plant growth, depends on the dose used, and the duration of exposure and sensitivity of the organism to the compound (Call and Hague, 1962; Van Gundy et al.,

1972; Munnecke and Van Gundy, 1979; Rozman and Doull, 2000). Persistence of added toxic compounds is desired in the short-term to provide a dose and duration to kill the target organism (Lawrence and McLean, 2000; Haydock et al., 2006) but in longer-term, persistence is not desired as it increases the chance of non-target organisms to be killed and can prevent repopulation of soil in the likely event beneficial organisms are also killed (Haydock et al., 2006). The persistence of VFA in acidified LHM added to soil or involvement of sorption and volatilization is currently unknown, though clearly important as persistence for days and wk increases the likelihood of acting as a fumigant and negatively affecting soil food webs.

Analysis of taxa abundance for nematode communities in soil, sometimes referred to as nematode faunal analysis (Ferris et al., 2001), could be a means to indicate how LHM and acidified LHM result in the presence of opportunistic bacterial-feeding nematodes. Nematodes faunal analysis has been shown to be an excellent tool in assessing the structure and function of soil food webs and their response to altered soil conditions (Freckman and Ettema, 1993; De Ruiter et al., 1994; Ferris et al., 1996; Bongers, 1999; Bongers and Ferris, 1999; Ferris et al., 2001). This is due to characteristics of nematodes including: (a) having high diversity and abundance in soil environments with complex food webs; (b) having a permeable cuticle in contact with dissolved constituents of soil water or volatiles in soil atmosphere; (c) some being able to become active and rapidly reproduce to feed on the proliferation of microbes following an increase in available carbon substrates; (d) some are slow growing and reproduce slowly, thus their establishment following disturbance is slow; (e) they are relatively easy to isolate and identify to the genus taxon, (f) they occupy key trophic guilds in soil food

webs, and (g) there is a clear relationship between nematode feeding structures and their food resource (Bongers and Ferris, 1999).

As part of a research program to determine if LHM is an option for farmers to control *Pratylenchus* spp. and other plant pathogens and pests, soil microcosm experiments and nematode faunal analysis were conducted to determine: (i) if LHM and its acidification increases opportunistic nematodes through addition of readily decomposable materials or through partial sterilization of soil, (ii) if persistence of VFA in soil receiving acidified LHM is of short or long duration, and (iii) if low persistence of VFA in soil is due to biological degradation of the compounds.

6.3 Materials and Methods

6.3.1 Soil:

Cattle grazed, native mixed-grass prairie soil harboring a diverse range of nematode genera and trophic guilds was collected in the summer of 2007 from the Yellow Quill Prairie Preserve near the town of Shilo, in south central Manitoba. The soil was collected as three separate blocks (50 x 50 x 15 cm h.) about 10 m from each other. Each soil block was placed in a polyethylene bag and brought to the laboratory in an ice chest, where it was stored (< 1 week) at 5 °C until used. The soil was sandy loam in texture and had 6.7% organic matter content, 16.5% gravimetric moisture and pH (1:2 soil: H₂O ratio) of 7.5.

6.3.2 Liquid Hog Manure:

LHM was collected from an earthen storage lagoon at a commercial finisher hog farm in southeastern Manitoba. The manure had a pH of 7.2, 2.7% dry matter, 0.6% total

N, 0.01% P and 0.25% K. It was centrifuged (10 min at 3,400 x g) to remove particulates. The manure had a total volatile fatty acid concentration (ionized plus non-ionized forms) of 347 mM (acetic = 190 mM, propionic = 51 mM, isobutyric = 23 mM, *n*-butyric = 54, isovaleric = 13 mM, *n*-caproic = 9 mM and *n*-valeric = 9 mM). The manure was frozen at – 20°C until used.

6.3.3 *Pratylenchus* spp.:

A soil naturally infested with high populations of two root-lesion nematode species, *P. penetrans* and *P. crenatus* Loof, was collected from a commercial potato field in the province of Prince Edward Island and reared on common mint (*Mentha spicata* L.) in a greenhouse. *Pratylenchus* spp. present in the soil were identified using species-specific polymerase chain reaction (PCR) primers (Al-Banna et al., 2004) and species identification confirmation by molecular sequencing of PCR products and sequence comparison to the GenBank BLAST database (Mahran et al., *in press*). Nematodes were extracted from soil using Cobb's sieving and decanting followed by sugar flotation (Ingham, 1994) 24 hr prior to the experiment. The density of the resulting mixed population of juveniles and adults of *Pratylenchus* spp. was adjusted in dH₂O and added to the soil in microcosms to 1,000 nematodes kg⁻¹ dry soil equivalent. The *Pratylenchus* spp. suspension added contained eggs; however, their density was not determined. *Pratylenchus penetrans* and *P. crenatus*, are common nematode pests of potato in central and eastern Canada though not present in Manitoba. They were added to the soil to provide levels of a common nematode pest of great economic importance.

6.3.4 Microcosm:

Each of the three soil collections was passed through a 2 mm mesh screen (USA Standard Test Sieve) to remove stones, roots, and debris. The collections remained separate from each other to serve as independent replicates in soil microcosm experiments.

Soil microcosms were prepared by adding 90 g fresh soil in a 118 ml polyethylene specimen storage container (6.4 cm d. x 6.4 cm h.) (Fisher Scientific Canada Ltd., Edmonton, Canada). The container lid had 20, 0.1 cm diam. holes punctured into it, to prevent anaerobic conditions during incubation. Different combinations of sulfuric acid 98% (equivalent to 4.9 mg H₂SO₄ ml⁻¹) (Fisher Scientific Canada Ltd.) and LHM were applied to microcosms to provide the following treatments: non-treated control, acid (initial soil pH reduced to 5.5 using sulfuric acid 98%), LHM (15% of soil water volume at 30% of soil field capacity equivalent to 40,500 liters ha⁻¹) and acidified LHM mixtures to produce concentrations of 5%, 10%, and 15% volume of manure to soil water volume. At an assumed soil bulk density of 1.3 Mg m⁻³, typical depth of field application (15 cm) and gravimetric moisture content of the soil, acidified LHM added at 5%, 10%, and 15% rates are equivalent to 13,500, 27,000, and 40,500 liters ha⁻¹, respectively. The amounts of sulfuric acid needed to bring initial soil pH and the LHM down to the target pH was determined by titrating soil and LHM with various amounts of acid. Following application, the soil gravimetric moisture content was adjusted to 30% of field capacity (Cassel and Nielsen, 1986) using dH₂O. Microcosms were arranged in a completely randomized design comprising three independent replicates per treatment and were

placed in an incubator at 22°C in the dark (Iso-temp incubator model 304; Fisher Scientific Canada Ltd.).

