

**Survey and Development of a Molecular Test for Soybean Cyst Nematode
(*Heterodera glycines* Ichinohe) in Manitoba**

By

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Abstract

Ghavami Shirehjin Nazanin, Ph.D., The University of Manitoba, April 2024. Survey and Development of a Molecular Test for Soybean Cyst Nematode (*Heterodera glycines* Ichinohe) in Manitoba. Advisor: Dr. M. Tenuta.

Soybean Cyst Nematode (SCN), *Heterodera glycines* (Ichinohe 1952), recognized as a major global soybean pest, is already established in the Canadian provinces of Ontario and Quebec. This study anticipated its imminent presence in Manitoba, driven by its rapid spread from U.S. counties bordering the province. The research objectives were to a) survey SCN presence in Manitoba soybean fields, b) develop a molecular method for direct soil quantification of *H. glycines*, and c) identify native cyst nematodes in Manitoba Prairie Preserves. The survey of 30 commercial soybean fields in Manitoba revealed four positive fields for SCN using morphological characters of cysts, PCR with species-specific primers for *H. glycines* (COXIII and SCAR), and DNA sequencing of four regions of the genome, marking the first report of *H. glycines* in Manitoba. In 2021, an additional soybean field in Manitoba showed SCN symptoms and *H. glycines* females on the soybean roots. A molecular test employing a SYBR Green I-based real-time qPCR assay using species-specific primers for *H. glycines* (COXIII and SCAR) was developed, offering a reliable, sensitive and specific means to detect and quantify *H. glycines* eggs directly in soil DNA extracts of Manitoba and Ontario samples. This developed method eliminates the need for labour-intensive microscopic identification and egg counting of *H. glycines* in soils. Soil sampling in three Prairie Preserves of Manitoba revealed that, out of the prairies sampled, only one had nematode cysts. The cysts were extracted from the rhizosphere soil of Prairie Cordgrass located in the Manitoba Tall Grass Prairie Preserve. Using morphological and molecular analysis, which involved PCR amplification and sequencing of four regions of the genome (ITS rDNA, D2-D3 expansion segments of 28S rDNA; 18S rDNA, and a fragment of Mt-DNA (COI)), the recovered cyst

nematodes were confirmed to be *Punctodera stonei*. This finding marks the first report of *P. stonei* in Manitoba and only the second in Canada. The findings of this study provide valuable insights into SCN presence in Manitoba, present a reliable molecular method for *H. glycines* quantification in soils, and unveil the presence of a cyst nematode species in a native prairie of Manitoba.

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	Base Pair(s)
°C	Degrees Celsius
CCN	Cereal Cyst Nematodes
cm	Centimetre
cm ³	Cubic Centimetre
COXIII	Cytochrome Oxidase Subunit III
COI	Cytochrome c Oxidase Subunit I
Cq	Quantification Cycle
sd	Standard Deviation
SEM	Standard Error of Mean
dH ₂ O	distilled Water
diam	Diameter
DNA	Deoxyribonucleic Acid
E	Efficiency
ed.	Editors
e.g.	Exempli Gratia
Fig.	Figure
FI	Female Index
g	Gram
>	Greater than
HG-Type	<i>Heterodera glycines</i> Type Test
hr	Hour(s)
hsp90	Heat shock protein
ITS	Internal Transcribed Spacer
J1	First-stage Nematode (Juvenile)
J2	Second-stage Nematode (Juvenile)
J3	Third-stage Nematode (Juvenile)
J4	Fourth-stage nematode
kg	Kilogram
L	Length
<	Less than
MB	Manitoba
min	Minute(s)
mL	Millilitre
mm	Millimeter
μM	Micromolar

μm	Micrometer
μL	Microliter
Mt-DNA	Mitochondrial DNA
MTGPP	Manitoba Tall Grass Prairie Preserve
n	Number
NCBI	The National Centre for Biotechnology Information
NCCC	Nature Conservancy of Canada
ng	Nanogram
NTC	No Template Control
OMAFRA	Ontario Ministry of Agriculture, Food and Rural Affairs
ON	Ontario
PCN	Potato Cyst Nematodes
PCR	Polymerase Chain Reaction
%	Percentage
PI	Plant Introduction
PPN	Plant-Parasitic Nematodes
qPCR	Quantitative Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RM	Rural Municipality
RMNP	Riding Mountain National Park
RNA	Ribonucleic Acid
rpm	Resolutions per minute
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
R ²	Coefficient of Determination
SCAR	Sequence Characterized Amplified Region
SCN	Soybean Cyst Nematode
sec	Second(s)
spp.	Species
U.S.	United States
USA	United States of America
USDA	United States Department of Agriculture
V	Volts
var.	Cultivar
W	Width
x g	Gravity
18S	Small nuclear ribosomal subunit
28S	Large nuclear ribosomal subunit

Contributors to the Thesis

The data chapters 2, 3, and 4 were co-authored with Dr. Mario Tenuta, the candidate's supervisor from the University of Manitoba. The experimental design, sampling strategy and statistical analyses were developed collaboratively by Dr. Mario Tenuta, the candidate, the guidance of the internal committee members (Dr. Francis Zvomuya and Dr. Robert Gulden), and Albert Tenuta (Ontario Ministry of Agriculture, Food and Rural Affairs, Ridgeway, ON). The selection of study fields in Manitoba and Ontario (Chapters 2 and 3) was accomplished with the assistance of Dennis Lange (Manitoba Agriculture, Food and Rural Development, Altona, MB) and Albert Tenuta, respectively.

Field samplings for all studies were conducted by the candidate, with specific assistance as follows: Lanny Gardiner, a field technician at the Soil Ecology Laboratory, in Chapter 2; Katie Webb (technician at the Soil Ecology Laboratory) and Albert Tenuta in Chapter 3; and additional assistance from summer students at the Soil Ecology Laboratory. Laboratory analyses for all chapters were performed by the candidate, with training and technical guidance provided by Terri Fairman, a molecular lab technician at the Soil Ecology Laboratory.

1.0 General Introduction

1.1. The Origin and History of Soybean (*Glycine max* L. Merr.)

Soybean [*Glycine max* (L.) Merr.] originated in Eastern Asia, where it is believed to have been domesticated around 1100 BC by farmers in Northeast China from the annual wild soybean (*G. soja*) (Piper and Morse 1923; Hymowitz 1970, 1990). The word *soy* first appeared in a Japanese dictionary in 1597, inspired by the Japanese word *sboyu* (Shurtleff and Aoyagi 1983).

Soybean is an annual herbaceous legume that has become reputed as a source of oil and protein, used as food for human consumption (Zarkadas et al. 1993), animal fodder (Manyong et al. 1996), and many industrial products. Moreover, soybean exhibits the ability to fix nitrogen as a consequence of symbiotic interaction between the roots and bacteria (Dorff 2007).

Initially, the distribution of soybean developed among neighbouring countries of China, including Japan, Indonesia, Philippines, Vietnam, Thailand, Malaysia, Burma, Nepal, and India (Hymowitz 1990). Subsequently, soybean and soy products rapidly made their way into Europe (Hymowitz 2008).

It is believed the first introduction of soybean to North America was in 1765 when Samuel Bowen, a sailor from the East India Company, brought seeds from China to Savannah (Georgia) (Hymowitz and Harlan 1983). Benjamin Franklin played a significant role in introducing soybean to the USA, sending seeds from London, England, to Philadelphia in 1770 (Hymowitz and Harlan 1983). Although soybean reached Canada in the mid-1800s and was planted at the Ontario Agricultural College, it did not become a commercial crop until the 1920s when a crushing plant was established in southern Ontario, which has growing conditions suitable for soybean production (Dorff 2007).

1.2. Soybean Production and Economy in North America and the World

According to the United States Department of Agriculture (USDA), Soybean is the second most important legume grown in the United States regarding acreage and production. The United States annually produces 34% of the world's total soybean production, with a total value of about \$45.7 billion in 2022, which makes the USA second in soybean production in the world after Brazil with 36%, followed by Argentina (12%), and China (5%) (Soystats 2022). With this, North and South America accounted for around 80% of the total soybean produced in the world.

Canadian farmers have grown soybeans for more than 70 years, but until the mid-1970s, soybeans were restricted by climate, primarily to southern Ontario. In recent years, advancements in innovation, such as the development of new early maturing soybean varieties, opened up more widespread growing possibilities across Canada for this crop (Dorff 2007).

Over the past decade, there has been a significant increase in the seeded area for soybean in Canada, growing from 1.68 million hectares in 2012 to 2.13 million hectares in 2022 (Soy Canada 2022). This upward trend is expected to continue, with estimations suggesting that by 2027, the suitable land for soybean production in Western Canada will surpass 14 million hectares. This expansion is driven by the introduction of new short-term, high-yielding soybean varieties into the market (Soy Canada 2017).

Today soybean has become a common part of the rotation on many farms in Manitoba and Quebec, as well as parts of the Maritimes, southeast Saskatchewan and southern Alberta. This makes soybean rank third among Canada's principal crops in terms of acreage and farm cash receipts (Soy Canada 2022).

In the early 1900s, the United States introduced soybean cultivation to Manitoba, and in the past decade, its growth has expanded significantly. Among Canadian provinces, Manitoba holds

the second position in terms of total seeded area, experiencing a remarkable 98% increase from 0.23 million hectares in 2011 to 0.46 million hectares in 2022 (Soy Canada 2022). This growth has been driven by the development of new early maturing and glyphosate-resistant varieties, which have lower production costs due to reduced N-fertilizer requirements. Additionally, double inoculation technologies, along with the ease of cultivation and harvesting, have facilitated the extension of soybean cultivation to northern and western areas of the province beyond the traditional Red River Valley region.

Soybean production faces various abiotic (such as low temperature, salt toxicity, water stress, and nutritional deficiency) and biotic (including weeds, insect pests, plant-parasitic nematodes, and diseases) stresses worldwide, which have a significant impact on its yield (Hartman et al. 2011). Notably, Soybean Cyst Nematode (SCN), seedling diseases, Sudden Death Syndrome (SDS) (caused by *Fusarium virguliforme* Donnell and Aoki), Phytophthora root and stem rot (caused by *Phytophthora sojae* Kaufm. and Gerd.), and charcoal rot (caused by *Macrophomina phaseolina* Tassi Goid) are the main contributors to yield losses in soybean crops. Between 2010 and 2014, the average economic loss attributed to all soybean diseases across U.S. states and Ontario was estimated at \$60.66 USD per acre. Among these yield-reducing factors, plant-parasitic nematodes, specifically SCN, plays a leading role (Allen et al. 2017).

1.3. Plant-Parasitic Nematodes (PPNs)

Nematodes are microscopic worms and represent one of the most numerous multi-cellular organisms within the animal kingdom. Within terrestrial nematode communities, there are eight identified feeding groups, including predators, animal parasites, omnivores, plant, fungal, bacterial, eukaryote, and substrate feeders. Among these, plant-parasitic nematodes (PPNs) pose a significant threat to global crop production (Yeates et al. 1993; Handoo 1998). Most PPNs are

small, ranging from 0.3-1.0 mm in size, although some can grow up to 4.0 mm long. In some species, female nematodes become swollen and take on various shapes like pear, lemon, or kidney shapes at maturity (Agrios 2005). The well-known plant-parasitic nematodes include root-knot nematodes (*Meloidogyne* spp.), cyst nematodes (*Globodera* spp. and *Heterodera* spp.), root-lesion nematodes (*Pratylenchus* spp.), stem nematodes (*Ditylenchus* spp.), and root-rot nematodes (*Hirschmanniella* spp.). Among these, the world's most destructive agricultural pests are root-knot nematodes and cyst nematodes (Curtis 2007).

The PPNs utilize a needle-like structure called a stylet to puncture the cell walls of host plants and extract their contents, leading to crop damage (Agrios 2005). Damage initiated by distinctive plant-parasitic nematodes can be a threat to crop production, resulting in a reduction in both the quality and quantity of yield (Handoo 1998).

The extent of damage caused by plant-parasitic nematodes can vary based on several factors, including nematode species, population size, host plant susceptibility, cropping systems, temperatures, time of the growing season, geographical region, and soil characteristics such as texture, pH, and fertility. Additionally, the presence of other organisms contributing to the damage can also influence the impact of PPNs on yield losses (Zirakparvar 1985; Back et al. 2002). In some cases, yield losses can reach as high as 30-100%. These nematodes collectively account for an annual global yield loss of over \$80 billion (Nicol et al. 2011).

Canada's agroecosystems have historically experienced a lower impact from PPNs compared to other nations due to their cooler climate and harsh winters, which directly and indirectly hinder the survival of most pathogenic nematode species. The climate, particularly in eastern Canada, also encourages diverse crop rotations and discourages monoculturing, limiting the population buildup of PPNs (Subbotin and Chitambar 2018).

1.4. The Cyst Nematodes

Cyst-forming nematodes are obligatory endoparasites that require the establishment of a permanent feeding site, known as a syncytium, within the roots of their host plants for their development. The females of these nematodes share a special capability of forming a protective cyst, transforming their cuticle into a firm shell to protect their eggs and second-stage juveniles (Perry et al. 2018).

This group of nematodes belongs to the subfamily *Heteroderinae* and is divided into eight genera: *Heterodera*, *Globodera*, *Punctodera*, *Cactodera*, *Dolichodera*, *Betulodera*, *Paradolichodera*, and *Vittatidera* (Wouts and Baldwin 1998; Subbotin et al. 2018). To date, there are a total of 121 recognized species within these genera (Perry et al. 2018). Among these cyst-forming nematodes, the economically important species occur within the *Heterodera* and *Globodera* genus. Notably, Soybean Cyst Nematodes (SCN) (*Heterodera glycines*), Potato Cyst Nematodes (PCN) (*Globodera pallida* and *G. rostochiensis*), and Cereal Cyst Nematodes (CCN) (*Heterodera avenae* and *H. filipjevi*) are considered the most detrimental species (Jones et al. 2013).

The first cyst nematode was reported by Schacht (1859) in sugar beets, and in 1871, Schmidt classified it as *Heterodera schachtii*. In North America, Thorne described the first species of cyst-forming nematodes, *Heterodera punctata*, in 1928 in Saskatchewan, Canada.

1.5. Major Nematode Species Affecting Soybean

Wide range of nematode species (*Meloidogyne*, *Heterodera*, *Pratylenchus*, *Rotylenchulus*, *Hoplolaimus*, *Belonolaimus*, *Helicotylenchus*, *Tylenchorhynchus*, *Scutellonema*, *Paratrichodorus*, *Criconea* and *Xiphinema*) (Schmitt 1985; Sikora et al. 1990) have been linked with soybean; however, only a few of them affect soybean production significantly around the globe. Of these, *Meloidogyne* species (root-knot nematodes) and *Heterodera glycines* (Ichinohe) (Schmitt 1985; Sikora et al. 1990; Fourie et al. 2001) stand out as causing substantial economic damage to soybean crops. Among all these nematodes, Soybean Cyst Nematode (SCN) has been identified as the most detrimental, leading to the highest estimated yield losses (Niblack 2005; Wrather and Koenning 2006).

1.6. Soybean Cyst Nematode (*Heterodera glycines* Ichinohe; SCN)

Soybean cyst nematode, belonging to the phylum Nematode, order *Tylenchida*, and family *Heteroderidae*, was first identified by Ichinohe in 1952 as *H. glycines*, a distinct species. It is part of the genus *Heterodera*, along with *H. avenae*, *H. schachtii*, *H. trifolii*, *H. gottingiana*, *H. cajani*, and *H. zae*. These species form cysts, which are thick-walled dead females protecting eggs. *H. glycines* is a sedentary root endoparasite that parasitizes soybean roots (Niblack et al. 2006), impeding the movement of water and nutrients from the roots to the shoots and carbohydrates from the shoots to the roots. Adult females undergo changes in appearance, starting as white and swollen when young and turning yellow with age. Upon death, their body wall hardens, forming a dark brown cyst that contains 50-200 eggs. These cysts can persist in the soil for several years and are spread by water, wind, soil peds, and machinery. The adult male nematodes are vermiform and can be found in the soil, measuring approximately 480 µm in length (Ichinohe 1952). They play a crucial role in the reproduction process. The development of soybean cyst nematode occurs within

a temperature range of 18-32°C, with the optimal development between 24-28°C. At temperatures exceeding 33°C, no development takes place, and below 16°C, progress is significantly slowed (Singh 2010).

The most easily diagnosed stage of SCN is the adult female stage. Cysts of *Heterodera* exhibit a lemon-shaped appearance with a protuberant neck and cone structure. These cysts range in length from 340 to 920 µm and in width from 320 to 610 µm, featuring a distinctive zig-zag pattern of ridges on their cuticle surface. Initially, young cysts have an intact vulval area, while mature specimens show a missing thin-walled cuticle in the cone, resulting in an open fenestra. This fenestra is crossed by the vulval bridge, separating it into two semifenestrae, commonly known as ambifenestrate (Wouts and Baldwin 1998). As the females develop, they swell and undergo molting throughout their juvenile life stages until they protrude from the plant roots. In warmer climates, they can produce up to four generations per season, while cooler climates allow for as few as two generations (Gao et al. 2006).

Soybean cyst nematode has been identified as the main pathogen responsible for reducing soybean yields in North America (Winstead et al. 1955). The nematode has recently been ranked as the most detrimental soybean pathogen in both the United States and Canada (Allen et al. 2017).

1.6.1. Origin and Distribution of SCN

It is believed that SCN originated in Asia, specifically in China and Japan, where it was associated with soybeans back in the 1880s (Hymowitz 1970). Subsequently, SCN was reported in other regions, including Africa, South America, Europe, and the United States of America (Winstead et al. 1955). Reports of SCN presence also emerged in Canada, Italy, and Iran more recently (Yu 2011).

In the United States, SCN was first detected in 1954, specifically in North Carolina (Winstead et al. 1955). It is believed that the nematode was introduced via soybean seeds and flower bulbs of *narcissus* and *gladiolus* from Japan (Spears 1955). Since then, SCN has been detected in almost every soybean-producing state in the United States (Tylka and Marett 2014), with its distribution extending into Canada due to the expansion and intensification of soybean cultivation. The nematode's presence was reported in Minnesota and North Dakota (along the Manitoba border) in 1978 (MacDonald et al. 1980) and 2003, respectively (Bradley et al. 2004).

In southwest Ontario, Canada, SCN was first detected in 1987 (Anderson et al. 1988) and has since spread northwards and northeastwards, reaching Quebec in 2013 (Mimee et al. 2014). However, in all soybean-producing regions of Quebec, the pest population densities were reported to be relatively low (Mimee et al. 2016).

The transmission of the SCN can happen by water, wind, farm equipment, and the movement of animals (birds) (Riggs 1977; Niblack and Tylka 2016). Once introduced into a field, it can take between 5-9 years for SCN to become a lasting issue in that field (Davis and Tylka 2021).

1.6.2. Life Cycle and Infection Process of SCN

Under ideal field conditions, the complete life cycle of SCN from one generation to another spans 21 to 28 days. These conditions include having a suitable host plant, adequate water (at approximately field capacity), and a soil temperature ranging from about 24°C to 28°C. Other environmental factors, such as soil texture, pH level, and the presence of soybean root exudates, also play a role in influencing the nematode's life cycle. Depending on the specific combination of these factors, it becomes possible to have multiple generations of SCN within a single growing season (Hill and Schmitt 1989; Heatherly and Young 1991; Yen et al. 1995).

The life cycle of SCN comprises six stages, including four juvenile stages (J1-J4), the egg stage, and the adult stage (Davis and Tylka 2021). Starting with embryogenesis and molting within the egg to form the first-stage juvenile (J1) (Niblack 2005), the nematode then molts to the second-stage juvenile (J2) in the egg. Once the host plant is present, the J2 hatches from the egg in the soil (Sharma and Sharma 1998), migrates toward the roots of soybean plants, and uses a specialized penetrating organ called a stylet to enter the fine epidermal root tissue (Lauritis et al. 1983). It is believed that exudates from soybean roots secrete the hatching signals for SCN. Upon reaching the root vascular tissue, the nematode establishes a feeding site known as a syncytium. A syncytium is a permanent, active feeding site formed by the nematode's chemical excretions, involving several enlarged plant cells that become one (Noel 2004). At this stage, the juveniles become sedentary and continue feeding on the syncytium. They undergo further molts to progress to the next stages and ultimately reach reproductive maturity by differentiating sexually into female or male adults. The male nematodes exit the root and become free-living, vermiform nematodes in the soil, actively searching for females present on the root surface for sexual reproduction. On the other hand, the females continue to feed and take on a lemon-shaped appearance (cyst). They remain loosely connected to the syncytium, with their visible bodies protruding from the root. Upon the females' death, the cyst turns brown and becomes hardened due to the production of antimicrobial compounds such as chitinase and polyphenol oxidase, which serve to protect the eggs. The cyst remains in the soil for several years, housing viable eggs ready to hatch during the next growing season when soybeans are planted (Niblack et al. 2006; Melito et al. 2010).

1.6.3. Symptoms and Detection of SCN

Symptoms of SCN damage are often confused with other pest and disease indicators, environmental factors as well as problems related to management, such as drought and water

stresses, nutrient deficiencies, herbicide injury, etc. This may lead to late detection of the nematode infestation until its population builds up. The soybean plant infected with SCN shows symptoms in the shoot and root systems. Depending on the level of infestation (high population densities), the symptoms vary from light to intense and impact the growth and yield of soybean. Typical above-ground symptoms of SCN include stunting, yellow-green leaf colour, midday wilt, and premature defoliation compared to healthy plants (Niblack 1999); however, sometimes the infestation may not be detected in the early stages due to a lack of above-ground symptoms (Wang et al. 2003), although yield losses up to 30% may occur in asymptomatic plants (Noel 1992; Noel and Edwards 1996). The roots that are infected with a high number of SCN females are poorly developed and stunted. In addition, the infection inhibits nodulation and reduces total nitrogen fixation (Noel 2004).

The intensity of yield losses is influenced by the number of nematodes, the cultivar is grown, environmental conditions, cultural practices, past disease history, the virulence of the nematode population, and the presence of interacting organisms (e.g., *Verticillium* species) (Whitehead 1998; Mueller et al. 2016). Close to the field entrance where machinery moves into the field and areas where other fields' soil is deposited by wind or water, tend to be the first locations where SCN establishes.

Due to this invisible cause of yield loss and misleading symptoms, a close examination of the roots (6 weeks after planting) is crucial to detect small, white to yellow females attached to the root surface. Additionally, soil sampling (preferably at post-harvest) for the presence of nematode cysts/juveniles in soil extracts provides the most reliable confirming and monitoring methods (Niblack 2005; Faghihi and Ferris 2006).

The preferred life stage for SCN evaluation by laboratories is the cyst stage. The risk for SCN in a soil sample is assessed based on SCN egg numbers/100 cm³ soil, which is a rational predictor of yield loss (Koenning et al. 1995; Niblack 2005). There are several molecular techniques as well which can detect SCN effectively, including Polymerase Chain Reaction (PCR) and Real-time quantitative PCR (Hooper et al. 2005; Sankaran et al. 2010).

1.6.4. Hosts of SCN

SCN is capable of reproducing on a wide host range, particularly within the family *Leguminosae*. While soybean serves as its main host, SCN can also infect other crops such as turnip (*Brassica rapa* L.), alfalfa (*Medicago sativa* L.), common bean (*P. vulgaris* L.), hairy vetch (*Vicia villosa* Roth.), cowpea (*Vigna unguiculata*), tomato (*Lycopersicon esculentum* Mill.), white lupine (*Lupinus albus* L.), purple bush bean (*Macroptilium atropurpurea*), wild bush bean (*Macroptilium lathyroides*), tepary bean (*P. acutifolius* A.), and adzuki bean (*Vigna angularis* Willd.) (Riggs 1987). Greenhouse studies have revealed that legumes like Canada tick clover (*Desmodium canadense* L.), hairy vetch (*Vicia villosa* Roth.), lima bean (*Phaseolus lunatus* L.), little marvel pea (*Pisum sativum* L.), strawberry clover (*Trifolium fragiferum* L.), string bean (*Phaseolus vulgaris* L.) and tender bean (*Phaseolus vulgaris* L.) are also considered favourable hosts for SCN. Several field bean (*Phaseolus vulgaris* L.) market classes including Black bean (var. Condor, Eclipse, Jaguar, and T-39), Kidney bean (var. Cal Early, Chinook 2000, Montcalm, Pink Panther, and Red Hawk), Navy bean (var. Cirrus, Ensign, Mayflower, Navigator, Norstar, Premiere, Seahawk, and Vista), and Pinto bean (var. Buster, Maverick, Rally, Remington, Othello, GTS-900, Topaz, and Winchester) have been reported as good hosts for SCN as well (Poromarto and Nelson 2009; Poromarto et al. 2011; Yan et al. 2017).

Weeds can also function as alternative hosts for SCN, influencing strategies for managing this pest. Weed hosts that facilitate high reproduction of SCN could reduce the efficacy of crop rotation. SCN can attack numerous weed species belonging to at least 23 plant families including *Asteraceae*, *Brassicaceae*, and *Fabaceae*. In recent studies, Poromarto et al. (2015) reported 26 of the weed species in 11 plant families as newly identified SCN hosts, with henbit (*Lamium amplexicaule* L.) (Epps and Chambers 1958, Riggs and Hamblen 1962; Creech et al. 2007; Poromarto et al. 2015; Werle et al. 2015), common purslane (*Portulaca oleracea* L.) (Riggs and Hamblen 1966; Poromarto et al. 2015), purple deadnettle (*Lamium purpureum* L.) (Riggs and Hamblen 1962; Creech et al. 2007; Poromarto et al. 2015; Werle et al. 2015), and field pennycress (*Thalpsi arvense* L.) (Venkatesh et al. 2000; Poromarto et al. 2015) being the major alternative weed hosts commonly found in different states across the United States.

1.6.5. Virulence of SCN

As scientists began to recognize the genetic variability among populations of *H. glycines*, several studies (Smart 1964; Golden and Epps 1965; Riggs et al. 1968; Sugiyama et al. 1968; Miller 1967, 1969, 1970) highlighted the need to provide a means of evaluating this distinctive type of variability. Nematologists and soybean breeders have been using a bioassay since 1969 to characterize this diversity. The term race has been adopted to describe *H. glycines* populations that differ in their ability to develop on a specific differential set of soybean cultivars, and this race classification was developed in 1970 (Golden et al. 1970). Later Riggs and Schmidt (1988) enhanced the race test using four indicator soybean genotypes (Pickett, Peking, PI 88788, and PI 90763). In 2002 (Niblack et al.), a modified classification system called the “HG-Type” test (HG = *H. glycines*) replaced the race classification, aiming to define better the diversity in virulence phenotypes of SCN populations (Table 1.1.). The “HG-Type” system represents which population

of SCN would be able to infect any genetic sources of soybean resistance (Davis and Tylka 2021). The revised classification retains a similar methodology to the race test but expands to include seven Plant Introductions (PIs) as sources of the SCN resistance line. PI is a universal method for classifying plant genotypes.

In the "HG-Type" system, the virulence of an SCN population on an indicator line or source of resistance is evaluated based on the Female Index (FI). The calculation of FI involves taking the average number of SCN females produced on an indicator line, dividing it by the average number of females on a standard susceptible line, and then multiplying the result by 100. A higher FI indicates better reproductive ability of the SCN population on the source of resistance, while a low FI indicates poor reproduction on that line. If the FI is equal to or higher than 10% on any indicator line, the SCN population is considered virulent on that source of resistance (Niblack et al. 2002).

The Schmitt and Shannon scale (Schmitt and Shannon 1992) classifies SCN resistance in soybean plants as follows: a SCN population with an FI greater than 30 on any line indicates moderate susceptibility of the indicator line to the SCN population, while an FI between 10 and 30 classifies a line as moderately resistant to SCN population tested. Lines with an FI of 60 or higher are considered susceptible to SCN.

Table1. 1. HG-Type classification system (Niblack et al. 2002).

HG-Type Classification								
Indicator Lines	0	1	2	3	4	5	6	7
1. PI 548402		+						
2. PI 88788			+					
3. PI 90763				+				
4. PI 437654					+			
5. PI 209332						+		
6. PI 89772							+	
7. PI 548316								+

The SCN population is considered virulent on an indicator line (an HG-Type is classified) if the FI is equal to or higher than 10% on any indicator line.

1.6.6. Identification of SCN

For effective nematode management in sustainable agriculture, precise and trustworthy identification and quantification of plant-parasitic nematodes are crucial (Clapp et al. 2000). Traditionally, the identification of *Heterodera* species has relied on morphological and morphometric characteristics of cysts, juveniles, and occasionally males (Rivoal et al. 2003). These procedures are laborious, time-consuming, subject to variability among analysts, and could result in the misidentification of other *Heterodera* species present in soils (e.g., *H. avenae*, *H. schachtii*, and *H. trifoli*). To overcome the limitations of conventional methods, modern DNA-based detection methods have been increasingly replacing microscopic techniques, supplementing classical taxonomy to ensure accurate results (Yu 2008).

1.6.6.1. Morphological Identification

The common techniques for identifying nematodes involve soil extraction followed by microscopic examination, focusing on their morphological and morphometric characteristics (Bongers and Bongers 1998; Clapp et al. 2000; Madani et al. 2005; Berry et al. 2008). This process includes recovering cysts from the soil, counting them under a microscope and determining whether they belong to the genus *Heterodera*. A useful key exists for identifying both cyst and non-cyst forming genera within the *Heteroderinae* subfamily (Baldwin and Mundo-Ocampo 1991). Additionally, this key is beneficial in identifying species within the *Heterodera* genus (Wouts and Baldwin 1998). Various standard characteristics are used to classify these species, such as the size and lemon-shaped structure of the cyst, the vulval cone structure, vulval slit length, fenestral shape, the presence or absence of bullae under a bridge in the vulval cone, the length of second-stage juveniles, and the shape of their stylet knobs (Mulvey 1972; Mulvey and Golden 1983; Wouts and Weischer 1977; Cook 1982).

However, the identification of cyst nematodes to the species level based on morphology can be challenging due to the vast number of species, their highly variable morphology, and the requirement for specialized expertise. Particularly within the *schachtii* group of cyst nematodes, to which *H. glycines* belongs, only slight differences in morphological and morphometrical features distinguish the species (Sikora and Paul 1986). To achieve accurate nematode diagnostics, it is crucial to combine morphological and molecular identification methods.

1.6.6.2. Molecular Identification

Unlike morphological identification methods, molecular tools offer the advantage of being applicable without expertise in nematode morphology. Molecular approaches are known for their cost-effectiveness, speed, accuracy, reliability, and ease of implementation (Yan et al. 2008; Abebe et al. 2011; Castagnone-Sereno et al. 2011). Various molecular techniques have been used for identifying cyst nematodes. These methods include Restriction Fragment Length Polymorphism (RFLP) (Bekal et al. 1997; Szalanski et al. 1997; Subbotin et al. 2000; Zheng et al. 2000; Amiri et al. 2002; Maafi et al. 2003; Ou et al. 2008b; Ko et al. 2019), Polymerase Chain Reaction (PCR) with species-specific primers (Subbotin et al. 2001; Ou et al. 2008a; Toumi et al. 2013; Baidoo 2017), and DNA sequencing (Szalanski et al. 1997; Zheng et al. 2000; Madani et al. 2007).

Moreover, real-time quantitative Polymerase Chain Reaction (qPCR) has gained popularity for its ability to rapidly and accurately quantify various organisms, including cyst nematodes (Fusco et al. 2011; Min et al. 2012; Mimee et al. 2015; Braun-Kiewnick and Kiewnick 2018). Quantitative PCR enables real-time observation of the sample during the PCR process by using highly-specific hybridization probes (TaqMan) or less-specific double-stranded dyes such as SYBR green I. In this technique, the fluorescence level generated by the dye or probe is directly proportional to the amount of amplified DNA. The fluorescence is measured after each cycle, and the cycle threshold

(Ct) value, indicating the cycle at which the fluorescence signal surpasses a set threshold, is determined. Lower Ct values correspond to higher initial concentrations of the target DNA in the sample. Recently, the term Ct has been replaced with quantification cycle (Cq). Standard curves or DNA standards of known quantities are often used to relate Ct values to the quantity of the target DNA in the original sample (Kingsnorth et al. 2003).

Recently, the combination of species-specific primers and qPCR techniques has enabled the simultaneous identification and quantification of *H. glycines* (Goto et al. 2009; Min et al. 2012; Ye et al. 2012; Li et al. 2014; Ko et al. 2019; Shirai and Toyota 2019). Additionally, these methods have proven effective in detecting other cyst-forming nematodes (Madani et al. 2005; Toyota et al. 2008; Quader et al. 2008; Goto et al. 2010; Toumi et al. 2015).

Several qPCR assays have been developed for the quantitative detection of *Heterodera* species, using primers targeting the ITS region of rDNA (Madani et al. 2004; Goto et al. 2009; Shirai and Toyota 2019). However, the ITS-rDNA region may not always be ideal for species-specific primer design. Ferris et al. (1999) noticed the presence of closely related species when comparing ITS-rDNA sequences of two morphologically similar yet geographically distinct *H. latipons* isolates from Israel and Russia. As a result, the COI gene has been employed in several studies for DNA barcoding within the *Heteroderidae* family (Vovlas et al. 2015; Powers et al. 2018; Subbotin et al. 2018; Ko et al. 2019). DNA barcoding involves the identification of species based on their unique DNA sequence fragment from a specific gene (barcodes). According to Subbotin et al. (2018), the COI gene serves as a reliable barcoding marker due to its distinct sequence for each *Heterodera* species, facilitating accurate identification.

Recent studies have been centred around the direct extraction of DNA from soil to identify and quantify plant-parasitic nematodes using qPCR assays (Briely et al. 2009; Goto et al. 2009; Min

et al. 2011; Li et al. 2014; Baidoo et al. 2017; Arora et al. 2020; Baidoo and Yan 2021). A number of these studies have outlined the use of qPCR in conjunction with species-specific primers, offering a reliable and valuable approach for the specific detection of *H. glycines* population and rapidly quantifying its abundance in soil (Goto et al. 2009; Li et al. 2014; Shirai and Toyota 2019; Baidoo and Yan 2021). These advanced molecular methodologies offer effective alternatives to the conventional methods of microscopic identification and quantification of SCN within soil samples.

1.6.7. Management of SCN

There are various practices beneficial in SCN management. These include genetic resistance, cultural methods, chemical control, and integrated management approaches (Concibido et al. 2004; Conley et al. 2011; Grabau 2013; Li et al. 2011).

1.6.7.1. Genetic Resistance

Employing soybean varieties that are resistant to SCN is the most effective and economically viable strategy for managing the nematode. However, the success of these resistant varieties depends on the genetic variability of the SCN population (Niblack et al. 2002). Many SCN-resistant soybean cultivars have been released, but over 90% of them have derived their resistance from PI 88788, Peking, PI 437654, or combinations of these sources (Tylka 2015; Concibido et al. 2004; Schmitt et al. 2004; Shannon et al. 2004; Faghihi and Ferris 2006; Tylka and Mullaney 2015). Consequently, continuous use of SCN-resistant soybean varieties with a single source of resistance has led to significant shifts in nematode populations, resulting in SCN populations adapted to reproduce on these specific sources of resistance (Niblack 2005; Tylka and Mullaney 2015). To overcome this resistance-breaking, alternately use of cultivars with different sources of SCN resistance is recommended (Tylka 1994).

1.6.7.2. Cultural Management

Crop rotation is another possible tool to manage SCN population density. Rotating soybeans with a non-host crop has proven beneficial for many growers. Once there are no suitable host roots, the nematode cannot mature and reproduce, leading to a notable reduction in the SCN population in the soil. It is recommended to rotate with non-host crops like corn (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), oat (*Avena sativa* L.), rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and sunflower (*Helianthus annuus* L.), as this serves as a valuable cultural practice (Lima et al. 2017). In some cases, using a resistant soybean cultivar instead of a non-host crop can be an option. The host status of crop species, the number of years of rotation, and geographical location are all factors that influence the effectiveness of the rotation system (Schmitt 1991; Koenning et al. 1993). Another helpful cultural method for controlling soybean cyst nematode is incorporating cover crops into the rotation, such as annual ryegrass (*Lolium multiflorum* L.) and winter annual weeds, like purple deadnettle and henbit (*Lamium amplexicaule* L.) (Riggs 1992; Riggs and Wrather 1992; Venkatesh 2000; Riga et al. 2001; Gibson et al. 2005; Creech et al. 2008).

1.6.7.3. Chemical Control

The application of nematicides and pesticides can offer some level of control against Soybean Cyst Nematode (SCN) by reducing the infestation of soybean roots by juveniles and preventing their early season feeding and growth. While they might offer short-term control of the pest, they generally do not ensure season-long control (Wrather et al. 1984; Chen et al. 2001; Tylka 2012). In addition, this approach can be expensive, and its effectiveness may vary depending on soil conditions, temperatures, and rainfall (Schmitt et al. 1991). Nematicides can be classified according to their method of application, mode of action, and chemical composition (Haydock et al. 2006). Almost all nematicides are highly toxic, especially non-fumigants like aldicarb (Temik or Bolster) and oxamyl (Vydate), which are nerve poisons (Davis and Tylka 2021). Consequently,

the widespread use of these chemicals for SCN control is no longer widely recommended (Riggs and Schuster 1998). Using seed treatment presents a more economically viable option for managing SCN, as it has limited mobility, particularly in crops like soybeans (Haydock et al. 2006; Grabau 2017). Nonetheless, the effectiveness of seed treatments in field studies has shown variation, potentially due to environmental conditions or initial nematode densities (Wheeler et al. 2013; Tylka et al. 2015).

1.6.7.4. Integration of Management Strategies

Integration of control strategies for SCN is the best choice for efficient long-term management of the SCN. Among the above-mentioned management options, the combination of soybean-resistant cultivars and non-host crops in the crop rotation has been identified as the most effective, and affordable means to lower the density of *H. glycines* in the soil (Niblack et al. 2003; Niblack and Chen 2004; Schmitt et al. 2004; Hershman 2010; Oyekanmi and Fawole 2010; Conley et al. 2011). The successful implementation of these integrated pest management methods relies on precise data regarding the level of SCN infestation.

1.7. Thesis Justifications and Objectives

Soybean Cyst Nematode (SCN), *Heterodera glycines* (Ichinohe 1952), holds significant global importance as a primary threat to soybean crops. In the years spanning from 2017 to 2020, the impact of SCN infestations on soybean expanded, affecting a growing number of counties in both the United States and Canada. This observation strongly suggests an ongoing and consistent expansion of SCN distribution within these two countries (Tylka and Marett 2021).

In Canada, SCN has currently been identified in the provinces of Ontario and Quebec. Its presence in Manitoba is anticipated due to its rapid spread from bordering U.S. counties, namely North Dakota and Minnesota. As previously mentioned, early-stage SCN infestation may not be

detected due to a lack of visible symptoms (Wang et al. 2003). To effectively monitor SCN and to prevent yield losses, it becomes necessary to conduct soil sampling, preferably post-harvest, to confirm the nematode's presence (Niblack 2005; Faghihi and Ferris 2006).

Consequently, it is important to continue surveys to detect new SCN infestations. Monitoring the spread and abundance of SCN stands as the primary measure in effectively managing this pathogen (Castillo and Vovlas 2007; Sato et al. 2009). The most valuable diagnostic tool for this purpose involves quantifying the density of SCN eggs per unit volume of soil.

Conventionally, the methods of identifying and quantifying *Heterodera* species involve recovering cysts from the soil, extracting eggs from cysts, morphologically identifying the nematodes, and conducting a microscopic count (Noel 1985; Ingham 1994). These protocols are labour-intensive, time-demanding, susceptible to variations among analysts, and come with the risk of misidentification of other *Heterodera* species. This emphasizes the need for reliable and rapid methods for identifying and quantifying plant-parasitic nematodes in soil samples. Such data will provide valuable insights for growers, helping them determine the optimal practices for managing this pest.

Moreover, our understanding of cyst nematode distribution within Canada's native prairies is rather limited. It is possible that nematodes in cultivated fields can trace their origins back to these native prairies. Thus, studying these native prairies can offer insights into the contemporary agricultural and environmental challenges we face.

This thesis addresses three main objectives:

- (i) Surveying soybean fields in Manitoba for the presence of the soybean cyst nematode
- (ii) Developing a molecular method for quantifying *H. glycines* directly in the soil, and
- (iii) Identifying native cyst nematodes in the prairie preserves of Manitoba

1.8. Structure of Thesis

This thesis comprises five chapters each addressing different research objectives. The initial chapter (**Chapter 1**) serves as a general introduction chapter that describes the origin and global production of soybean with particular emphasis on North America. Furthermore, the chapter provides an understanding of plant-parasitic nematodes, with a focus on the various aspects of the soybean cyst nematode, *Heterodera glycines*. It elaborates on its origin and distribution, life cycle, symptoms, host plants, and techniques employed for its identification and management. Chapter One also outlines the research objectives and the overall structure of the thesis. Subsequent to this introduction are three chapters written in the form of scientific journal manuscripts. **Chapter 2** investigates the presence of *H. glycines* in Manitoba soybean fields. The methodologies employed in this chapter include both morphological and molecular methods for the identification of *H. glycines* in soil samples collected from 30 soybean fields, predominantly along the MB/U.S. border. This chapter focuses on the first objective of the thesis, detailing the method of cyst extraction from the soil, morphological analysis of the cysts, PCR using previously proven *H. glycines*-specific primers (CoxIII and SCAR), and DNA sequencing of different regions of the genome. Expanding upon the foundation established in Chapter 2, the subsequent chapter (**Chapter 3**) presents a developed molecular method for quantifying *H. glycines* directly in the soil. This involves optimized DNA extraction from soil debris obtained through the wet-sieving method, and a SYBR Green I-based real-time quantitative polymerase chain reaction (qPCR) assay using species-specific primers for *H. glycines* (COXIII and SCAR). Furthermore, this chapter compares the number of *H. glycines* eggs quantified by the traditional microscopy method with numbers estimated by qPCR in naturally infested field soils. The fourth chapter (**Chapter 4**) employs the cyst and DNA

extraction techniques, along with morphological and molecular identification methods described in Chapter 2. This chapter investigates the presence of cyst nematodes in three native prairies of Manitoba, including Manitoba Tall Grass Prairie Preserve, Yellow Quill Prairie, and Baldy Prairie at Riding Mountain National Park. The identification process involves assessing cyst nematodes based on cyst morphology, PCR using universal primer sets for cyst nematodes, and sequencing the rDNA of different regions of the genome (ITS, D2A-D3B, 18S, and the mitochondrial DNA gene, COI). The structure of this thesis follows the "Sandwich" style outlined by the Faculty of Graduate Studies and the Department of Soil Science at the University of Manitoba. The data chapters follow a manuscript format consistent with the guidelines of the Canadian Journal of Plant Pathology, where they are scheduled for publication. Following these research chapters, **Chapter 5** serves as an overall synthesis. Here, the study's key findings are summarized, challenges and unexpected findings encountered throughout the research are discussed, and recommendations for future studies in this field are provided.