Soil samples were extracted 24 hr, three days, and one, two, three and four wk following experimental setup to examine changes in nematode communities as detailed by Forge and Tenuta (2008) and to determine VFA concentration in soil. Fifty g of soil from each microcosm was used for extraction of nematodes using Cobb's sieving and decanting followed by sugar flotation (Ingham, 1994). Live nematodes were collected in water in a 15 ml centrifuge tube, excess water was removed, and an equal volume of hot (80 °C) buffered formalin solution (pH 7.0) (Humason, 1972) was added to the nematode suspension where they were preserved until identified to the genus level. Total count of nematodes was determined using a stereo microscope (40x magnification). The first 100 nematodes encountered in each sample were then identified using an inverted compound microscope (400 x magnification), using the diagnostic keys of Bongers (1988). The abundance of each genus in a microcosm was calculated by multiplying the fraction of nematodes of that genus in the identified 100 nematodes by the total count of nematodes (Ferris et al., 1996). The nematode genera were assigned to a colonizer-persister value (c-p value) according to Bongers (1990) where nematode families are classified on a colonizer-persister (c-p) scale ranging from one to five. Opportunistic bacterial-feeding nematodes having similar characteristics to r-strategists were assigned to group, c-p 1. Higher c-p groups were ordered based on increasing reliance upon a k-strategy of life-history. Bacterial- and fungal-feeding nematodes mainly comprised group c-p 2 nematodes, and omnivorous and carnivorous nematodes mainly comprised group c-p 3 through 5 nematodes.

6.3.5 Food web indices:

Food web indices computed included the enrichment and structure indices (Ferris et al., 2001). The structure trajectory is a weighted abundance of larger, slower-reproducing bacterivore, fungivore, omnivore and carnivore c-p 3 to 5 value nematodes. The structure index was calculated as $100 \times (s/(s + b))$, with b calculated as $\sum kbnb$, where kb are the weightings assigned to guilds that indicate basal characteristics of the food web and nb are the abundances of nematodes in those guilds, the s component was calculated similarly, using those guilds having weightings assigned to the structure index. The enrichment trajectory is a weighted abundance of opportunistic bacteriovorous c-p 1 and fungivorous c-p 2 nematodes species that respond rapidly to prey resources. The enrichment index was calculated as $100 \times (e/(e + b))$. The e component was calculated as previously mentioned using those guilds indicating enrichment.

6.3.6 Volatile fatty acids persistence and fate in soil:

The persistence of VFA in soil and its subjugation to biological degradation and sorption was examined in a separate microcosm experiment. The collections of grazed mixed-grass prairie soil used for the previous experiment were also used here. The soil was split into two parts, one part (2 kg) was used without sterilization and the other part (2 kg) was autoclaved (at 121°C and 15 psi) for 45 min and the autoclaving repeated after three days to kill germinated heat-resistant spores. The LHM used in the previous microcosm experiment was also used here. To the non-sterile soil, LHM was added without sterilization. To sterile soil, LHM was sterilized by first centrifugation (30 min at 11,500 x g) to remove particulates and supernatant collected into sterile polyethylene 50 ml conical tubes (Fisher Scientific Canada Ltd.). The supernatant was then filtered

through Whatman binder-free glass microfiber filters (9.0 cm d.; Fisher Scientific Canada Ltd.) to remove fine particulates. The filtrate was then filter-sterilized using Nalgene disposable sterile analytical filter units (pore size 0.2 μm ; Fisher Scientific Canada Ltd.). The sterile LHM was then stored at 5°C in sterile polyethylene 50 ml conical tubes until used. Microcosms were set up as described previously except 30 g fresh soil was used and all polyethylene containers were sterilized using 95% ETOH (Fisher Scientific, Canada Ltd.) prior to use. Treatments were non-treated control, acid (initial soil pH reduced to 5.5 using sulfuric acid 98%), and acidified LHM (initial soil pH reduced to 5.5 plus 15% of soil water volume at 30% of soil field capacity) treatments. Sterile water was added to all treatments to bring soil moisture to 30% of field capacity.

Microcosms were destructively sampled after setup (0 hr) and every 24 hr for seven days following experiment setup to observe changes in volatile fatty acid concentration in soil. Persistence of VFA with the sterile acidified LHM treatment was taken to indicate a lack of biological degradation, sorption, and volatilization. In contrast, lack of persistence of VFA in the non-sterile treatments only was taken to indicate biological degradation of the compounds.

6.3.7 Volatile fatty acids determination and concentration in soil:

In order to determine the concentration of VFA in soil, 15 ml dH_2O was added to 15 g moist soil in 50 ml polyethylene conical tubes. The tubes were then shaken for 30 min on their side at 2,000 rpm at 5 °C using a refrigerated incubator shaker (New Brunswick Scientific Co., Edison, USA). The tubes were then centrifuged (5 min at 5000 x g) and the supernatant analyzed for VFA concentration. Individual volatile fatty acid compounds from C2 (acetic) to C6 (*n*-caproic) were determined using chemical-

suppression ion-exclusion chromatography and conductivity detection (Tenuta et al., 2002) using a Dionex ion chromatography system, ICS-1000 (Dionex Corp., Sunnyvale, USA). The chromatograph was equipped with an IonPac ICE-AS1 (9 x 280 250mm) analytical column and anion micro-membrane suppressor (AMMS-ICE II). Soil extracts contained in 1 ml vials with filter caps (Dionex Corp.) were injected (25µl) using an automated sampler (AS40, Dionex Corp.). For analysis, 1 mM heptafluorobutyric acid (Acros Organics USA, Morris Plains, USA) with 5% acetonitrile (v v.⁻¹) was used as the eluent. Commercially available individual VFA (≥ 99% purity; acetic acid, Fisher Scientific Canada Ltd.; propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, and *n*-valeric acid, Sigma-Aldrich; and *n*-caproic acid; Acros Organics) were used to calibrate the response of the conductivity cell to individual volatile fatty acid concentration using eight point response curves. The concentration of volatile fatty acid in soil solution for each soil sample was determined by multiplying its concentration in the supernatant by a dilution factor that is estimated based on the soil moisture content of soil and the volume of water added to it (15 ml).

6.3.8 Statistical analysis:

Results for both microcosm experiments were tested for normality using the Shapiro-Wilk test prior to analysis of variance using the Proc GLM procedure of the Statistical Analysis Software (SAS Institute Inc., Cary, USA). Treatment means were compared using Tukey's Multiple Comparison test ($P < 0.05$). Data for nematode indices data were square-root transformed prior to analysis of variance and comparison of means.

6.4 Results

6.4.1 Nematode population response:

Total nematode population: Total nematode population steadily declined over the course of the experiment in the non-treated control and acid treatments with that of the acid treatment being lowest of all treatments over the course of the experiment (Fig. 6.1a). For the LHM treatment, total nematode population declined over the first seven days following application then increased steadily and by 28 days it yielded the highest total nematode population among all treatments (Fig. 6.1a). Acidified LHM resulted in a decline in the total nematode population over the first three days with lower population with increasing concentration of LHM. Thereafter, total nematode population of acidified manure treatments increased over the period of the study and by 28 days were lower than that for the LHM alone treatment (Fig. 6.1a).

c-p 1 nematodes: The population of opportunistic (c-p 1) nematodes decreased from about 1,500 to 250 individuals kg^{-1} soil by the end of the experiment for the acid only treatment (Fig. 6.1b). Population for the non-treated control increased at three days and thereafter declined to a similar level of that for the acid alone treatment (Fig. 6.1b). The LHM treatment resulted in an increase by four wk with the population being 11,000 individuals kg^{-1} soil or a 25-fold increase compared to the non-treated control. Individuals in the genera *Diploscapter* and *Mesorhabditis* were mainly responsible for the increase in population of c-p 1 nematodes observed in the LHM treatment. With acidified LHM treatment, addition of 5% resulted in an increase within one day of addition followed by a decline to that of all other treatments by one week (Fig. 6.1b). Thereafter, populations increased for all acidified liquid hog manure treatments to a level

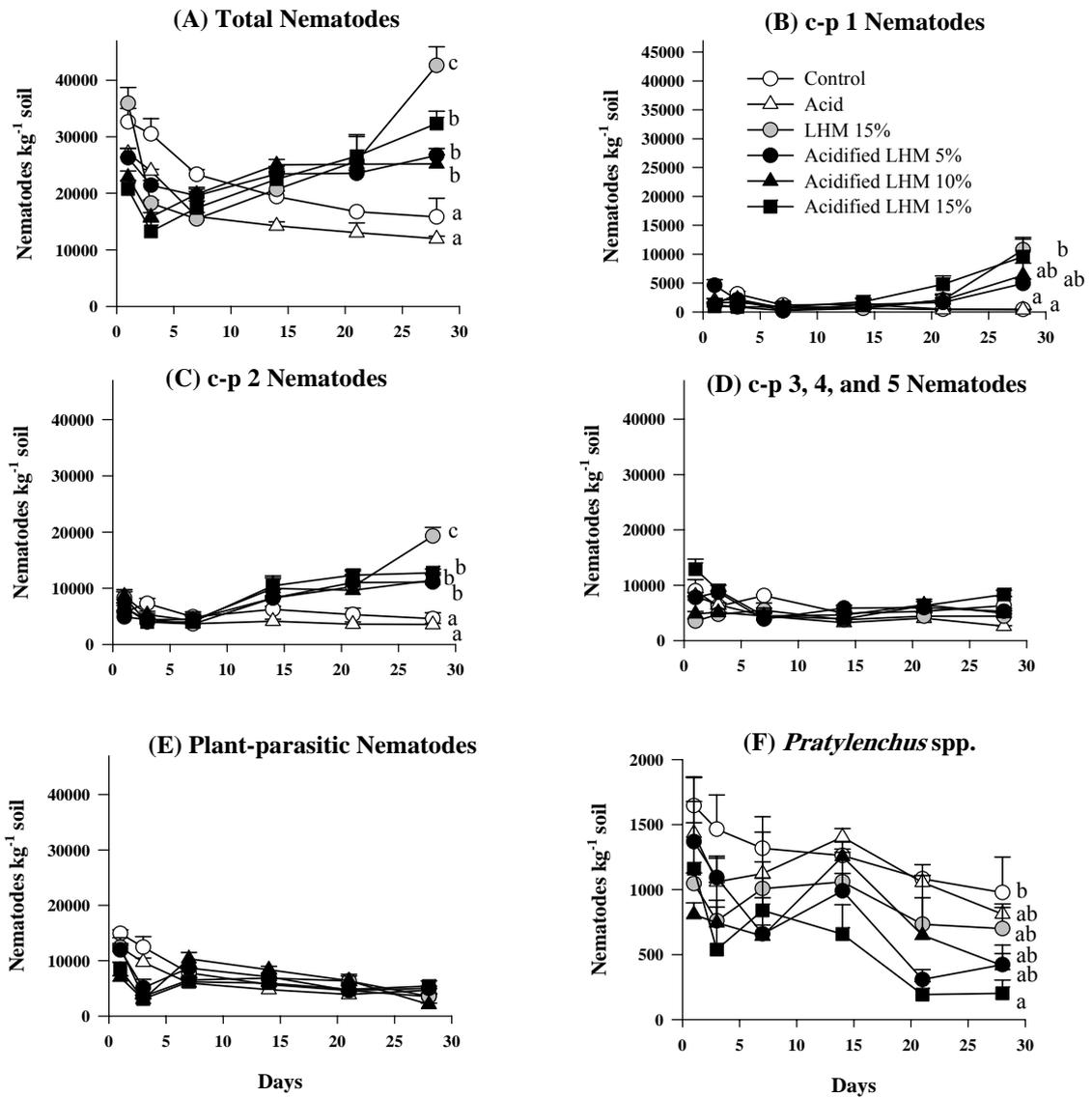


Figure 6.1 Population of: (A), total nematodes; life-history groups, (B), c-p 1 nematodes; (C), c-p 2 nematodes; (D), c-p 3, 4, and 5 nematodes; and (E), plant-parasitic nematodes; and the plant-parasitic nematode (F), *Pratylenchus* spp. in the microcosm experiment. Treatments are control (non-treated), acid alone (initial soil pH was reduced to 5.5), liquid hog manure (LHM) 15% and acidified LHM 5%, 10%, and 15% (initial soil pH reduced to 5.5 plus 5%, 10%, and 15% LHM v^{-1} soil water, respectively) over a 28 day period. Data shown are the mean of three independent replicates are + 1 standard error of the mean. Plots followed by different letters are significantly different from one another ($P < 0.05$) as determined by Tukey's multiple comparison test.

similar ($P > 0.05$) to that for the LHM alone treatment, though numerically populations were greater with increasing concentration of applied acidified LHM. Individuals in the genera, *Diploscapter* and *Mesorhabditis*, were responsible for the increase in c-p 1 nematode population in acidified LHM treatments.

c-p 2 nematodes: Population of c-p 2 nematodes tended to be higher in the non-treated control compared to acid alone treatment though statistically similar ($P > 0.05$) by the end of the experiment (Fig. 6.1c). In the LHM treatment, c-p 2 nematodes declined over the first seven days of the experiment and then started to increase attaining 20,000 individuals kg^{-1} soil by the end of the experiment (Fig. 6.1c). Bacterial-feeding nematodes of the genera *Chiloplacus*, *Eucephalobus*, *Acrobeles*, and of the Plectidae and fungal-feeding nematodes of the genera *Aphelenchus* and *Aphelenchoides* were the c-p 2 nematodes that increased as a result of LHM treatment. There was a sharp increase in the population of c-p 2 nematodes after 21 days due to an increase in individuals in the genus *Chiloplacus* and to a lesser extent in the genus *Acrobeles*. A similar trend in population occurred with acidified LHM treatments except these treatments had populations intermediate of the LHM alone, and the non-treated control and acid alone treatments. Bacterial-feeders belonging to the c-p 2 nematode genera *Chiloplacus* and *Acrobeles* increased with acidified LHM treatment.

c-p 3, 4, and 5 nematodes: Compared to the response of c-p 1 and c-p 2 nematodes, populations of c-p 3, 4 and 5 nematodes collectively were not as responsive to treatments (Fig. 6.1d). There were no differences ($P > 0.05$) in their population among all treatments by 28 days. Two trends in populations however were evident; acid alone resulted in a declining population during the two wk following treatment with the

population remaining numerically lower than other treatments by the end of the experiment. Acidified LHM 15% resulted in a decline in population up to one week and then the highest numeric population at the end of the experiment compared to other treatments. The genera of nematodes comprising the colonizer-persister groups c-p 3, 4 and 5 were the bacterial-feeding genera, *Rhabdolaimus*, *Teratocephalus* and *Ethmolaimus*, the fungal-feeding genus, *Tylencholaimus*, and omnivorous nematodes of the Qudsinematidae.