1.9. References

- Abebe E, Makete T, Thomas WK. 2011. A critique of current methods in nematode taxonomy. *Afr J Biotechnol.* 10(3):312-323.
- Agrios GN. 2005. *Plant pathology*. 5th ed. Burlington (MA): Academic Press.
- Allen TW, Bradley CA, Sisson AJ, Byamukama E, Chilvers MI, Coker CM, Collins AA, Damicone JP, Dorrance AE, Dufault NS, Esker PD. 2017. Soybean yield loss estimates due to diseases in the United States and Ontario, Canada, from 2010 to 2014. *Plant Health Prog.* 18(1):19-27.
- Amiri S, Subbotin SA, Moens M. 2002. Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *Eur J Plant Pathol.* 108:497-506.
- Anderson TR, Welacky TW, Olechowski HT, Ablett G, Ebsary BA. 1988. First report of *Heterodera glycines* on soybeans in Ontario, Canada. *Plant Dis.* 72:453.
- Arora D, Yan G, Baidoo R. 2020. Developing a real-time PCR assay for direct detection and quantification of *Pratylenchus scribneri* in field soil. *Nematology.* 22(7):733-744.
- Back MA, Haydock PPJ, Jenkinson P. 2002. Disease complexes involving plant parasitic nematodes and soilborne pathogens. *Plant Pathol.* 51(6):683-697.
- Baidoo R, Yan G. 2021. Developing a real-time PCR assay for direct identification and quantification of soybean cyst nematode, *Heterodera glycines*, in soil and its discrimination from sugar beet cyst nematode, *Heterodera schachtii*. *Plant Dis.* 105(12):3848-3857.
- Baidoo R, Yan G, Nelson B, Skantar AM, Chen S. 2017. Use of chemical flocculation and nested PCR for *Heterodera glycines* detection in DNA extracts from field soils with low population densities. *Plant Dis.* 101(7):1153-1161.

- Baldwin JG, Mundo -Ocampo M. 1991. *Heteroderinae*, cyst- and non-cyst forming nematodes. In: Nickle WR, editor. Manual of Agricultural Nematology. New York (NY): Marcel Dekker; p. 275-362.
- Bekal S, Gauthier JP, Rivoal R. 1997. Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region. *Genome*. 40(4):479-486.
- Berry SD, Fargette M, Spaull VW, Morand S, Cadet P. 2008. Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Mol Cell Probes*. 22(3):168-176.
- Bongers T, Bongers M. 1998. Functional diversity of nematodes. *Appl Soil Ecol*. 10(3):239-251.
- Bradley CA, Biller CR, Nelson BD. 2004. First report of soybean cyst nematode (*Heterodera glycines*) on soybean in North Dakota. *Plant Dis*. 88(11):1287-1287.
- Braun-Kiewnick A, Kiewnick S. 2018. Real-time PCR, a great tool for fast identification, sensitive detection and quantification of important plant-parasitic nematodes. *Eur J Plant Pathol*. 152:271-283.
- Bulman SR, Marshall JW. 1997. Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *N Z J Crop Hortic*. 25(2):123-129.
- Castagnone-Sereno P, Skantar A, Robertson L. 2011. Molecular tools for diagnostics. In: Jones J, Gheysen G, Fenoll C, editors. Genomics and molecular genetics of plant-nematode interactions. The Netherlands: Springer; P. 443-464.
- Castillo P, Vovlas N. 2007. *Pratylenchus* (Nematoda: *Pratylenchidae*): diagnosis, biology, pathogenicity and management. Boston (IL): Brill. 6:305-324.

- Clapp JP, Van Der Stoel CD, Van Der Putten WH. 2000. Rapid identification of cyst (*Heterodera spp.*, *Globodera spp.*) and root-knot (*Meloidogyne spp.*) nematodes on the basis of ITS2 sequence variation detected by PCR-single-strand conformational polymorphism (PCR-SSCP) in cultures and field samples. *Mol Ecol.* 9(9):1223-1232.
- Concibido VC, Diers BW, Arelli PR. 2004. A decade of QTL mapping for cyst nematode resistance in soybean. *Crop Sci.* 44(4):1121-1131.
- Conley SP, Gaska JM, Pedersen P, Esker P. 2011. Soybean yield and *Heterodera glycines* response to rotation, tillage, and genetic resistance. *Agron J.* 103(6):1604-1609.
- Cook R. 1982. Cereal and grass hosts of some gramineous cyst nematodes. *EPPO Bull.* 12(4):399-411.
- Creech JE, Webb JS, Young BG, Bond JP, Harrison SK, Ferris VR, Faghihi J, Westphal A, Johnson WG. 2007. Development of soybean cyst nematode on henbit (*Lamium amplexicaule*) and purple deadnettle (*Lamium purpureum*). *Weed Technol.* 21(4):1064-1070.
- Creech JE, Westphal A, Ferris VR, Faghihi J, Vyn TJ, Santini JB, Johnson WG. 2008. Influence of winter annual weed management and crop rotation on soybean cyst nematode (*Heterodera glycines*) and winter annual weeds. *Weed Sci.* 56(1):103-111.
- Curtis RH. 2007. Plant parasitic nematode proteins and the host-parasite interaction. *Briefings in Functional Genomics and Proteomics.* 6(1):50-58.
- Davis EL, Tylka GL. 2021. Soybean cyst nematode disease. The plant health instructor. [Accessed 2023 Aug 5].
<https://www.apsnet.org/edcenter/disandpath/nematode/pdlessons/Pages/Soybeancystnematode.aspx>

- Dorff E. 2007. The soybean, agriculture's jack-of-all-trades, is gaining ground across Canada. Statistics Canada. [Accessed 2022 Jan 27].
<https://www150.statcan.gc.ca/n1/en/pub/96-325-x/2007000/article/10369-eng.pdf?st=6wd-Sqjq>
- Epps JM, Chambers AY. 1958. New host records for *Heterodera glycines*; including one host in the Labiatae. Plant Dis. Rep.; 42(2):194.
- Faghihi J, Ferris VR. 2006. Soybean cyst nematode. Department of Entomology. Purdue University. [Accessed 2022 May 3].
<https://ag.purdue.edu/departments/entm/extension/index.html>
- Ferris VR, Subbotin SA, Ireholm A, Spiegel Y, Faghihi J, Ferris JM. 1999. Ribosomal DNA sequence analysis of *Heterodera filipjevi* and *H. latipons* isolates from Russia and comparisons with other nematode isolates. Russ J Nematol. 7(2):121-125.
- Fusco V, Quero GM, Morea M, Blaiotta G, Visconti A. 2011. Rapid and reliable identification of *Staphylococcus aureus* harbouring the enterotoxin gene cluster (egc) and quantitative detection in raw milk by real-time PCR. Int J Food Microbiol. 144(3):528-537.
- Gao X, Jackson TA, Hartman GL, Niblack TL. 2006. Interactions between the soybean cyst nematode and *Fusarium solani* f. sp. *glycines* based on greenhouse factorial experiments. Phytopathology. 96(12):1409-1415.
- Gibson KD, Johnson WG, Hillger DE. 2005. Farmer perceptions of problematic corn and soybean weeds in Indiana. Weed Technol. 19(4):1065-1070.
- Golden AM, Epps JM. 1965. Morphological variations in soybean cyst nematodes. Nematologica. 11:38.
- Golden AM. 1970. Terminology and identity of infraspecific forms of the soybean cyst nematode (*Heterodera glycines*). Plant Dis. 54(7):544-546.

- Goto K, Sato E, Toyota K. 2009. A novel detection method for the soybean cyst nematode *Heterodera glycines* Ichinohe using soil compaction and real-time PCR. Jpn J Nematol. 39:1-7.
- Goto K, Sato E, Gang L, Toyota K, Sugito T. 2010. Comparison of calibration curves prepared by soil compaction and ball milling methods for direct quantification of the potato cyst nematode *Globodera rostochiensis* in soil. Jpn J Nematol. 40(2):41-45.
- Grabau Z. 2017. Fumigant and non-fumigant nematicides labeled for agronomic crops in Florida. Department of Entomology and Nematology, University of Florida, IFAS Extension. ENY-065/IN1152.
- Grabau ZJ. 2013. Management strategies for control of soybean cyst nematode and their effect on the nematode community [master's thesis]. Minneapolis (MN): University of Minnesota.
- Handoo ZA. 1998. Plant-parasitic nematodes. USDA, ARS, Nematology Laboratory. [Accessed 2020 Sep 12].
<https://usda.library.cornell.edu/usda/current/CropProdSu>
- Hartman GL, West ED, Herman TK. 2011. Crops that feed the World 2. Soybean-worldwide production, use, and constraints caused by pathogens and pests. Food Secur. 3(1):5-17.
- Haydock PPJ, Woods SR, Grove IG, Hare MC. 2006. Chemical control of nematodes: plant nematology. Wallingford (UK): CABI; p. 392-410.
- Heatherly LG, Young LD. 1991. Soybean and soybean cyst nematode response to soil water content in loam and clay soils. Crop Sci. 31(1):191-196.
- Hershman DE. 2010. 2015 Soybean Cyst Nematode (SCN) management recommendations for Kentucky. Lexington (KY): University of Kentucky, Plant Pathology Extension.
- Hill NS, Schmitt DP. 1989. Influence of temperature and soybean phenology on dormancy induction of *Heterodera glycines*. J Nematol. 21(3):361-369.

- Hooper DJ, Hallmann J, Subbotin SA. 2005. Methods for extraction, processing and detection of plant and soil nematodes: Plant parasitic nematodes in subtropical and tropical agriculture. Wallingford (UK): CABI Publishing; p. 53-86.
- Hymowitz T, Harlan JR. 1983. Introduction of soybean to North America by Samuel Bowen in 1765. *Econ Bot.* 37(4):371-379.
- Hymowitz T. 1970. On the domestication of the soybean. *Econ Bot.* 24(4):408-421.
- Hymowitz T. 1990. Soybeans: The success story. advances in new crops. Proceedings of the first national symposium 'New crops: research, development, economics'; October 23-26; Indianapolis. Indiana: Timber Press; p. 159-163.
- Hymowitz T. 2008. The history of the soybean. In Soybeans (pp. 1-31). AOCS Press.
- Ichinohe M. 1952. On the soybean nematode, *Heterodera glycines* n. sp., from Japan. *Magazine of Appl Zool.* 17:1-4.
- Ingham RE. 1994. Nematodes: methods of soil analysis, part 2. Microbiological and biochemical properties no. 5. Madison (WI): Soil Society of America; p. 459–490.
- Iowa State University. (n.d.). Extraction of SCN eggs and cysts. [Accessed 2019 May 15].
<https://scn.plantpath.iastate.edu/extraction-scn-eggs-cysts>.
- Jones JT, Haegeman A, Danchin EG, Gaur HS, Helder J, Jones MG, Kikuchi T, Manzanilla-López R, Palomares-Rius JE, Wesemael WM, Perry RN. 2013. Top 10 plant-parasitic nematodes in molecular plant pathology. *Mol Plant Pathol.* 14(9):946-961.
- Kingsnorth CS, Kingsnorth AJ, Lyons PA, Chwarszczynska DM, Asher MJ. 2003. Real-time analysis of *Polymyxa betae* GST expression in infected sugar beet. *Mol Plant Pathol.* 4(3):171-176.
- Koenning SR, Schmitt DP, Barker KR. 1993. Effects of cropping systems on population density of *Heterodera glycines* and soybean yield. *St Paul. Plant Dis.* 77(8):780-786.

- Koenning SR, Schmitt DP, Barker KR, Gumpertz ML. 1995. Impact of crop rotation and tillage system on *Heterodera glycines* population density and soybean yield. *Plant Dis.* 79:282–286.
- Ko HR, Kang H, Park EH, Kim EH, Lee JK. 2019. Identification of *Heterodera glycines* (Tylenchida; *Heteroderidae*) Using qPCR. *Plant Pathol J.* 35(6):654-661.
- Lauritis JA, Rebois RV, Graney LS. 1983. Development of *Heterodera glycines* Ichinohe on soybean, *Glycine max* (L.) Merr., under gnotobiotic conditions. *J Nematol.* 15(2):272-281.
- Li J, Todd TC, Lee J, Trick HN. 2011. Biotechnological application of functional genomics towards plant-parasitic nematode control. *Plant Biotechnol J.* 9(9):936-944.
- Li Y, Lawrence GW, Lu S, Balbalian C, Klink VP. 2014. Quantitative field testing *Heterodera glycines* from metagenomic DNA samples isolated directly from soil under agronomic production. *PLoS One.* 9(2): e89887.
- Lima FS, Correa VR, Nogueira SR, Santos PR. 2017. Nematodes affecting soybean and sustainable practices for their management: Soybean- the basis of yield, biomass and productivity. London (UK): InTech Open; p. 95-110.
- Maafi ZT, Subbotin S, Moens M. 2003. Molecular identification of cyst-forming nematodes (*Heteroderidae*) from Iran and a phylogeny based on ITS-rDNA sequences. *Nematology.* 5(1):99-111.
- MacDonald DH, Noel GR, Lueschen WE. 1980. Soybean cyst nematode, *Heterodera glycines*, in Minnesota. *Plant Dis.* 64(3):319-321.
- Madani M, Kyndt T, Colpaert N, Subbotin SA, Gheysen G, Moens M. 2007. Polymorphism among sugar beet cyst nematode *Heterodera schachtii* populations as inferred from AFLP and ITS-rDNA gene analyses. *Russ J Nematol.* 15(2):117-128.

- Madani M, Subbotin SA, Moens M. 2005. Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using real-time PCR with SYBR green I dye. *Mol Cell Probes*. 19(2):81-86.
- Madani M, Vovlas N, Castillo P, Subbotin SA, Moens M. 2004. Molecular characterization of cyst nematode Species (*Heterodera* spp.) from the Mediterranean Basin using RFLPs and sequences of ITS-rDNA. *J Phytopathol*. 152(4):229-234.
- Manyong VM, Otite O, Dashiell K. 1998. Spread of new soybean varieties in a traditional soybean growing area of Nigeria: towards sustainable farming systems in sub-saharan Africa, Ouagadougou: AAFSRET; p. 151-162.
- McDonald A, Fourie H, Loots G. 2001. Plant-parasitic nematodes in field crops in South Africa. 6. Soybean. *Nematology*. 3(5):447-454.
- Melito S, Heuberger AL, Cook D, Diers BW, MacGuidwin AE, Bent AF. 2010. A nematode demographics assay in transgenic roots reveals no significant impacts of the Rhg1 locus LRR-kinase on soybean cyst nematode resistance. *BMC Plant Biol*. 10:1-4.
- Miller LI. 1967. Development of 11 isolates of *Heterodera glycines* on six legumes. *Phytopathol*. 57(7):647.
- Miller LI. 1970. Differentiation of eleven isolates as races of the soybean cyst nematode. *Phytopathol*. 60:6.
- Miller LI. 1969. Physiologic variation of six isolates of *Heterodera glycines*. *Phytopathol*. 59(2):1558
- Mimee B, Duceppe MO, Véronneau PY, Lafond-Lapalme J, Jean M, Belzile F, Bélair G. 2015. A new method for studying population genetics of cyst nematodes based on Pool-Seq and genomewide allele frequency analysis. *Mol Ecol Resour*. 15(6):1356-1365.

- Mimee B, Gagnon AÈ, Colton-Gagnon K, Tremblay É. 2016. Portrait de la situation du nématode à kyste du soja (*Heterodera glycines*) au Québec (2013-2015). *Phytoprotection*. 96(1):33-42.
- Mimee B, Peng H, Popovic V, Yu Q, Duceppe MO, Tétreault MP, Belair G. 2014. First report of soybean cyst nematode (*Heterodera glycines* Ichinohe) on soybean in the province of Quebec, Canada. *Plant Dis*. 98(3):429-429.
- Min YY, Toyota K, Sato E. 2012. A novel nematode diagnostic method using the direct quantification of major plant-parasitic nematodes in soil by real-time PCR. *Nematology*. 14(3):265-276.
- Min YY, Toyota K, Goto K, Sato E, Mizuguchi S, Abe N, Nakano A, Sawada E. 2011. Development of a direct quantitative detection method for *Meloidogyne incognita* in sandy soils and its application to sweet potato cultivated fields in Tokushima prefecture, Japan. *Nematology*. 13(1):95-102.
- Mueller D. 2016. A farmer's guide to soybean diseases. St. Paul (MN): American Phytopathological Society.
- Mulholland V, Carde L, O'Donnell KJ. 1996. Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. *Proceedings of the Monographs-British Crop Protection Council symposium*; Farnham. p. 247-252.
- Mulvey RH. 1972. Identification of *Heterodera* cysts by terminal and cone top structures. *Can J Zool*. 50(10):1277-1292.
- Mulvey RH, Golden AM. 1983. An illustrated key to the cyst-forming genera and species of *Heteroderidae* in the western hemisphere with species morphometrics and distribution. *J Nematol*. 15:1-59.
- Niblack TL, Chen SY. 2004. Cropping systems and crop management practices: biology and management of soybean cyst nematode. 2nd ed. Marceline (MO); P. 181-206

Niblack TL, Tylka GL. 2016. Soybean cyst nematode management guide. 5th ed. Columbia, University of Missouri: North Central Soybean Research Program Publication. [Accessed 2022 May 21].

http://soybeanresearchinfo.com/pdf_docs/SCNGuide_5thEd.pdf

Niblack TL, Arelli PR, Noel GR, Opperman CH, Orf JH, Schmitt DP, Shannon JG, Tylka GL. 2002.

A revised classification scheme for genetically diverse populations of *Heterodera glycines*. J Nematol. 34(4):279-288.

Niblack TL, Lambert KN, Tylka GL. 2006. A model plant pathogen from the kingdom animalia:

Heterodera glycines, the soybean cyst nematode. Annu Rev Phytopathol. 44:283-303.

Niblack TL. 1999. Management of soybean cyst nematode with cultural practices. Proceedings of the

National Soybean Cyst Nematode Conference; Orlando. Florida (FL). p. 2-2.

Niblack TL. 2005. Soybean cyst nematode management reconsidered. Plant Dis. 89(10):1020-1026.

Niblack TL, Wrather JA, Heinz RD, Donald PA. 2003. Distribution and virulence phenotypes of

Heterodera glycines in Missouri. Plant Dis. 87(8):929-932.

Nicol JM, Turner SJ, Coyne DL, Den Nijs L, Hockland S, Maafi ZT. 2011. Current nematode threats

to world agriculture: genomics and molecular genetics of plant-nematode interactions. The Netherlands. Springer; p. 21-43.

Noel GR. 1985. The soybean cyst nematode. In: Lamberti F, Taylor CE, editors. Cyst Nematodes.

New York (NY): Plenum Press. 121:257-268.

Noel GR, Edwards DI. 1996. Population development of *Heterodera glycines* and soybean yield in

soybean-maize rotations following introduction into a noninfested field. J Nematol. 28(3):335-342.

- Noel GR. 1992. History, distribution, and economics: biology and management of the soybean cyst nematode. St. Paul (MN). American Phytopathological Society; p. 1-13.
- Noel GR. 2004. Response to infection: biology and management of soybean cyst nematode, 2nd ed. Marceline: Schmitt & Associates of Marceline; p.131-151.
- Ou S, Peng D, Liu X, Li Y, Moens M. 2008a. Identification of *Heterodera glycines* using PCR with sequence characterised amplified region (SCAR) primers. *Nematology*. 10(3):397-403.
- Ou SQ, Peng DL, Li Y, Wang YJ. 2008b. Restriction fragment length polymorphism and sequences analysis of rDNA-ITS region of cereal cyst nematode (*Heterodera avenae*) on wheat from Zhengzhou. *Acta Phytopathol Sin*. (38):407-413.
- Oyekanmi EO, Fawole B. 2010. Nematodes of soybean and their management: the soybean, botany, production and uses. Wallingford (UK): CABI; p. 325-344.
- Perry RN, Moens M, Jones JT. editors. 2018. Cyst nematodes: life cycle and economic importance. Wallingford (UK): CAB International.
- Petersen DJ, Zijlstra C, Wishart J, Blok V, Vrain TC. 1997. Specific probes efficiently distinguish root-knot nematode species using signature sequences in the ribosomal intergenic spacer. *Fundam Appl Nematol*. 20(6):619-626.
- Piper CV, Morse WJ. 1923. The soybean. McGraw-Hill Book Company, Incorporated. New York (NY).
- Poromarto SH, Nelson BD. 2009. Reproduction of soybean cyst nematode on dry bean cultivars adapted to North Dakota and northern Minnesota. *Plant Dis*. 93(5):507-511.
- Poromarto SH, Gramig GG, Nelson Jr BD, Jain S. 2015. Evaluation of weed species from the Northern Great Plains as hosts of soybean cyst nematode. *Plant Health Prog*. 16(1):23-28.

- Poromarto SH. 2011. Studies on the biology of soybean cyst nematode [dissertation]. Fargo (ND): North Dakota State University.
- Powers T, Harris T, Higgins R, Mullin P, Powers K. 2018. Discovery and identification of *Meloidogyne* Species using COI DNA barcoding. *J. Nematol.* 50(3):399-412.
- Quader M, Nambiar L, Cunnington J. 2008. Conventional and real-time PCR-based species identification and diversity of potato cyst nematodes (*Globodera* spp.) from Victoria, Australia. *Nematology.* 10(4):471-478.
- Riga E, Topp E, Potter J, Welacky T, Anderson T, Tenuta A. 2001. The impact of plant residues on the soybean cyst nematode, *Heterodera glycines*. *Can J Plant Pathol.* 23(2):168-173.
- Riggs RD, Schmitt DP. 1988. Complete characterization of the race scheme for *Heterodera glycines*. *J Nematol.* 20(3):392-395.
- Riggs RD, Slack DA, Hamblen ML. 1968. New biotype of soybean cyst nematode. *Arkansas Farm Research*; 17(5):11.
- Riggs RD, Hamblen ML. 1962. Soybean-cyst nematode host studies in the family *Leguminosae*: Fayetteville (AR): Arkansas Agricultural Experiment Station. Report Series: 110.
- Riggs RD, Schuster RP. 1998. Management. In: Sharma SB, editor. *The cyst nematodes*. Boston, London: Kluwer Academic Publishers; p. 388-416.
- Riggs RD, Wrather JA. 1992. Host range, biology and management of the soybean cyst nematode. St. Paul (MN): American Phytopathological Society; p. 107-114.
- Riggs RD. 1987. Nonhost root penetration by soybean cyst nematode. *J Nematol.* 19(2):251-254.
- Riggs RD. 1977. Worldwide distribution of soybean-cyst nematode and its economic importance. *J Nematol.* 9(1):34-39.

- Sankaran S, Mishra A, Ehsani R, Davis C. 2010. A review of advanced techniques for detecting plant diseases. *Comput Electron Agric.* 72(1):1-13.
- Sato E, Min YY, Toyota K, Takada A. 2009. Relationships between the damage to radish caused by the root-lesion nematode *Pratylenchus penetrans*, its density prior to cultivation and the soil nematode community structure evaluated by polymerase chain reaction-denaturing gradient gel electrophoresis. *Soil Sci Plant Nutr.* 55(4):478-484.
- Schacht H. 1859. Über einige Feinde der Rübenfelder. *Zeitschrift Vereines Rübenzucker-Industrie Zollverein.* 9:175-179.
- Schmidt A. 1871. About the beet nematodes. *Zollverein.* 21:1-19
- Schmitt DP. 1991. Management of *Heterodera glycines* by cropping and cultural practices. *J Nematol.* 23(3):348-352.
- Schmitt DP. 1985. Plant-parasitic nematodes associated with soybeans. *Proceedings of the World Soybean Research Conference III.* Boulder (CO): Westview Press; p. 541-546.
- Schmitt DP, Shannon G. 1992. Differentiating soybean responses to *Heterodera glycines* races. *Crop Sci.* 32(1):275-277.
- Schmitt DP, Riggs RD, Wrather JA, editors. 2004. *Biology and management of soybean cyst nematode.* Marceline: Schmitt and Associates of Marceline; p. 60-66.
- Shannon JG, Arelli PR, Young LD. 2004. Breeding for soybean cyst nematode resistance. In: Riggs RD, Wrather JA, editors. *Biology and management of soybean cyst nematode.* St. Paul (MN): American Phytopathological Society; p.155-180.
- Sharma SB, Sharma R. 1998. *Hatch and emergence: the cyst nematodes.* The Netherlands: Springer; p. 191-216.

- Shirai S, Toyota K. 2019. Optimisation of a species-specific primer set to quantify the soybean cyst nematode, *Heterodera glycines*, in soil using real-time PCR. *Nematology*. 21(10):1037-1042.
- Shurtleff W, Aoyagi A. 1983. The soyfoods industry and market. Directory and databook, Soyfood Center.
- Sikora RA, Greco N. 1990. Nematode parasites of food legumes: plant parasitic nematodes in subtropical and tropical agriculture. Wallingford (UK): CAB International; p.181-235.
- Sikora RA, Paul WT. 1986. An analysis of the *Heterodera trifolii* complex and other species in the *schachtii* group attacking legumes. *Cyst Nematodes*. Boston (MA): Springer; p. 293-313.
- Singh G, ed. 2010. The soybean: botany, production and uses. Oxfordshire (UK): CABI Publishing.
- Smart Jr GC. 1964. Physiological strains and one additional host of the soybean cyst nematode, *Heterodera glycines*. *Plant Dis*. 48:542-543.
- Smith GS, Niblack TL, Minor HC. 1991. Response of soybean cultivars to aldicarb in *Heterodera glycines*-infested soils in Missouri. *J Nematol*. 23:693-698.
- Soy Canada. 2017. 10 million acres of opportunity. [Accessed 2022 May 11].
<http://soycanada.ca/wp-content/uploads/2017/04/10-Million-Acres-of-Opportunity-Discussion-Paper.pdf>
- Soy Canada. 2022. Canadian soybean industry. [Accessed 2023 Apr 10].
<https://soycanada.ca/industry/statistics/at-a-glance/>
- Soystats. 2022. A reference guide to important soybean facts and figures. American Soybean Association. [Accessed 2023 May 11].
<https://soygrowers.com/wp-content/uploads/2022/06/22ASA-002-Soy-Stats-Final-WEB.pdf>
- Spears JR. 1955. Information regarding the soybean cyst nematode. USDA, ARS, Plant Pest Control Branch, July 29, 1955.

- Subbotin SA, Chitambar JJ, editorss. 2018. Plant parasitic nematodes in sustainable agriculture of North America. Vol. 2, northeastern, midwestern, and southern USA. New York (NY): Springer.
- Subbotin SA, Peng D, Moens M. 2001. A rapid method for the identification of the soybean cyst nematode *Heterodera glycines* using duplex PCR. *Nematology*. 3(4):365-371.
- Subbotin SA, Toumi F, Elekçioğlu IH, Waeyenberge L, Maafi ZT. 2018. DNA barcoding, phylogeny and phylogeography of the cyst nematode species of the *Avenae* group from the genus *Heterodera* (*Tylenchida: Heteroderidae*). *Nematology*. 20(7):671-702.
- Subbotin SA, Waeyenberge L, Moens M. 2000. Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: *Heteroderidae*) based on the ribosomal DNA-RFLP. *Nematology*. 2(2):153-164.
- Sugiyama S, Hiroma K. 1968. Studies on the resistance of soybean varieties to the soybean cyst nematode. I. Comparison of Varietal Responses. *Jpn J Breed*. 18(2):80-87.
- Szalanski AL, Sui DD, Harris TS, Powers TO. 1997. Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *J Nematol*. 29(3):255-267.
- Thorne G. 1928. *Heterodera punctata* n. sp. A nematode parasitic on wheat roots from Saskatchewan. *Sci Agric*. 8(11):707-710.
- Toumi F, Waeyenberge L, Viaene N, Dababat AA, Nicol JM, Ogbonnaya FC, Moens M. 2015. Development of qPCR assays for quantitative detection of *Heterodera avenae* and *H. latipons*. *Eur J Plant Pathol*. 143:305-316.
- Toyota K, Shirakashi T, Sato E, Wada S, Min YY. 2008. Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. *Soil Sci Plant Nutr*. 54(1):72-76.

- Tylka GL. 2012. Soybean cyst nematode field guide, 2nd edition. Iowa State University extension and outreach. [Accessed 2019 Jan 11].
<https://store.extension.iastate.edu/product/12854>
- Tylka GL, Marett CC. 2014. Distribution of the soybean cyst nematode, *Heterodera glycines*, in the United States and Canada: 1954 to 2014. Plant Health Prog. 15(2):85-87.
- Tylka GL, Marett C.C. 2021. Known distribution of the Soybean Cyst Nematode, *Heterodera glycines*, in the United States and Canada in 2020. Plant Health Prog. 22(1):72-74.
- Tylka GL, Mullaney MP. 2015. Soybean cyst nematode-resistant soybeans for Iowa. Ext Publ. PM, 1649. Iowa State University Extension, Ames.
- Uehara T, Mizukubo T, Kushida A, Momota Y. 1998. Identification of *Pratylenchus coffeae* and *P. loosi* using specific primers for PCR amplification of ribosomal DNA. Nematologica. 44(4):357-368.
- Venkatesh R, Harrison SK, Riedel RM. 2000. Weed hosts of Soybean Cyst Nematode (*Heterodera glycines*) in Ohio 1. Weed Technol. 14(1):56-160.
- Vovlas N, Vovlas A, Leonetti P, Liébanas G, Castillo P, Subbotin SA, Palomares Rius JE. 2015. Parasitism effects on white clover by root-knot and cyst nematodes and molecular separation of *Heterodera daverti* from *H. trifolii*. Eur J Plant Pathol. 143:833-845.
- Wang J, Niblack TL, Tremain JA, Wiebold WJ, Tylka GL, Marett CC, Noel GR, Myers O, Schmidt ME. 2003. Soybean cyst nematode reduces soybean yield without causing obvious aboveground symptoms. Plant Dis. 87(6):623-628.
- Werle R, Giesler LJ, Bernards ML, Lindquist JL. 2015. Likelihood of soybean cyst nematode (*Heterodera glycines*) reproduction on henbit (*Lamium amplexicaule*) roots in Nebraska. Weed Technol. 29(1):35-41.

- Wheeler TA, Lawrence KS, Porter DO, Keeling W, Mullinix Jr BG. 2013. The relationship between environmental variables and response of cotton to nematicides. *J Nematol.* 45(1):8-16.
- Whitehead AG. 1998. *Plant Nematode Control*. Wallingford (UK): CAB International.
- Winstead NN, Skotland CB, Sasser JN. 1955. Soybean cyst nematode in North Carolina. *Plant Dis.* 39(1):9-11.
- Wouts WM, Baldwin JG. 1998. *Taxonomy and identification: the cyst nematodes*. Dordrecht: Springer; p. 83-122.
- Wouts WM, Weischer B. 1977. A classification of fifteen species of *Heteroderinae* commonly occurring in Western Europe based on the larval characteristics. *Nematologica.* 23(3):289-310.
- Wrather JA, Anand SC, Dropkin VH. 1984. Soybean cyst nematode control. *Plant Dis.* 68:829-833.
- Wrather JA, Koenning SR. 2006. Estimates of disease effects on soybean yields in the United States 2003 to 2005. *J Nematol.* 38(2):173-180.
- Yan GP, Plaisance A, Chowdhury I, Baidoo R, Upadhaya A, Pasche J, Markell S, Nelson B, Chen S. 2017. First report of the soybean cyst nematode *Heterodera glycines* infecting dry bean (*Phaseolus vulgaris* L.) in a commercial field in Minnesota. *Plant Dis.* 101(2):391-391.
- Yan, G, Smiley RW, Okubara PA, Skantar A, Easley SA, Sheedy JG, Thompson AL. 2008. Detection and discrimination of *Pratylenchus neglectus* and *P. thornei* in DNA extracts from soil. *Plant Dis.* 92(11):1480–1487.
- Yeates GW, Bongers TD, De Goede RGM, Freckman DW, Georgieva SS. 1993. Feeding habits in soil nematode families and genera-an outline for soil ecologists. *J Nematol.* 25(3):315-33.
- Yen JH, Niblack TL, Wiebold WJ. 1995. Dormancy of *Heterodera glycines* in Missouri. *J Nematol.* 27(2):153-163.

- Ye W. 2012. Development of primetime-real-time PCR for species identification of soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) in North Carolina. J Nematol. 44(3):284-290.
- Yu Q. 2011. Soybean cyst nematode (*Heterodera glycines* Ichinohe). In: El-Shemy H, editor. Soybean physiology and biochemistry. New York (NY): InTech; p. 461-474.
- Zarkadas CG, Yu Z, Voldeng HD, Minero-Amador A. 1993. Assessment of the protein quality of a new high-protein soybean cultivar by amino acid analysis. J Agric Food Chem. 41(4):616-623.
- Zheng J, Subbotin SA, Waeyenberge L, Moens M. 2000. Molecular characterisation of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. Russ J Nematol. 8(2):109-114.
- Zirakparvar ME. 1985. Chemical control of nematodes attacking soybeans. Proceedings of the World Soybean Research Conference; Boulder (CO): Westview Press; pp. 523-527.

2.0 Survey and First Report of the Soybean Cyst Nematode (*Heterodera glycines* Ichinohe) in Soybean Fields in the Province of Manitoba, Canada

2.1. Abstract

Soybean Cyst Nematode (SCN), *Heterodera glycines* (Ichinohe 1952), is the major soybean (*Glycine max* (L.) Merr.) pest worldwide. In Canada, SCN is present in Ontario and Quebec. Past surveys conducted in Manitoba failed to report the pest. Nevertheless, the nematode is expected to be present in Manitoba as it spreads rapidly northwards in North Dakota and Minnesota. Thus, the objective of this study was to survey soybean fields in Manitoba for the presence of SCN. In October 2017, soil samples were collected from 30 commercial soybean fields in Manitoba. A modified Fenwick elutriator based on the USDA cyst extractor was used to recover cysts. Cysts were identified based on morphological characters, PCR with species-specific primers for *H. glycines* (COXIII and SCAR), and DNA sequencing of four regions of the genome. Four fields were positive for SCN and had 64, 21, 86, and 40 eggs/2.2 kg of soil (2.9, 0.95, 3.9, and 1.81 eggs/100 g soil). In July 2021, an additional soybean field showing stunting and yellowing symptoms in soybean and swollen nematode females on roots was sampled. The SCN population density of samples collected from the symptomatic area by the field entrance was 7,797 eggs/100 cm³ soil in the area centre and declined to 0 in the asymptomatic areas of the field. The population of SCN from this field was of HG type 7 (race 3). Three positive fields were in the rural municipalities of Rhineland, Emerson-Franklin, and Montcalm, and 27, 17 and 16 km, respectively, from the Red River border crossing and an area affected by spring flooding. One field was in RM of Norfolk-Treherne, and the symptomatic field was in Thompson municipality, being 138 and 85 km, respectively, from the Red River border crossing and unaffected by floodwater from the USA. This study reports the first finding of *H. glycines* and SCN disease in Manitoba.

Keywords: Soybean, Soybean Cyst Nematode (SCN), *Heterodera glycines*, species-specific primers.

2.2. Introduction

The Soybean Cyst Nematode (SCN), *Heterodera glycines* (Ichinohe 1952) is one of the most devastating parasites of soybean (*Glycine max* (L.) Merr.) in all major soybean growing regions, including Canada and the United States (Allen et al. 2017). SCN was first documented in 1915 in Japan by Hori (1915), and it was recognized as a new species, *Heterodera glycines*. The nematode has been reported in North America: Canada (Ontario, Quebec) (Anderson et al. 1988; Mimee et al. 2014), many states in the U.S. (Alabama, Arkansas, Delaware, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maryland, Minnesota, Michigan, Mississippi, Missouri, Nebraska, New Jersey, New York, North Carolina, North Dakota, Ohio, Oklahoma, Pennsylvania, South Carolina, South Dakota, Tennessee, Texas, Virginia, and Wisconsin) (Tylka and Marett 2021); South America: Argentina, Brazil, Chile, Colombia, Ecuador (Riggs 1977; Noel 1985; Baldwin and Mundo-Ocampo 1991; Mendes and Dickson 1993; Evans and Rowe 1998); Asia: China (Hebei, Hubei, Heilongjiang, Henan, Jiangsu, Liaoning) (Liu et al. 1997), Indonesia (Java), the Korean peninsula (Yokoo 1936), Japan (Ichinohe 1952), Iran (Maafi et al. 1999), Russia (Amur District in the Far East) (Eroshenko et al. 1990), Europe (Italy) (Manachini 2000), and Africa: Egypt (Diab 1968). Basically, SCN is present in every major region growing soybean.

Soybean cyst nematode has moved rapidly throughout the southeast, northeast, and midwestern regions of the U.S. and currently occurs in soybean-growing areas of both the United States and Canada. To date, in Canada, SCN has been reported in the provinces of Ontario and Quebec. SCN was first reported in Canada in 1987 (Anderson et al. 1988) in southwestern Ontario adjacent to Michigan. Since then, the pest has moved northeastward within that province, and in 2013, it was

found in Quebec (Mimee et al. 2014). Meanwhile, in all soybean-producing areas of Quebec, low population densities of the pest were reported soon after (Mimee et al. 2016). Minnesota and North Dakota bordering Manitoba were reported with SCN in 1978 (MacDonald et al. 1980) and 2003 (Bradley et al. 2004), respectively. In either state, the pest continues to spread among soybean-producing regions along the Red River, which flows northward towards counties adjacent to Manitoba. As a result, the areas in Manitoba that cultivate soybeans in the Red River Valley face the risk of infestation by SCN. Notably, SCN is currently present in six of eight counties of North Dakota bordering Manitoba (Anonymous 2022) and two of three agricultural counties of Minnesota bordering Manitoba (Tylka and Maret 2021).

Soybean cultivation in Canada has significantly expanded over the last decade. Manitoba ranks second among other Canadian provinces in the total seeded area, with an increase of approximately 130% from 0.23 million hectares in 2011 to 0.53 million hectares in 2021 (Soy Canada 2021). This increase has been attributed to the development of early maturing, glyphosate-resistant varieties with low production costs (reflecting a reduced need for N-fertilizer), double inoculation technologies, ease of cultivation, and harvesting. Double inoculation involves employing two inoculant formulations or placement methods. A typical approach for double inoculation entails using seed-applied liquid inoculant along with a granular or liquid in-furrow application to optimize nodulation and enhance soybean performance (Muldoon et al. 1980). These technologies made it possible to grow soybeans in northern and western areas of the province instead of only in the Red River Valley region. Field soil surveys in Manitoba for SCN egg recovery were conducted in 2012 and 2013 (Madani et al. 2023) and 2015 (unpublished). A total of 76 commercial fields were sampled with no cysts or eggs of *H. glycines* observed. With the rapid spread of SCN northward along the Red River in North Dakota and Minnesota and the substantial increase in

soybean production, it is expected that SCN will eventually be present in Manitoba. Early detection of new pests is critical for mitigation of the negative effects of that pest.

Heterodera glycines does not cause specific symptoms on crop hosts, but patches of poor growth can be observed in a soybean field when *H. glycines* has parasitized soybean plants. Soybean plants in these patches may show stunting, chlorosis, wilting, or loss of leaves with reduced seed production. There is often an abrupt transition between affected and non-affected parts of the field, and the middle of the affected areas are often the most severely damaged. Field machinery entrances and water courses in and adjacent to fields can be areas of the introduction of SCN. Wind is also thought to move nematode cysts to new fields (Riggs 1977; Niblack and Tylka 2016).

The soybean cyst nematode may cause yield losses of up to 30% in soybeans (Noel 1992; Noel and Edwards 1996). In many cases, the SCN infestation may not be detected early due to a lack of visible symptoms (Wang et al. 2003). To effectively monitor SCN and to prevent yield losses, there is a need for close examination of roots (six weeks after planting) and soil sampling (preferably post-harvest) for the presence of small, white to yellow females attached to the root surface and nematode cyst in soil extracts (Niblack 2005; Faghihi and Ferris 2006).

Detecting infested fields with the nematode early is crucial to reduce the spread of SCN and its associated yield losses. Once infested fields are identified, control measures can be implemented to prevent the nematode from spreading to other fields of farming regions. Conventional methods to identify SCN include using nematode morphology, that is, morphometric characters such as vulval cone structure, body and fenestra length, and width. SCN and other plant-parasitic nematodes can also be identified using DNA-based methods such as polymerase chain reaction (PCR) with species-specific primers (Subbotin et al. 2001; Ou et al. 2008) and DNA sequencing

(Szalanski et al. 1997; Zheng et al. 2000; Madani et al. 2007). Molecular techniques have since replaced morphological methods for their efficiency, reliability, and accuracy (Sato et al. 2007; Toyota et al. 2008; Donn et al. 2008; Okada and Oba 2008). The Canadian Food Inspection Agency recently delisted SCN as a regulatory pest; therefore, it is up to growers and provinces to conduct surveys for the pest. The objective of this study was to survey soybean fields in Manitoba for the presence of the soybean cyst nematode. We report here the first confirmation of SCN in commercial fields in the province of Manitoba, Canada.

2.3. Materials and Methods

2.3.1. Soil Sampling

2.3.1.1. Survey

In October 2017, soil samples were collected from 30 commercial soybean fields in Manitoba, mainly near the border with North Dakota and Minnesota flanking the Red River (Fig. 2.1). The fields had a history of at least three years of soybean crops. Each field was partitioned into three areas based on the risk of establishing SCN: entrances, depressions, and remaining field areas. A minimum of 15 soil cores (5 cm diameter $\times$ 15 cm deep) from each area were taken using a Dutch auger. The 15 soil cores were combined to a single composite sample for a total of 90 composite soil samples. To verify the presence of the pest, the fields in which the nematode was found were resampled in July 2019 using the same method. The roots of arbitrarily selected plants were also examined in 2019 with a stereoscope for the presence of SCN females.