Plant-parasitic nematodes: The populations of plant-parasitic nematodes steadily declined over the first two wk of the experiment in the non-treated control and acid alone treatments (Fig. 6.1e). The plant-parasitic nematode population tended to be lower in the acid alone than the non-treated control over the course of the experiment. All LHM treatments (acidified and non-acidified) resulted in a decrease in plant-parasitic nematode population by day three with increasing manure concentration. A rebound in numbers occurred by one week, with numbers then having declined to the end of the experiment. The rebound and subsequent decline in population was due to Tylinchid nematodes, of the genera *Filenchus* and *Tylenchus*. By end of the experiment, populations of plant-parasitic nematodes were similar for all treatments ($P > 0.05$).

Pratylenchus spp.: *Pratylenchus* spp. steadily declined over the course of the experiment in the non-treated control (Fig. 6.1f). In the acid treatment, *Pratylenchus* spp. declined over the first three days before rebounding at 14 days then declining to the end of the experiment. Addition of LHM alone resulted in a lower population compared to the non-treated control throughout the experiment. Acidified LHM treatments resulted in numerically lower populations than the non-treated control and by the end of the

experiment acidified 15% had a lower ($P < 0.05$) population than the non-treated control. For plant-parasitic and *Pratylenchus* spp. nematodes, a population decline with manure treatment was consistent with volatile fatty acid toxicity, a rebound in population at two wk with hatching of eggs and decline to four wk with absence of plant hosts.

6.4.2 Nematode faunal analysis:

At 24 hr following treatment application, food webs in all treatments were moderately enriched and highly structured as indicated by nematode faunal analysis. Both the enrichment and structure index values were different after 28 days compared to that at one day following treatment. After 28 days, the enrichment index declined in the non-treated control and the structure index remained almost the same. In the acid treatment, both the enrichment and the structure indices declined by 28 days following treatments application. In the LHM and acidified LHM treatments, the enrichment index increased ($P < 0.05$) and the structure index decreased ($P < 0.05$), yielding a soil food web indicated to be highly enriched and moderately structured (Fig. 6.2).

6.4.3 Volatile fatty acids in soil:

No VFA were detected in soil in the non-treated control and acid alone treatments (Fig. 6.3). The concentration (mM) of VFA in soil at 0 hr increased with increasing concentration of LHM applied. VFA in soil declined steadily over time and were not detected in soil at three days in the acidified LHM (5%, 10%, and 15%) treatments. With LHM 15% alone, VFA were almost undetectable at seven days.

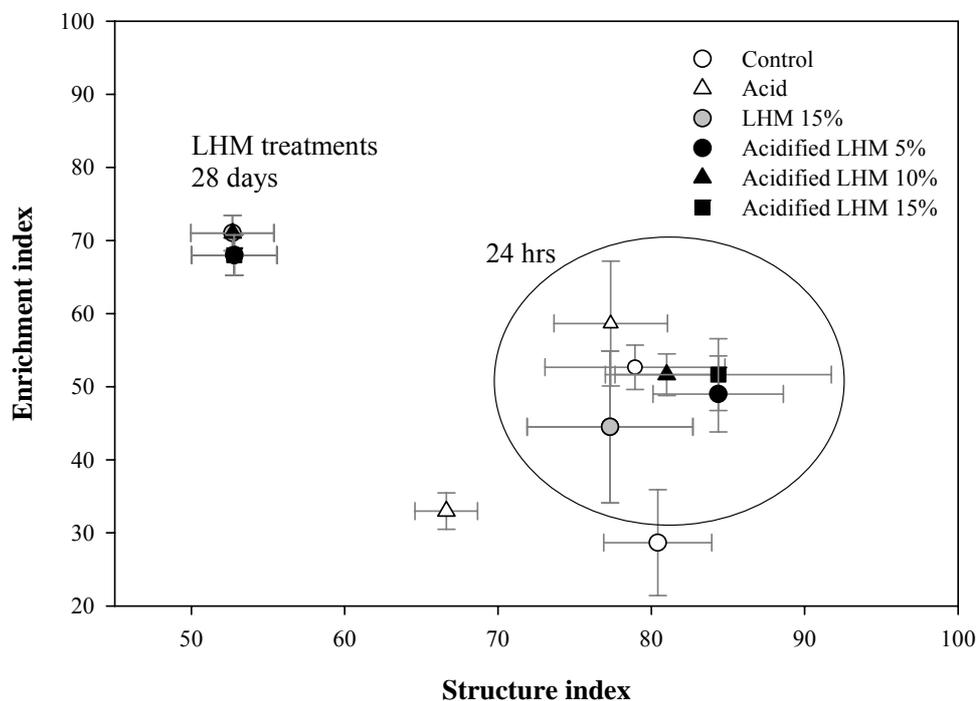


Figure 6.2 Results of nematode faunal analysis showing nematode community structure and enrichment conditions of the soil food web at 24 hr and 28 days following application of treatments. Treatments are control (non-amended treatment), acid alone (initial soil pH reduced to 5.5), liquid hog manure (LHM) 15% (15% LHM v v⁻¹ soil water), and acidified LHM 5%, 10%, and 15% (initial soil pH reduced to 5.5 plus 5%, 10%, and 15% LHM v v⁻¹ soil water, respectively). Data shown are the mean of three independent replicates ± 1 standard error of the mean in the x- and y-axis directions.

6.4.4 Volatile fatty acids persistence in soil:

Addition of acidified and non-acidified LHM at 15% was expected to provide a concentration of 61.3 mM VFA in soil solution. Immediately after setup of the experiment, the recovery of VFA in the acidified LHM 15% treatment was 59.0 mM under sterile conditions and 57.2 mM under non-sterile conditions (Fig. 6.4), being approximately 96.2% and 93.3% of added VFA, respectively. However, at four days VFA added were not recovered from soil treated with the acidified LHM (Fig. 6.4).

Under sterile conditions, VFA persisted throughout the experiment. Autoclaving seems to have resulted in low concentrations of VFA being recovered as evident in 3.0 and 2.3 mM concentration in the non-treated control and acid alone treatments, respectively, and being similar to elevated recovery of VFA from sterile compared to non-sterile acidified LHM treatments (Fig. 6.4).

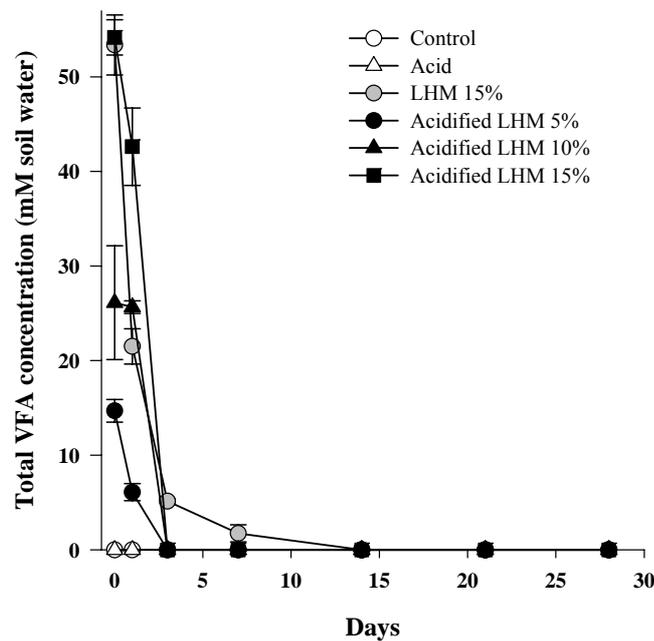


Figure 6.3 Total (ionized plus non ionized forms) volatile fatty acids (VFA) concentration (mM in soil water) over a 28 day period in soil receiving the treatments, control (non-amended treatment), acid alone (initial soil pH reduced to 5.5), liquid hog manure (LHM) 15% (15% LHM v v⁻¹ soil water), and acidified LHM 5%, 10%, and 15% (initial soil pH reduced to 5.5 plus 5%, 10%, and 15% LHM v v⁻¹ soil water, respectively). Data shown are the mean of three independent replicates \pm 1 standard error of the mean.