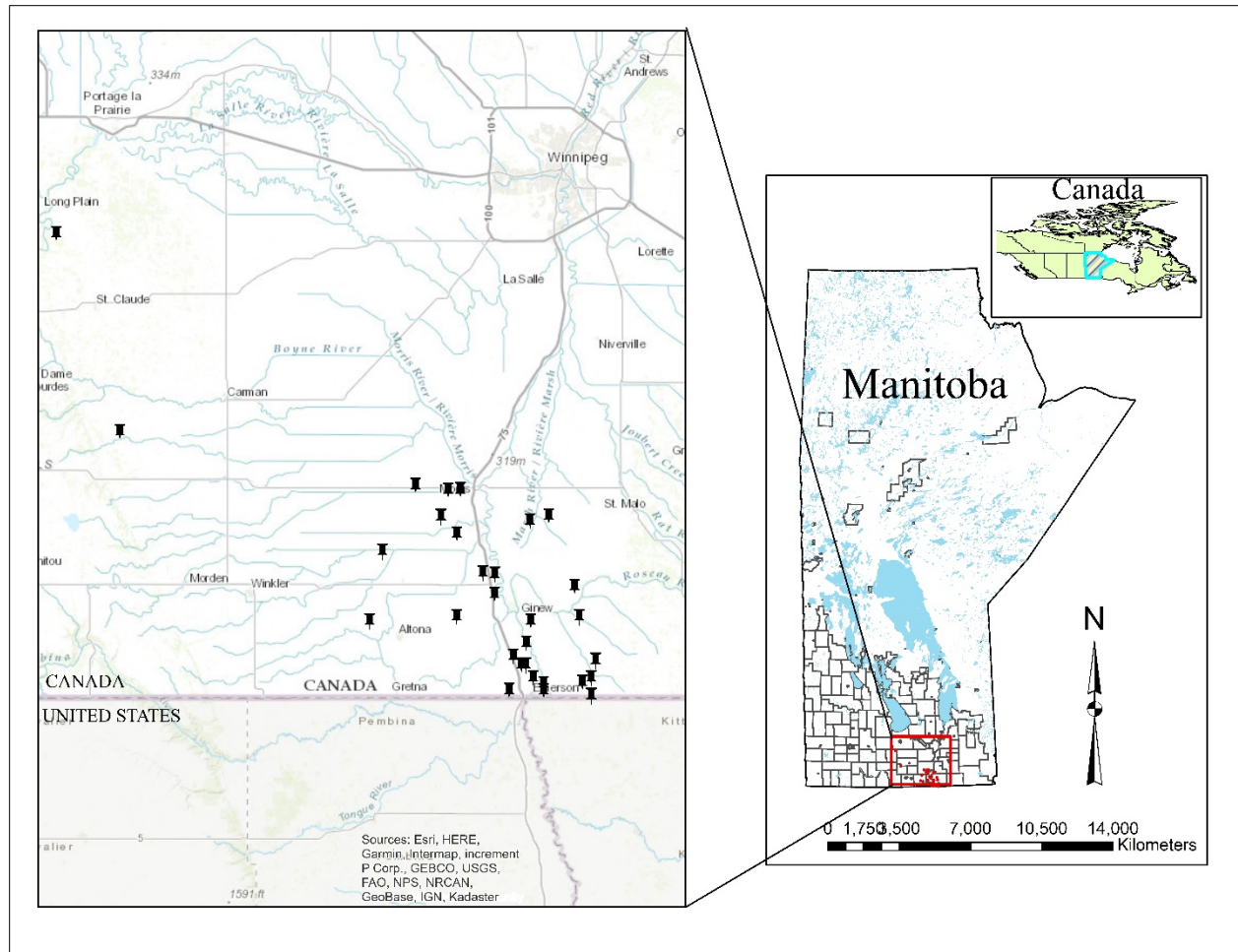


Fig. 2. 1. Locations of the soybean fields sampled in Manitoba 2017-2021

2.3.1.2. Symptomatic Field in 2021

In early July 2021, a local crop consultant reported to us a soybean field in south-central Manitoba exhibiting chlorosis, stunting and females on roots. A visitation on July 8, 2021, confirmed a headland area of approximately 60 m by 15 m with the above symptoms. The affected area was along the field edge by a machinery entranceway and gravel road. A composite soil sample and soybean roots from the affected area were brought to the Soil Ecology Laboratory for analysis. Cysts of nematodes were isolated from three soil subsamples, and egg densities were determined. Roots were examined with a stereoscope for adult cyst nematode females.

A resampling of the headland area was conducted on September 3, 2021, to determine the coincidence of the symptomatic area and soil densities of SCN eggs. A grid (10 by 6 m) was laid out using measuring tape, covering a total area of 70 m along the side road by 24 m into the field. The area extended 5 to 10 m beyond the symptomatic soybean area. At each grid intersection, soil as a composite of three collections using a Dutch auger (0-15 cm) was obtained for a total of 32 composite samples. The 20 and 50 m grid intersection points were not sampled out of concern for the ability to not be able to process samples in a timely schedule. The soil was processed in the laboratory for the determination of egg densities.

2.3.2. Cyst and Egg Recovery

2.3.2.1. Survey

A modified Fenwick elutriator based on the USDA Cyst Extractor (Fenwick 1940; Roach et al. 1968) in the Soil Ecology Laboratory was used to recover cysts from the soil samples in 2017 survey. The soil samples were air-dried, and then 2.2 kg of each sample was soaked in water for one hour and processed with the elutriator with nested sieves (20-mesh (850 μ m) over 60-mesh (250 μ m)). Cysts were separated from recovered debris by ethanol flotation (Seinhorst 1974) to

facilitate cyst isolation under the microscope (Fig. 2.2). The recovery efficiency of the elutriation and cyst isolation was assessed by adding 100 cysts into three soil samples (each 2.2 kg) before initiating the cyst recovery procedure.

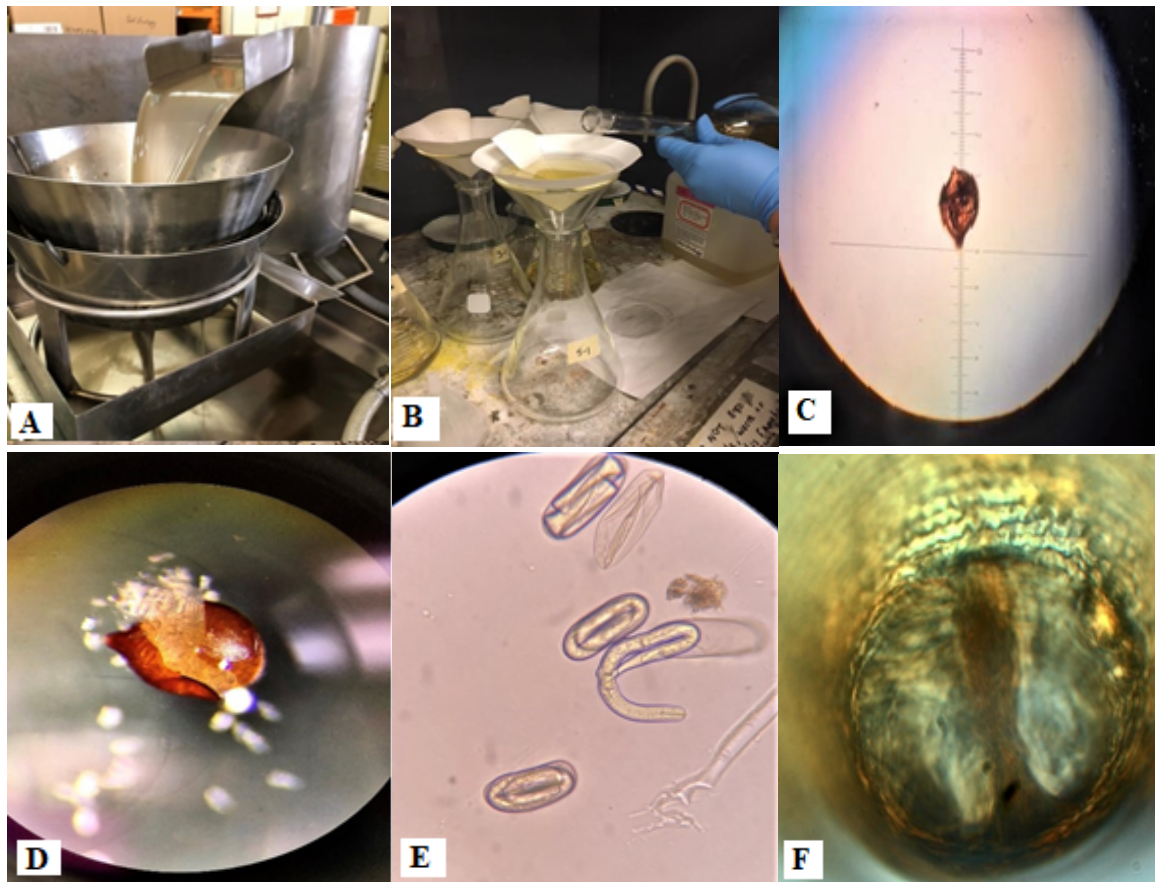


Fig. 2. 2. A) Modified Fenwick elutriator, B) Ethanol Flotation, C) Measuring length of the cyst, D) *Heterodera* spp. cyst broken open to expose eggs and juveniles within, E) Second stage juvenile of SCN hatching from its egg, and F) Compound microscope image of vulval cone structure.

2.3.2.2. Symptomatic Field in 2021

Cysts were recovered from soil samples from grid sampling by a modified wet-sieving and decanting method (Gerdemann 1955). Anticipating a high SCN density in the symptomatic field, the wet-sieving method was chosen. Unlike survey experiments, which typically demand larger soil volumes to recover an adequate number of nematodes, the wet-sieving method proves advantageous when dealing with high-density SCN infestations. This method ensures a comprehensive examination of SCN distribution in highly-infested field soils by analyzing a smaller soil sample size. In this approach, the top sieve (850 μm) caught root and soil debris while the bottom (250 μm) retained cysts and cyst-size debris. Each composite sample was thoroughly mixed, and a subsample of 100 cm^3 was obtained by the water volumetric displacement method and used to determine the SCN egg density. The subsamples passed through a series of #20 (850 μm) and #60 (250 μm) mesh screens. Eggs were released from cysts by manually grinding material on the smallest screen using a rubber stopper and washing it into a screen tray. Another set of two sieves with finer mesh was set up to collect the eggs. The eggs were collected on a #500 (25 μm) mesh screen, which was placed below a screen with a mesh size of #200 (75 μm). Subsequently, the isolated eggs were stained with acid fuchsin (0.003 g ml^{-1}) and heated to boiling. Two ml of thoroughly mixed and cooled sample was transferred into a counting slide using a glass Pasteur pipette. Stained eggs were then counted using a compound microscope (Olympus BX51, Olympus Canada Inc., Richmond Hill, ON) at 10 $\times$ magnification (Niblack et al. 1993). Results are reported as the number of eggs/100 cm^3 soil.

2.3.3. Cyst Morphology

Extracted cysts were collected and counted under a microscope and observed if lemon-shaped and thus possibly belonging to the genus *Heterodera*. Slides of cone tops were prepared in a drop of

melted glycerin-gelatin (Correia et al. 1997). Images of individual cysts and cone tops were obtained using a compound microscope (Olympus BX51, Olympus Canada, Inc., Richmond Hill, ON) equipped with a digital imaging camera (QColor3, Olympus, Tokyo, Japan) and an Image-Pro Plus 6.2 software (Media Cybernetics, USA), in transmitted light at 4× and 40× magnifications (Fig. 2.2). Cysts of *Heterodera* were then classified to *H. glycines* using identification keys (Hirschmann 1956; Subbotin et al. 2010). Generally, the identification of cysts is based on both morphological and morphometrical characters (Mulvey 1972; Mulvey and Golden 1983; Golden 1986; Wouts and Baldwin 1998). The characters used included: vulval cone structure, lemon-shaped body, as well as measurements of body length (L) including neck, body width (W), the ratio of body length and body width (L/W), fenestra length and width, and vulval slit.

2.3.4. Molecular Identification

2.3.4.1. Species-Specific PCR

2.3.4.1.1. Survey

DNA was extracted from the suspension of juveniles and eggs recovered from a single cyst using the Proteinase K method (Subbotin et al. 2000) and crushing with glass beads. The extraction was conducted in a 65 µl volume containing 50µl tail lysis buffer (Viagen Biotech, Los Angeles, CA, USA), 13 µl sterile diH₂O of eggs and juveniles from a cyst, three 1mm glass beads (autoclaved) to mechanically disrupt eggs and juveniles, and 2µl of 100 mg Proteinase K (Fisher Bioreagents, Germany). The contents of each tube were vortexed briefly at low speed (Vortex Genie, Scientific Industries, Inc., Bohemia, NY, USA), centrifuged at 2,200 xg for 1 min (Mini Centrifuge, Fisher Scientific) and stored overnight in a freezer at -20°C. The next day, samples were incubated at 60°C (1 hr), followed by 95°C (15 min), to deactivate Proteinase K, using a thermocycler (Eppendorf CF2060, Master-cycler Pro-S, Hamburg, Germany). The quality and quantity of each

DNA extraction were verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and then the extracts were stored at -20°C before amplification by conventional PCR.

The identity of potential SCN cysts was identified using two species-specific primer sets in a duplex reaction with universal primers in conventional PCR. Duplex reactions had a primer set D3A-D3B (Table 2.1) for amplification of the D3 region of the 28S rDNA (Al-Banna et al. 1997; Nadler et al. 1999; Subbotin et al. 2001) and one of two species-specific primer sets for amplification of the characterized amplified regions (SCAR) (Ou et al. 2008; Subbotin et al. 2001) or mitochondrial DNA (Mt-DNA) gene (COXIII) (Madani et al. 2013, Table 2.1). Species-specific amplification of the COXIII gene was conducted using forward 65F1 and reverse 66R1 primers and the SCAR region was amplified using forward SCNF1 and reverse SCNR1 primers. The primer sets and the modified PCR amplification conditions used in the assays are given in Tables 2.1 and 2.2, respectively. A total reaction volume of 25.5 µl of the PCR reaction mixture was prepared having 16.8 µl autoclaved diH₂O, 2.5 µl of 10X Dream Taq buffer, 1.0 µl dNTPs, 1.5 µl 25 mM MgCl₂, 0.2 µl DreamTaq DNA Polymerase (Thermo Scientific, Waltham, MA, USA), and 0.5 µl of each forward and reverse primers (10 µM) (D3A, D3B, SCNF1, and SCNR1 in SCAR amplification and D3A, D3B, 65F1, and 66R1 in COXIII amplification). Nematode DNA (1.5 µl) was then added to a PCR reaction tube, tube contents were mixed, and amplified using the thermocycler. Primer concentration and annealing temperature were optimized previously in the Soil Ecology Laboratory by performing a gradient PCR and visualizing the PCR products. Following amplification, the amplified products were then separated by gel electrophoresis using 1.2% agarose gel (Invitrogen, Carlsbad, CA, USA) stained with 2.5 µl of 10,000 × concentrated GelRed fluorescent nucleic acid gel stain (Biotium, Fremont, CA, USA) at 85 V for 90 min in a

horizontal gel box (Fisherbrand, Fisher Scientific, Nepean, ON). The amplification products were then observed under UV light (G: BOX F3, Syngene, Frederick, MD, USA). Amplified bands were compared to a 100 bp DNA ladder (ProMega, Madison, WI, USA) to obtain fragment sizes. PCR products from reactions that produced bands with expected sizes were purified using a QIAquick PCR Purification Kit (250) (QIAGEN, Hilden, Germany). In cases where some DNA samples displayed double bands or primer dimers with universal primers, the appropriate fragments were carefully excised from the agarose gel and subsequently purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany).

2.3.4.1.2. Symptomatic Field in 2021

DNA was extracted from the suspension of eggs and juveniles of four arbitrarily selected nematode cysts collected from the highly infested field observed in 2021. Additionally, DNA extraction was performed on three females recovered from the roots of soybean (S007-Y4, Syngenta, Guelph, ON) plants grown in $3.8 \times 21.6 \text{ cm}^2$ Cone-tainers (Stuewe & Sons, Tangent, OR, USA) trays that were inoculated with SCN eggs obtained from the same infested field. Details of the growth conditions are provided in Section 2.3.5. The DNA extraction method including proteinase K was used on the whole samples prepared above. Both universal ITS (Powers et al. 1997) and NEM-18S (Floyd et al. 2005) primer sets, along with species-specific primer sets (COXIII and SCAR), were used in conventional PCR reactions. Monoplex PCR, using one primer set per reaction, was conducted to streamline the sample preparation for sequencing. For the ITS primer set, the PCR reaction mixture (total reaction volume of 25 μl) included 17.3 μl sterile water, 2.5 μl of 10X Dream Taq buffer, 1 μl 25 mM MgCl_2 , 1.0 μl dNTPs, 0.25 μl Dream Taq Polymerase and 0.5 μl of each primer. Subsequently, 2 μl of the thawed nematode DNA was added to the PCR reaction tubes and run in the thermocycler. For the NEM-18S primer set a total reaction volume of 40 μl of

the PCR reaction mixture was prepared having 28.2 μ l sterile water, 4 μ l of 10X Dream Taq buffer, 1.6 μ l dNTPs, 0.25 μ l Dream Taq DNA Polymerase and 2 μ l of each primer. To achieve higher yields in the PCR process, adjustments have been made to various aspects of the PCR amplification, including the reaction volume, cycle number, the amount of DNA template, and primer concentrations. The reaction mixture for the SCAR and COXIII primer sets remained the same as above. The primer sets and the modified PCR amplification conditions are provided in Tables 2.1 and 2.2, respectively. Following amplification, the amplified products from all reactions were visualized on a gel using the same conditions as described above.

Table 2. 1. Universal and species-specific primer sets used in a polymerase chain reaction (PCR) and sequencing.

Primer set	Region/ Species	Primer Sequence	PCR product size (bp)	Reference
D3A_D3B	28S rDNA (universal)	GACCCGTCTTGAAACACGGA TCGGAAGGAACCAGCTACTA	345	Al-Banna et al. 1997
ITS-F ITS-R	ITS (universal)	5'-TTGATTACGTCCCTGCCCTTT-3' 5'-ACGAGCCGAGTGATCCACCG-3'	850	Powers et al. 1997
NEM-18S-F_ NEM-18S-R	18S (universal)	CGCGAATRGCTCATTACAACAGC GGGCGGTATCTGATCGCC	900	Floyd et al. 2005
SCNFI_SCNRI	SCAR <i>H. glycines</i>	GGACCCTGACCAGAAAGTTTCCGC GGACCCTGACGAGTTATGGGCCCCG	477	Ou et al. 2008
65F1_66R1	Mt-DNA-COXIII <i>H. glycines</i>	5'-TGAGGCTTTAGTTGGCTTTCA-3' 5'-TGAGAAAAGGTAATAACCGAAGC-3'	252	Madani et al. 2013

Table 2. 2. Thermal profiles used in PCR assays.

Program	Primers used	Modified amplification conditions	Reference
ITS	ITS-F ITS-R	94°C 5 min; 35 cycles at 94°C 30 sec, 58°C 30 sec, 72°C 1 min, and a final extension 72°C 10 min	Powers et al. 1997
NEM-18S	NEM-18S-F NEM-18S-R	94°C 5min; 35 cycles at 94°C 30 sec, 54°C 30 sec, 72°C 1 min, and a final extension 72°C 10 min	Floyd et al. 2005
Duplex 2	D3A_D3B_ SCNFI_SCNRI	94°C 4 min; 35 cycles at 94°C 30 sec, 66°C 1min, 72°C 2 min, and a final extension 72°C 10 min	Ou et al. 2008
Duplex 4	D3A_D3B_ 65F1_66R1	94°C 4 min; 35 cycles at 94°C 30 sec, 57°C 30 sec min, 72°C 1 min, and a final extension 72°C 10 min	Madani et al. 2013

2.3.4.2. Sequencing

The identity of the nematode as *H. glycines* was further confirmed by sequencing the Ribosomal DNA of the ITS, 18S, SCAR, and Mt-DNA, COXIII genes. The primers used for the sequencing process were the same as those used in the PCR assays. The purified PCR products were sent to Macrogen Inc. (now Psomagen, Rockville, MD, USA). To determine the closest match to *H. glycines*, all nucleotide sequences were compared to the GenBank database using the basic local alignment search tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI). Match in the database was based on lowest expected value (E-value) and highest percent identity match.

2.3.5. Reproduction Assays

2.3.5.1. Pot

Two approaches were used to confirm that cysts identified from the five fields in Manitoba as SCN were capable of parasitizing a susceptible variety of soybean (S007-Y4, Syngenta, Guelph, ON). The first used soybean grown in 16 cm polyethylene plastic pots filled with two kilograms of a 2 (positive SCN soil):1 (fine sand) mixture. One soybean plant was grown in each pot for two growth cycles, each lasting 60 days. Plants were grown at $25 \pm 2^\circ\text{C}$ during 16 hr of light and 18°C during 8 hr of darkness with humidity 65-70% (TPRB-149 growth room, Bio Chambers Inc., Winnipeg, MB), and a light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. The crops were fertilized two and four weeks after emerging, using Miracle-Gro (20:20:20), a water-soluble fertilizer. Each application involved dissolving one teaspoon of fertilizer in 4 l of water. Soil from two fields from southern Ontario infested with SCN was used as positive controls. These Ontario soils had SCN population levels of 3,700 and 6,100 eggs/100 cm^3 soil. Two field soils from Manitoba which had not been planted with soybeans in the past five years and were confirmed to be free from SCN by sieving and

microscopic analysis were used as negative controls. At the end of the assay, the plant roots were examined for lemon-shaped females, and the cysts were identified using species-specific PCR and sequencing of the four regions of the genome. Each of the five SCN positive fields had three replicate pots.

2.3.5.2. Cone-tainer

The second approach involved adding eggs from the five SCN positive fields. Here the same susceptible soybean variety was used but grown in SC10 Cone-tainer (3.8 cm diameter, 21.6 cm deep) seed starter tubes (Stuewe and Sons, Inc., Tangent, OR, USA). The Cone-tainers were filled with a 1(fine sand): 2 (Miracle-Gro (Scotts Miracle-Gro, Marysville, OH, USA)) mixture. A deep hole was made at the surface of each Cone-tainer, into which one germinated soybean seed was transferred and 7 days later inoculated with 1,000 eggs and juveniles suspended in 1 ml H₂O obtained from freshly extracted SCN cysts from soil using a pipet. A control consisting of uninoculated soybean was included. Seedlings were allowed to develop over three to four weeks as above.

After 30 days, the Cone-tainers were submerged in water and the roots were carefully recovered. Then the roots were washed using a high-pressure spray of water onto nested screens, #20 screen (850 µm) on top, #60 screen (250 µm) at the bottom. After a thorough rinse of the #20 screen, the debris collected on the #60 screen was examined using a stereo microscope and females were handpicked using a forceps. Eggs or juveniles from recovered females were extracted for DNA and identified using species-specific PCR. The regions of the genome ITS, 18S, SCAR, and COXIII were also sequenced for nematode species identification.

2.3.6. HG-Typing

Soil (4 kg) collected from the symptomatic field in 2021 was sent to SCN Diagnostics, University of Missouri (Columbia, MO, USA), for typing according to Niblack et al. (2002). Seven soybean indicator genotypes of various sources of resistance to *H. glycines* were grown in Cone-tainer pots filled with sand under controlled greenhouse conditions at 23 °C to 28 °C and a 16-hr photoperiod. The lines were a susceptible variety, Lee74, and indicator lines of resistance PI548402 (Peking), PI 88788, PI 90763, PI 437654, PI 209332, PI 89772, and PI 548316 (Cloud). Though the HG-type test has superseded it, the SCN race typing of population resistance is still in use. Thus, the variety ‘Pickett’ was also included in the assay. The soybean genotypes used in the race test included ‘Pickett’, ‘Peking’, PI 88788, and PI 90763 indicator lines (Riggs and Schmitt 1988).

For each line, four individual seedlings were grown in separate Cone-tainers of sterilized sandy soil. Seven days after emergence two aliquots of 0.5 ml suspension containing 500 eggs (J2) each recovered from the sample soil were added into the soil around each seedling. The Cone-tainers of inoculated seedlings were submerged in water tables set to 27 °C for 30 days. The bath maintained an optimal temperature for SCN reproduction. Females were removed from roots by washing with a strong jet of water on nested screens, #20 screen (850 µm) over #60 screen (250 µm). All trapped females on the #60 screen were counted under a microscope following a thorough rinse of the #20 screen. The ability of the SCN population to reproduce on a particular soybean genotype is reported as the Female Index (FI). The FI for each line was obtained as follows: $FI = (\text{mean number of females on each indicator line} / \text{mean number of females on the susceptible Lee74 line after 30 days}) \times 100$. An indicator line with FI between 10 and 30 was considered moderately resistant to the SCN population, while those with $FI < 10$ were considered resistant, and they were respectively assigned the labels + and – as part of the race scheme.

2.4. Results

2.4.1. Survey

2.4.1.1. Cyst and Egg Densities

A total of 42 nematode cysts were obtained from 17 samples collected from 12 out of the 30 fields sampled in 2017. Only one to a few cysts were recovered from each of the 17 composite samples. Of the 42 cysts, 21 were intact, brown and lemon-shaped, possibly indicating *H. glycines*, making them suitable for microscopic measurements. These selected potential SCN cysts were recovered from just seven fields. The other cysts (n=21) recovered were round, damaged and empty. The recovery efficiency of the elutriation and cyst isolation was determined to be 70%.

2.4.1.2. Morphological Identification

The intact lemon-shaped cysts (n=21) from seven fields were ambifenestrate for vulval cone structures, consistent with being *H. glycines* (Fig. 2.2). Measurements (mean $\mu\text{m} \pm \text{sd}$) of the 21 cysts were as follows: body length (L) ($552.38 \pm 89.05 \mu\text{m}$), body width (W) ($370.23 \pm 77.70 \mu\text{m}$) and ratio of body length and width (L/W) (1.51 ± 0.20). In addition, measures for fenestra length (n=13, $57.92 \pm 6.27 \mu\text{m}$) and width ($43.61 \pm 4.13 \mu\text{m}$), and vulval slit (n=8, $43.31 \pm 4.83 \mu\text{m}$) were taken where possible. Together, the measurements were within the range for *H. glycines* (Table 2.3) given by Hirschmann (1956) and Subbotin et al. (2010). The round cysts recovered all had circumfenestrate cone top pattern.

Table 2. 3. Morphometrics of SCN cysts extracted from soybean fields sampled in 2017 and 2021 in Manitoba in comparison with previous studies by Hirschmann (1956) and Subbotin et al. (2010).

Characters	Body length (n=25)	Body width (n=25)	Length/ Width (n=25)	Fenestra length (n=17)	Fenestra width (n=17)	Vulval slit (n=8)	Vulval cone structure (n=21)
Cysts (n)							
This study	564 ± 87.82 (400-725)	386 ± 83.56 (225-550)	1.48 ± 0.20 (1.2-2.1)	57.35 ± 6.07 (48-68)	43.68 ± 4.06 (36-48)	43.31 ± 4.83 (34-51)	Ambifenestrates
Hirschmann (1956)	(340-920)	(200-560)	(1.2-2.1)	(37-65)	(33-48)	(43-56)	Ambifenestrates
Subbotin et al. (2010)	(474-709)	(327-535)	(1.3-1.7)	(34-58)	(16-41)	(38-50)	Ambifenestrates

all measurements are given in µm: Mean ± sd (range)

2.4.1.3. Molecular Identification

2.4.1.3.1. Species-Specific PCR

Based on duplex PCR amplification, all intact lemon-shaped cysts (n=21) had successful universal primer (D3A-D3B) amplification and of those 14 and 10 cysts had successful amplification of COXIII gene and SCAR region, respectively. The universal primer produced 345 bp fragments together with each of the 252 bp and 477 bp amplicons when COXIII and SCAR were used, respectively (Fig 2.3). These fragment sizes were consistent with those associated with *H. glycines*. The DNA samples obtained from nine cysts recovered from four of the seven fields showed sharp bands of appropriate sizes specific to *H. glycines* on the gel when both sets of species-specific primers were used for amplification. The PCR products of the selected samples were purified and chosen for subsequent sequencing analysis.

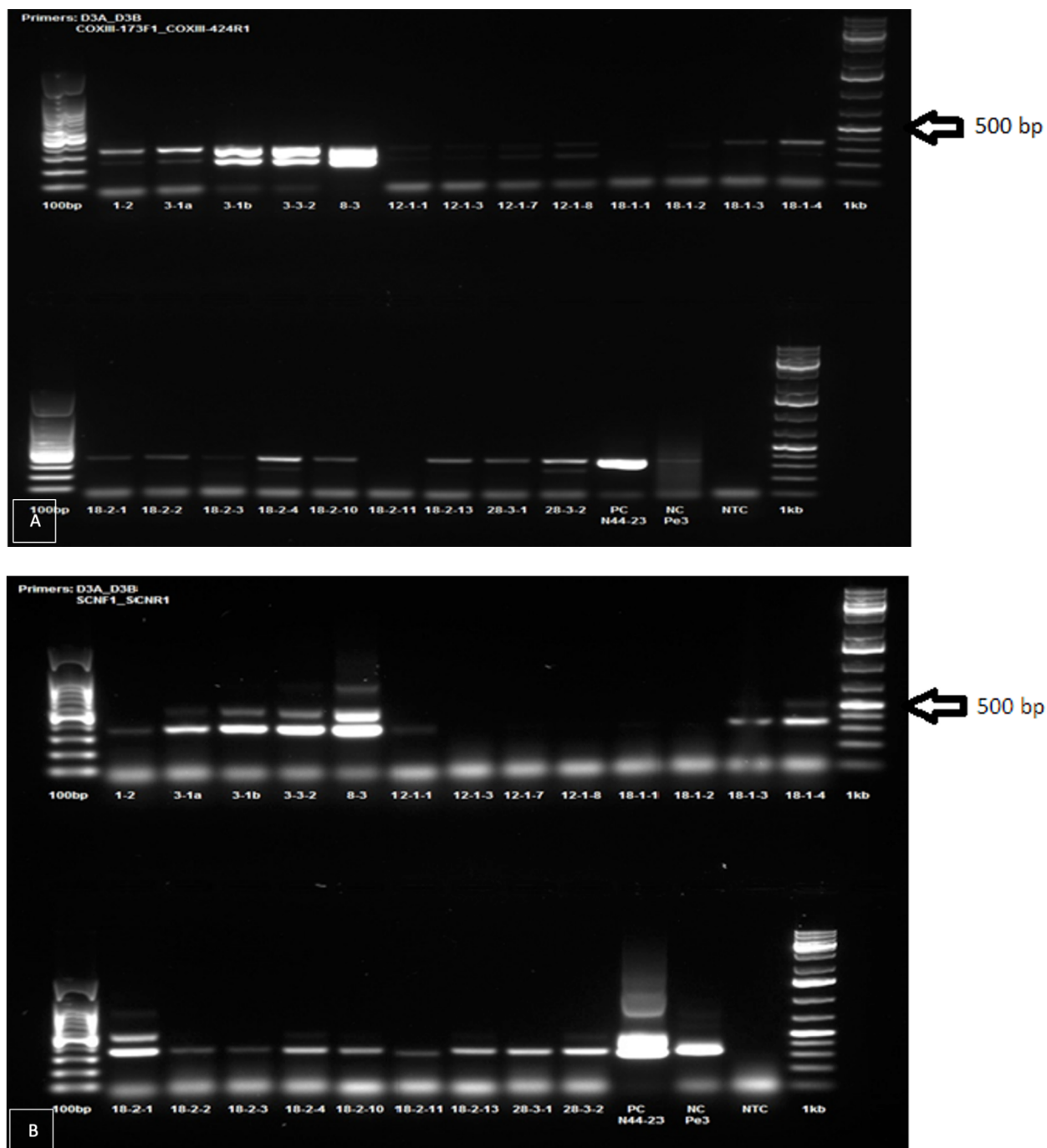


Fig. 2. 3. Agarose gel electrophoresis pattern of duplex PCR products amplified using universal (D3A-D3B, (345 bp)) and *H. glycines* specific primers, A) COXIII (252 bp), and B) SCAR (474 bp) with DNA extracted from eggs of the single cyst. Lanes: 100 bp DNA ladder, 1-2, 3-1a, 3-1b, ..., 28-3,1, and 28-3,2 are cyst individuals extracted from soil sampled in 2017. The following last three lanes: positive control (PC) *H. glycines*, Ontario population, negative control (NC) plant-parasitic nematode other than *H. glycines*, *H. schachtii*, and non-template control (NTC)

2.4.1.3.2. Sequencing

Purified PCR products of 14 cysts were submitted for sequencing, with only nine cysts having good read length and being identified as *H. glycines*. Sequencing of the ITS and 18S genes was successful for all nine cysts, however, SCAR and COXIII yielded only five and four successful sequencings, respectively (Table 2.4). The ITS and 18S gene sequences highly matched that of SCN accessions in the NCBI database (>99.09%). Identity matches to *H. glycines* accessions were greater than 93.49% and 96.35% for SCAR and COXIII. The sequences for each of the four target areas have been submitted to GenBank with the following accession numbers: ITS (OR265515 - OR265519); 18S (OR265522 - OR265526); SCAR (OR291425 - OR291426); and COXIII (OR291423). The sequencing results aligned with the findings obtained through PCR assays using species-specific primers.

Table 2. 4. NCBI BLAST results for the ITS, 18S, SCAR, and COXIII gene sequences of *H. glycines* recovered from field soils sampled in 2017.

Nematode individual	Match	Gene	Accession	Identity (%)	E-value	Query cover (%)	Read length (bp)
3-1	<i>H.glycines</i>	ITS	CP049713.1	100	0.0	100	679
		18S	MK292129.1	99.88	0.0	100	860
		SCAR	CP049710.1	93.49	2e-171	99	442
3-3,2	<i>H.glycines</i>	ITS	CP049713.1	99.85	0.0	100	691
		18S	MK292129.1	99.82	0.0	100	553
		SCAR	CP049710.1	94.13	1e-169	93	458
8-3	<i>H.glycines</i>	ITS	CP049713.1	99.85	0.0	100	690
		18S	MK292129.1	100	0.0	100	793
		COXIII	HM640930.1	98.80	6e-120	100	249
18-1,3	<i>H.glycines</i>	ITS	CP049713.1	100	0.0	100	588
		18S	MK292129.1	99.68	5e-158	100	746
		COXIII	HM640930.1	97.95	8e-89	99	193
18-1,4	<i>H.glycines</i>	ITS	CP049713.1	99.79	0.0	97	493
		18S	MK292129.1	99.76	0.0	100	850
		SCAR	CP049709.1	95.24	0.0	99	431
18-2,4	<i>H.glycines</i>	ITS	CP049713.1	99.81	0.0	100	526
		18S	MK292129.1	99.75	0.0	100	829
		SCAR	CP049709.1	96.14	0.0	100	446
18-2,10	<i>H.glycines</i>	ITS	CP049713.1	100	0.0	100	681
		18S	MK292129.1	99.46	0.0	100	551
		COXIII	HM640930.1	96.35	1e-94	99	193
18-2,13	<i>H.glycines</i>	ITS	CP049713.1	100	0.0	100	588
		18S	MK292129.1	99.09	1e-163	99	897
		SCAR	CP049710.1	95.16	0.0	90	460
28-3,2	<i>H.glycines</i>	ITS	CP049713.1	99.52	0.0	100	639
		18S	MK292129.1	100	0.0	100	845
		COXIII	HM640930.1	98.54	1e-95	99	206

2.4.1.4. Reproduction Assays

Thirty and 60 days after planting female cysts were not observed on roots grown in pots with soil from the four positive SCN fields in 2017. Similarly, soybean inoculated with a small number of SCN juveniles (~ 200 eggs/juveniles) and grown in Cone-tainers did not have females on roots. Growing SCN susceptible genotype (S007-Y4, Syngenta, Guelph, ON) in Ontario soil with higher levels of SCN eggs (3,700 and 6,100 eggs/100 cm³ soil) and inoculating them with a higher number of eggs/juveniles (1,000 eggs/juveniles) recovered from these highly infested soils showed proliferation of females on roots.

2.4.2. Symptomatic Field in 2021

2.4.2.1. Cyst and Egg Densities

2.4.2.1.1. Composite Samples

The field area with soybean stunted and chlorosis had a moderate infestation level, with mean egg counts of $1,389 \pm 3.75$ eggs/100 cm³ soil (mean $\pm$ SEM, n=3).

2.4.2.1.2. Grid Samples

The affected field area had the highest numbers of cysts and eggs in the middle of the affected patch and grid sampled. The cysts and egg count of the patch ranged from 0 to 110 cysts and 0 to 7,797 eggs/100 cm³ soil (Fig. 2.4). Samples with no cysts occurred at the edge of the sampled grid and into the unaffected area of the field.

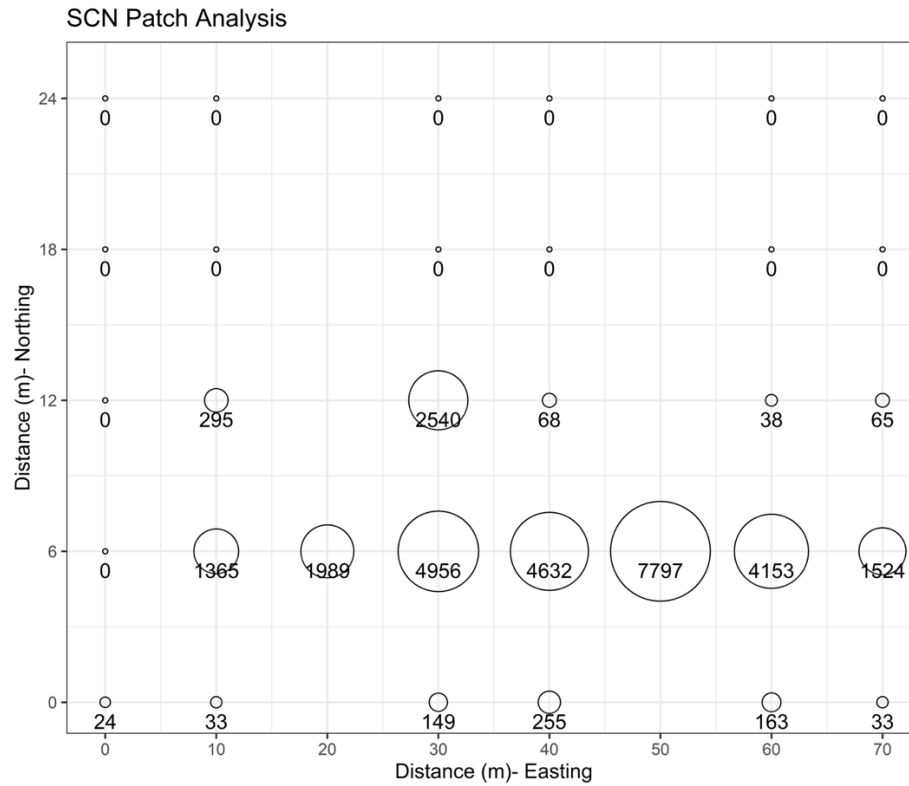


Fig. 2. 4. Distribution of SCN at the infested patch. Values within and below circles indicate the number of eggs/100 cm³ of soil. The symptomatic area covered ~ 50% of the sampled area (1680 m²). The size of the circles corresponds to the number of *H. glycines* eggs present in soil. Larger circles indicate higher densities of eggs, while smaller circles indicate lower densities.

2.4.2.2. Morphological Identification

Measurements of lemon-shaped cysts (mean $\mu\text{m} \pm \text{sd}$, $n=4$) were as follows: body length (L, 625 ± 54.0), body width (W, 468.8 ± 68.8), ratio L/W (1.34 ± 0.11), fenestral length (55.51 ± 5.765) and width (43.92 ± 4.454). These measurements all fell within the range for *H. glycines* given by Hirschmann (1956) and Subbotin et al. (2010).

2.4.2.3. Molecular Identification

2.4.2.3.1. Species-Specific PCR

Species-specific PCR of four cysts and three females produced expected single bands for universal primers ITS and NEM-18S, 850 and 900 bp, respectively. Additionally, expected amplicon fragments of 252 bp for COXIII and 477 bp for SCAR were also observed and consistent for *H. glycines* (Fig 2.5).

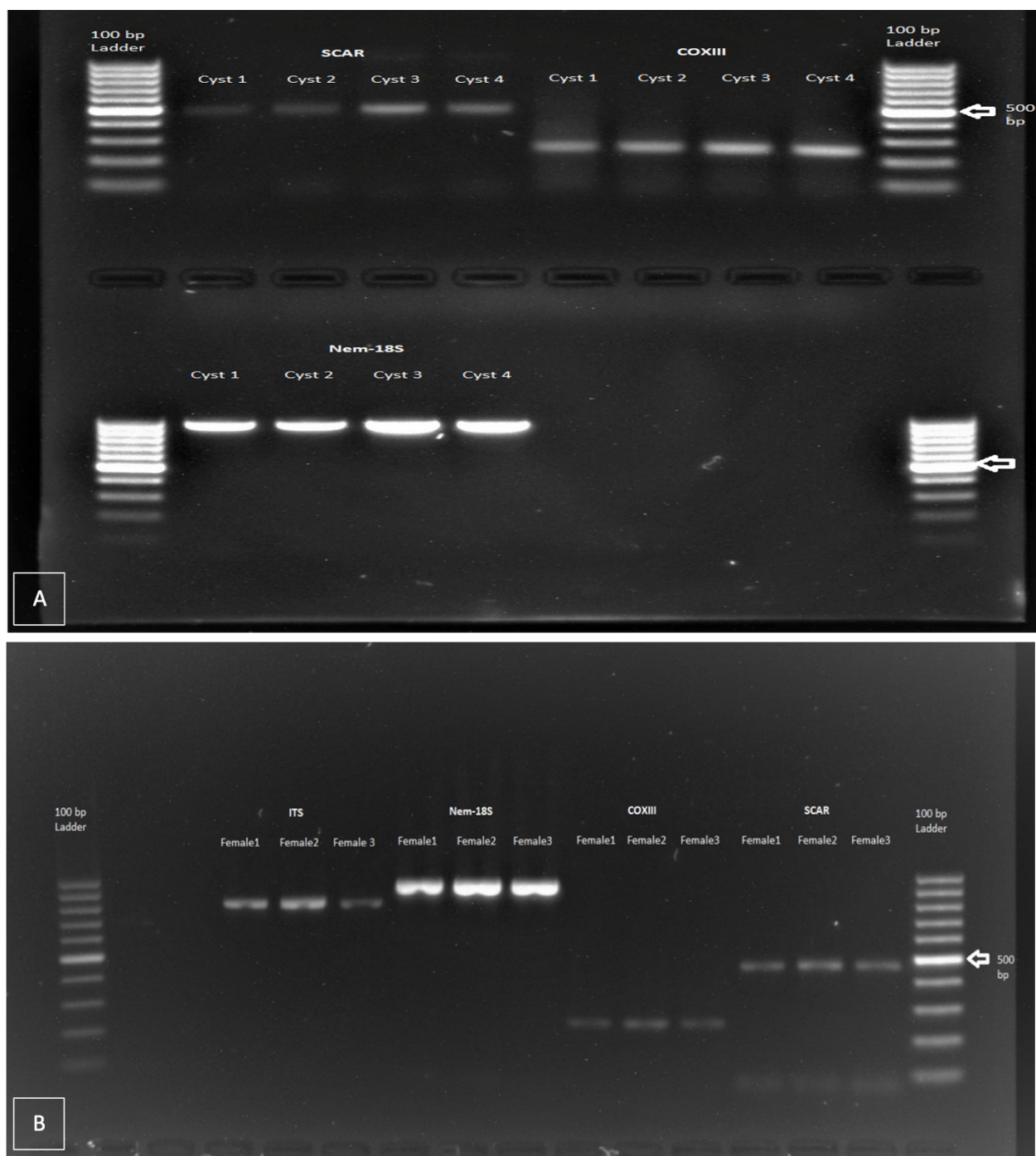


Fig. 2. 5. PCR amplification products obtained using universal primers (ITS (850 bp); Nem-18S (900 bp)), and *H. glycines* specific primers (COXIII (252 bp); SCAR (474 bp)) from DNA extracted from eggs of the A) single cyst recovered from the recently infected field soil, and B) females separated from roots of soybeans planted on the same soil in the growth chamber.

2.4.2.3.2. Sequencing

Three cysts from the affected area soil and three females from the Cone-tainer assay tested positive for *H. glycines* using species-specific PCR assay. Upon sequencing, all samples showed a strong match with *H. glycines*, with identity percentages ranging from 98-100% and E-values of zero for the ITS and 18S regions of Genbank accessions CP049713.1 and MK292129.1, respectively. They also had identity matches of 98-100% (COXIII) and >92% (SCAR) to accessions HM640930.1 and CP049709.1 of *H. glycines* in GenBank, respectively (Table 2.5). The sequences corresponding to the four target genes or regions are new additions to the GenBank (OR265520 - OR265521 ITS, OR265527 - OR265528 18S, OR291427 - OR291428 SCAR, and OR291424 COXIII).

Table 2. 5. NCBI BLAST results for the ITS, 18S, SCAR, and COXIII gene sequences of *H. glycines*' cysts recovered from the symptomatic field soil (2021), and females separated from roots of soybeans planted in the growth chamber.

Nematode individual	Match	Gene	Accession	Identity (%)	E-value	Query cover (%)	Read length (bp)
Cyst 1	<i>H.glycines</i>	ITS	CP049713.1	100	0.0	100	667
		18S	MK292129.1	99.82	0.0	100	551
		COXIII	HM640930.1	98.96	2e-90	99	197
Cyst 2	<i>H.glycines</i>	ITS	CP049713.1	98.61	0.0	95	689
		18S	MK292129.1	99.65	0.0	100	864
		COXIII	HM640930.1	100	1e-94	97	129
		SCAR	CP049709.1	95.24	2e-157	99	377
Cyst 3	<i>H.glycines</i>	ITS	CP049713.1	98.24	0.0	98	639
		18S	MK292129.1	99.32	0.0	98	752
		SCAR	CP049709.1	97.20	0.0	100	405
Female 1	<i>H.glycines</i>	ITS	CP049713.1	100	0.0	100	690
		18S	MK292129.1	100	0.0	100	830
		COXIII	HM640930.1	98.58	2e-99	99	212
		SCAR	CP049709.1	92.98	2e-168	99	418
Female 2	<i>H.glycines</i>	ITS	CP049713.1	100	0.0	100	676
		18S	MK292129.1	100	0.0	100	851
		COXIII	HM640930.1	99.51	9e-99	99	207
		SCAR	CP049709.1	92.92	7e-142	100	353
Female 3	<i>H.glycines</i>	ITS	CP049713.1	99.85	0.0	100	689
		18S	MK292129.1	100	0.0	97	869
		COXIII	HM640930.1	99.51	1e-98	90	227
		SCAR	CP049709.1	93.00	9e-100	98	262

2.4.2.4. Reproduction Assay

Soil from the symptomatic field in 2021 had much higher levels of *H. glycines* eggs than positive fields from the 2017 survey. With mean egg counts of 1,389 eggs/100cm³, females were abundant on the roots of soybean grown in pots (Fig. 2.6). Similarly, 1,000 eggs and juveniles added to soybean in Cone-tainers resulted in many females on roots. Further, plants were stunted and chlorotic in Cone-tainers inoculated with *H. glycines* (Fig. 2.6). Females were not found on plants from non-SCN soil or when not inoculated with *H. glycines*.

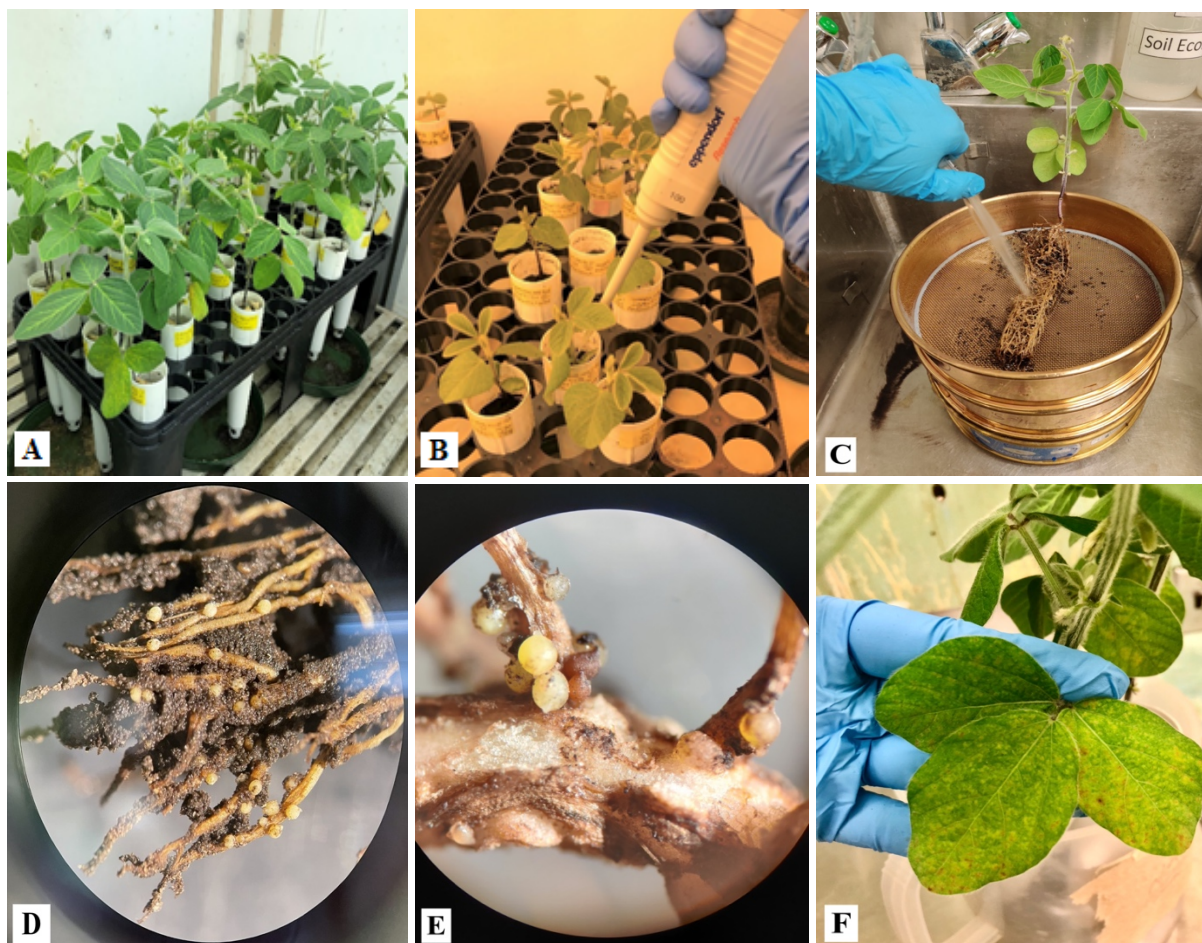


Fig. 2. 6. A) Soybean plants grown in cone-tainers under growth-chamber condition, B) The process of inoculation, C) Soybean roots washed over sieves to collect SCN females, D, E) The adult SCN females on infected soybean roots directly planted in soil from a field and inoculated with eggs/juveniles extracted from the field and F) Foliar symptoms of SCN infection on soybean planted directly in soil from a positive field and inoculated with *H. glycines* eggs/juveniles.

2.4.2.5. HG-typing

The SCN population of the field located in south-central Manitoba displayed varying reproduction abilities on soybean resistance lines. According to the Schmitt and Shannon scale (1992), indicator lines PI548402 (Peking), PI 88788, PI 90763, PI 437654, PI 209332, PI 89772, and Pickett are considered resistant to the population tested in this study as their FI were below 10% (Table 2.6). However, PI 548316 (Cloud) (indicator line #7), with FI between 10 and 30 (14.6 %), is moderately resistant for the SCN population. Based on these findings, the SCN population recovered is HG type 7 and race 3. HG type 7, being the population, has parasitism genes to the #7 PI line (PI 548316). The population is race 3 based on the fact that it had FI<10% on indicator lines Pickett, Peking, PI 88788, and PI 90763 (Table 2.6).

Table 2. 6. Virulence phenotypes of SCN population collected in Miami, Manitoba in 2021.

Indicator Line= Sources of Resistance	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Female index (FI)^a	FI≥10%
1) PI 548402 (Peking)	0	0	0	0	0	0%	- ^c
2) PI 88788	4	16	8	15	7	4.2%	-
3) PI 90763	0	0	1	0	0	0%	-
4) PI 437654	0	0	1	0	0	0%	-
5) PI 209322	1	5	10	2	3	1.8%	-
6) PI 89772	0	0	0	0	0	0%	-
7) PI 548316 (cloud)	34	33	41	32	X	14.6%	+ ^b
Pickett	1	3	8	3	0	1.6%	-

^aThe Female Index (FI) is calculated by taking the mean number of SCN females produced on an indicator line divided by the mean number of females on a standard susceptible (Lee 74) and multiplied by 100.

^bA + sign for races in the table above indicates a female index ≥ 10

^cA - sign indicates a female index < 10 .

2.4.3. SCN Distribution in Manitoba

H. glycines is currently present in five fields in Manitoba. Notably, these positive fields were distributed across five of the 18 rural municipalities in southern Manitoba, including all the areas sampled in both past surveys (which did not find the nematode) and the current study (Fig. 2.7). In the 2017 survey, *H. glycines* was detected in four fields, with egg numbers of 64, 21, 86, and 40 eggs /2.2 kg of soil (2.9, 0.95, 3.9, and 1.81 eggs/100 g soil) in the RMs of Norfolk-Treherne, Emerson-Franklin, Rhineland, and Montcalm, respectively. In the RM of Thompson, one field showed higher mean egg counts, reaching 7,797 eggs/100 cm³ soil in the central area of an affected headland.

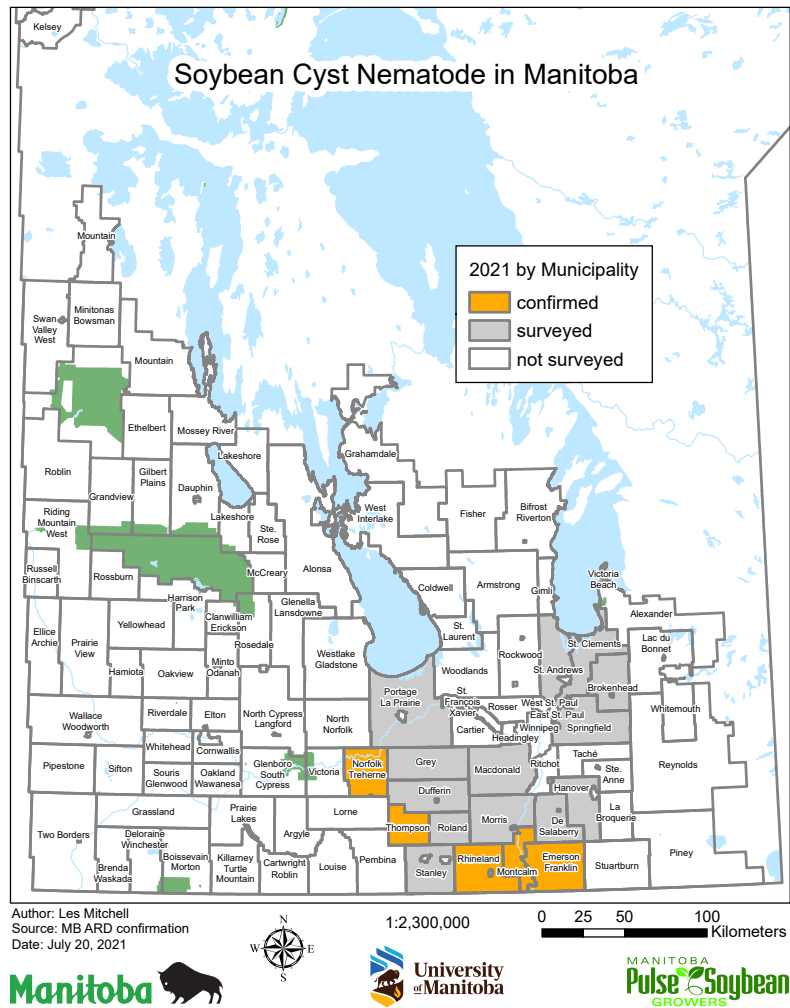


Fig. 2. 7. Map of rural municipalities where Soybean Cyst Nematode was found in soil samples surveyed from 2012 to 2021 in Manitoba (Mitchell 2021).

2.5. Discussion

This study presents the first reporting evidence of SCN in commercial soybean fields in Manitoba. Morphological measurements, two species-specific PCR assays, and sequences of four DNA genes or regions provided consensus of *H. glycines* being present in five fields in Manitoba. One field with a moderate density of *H. glycines* eggs and showing symptoms of SCN in soybean also confirmed the nematode was capable of reproduction on a susceptible variety in growth rooms assays.

Previous surveys for *H. glycines* in Manitoba were conducted in 2012 and 2013 (Madani et al. 2023) and in 2015 (unpublished) across 76 commercial soybean fields, and did not find the nematode. However, in our present study, as soybean acreage in Manitoba has expanded and the number of years soybean has grown in fields has increased, SCN is now present and moreover, above-ground symptoms are apparent. It is expected that the prevalence and severity of disease by SCN will continue to rise as more years of soybean cultivation accumulate in these fields.

Here, *H. glycines* was found in five fields. During the 2017 survey, *H. glycines* was present in four fields, with very low egg numbers (less than four eggs/100 g soil) in the RMs of Norfolk-Treherne, Emerson-Franklin, Rhineland, and Montcalm, respectively. A field in the RM of Thompson showed higher mean egg counts ($\sim 7,000$ eggs/100 cm³ soil) in the centre of a symptomatic headland area.

According to the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA), the risk level is considered low when the nematode eggs count in 100 cm³ of soil is below 1,000. In such cases, susceptible varieties may experience yield losses ranging from 0 to 20% under Ontario conditions. To minimize this risk, it is recommended to either grow a resistant variety or rotate the field with a non-host crop such as corn or wheat. In fields with a moderate to high-risk level (1,000

to 10,000 eggs/100 cm³ soil), potential yield losses can range from 20 to 50%. SCN-resistant varieties, particularly those containing the most common source of resistance in available cultivars, PI88788, are likely to experience yield loss in these moderate to high-risk infected soils. In situations where the nematode egg count exceeds 10,000 eggs/100 cm³ soil, it is advisable to rotate to non-host crops for two years. Subsequently, a soil sample should be taken to assess whether the nematode populations have adequately declined to enable the cultivation of soybeans (Welacky and Tenuta 2014).

It is important to note that the positive fields in Manitoba were geographically distant from each other, suggesting that there may be other fields not included in this study where SCN could potentially be present. Three positive fields in the rural municipalities of Rhineland, Emerson-Franklin, and Montcalm, located adjacent to the North Dakota or Minnesota border, were 27, 17 and 16 km, respectively, from the Red River border crossing and an area affected by spring flooding. This suggests a possible northerly dispersal of SCN through flooding. The positive field in the rural municipality of Norfolk-Treherne is located 138 km away from the Red River border crossing and is unaffected by floodwater from the United States. It receives river water flowing eastward from Saskatchewan, not from the south by way of the Red River, suggesting that the dispersal of *H. glycines* likely occurred through the introduction of infested soil. Similarly, the symptomatic field in the RM of Thompson in 2021 did not have water courses flowing north from the U.S. and is located 85 km away from the Red River border. The translocation means introducing *H. glycines* in the fields not associated with the flooding of the Red River can be many. These include the introduction of the nematode from used farm equipment in soil on farm seeding and tillage equipment, and farm vehicles (pickup trucks, grain trucks, tractors) obtained from the United States. Noteworthy, the most recently used farm equipment has been used on the

symptomatic field was a planter purchased from the U.S. and employed for the 2021 soybean growing year. Indeed, the affected area of the field was located at a machinery entrance.

The observation of the symptomatic field in early July, 2021, followed typical conditions for the manifestation of SCN in a new region (Pembina Valley, south-central Manitoba). The affected area was by an entranceway where soil on farm vehicles and machinery could introduce soil containing *H. glycines* cysts. Soil conditions were extremely dry due to only 197 mm of total precipitation having occurred from October through July compared to the expected normal of 343 mm (Carman, Manitoba, ID: 5020551) (Environment and Climate Change Canada (ECCC) 2023).

The dry conditions in Manitoba ranged from moderate to severe throughout May, June, and July 2021, reaching extreme drought (D3) or exceptional drought (D4) conditions in July, affecting nearly the entire region (Manitoba government 2021). According to data from Environment and Climate Change Canada (2023), the total rainfall in July was 61.9 mm less than the monthly normal (78.6 mm) for the weather station in the region.

Moreover, the soil from the affected field has low water holding capacity as it is mapped to the Willowcrest Series (Michalyna et al. 1988) of coarse texture, fine sand or loamy fine sand (Manitoba Agriculture, Food and Rural Initiatives 2010). By observation, the SCN female-laden root systems in the affected area were less extensive than in the non-affected field area. Together, soil of low water holding capacity, drought and compromised root system by SCN culminated into manifestation of SCN disease symptoms.

During dry periods, particularly in sandy soils, the likelihood of yield reduction due to nematode infestation rises significantly. Welacky and Tenuta (2014) reported that in sandy soils, a moderate level of infestation (1,000 eggs/100 cm³ soil) has the potential to cause yield losses between 20% and 50% in susceptible soybean varieties, while in fine-textured soils, an equivalent yield loss

potential occurs with a higher egg density in the soil (2,000 eggs/100 cm³ soil). It is important to note that both heavy and light-textured soils are equally susceptible to nematode establishment. However, the interaction between moisture stress and nematode damage becomes more pronounced in lighter textured soils compared to heavier ones due to the drainage characteristics of finer-textured soils, which can create anaerobic conditions hindering nematode activity (Vrain and Keng 1986).

Visitation to the symptomatic field in early September 2021, showed the symptomatic area to be extremely weed infested at its centre and soybean at the fringes in the early reproductive phase, foliage green and non-chlorotic and the canopy closed compared to the rest of the field having completed reproduction phase (Fig. 2.8). At harvest mid-October, the previously symptomatic area did not complete the reproductive phase and did not yield due to SCN infestation. Precipitation from July through October was near normal, 191 mm compared to the expected 246 mm (Carman U of M CS, Manitoba, ID: 502I001) (ECCC 2023). A recovery in SCN disease symptoms is not unusual with abundant soil moisture (Johnson et al. 1994). However, the recovery did not result in soybean yield by the onset of frost conditions on October 20, 2021.

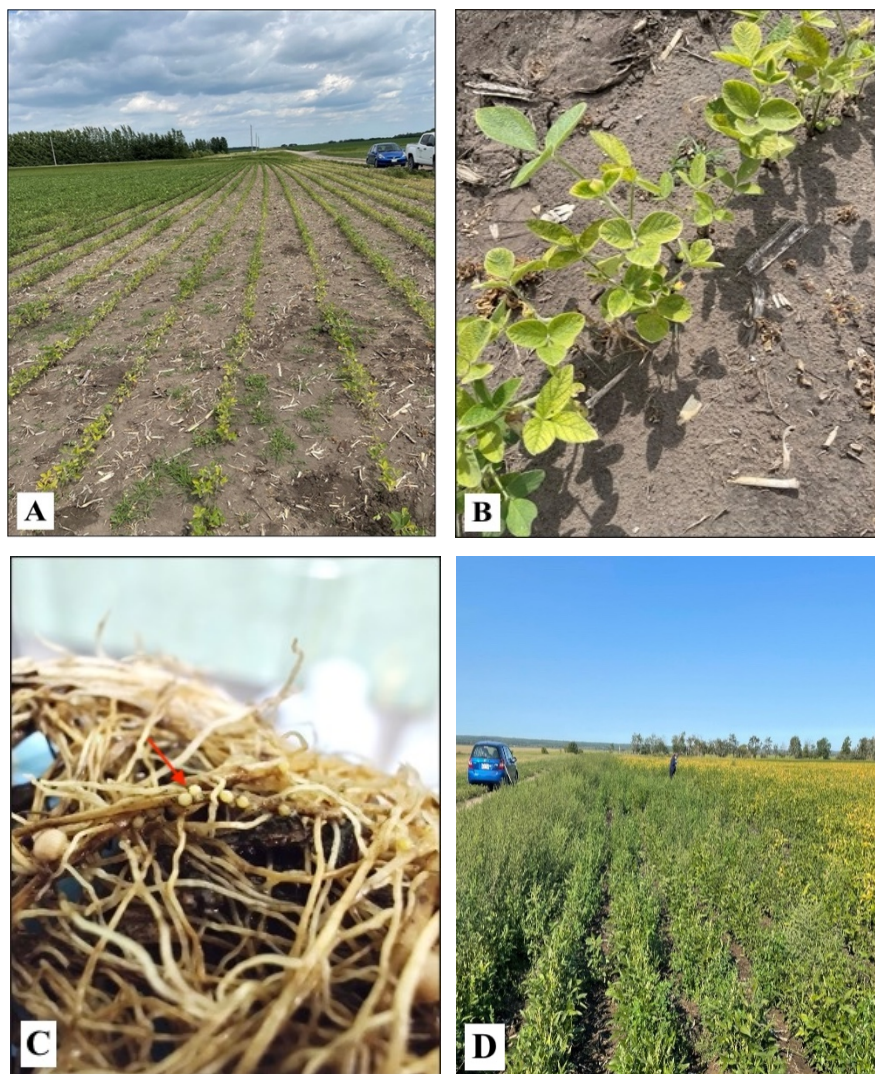


Fig. 2. 8. Symptomatic soybean field in 2021, A) Symptoms of SCN damage in the headland area (July 8, 2021), B) Stunted and chlorotic soybean plants caused by SCN, C) The SCN females on infected soybean roots randomly collected from an affected area of the field, D) Second visitation from the field (September 3, 2021) showing significant weed infestation and soybean in the early reproductive phase in the affected area, compared to the rest of the field progressing towards maturity.

The HG-type analysis of the soil samples collected from the field in the RM of Thompson indicated the population of SCN as HG type 7 and race 3. This is not surprising since race 3 is the most prevalent race in the northern states of the U.S. (Yu 2011). The reproduction of SCN was greater (>10%) on the PI 548316-derived soybean cultivar compared to PI 209332 and PI 88788, and the population was unable to reproduce on any other differential lines. These results align with previous studies conducted in North America, particularly in states adjacent to Manitoba, such as North Dakota (Chowdhury et al. 2017) and Minnesota (Zheng et al. 2006), where PI 548316 (indicator line 7) was found to be a suitable host for SCN populations there. Similar findings were in South Dakota (Acharya et al. 2016), Kentucky (Hershman et al. 2008), and Iowa (Beeman et al. 2016), where PI 548316 (#7) also was found to be a suitable host for local populations of SCN.

The correlation between the pathogenicity of our tested SCN population on PI 548316, PI 209332 and PI 88788 aligns with findings from Colgrove and Niblack (2008). Their research indicated that the breakdown of resistance to PI 548316 (indicator line 7) is associated with the eventual breakdown of resistance in PI 88788 (indicator line 2) and PI 209332 (indicator line 5). Likewise, FI on Peking, PI 90763, and PI 89772 are highly correlated. These findings suggest that in Manitoba, there is evidence of the initiation of resistance development in Manitoba SCN populations.

In North America, planting resistant cultivars and implementing crop rotation with non-host crops are the main practices for SCN management (Niblack and Chen 2004). However, the effectiveness of resistant soybean varieties relies on genetic variability, that is, the presence of new virulent populations or HG types of the SCN population (Niblack et al. 2002). Based on the findings of Zheng and Chen (2011) in Minnesota, the pathogenicity of the SCN on PI 88788 had increased over five years. As a result, original race 3 (HG type 0 and 7) changed to race 1 (HG

type 2.7 or 2.5.7) either in monoculture or rotation with other soybean cultivars and corn. This is consistent with recent studies that have reported that HG type 2.5.7 is the most common HG type for SCN populations in U.S. counties. This HG type which can prevail the resistance in PI88788-derived cultivars is also reported in Essex County in Ontario (Tenuta and Welacky 2016). To overcome that resistance-breaking, alternately use of cultivars with different sources of SCN resistance is recommended (Tylka 1994).

SCN doesn't only affect soybean but also other members of the *Leguminosae* family, such as dry beans (*P. vulgaris*) (Abawi and Jacobsen 1984). Previous studies conducted in the U.S. and Canada have shown that the SCN can infect dry beans, causing yield losses in both field and controlled environments (Poromarto et al. 2009; Yan et al. 2017; Zhang 2018; Katsande 2019). Poromarto et al. conducted experiments in 2009 and found that the *H. glycines* could reproduce on the roots of various commercially grown or tested dry bean cultivars in North Dakota and northern Minnesota. They reported that all 24 dry bean cultivars in the four bean market classes were susceptible to the development of *H. glycines* females. In 2016, the presence of SCN was observed for the first time in a commercial dry bean field in Minnesota, where patches of stunted and yellowish plants were noticed in dark red kidney beans (Yan et al. 2017). According to Zhang (2018) and Katsande (2019), dry beans in infested research sites in Canada were found to be susceptible to SCN infection. However, it was not until August 2018 that the first occurrence of SCN infection with visible above-ground symptoms was reported in a commercial dry bean field in Bruce County, Ontario, Canada (Trueman et al. 2022). Canada's largest producer of dry beans is Manitoba, accounting for 42% of the country's production in the 2021-2022 crop years, followed by Ontario with 37% of total production (Goodwin and McDougall 2022). Consequently, the presence of SCN in commercial dry bean fields poses a significant risk to the industry in Manitoba.

A range of practices can be employed to manage SCN, such as rotating soybeans with a non-host crop, growing SCN-resistant soybean varieties, using cover crops, reducing tillage, controlling host weed species, and using a nematicide seed treatments (Concibido et al. 2004; Conley et al. 2011; Grabau 2013; Li et al. 2011). These integrated pest management methods rely on accurate data regarding the extent of SCN infestation in order to be effectively implemented. The most valuable diagnostic tool for managing SCN is determining the density of *H. glycines* eggs per unit volume of soil, as it provides crucial information for decision-making. Therefore, continuous surveys to monitor the spread and abundance of SCN are of significant importance.

Of note, the manifestation of SCN symptoms in the symptomatic field in 2021 can be also linked to various factors within the crop rotation history. Firstly, in 2021, the field was in cereal-grain legume-oilseed rotation, with 2012 being the first year soybean was grown. Since 2009, the rotation included susceptible crops like soybean (cultivated in 2012, 2015, and 2017) and navy bean (in 2019). Importantly, all grown soybean varieties in this field, including S007-A2XS in 2021, were non-SCN resistant.

Before 2009, the crop rotation included a one-in-four-year dry bean crop. In addition to the susceptible crops, non-susceptible crops such as canola (cultivated in 2009, 2011, and 2014), grain corn (in 2010, 2013, 2016, and 2020), and spring wheat (in 2018) were also part of the crop rotation. However, the consistent cultivation of susceptible crops over the years likely contributed to the establishment of SCN population in this field. This emphasizes the importance of understanding crop rotations and the selection of resistant varieties in effectively managing SCN.

Farmers in Manitoba need to be aware of the presence of SCN and take measures to prevent its spread and population increase. SCN can spread rapidly through water and wind dispersal, as well as through the movement of farm equipment and animals, such as birds (Riggs 1977; Niblack and

Tylka 2016). Once introduced into a field, it can take 5-9 years for SCN to establish and become a persistent problem in an area (Davis and Tylka 2021). From 2017 to 2020, there was an increase in the number of counties affected by SCN infestations in the United States and Canada. In the U.S., 55 new counties across 11 states reported SCN infestations during this period. Similarly, in Canada, SCN was found in 24 new counties and rural municipalities in the provinces of Manitoba, Ontario, and Quebec. These findings indicate a consistent expansion of SCN distribution throughout both countries. It is inevitable that this pest will continue to spread within soybean-producing areas in the future (Tylka and Marett 2021). With SCN now detected in five rural municipalities in Manitoba, along with its substantial presence in one particular field causing yield reductions in the affected area, it is crucial to continue scouting and soil sampling to detect new SCN infestations and minimize the risk of transferring the pest between fields. This serves as the initial measure in the efficient management of this pathogen.

Considering the current low to moderate population densities of SCN in Manitoba, we recommend that farmers utilize SCN-resistant soybean varieties and practice crop rotations with non-host crops such as barley, oats, corn, wheat, and canola. Though the percentage of SCN reproduction on PI 88788 in the population tested in this study was not as high as has been reported in the northern states bordering Canada, it still likely shows the start of SCN population build-up on PI 88788, the most widely used source of resistance in more than 90% of available cultivars (Tylka and Mullaney 2015; Patil et al. 2019). So, it can be said that soybean cultivars with the PI 548402 (Peking), PI 90763, PI 437654, and PI 89772 sources of SCN resistance would be most effective for managing the pest in the currently infested area. Particularly PI437654 has been shown to be resistant to all HG types due to containing a different resistant background than that of PI88788 or Peking (Patil et al. 2019, Diers and Arelli 1999). The Seed Manitoba guide

(Anonymous 2023) listed 76 herbicide-resistant soybean varieties for maturity groups 0 to 3 in eastern and western Manitoba, of which 38 are resistant to SCN. However, the majority of the available varieties rely on resistance genes sourced from PI 88788. This underscores the importance of introducing a wider range of the source of resistance in the existing soybean varieties. The findings from this study will provide valuable insights into the current situation of soybean cyst nematode in Manitoba and aid in determining the optimal practices for managing this pest.

2.6. References

- Abawi GS, Jacobsen BJ. 1984. Effect of initial inoculum densities of *Heterodera glycines* on growth of soybean and kidney bean and their efficiency as hosts under greenhouse conditions. *Phytopathology*. 74(12):1470-1474.
- Acharya K, Tande C, Byamukama E. 2016. Determination of *Heterodera glycines* virulence phenotypes occurring in South Dakota. *Plant Dis*. 100(11):2281-2286.
- Al-Banna L, Williamson V, Gardner SL. 1997. Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. *Mol Phylogenet Evol*. 7(1):94-102.
- Allen TW, Bradley CA, Sisson AJ, Byamukama E, Chilvers MI, Coker CM, Collins AA, Damicone JP, Dorrance AE, Dufault NS, Esker PD. 2017. Soybean yield loss estimates due to diseases in the United States and Ontario, Canada, from 2010 to 2014. *Plant Health Prog*. 18(1): 19-27.
- Anderson TR, Welacky TW, Olechowski HT, Ablett G, Ebsary BA. 1988. First report of *Heterodera glycines* on soybeans in Ontario, Canada. *Plant Dis*. 72:453.
- Anonymous 2022. Summary of soybean cyst nematode survey: 2013-2021. North Dakota State University. [Accessed 2023 Dec 19].
<https://www.ndsu.edu/agriculture/ag-hub/ag-topics/crop-production/crop-pest-report/plant-pathology/summary-soybean-cyst-nematode>
- Anonymous 2023. Variety selection and growers source guide. Seed Manitoba. [Accessed 2023 July 26].
<https://www.seedmb.ca/wp-content/uploads/2022/12/Seed-Manitoba-2023-Digital-Edition.pdf>
- Baldwin JG, Mundo -Ocampo M. 1991. *Heteroderinae*, cyst- and non-cyst forming nematodes. In: Nickle WR, editor. *Manual of Agricultural Nematology*. New York (NY): Marcel Dekker; p. 275-362.

- Beeman AQ, Harbach CJ, Marett CC, Tylka GL. 2016. Soybean cyst nematode HG Type test results differ among multiple samples from the same field but the management implications are the same. *Plant Health Prog.* 17(3):160-162.
- Bradley CA, Biller CR, Nelson BD. 2004. First report of soybean cyst nematode (*Heterodera glycines*) on soybean in North Dakota. *Plant Dis.* 88(11):1287-1287.
- Chen S. 2011. Soybean cyst nematode management guide. University of Minnesota Extension. [Accessed 2021 Sep 8].
<https://extension.umn.edu/soybean-pest-management/soybean-cyst-nematode-management-guide#spread-in-minnesota-1506960>
- Chowdhury I, Yan G, Plaisance A. 2017. Characterizing virulence phenotypes of soybean cyst nematode (*Heterodera glycines*) in infested fields of North Dakota. *Phytopathology.* 107(1):3-4.
- Colgrove AL, Niblack TL. 2008. Correlation of female indices from virulence assays on inbred lines and field populations of *Heterodera glycines*. *J Nematol.* 40(1):39-45.
- Concibido VC, Diers BW, Arelli PR. 2004. A decade of QTL mapping for cyst nematode resistance in soybean. *Crop Sci.* 44(4):1121-1131.
- Conley SP, Gaska JM, Pedersen P, Esker P. 2011. Soybean yield and *Heterodera glycines* response to rotation, tillage, and genetic resistance. *Agron J.* 103(6):1604-1609.
- Correia FJ, Abrantes IM. 1997. An improved technique for mounting *Heterodera* cysts for light microscopy. *Nematologica.* 43(6):507-509.
- Davis EL, Tylka GL. 2021. Soybean cyst nematode disease. The plant health instructor. [Accessed 2022 Mar 10].

<https://www.apsnet.org/edcenter/disandpath/nematode/pdlessons/Pages/Soybeancystnematode.aspx>

- Diab KL. 1968. Occurrence of *Heterodera glycines* from the Golden Island, Giza, UAR. Nematologica. 14(1):148.
- Diers BW, Arelli PR. 1999. Management of parasitic nematodes of soybean through genetic resistance. Proceedings of the 6th world soybean research conference; Chicago (IL); p. 4-7.
- Donn S, Griffiths BS, Neilson R, Daniell TJ. 2008. DNA extraction from soil nematodes for multi-sample community studies. Appl Soil Ecol. 38(1):20-26.
- [ECCC] Environment and Climate Change Canada. 2023. 1981-2010 climate normals and averages. Canadian climate normals. [Accessed 2023 June 10].
https://climate.weather.gc.ca/climate_normals/results_1981_2010_e.html?searchType=stnProv&lstProvince=MB&txtCentralLatMin=0&txtCentralLatSec=0&txtCentralLongMin=0&txtCentralLongSec=0&stnID=3582&dispBack=0
- Eroshenko AS, Kazachenko IP, Volkova TV. 1990. Distribution and damage induced by the soybean cyst nematode in the Primorsky territory. Zashita rastenii na Dalnem Vostoke. Vladivostok, Russia DVO AN SSSR; p. 90-100.
- Evans K, Rowe JA. 1998. Distribution and economic importance. In: Sharma SB, editor. The cyst nematodes. The Netherlands: Springer; p. 1-30.
- Faghihi J, Ferris VR. 2006. Soybean cyst nematode. Department of Entomology. Purdue University. [Accessed 2023 Feb 10].
<https://ag.purdue.edu/departement/entm/extension/index.html>
- Fenwick DW. 1940. Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. J Helminthol. 18(4):155-172.

- Floyd RM, Rogers AD, Lambshhead PJD, Smith CR. 2005. Nematode-specific PCR primers for the 18S small subunit rRNA gene. *Mol Ecol Notes*. 5(3):611-612.
- Gerdemann JW. 1955. Relation of a large soil-borne spore to phycomycetous mycorrhizal infections. *Mycologia*. 47:619-632.
- Goodwin J, McDougall T. 2022. Canada: Outlook for principal field crops. Agric. Agri-Food Can. [Accessed 2023 Jan 15].
https://agriculture.canada.ca/sites/default/files/documents/2022-07/Canada%20Outlook%20for%20Principal%20Field%20Crops_202207.pdf
- Grabau ZJ. 2013. Management strategies for control of soybean cyst nematode and their effect on the nematode community [master's thesis]. Minneapolis (MN): University of Minnesota.
- Hershman DE, Heinz RD, Kennedy BS. 2008. Soybean cyst nematode, *Heterodera glycines*, populations adapting to resistant soybean cultivars in Kentucky. *Plant Dis*. 92(10):1475-1475.
- Hirschmann H. 1956. Comparative morphological studies on the soybean cyst nematode, *Heterodera glycines* and the clover cyst nematode, *H. trifolii* (Nematoda: *Heteroderidae*). *Proc Helminthol Soc Wash*. 23(2):140-151.
- Hori S. 1915. Phytopathological notes. Sick soil of soybean caused by a nematode. *J Plant Protect*. 2:927-930.
- Ichinohe M. 1952. On the soybean nematode, *Heterodera glycines* n. sp., from Japan. *Magazine of Appl Zool*. 17:1-4.
- Johnson AB, Scott HD, Riggs RD. 1994. Response of soybean in cyst nematode-infested soils at three soil-water regimes. *J Nematol*. (26):329-335.

- Katsande TT. 2019. Evaluating seed treatments for the management of soybean cyst nematode (*Heterodera glycines* Ichinohe) in dry bean (*Phaseolus vulgaris* L.) [dissertation]. Guelph (ON): University of Guelph.
- Kumari S, Subbotin SA. 2012. Molecular characterization and diagnostic of stubby root and virus vector nematodes of the family *Trichodoridae* (Nematode: *Triplonchida*) using ribosomal RNA genes. *Plant Pathol.* 61(6):1021-1031.
- Li J, Todd TC, Lee J, Trick HN. 2011. Biotechnological application of functional genomics towards plant-parasitic nematode control. *Plant Biotechnol J.* 9(9):936-944.
- Liu X, Li J, Zhang D. 1997. History and status of soybean cyst nematode in China. *Int J Nematol.* 7(1):18-25.
- Maafi ZT, Geraert E, Kheiri A, Sturhan D. 1999. Occurrence of soybean cyst nematode *Heterodera glycines* Ichinohe, 1952 in Iran. *Iran J Plant Pathol.* 35:63-64.
- MacDonald DH, Noel GR, Lueschen WE. 1980. Soybean cyst nematode, *Heterodera glycines*, in Minnesota. *Plant Dis.* 64(3):319-321.
- Madani M, Kyndt T, Colpaert N, Subbotin, SA, Gheysen G, Moens M. 2007. Polymorphism among sugar beet cyst nematode *Heterodera schachtii* populations as inferred from AFLP and ITS-rDNA gene analyses. *Russ J Nematol.* 15(2):117-128.
- Madani M, Tenuta M, Castillo P. 2023. The occurrence of cyst nematodes in agricultural fields of southern Manitoba. *Can J Plant Pathol.* doi:[10.1080/07060661.2023.2293128](https://doi.org/10.1080/07060661.2023.2293128).
- Madani M, Tenuta M, Welacky T, Tenuta A. 2013. Molecular identification of populations of the soybean cyst nematode (*Heterodera glycines*) from Ontario. Poster session presented at: CPS Annual Meeting; June 16-19; Edmonton, AB.

- Manachini B. 2000. First report of *Heterodera glycines* Ichinohe on soybean in Italy. Boll Zool Agrar Bachic. 32(3):261-267.
- Manitoba Agriculture, Food and Rural Initiatives. 2010. Soil Series Description. [Accessed 2023 Dec 18].
https://agrimaps.gov.mb.ca/agrimaps/extras/info/Soil_Series_Descriptions.pdf
- Manitoba Government. 2021. Water availability and drought conditions report. [Accessed 2022 May 11].
https://www.gov.mb.ca/sd/pubs/water/drought/2021/drought_conditions_report_july_2021.pdf
- Mendes ML, Dickson DW. 1993. *Heterodera glycines* found on soybean in Brazil. Plant Dis. 77:499-500.
- Michalyna W, Podolosky G, Jacques ES. 1988. Soils of the rural municipalities of Grey, Dufferin, Roland, Thompson and part of Stanley. Manitoba Department of Agriculture. Department of Soil Science. University of Manitoba. Soils Report No. D60. [Accessed 2023 Dec18].
https://sis.agr.gc.ca/cansis/publications/surveys/mb/mbd60/mbd60_report.pdf
- Mitchell L. 2021. Soybean Cyst Nematode in Manitoba. Manitoba Agriculture, Winnipeg, Manitoba. Canada. [Accessed 2021 Dec 15].
<https://www.gov.mb.ca/agriculture/crops/seasonal-reports/Pubs/soybean-cyst-nematode-map-2021.pdf>
- Mimee B, Gagnon AÈ, Colton-Gagnon K, Tremblay É. 2016. Portrait de la situation du nématode à kyste du soja (*Heterodera glycines*) au Québec (2013-2015). Phytoprotection. 96(1):33-42.
- Mimee B, Peng H, Popovic V, Yu Q, Duceppe MO, Tétreault MP, Belair G. 2014. First report of soybean cyst nematode (*Heterodera glycines* Ichinohe) on soybean in the province of Quebec, Canada. Plant Dis. 98(3):429-429.