6.5 Discussion

In this study we investigated if an increase in opportunistic nematodes following addition of LHM to control *Pratylenchus* spp. was a direct result of addition of readily available decomposable materials or indirect from first killing of soil organisms followed

by proliferation of opportunistic organisms. The results show that the total nematode population declined following application of LHM and acidified LHM. This decline was not specific to a certain trophic group but was general including c-p 1, c-p 2, c-p 3, 4, and 5, and plant-parasitic nematodes. The decline increased with increasing LHM concentration being greatest for the LHM 15% ($v v^{-1}$ soil water) rate. The decline in nematode population was attributed to the presence of LHM as nematode population did not decline to the same extent in the non-treated control or acid alone treatments.

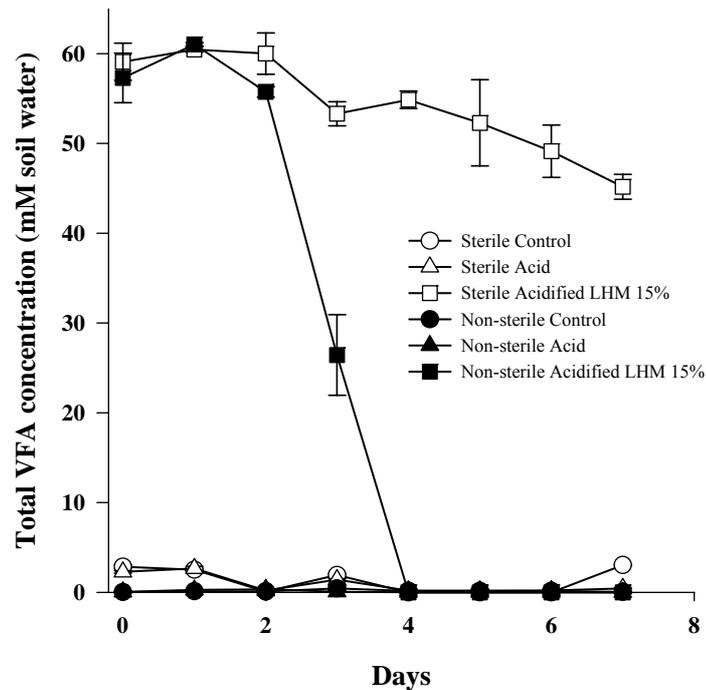


Figure 6.4 Total (ionized plus non ionized forms) volatile fatty acids (VFA) concentration (mM in soil water) over a seven day period in soil receiving the treatments, control (non-amended treatment), acid alone (initial soil pH reduced to 5.5), and acidified liquid hog manure (LHM) (initial soil pH reduced to 5.5 plus 15% LHM $v v^{-1}$ soil water) under sterile and non-sterile conditions. Data shown are the mean of three independent replicates \pm 1 standard error of the mean.

Presence of VFA in soil was consistent with mortality of nematodes. VFA in LHM were responsible for mortality of the plant-parasitic nematodes, *Pratylenchus* spp., *Heterodera glycines* Ichinohe, *Tylenchorhynchus* spp., and the wilt fungus, *V. dahliae* (Tenuta et al., 2002; McElderry et al., 2005; Xiao et al., 2007 and 2008) and their effectiveness increase by acidification (Tenuta et al., 2002; Mahran et al., 2008). Acidification formed non-ionized forms of VFA that killed *P. penetrans* and microsclerotia of *V. dahliae* in laboratory solution exposure studies (Tenuta et al., 2002; Mahran et al., 2008).

The effect of VFA in LHM and acidified LHM was not specific to a certain life-history group of nematodes but was general to all nematodes including plant-parasitic ones. Although there was a decline in nematode population, they were not entirely eliminated and were still detectable following LHM and acidified LHM application. Accordingly, VFA in LHM, with the rates and at the pH used in this experiment, did not seem to act as a chemical fumigant eliminating all nematodes and causing a “biological vacuum” in soil, where nematodes gets eliminated and not detected for a period of time (Yeates et al., 1991; Ettema and Bongers, 1993; Wang et al., 2006). The nematode population started to increase rapidly by one week following LHM and acidified LHM application, which corresponded to loss of VFA from soil, yielding higher total numbers of nematodes by 28 days. Opportunistic c-p1 and c-p 2 nematodes were primarily responsible for the increase in total nematode population in soil. The increase in population of these nematodes in relation to acidified and non-acidified LHM treatment was likely in response to enrichment of the soil environment with readily degradable compounds in LHM, such as VFA, and perhaps mortality of some soil organisms by

VFA. These enrichment-opportunist nematodes have increased in soil rapidly following the addition of decomposable materials (Bongers and Bongers, 1998; Ferris and Matute, 2003) or availability of organic resources from mortality of organisms (Ferris et al., 2001). An increase in enrichment-opportunistic nematodes of c-p 1 indicates stimulation by “bottom-up” processes resulting from an increase in microbial biomass for feeding by enrichment-opportunistic nematodes. In contrast, population of nematodes of higher trophic levels (c-p 3, 4, and 5) did not change.

The population of plant-parasitic nematodes declined over the first three to seven days following application of LHM and acidified LHM. By the end of the experiment, 28 days following treatment application, populations of plant-parasitic nematodes were not different between manure and the non-treated control and acid alone treatments. That could be attributed to the fact that plant-parasitic nematodes are dependent on plant hosts for nourishment and they were effectively starved in their absence (Viaene et al., 2006).

The population of *Pratylenchus* spp. did rebound from initially depressed levels following acidified and non-acidified LHM by 7-14 days before declining again to the end of the experiment. This increase observed in the *Pratylenchus* spp. population in the non-acidified and acidified LHM treatments is possibly related to the hatching of the nematodes eggs that were added with the nematodes at experimental set up. The optimum pH for *P. penetrans* reproduction, and in turn egg hatching, is 5.2 to 6.4 (Morgan and MacLean, 1968; Willis, 1972). The pH of soil receiving these treatments fell within this optimum range for *P. penetrans* reproduction.

The survival of higher trophic nematodes belonging to colonizer-persister groups, c-p 3, 4, and 5, were not significantly different between acidified and non-acidified LHM

treatments by the end of the experiment. This observation is surprising as these nematodes have been shown to be more sensitive to different stressors such as: metals (Zullini and Peretti, 1986; Korthals et al., 1996 and 2000; Georgieva et al., 2002), acidification (Dmowska, 1993; Ruess et al., 1996); nematicides (Smolik, 1983), and nitrogen compounds (Tenuta and Ferris, 2004). VFA were expected to be a stressor being shown to be lethal to *V. dahliae* (Conn and Lazarovits, 2000; Conn et al., 2005; Tenuta et al., 2002), *P. penetrans* (Mahran et al., 2008), *H. glycines* (Xiao et al., 2007, 2008), and *Tylenchorhynchus* spp. (McElderry et al., 2005). Perhaps that the finding here that VFA did not persist in soil, being four days, was not long enough to kill these nematodes. The tolerance to stress of persister nematodes (carnivores and omnivores) has been previously reported. In a field study, omnivore populations increased in soil 28 days following incorporation of the plant-parasitic suppressive green manure, sunn hemp (*Crotalaria juncea* L.), into soil (Wang et al., 2004). Alternatively, it is possible that soil handling and preparations for microcosms could have killed sensitive higher c-p nematodes as these have been shown to be sensitive to physical disturbance (Lenz and Eisenbies, 2000; Fiscus and Neher, 2002; Rahman et al., 2007).

Availability of organic resources from external input, mortality of organisms, or favorable shifts in the environment (Odum, 1985) cause a flush of microbial activity that enhance opportunistic bacterial-feeding nematodes which in turn result in an enriched food web with a high enrichment index (Ferris et al., 2001). The introduction of organic resources to soil represents a “bottom-up” constraint on the size and activity of the food web. The presence of high c-p nematodes that use the opportunistic nematodes as a food source and competition among trophic levels provide “top-down” regulation of food web

structure and function (Ferris et al., 2001) and over time result in a structured food web. In the circumstance that death of the general nematode community occurs following fumigation, dead organisms become a resource leading to flush of microbial activity that enhance opportunistic bacterial-feeding nematodes (Ferris et al., 2001). In the absence of high c-p nematodes, that are sensitive to fumigation, the food web becomes enriched and the structure index becomes very low (Wang et al., 2006). In our experiment, the soil food web condition was moderately enriched but highly structured one day following application of treatments. However, 28 days following the addition of LHM and acidified LHM the nematode community became highly enriched but poorly structured compared to non-treated control and acid alone treatments. Application of LHM and acidified LHM lead to an increase in the population of opportunistic nematodes due to the availability of organic resources. However, no corresponding increase in population of high c-p nematodes lead to a reduction in the structure index for the acidified and non-acidified LHM treatments (Ferris et al., 2001; Fiscus and Neher, 2002). The effect of the three different concentrations of acidified LHM and LHM used in this experiment on the soil food web was similar between treatments.

Nematode taxa at different trophic and life-history groups responded differently to the application of acidified and non-acidified LHM. By 28 days following treatments application, opportunistic c-p 1 nematodes of the genera *Diploscapter* and *Mesorhabditis* was dominant in soil and the population of the genus *Monhystera* declined. Population of the bacterial-feeding c-p 2 nematodes of the genera *Acrobeles* and *Chiloplacus* increased in soil while individuals of the genus *Plectus* was not detected in soil in LHM and acidified LHM 10% and 15% treatments. The population of the genus *Wilsonema*

generally was not affected by the treatments. Taxa of the persister nematodes, c-p 3, 4, and 5, also varied in their response to the treatments. The population of the bacterial-feeding nematodes of the genus *Ethmolaimus* increased in soil in response to LHM treatments but the population of nematodes of the genus *Tylencholaimus* declined.

The recovery of VFA immediately after application of acidified LHM 15% under sterile and non-sterile conditions was approximately 96.2% and 93.3% of added VFA, respectively. Accordingly, the majority of VFA added to soil in LHM were recovered and thus sorption of VFA to soil was minimal. Water was used as an extractant and thus only soluble VFA are recovered and measured. Acetic acid, the major volatile fatty acid in LHM, has been shown to be sorbed to marine sediments (Shiba et al., 2001) and onto aluminum (Alliot et al., 2005) thus likely possible in soil. However, in the soil used here, sorption does not seem to have been appreciable.

VFA persisted in soil under sterile conditions for longer periods than under non-sterile conditions. The concentration of VFA did not decline over the first 24 hr following application under non-sterile conditions and for the first three days under sterile conditions. That indicates that the major route of loss for VFA in soil is likely due to microbial degradation and not sorption, which if to occur, would have occurred soon after application. Various microorganisms are able to degrade VFA in LHM and use them as a source of carbon and energy (Zhu, 2000). These microorganisms vary in their abilities to degrade VFA in LHM (Bourque et al., 1987; Jolicoeur and Morin, 1987). While some bacteria such as *Acinetobacter calcoaceticus* (Beijerinck) Bouvet and Grimont, *Alcaligenes faecalis* Castellani and Chalmers, and *Arthrobacter flavescens* Lochhead, are capable of degrading all types of VFA in LHM (Bourque et al., 1987; Jolicoeur and

Morin, 1987), others such as *Corynebacterium glutamicum* (Kinoshita) Abe and *Micrococcus* spp. can only degrade acetic and propionic acids (Bourque et al., 1987).

Microbial biomass, enzyme activities and microbial respiration, have been found to increase upon application of anaerobically stored liquid animal manures to soil (Paul and Beauchamp, 1996; Lalande et al., 1998; Zaman et al., 1999; Chantigny, 2001). Application of liquid animal manures to field plots increased microbial respiration for the first week presumably due to the rapid oxidation of VFA present in slurry (Tenuta et al., 2000; Chantigny et al., 2001). Rochette et al. (2000) showed that the decomposition of LHM applied to field plots was rapid with one-half of the total annual CO₂ emissions from soil having occurred during the week after manure application.

In conclusion, the increase in opportunistic nematodes in soil following application of LHM and acidified LHM was in response to enrichment of the soil environment likely resulting from addition of readily available decomposable materials in LHM and mortality of some organisms which in turn release readily decomposable materials. The large increase in the population of opportunistic nematodes 28 days following the application of LHM and acidified LHM with no corresponding increase in high c-p nematodes resulted in a poorly structured food web indicating the lack of higher trophic links. VFA in LHM persisted in soil for only four days and their major loss was due to biological degradation. LHM alone or when acidified does not behave as a chemical fumigant which eliminates soil trophic interactions. Why acidified LHM is as an effective method to control plant-parasitic nematodes including *Pratylenchus* spp. without having the same effect on other trophic nematode is worthy of further

investigation. This is because controlling plant-parasitic nematodes without killing all soil organisms or nematodes is desirable in the promotion of soil health.

7.0 GENERAL DISCUSSION

The main objective of this thesis was to appraise the use of LHM as a low-cost, effective, and environmentally safe strategy to control *Pratylenchus* spp. in potato fields. The thesis included a survey of potato fields in Manitoba through which the prevalence and species of *Pratylenchus* spp. in Manitoba potato fields were determined. In addition, using a laboratory solution study, I tested if VFA are the constituents in LHM that account for the manure toxicity to *Pratylenchus* spp. under acidic conditions. The efficacy of LHM in killing *Pratylenchus* spp. in soil was determined using micro-plot and field studies. Moreover, the hypothesis that acidification improves LHM efficacy in killing *Pratylenchus* spp. was tested. The impact of LHM on non-target nematodes and soil food webs were examined using a microcosm experiment and nematode faunal analysis.

Accurate diagnosis of the species of *Pratylenchus* spp. is a key factor for successful crop management (Castillo and Vovlas, 2007). Since there was no information available in the current literature on the species of *Pratylenchus* spp. present in Manitoba potato fields, *Pratylenchus* spp. identification to species level was addressed using both morphometrics and molecular techniques. Results of the morphometrics and molecular diagnostics indicated that the *Pratylenchus* species present in Manitoba is *P. neglectus*. To the best of my knowledge, this is the first time the species of *Pratylenchus* has been identified in Manitoba potato fields.