- Muldoon JF, Hume DJ, Beversdorf WD. 1980. Effects of seed- and soil-applied *Rhizobium japonicum* inoculants on soybean in Ontario. Can J Plant Sci. 60(2):399-409.
- Mulvey RH. 1972. Identification of *Heterodera* cysts by terminal and cone top structures. Can J Zool. 50(10):1277-1292.
- Mulvey RH, Golden AM. 1983. An illustrated key to the cyst-forming genera and species of *Heteroderidae* in the western hemisphere with species morphometrics and distribution. J Nematol. 15(1):1-59.
- Nadler S, Felix MA, Frisse L, Sternberg P, De Ley P, Thomas WK. 1999. Molecular and morphological characterisation of two reproductively isolated species with mirror-image anatomy (Nematoda: *Cephalobidae*). Nematology. 1(6):591-612.
- Niblack TL. 2005. Soybean cyst nematode management reconsidered. Plant Dis. 89(10):1020-1026.
- Niblack TL, Chen SY. 2004. Cropping systems and crop management practices: biology and management of soybean cyst nematode. 2nd ed. Marceline (MO); P. 181-206
- Niblack TL, Arelli PR, Noel GR, Opperman CH, Orf JH, Schmitt DP, Shannon JG, Tylka, GL. 2002. A revised classification scheme for genetically diverse populations of *Heterodera glycines*. J Nematol. 34(4):279-288.
- Niblack TL, Heinz RD, Smith GS, Donald PA. 1993. Distribution, density, and diversity of *Heterodera glycines* in Missouri. J Nematol. 25(4):880-886.
- Niblack TL, Tylka GL. 2016. Soybean cyst nematode management guide. 5th ed. Columbia, University of Missouri: North Central Soybean Research Program Publication. [Accessed 2021 Aug 26].

http://soybeanresearchinfo.com/pdf_docs/SCNGuide_5thEd.pdf

- Noel GR. 1985. The soybean cyst nematode. In: Lamberti F, Taylor CE, editors. Cyst Nematodes. New York (NY): Plenum Press. 121:257-268.
- Noel GR. 1992. History, distribution, and economics: biology and management of the soybean cyst nematode. St. Paul (MN). American Phytopathological Society; p. 1-13.
- Noel GR, Edwards DI. 1996. Population development of *Heterodera glycines* and soybean yield in soybean-maize rotations following introduction into a noninfested field. J Nematol. 28(3): 335-342.
- Okada H, Oba H. 2008. Comparison of nematode community similarities assessed by polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE) and by morphological identification. Nematology. 10(5):689-700.
- Ou S, Peng D, Liu X, Li Y, Moens M. 2008. Identification of *Heterodera glycines* using PCR with sequence characterised amplified region (SCAR) primers. Nematology. 10(3):397-403.
- Patil GB, Lakhssassi N, Wan J, Song L, Zhou Z, Klepadlo M, Vuong TD, Stec AO, Kahil SS, Colantonio V, Valliyodan B. 2019. Whole-genome re-sequencing reveals the impact of the interaction of copy number variants of the rhg1 and Rhg4 genes on broad-based resistance to soybean cyst nematode. Plant Biotechnol J. 17(8):1595-1611.
- Poromarto SH, Nelson BD. 2009. Reproduction of soybean cyst nematode on dry bean cultivars adapted to North Dakota and northern Minnesota. Plant Dis. 93(5):507-511.
- Powers TO, Todd TC, Burnell AM, Murray PCB, Fleming CC, Szalanski AL, Adams BA, Harris TS. 1997. The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. J Nematol. 29(4):441-450.

- Riggs RD. 1977. Worldwide distribution of soybean-cyst nematode and its economic importance. *J Nematol.* 9(1):34-39.
- Roach BA, Spears JF, Kulik MM, Gingrich SF. 1968. The golden nematode handbook: survey, laboratory, control, and quarantine procedures. US Agricultural Research Service. No; p. 351-355.
- Sato E, Min YY, Shirakashi T, Wada S, Toyota K. 2007. Detection of the root-lesion nematode, *Pratylenchus penetrans* (Cobb), in a nematode community using real-time PCR1. *Jpn J Nematol.* 37(2):87-92.
- Schmitt DP, Shannon G. 1992. Differentiating soybean responses to *Heterodera glycines* races. *Crop Sci.* 32(1):275-277.
- Seinhorst JW. 1974. Separation of *Heterodera* cysts from dry organic debris using ethanol. *Nematologica.* 20:367-369.
- Soy Canada. 2017. 10 million acres of opportunity. [Accessed 2022 Dec 8]. <http://soycanada.ca/wp-content/uploads/2017/04/10-Million-Acres-of-Opportunity-Discussion-Paper.pdf>
- Soy Canada. 2021. Canadian Soybean Production. [Accessed 2023 Feb 5]. <http://soycanada.ca/statistics/production/>
- Subbotin SA, Mundo-Ocampo M, Baldwin JG. 2010. Systematics of cyst nematodes (Nematoda: *Heteroderinae*): Nematology Monographs and Perspectives. In: Hunt DJ, Perry RN, editors. Leiden, The Netherlands: Brill. 8:1-351.
- Subbotin SA, Peng D, Moens M. 2001. A rapid method for the identification of the soybean cyst nematode *Heterodera glycines* using duplex PCR. *Nematology.* 3(4):365-371.

- Subbotin S, Waeyenberge L, Moens M. 2000. Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: *Heteroderidae*) based on the ribosomal DNA-RFLP. *Nematology*. 2(2):153-164.
- Szalanski AL, Sui DD, Harris TS, Powers TO. 1997. Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *J Nematol*. 29(3):255-267.
- Tenuta A, Welacky T. 2016. Resisting soybean cyst nematode. [Accessed 2022 Oct 15]. <https://www.topcropmanager.com/resisting-soybean-cyst-nematode-18324/>
- Toyota K, Shirakashi T, Sato E, Wada S, Min YY. 2008. Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. *Soil Sci Plant Nutr*. 54(1):72-76.
- Trueman C, Blauel T, Abaya A, Zhang K, Gillard C. 2022. First report of *Heterodera glycines* infecting commercial dry beans (*Phaseolus vulgaris*) in Canada. *Can J Plant Sci*. 102(4):935-939.
- Tylka GL, Marett CC. 2014. Distribution of the soybean cyst nematode, *Heterodera glycines*, in the United States and Canada: 1954 to 2014. *Plant Health Prog*. 15(2):85-87.
- Tylka GL, Marett CC. 2021. Known Distribution of the Soybean Cyst Nematode, *Heterodera glycines*, in the United States and Canada in 2020. *Plant Health Prog*. 22(1):72-74.
- Tylka GL, Mullaney MP. 2015. Soybean cyst nematode-resistant soybeans for Iowa. Ext Publ. PM, 1649. Iowa State University Extension, Ames.
- Vrain TC, Keng JC. 1986. Application of nonvolatile nematicides through a trickle irrigation system to control *Pratylenchus penetrans* in raspberries. *Can J Plant Pathol*. 8(1):97-101.

- Wang J, Niblack TL, Tremain JA, Wiebold WJ, Tylka GL, Marett CC, Noel GR, Myers O, Schmidt ME. 2003. Soybean cyst nematode reduces soybean yield without causing obvious aboveground symptoms. *Plant Dis.* 87(6):623-628.
- Welacky T, Tenuta A. 2014. Fall is the time to sample for Soybean Cyst Nematode. *Agriculture and Agri-Food Canada and OMAFRA*. [Accessed 2023 Apr 21]. <http://omaf.gov.on.ca/english/crops/field/news/croptalk/2019/ct-1119a5.htm>
- Wouts WM, Baldwin JG. 1998. *Taxonomy and identification: the cyst nematodes*. Dordrecht: Springer; p. 83-122.
- Yan GP, Plaisance A, Chowdhury I, Baidoo R, Upadhyaya A, Pasche J, Markell S, Nelson B, Chen S. 2017. First report of the soybean cyst nematode *Heterodera glycines* infecting dry bean (*Phaseolus vulgaris* L.) in a commercial field in Minnesota. *Plant Dis.* 101(2):391-391.
- Yokoo T. 1936. Host plants of *Heterodera schachtii* Schmidt and some instructions. *Korea Agricultural Experiment Station Bulletin*. 8:47-174.
- Yu Q. 2011. Soybean cyst nematode (*Heterodera glycines* Ichinohe). In: El-Shemy H, editor. *Soybean physiology and biochemistry*. New York (NY): InTech; p. 461-474.
- Zhang K. 2018. *Assessment of Dry Bean (Phaseolus vulgaris L.) Susceptibility to Soybean Cyst Nematode (Heterodera glycines Ichinohe) and the effects of biological and chemical control in a controlled environment [dissertation]*. Guelph (ON): University of Guelph.
- Zheng J, Li Y, Chen S. 2006. Characterization of the virulence phenotypes of *Heterodera glycines* in Minnesota. *J Nematol.* 38(3):383-390.
- Zheng JW, Chen SY. 2011. Estimation of virulence type and level of soybean cyst nematode field populations in response to resistant cultivars. *J. Entomol Nematol.* 3:37-43.

3.0 Development of a Molecular Test for the Quantification of Soybean Cyst Nematode (*Heterodera glycines* Ichinohe) in Soil

3.1. Abstract

Soybean Cyst Nematode (SCN), *Heterodera glycines* (Ichinohe 1952), is one of the most devastating disease/pest organisms of soybean worldwide. SCN is common in soybean fields in Ontario and increasingly so in Quebec. Its presence has recently been reported in Manitoba in 2019. Traditional methods of identifying and counting *H. glycines* eggs are laborious and require much training. This study aimed to develop an alternative molecular method for quantifying *H. glycines* eggs in soil. Naturally infested fields with *H. glycines* covering different soil types and SCN levels were sampled from southern Ontario and Manitoba. The extraction methods for cysts, eggs, and DNA from soil were optimized. SYBR Green I-based real-time quantitative polymerase chain reaction (qPCR) assays were developed using the previously reported species-specific primer set SCAR and a new species-specific primer set based on the COXIII gene. Calibration curves were established by adding different numbers of *H. glycines* eggs (10, 100, 500, and 1,000) in triplicate to suspension and autoclaved soil debris, in which the DNA was extracted using the PowerSoil DNA Isolation Kit. The microscopic egg counts in 21 naturally infested composite soil samples were compared to those estimated by qPCR using equations obtained from calibration curves. The calibration curves showed high linearity between the mean C_q values and the number of eggs added in both nematode suspensions and soil debris at all the inoculation levels for both primer sets. The melt curves confirmed the assay's species-specificity. There was a strong relationship between the egg counts quantified by the qPCR and the conventional method. The qPCR assay developed in this study indicates a sensitive, specific and rapid detection and quantification of *H. glycines* from field soil.

Keywords: Soybean Cyst Nematode (SCN), *Heterodera glycines*, Real-time qPCR, Species-specific primer.

3.2. Introduction

Soybean production worldwide is restricted by various abiotic and biotic stresses, including low temperature, water stress, nutrient deficiency, plant-parasitic nematodes, and disease (Hartman et al. 2011). Plant-parasitic nematodes, specifically, Soybean Cyst Nematodes (SCN), *Heterodera glycines*, play a significant impact on soybean yield losses among these factors (Allen et al. 2017). *H. glycines* was initially detected in Canada in 1987 in southwest Ontario (Anderson et al. 1988) and has since spread to the north and northeastwards within that province, and in 2013, it was found in Quebec (Mimee et al. 2014).

In a recent survey conducted in our laboratory, *H. glycines* was found for the first time in Manitoba soybean fields in 2019. Before this study, no information on the prevalence of SCN in Manitoba soybean fields was available. The initial density of plant-parasitic nematodes in the soil is a crucial factor in the level of damage to the host plant (Castillo and Vovlas 2007; Sato et al. 2009), emphasizing the need for reliable and rapid identification and quantification of plant-parasitic nematodes in soil samples for effective pest management.

Traditionally, the identification of *Heterodera* species has relied on morphological and morphometric characteristics of cysts, juveniles, and occasionally males (Rivoal et al. 2003). This process includes recovering cysts from the soil, extracting eggs from cysts, morphologically identifying the nematodes, and counting them under a microscope (Ingham 1994; Noel 1985). These procedures are laborious, time-consuming, prone to variations among analysts, and lead to the possibility of misidentification among other *Heterodera* species present in Canadian soybean field soils (e.g., *H. avenae*, *H. schachtii*, and *H. trifoli*).

Various molecular techniques, such as restriction fragment length polymorphism (RFLP) based on polymerase chain reaction (PCR) targeting the internal transcribed spacer (ITS) or the

cytochrome oxidase subunit I (COI) (Subbotin et al. 2000; Zheng et al. 2000; Amiri et al. 2002; Maafi et al. 2003; Ou et al. 2008; Ko et al. 2017) as well as PCR using species-specific primers of the COI region or sequence characterized amplified region (SCAR) markers (Ou et al. 2008b; Toumi et al. 2013; Baidoo 2017), have been used to identify cyst forming nematodes. In addition, quantitative Polymerase Chain Reaction (qPCR) is becoming increasingly popular for the quick and accurate quantitative detection of a wide variety of organisms, including nematodes (Fusco et al. 2011; Min et al. 2012; Braun-Kiewnick and Kiewnick 2018).

Recently, the use of species-specific primers in conjunction with qPCR has enabled the simultaneous identification and quantification of *H. glycines* (Goto et al. 2009; Min et al. 2012; Ye et al. 2012; Li et al. 2014; Ko et al. 2019; Shirai and Toyota 2019) and other cyst forming nematodes (Madani et al. 2005; Toyota et al. 2008; Quader et al. 2008; Goto et al. 2010; Toumi et al. 2015). Several qPCR assays have been developed for quantitative detection of *Heterodera* species using ITS region-based primers. In a study conducted by Madani et al. (2004), a qPCR assay was developed to detect *H. schachtii* by targeting the ITS-rDNA gene. Similarly, Goto et al. (2009) used the same DNA region for qPCR-based quantitative detection of the *H. glycines*. In another study, Shirai and Toyota (2019) optimized the previously developed ITS-based species-specific primer set to increase its specificity in quantifying *H. glycines*.

The ITS-rDNA region is not always suitable for the development of qPCR primers. Ferris et al. (1999) observed the presence of sibling species when they compared the ITS-rDNA sequences of two morphologically similar but geographically separated *H. latipons* isolates from Israel and Russia. Subsequently, several studies have utilized the COI gene for DNA barcoding of the *Heteroderidae* family (Vovlas et al. 2015; Powers et al. 2018; Subbotin et al. 2018; Ko et al. 2019). As stated by Subbotin et al. (2018), the COI gene is an effective barcoding marker as each

Heterodera species has a distinct sequence, providing an accurate identification tool. Recent studies have focused on identifying and quantifying plant-parasitic nematodes from DNA extracted directly from the soil using qPCR assay (Briely et al. 2009; Goto et al. 2009; Min et al. 2011; li et al. 2014; Baidoo et al. 2017; Arora et al. 2020; Baidoo and Yan 2021). These molecular techniques provide viable alternatives to the traditional microscopic detection and quantification of the SCN in soil samples.

While various studies have described the use of qPCR in combination with species-specific primers for the quantitative detection of *H. glycines* in soil (Goto et al. 2009; Li et al. 2014; Shirai and Toyota 2019; Baidoo and Yan 2021), few have explored the relationship between the qPCR-estimated egg quantity and the number of eggs counted under the microscope (Baidoo and Yan 2021). Moreover, to the best of our knowledge, there is no molecular method for quantifying *H. glycines* eggs directly in soil extracts reported for the Canadian populations of SCN.

To address this gap, this study developed SYBR Green I -based real-time qPCR protocols using the previously reported species-specific primers SCAR (Ou et al. 2008) and COXIII, which our laboratory has shown that it is specific to the Ontario population of SCN (Madani et al. 2013). Notably, these selected primers have not been applied to quantify DNA extracted directly from soil using real-time PCR. By employing these techniques, we aimed to eliminate the laborious and time-consuming steps of microscopic identification and quantification of *H. glycines* in soil samples. Furthermore, we have validated the present methodology for determining the number of SCN eggs in naturally infested Manitoba field soils.

3.3. Materials and Methods

3.3.1. Soil Sampling

In July 2019, a total of 20 fields naturally infested with *Heterodera glycines* were sampled in southern Ontario, covering various SCN levels and soil types in collaboration with Albert Tenuta (OMAFRA, Ridgeway, ON) (Fig. 3.1). The purpose of this sampling was to optimize the extraction methods for cysts, eggs, and DNA, as well as PCR reaction protocols. The DNA obtained from these field soils was also used for testing the specificity of the selected qPCR primers when applied to different soil DNA extracts. In addition, of these fields, 11 were selected based on SCN levels and soil types (sandy loam, loamy sand, silt loam, and silty clay loam), to validate the developed qPCR method for quantifying SCN eggs in naturally infested field soils. Three to four soil samples as a composite of at least 15 points were randomly collected from each field using a core from a depth of 0-15 cm, totalling 71 composite samples. The collected soils were placed in labelled plastic bags, transported to the Soil Ecology Laboratory at the University of Manitoba, and stored in the walk-in cooler at 4°C until use. The presence and density of *H. glycines* were determined in each sample using both traditional and molecular quantification methods described below.

In September 2021, another sampling was conducted in an infested patch of a soybean field in south-central Manitoba (sandy loam texture), where the above-ground symptoms of SCN infestation were observed for the first time in the province. A grid (10 by 6 m) was laid out using measuring tape, covering a total area of 70 m along the side road by 24 m into the field. The area extended 5 to 10 m beyond the symptomatic plant area. At each grid intersection, soil as a composite of three collections using a Dutch auger (5 cm diameter × 15 cm deep) was obtained for a total of 32 composite samples. Of these samples, 10 were selected based on nematode levels and the recovered eggs were used to generate calibration curves. Furthermore, the selected soil

samples were also used to validate the qPCR method for estimating *H. glycines* eggs number in naturally infested field soils.

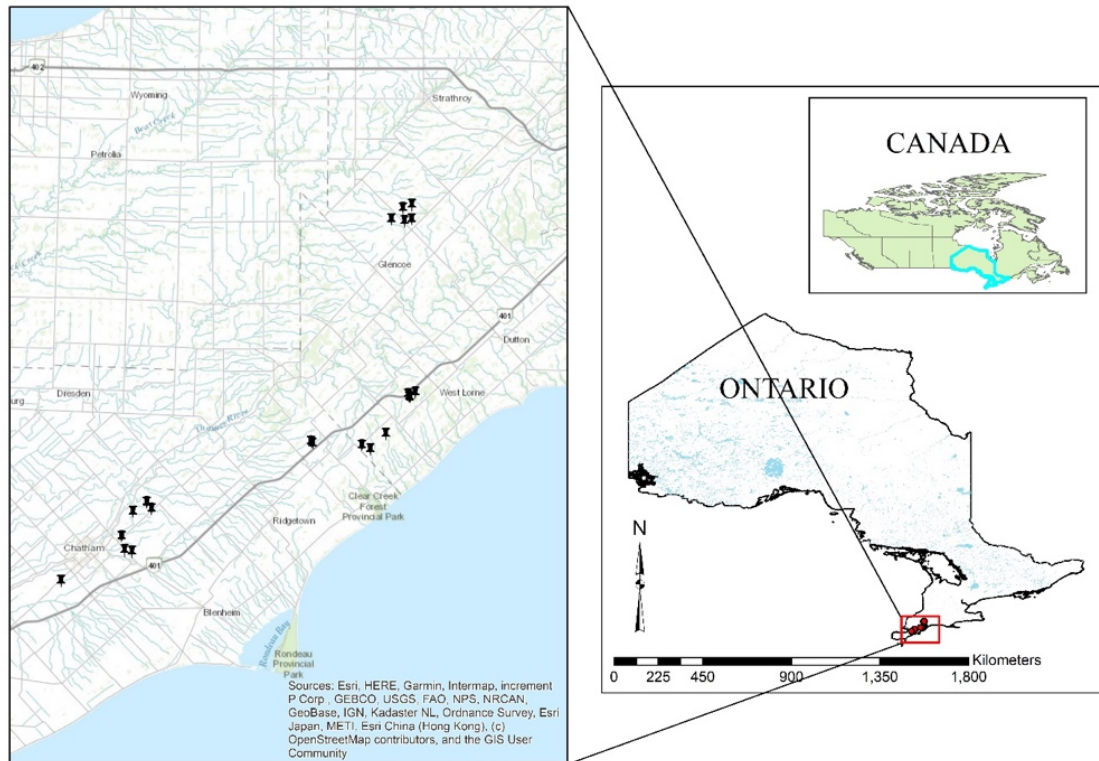


Fig. 3. 1. Sampled fields in southern Ontario covering a range of SCN levels and soil textures.

3.3.2. Cyst and Egg Recovery

To extract cysts from each composite soil sample, a water volumetric displacement method was used to measure a 100 cm³ subsample from each composite field soil sample. Two separate extractions were conducted simultaneously for each sample using the wet-sieving method (Gerdemann 1955) with some modifications. One extraction was used for the traditional egg quantification method by microscopy, while the other extraction was subjected to DNA extraction from soil debris. Briefly, the wet-sieving method involved using two sieves of different mesh sizes (#20 (850 µm) and #60 (250 µm)) to capture root and soil particles, as well as cysts and debris of cyst size. The cysts were ground manually over the #60 mesh screen, and the ground material was sieved through a stack of sieves (#200 (74 µm), #500 (25 µm)) to separate the eggs from the cyst exterior. The captured debris on the #500 mesh sieve was then used for both counting the number of SCN eggs by microscopy and DNA extraction (Fig. 3.2).

The captured material on the #500 (25 µm) screen was scraped after being allowed to dry at room temperature and transferred to an extraction bead tube to extract DNA. To count eggs, the captured debris on the other #500 (25 µm) mesh screen (obtained after repeating the procedure with a second soil suspension) was thoroughly rinsed into a 250 ml beaker, using as little water as possible. The volume of the egg suspension in the beaker was adjusted to 100 ml. To stain the eggs, 4-5 drops of 1M HCl and two Pasteur pipets of acid fuchsin stain (0.003 g ml⁻¹) were added to a beaker, followed by heating the solution on a hot plate until it was about to boil. Two ml of thoroughly mixed and cooled sample was transferred into a counting slide using a glass Pasteur pipette to quantify the egg numbers under a compound microscope (Olympus BX51, Olympus Canada, Inc., Richmond Hill, ON) at 10× magnification.

The total count and volume of the extract were recorded. The counts were then converted to the egg numbers/100 cm³ soil. The counting was done in three replicates for each solution. The modifications made to the laboratory method of soil processing for SCN extraction and egg counts described by Gerdemann (1955) included adding repetition to a few steps.

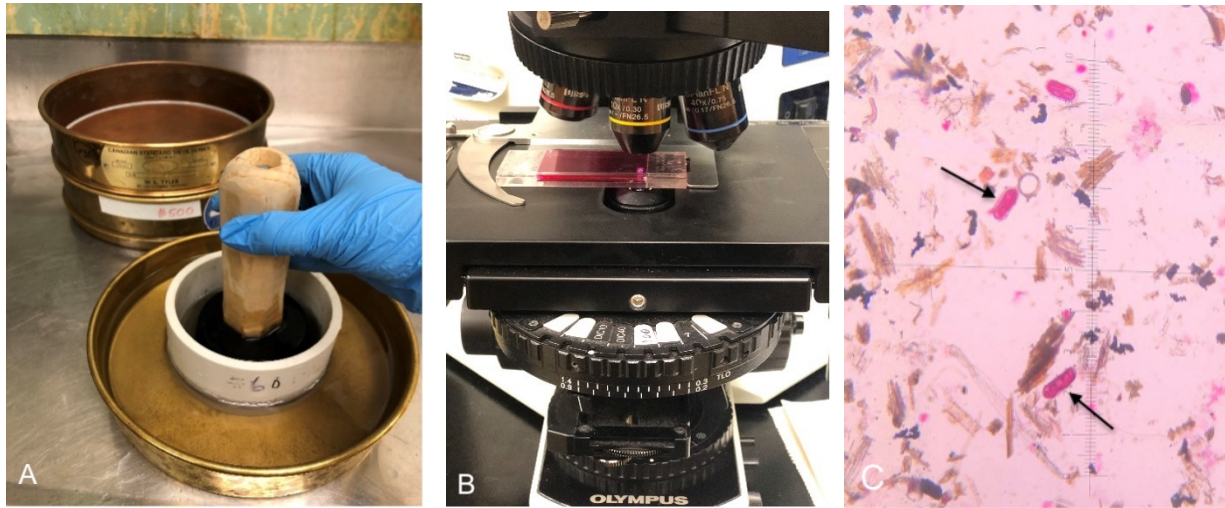


Fig. 3. 2. Images of the current (traditional) test approach to quantify the number of eggs in soil. A) Manually grinding cysts over the #60 mesh screen, B) Counting the number of stained eggs under the compound microscope, and C) Stained SCN eggs in a nematode counting slide viewed under the microscope.

3.3.3. DNA Extraction from a Single Cyst

Cysts were recovered manually using the above-described wet-sieving method. To extract DNA, the eggs from a single cyst were used, and the Proteinase K method (Subbotin et al. 2000) was centrifuge volume consisting 50 µl lysis buffer (tail buffer) (Viagen Biotech, Los Angeles, CA, USA), 13 µl sterile diH₂O, with eggs and juveniles from each cyst suspended in it, three 1 mm glass beads (autoclaved) to mechanically disrupt eggs, and 2 µl of 100 mg Proteinase K (Fisher Bioreagents, Germany). The mixture was lightly vortexed (Vortex Genie, Scientific Industries, Inc., Bohemia, NY, USA), centrifuged at 2,200 xg for 1 min (Mini Centrifuge, Fisher Scientific) and stored overnight at -20°C. The following day, samples were incubated at 60°C, followed by 15 minutes at 95°C in a thermocycler (Eppendorf CF2060, Master-cycler Pro-S, Hamburg, Germany). The extracted DNA was preserved and used as a template in the subsequent real-time PCR assay.

3.3.4. DNA Extraction from Soil

To analyze the DNA of *H. glycines* in artificially infested soil and natural field soil, the PowerSoil DNA Isolation Kit (MOBIO Laboratories, Solana Beach, CA) was used to extract total DNA from the soil. The DNA was extracted from the soil debris that was captured on the #500 mesh screen (25 µm) following the wet-sieving and egg extraction method applied to 100 cm³ of each soil sample (debris of 100 cm³ of soil equals approximately 150-200 g of soil depending on the soil texture). To do this, a 600 g representative sample of each homogenized composite soil sample was collected and divided into three 200 g subsamples.

The extraction followed the manufacturer's instructions with some modifications. The method involved extracting DNA from soil debris by weighing up to 0.2 g of the debris and transferring it into power-bead tubes. Sixty µl of solution C1 was added to the power-bead tubes, which were then mixed using a mini-beadbeater¹⁶ (BioSpec Products, Bartlesville, OK) for 2 minutes at 3450

strokes/min, followed by an additional 2 minutes of bead beating after one-minute cooling. The tubes were then centrifuged at 11,000 xg for 30 seconds, and approximately 650 µl of the resulting supernatant was transferred into new sterile collection tubes. Solution C2 (250 µl) was added to the supernatant, and the tubes were vortexed for 5 seconds and incubated at 4 °C for 5 minutes. After centrifugation at 11,000 xg for 1 minute, up to 650 µl of the supernatant was transferred to fresh collection tubes, and 200 µl of solution C3 was added before vortexing for 5 seconds and incubating at 4 °C for 5 minutes. This was followed by another centrifugation step at 11,000 x g for 1 minute. 750 µl of supernatant was again transferred into fresh sterile collection tubes, which were then vortexed for 5 seconds after adding 1,000 µl of solution C4. Subsequently, 650 µl solution from the collection tube was loaded onto clean Spin Filter tubes and centrifuged for one minute at 10,000 xg. This process was repeated three times until all samples had been processed. The Spin Filter tubes were then filled with 500 µl of solution C5, and centrifuged for 30 seconds at 10,000 xg. Again, the tubes were centrifuged for one minute at 10,000 x g, but this time the detachable spin filter was removed from the Spin Filter tubes and placed into fresh, fully labelled collection tubes. In the final step, 50 µl of solution C6 was added to the centre of the white membrane filter of the spin filter and the tubes were left to stand for 2 minutes. After two minutes, the tubes were centrifuged for thirty seconds at 10,000 xg, and the Spin Filters were discarded because the DNA had eluted into the collection tubes.

Using solution C6 as a blank, the concentration (ng µl⁻¹) and purity (260/280 ratio) of the extracted DNA were quantified using the Qubit 4 Fluorometer (Invitrogen, Thermo Fisher Scientific, Singapore) and the NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). Following purity measurements, DNA samples were stored at -20°C until the subsequent real-time quantitative PCR step.

In the above-outlined protocol, I combined grinding soil debris containing cysts on a sieve with an extended bead beating time to ensure the proper crushing of all nematode cysts and extracting a sufficient amount of DNA. In the centrifugation steps of the Spin Filter, the original protocol of 10000 x g was optimized to 11000 x g. Additionally, I reduced the load volume of 675 µl to 650 µl on the Spin Filter to prevent solution splashing from the overfilled tubes onto the centrifuge interior. These adjustments improved the 260/280 ratio in the extracted DNA, resulting in a higher purity.

3.3.5. DNA Extraction from an Artificially Infested Nematode Suspension

Different numbers of *H. glycines* eggs (10, 100, 500, and 1,000), obtained with the sieving method from a symptomatic Manitoba soybean field, were added to sterile distilled water in three replicates. The nematode suspensions were allowed to settle in a fridge at 4°C overnight. After the eggs had settled at the bottom, the excess water was removed, leaving behind 100 µl of a concentrated nematode suspension. This concentrated nematode suspension was then transferred into power-bead tubes, and the DNA extraction process was carried out using the PowerSoil DNA Isolation Kit (MOBIO Laboratories, Solana Beach, CA), following the previously described procedure.

3.3.6. Real-time PCR

The DNA samples extracted earlier were analyzed using SYBR Green I-based real-time PCR assay with primer sets previously reported specific to SCN, COXIII (Madani et al. 2013) and SCAR (Ou et al. 2008). The DNA samples were first thawed and then diluted 10-fold with autoclaved distilled water. A total of 20 µl of qPCR reaction mixture was prepared for each sample in Bio-Rad optical 0.2 ml 8-cap flat tubes and strips (Bio-Rad Laboratories, Hercules, CA). For the SCAR primer set, the qPCR reaction mixture included 10 µl of EvaGreen dye, a pre-made PCR master mix (BioRad,

SsoFast, EvaGreen Supermix) (Bio-Rad Laboratories), 0.20 μ M of forward SCNF1(5'-GGACCCTGACCAAAAAGTTTCCGC-3') and reverse SCNR1 (5'-GGACCCTGACGAGTTATGGGCCCCG-3') primers (10 μ M), 2 μ l of DNA template for a total volume of 20 μ l with nuclease-free water. The qPCR assay was conducted using a CFX96 detection system (Bio-Rad Laboratories) programmed for 37 cycles of an initial melt at 98°C for 2 minutes, followed by 98°C for five seconds and an annealing temperature of 60°C for 45 seconds. Similarly, for the COXIII primer set, the qPCR reaction mixture included 10 μ l of EvaGreen dye, 0.6 μ l of 10 μ M of each of the forward COXIII-F (5'-TGAGGCTTTAGTTGGCTTTCA-3') and reverse COXIII-R (5'-TGAGAAAAGGTAATAACCGAAGC-3') primers (10 μ M), 6.8 μ l of nuclease-free water, and 2 μ l of DNA template. The Bio-Rad PCR conditions (40 cycles) included 98°C for 2min, followed by 98°C for five seconds, and an annealing temperature of 55°C for 30 seconds. After the completion of the cycles, an amplicon melting profile specific to each gene was conducted to distinguish between potential primer dimer and nonspecific amplification products. This was achieved by cooling the samples to 60°C and gradually increasing the temperature to 95°C at a rate of 0.5 °C every 18 seconds and recording fluorescence at each temperature.

All reactions in this study were amplified in four technical replicates for each sample. The assay control included DNA from *Heterodera glycines* recovered from pot cultures as the positive control, while DNA from closely related *Heterodera* species (*H. schachtii*, *H. trifolii*,) and other common plant-parasitic nematodes (*P. penetrans*) as well as a non-template control (sterile distilled water instead of a DNA template), served as negative controls.

3.3.7. Samples Preparation for Various Analysis

A single *H. glycines* cyst and two highly SCN-infested soil samples, selected from Ontario and Manitoba SCN populations, were used to evaluate the sensitivity, specificity, and efficiency of *H.*

glycines-specific primers (SCAR and COXIII) in real-time qPCR. A dilution series of the concentrated DNA extracted from these samples was then used to generate standard curves.

DNA samples obtained from different numbers of *H. glycines* eggs, simulating the variability that can be expected in natural field samples, were also used to assess the sensitivity and specificity of these species-specific primers. In addition to the evaluation of primer efficiency, these DNA samples were used to determine the quantitative nature of the DNA extraction method and qPCR assays in terms of egg numbers. The mentioned samples were prepared by inoculating different numbers of *H. glycines* eggs obtained from a Manitoba field to the suspension and autoclaved soil debris. DNA extracted from these artificially infested samples was then used to generate calibration curves.

Naturally infested field soils from Ontario and Manitoba, covering diverse *H. glycines* densities and soil types, were selected to determine whether the developed qPCR assay could quantify the number of *H. glycines* eggs in field soils. To validate the developed molecular method, the egg numbers estimated by microscopy were compared with those estimated using the qPCR method in these naturally infested soils.

3.3.8. Primer Evaluation and Generating Standard Curves using DNA Extracted from a Single Cyst and Highly Infested Soil

Two primer pairs, SCAR and COXIII, that have previously demonstrated specificity for *H. glycines* were evaluated for their sensitivity, specificity, and efficiency using the real-time qPCR method. The effectiveness of each primer pair was evaluated based on their ability to amplify the target DNA and their high amplification efficiency.

The SCNF1/SCNR1 primer set, designed by Ou et al. (2008), targets a 477 bp randomly amplified polymorphic DNA-sequence characterized region (RAPD-SCAR). The COXIII-

F/COXIII-R primer set, designed by Madani et al. (2013) in the Soil Ecology Laboratory, targets a 252 bp fragment of the *H. glycines* mitochondrial DNA (Mt-DNA) gene.

DNA was extracted from a single cyst and highly-infested soil samples from two SCN populations (ON and MB). To determine the minimum amount of *H. glycines* DNA that could potentially be quantified specifically in a single cyst using the developed qPCR method and selected species-specific primers, DNA was extracted from 30 eggs collected from three distinct cysts obtained from Ontario populations of *H. glycines* using the modified proteinase K technique outlined above. The DNA was then subjected to quantitative PCR using the species-specific primers, and the resulting Cq values from four replicates were averaged and compared for each population. Due to the similarity of the Cq values, the DNA of one population was used to determine the detection limit for each primer set. A 10-fold dilution series at seven concentrations (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7}) was prepared using the selected DNA template.

To determine the ability of the primers to detect and quantify the minimum concentration of DNA in soil, the same dilution series was prepared this time from 1:10 diluted *H. glycines* DNA extracted directly from soil samples collected from highly infested fields in Ontario and Manitoba (Approximately 25,000 eggs/100 cm³ soil). To cover the range of concentrations across all samples, soil samples with the highest level of infestation were selected.

The above series of serial dilutions were used in real-time PCR reactions with the SCAR and COXIII primer sets under the aforementioned qPCR assay conditions. In both experiments, each run also included a positive DNA control (DNA from *Heterodera glycines*), a negative DNA control (DNA from closely related *Heterodera* species (*H. schachtii*, *H. trifolii*), and other common plant-parasitic nematodes (*P. penetrans*) to examine the specificity of qPCR primers. In addition, each run contained a non-template control (NTC). Using Bio-Rad CFX Manager Software V3.0,

standard curves were generated by plotting logarithmic values of DNA concentration versus the average C_q values. The coefficient of determination (R²) was then calculated, and the amplification efficiency (E) was calculated using the formula 3.1, where m is the slope of the equation obtained from standard curves. To expand the specificity evaluation, the qPCR product identity was confirmed by electrophoresis on a 1.2% agarose gel. The gel was run at 85 V for 90 minutes in a gel box (Fisherbrand, Fisher Scientific, Nepean, ON). Amplified bands were compared to a 100 bp DNA ladder (ProMega, Madison, WI, USA) to determine fragment sizes.

$$E = 10^{\left(\frac{1}{-m}\right)} - 1 \quad (3.1)$$

3.3.9. Generation of Calibration Curves from Artificially Infested Soil Debris and Nematode Suspension

Using suspension and soil debris inoculated with different numbers of *H. glycines* eggs, the sensitivity and quantification of the DNA extraction (PowerSoil DNA Isolation Kit) and qPCR method were assessed. In addition, the artificially infested soil was used to create a calibration curve that relates the C_q value to the number of *H. glycines* /100 cm³ soil. To this end, the curves were generated using *H. glycines* eggs recovered from a symptomatic Manitoba soybean field in 2021. The species identity of nematodes extracted from this field was earlier confirmed morphologically and molecularly by the amplification and sequencing of four regions of the genome as described in the previous chapter (see Section 2.4.2).

The autoclaved soil debris retained on a #500 (25µm) mesh sieve after applying the sieving method on a 100 cm³ soil sample was used for inoculation in all the experiments. The soil was collected from a field in Manitoba and confirmed to be *H. glycines*-free. In separate experiments, different numbers (10, 100, 500, and 1,000) of *H. glycines* eggs, obtained with the sieving method,

were added in triplicate to suspension and 0.2 g of an autoclaved silty clay soil debris from which genomic DNA was extracted using the PowerSoil DNA Isolation Kit exactly as mentioned earlier.

The above set of artificially infested soil and suspension were then subjected to qPCR amplification using the SCN-specific SCAR and COXIII primer sets described above. Reactions with distilled water in place of DNA and autoclaved non-inoculated soil debris were used as negative controls. A previously confirmed *H. glycines* DNA from a pot culture was also included as a positive control. The reaction was run in four technical replicates for each of the three independent biological replicates. This resulted in twelve real-time PCR observations per infestation level.

Calibration curves were generated by plotting the log number of *H. glycines* eggs used to infest 0.2 g of soil debris and suspension versus the average C_q values from each infestation level for both SCAR and COXIII primer sets. The sensitivity of the qPCR assays was determined by detecting C_q values (specific amplification) at the minimum level of infestation.

3.3.10. Detection and Quantification of *H. glycines* in Naturally Infested Field Soils

A combined set of 11 composite soil samples from Ontario fields (belonging to different textural groups) and 10 composite soil samples from a SCN-infested patch of a soybean field in Manitoba (with sandy loam soil texture), covering a wide range of SCN levels, were selected. The soil sampling aimed to determine whether the qPCR assay could quantify the number of eggs in naturally infested field soils covering different SCN populations, densities, and soil types.

As explained earlier, the approximate 200 g (100 cm³) subsample was used in triplicate for the sieving process. A total of 600 g of each composite soil after thoroughly mixing the samples was utilized in this experiment. SCN eggs were counted in three replicates in each subsample as described above (cyst and egg recovery section) and expressed as the average egg numbers/100 cm³ soil. Three independent DNA extractions (using the MoBio PowerSoil DNA isolation kit) were conducted for each sample using up to 0.2 g of captured soil debris on the 500-mesh (25 µm) sieve, in which the sieving and grinding method was applied, and eggs were counted.

A qPCR was then set up with four technical replicates for each sample. The average C_q values from each composite sample were plotted against the log of the traditional egg counts for both SCAR and COXIII primer sets. To investigate the ability to quantify *H. glycines* egg numbers in field soils by the real-time PCR method, egg counts of the naturally infested soil determined by traditional egg extraction and quantification by microscopy were compared to numbers determined by C_q values. Using equations derived from the calibration curves, the number of eggs per 100 cm³ of each soil sample (x) was estimated employing formula 3.2. The qPCR estimates obtained using the calibration curve equation were then plotted against the number of *H. glycines* eggs quantified by microscopy for each primer set. The final egg numbers of SCN quantified (per 100 cm³ soil) were reported as the mean of three biological and four technical replicates.

$$X = 10^{\left(\frac{\text{mean } Cq \text{ of the sample} - \text{intercept}}{\text{slope}}\right)} \quad (3.2)$$

3.3.11. Statistical Analysis

Standard curves were generated by plotting logarithmic DNA concentration values versus the average Cq values obtained from real-time qPCR using Bio-Rad CFX Manager Software V3.0. The coefficient of determination (R^2) was then determined, and the amplification efficiency (E) was calculated using the formula (3.1), where m is the slope of the equation obtained from standard curves. The relationship between the mean Cq values and the logarithmic values of numbers of the *H. glycines* eggs added to the suspension and soil debris (calibration curves), as well as the relationship between estimated egg numbers quantified by microscopy and qPCR in naturally infested field soils for both primer sets (SCAR and COXIII), were examined using linear regression analysis in the software Excel Statistics 2021.

Analysis of variance (ANOVA) was conducted to investigate the application of different quantification methods (traditional microscopy and real-time qPCR), and different primer sets (SCAR and COXIII) in estimating the number of *H. glycines* eggs within each naturally infested soil in Manitoba and Ontario. The analysis was performed using the PROC GLIMMIX procedure (Version 9.4; SAS Institute, Cary, NC) with the Poisson distribution. Furthermore, the assumption of ANOVA was met by using the PROC UNIVARIATE procedure of SAS for the normality of the residuals (Shapiro-Wilk). In the model statement, the methods of *H. glycines* eggs quantification and the different primer sets were modelled as fixed effects, while the replications were treated as random effects. The Cq values from different inoculation levels of *H. glycines* eggs, the number of *H. glycines* eggs determined by two quantification methods, and the number of *H. glycines* eggs estimated by two primer sets were compared using the least-square means (LSmeans) statement based on the Tukey-Kramer test with a probability level for the significance of 0.05.