The geographic distribution of *Pratylenchus* spp. is dependent on their introduction through infected plant material, the prevalence of suitable plant host that support their reproduction, and physical factors (e.g. soil texture) (Castillo and Vovlas,

2007; Duncan and Moens, 2006). *Pratylenchus penetrans* is the most widely distributed species of root-lesion nematode in potato growing areas of the United States and Canada (Rowe et al., 1987; Kimpinski, 1979), and the observation that *P. penetrans* was not found in the Manitoba potato fields surveyed is surprising.

Several factors could explain the observation that *P. penetrans* was not found in the Manitoba potato fields surveyed. Possibly that *P. penetrans* was not introduced to potato fields of Manitoba. Alternatively, it has been introduced but did not survive because the soils in the potato fields we surveyed was suppressive to its survival due to the presence of antagonistic microorganisms that has not been studied. The absence of suitable plant host to support its reproduction or the presence of unfavorable abiotic factors (i.e. soil texture) could also be factors. *Pratylenchus penetrans* occurred more frequently in fruit and tobacco growing areas of Ontario and has thus been concluded to be a fruit and tobacco parasite (Potter and Townshend, 1973). In the potato fields surveyed, potato is normally rotated with cereals and canola that may be unsuitable hosts for *P. penetrans*.

Soil type also affects *Pratylenchus* spp. distribution in potato fields within a region (Florini et al., 1987). *Pratylenchus penetrans* is found more frequently in lighter sandy soils (Loof, 1978; Florini et al., 1987) with higher availability of oxygen (Castillo and Vovlas, 2007). The soil texture in the potato fields surveyed in Manitoba that harbored high *Pratylenchus* spp. numbers were loam and loamy sand; thus, perhaps less suitable to the survival of *P. penetrans*.

The total acreage that we surveyed was relatively small compared to the total acreage of potato in Manitoba. *Pratylenchus penetrans* could be present in other potato fields that we did not cover in the survey. Moreover, molecular identification was performed on a limited number of fields and 100 nematodes from each field, which may have reduced the chance of detecting *P. penetrans* if present in low numbers.

Pratylenchus neglectus was the only root-lesion nematode found in the Manitoba potato fields surveyed. *Pratylenchus neglectus* was the most predominant *Pratylenchus* spp. present in cereal and forage crops in Ontario and thus concluded that it is a cereal and forage parasite (Potter and Townshend, 1973). Canola as well has been shown to be a good host for *P. neglectus* (Taylor et al., 2000; Fatemy et al., 2006). In the potato fields sampled in this survey, cereals and canola are normally rotated with potato and thus favoring the presence of *P. neglectus*. *Pratylenchus penetrans* was also not prevalent in potato growing regions of Australia, where wheat is rotated with potato, where it was only detected in two of 77 potato fields surveyed; whereas, other species of *Pratylenchus* were detected in 87% of the fields and on roots in 92% of the fields (Harding and Wicks, 2007).

Pratylenchus neglectus is found more frequently in loamy soils (Loof, 1978) and its pathogenicity and reproduction is greatest in sandy loam and loamy sand soil (Griffin, 1996; Townshend, 1972; Olthof, 1990). The soil texture in potato fields that harbored high *Pratylenchus* spp. numbers in the survey was loam and loamy sand; thus, favoring *P. neglectus*.

I do not believe that temperature is a factor in favoring the presence of *P. neglectus* rather than *P. penetrans* in the potato fields surveyed. Both *P. penetrans* and *P.*

neglectus showed to have the same sensitivity to low temperatures (-2 °C) (Townshend, 1973).

Little is known about the effect of *P. neglectus* on potato (Hafez et al., 1999) and reported results are conflicting (Brodie, 1984; Olthof, 1990; Davis et al., 1992; Hafez et al., 1999). The suitability of potato cv. Russet Burbank as a host for populations of *P. neglectus* in Manitoba potato fields was investigated. *Pratylenchus neglectus* populations from two potato fields in Manitoba did not propagate on potato plants under field or growth chamber conditions. Consequently, *P. neglectus* does not seem to be a restraint to potato production in Manitoba. These findings are of great importance to current potato producers in Manitoba for successful management of potato early dying disease complex.

My results show that *P. neglectus* does not seem to be a limitation to potato production in Manitoba; therefore, *P. penetrans* was used in the successive studies of assessing the use of LHM to control *Pratylenchus* spp. in potato fields. *Pratylenchus penetrans* is the most widely spread and economically important species in soils regularly cropped to potato in eastern United States and Canada (Rowe et al., 1987; Kimpinski, 1979). It causes serious damage to potato crops through either direct infection (Oostenbrink, 1954) or through interaction with *V. dahliae* leading to potato early dying disease complex (Martin et al., 1982; MacGuidwin and Rouse, 1990; Rowe and Powelson, 2002).

LHM was shown previously to control plant pathogens through various constituents and/or mechanisms including the effects of ammonia, nitrous acid (Conn et al., 2005), and VFA (Tenuta et al., 2002; Conn et al., 2005). Using laboratory solution

exposure studies, I found that VFA could account for the toxicity of the manure to *P. penetrans* under acidic conditions. Accordingly, *P. penetrans* can be added to the list of pathogens that VFA in LHM are capable of controlling making it a broad-spectrum control strategy.

The nature of interaction between individual VFA in LHM has not been addressed before; thus, it was addressed in this study to predict the manure's overall impact on *P. penetrans*. Individual VFA in LHM did not appear to interact synergistically with each other, so their effect is likely additive. This additive effect is explained by their shared common mode of action, which is the uncoupling of the substrate transport and oxidative phosphorylation from the electron transport system leading to the inhibition of the uptake of essential substrates such as amino acids, organic acids, and phosphate (Freese et al., 1973). Accordingly, the overall effect of VFA mixture in LHM in killing *P. penetrans* can be estimated by simply summing up the lethal effects of individual concentrations of VFA present. This finding is of importance in the practical application of LHM, as it will make the calculation of the rate of LHM application to control *P. penetrans* simpler. To the best of my knowledge, this was the first time the toxicological nature of potential interactions among VFA was addressed. In addition, the methodology used to address the interaction of individual VFA could be applied to examine the interaction among constituents of other mixture compounds (e.g. other organic amendments and green manures) that are used for control of plant pathogens.

LHM and acidified LHM proved to be effective in killing *Pratylenchus* spp. in soil as evident in micro-plot and field experiments. Accordingly, they have the potential to compete with commercially available nematicides. Acidification enhanced the ability

of LHM to kill *Pratylenchus* spp., especially when it has low VFA content; however, its practical use by producers is perhaps limited. Addition rates of sulfuric acid will need to be monitored carefully to prevent excessive acidification of soil that may lead to increasing the availability of some elements (e.g. Fe and Mn) to toxic levels to the crops. At the same time, acidity may cause reduction in the availability of some nutrients (e.g., Ca, Mg, and P) (Martini and Mutters, 1985a; Martini and Mutters, 1985b).

The results of the micro-plot and field experiments indicate that variation in the total VFA content in LHM may cause inconsistency in its efficacy in controlling *Pratylenchus* spp. if to be applied without acidification. Moreover, results of the bioassays showed that individual VFA in LHM vary in their lethality to *P. penetrans* with *n*-valeric acid being the most toxic (LC₉₅= 6.8 mM) and isobutyric acid being the least toxic (LC₉₅= 45.7 mM). This indicates that not only the variation in total VFA contents may cause inconsistency in LHM efficacy, but the variation in individual VFA proportions can cause inconsistency as well. Variation in VFA concentrations from different operations was documented in southwestern Ontario, Canada, where it was found that LHM from finishing hog operations had sufficient VFA to kill microsclerotia of *V. dahliae* while those from sow operations did not (Conn et al., 2007). This inconsistency in effectiveness is considered a limitation to the use of LHM as a control product. However, it could be surmounted by determining the VFA content and profile in stored LHM within operations if to be used as a pest management strategy.

LHM and acidified LHM are economically competitive to currently available fumigants used for *Pratylenchus* spp. control. The cost of LHM application based on the rate used in the micro-plot and field studies (58,500 L ha⁻¹) is estimated at \$533 USD ha⁻¹

¹. In case that acid needs to be added by the rate used in the micro-plot and field experiments, the total cost of acidified LHM application (58,500 L ha⁻¹) would be \$953 USD ha⁻¹ if the LHM was only available for a cost. However, the cost of a nematicide (e.g. Vapam (metam sodium)) application to control *Pratylenchus* spp. is approximately \$1,400 USD ha⁻¹ (based on 2008 prices and rate of application of 700 L ha⁻¹) (Dr. Robert Wick, personal communication). Accordingly, LHM application alone or acidified is an economically competitive option for *Pratylenchus* spp. control compared to nematicides.

An ideal amendment to control plant pathogens and pests should not be lethal to non-target beneficial organisms in the soil food web while still being highly toxic to target organisms. The effect of VFA in LHM and acidified LHM on nematode communities in soil as indicators of soil health was addressed using nematode faunal analysis and a microcosm experiment. VFA in LHM, and acidified LHM did not seem to act as a fumigant that eliminates all nematodes and causes “biological vacuum” in soil where nematodes are not detected for a period of time (Yeates et al., 1991; Ettema and Bongers, 1993; Wang et al., 2006). LHM and acidified LHM enriched the soil environment with readily degradable compounds and mortality of some soil organisms by VFA was observed that led to increase in the opportunistic c-p1 and c-p 2 nematodes. More research is needed to elucidate why LHM and Acidified LHM killed plant-parasitic nematodes including *Pratylenchus* spp. without having the same effect on other trophic nematodes.

Liquid hog manure application carries some environmental and health concerns. Field application of LHM may lead to leaching and runoff losses of estrogenic and endocrine-disrupting compounds (e.g. 17-b-estradiol, estrone, and equol) present in the

manure to groundwater or aquatic environments (Burnison et al., 2003). These compounds have long-term adverse effects on growth, development, and reproduction of fish and wildlife (Guillette, 1995). It may also cause runoff losses of antibiotics (e.g. chlortetracycline and tylosin) into groundwater that may lead to the development of resistant strains of bacteria to these antibiotics (Dolliver and Gupta, 2008). In addition, LHM application may help in transmitting human pathogenic bacteria (*Escherichia coli*, *Salmonella* spp., *Yersinia enterocolitica* and *Cryptosporidium* spp.) that are present in the manure through runoff to groundwater (Cote et al., 2006). Application of LHM with high rates can cause nitrate contamination of drinking water (Elmi et al., 2005) and nutrient loss to surface water (e.g. phosphorus) (Royer et al., 2003). Producers using LHM as a control strategy for *Pratylenchus* spp. should follow the guidelines and regulations of the environmental protection authorities to maintain the balance between pest control and environmental protection.

LHM can be applied to soil to control *Pratylenchus* spp. either in the fall, when nematode populations are at their peak, or in the spring, targeting the nematode life stages present in soil at that period of the year. These application times were chosen based on LHM availability and producers' needs for its recycle use in land. As producers need to empty their lagoons in the fall, in order to create space for the storage of the manure produced over the winter, or in the spring after lagoons have been filled up over the winter. LHM application technique greatly determines the efficacy of LHM in controlling *Pratylenchus* spp. It is very important to minimize the loss of VFA due to volatilization. Injecting the manure into the soil is the most appropriate method of application to reduce losses of VFA. The injection of manure can be done with low disturbance coulters or

knives. Row spacing of less than 45 cm should be used to get good distribution and minimize the volume in each row.

In conclusion, VFA are the constituents in LHM that account for its toxicity to *Pratylenchus* spp. LHM is an effective, and low cost strategy to control *Pratylenchus* spp. Acidification seemed to enhance LHM to kill *Pratylenchus* spp. when VFA concentration of LHM is low. LHM did not act as a fumigant in soil; however, its application may carry some environmental risks such as nitrate contamination of drinking water, pathogen transfer to surface and ground water, and greenhouse gas emissions that needs to be minimized through management practices for a safer usage of LHM.

Recommendations for future research

Identification of *Pratylenchus* spp. in Manitoba potato fields show that the species prevalent in potato fields is *P. neglectus*. *Pratylenchus* spp. populations from two potato fields in Manitoba did not propagate on potato cv. Russet Burbank. However, high *Pratylenchus* spp. population levels were detected in some potato fields that are indicative of the nematode being feeding on hosts other than potato. Future research is needed to examine whether *Pratylenchus* spp. in potato fields are feeding on other crops that are normally in rotation with potato in Manitoba. Wheat and canola are commonly rotated with potato in Manitoba and were shown to be damaged by *Pratylenchus* spp. Determining whether *Pratylenchus* spp. in Manitoba is causing damage to other crops in rotation with potato is of importance to producers as to whether they apply nematodes management strategies.

Pratylenchus neglectus in Manitoba did not propagate on potato cv. Russet Burbank. These results are conflicting with those by Hafez et al., (1999) that a *P. neglectus* population from Ontario, Canada, was capable of infecting potato plants and interacting synergistically with *V. dahliae*. Accordingly, determination of the genotype of *P. neglectus* from Manitoba using molecular techniques, such as PCR–RFLP of the ITS region of the ribosomal RNA gene, is essential to distinguish it from other populations of *P. neglectus* in Ontario and other regions of North America.

The effect of VFA in LHM on *P. penetrans* was determined using solution exposure studies. Understanding the effect of VFA in LHM on other plant-parasitic nematodes is needed to determine if it can be applied to control other economically

important nematodes. The same methodology used here can be used to determine whether VFA in LHM have the same effect on other plant-parasitic nematodes.

Acidity seemed to increase the efficacy of LHM in killing *Pratylenchus* spp. when total VFA content is low. The effect of other soil properties (e.g. organic matter content and soil texture) on the efficacy of VFA in LHM to kill *Pratylenchus* spp. is unknown though needed to determine whether LHM will be effective in killing *Pratylenchus* spp. under different soil conditions. Accordingly, greenhouse screening of potato soils from various provinces is required to determine the effectiveness of LHM in controlling *Pratylenchus* spp. under different soil conditions.

The microcosm and faunal analysis experiments show that LHM and Acidified LHM are effective in killing plant-parasitic nematodes including *Pratylenchus* spp. without having the same effect on other trophic nematodes. Understanding why the effect of LHM and Acidified LHM is specific to plant-parasitic nematodes and not general to all trophic levels is worthy of further investigation. This is because controlling plant-parasitic nematodes without killing other beneficial nematodes in soil is desirable in the promotion of soil health.

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