3.4. Results

3.4.1. Detection of *H. glycines* in Different Field Soils of Ontario and Manitoba

Both conventional (data not presented) and qPCR assays confirmed the presence of *H. glycines* in soil samples collected from soybean fields in southern Ontario and Manitoba used for further analysis. The qPCR assay showed a specific amplification for soil DNA extracts of Ontario populations of *H. glycines* obtained from different fields regardless of infestation level, soil type, or primer used. The positive soil DNA extracts provided a single melting peak at 75 ± 0.5 °C and 80.5 ± 0.5 °C for COXIII and SCAR, respectively, suggesting the primers bind to their specific DNA target and not to other DNA targets or primer-dimers (Fig. 3.3 A and B). The lowest and highest C_q values were obtained, ranging from 17 to 29 cycles. Nematodes extracted from an infested patch of a soybean field in Manitoba were previously identified to the species level in the previous study based on morphological and molecular analyses and sequencing of four different regions of the genome (ITS, 18S, COXIII, and SCAR). Data blast revealed a sequence similarity of 98-100% with *H. glycines* samples deposited in GenBank (e.g., Accession Nos: CP049713.1, MK292129.1, and HM640930.1). Additionally, when the soil DNA extracts from the previously confirmed SCN-positive Manitoba field were assayed with qPCR, a positive signal with a single melting peak was observed in almost all samples at 75 ± 0.5 °C and 80.5 ± 0.5 °C when amplified using COXIII and SCAR primers, respectively (Fig. 3.3 C and D). However, one field soil sample, S2, having a count of 33 eggs/ 100 cm³ soil, did not produce the *H. glycines*-specific amplicon in all replicates of melting curve analyses when the DNA was amplified with the SCAR primer set (Fig. 3.3 C). Yet, upon visualizing the qPCR products of this sample on a gel, single faint band with the correct size were observed. In contrast, the amplification of DNA extracted from S2 was specific in all replicates when the COXIII primer set was used.

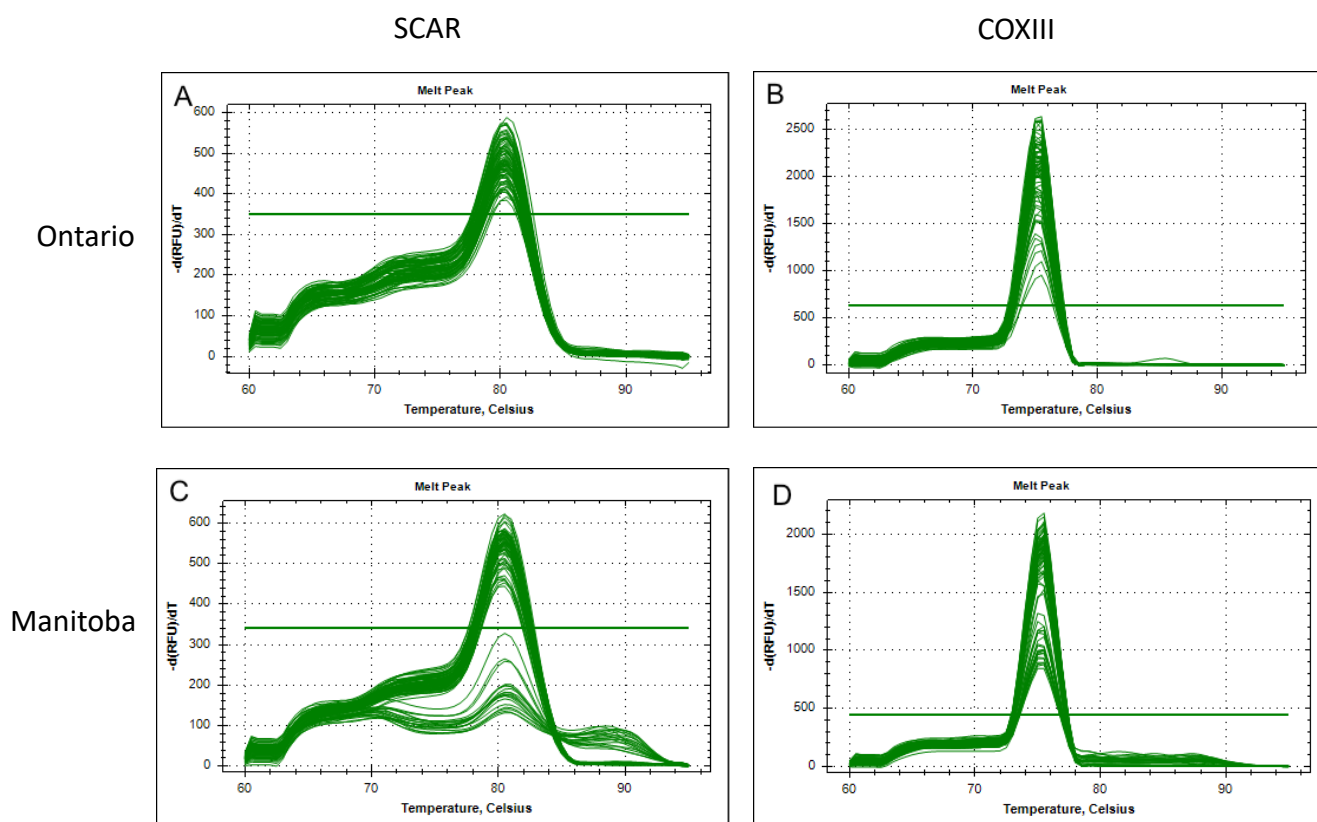


Fig. 3. 3. Real-time qPCR analysis. Melting curve profiles (fluorescence versus temperature) (Line: Threshold) of A, B) Ontario and C, D) Manitoba field soils resulted in single peaks at 75 ± 0.5 °C (COXIII) and 80.5 ± 0.5 °C (SCAR) for DNA extracted from naturally infested soils with varying number of SCN.

3.4.2. Primer Evaluation and Generating Standard Curves using DNA Extracted from a Single Cyst

The sensitivity and specificity of the SCN-specific qPCR primers (COXIII and SCAR) were determined in a serially diluted suspension containing SCN eggs. The assay was able to specifically amplify the DNA of 30 eggs of a single *H. glycines* cyst diluted five times. The dilution series ranged from 30 eggs down to an equivalent of 0.0003 of an egg (30, 3, 0.3, 0.03, 0.003, 0.0003). The C_q values in both assays increased proportionally with the dilution rate. The reaction efficiency (E) was calculated using the formula 3.1 and was estimated as 98.8 % for SCAR and 101.3 % for COXIII (Fig. 3.4 A and C). The specific SYBR fluorescent signal was not detected in all four replicates of reactions containing template DNA at the 6th dilution and the NTC for both primers. This suggests these primer sets could potentially detect an equivalent of 0.003 *H. glycines* egg of a single cyst containing 30 eggs in the real-time PCR assay. As shown in the melting curve analysis presented in Fig. 3.4 B and D, single peaks were observed at 80.5±0.5 °C (SCAR) and 75±0.5 °C (COXIII) for positive extracts of *H. glycines* (except the 6th dilution with SCAR). DNA from species other than SCN did not produce specific amplification when amplified with both primers. Both assays were able to detect DNA in dilutions (ten-fold each) from DNA originating from 30 eggs of *H. glycines*. Overall, the results from the qPCR assay confirmed the SCAR and COXIII primer sets sensitivity and specificity for *H. glycines* determined by Ou et al. (2008) and Madani et al. (2013).

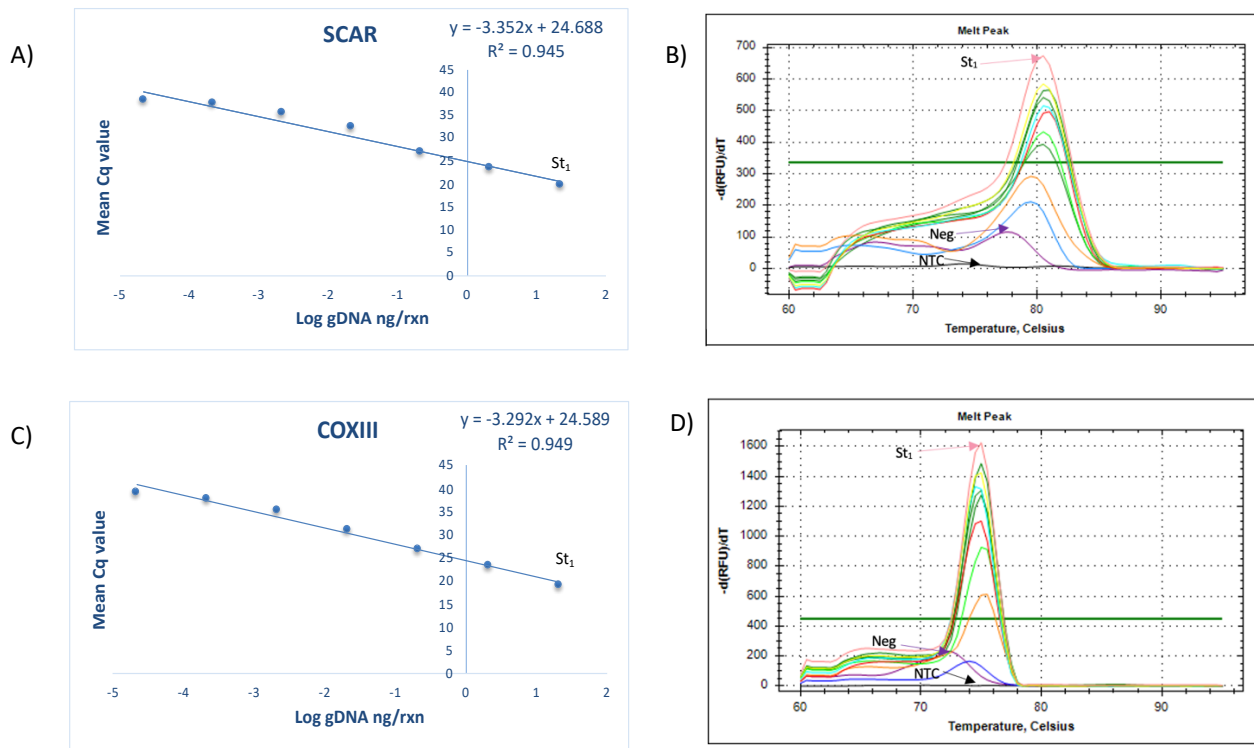


Fig. 3. 4. Real-time qPCR analysis. Standard curves of A) SCAR and C) COXIII for *H. glycines*: quantification cycle number (Cq) plotted against the dilution series (10-fold each) of 1:10 diluted DNA extracted from 30 eggs using Proteinase K method. Melting curve profiles (fluorescence versus temperature) of B) SCAR and D) COXIII resulted in single peaks at 80.5 ± 0.5 °C (SCAR) and 75 ± 0.5 °C (COXIII) for the dilution series of DNA extracted from 30 eggs of *H. glycines*. R^2 : coefficient of determination. No amplification was observed in no-template controls (NTC).

3.4.3. Primer Evaluation and Generating Standard Curves using DNA Extracted Directly from the Soil

When the 1:10 diluted DNA extracted directly from highly infested Ontario and Manitoba soil samples ($\sim 25,000$ eggs/100 cm³ soil) were serially diluted and amplified with the SCN-specific SCAR and COXIII primer sets, the real-time PCR with both primer sets was able to detect DNA in five dilutions (10-fold each). The relation between the average quantification cycle number (C_q) values and the logarithm of the serially diluted DNA concentration extracted from soil showed a linear response along the 10-fold dilution series of the DNA originating from Ontario soil with C_q values ranging from 18.2 ± 0.15 (mean $\pm$ sd) to 30.46 ± 0.45 and 17.5 ± 0.27 to 29.9 ± 0.52 when the DNA amplified using SCAR and COXIII primer sets, respectively.

The regression equation for the SCAR standard curve using DNA extracted from Ontario soil was $y = -3.256x + 23.712$, with an R² of (coefficient of determination) 0.9839, slope -3.256, and $P < 0.001$. The dilution series of the same DNA amplified with COXIII primer also resulted in a standard curve showing highly significant linearity between the C_q value and the dilution rate, as described by the equation $y = -3.313x + 23.097$, with an R² = 0.9837; slope = -3.313; and $P < 0.001$. The reaction efficiency (E) was estimated as 102.82 % for SCAR and 100.37 % for COXIII (Fig. 3.5 A and B).

A similar dilution series was also generated using DNA extracted from Manitoba soil and was assayed in qPCR. The equation for the standard curve was described as $y = -3.3694x + 23.619$, with an R² value of 0.9802, $P < 0.001$ and efficiency (E) of 98.07% when the SCAR primer set was used. Each dilution level was also used to generate a standard curve using a COXIII primer set. The regression equation for COXIII was $y = -3.37x + 22.809$ with an R² = 0.9867, $P < 0.001$, and E = 98.032 % (Fig. 3.5 C and D).

The melt temperature of the amplified products from each dilution level was within the range of the melt temperatures of the positive controls (80.5 ± 0.5 °C (SCAR) and 75 ± 0.5 °C (COXIII)) for both of the SCN populations from ON and MB. No amplification signals were detected in no-template controls (NTC). According to the electrophoresis results, a single expected fragment of 474 bp for SCAR and 252 bp for COXIII was observed in the dilution series for both populations but not for the NTC (Fig. 3.6 A, B and C).

Since the amplification efficiency for DNA of Ontario soil extracts, 102.82 % and 100.37 %, was very similar to that for DNA of Manitoba soil, 98.07 % and 98.032 % (SCAR and COXIII), the R^2 value was high and within the acceptable range, and specific amplification was obtained for populations of *H. glycines* equivalent of as low as one *H. glycines* egg per 100 cm³ soil, the SCAR and COXIII primer sets showed same sensitivity for both populations and were considered suitable for quantitative detection of different populations of *H. glycines* in soil samples using real-time PCR assay.

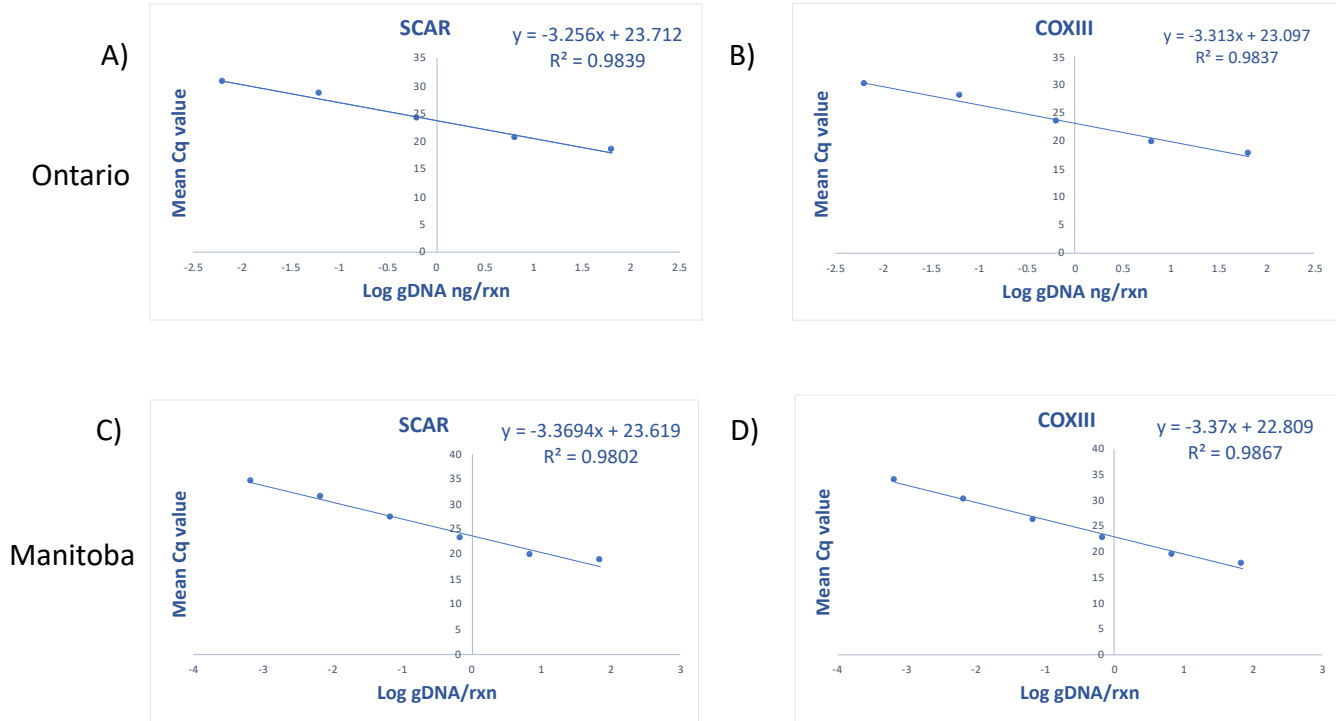


Fig. 3. 5. Real-time qPCR analysis. Standard curves of SCAR and COXIII primer sets for *H. glycines*: quantification cycle number (Cq) plotted against the dilution series (10-fold each) of 1:10 diluted DNA extracted from highly SCN-infested A, B) Ontario and C, D) Manitoba field soils. R^2 : coefficient of determination.

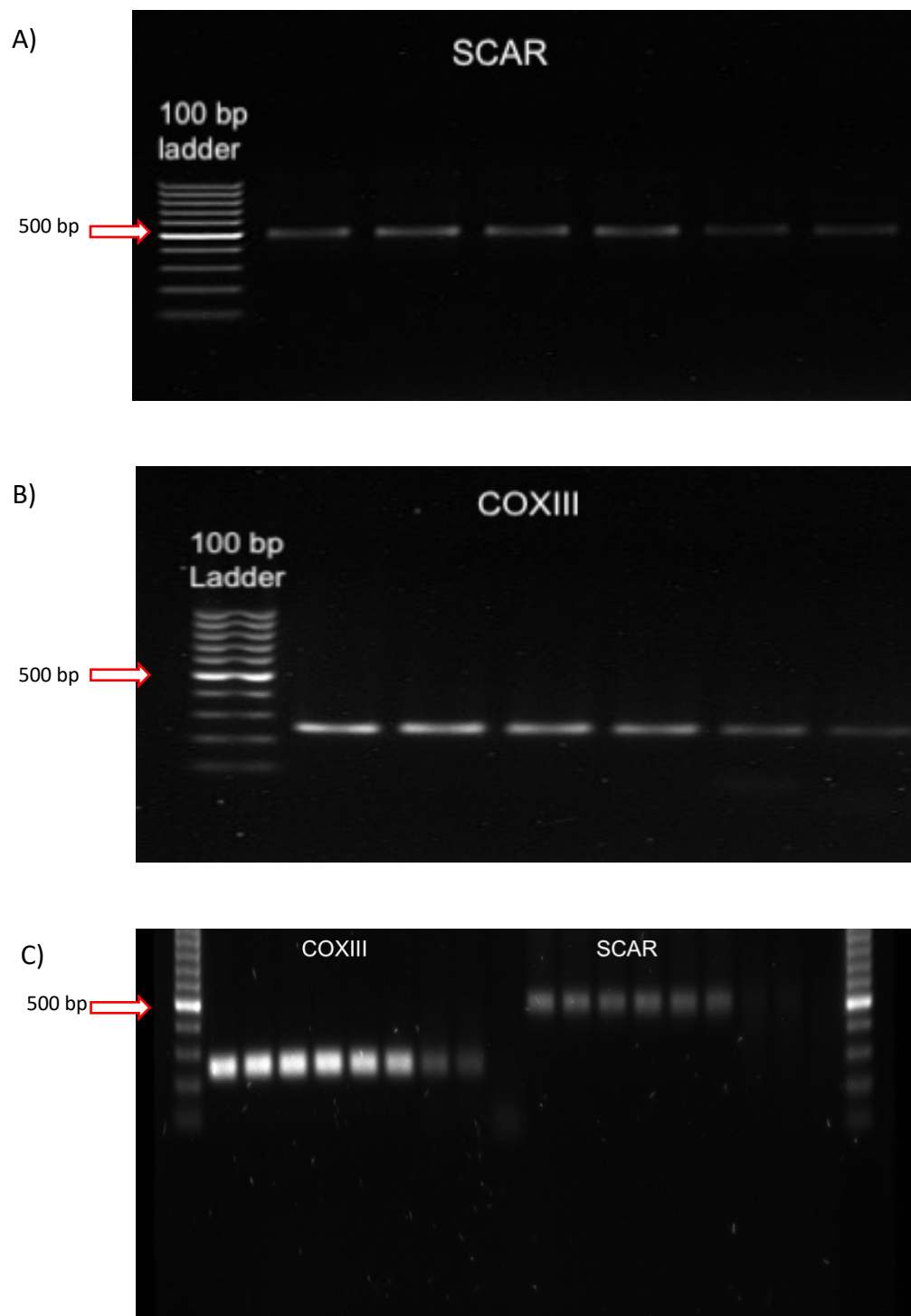


Fig. 3. 6. A gel electrophoresis results of the qPCR products showing the specificity of the SCAR (474 bp) and COXIII (252 bp) primer sets using DNA extracted from highly SCN-infested A, B) Ontario and C) Manitoba soils.

3.4.4. Generation of Calibration Curves from Artificially Infested Nematode Suspension and Soil Debris

The averaged Cq value of the three different DNA extraction replications (12 total observations) from the nematode suspensions containing 10, 100, 500, and 1,000 eggs of SCN amplified with SCAR primer set was 28.17, 23.89, 19, and 16.47, respectively, while with COXIII the averaged Cq values were 26.76, 24.06, 19.33 and 16.55, respectively. Highly significant linearity existed between the log number of eggs added and mean Cq values when the SCAR primer set was used ($R^2 = 0.9879$, $P < 0.001$). Each dilution level amplified with COXIII primer also resulted in a highly significant linearity between the Cq value and the inoculation level ($R^2 = 0.9737$, $P < 0.001$) (Fig. 3.7 A and C). No DNA melting curve was observed for the negative controls, in which distilled water was used as a template instead of DNA. Gel analysis data additionally confirmed that the detected fluorescent signal was attributed to the presence of primer dimers in no-template controls. The PCR products obtained in real-time and visualized on agarose gel showed only a single band of the correct size, with the absence of primer dimers for all positive (inoculated) samples.

The qPCR assays with SCAR and COXIII showed specific amplification for the minimum level of inoculation (10 eggs) to nematode suspension. The similarity between the average Cq values of the three different DNA extractions from suspensions containing the same number of eggs indicates the stability of the DNA extraction method. In addition, the DNA concentration of suspensions increased with the number of inoculated eggs, demonstrating that the DNA extraction method was quantitative.

Calibration curves were generated for *H. glycines* quantification directly from the soil. When DNA was extracted from the soil debris (up to 0.2 g) (retained on a #500 mesh screen after the

wet-sieving method) containing different numbers of *H. glycines* eggs (10, 100, 500, and 1,000) and used as templates for real-time PCR using SCAR primer, Cq values were 29.18 ± 0.5 (mean $\pm$ SD), 25.8 ± 0.3 , 23.13 ± 0.1 , and 21.5 ± 0.2 , respectively. The average Cq values for DNA amplified using the COXIII primer set were recorded as 28.54 ± 0.4 (mean $\pm$ SD), 24.95 ± 0.3 , 21.67 ± 0.3 , and 20.62 ± 0.3 , respectively. A highly significant negative relationship existed between the Cq values and the number of eggs added for both primer sets ($R^2 = 0.9966$, $P < 0.001$ for SCAR and $R^2 = 0.9817$, $P < 0.001$ for COXIII) (Fig. 3.7 B and D). Specific melting curve profiles (fluorescence versus temperature) of the qPCR test resulted in single peaks at 80.5 ± 0.5 °C (SCAR) and 75 ± 0.5 °C (COXIII) for the DNA extracted from all infestation levels, excluding non-inoculated suspension and soil debris (Fig. 3.8). Specific amplification for the minimum level of infestation, 10 eggs into 0.2 g of soil, extrapolates that the real-time PCR assays using both primers could detect the Cq values at *H. glycines* density of as low as 10 eggs/100cm³ soil.

The calibration and melting curves demonstrate that the SCAR and COXIII qPCR primers are able to quantitatively and specifically measure the different numbers of *H. glycines* eggs within the suspension and soil debris. Calibration curves of inoculated soil debris were nearly identical for both primer sets, with the mean Cq values lower for the COXIII primer set across all inoculation levels. In other words, COXIII may enable more sensitive quantification of *H. glycines* in soils. However, the mean Cq values did not show a significant difference ($P = 0.711$) between both primer sets. When the Cq values in the soil debris inoculated with different numbers of *H. glycines* eggs were compared with that in the suspension containing an equivalent number of SCN eggs, the difference in Cq values was not significant for both primer sets ($P = 0.342$ for SCAR and $P = 0.390$ for COXIII). These findings might indicate a low probability of the presence of inhibitors in the PCR reaction for both primer sets.

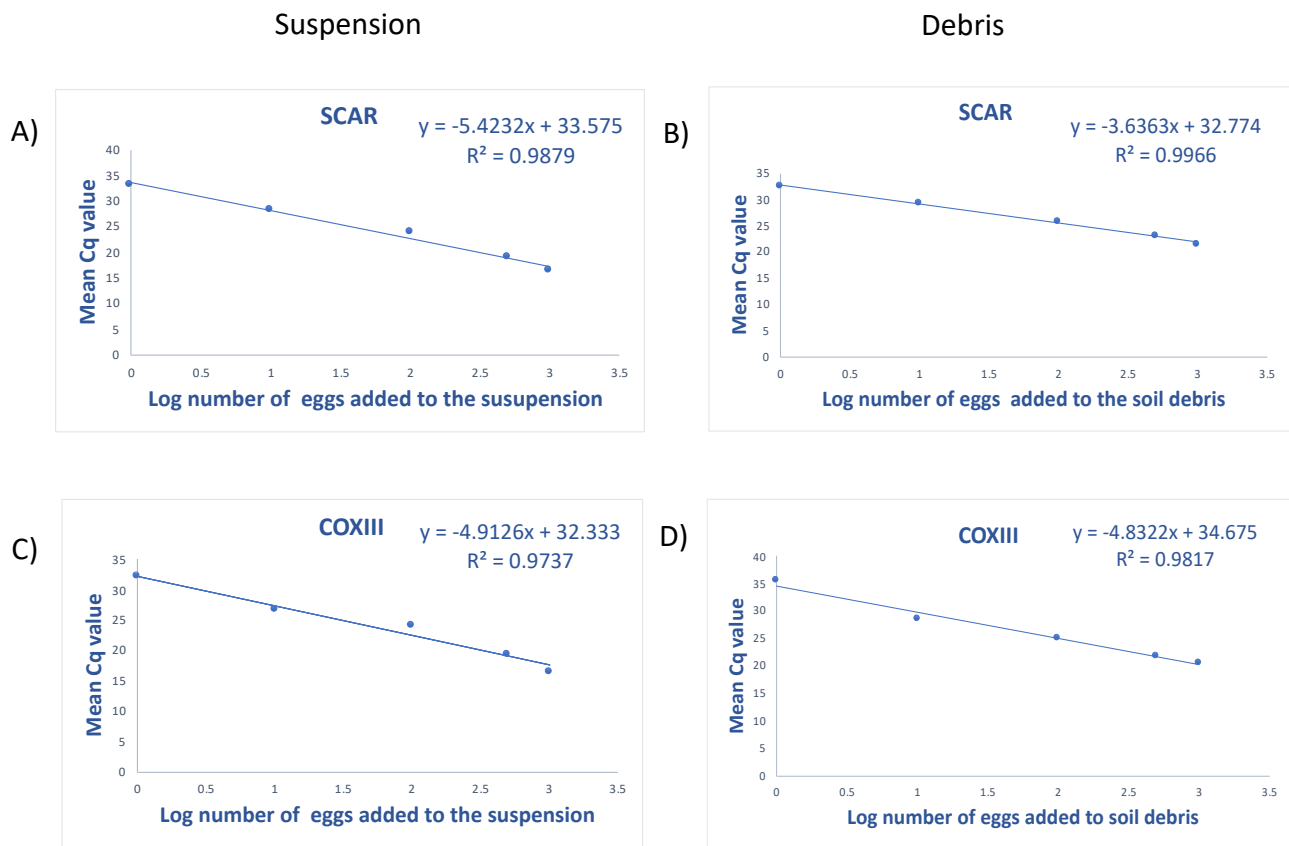
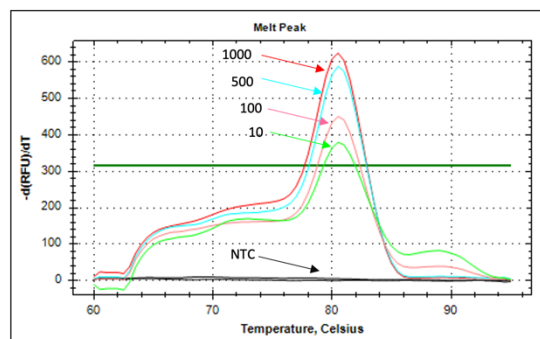


Fig. 3. 7. Real-time qPCR analysis. Relationship between the quantification cycle number (Cq) and log number of SCN eggs (0, 10, 100, 500, and 1,000) added to the A, C) Suspension and B, D) Soil debris for the SCAR and COXIII primer sets. Each dot represents the mean of three biological replicates (DNA extraction) and four technical replicates (qPCR run). R^2 : coefficient of determination.

A) SCAR



B) COXIII

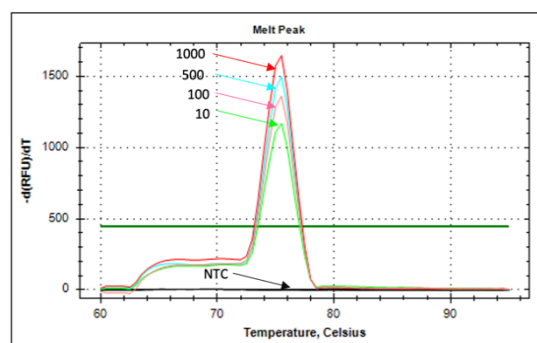


Fig. 3. 8. Real-time qPCR analysis. Melting curve profiles (fluorescence versus temperature) of A) SCAR and B) COXIII resulted in single peaks at 80.5 ± 0.5 °C (SCAR) and 75 ± 0.5 °C (COXIII) for the DNA extracted from the soil debris inoculated with the different number of *H. glycines* eggs (10, 100, 500, and 1,000). No specific amplification was observed in non-infested soil.

3.4.5. Direct Detection and Quantification of *H. glycines* in Naturally Infested Field Soils of Manitoba

Regression analysis was conducted to determine the relationship between the mean C_q values from qPCR and the egg numbers determined by wet-sieving extraction and microscopic counting for the 10 soil samples collected from the Manitoba field. The average microscopic count (average of three extractions and three counting replicates per soil sample) in naturally infested field soils ranged from 33 to 24,566 eggs/ 100 cm³ soil with one sample, S1, showing no detectable *H. glycines* egg (Table 3.1). The average C_q values from qPCR were 17.03 to 32.75 and 16.52 to 35.56 for SCAR and COXIII primers, respectively. There was a negative relationship between C_q values from the qPCR assay and microscopy-based counts of *H. glycines* eggs for both primer sets, indicating that the two methods were in close agreement (Fig. 3.9).

One of the 10 field soil samples, S2, having a count of 33 eggs/ 100 cm³ soil, did not produce the *H. glycines*-specific amplicon in all replicates of melting curve analyses when the DNA was amplified with the SCAR primer set. This result aligns with the findings of Baidoo et al. (2017), who noted low detection efficiency and consistency when the SCNF1/SCNR1 (SCAR) primer set was used to amplify DNA in PCR from soil samples with low population densities (< 349 eggs/ 200 g soil). However, when the qPCR products of this sample (S2) were visualized on a gel, a faint single band with the right size were observed. The amplification of DNA extracted from S2 was specific in all replicates when the COXIII primer set was used. This result suggests that soils infested with low densities of *H. glycines* may be misinterpreted if the SCAR primer set was used. The remaining samples all produced specific amplification for both primers (single melting curve at 80.5±0.5 °C and 75±0.5 °C for SCAR and COXIII, respectively). Both SCN-specific primer

pairs used in our developed qPCR assay were able to identify and quantify SCN directly from field soil samples.

Table 3. 1. Comparison between the traditional microscopic counts and qPCR estimates of *H. glycines* eggs in different natural field soils of Manitoba (sandy loam soil texture) and Ontario (different soil textural group).

Field soil ^a	Microscopic estimation ^b	qPCR estimation ^c		<i>P</i> -value		<i>P</i> -value
	(#eggs/100cm ³ soil)	(eggs/100cm ³ soil)		(Comparison between #eggs estimated by two quantification methods)		(Comparison between #eggs estimated by the primers)
Manitoba		SCAR	COXIII	SCAR	COXIII	
S1	0	1.01	0.65	0.99	0.99	1.00
S2	33	2.38	2.37	0.29	0.29	1.00
S3	67	2.71	5.09	0.23	0.21	0.86
S4	120	88.78	105.43	0.62	0.90	0.91
S5	466	636.3	525.29	0.17	0.37	0.30
S6	1,833	2,927	1,449.5	0.06	0.19	0.06
S7	4,616	5,342	2,379.3	0.09	0.06	0.03*
S8	5,216	7,473	3,227.74	0.06	0.06	0.02*
S9	16,550	17,907	4,909.28	0.07	0.01*	0.01*
S10	24,556	21,381	5,717.99	0.09	0.01*	0.01*
Ontario						
F1	104	11.33	18.87	0.17	0.32	0.70
F2	496	359.83	338.85	0.53	0.45	0.99
F3	550	503.35	520.31	0.97	0.99	0.99
F4	870	299.46	229.24	0.08	0.08	0.51
F5	1,611	2,215.31	1,375.47	0.11	0.32	0.08
F6	1,948	2,360.13	1,994.69	0.13	0.39	0.19
F7	2,296	2,578.93	1,594.43	0.17	0.11	0.07
F8	2,419	2,872.07	2,112.07	0.08	0.99	0.08
F9	2,655	1,634.63	1,280.58	0.08	0.06	0.24
F10	3,292	2,132.71	1,349.50	0.23	0.06	0.06
F11	3,305	2,945.75	2,495.42	0.32	0.12	0.20

^a The naturally infested field soil samples were collected from the symptomatic soybean field in Manitoba and different fields in southern Ontario

^b Mean number of eggs/100cm³ soil estimated by traditional microscopic counting based on the average of three wet-sieving extractions and three counting replicates per soil sample

^c Mean number of eggs/100cm³ soil estimated by qPCR based on the mean cycle threshold (Cq) value from three independent biological replicates, each analyzed in four technical replicates per soil sample

*Indicates significance at $P < 0.05$

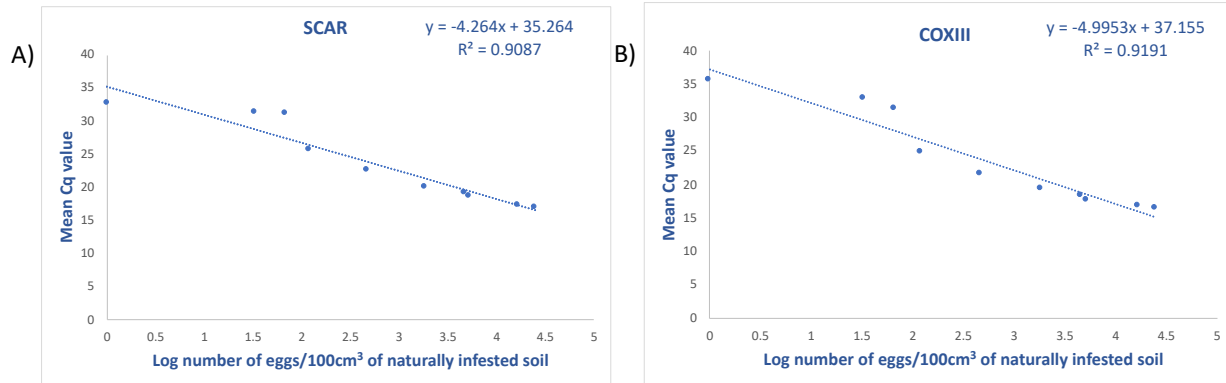


Fig. 3. 9. Real-time qPCR analysis. Relationship between the number of SCN eggs/100 cm³ soil determined in 10 naturally infested Manitoba field soils (sandy loam texture) under the microscope and quantification cycle number (Cq) for the A) SCAR and B) COXIII primer sets. Each Cq value represents the average of three biological replicates (DNA extraction) and four technical replicates (qPCR run), and the corresponding traditional counting represents the average of three wet-sieving extractions and three counting replicates per sample. R²: coefficient of determination.

3.4.6. Relationship between the Number of *H. glycines* Eggs Quantified by Microscopy and qPCR in Naturally Infested Field Soils

The developed qPCR assays were further validated by comparing the estimates of *H. glycines* eggs in field soil samples obtained by traditional microscopic counting and the numbers calculated using the soil calibration curve equation. By substituting the C_q values of DNA extracted from the naturally infested soil in the equation obtained in Fig. 3.7, the number of SCN eggs in 100 cm³ of the soil was estimated. Using that estimate, a linear regression was conducted between the counted *H. glycines* using the microscopy method and the qPCR estimate (Fig. 3.10).

In Manitoba field soils, the SCN densities varied from 0 to 5,000 eggs/ 100 cm³ soil, except for two exceptional samples (S9 and S10), where the egg counts were notably higher, reaching as many as 16,000 and 24,000 eggs/100 cm³ soil. In the field soils of Ontario, the egg counts ranged from 100 to 3,000 eggs/ 100 cm³ soil (Table 3.1). According to the qPCR estimates, all 21 composite samples tested positive for SCN when both primers were used, whereas one of the Manitoba samples tested negative for SCN (S1) using the conventional counting method.

The qPCR analysis, using the COXIII primer set, generally resulted in lower egg counts compared to the conventional counting method in both Manitoba and Ontario soils. Nonetheless, there were no statistically significant differences between the estimated number of eggs from the two quantification methods within each sample in all Ontario field soils. Similarly, no significant difference was observed within each Manitoba soil sample with less than about 5,000 eggs/ 100 cm³ soil (Table 3.1). This underestimation was only significant ($P < 0.05$) in the very highly infested soil samples from Manitoba (S9 and S10). This notable variability in estimating egg numbers between the two quantification methods in heavily infested soils is likely attributed to a high concentration of DNA extracted or reduction in DNA extraction efficiency and the variation

in nematode DNA at various developmental stages existing in naturally infested field soils (Yan et al. 2013; Baidoo et al. 2017). Additionally, the COXIII primer set (Mt-DNA gene) is often more susceptible to PCR inhibitors compared to nuclear DNA due to its smaller size. These factors consequently affect the accuracy of qPCR quantification.

In soil samples where DNA was amplified with the SCAR primer set, the qPCR method's estimates of *H. glycines* eggs were not significantly different from those obtained through microscopic counting within all Ontario and Manitoba soil samples naturally infested with varying levels of *H. glycines* (Table 3.1). Despite these differences, there was a relatively strong positive relationship between the number of eggs quantified using qPCR and traditional microscopic counting, regardless of the soil types, nematode population densities, and primer sets used ($R^2 = 0.9745$ and 0.7507 , $P < 0.001$ for SCAR and $R^2 = 0.8895$ and 0.7174 , $P < 0.001$ for COXIII) (Fig. 3.10).

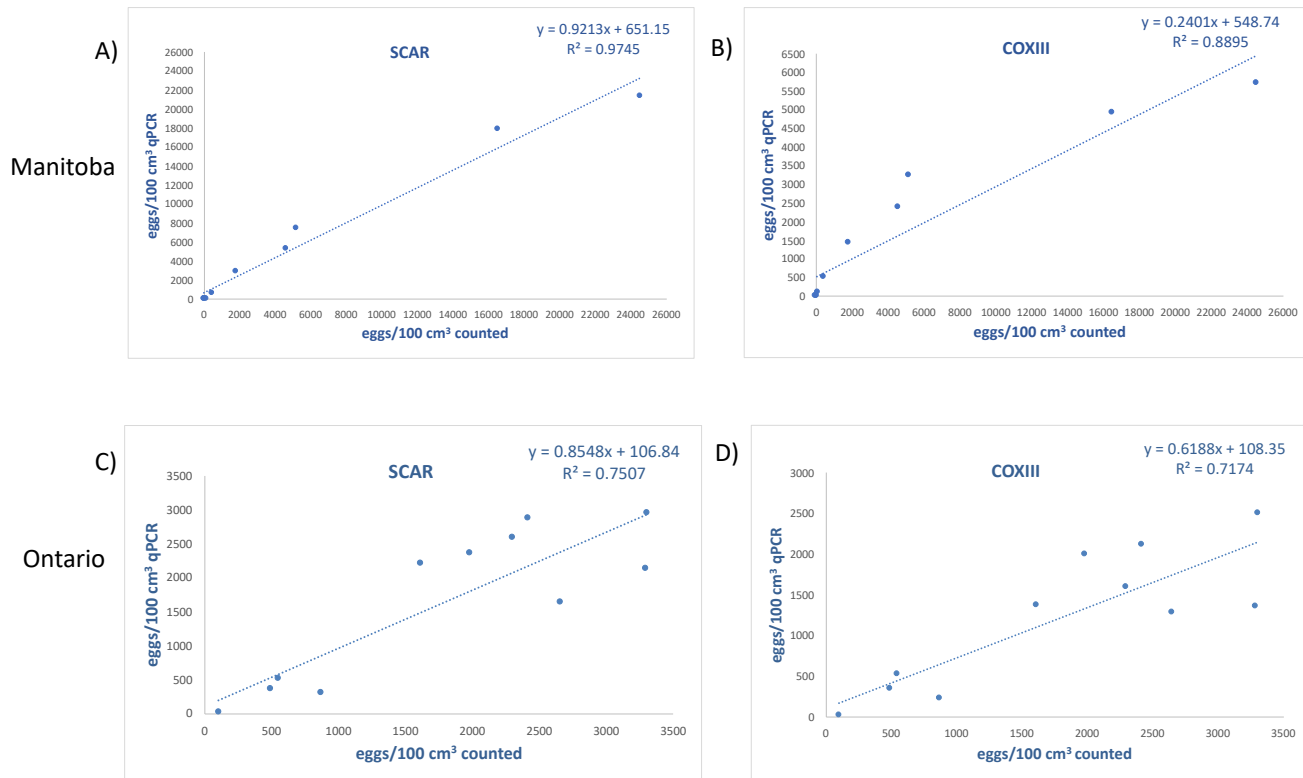


Fig. 3. 10. Relationship between the number of *H. glycines* eggs/100 cm³ soil in naturally infested field soils of A, B) Manitoba (sandy loam soil texture) and C, D) Ontario (different soil textural group) counted under the microscope and qPCR estimates of the same samples using the SCAR and COXIII primer sets. Each dot for qPCR represents the average of three biological replicates (DNA extraction) and four technical replicates (qPCR run), and the corresponding dot for traditional counting represents the average of three wet-sieving extractions and three counting replicates per sample. R^2 : coefficient of determination.

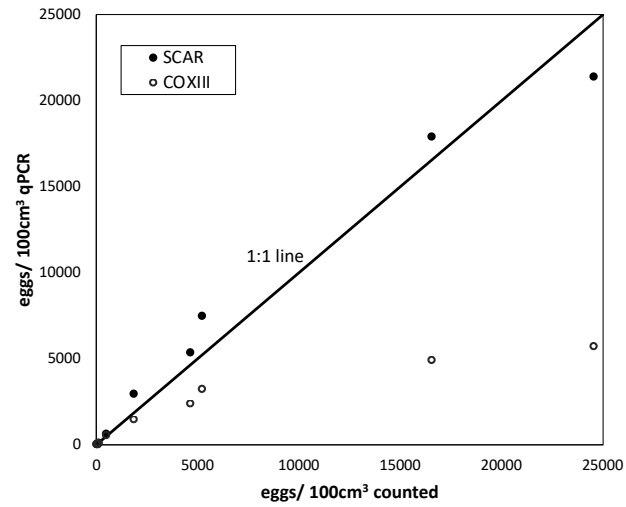
3.4.7. Comparison of the qPCR Estimates of *H. glycines* Eggs between SCAR and COXIII

Primer Sets

In comparison between SCAR and COXIII primer sets of qPCR-estimated egg density in 10 naturally infested field soils of Manitoba, all characterized by a sandy loam texture, no significant difference was observed between the numbers determined by the two primers within soils with less than about 5,000 eggs/100cm³ soil (Table 3.1). However, in naturally infested soil samples with egg densities exceeding 5000 eggs/100cm³ soil, the difference between qPCR estimates of eggs by the SCAR and COXIII was significantly different ($P < 0.05$). This difference became more pronounced in very highly infested soils with more than 15,000 eggs/100cm³ soil (Fig. 3.11 A).

In naturally infested field soils from Ontario, covering a variety of soil types, there was no significant difference between the number of *H. glycines* eggs determined by the two primers within all soil samples (egg counts ranging from 100 to 3,000 eggs/ 100 cm³ soil) (Table 3.1). However, in Ontario soil samples with egg count exceeding 1,000 eggs/100cm³ soil, greater variability was observed in the qPCR estimates of eggs between the two primer sets (Fig. 3.11 B).

A) Manitoba



B) Ontario

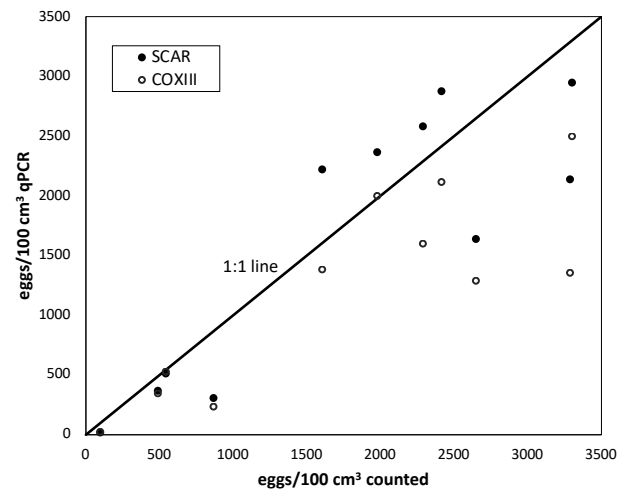


Fig. 3. 11. Comparison of the qPCR estimates of *H. glycines* eggs/100 cm³ soil between SCAR and COXIII primer sets in naturally infested field soils of A) Manitoba (sandy loam soil texture) and B) Ontario (different soil textural group). A scatter plot with 1:1 line.

3.5. Discussion

This research presents a SYBR Green I-based qPCR method for quantifying *H. glycines* directly from field soils. To achieve this, the assay used the soil debris obtained from the sieving method to extract DNA and applied previously reported species-specific primers, COXIII and SCAR, which target the 252 bp of the COI sequence of Mt-DNA and the 477 bp of the RAPD-SCAR marker, respectively (Ou et al. 2008; Madani et al. 2013). These primers were selected based on their ability to detect low quantities of DNA in SCN populations and their consistency across dilution series and qPCR reactions.

However, while previous studies evaluated the specificity and sensitivity of these primers only in DNA extracted from nematode individuals in nematode suspensions, this study tested them in DNA extracts from local *H. glycines* egg suspensions and soils to confirm their suitability for direct quantification of *H. glycines* in field soils. To the best of our knowledge, this is the first time that the COXIII and SCAR primer sets have been used to quantify *H. glycines* directly in soil DNA extracts from natural fields using qPCR assay.

The qPCR assays developed in this study were shown to be sensitive and specific in both artificially and naturally infested soil samples with *H. glycines* eggs, detecting as few as 10 eggs /100cm³ soil. In comparison to other studies, the sensitivity of these assays was comparable to the qPCR assay developed by Baidoo and Yan (2021), which was able to detect 1 *H. glycines* egg in 20 g of North Dakota field soil (10 eggs/200 g soil). However, the current method had a higher detection limit than previously reported studies, such as the one by Baidoo et al. (2017) which used a PCR method targeting the SCAR genes to detect *H. glycines* in soil. They reported a detection limit of as low as 20 *H. glycines* eggs/200g soil; however, it was noted that not all the samples with a low SCN population produced visible bands when visualized on a gel. Another study by

Goto et al. (2009) used a combination of compaction and qPCR to quantify *H. glycines* directly in the soil, with a minimum detection limit of 10 J2 of *H. glycines* per 20 g of soil (equivalent to 100 *H. glycines* /200 g). The primers used in this study were also able to detect *H. schachtii* and *H. trifolii* with equal efficiency. To validate the soil calibration curve generated by the developed qPCR assay, naturally infested field soils were used.

When the soil calibration curve developed in this study was tested to estimate the number of *H. glycines* eggs in naturally infested fields in Manitoba and Ontario, a strong relationship was observed between the number of eggs counted under the microscope and those quantified using the soil calibration curve and qPCR for both primer sets. However, some variation was observed between the two methods of estimating *H. glycines* eggs, with higher variation observed in samples with very low and very high population densities. The variations in samples with these SCN population densities could result from differences in DNA concentration, reductions in DNA extraction efficiency, and different amounts of DNA recovered during the extraction process, ultimately affecting the precision of qPCR quantification. In support of this hypothesis, Khanna and Stotzky (1992) and Goto et al. (2009) have reported greater binding of DNA to soil particles or humic substances at lower DNA concentrations. Yan et al. (2013) and Baidoo et al. (2017) found that the variability in estimating egg numbers between the two quantification methods in heavily infested soils is likely linked to the variation in nematode DNA at various developmental stages present in naturally infested field soils (Yan et al. 2013; Baidoo et al. 2017). Supporting this, Cunha et al. (1999) observed an increase in the number of cells in nematodes during growth. Additionally, Sato et al. (2007) reported significant differences in Cq values of *P. penetrans* among different developmental stages of the nematode. Nonetheless, Baidoo and Yan (2021) found no significant difference in the DNA contents between SCN J2 and eggs. They concluded that either eggs,

juveniles (J2), or a mixed population can be used for the qPCR estimation. However, it is worth noting that they observed a greater variation in Cq values in samples dominated by eggs compared to J2s. These differences in Cq values might be due to the variation in copy numbers between different developmental stages of the nematode. However, a study investigating the developmental regulation of mitochondrial genome copy number in the free-living nematode *Caenorhabditis elegans* found that the Mt-DNA copy number of an embryo remains constant during the early stages of development, increasing with sexual maturation and closely linked to reproduction (Tsang and Lemire 2002).

The results of the current study also revealed that in highly infested soils, the primers demonstrated different outcomes. Specifically, the SCAR primer set showed no significant difference between traditional and molecular egg estimates, whereas the COXIII primer showed a significant difference. This observed variability could be linked to the smaller size of the Mt-DNA compared to nuclear DNA, which makes it more susceptible to potential inhibition and lower DNA extraction efficiency.

Results of comparisons among primer sets suggest that the SCAR primer set could accurately quantify *H. glycines* in soils with high SCN infestation, while the suitability of the COXIII primer set is higher in less-infected soils. However, the densities below 5,000 eggs/100cm³ soil are more realistic in Manitoba's fields than those exceeding 5,000 eggs/100cm³. Therefore, both primer sets can be used reliably to estimate SCN numbers using qPCR in the province.

The manual method of wet-sieving and egg extraction can be performed in any laboratory without the need for specialized machinery. This method takes 15 minutes per sample to obtain ground soil debris, which is comparable to the time needed for compacting with automated tools. Additionally, this developed method uses a larger sample size of approximately 200 g of soil to

obtain a more representative sample, whereas the majority of other reported methods use smaller soil samples, which may not be as representative (Ophel-Keller et al. 2008). As a result, the qPCR assays we developed can quickly and accurately process multiple soil samples in a single day, including wet-sieving, DNA extraction, sensitive identification and quantification of *H. glycines*. The method eliminates the need for time-consuming microscopic identification and egg counting.

In this study, we used non-infested silty clay texture soil debris to generate the calibration curve, which we then used to estimate the density of *H. glycines* eggs in Manitoba and Ontario field soils with different soil textural groups. The equation we derived from the calibration curve was reliable in estimating egg density in those field soils, suggesting that these curves can be used to quantify the *H. glycines* in different soil types. These findings align with those of previous research conducted by Brierley et al. (2009), who found that the type of soil did not affect the recovery of soil-borne potato pathogens *C. coccodes*. In another study, Goto et al. (2010) investigated the effect of soil pre-treatment (either soil compaction or ball mill pulverization) on PCN quantification using qPCR assay in three different soil types and found that their calibration curve was applicable to different soil texture classes when the soil was pre-treated with ball milling. However, Sato et al. (2010) suggested that creating a calibration curve for each soil type may be necessary due to differences in DNA extraction and PCR efficiency. Similarly, Baidoo and Yan (2021) found that soil texture is an important consideration when estimating nematodes directly from soil using qPCR.

Although our method of wet-sieving and DNA extraction from soil debris did not appear to affect qPCR amplification efficiency across different soils, it is important to exercise caution as the calibration curve we developed may not accurately reflect all other soil types and conditions.

Additional experiments are required to investigate the influence of soil texture on the reliability of qPCR quantification.

The present study found that a technique combining DNA extraction from ground soil debris obtained through the sieving method and a SYBR Green I-based qPCR assay using species-specific primers (either SCAR or COXIII) can be used to detect and quantify SCN in both artificially and naturally infested soil samples. The damage threshold for SCN varies depending on soil and environmental factors, making it difficult to apply to individual fields with confidence. However, the minimum detection sensitivity achieved in this study was much lower than the threshold levels used in commercial laboratories in Illinois and other U.S. states (3 and 1 cyst/100cm³ soil, respectively, according to Niblack 2005). This suggests that this newly developed assay can be helpful in devising strategies to manage nematodes. With nematode populations fluctuating annually, this assay will help growers make informed decisions regarding crop selection and cultivars.

3.6. References

- Allen TW, Bra A, Sisson AJ, Byamukama E, Chilvers MI, Coker CM, Collins, AA, Damicone JP, Dorrance AE, Dufault NS, Esker PD. 2017. Soybean yield loss estimates due to diseases in the United States and Ontario, Canada, from 2010 to 2014. *Plant Health Prog.* 18(1):19-27.
- Amiri S, Subbotin SA, Moens M. 2002. Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *Eur J Plant Pathol.* 108:497-506.
- Anderson TR, Welacky TW, Olechowski HT, Ablett G, Ebsary BA. 1988. First report of *Heterodera glycines* on soybeans in Ontario, Canada. *Plant Dis.* 72:453.
- Arora D, Yan G, Baidoo R. 2020. Developing a real-time PCR assay for direct detection and quantification of *Pratylenchus scribneri* in field soil. *Nematology.* 22(7):733-744.
- Baidoo R, Yan G. 2021. Developing a real-time PCR assay for direct identification and quantification of soybean cyst nematode, *Heterodera glycines*, in soil and its discrimination from sugar beet cyst nematode, *Heterodera schachtii*. *Plant Dis.* 105(12):3848-3857.
- Baidoo R, Yan G, Nelson B, Skantar AM, Chen S. 2017. Use of chemical flocculation and nested PCR for *Heterodera glycines* detection in DNA extracts from field soils with low population densities. *Plant Dis.* 101(7):1153-1161.
- Braun-Kiewnick A, Kiewnick S. 2018. Real-time PCR, a great tool for fast identification, sensitive detection and quantification of important plant-parasitic nematodes. *Eur J Plant Pathol.* 152:271-283.
- Briarly RA, Boag B, Neilson R. 2009. An assessment of methods for the extraction of plant nematodes from soil and the enumeration of nematode populations. *Soil Research.* 47(3):211–219.

- Castillo P, Vovlas N. 2007. *Pratylenchus* (Nematoda: Pratylenchidae): diagnosis, biology, pathogenicity and management. Boston (IL): Brill. 6:305-324.
- Cunha A, Azevedo RB, Emmons SW, Leroi AM. 1999. Variable cell number in nematodes. *Nature*. 402(6759):253-253.
- Ferris VR, Subbotin SA, Ireholm A, Spiegel Y, Faghihi J, Ferris JM. 1999. Ribosomal DNA sequence analysis of *Heterodera filipjevi* and *H. latipons* isolates from Russia and comparisons with other nematode isolates. *Russ J Nematol*. 7(2):121-125.
- Fusco V, Quero GM, Morea M, Blaiotta G, Visconti A. 2011. Rapid and reliable identification of *Staphylococcus aureus* harbouring the enterotoxin gene cluster (egc) and quantitative detection in raw milk by real-time PCR. *Int J Food Microbiol*. 144(3):528-537.
- Gerdemann JW. 1955. Relation of a large soil-borne spore to phycomycetous mycorrhizal infections. *Mycologia*. 47:619-632.
- Goto K, Sato E, Toyota K. 2009. A novel detection method for the soybean cyst nematode *Heterodera glycines* Ichinohe using soil compaction and real-time PCR. *Jpn J Nematol*. 39:2-7.
- Goto K, Sato E, Gang L, Toyota K, Sugito T. 2010. Comparison of calibration curves prepared by soil compaction and ball milling methods for direct quantification of the potato cyst nematode *Globodera rostochiensis* in soil. *Jpn J Nematol*. 40(2):41-45.
- Hartman GL, West ED, Herman TK. 2011. Crops that feed the world 2. Soybean-worldwide production, use, and constraints caused by pathogens and pests. *Food Secur*. 3(1):5-17.
- Ingham RE. 1994. Nematodes: methods of soil analysis, part 2. Microbiological and biochemical properties no. 5. Madison (WI): Soil Society of America; p. 459–490.

- Khanna M, Stotzky G. 1992. Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. Appl Environ Microbiol. 58(6):1930-1939.
- Ko HR, Kang H, Park EH, Kim EH, Lee JK. 2019. Identification of *Heterodera glycines* (Tylenchida; Heteroderidae) Using qPCR. Plant Pathol J. 35(6):654-661.
- Li Y, Lawrence GW, Lu S, Balbalian C, Klink VP. 2014. Quantitative field testing *Heterodera glycines* from metagenomic DNA samples isolated directly from soil under agronomic production. PLOS One. 9(2):e89887.
- Maafi ZT, Subbotin S, Moens M. 2003. Molecular identification of cyst-forming nematodes (*Heteroderidae*) from Iran and a phylogeny based on ITS-rDNA sequences. Nematology. 5(1):99-111.
- Madani M, Subbotin SA, Moens M. 2005. Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using real-time PCR with SYBR green I dye. Mol Cell Probes. 19(2):81-86.
- Madani M, Vovlas N, Castillo P, Subbotin SA, Moens M. 2004. Molecular characterization of cyst nematode Species (*Heterodera* spp.) from the Mediterranean Basin using RFLPs and sequences of ITS-rDNA. J Phytopathol. 152(4):229-234.
- Mimee B, Peng H, Popovic V, Yu Q, Duceppe MO, Tétreault MP, Belair G. 2014. First report of soybean cyst nematode (*Heterodera glycines* Ichinohe) on soybean in the province of Quebec, Canada. Plant Dis. 98(3):429-429.
- Min YY, Toyota K, Sato E. 2012. A novel nematode diagnostic method using the direct quantification of major plant-parasitic nematodes in soil by real-time PCR. Nematology. 14(3):265-276.

- Min YY, Toyota K, Goto K, Sato E, Mizuguchi S, Abe N, Nakano A, Sawada E. 2011. Development of a direct quantitative detection method for *Meloidogyne incognita* in sandy soils and its application to sweet potato cultivated fields in Tokushima prefecture, Jpn Nematol. 13(1):95-102.
- Niblack TL. 2005. Soybean cyst nematode management reconsidered. Plant Dis. 89(10):1020-1026.
- Noel GR. 1985. The soybean cyst nematode. In: Lamberti F, Taylor CE, editors. Cyst Nematodes. New York (NY): Plenum Press. 121:257-268.
- Ophel-Keller K, McKay A, Hartley D, Curran J. 2008. Development of a routine DNA-based testing service for soilborne diseases in Australia. Australas Plant Pathol. 37(3):243-253.
- Ou SQ, Peng DL, Li Y, Wang YJ. 2008a. Restriction fragment length polymorphism and sequences analysis of rDNA-ITS region of cereal cyst nematode (*Heterodera avenae*) on wheat from Zhengzhou. Acta Phytopathol Sin. 38:407-413.
- Ou S, Peng D, Liu X, Li Y, Moens M. 2008b. Identification of *Heterodera glycines* using PCR with sequence characterised amplified region (SCAR) primers. Nematology. 10(3):397-403.
- Powers T, Harris T, Higgins R, Mullin P, Powers K. 2018. Discovery and identification of *Meloidogyne* species using COI DNA barcoding. J Nematol. 50(3):399-412.
- Quader M, Nambiar L, Cunnington J. 2008. Conventional and real-time PCR-based species identification and diversity of potato cyst nematodes (*Globodera* spp.) from Victoria, Australia. Nematology. 10(4):471-478.

- Rivoal R, Valette S, Bekal S, Gauthier JP, Yahyaoui A. 2003. Genetic and phenotypic diversity in the graminaceous cyst nematode complex, inferred from PCR-RFLP of ribosomal DNA and morphometric analysis. *Eur J Plant Pathol.* 109:227-241.
- Sato E, Goto K, Min Y, Toyota K, Suzuki C. 2010. Quantitative detection of *Pratylenchus penetrans* from soil using soil compaction and real-time PCR. *Nematol Res.* 40(1):1-6.
- Sato E, Min YY, Toyota K, Takada A. 2009. Relationships between the damage to radish caused by the root-lesion nematode *Pratylenchus penetrans*, its density prior to cultivation and the soil nematode community structure evaluated by polymerase chain reaction-denaturing gradient gel electrophoresis. *Soil Sci Plant Nutr.* 55(4):478-484.
- Shirai S, Toyota K. 2019. Optimisation of a species-specific primer set to quantify the soybean cyst nematode, *Heterodera glycines*, in soil using real-time PCR. *Nematology.* 21(10):1037-1042.
- Subbotin SA, Toumi F, Elekçioğlu IH, Waeyenberge L, Maafi ZT. 2018. DNA barcoding, phylogeny and phylogeography of the cyst nematode species of the *Avenae* group from the genus *Heterodera* (Tylenchida: Heteroderidae). *Nematology.* 20(7):671-702.
- Subbotin SA, Waeyenberge L, Moens M. 2000. Identification of cyst-forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLP. *Nematology.* 2(2):153-164.
- Toumi F, Waeyenberge L, Viaen, Dababat AA, Nico JM, Ogbonnaya FC, Moens M. 2015. Development of qPCR assays for quantitative detection of *Heterodera avenae* and *H. latipons*. *Eur J Plant Pathol.* 143:305-316.

- Toyota K, Shirakashi T, Sato E, Wada S, Min YY. 2008. Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. Soil Sci Plant Nutr. 54(1):72-76.
- Tsang WY, Lemire BD. 2002. Mitochondrial genome content is regulated during nematode development. Biochem Biophys Res Commun. 291(1):8-16.
- Vovlas N, Vovlas A, Leonetti P, Liébanas G, Castillo P, Subbotin SA, Palomares Rius JE. 2015. Parasitism effects on white clover by root-knot and cyst nematodes and molecular separation of *Heterodera daverti* from *H. trifolii*. Eur J Plant Pathol. 143:833-845.
- Yan G, Smiley RW, Okubara PA, Skantar AM, Reardon CL. 2013. Developing a real-time PCR assay for detection and quantification of *Pratylenchus neglectus* in soil. Plant Dis. 97(6):757-764.
- Ye W. 2012. Development of primetime-real-time PCR for species identification of soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) in North Carolina. J Nematol. 44(3):284-290.
- Zheng J, Subboth SA, Waeyenberge L, Moens M. 2000. Molecular characterization of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. Russ J Nematol. 8(2):109-113.

4.0 Morphological and Molecular Identification of Native Cyst Nematodes in Prairie Preserves of Manitoba

4.1. Abstract

In October 2020, soil sampling was conducted in three Prairie Preserves located in Manitoba: Manitoba Tall Grass Prairie Preserve (MTGPP), Yellow Quill Mixed Grass Prairie, and Baldy Prairie at Riding Mountain National Park. Within each prairie, five 15×15 m areas were chosen, each separated by a minimum distance of 50 m. Soil samples, which were composites from 15-20 points, were collected to a depth of 20 cm around the roots of different native plants, depending on their presence at the site. A total of 20 composite samples were collected in the fall of 2020. An additional eight composite samples were taken in fall 2021 from the rhizosphere region of the same plants at the MTGPP. Cysts were extracted from each sample using a soil washing unit, a modified Fenwick elutriator developed by the USDA. Wet-sieving and decanting technique were also used in the cyst extraction process. Furthermore, in cases where nematode cysts were discovered in the samples, nematode juveniles were extracted using a modified Cobb's sieving and decanting method. The identification of nematode species was carried out based on the morphometrics and morphological characteristics of cysts and a limited number of second-stage juveniles recovered from the same cysts. To confirm cyst identification, molecular methods were employed. This involved performing PCR amplification on four regions of the genome (ITS rDNA, D2-D3 expansion segments of 28S rDNA; 18S rDNA, and a fragment of Mt-DNA (COI)). The purified PCR products were subsequently sequenced for all four regions of the genome. Out of the three prairies that were examined, nematode cysts were recovered exclusively in one of them, specifically within the MTGPP, in soil samples collected from the root area of prairie cordgrass. Based on morphological and molecular analysis, we identified the cyst nematodes recovered as

Punctodera stonei. To our knowledge, this marks the first report of *P. stonei* in Manitoba, and the second recorded case in Canada.

4.2. Introduction

Nematodes are microscopic worms and represent one of the most abundant groups of multi-cellular organisms. They are classified into eight identified feeding categories, including predators, animal parasites, omnivores, as well as plant, fungal, bacterial, eukaryote, and substrate feeders. Soil nematodes contribute significantly to ecosystems by impacting root biomass, facilitating nutrient cycling and shaping the functional composition of the soil microbiome (Neher 2010). The remarkable functional diversity among nematodes, coupled with their abundance, limited motility, and sensitivity to environmental factors, has also positioned this group as valuable bioindicators of soil health (Bongers and Ferris 1999; Ekschmitt et al. 2001; Griffiths et al. 2001).

However, Plant-Parasitic Nematodes (PPNs) pose a significant threat to numerous crops, ranging from native grasses to high-yielding hybrid corn varieties worldwide. Most PPNs are small, measuring between 0.3 to 1.0 mm, although some can reach a length of up to 4.0 mm. In mature females of certain PPN species, distinct swelling occurs, resulting in varied shapes such as pear, lemon, or kidney (cysts) (Agrios 2005). These PPNs employ a needle-like structure called a stylet to puncture the cell wall of host plants, withdrawing the cell contents and causing damage to the crop. Among PPNs, root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.), characterized as the world's most destructive agricultural pests (Curtis 2007).

All cyst nematodes, classified under the *Heteroderidae* family, are obligate endoparasites that feed from within the roots of their host plants. A distinguishing feature of cyst nematodes is the transformation of female cuticles into persistent and protective cysts for their eggs (Moens et al. 2018). Cyst nematodes parasitism is characterized by the creation of permanent feeding sites known as syncytia, achieved through the modification of root cells within the cortex, endodermis,

pericycle, and vascular parenchyma of host tissues. This parasitic activity can result in the suppression of plant growth and reduced crop yields (Jones 1981; Stone 1985; Baldwin and Mundo Ocampo 1991).

Cyst nematodes are classified into eight genera within the *Heteroderinae* subfamily: *Heterodera*, *Globodera*, *Cactodera*, *Dolichodera*, *Paradolichodera*, *Betulodera*, *Punctodera* and *Vittatidera* (Subbotin et al. 2010; Moens et al. 2018). The first instance of a cyst-forming nematode was reported by Schacht in 1859, affecting sugar beets with poor development. Subsequently, Schmidt (1871) provided a formal description, labeling it as *Heterodera schachtii*. *Heterodera punctata*, commonly referred to as the grass cyst nematode, marked the first discovery of a cyst nematode species in North America (Thorne 1928).

The genus *Punctodera* Mulvey and Stone, 1976, includes cyst-forming nematodes that parasitize both cultivated and wild-growing plants belonging to the *Graminae* family (Dobosz et al. 2013). *Punctodera stonei* (Brzeski 1998) is a species belonging to this genus (Mulvey and Stone 1976). Other species within the genus include *P. punctata* (Thorne 1928; Mulvey and Stone 1976), *P. matadorensis* (Mulvey and Stone 1976), *P. chaltoensis* (Stone et al. 1976), along with more recent additions like *P. mulveyi* (Kantor et al. 2020) and *P. achalensis* (Lax et al. 2021).

Thorne first described *P. punctata* in 1928 from wheat samples in Saskatchewan, Canada, initially categorizing it as *Heterodera punctata*. This nematode is native to the United Kingdom, where it has been found on *Agrostis* spp., and has also been reported on wheat in other parts of Canada (Grujicic 1969). *P. punctata* has been also found on prairie grasses from Lethbridge, Alberta; bent grass in Cap Tourmente, Quebec, and in soils across various Canadian areas, including Labrador, Newfoundland, Sable Island (off Nova Scotia), Manitoba, Ontario, and British Columbia (Baker 1957; Mulvey and Stone 1976). Furthermore, soils containing native grass roots

from Matador Ranch, Matador, Saskatchewan, Canada, were found to be infected with nematode cysts in 1971. Initially identified as *Heterodera punctata* by Mulvey in 1972, further detailed identification led to the recognition of a new species, *P. matadorensis*, by Mulvey and Stone in 1976. *P. matadorensis* has also been reported in North Dakota (Handoo et al. 2010), distinguished from *P. punctata* with larger cysts containing prominent bullae and a lack of parasitism on wheat (Sharma 1998). *P. chaltoensis* is a significant pest of corn, causing substantial maize losses in Mexico and being classified as a harmful organism in Brazil, Ecuador and South Korea (CAPS 2017).

The discovery of Stone's cyst nematode, *P. stonei*, can be traced back to its initial description from a grass lawn in Skirniewice, Poland, by Brzeski (1998). In Poland the genus *Punctodera* is encompasses two known species: *P. stonei*, a nematode parasite of root systems of wild-growing grasses (Brzeski 1998), and *P. punctata*, which inhabits the root area of weeds in spring cereal fields (Ishaq 1992). *P. stonei* has also been reported in various other regions, including England and Spain (Brzeski 1998), Germany (Brzeski 1998; Sturhan 2006), Slovakia (Sturhan and Lišková 2004), The Netherlands (Holterman et al. 2009) Greece (Fauna Europea 2012; Dobosz et al. 2013), Canada (Dobosz et al. 2013; Kantor et al. 2020), and USA (Kantor et al. 2022).

Subbotin et al. (2010) provided an extensive summary of the current knowledge regarding the morphology, biology, pathogenicity and geographical distribution of cyst nematodes. Furthermore, they provided an identification key to cysts and second-stage juveniles. The differentiation of *Punctodera* species relies on minor morphological and morphometric variations observed in both cysts and second-stage juveniles. Identifying *Punctodera* species through the morphological characterization of cysts and specific features of second-stage juveniles demands a high level of skill and expertise to effectively interpret these subtle differences, which can sometimes be

ambiguous. Morphometric measurements of these characteristics frequently reveal overlapping values among species, further complicating the morphological identification of cyst nematodes, particularly when distinguishing between *P. stonei* and *P. matadorensis*. Notably, *P. stonei* closely resembles *P. matadorensis*, with distinctions observed in the shape of the J2 stylet knobs, which are somewhat flat to slightly concave anteriorly in *P. stonei* as opposed to strongly concave anteriorly, and in the length of the hyaline region, which spans from 41-66 µm in *P. stonei* compared to 35-42 µm in *P. matadorensis* (Subbotin et al. 2010; Kantor et al. 2020).

The identification of cyst-forming nematodes is best achieved through a combination of both morphological and molecular methods, especially in cases where new introductions of these nematodes are suspected (EPPO 2017). The key DNA regions reported for the identification of cyst nematodes are found within nuclear ribosomal RNA genes. These include the 18S (the small nuclear ribosomal subunit), the 28S ribosomal DNA (the large ribosomal subunit), and notably, the internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2). The ITS1 and ITS2 are situated between 18S and 28S rRNA genes, respectively (Subbotin et al. 2001, 2020; Vallejo et al. 2021). These regions have proven to be highly suitable for both sequencing and for resolving relationships at the species level.

The ribosomal genes that flank the internal transcribed spacer region (ITS-rRNA) have a higher mutation rate compared to other rRNA regions, often offering adequate sequence variability to differentiate nematodes at the species level (Subbotin et al. 2000a; Zheng et al. 2000). Over the past decade, significant progress has been made in nucleic acid sequencing techniques, leading to the reporting and deposition of ITS-rRNA gene sequences for a considerable number of cyst nematode species in the GenBank database (Subbotin et al. 2010). A leading technique for identifying cyst nematodes involves comparing the gene sequences of unknown species with those

already published and stored in the GenBank database. This method enables the reliable and rapid identification of nematodes, allowing for differentiation between different nematode species and those closely related to them (Ferris et al. 1995; Thiéry and Mugniéry 1996; Orui 1997; Blok et al. 1998; Subbotin et al. 1999, 2000a; Sirca and Urek 2004; Madani et al. 2005, 2008, 2010; Uehara et al. 2005; Skantar et al. 2007).

Recent studies have unveiled the existence of multiple sibling species within currently defined plant-parasitic nematode species, such as *G. pallida* (Subbotin et al. 2000b; Madani et al. 2010), *H. avenae* (Subbotin et al. 2003), *P. punctata* (Mulvey 1972; Brzeski 1998; Subbotin et al. 2010; Kantor et al. 2020), and several others. However, in some instances, the ITS-rRNA sequences do not exhibit sufficient differences to distinguish among closely related species, such as *H. avenae* and *H. arenaria*, as well as *H. daveri* and *H. trifolii* (Subbotin et al. 2003; Subbotin 2010, unpublished). Therefore, a more confident distinction among closely related species will require combined analyses of the ITS region with other molecular regions.

Mitochondrial DNA (Mt-DNA) genes, characterized by their relatively higher mutation rates compared to rRNA genes, have also been introduced as valuable tools for diagnosing races and populations of cyst nematodes (Subbotin et al. 2010; Madani et al. 2010). The mitochondrial genome is a logical choice for developing species-specific diagnostic markers due to its presence in high copy numbers, consisting of rapidly evolving genes and conserved regions, all of which enable its broad phylogenetic application (Hyman 1988; Blouin 2002; Powers 2004).

Cyst nematode distribution within the native prairies of Canada, particularly within the prairie preserves of Manitoba, has not been examined thoroughly. It is feasible that nematodes in cultivated fields have their origins in these native prairies. Therefore, studies of the native prairie can provide insights into some of the agricultural/environmental challenges we face. Most of the

prior surveys were conducted many years ago and primarily focused on non-cyst-forming plant-parasitic nematodes. The objective of this research is to bridge this knowledge gap by identifying the cyst nematodes present in the prairie preserves of Manitoba.

The tallgrass prairie, known for its remarkable productivity, stands as North America's most endangered prairie ecosystem, primarily due to extensive conversion to agricultural use. Historically, the tallgrass prairie covered an area of about 577,500 km² stretching from southern Manitoba down to Texas (Reinking 2003). Today, the Manitoba Tall Grass Prairie Preserve (MTGPP) represents the largest remaining intact area of tallgrass prairie within Manitoba and it comprises the most extensive section of tallgrass prairie in Canada. Nevertheless, it is essential to note that the Preserve now occupies only a fraction (0.8%) of its original expanse of 6,000 km² (Koper et al. 2010).

The mixed grass prairie, historically a significant ecological community, once spanned an estimated 69 million hectares across the central region of North America (Bragg and Steuter 1996). Regrettably, it now stands as an endangered ecosystem, existing in the form of small, isolated fragments. Among these remnants, the Yellow Quill Mixed Grass Prairie Preserve is situated in southwestern Manitoba and is under the ownership of the Nature Conservancy of Canada (NCC). Assessments of the precise proportion of the original area that remains in Manitoba vary, with estimates ranging from less than 1% (Samson and Knopf 1994) to less than 18% (Sveinson et al. 2001).

Encompassing an area of approximately 3,000 km², the Riding Mountain National Park (RMNP) exhibits three overlapping vegetation zones, which include mixed-wood forests, boreal forests, and small remnants of the Great Plains rough-fescue grasslands (Parks Canada 2007). Rough fescue prairies have faced substantial declines in western Canada due to factors such as

land conversion, industrial development, the invasion of non-native plant species, and afforestation (Widenmaier and Strong 2010; Thorpe et al. 2015).

To identify cyst nematodes in the Manitoba prairie preserves characterized by distinct vegetation covers, including the Tall-grass, Mixed-grass, and Fescue grassland, we employed a combination of morphological and molecular methods. This included PCR and sequencing of specific genetic markers such as the ITS of rDNA, partial 18S rDNA, D2-D3 expansion segments of the 28S nuclear ribosomal DNA gene, and mitochondrial gene (COI).

4.3. Materials and Methods

4.3.1. Study Area

The research was conducted in three Manitoba prairie preserves distinguished by distinct vegetation covers, including the Manitoba Tall Grass Prairie Preserve (MTCPP), Yellow Quill Mixed Grass Prairie Preserve, and Baldy Prairie at Riding Mountain National Park (RMNP).

The Manitoba Tall Grass Prairie Preserve (MTCPP) is situated within the NE 30-01-06 EPM coordinates. In MTCPP, our sampling focused on the southern region of the Preserve, specifically the Prairie Shore Trail, extending for 1.5 km and lying approximately 2.5 km east of the town of Tolstoi. Dominant native species in this area include golden Indian grass (*Sorghastrum nutans* L.), prairie cordgrass (*Spartina pectinate*), prairie dropseed (*Sporobolus heterolepis*), and big bluestem (*Andropogon gerardii*).

The Yellow Quill Mixed Grass Prairie Preserve is approximately an 842-hectare prairie remnant in Manitoba (Sveinson et al. 2001), located approximately 20 km southeast of Brandon and 2 km north of the junction of the Souris and Assiniboine Rivers (SW26/27/28/29/34-8-16W). This Preserve is surrounded by agricultural and military-owned land and has been utilized for cattle grazing for several decades. The Yellow Quill Prairie itself is situated on Miniota sands,

characterized by well-drained soils with varying textures from sand to loamy sand (Ehrlich et al. 1957). The vegetation within this Preserve comprises a diverse array of grasses, sedges and wildflowers, including little bluestem (*Andropogon scoparius*), prairie clover (*Petalostemon* spp.), prairie sage (*Artemisia ludoviciana*), pasture sage (*Artemisia ludoviciana*), Aster (*Aster* spp.), and wild rose (*Rosa* spp.).

Riding Mountain National Park (RMNP), positioned in southwestern Manitoba, Canada (50°65' N, 99°97'W), is approximately 225 km northwest of the city of Winnipeg. The predominant soil types within the park fall within the Grey-Wooded or Grey-Black Transitional Zone, with the majority forming over surface deposits of glacial till (Ehrlich et al. 1956). In RMNP, we focused on the rough fescue grassland communities in the western section of the park, specifically in the vicinity of Baldy Lake and the Rosburn municipality within the Baldy prairie. The native plant cover in this area consisted of wheatgrass (*Agropyron* spp.), oat grass (*Dunthonia intermedia*), prairie sage (*Artemisia ludoviciana*), prairie clover (*Petalostemon* spp.), and wild rose (*Rosa* spp.).

4.3.2. Soil Sampling

In October 2020, soil samples were collected from five 15 × 15 m areas within each prairie. The five areas were distant by a minimum of 50 m. Within each designated area, we selected specific plant species, depending on the dominant plant cover on the site. Soil samples were taken from near the root zone to a depth of 0-20 cm, predominantly focusing on grasses and legumes, as these plant families tend to host a broader diversity of nematodes. For each plant genus within a designated area, we obtained 4-5 subsamples, adjusting the number based on their presence and density, ultimately creating a composite sample comprising 15-20 subsamples. Each composite sample corresponded to a specific plant genus and was collected using a Dutch auger of 5 cm diameter and a length of 15 cm. This resulted in each composite sample containing approximately

3 kg of soil. The plant genera selected for sampling within the three prairie preserves included big and little bluestem, golden Indian grass, prairie cordgrass, prairie dropseed, purple/white prairie clovers, prairie and pasture sage, aster, wild rose, oatgrass, wild licorice, and common grass (Fig. 4.1).

A resampling of the same property in the Manitoba Tall Grass Prairie Preserve was conducted in September 2021, maintaining consistency with the 2020 sampling procedure. A total of 20 composite samples were collected from three different prairies in the fall of 2020. Subsequent eight composite samples were taken in fall 2021, specifically from the rhizosphere region of the same plants located in the MTGPP. The collected samples were placed into labelled plastic bags transferred to the Soil Ecology Laboratory at the University of Manitoba, and stored in a cooler until further processing. To carry out these samplings, the necessary collection permits from prairie authorities were obtained.

In addition to soil samples, root samples were collected from various plant genera, corresponding to the areas where soil samples were taken from their root zone. The roots were examined under a dissecting microscope in the lab to detect any potential presence of female cysts.



Fig. 4. 1. Species of plants about which soil samples were taken for cyst extraction from native prairies of Manitoba, A) big bluestem, B) prairie wheatgrass, C) silver sage, D) prairie oatgrass, E) prairie cordgrass, F) golden Indian grass G) wild rose, H) prairie clover, I) Aster, J) wild licorice, K) pasture sage, L) little bluestem.

4.3.3. Cyst Recovery

Cyst extraction from the soil samples was carried out through a combination of methods, including a modified Fenwick elutriator inspired by the USDA cyst extractor (Fenwick 1940; Roach et al. 1968), as well as modified wet-sieving and decanting (Gerdemann 1955) techniques. The use of a substantial amount of soil with the elutriator was necessary due to the expected low population levels of cyst nematodes in our work. The second extraction approach which relied on the manual wet-sieving of fresh soil samples, was applied to secure viable eggs, as there was a concern that some cyst populations might lose their vitality during the drying process. Furthermore, to target potential populations that might produce smaller cysts, we employed a sieve with a finer mesh size compared to the one used in the Fenwick elutriator method within this manual cyst extraction technique.

4.3.3.1. Modified Fenwick Elutriator

Each composite sample was thoroughly mixed, and representative subsamples were obtained, while the remainder of the soil was set aside for subsequent extraction after being air-dried. 2.2 kg of each air-dried soil sample was used for extracting cysts using the soil washing unit, a modified Fenwick elutriator with nested sieves (20-mesh (850 μm) over 60-mesh (250 μm)) available in the Soil Ecology Laboratory. The air-dried soil was subjected to washing in the extractor, capturing cysts and plant debris on the 60-mesh screen. The debris on the 60-mesh sieve was examined for cyst nematodes under a microscope. Separation of cysts from debris is achieved by flotation in 95% ethanol (Seinhorst 1974) with some modifications. When cysts are extracted from substantial volumes of soil using the cyst extractor, it is common to encounter an excess of debris that is retained on the sieves. This large amount of soil debris generally increases the time required for the examination of each sample to recover cysts.

4.3.3.2. Wet-Sieving and Decanting

The remaining composite samples (fresh soils) were employed for the extraction of cysts using the manual wet-sieving method. A subsample of 100 cm³ from each sample (in two replicates) was passed through a series of sieves with #20 (850 µm) and #70 (212 µm) mesh sizes. The top sieve collected root and soil debris, while the bottom sieve trapped cysts and debris of cyst size. The recovered cysts were identified based on morphological characters and molecular analyses.

4.3.4. Nematode Juveniles Recovery

As juveniles of cyst nematodes may be found incidentally in soil extracts, in addition to the cyst extraction, nematode juveniles were extracted from the samples of a prairie in which cysts of nematodes were found, using modified Cobb's sieving and decanting, followed by the sugar flotation method (Ingham 1994). Approximately 100 g of fresh soil were weighed and placed into 500 ml plastic beakers filled with tap water. Employing another plastic beaker, the soil solutions were mixed back and forth around 10 times. A pair of U.S. Standard Test sieves, one with a mesh size of #45 (355 µm) and the other with #400 (38 µm) openings, were stacked together. The soil solutions were then passed through this sieve assembly, followed by a centrifugation step at 525 xg for 5 min (ST 8 centrifuge, ThermoFisher Scientific, Osterode, Germany). The supernatant was discarded and replaced with a solution of sucrose (454 g l⁻¹). Subsequently, the soil sucrose solutions were centrifuged at 176 xg for 1 min and 15 sec. In the final phase of the process, nematodes suspended in the sucrose solution were collected in a U.S. Standard Test with a mesh size of #500 (25 µm). Nematode counts were conducted for each extract using an inverted microscope (Motic AE21, Microscope World, Carlsbad, CA) and a Petri dish with a grid pattern.

4.3.5. Morphological Identification

The morphological identification of cyst nematode species was accomplished by examining the morphometrics and morphological features of both cyst and second-stage juveniles. We conducted species identification on cysts recovered from the prairie soil samples and a few second-stage juveniles recovered from the same cysts. Additionally, we examined the presence of cyst nematode juveniles in the soil samples obtained from a prairie where cysts had been recovered. This assessment was carried out by examining the morphological features of the specimens in soil extracts under an inverted microscope. To further enhance our understanding of whether the heteroderid J2 nematode was present in these same soil extracts, we subjected the first 100 nematodes on each slide to genus-level identification using a compound microscope.

4.3.5.1. Cyst Identification

Extracted cysts were examined under a microscope and identified using the identification key and description given by Subbotin et al. (2010). The morphological characters observed under the light microscope included; cyst shape, colour and the structure of cone tops. Additionally, morphometric measurements were conducted on the cysts, which included measuring their body length (L), body width (W), and the ratio of body length to body width (L/W).

To examine the structure of cone top, the cysts were cut off using a scalpel and placed in a few drops of distilled water. After cleaning, they were mounted within a drop of melted glycerin-gelatin mixture between slides. For measuring these morphometric characters, a compound microscope (Olympus BX51, Olympus Canada, Inc., Richmond Hill, ON) equipped with a digital imaging camera (QColor3, Olympus, Tokyo, Japan) and an Image-Pro Plus 6.2 software (Media Cybernetics, USA) was used. Our observations were made under transmitted light at 4× and 40× magnifications.

4.3.5.2. Second-stage Juvenile Identification

Species identification of juveniles recovered from cysts involved a thorough examination of their morphological characters, including the presence of a robust stylet, the shape of stylet knobs, and the shape of the tail and hyaline tail terminus. After conducting these morphological examinations, we proceeded to measure key morphometric characters of the J2s, including body length, stylet length, tail length and the length of the hyaline part. All these characters hold taxonomic significance for the diagnosis of cyst nematode species (Wouts and Weischer 1977; Wouts and Sturhan 1995; Subbotin et al. 2010; Perry et al. 2018).

In the soil samples where cysts were detected, we also examined cyst nematode juveniles (heteroderid J2s) recovered using Cobb's sieving method. This examination relied on the morphological features of the specimens during counts under an inverted microscope (Motic AE21, Microscope World, Carlsbad, CA), following an identification key in Mai et al. (1996). The heteroderid J2s were distinguished by their overall shape, which includes a rounded head continuous with the body contour and a tail that tapers to a point; the tail also includes a notable hyaline region (Baldwin and Handoo 2018).

To further clarify the presence of the heteroderid J2s, we prepared slides from the soil extracts already examined under an inverted microscope, and identified the first 100 juveniles in each slide to the genus level under the compound microscope. This classification was based on the criteria outlined in an identification key provided by Mai et al. (1996). The process of slide preparation commenced with the collection of the recovered juveniles from the soil extracts, which were placed into 15 ml conical vials. These vials underwent centrifugation at 684 xg for a duration of 5 min (International Equipment Co, USA) to ensure that all nematodes settled at the bottom. Following centrifugation, the supernatant was removed by pipetting, leaving the nematodes in 0.5 ml of water

within the vial. Drops of a nematode suspension from each vial were placed onto a microscope slide, covered with a coverslip and sealed with finger nail polish. The first 100 nematodes in each slide were identified to genus using a compound light microscope (40×-100× magnification).

4.3.6. Molecular Identification

4.3.6.1. DNA Extraction

Identification of cysts containing viable eggs and J2s was confirmed using molecular methods developed in the Soil Ecology Laboratory. DNA was extracted from the suspension of eggs and juveniles of 10 individual nematode cysts using the Proteinase K method (Subbotin et al. 2000) and crushing with glass beads. A single cyst was placed in a glass slide in double distilled water and crushed while being observed under a dissecting microscope. The resulting suspension of J2s and eggs was then transferred into a sterile Eppendorf tube. The extraction was conducted in volumes of 65 µl, comprising 50µl lysis buffer (tail buffer) (Viagen Biotech, Los Angeles, CA, USA), and 13 µl sterile diH₂O, with eggs and juveniles from each cyst suspended in it. Additionally, three autoclaved 1mm glass beads, and 2 µl of 100 mg Proteinase K (Fisher Bioreagents, Germany) were included. The content in the tubes was gently vortexed (Vortex Genie, Scientific Industries, Inc., Bohemia, NY, USA), followed by centrifugation (2,200 xg for 1 min, Mini Centrifuge, Fisher Scientific), and was then stored overnight at -20°C. The following day, samples were subjected to incubation at 60°C for 1 hour and then at 95°C for 15 minutes in a thermocycler (Eppendorf CF2060, Master-cycler Pro-S, Hamburg, Germany). To assess the quality and quantity of each DNA extraction, a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, DE, USA) was used. The DNA samples were then stored at -20°C before amplification through conventional PCR.

4.3.6.2. PCR Amplification

PCR amplification of DNA from 10 individual cysts by targeting four regions of the genome using different sets of primers was conducted. These regions included three rDNA gene fragments (ITS rDNA, D2-D3 expansion segments of 28S rDNA, and 18S rDNA) as well as one mitochondrial DNA (Mt-DNA) fragment (cytochrome oxidase subunit I (COI)). The amplification was carried out under the conditions specified in Table 4.1. The internal transcribed spacer (ITS) segment was amplified with primers ITS-F and ITS-R (Powers et al. 1997), while the 18S region was amplified with NEM-18S-F and NEM-18S-R primer sets (Floyd et al. 2005). For the 28S segment, the primers D2A and D3B (Nunn 1992) were used. To amplify the partial COI gene of Mt-DNA, Het-coxiF and Het-coxiR were used, following the protocol outlined in Subbotin et al. (2015). The primer sets used for PCR and DNA sequencing are detailed in Table 4.2.

For the D2A-D3B primer set, the PCR reaction mixture consisted of 34.6 µl autoclaved diH₂O, 5 µl of 10X Dream Taq buffer, 2 µl 25 mM MgCl₂, 2 µl dNTPs, 0.4 µl Dream Taq polymerase (Thermo Scientific, Waltham, MA, USA), and 1 µl of each primer (10 µM). 3 µl of thawed nematode DNA was then added to a PCR reaction tube, and the cycling process was performed using a thermocycler (Eppendorf CF2060, Master-cycler Pro-S, Hamburg, Germany). For the Het-coxi primer set, a total volume of 25 µl was prepared for the PCR reaction mixture, containing 15.8 µl sterile water, 2.5 µl 10X Dream Taq buffer, 1 µl dNTPs, 1 µl 25 mM MgCl₂, 0.2 µl Dream Taq polymerase, and 0.75 µl of each primer. The nematode DNA (3 µl) was then added to the PCR reaction tubes and cycled. The reaction mixture for the ITS and NEM-18S primer sets remained consistent with the earlier study described in the second chapter (see Section 2.3.4.1.2).

For all the examined regions of the genome, the resulting amplified products were loaded onto a 1.2% agarose gel (Invitrogen, Carlsbad, CA, USA) stained with 2.5 µl of 10,000X concentrated

GelRed fluorescent nucleic acid gel stain (Biotium, Fremont, CA, USA). The gel was run at 85 V for a duration of 90 min in a gel box (Fisherbrand, Fisher Scientific, Nepean, ON). To determine fragment sizes, the amplified bands were compared to a molecular weight marker of 100 bp DNA ladder (ProMega, Madison, WI, USA).

Table 4. 1. Thermal profiles used in PCR assays.

Primers used	Modified amplification conditions	Reference
ITS-F ITS-R	94°C 5 min; 35 cycles at 94°C 30 sec, 58°C 30 sec, 72°C 1 min, and a final extension 72°C 10 min	Powers et al. 1997
NEM-18S-F NEM-18S-R	94°C 5min; 35 cycles at 94°C 30 sec, 54°C 30 sec, 72°C 1 min, and a final extension 72°C 10 min	Floyd et al. 2005
D2A_D3B	95°C 3 min; 36 cycles at 95°C 1 min, 55°C 30 sec, 72°C 1 min, and a final extension 72°C 10 min	Subbotin et al. 2006
Het-COXiF Het-COXiR	94°C 4 min; 35 cycles at 94°C 30 sec, 57°C 30 sec min, 72°C 1 min, and a final extension 72°C 10 min	Subbotin et al. 2021

Table 4. 2. Primer sets used in a polymerase chain reaction (PCR) and sequencing.

Primer set	Region/ Species	Primer Sequence	PCR product size (bp)	Reference
D2A_D3B	28S rDNA (universal)	ACAAGTACCGTGAGGGAAAGTTG TCGGAAGGAACCAGCTACTA	800	Nunn 1992
ITS-F ITS-R	ITS (universal)	5'-TTGATTACGTCCTGCCCTTT-3' 5'-ACGAGCCGAGTGATCCACCG-3'	850	Powers et al. 1997
NEM-18S-F NEM-18S-R	18S (universal)	CGCGAATRGCTCATTACAACAGC GGGCGGTATCTGATCGCC	900	Floyd et al. 2005
Het-COXiF Het-COXiR	Mt-DNA-COI	5'- TAG TTGATCGTAATTTTAATGG -3' 5'- CCTAAACATAATGAAAATGWGC-3'	550	Subbotin et al. 2021

4.3.6.3. Sequencing

The identity of the cyst nematodes was further confirmed by sequencing (n = 5 cysts) the ribosomal DNA of the ITS, 18S, 28S, and Mt-DNA gene, COI. PCR products from reactions that produced high-yield single bands were purified using QIAquick PCR Purification Kit (250) (QIAGEN, Hilden, Germany) as described by the manufacturer's instructions. The purified PCR products were then sent to Macrogen Inc. (now Psomagen, Rockville, MD, USA) for sequencing to confirm our findings.

To determine the closest match to the cyst nematodes, all nucleotide sequences were blasted in the GenBank database. This comparison was based on two parameters: the expected value (E-value) and percent identity match. The percent identity signifies the percentage of base pairs that match the reference genome, typically falling within the range of 98% to 100% for a high degree of sequence similarity. The E-value, on the other hand, represents the likelihood of obtaining a random match of the same sequence size within the database. The closer the E-value is to zero, the more reliable it is.

4.4. Results

4.4.1. Cyst and Juvenile Densities

A total of 21 nematode cysts were recovered from two composite samples collected from about roots of prairie cordgrass in 2020 and 2021. Among the three native prairies sampled, nematode cysts were recovered only in the Manitoba Tallgrass Prairie Preserve. These cysts were extracted from soil samples taken from the root zone of prairie cordgrass in both sampling years. No cyst nematodes were found in soil samples from the other two prairies, regardless of the extraction methods used. Out of the total cysts recovered, 15 were intact, allowing for microscopic measurements, while the remaining cysts were damaged and empty. Notably, female cyst

nematodes were not observed on root samples among the various plant species, including the prairie cordgrass.

Furthermore, no heteroderid J2 (cyst nematode juveniles) were found in soil samples where nematode cysts were recovered using the modified Cobb's sieving and decanting /sugar flotation method. Similarly, cyst nematode juveniles were absent in soil samples collected from the rhizosphere area of other plants in the same prairie, where no cysts had been recovered.

4.4.2. Morphological Identification

4.4.2.1. Cysts

Fifteen intact cysts showed the following characteristics: they were ovoid in shape, ranged in colour from light to medium brown, and featured vulval and anal circumfenestral structures (Fig. 4.2). Measurements (range; mean $\pm$ sd) of these 15 cysts were as follows: body length (L) (0.47 to 0.65 mm; mean=0.54 $\pm$ 0.06), body width (W) (0.37 to 0.52 mm; mean=0.43 $\pm$ 0.05), and the ratio of body length and body width (L/W) (1.06 to 1.55 mm; mean=1.25 $\pm$ 0.13) (Table 4.3). These populations exhibited morphological and morphometric characters that were consistent with previously described populations of *Punctodera stonei* (Brzeski 1998), as documented in earlier studies (Handoo 2002; Subbotin et al. 2003; Subbotin et al. 2010; Kantor et al. 2022).

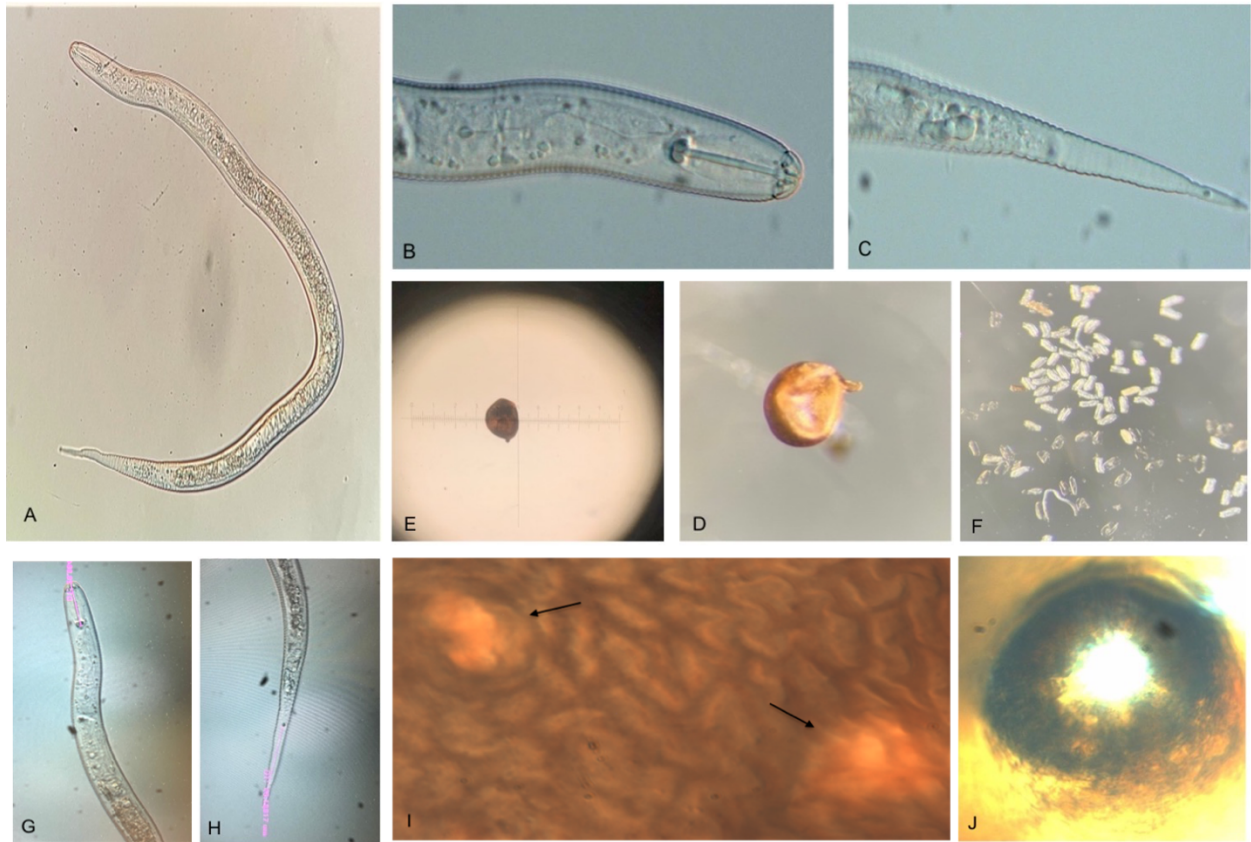


Fig. 4. 2. Microscopic images of *Punctodera stonei* extracted from Manitoba Tall Grass Prairie Preserve, A) image of whole second-stage juvenile (J2), B) J2 anterior region, C) J2 tail, D) entire cyst, E) measuring cyst size, F) eggs and juveniles G) stylet measurement, H) hyaline region of tail measurement, I) terminal region showing vulval and anal fenestra, J) circumfenestrate vulval cone structure.

Table 4. 3. Morphometrics of nematode cysts extracted from Manitoba Tallgrass Prairie Preserve sampled in 2020 and 2021 in comparison with previous studies by Subbotin et al. (2010) and Kantor et al. (2022).

Characters	Body length (n=15)	Body width (n=15)	Length/ Width (n=15)	Vulval structure (n=8)
Cysts (n)				
This study	0.54 ± 0.06 (0.47-0.65)	0.43 ± 0.05 (0.37-0.52)	1.25 ± 0.13 (1.06-1.55)	Circumfenestrate
Subbotin et al. (2010)	0.59 ± 0.06 (0.47-0.74)	0.44 ± 0.05 (0.37-0.55)	1.32 ± 0.1 (1.01-1.50)	Circumfenestrate
Kantor et al. (2022)	0.6 (0.5-0.7)	0.44 (0.4-0.6)	1.3 (1.0-1.5)	Circumfenestrate

all measurements are given in mm: Mean ± sd (range)

4.4.2.2. Juveniles

Second-stage juveniles (n=3) obtained from cysts recovered in soil samples collected at the Manitoba Tallgrass Prairie Preserve (root zone of prairie cordgrass) were observed under a light microscope and displayed distinctive morphological features. These features included slightly concave stylet knobs and tails that tapered uniformly to a narrowly rounded terminus. Notably, these tails featured a long hyaline region, constituting more than 50% of the tail's length (Fig. 4.2). The measurements taken from these J2s (n = 3), included lengths of body (L) (mean = 495.23 μm), stylet length (mean = 27.63 μm), tail length (mean = 91.75 μm), and hyaline part length (mean 48.42 μm). These morphometric and morphological characters are closely aligned with previously described populations of *Punctodera stonei* documented by Subbotin et al. (2010) and Kantor et al. (2022).

P. stonei specimen exhibit similarities to all known *Punctodera* species but can be differentiated from the other species mainly by their longer tail and hyaline region of the tail. In current study, *P. stonei* differs from *p. mulveyi* by having a longer J2 tail and a hyalin tail terminus of 91.75 μm and 48.42 μm on average, respectively, compared to 70 μm and 47 μm for *p. mulveyi*. Additionally, the J2 of *P. stonei* can be separated from that of *P. matadorensis* by having a slightly longer stylet (27.63 μm vs 24.6 μm) and flattish to slightly concave stylet knobs as opposed to strongly concave anterior knobs. The tail length of *P. stonei* is also longer, measuring 91.75 μm compared to 62 μm in *P. matadorensis*, and the hyaline tail terminus averaging 48.42 μm compared to 39.1 μm for *P. matadorensis* (Subbotin et al. 2010).

During the genus identification process of the first 100 juveniles under the microscope slide, several PPN genera, other than cyst nematodes, were observed in all composite samples. The nematodes detected in nearly all samples belonged to genera such as *Helicotylenchus* (spiral

nematodes), *Paratylenchus* (pin nematodes), *Cricconemella* (ring nematodes), *Aphelenchus*, *Hemicycliophora*, *Tylenchus*, and *Tylenchorhynchus*. *Hemicycliophora* and *Paratylenchus* were extracted less frequently, and their numbers were lower in comparison. It's worth noting that most of the observed nematodes were non-plant parasitic. The total nematode counts ranged from 153 to 291 per 100 g of soil. We did not attempt to estimate the population size of any specific genus.

4.4.3. Molecular Identification

4.4.3.1. PCR Amplification

Out of the intact cysts (n=15), only eight yielded DNA suitable for PCR analysis. Amplification of the DNA extracted from individual nematode cysts collected from Manitoba Tallgrass Prairie Preserve yielded single products with the following sizes: 850 bp for ITS and 950 bp for 18S. Additionally, single amplicon fragments of 850 base pairs for the 28S region (Fig. 4.3) and 550 base pairs for the mitochondrial COI (Fig. 4.3) were observed when using the Hetcoxi and D2A-D3B primers (Fig 4.4.). No PCR products were detected in the control lacking DNA template. For subsequent sequencing analysis, the high-yield PCR products of the selected samples (n =5 cysts) were purified and sent for sequencing.

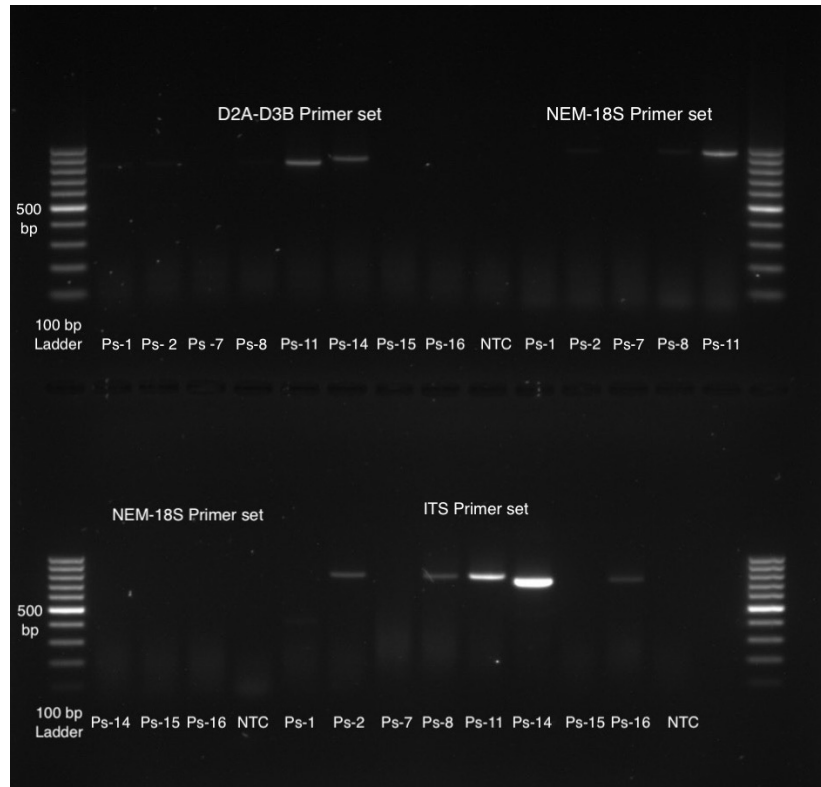


Fig. 4. 3. Agarose gel electrophoresis pattern of PCR products of genomic DNA extracted from the suspension of eggs and juveniles of single cysts amplified with D2A-D3B, NEM-18S, and ITS primer sets. Lanes: 100 bp DNA ladder, Ps-1, Ps-2, Ps-7, Ps-8, Ps-11, Ps-14, and Ps-16 are individual cysts recovered from the soil samples collected from Manitoba Tallgrass Prairie Preserve and about roots of prairie cordgrass.

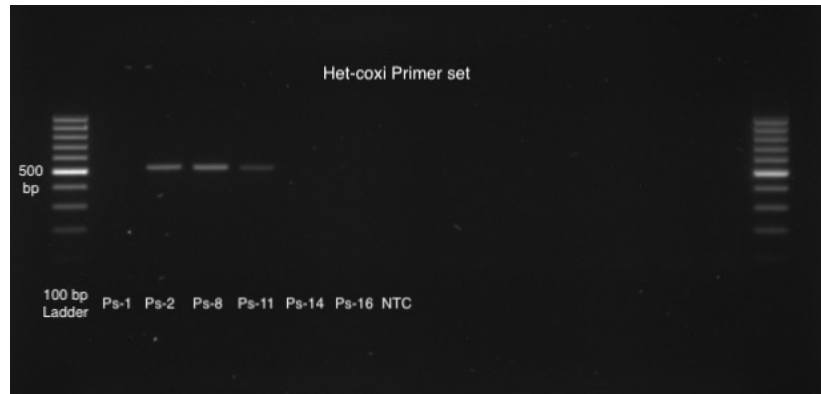


Fig. 4. 4. Agarose gel electrophoresis pattern of PCR products of genomic DNA extracted from the suspension of eggs and juveniles of single cysts amplified with Het-coxi primer set. Lanes: 100 bp DNA ladder, Ps-1, Ps-2, Ps-8, Ps-11, Ps-14, and Ps-16 are individual cysts recovered from the soil samples collected from Manitoba Tallgrass Prairie Preserve and about roots of Prairie Cord Grass.

4.4.3.2. Sequencing

Genomic DNA of five cysts was submitted for sequencing and all showed a strong match with *Punctodera stonei* (a nematode parasite associated with root systems of wild-growing grasses) (Brzeski 1998). These individuals had previously been identified as *Punctodera stonei* based on morphological and morphometric analysis. Sequencing of the ITS and 18S genes was each successful for four cysts, however, 28S, and COI regions yielded only one successful sequencing (Table 4.4). The ITS and 18S sequences displayed identity matches of greater than 97.81% and 98.89 %, respectively, to *Punctodera stonei* accessions (CP049713.1, MK292129.1, and JF895515.1).

A BLAST search conducted on NCBI using the sequences from the 28S and COI genes revealed a 98.80% and 95.12% identity match with the corresponding segments of *P. stonei* present in the database, with accession numbers JF895516.1 and MN267175.1, respectively. These sequencing outcomes were consistent with the findings derived from morphological analysis.

Table 4.4 Molecular identification of *Punctodera Stonei* recovered from the Manitoba Tallgrass Prairie Preserve, using DNA sequencing of the ITS, 18S, 28S, and COI regions based on identity match to the NCBI Blast database.

Nematode individual	Match	Gene	Accession	Identity (%)	E-value	Query cover (%)	Read length (bp)
Ps-11	<i>Punctodera stonei</i>	ITS	JF895515.1	98.41	0.0	99	558
		18S	JF895515.1	99.75	0.0	100	812
		28S	JF895516.1	98.80	0.0	98	680
		COI	MN267175.1	95.12	0.0	94	475
Ps-8	<i>Punctodera stonei</i>	ITS	CP049713.1	98.25	0.0	100	466
		18S	MK292129.1	98.89	0.0	99	814
T12-1	<i>Punctodera stonei</i>	ITS	JF895515.1	98.73	0.0	100	552
		18S	JF895515.1	99.50	0.0	100	819
T12-3	<i>Punctodera stonei</i>	ITS	JF895515.1	97.81	0.0	100	487
Ps-2	<i>Punctodera stonei</i>	18S	JF895515.1	99.88	0.0	99	817

4.5. Discussion

Based on morphological and molecular data provided in this study, we have identified the cyst nematodes recovered from the Manitoba Tallgrass Prairie Preserve as *Punctodera stonei*, commonly known as Stone's cyst nematode. To our knowledge, this is the first report of *P. stonei* in Manitoba and only the second in Canada.

The genus *Punctodera* Mulvey and Stone, 1976, consists of cyst-forming nematodes that parasitize both cultivated and wild-growing plants belonging to the *Graminae* family. Previously, *P. stonei* had been reported in Canada, specifically in turfgrass in Ottawa, Ontario (Dobsoz et al. 2013; Kantor et al. 2020). Additionally, in the United States, *P. stonei* was recently detected for the first time in Virginia, where it was found in the rhizosphere region of the common grass (*Festuca arundinacea* L.) based on morphological and molecular analysis of three genes (D2–D3 of 28S rRNA, ITS rRNA, and COI) (Kantor et al. 2022).

Morphologically, *Punctodera* cysts are distinct from other circumfenestrate cyst nematodes due to their spherical or subspherical shape with a rounded posterior end. More specifically, they are characterized by having vulval and anal fenestrae of nearly equal size. In our research, the presence of fenestration around the anus in the recovered cysts was a key distinguishing character used to identify them as belonging to the *Punctodera* genus.

The morphological and morphometric overlap among closely related *Punctodera* species can lead to potentially inaccurate identification. Therefore, it is crucial to complement specific identifications with the analysis of various molecular markers to strengthen the accuracy of diagnosis. Several researchers (Oostenbrink 1960; Mulvey 1972; Solovjeva and Vasiljeva 1973; Wouts et al. 1986; Brzeski 1998; Subbotin et al. 2010; Handoo and Subbotin 2018; Kantor et al. 2020), have suggested that *P. punctata* might actually represent a complex of several closely

related species. For instance, Brzeski (1998) reported that populations previously identified as *P. punctata* in locations such as Toppheide (Germany), Rothamsted (UK) (Wouts et al. 1986), and Jaen (Spain) (Castillo et al. 1989) should be reclassified as *P. stonei*. Similarly, morphological features of cysts and juveniles of other species of *Punctodera*, including *P. Mulveyi* and *P. matadorensis*, closely resemble those of *P. stonei*, making it challenging to differentiate these species solely based on morphology. In this context, a study conducted in the USA in 2020 is noteworthy. Initially, a cyst nematode found in turfgrass (*P. annua*) on golf course greens in Bandon, Coos County, Oregon, USA, was characterized and identified as *P. stonei* (Kantor et al. 2019). However, further examination through morphology, scanning electron microscopy (SEM), and molecular analysis using additional markers revealed significant differences from *P. stonei*. Consequently, this nematode was described as a new species, named *P. mulveyi* n. sp. (Kantor et al. 2020). The differentiation of *P. mulveyi* n. sp. from *P. matadorensis*, *P. punctata*, *P. stonei* and *P. chalcoensis* was accomplished through molecular analysis of ITS rDNA, heat shock protein90 and mitochondrial COI. The study noted that the 18S and 28S regions exhibited relatively conserved sequences with only few bp differences, and there were insufficient *Punctodera* species sequences available to strongly support the designation of a new species.

In this study, we aimed to strengthen the validity of our identification results by employing multiple molecular markers. A comparison of the rDNA and mitochondrial sequences obtained from our samples with sequences deposited in GenBank provided additional confirmation for species identification, alongside our analysis of morphological and morphometric features.

Considering that North America hosts a substantial diversity of closely related circumfenestrate cyst nematode species and genera, including 12 out of the 13 known *Cactodera* species, nearly all recognized *Punctodera* species, all *Betulodera* and *Dolichodera* species, as well as several

Globodera species, our study anticipated the retrieval of circumfenestrate cyst nematodes, specifically genus *Punctodera*, from native prairies. This expectation was based on the understanding that the *Punctodera* genus predominantly parasitizes grasses (Subbotin et al. 2010). Furthermore, within the *Punctodera* genus, it is known that *P. matadorensis*, *P. stonei* and *P. mulveyi* n. sp. are parasites of native grasses in North America. This aligns with the current study's report of the presence of *p. stonei* in a native prairie.

The prairies sampled in the current study had never undergone cultivation and had been designated as preserves. As a result, the likelihood of introduced populations of nematodes in these areas was minimal. Consequently, any nematode genus discovered in this region was likely native, suggesting that *P. stonei* might be more widespread than currently recognized. It could reasonably be anticipated that these nematodes might also be present in agricultural fields, lawns, and other cultivated areas beyond their presence in native prairies. This discovery aligns with the findings from our laboratory's soybean cyst nematode survey conducted from 2012 to 2017 in soybean fields across Manitoba, which reported the presence of *Punctodera* cyst nematodes in Manitoba fields.

In this study, the recovery of nematode cysts was limited to one specific prairie preserve and within the root zone of a particular plant genus. This occurrence may be linked to various factors influencing nematode resource availability, such as soil texture, root quantity and quality in the site. This also can be explained by the heterogeneous soil characteristics within the prairie and the clustered distribution pattern of nematodes. Prior researchers have highlighted the relationship between nematode genera and factors such as host plant species, root biomass, soil texture, pH, and organic matter within ecosystems (Schmitt and Norton 1972; De Deyn et al. 2004; DuPont et al. 2014). In a study conducted by Kimpinski and Welch (1971) to assess the nematode fauna in

southern Manitoba and the factors influencing nematode distribution and abundance, they concluded that the quantity of plant material was the most influential factor affecting nematode populations in Manitoba soils, with species like *Bromus inermis* L. (smooth brome) and *Poa compressa* L. (Canada bluegrass) comprising 88-95% of the vegetation.

In the context of host-based categorization, we identified *P. stonei* in samples collected from the roots of prairie cordgrass. However, it's worth noting that the ground cover consisted of a mix of plants, so the samples could have contained rhizosphere soils from sources other than the designated plants. Consequently, we couldn't confirm whether these plant parasites were exclusively feeding on a particular plant species.

Furthermore, our understanding of the host preferences of most nematodes in native prairie ecosystems is limited, making it challenging to determine if these findings are primarily influenced by the host plants, soil factors or climate. Moreover, most previous studies have primarily focused on *Punctodera* species and their associated plant hosts, typically recovering cysts and J2s from soil rather than from plant roots. However, identifying the specific host plant presents a challenge, necessitating a combination of experimental investigations and field observations of cyst nematodes on both plant roots and within the soil. Consequently, it becomes challenging to categorize nematodes within precise habitats and conclusively state that *P. stonei* exclusively feeds on prairie cordgrass. Therefore, it would be important to conduct further experimentation to examine the influential factors affecting the presence of cyst nematodes within our study area.

The low incidence of *Punctodera* cyst nematodes in our sampled prairie may be owing to the fact that only one generation occurs per growing season due to the low reproductive potential of *Punctodera*, specifically *P. punctata*, while the situation remains unknown for other species (Kantor et al. 2020). The absence of female cysts on the roots of prairie cordgrass is likely tied to

the timing of our sampling, which was conducted in mid-fall, late in the growing cycle when the number of cysts is at its peak in the soil rather than on plant roots.

Despite the agricultural significance of certain *Punctodera* species, there is limited information available about these organisms. Moreover, the methods for distinguishing between species within this genus, as outlined in identification keys, have traditionally relied heavily on the country or continent of origin and the host plant. However, recent research has unveiled broader origins and host plant associations for some species. For instance, *P. matadorensis*, was initially found in Canada parasitizing grasses (Subbotin et al. 2010). This species was also documented in North Dakota, USA, as reported by Handoo et al. (2010). Similarly, *P. stonei*, previously reported as a turfgrass parasite in Ottawa, Canada, marked its first appearance in North America and beyond Europe (Dobosz et al. 2013, Kantor et al. 2020). So, studies like what we present here expands our understanding of this species' geographical distribution. Such information also highlights that many nematodes found in agricultural crop fields in Manitoba were actually native to the local soil.

It is crucial to investigate populations of this species from various geographical locations, subjecting them to thorough molecular and morphological analyses to assess the reliability of diagnostic characteristics and the relationships between geographically diverse groups. Similar studies in other native prairies could provide valuable insights into soil communities and population dynamics in Manitoba. Additionally, it may be worthwhile to re-examine previous findings of the *Punctodera* species, specifically *P. punctata* in Canada by conducting more comprehensive morphological and molecular analysis.

4.6. References

- Agrios GN. 2005. Plant pathology. 5th ed. Burlington (MA): Academic Press.
- Baker AD. 1957. Notes on some nematodes in Canada, 1956. Nematode investigations section, entomology laboratory, Ottawa (ON). Canadian Insect Pest Review. 34:120-122.
- Baldwin JG, Handoo ZA. 2018. General morphology of cyst nematodes: cyst nematodes. Wallingford (UK): CAB International; p. 337-364.
- Baldwin JG, Mundo -Ocampo M. 1991. *Heteroderinae*, cyst- and non-cyst forming nematodes. In: Nickle WR, editor. Manual of Agricultural Nematology. New York (NY): Marcel Dekker; p. 275-362.
- Blok VC, Malloch G, Harrower B, Phillips MS, VrainTC. 1998. Intraspecific variation in ribosomal DNA in populations of the potato cyst nematode *Globodera pallida*. J Nematol. 30:262–274.
- Blouin MS. 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Int J Parasitol. 32:52731.
- Bongers T, Ferris H. 1999. Nematode community structure as a bioindicator in environmental monitoring. Trends Ecol Evol. 14:224–228.
- Bragg TB, Steuter AA. 1996. Prairie Conservation: preserving North America's most endangered ecosystem. In: Samson FB, Knopf FL. Prairie Ecology - The Mixed Prairie. Washington (DC): Island Press.
- Brzeski MW. 1998. Nematodes of *Tylenchina* in Poland and temperate Europe. Warsaw, Poland: Museum and Institute of Zoology, Polish Academy of Sciences; p. 398.

- CAPS. 2017. CAPS program resource and collaboration site. Cyst nematode survey reference-2017. *Punctodera chalcoensis*. [Accessed 2020 Aug 15]. <http://download.ceris.purdue.edu/file/3054>
- Castillo P, Romero MD, Barcina AG. 1989. New record of *Punctodera punctata* from Spain. Nematol Mediterr. 17:67-70
- Curtis RH. 2007. Plant parasitic nematode proteins and the host–parasite interaction. Brief Funct Genomics Proteomics. 6(1):50-58.
- De Deyn GB, Raaijmakers CE, Van Ruijven J, Berendse F, Van Der Putten WH. 2004. Plant species identity and diversity effects on different trophic levels of nematodes in the soil food web. Oikos. 106(3):576-586.
- Dobosz R, Winiszewska G, Malewski T, Rybarczyk-Mydlowska K, Tereba A, Kowalewska K, Gawlak M, Bogdanowicz W. 2013. Morphological and molecular features of *Punctodera stonei* Brzeski, 1998 (Nematoda: *Heteroderidae*)—species associated with roots of grasses. Ann Zool. 63(2):157-162.
- DuPont ST, Beniston J, Glover JD, Hodson A, Culman SW, Lal R, Ferris H. 2014. Root traits and soil properties in harvested perennial grassland, annual wheat, and never-tilled annual wheat. Plant Soil. 381:405-420.
- Ehrlich WA, Poyser EA, Pratt LE. 1957. Reconnaissance survey of Carberry area in Manitoba. Manitoba (MB): Canada Department of Agriculture, Provincial Department of Agriculture and Soils Department, The University of Manitoba. Report No.: 7.
- Ehrlich WA, Pratt LE, Poyser, EA. 1956. Report of reconnaissance soil survey of Rossburn and Virden map sheet areas. Manitoba (MB): Canada Department of Agriculture, Provincial Department of Agriculture and Soils Department, The University of Manitoba. Report No.: 6.

- Ekschmitt K, Bakonyi G, Bongers M, Bongers T, Boström S, Dogan H, Harrison A, Nagy P, Donnell AG, Papatheodorou EM, Sohlenius B, Stamou GP, Wolters V. 2001. Nematode community structure as indicator of soil functioning in European grassland soils. *Eur J Soil Biol.* 37:263–268.
- Fauna Europaea. 2012. fauna Europaea version 2.5. Web Service. [Accessed 2023 Jan 8]. <http://www.faunaeur.org>.
- Fenwick DW. 1940. Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. *J Helminthol.* 18(4):155-172.
- Ferris VR, Miller LI, Faghihi J, Ferris JM. 1995. Ribosomal DNA comparisons of *Globodera* from two continents. *J Nematol.* 27:273–283.
- Floyd RM, Rogers AD, Lambshhead PJD, Smith CR. 2005. Nematode-specific PCR primers for the 18S small subunit rRNA gene. *Mol Ecol Notes.* 5(3):611-612.
- Gerdemann JW. 1955. Relation of a large soil-borne spore to phycomycetous mycorrhizal infections. *Mycologia.* 47:619-632.
- Griffiths BS, Bonkowski M, Roy J, Ritz K. 2001. Functional stability, substrate utilisation and biological indicators of soils following environmental impacts. *Appl Soil Ecol.* 16:49–61.
- Grujicic G. 1969. Occurrence and pathogenicity of parasitic nematodes on *Maizein Serbia*. *Savremena Polijoprivreda.* 17:667-72.
- Handoo ZA. 2002. A key and compendium to species of the *Heterodera avenae* group (Nematoda: *Heteroderidae*). *J Nematol.* 34(3):250–262.
- Handoo ZA, Subbotin SA. 2018. Taxonomy, identification and principal species: In: Perry RN, Moens M, Jones JT, editors. *Cyst Nematodes*. Wallingford (UK): CAB International; p. 365-398.

- Handoo ZA, Skantar AM, Chitwood DJ, Carta LK. 2010. First report of the cyst nematode *Punctodera matadorensis* in the United States. J Nematol. 42(3):246-247.
- Hyman BC. 1988. Nematode mitochondrial DNA: anomalies and applications. J Nematol. 20:523–31.
- Holterman M, Karssen G, Van Den Elsen S, Van Megen H, Bakker J, Helder J. 2009. Small subunit rDNA-based phylogeny of the *Tylenchida* sheds light on relationships among some high-impact plant-parasitic nematodes and the evolution of plant feeding. Phytopathology. 99:227–235.
- Ingham RE. 1994. Nematodes: methods of soil analysis, part 2. Microbiological and biochemical properties no. 5. Madison (WI): Soil Society of America; p. 459-490.
- Jones MGK. 1981. The development and function of plant cells modified by endoparasitic nematodes. Plant Parasitic Nematodes. 3:255-279.
- Kantor MR, Handoo ZA, Skantar AM, Hult MN, Ingham RE, Wade NM, Ye W, Bauchan GR, Mowery JD. 2020. Morphological and molecular characterisation of *Punctodera mulveyi* n. sp. (Nematoda: *Punctoderidae*) from a golf course green in Oregon, USA, with a key to species of *Punctodera*. Nematology. 23(6):667-683.
- Kantor MR, Subbotin SA, Huse G, Handoo ZA. 2022. Morphological and Molecular Characterization of *Punctodera stonei* Brzeski, 1998 (Nematoda: *Heteroderidae*) from Virginia, USA. J Nematol. 54(1):20220008.
- Kimpinski J, Welch HE. 1971. The ecology of nematodes in Manitoba soils. Nematologica, 17(2):308-318.
- Koper N, Mozel KE, Henderson DC. 2010. Recent declines in northern tall-grass prairies and effects of patch structure on community persistence. Biol Conserv. 143(1):220-229.

- Lax P, Rondan Dueñas JC, Andrade AJ, Doucet ME. 2021. *Punctodera achalensis* n. sp. (Nematoda: *Punctoderidae*), a new cyst nematode from Argentina. Eur J Plant Pathol. 161:397-409.
- Madani M, Subbotin SA, Ward LJ, Debor SH. 2010. Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome b genes. Can J Plant Pathol. 32:252-263.
- Madani M, Vovlas N, Castillo P, Subbotin SA, Moens M. 2004. Molecular characterization of cyst nematode species (*Heterodera* spp.) from the Mediterranean basin using RFLPs and sequences of ITS-rDNA. J Phytopathol. 152(4):229-234.
- Mai W, Mullin PG, Howard LH. 1996. Plant-Parasitic Nematodes: a pictorial key to genera. New York (NY): Cornell University Press.
- Moens M, Perry RN, Jones JT. 2018. Cyst nematodes: life cycle and economic importance. Wallingford UK: CAB International; p. 1-26
- Mulvey RH. 1972. Identification of *Heterodera* cysts by terminal and cone top structures. Can J Zool. 50:1277-1292.
- Mulvey RH, Stone AR. 1976. Description of *Punctodera matadorensis* n. gen., n. sp. (Nematoda: *Heteroderidae*) from Saskatchewan with lists of species and generic diagnoses of *Globodera* (n. rank), *Heterodera*, and *Sarisodera*. Can J Zool. 54(5):772-785.
- Neher DA. 2010. Ecology of plant and free-living nematodes in natural and agricultural soil. Annu Rev Phytopathol. 48:371-394
- Nunn GB. 1992. Nematode molecular evolution [dissertation]. Nottingham (UK): University of Nottingham.

- EPPO. 2017. *Globodera rostochiensis* and *Globodera pallida* PM 7/40 (4). OEPP/EPPO Bull. 47:174-197.
- Oostenbrink M. 1960. Estimating nematode populations by some selected methods. In: Sasser JN, Jenkins VR, editors. Nematology. Chapel Hill (NC): The University of North Carolina Press; p. 85-102.
- Orui Y. 1997. Discrimination of *Globodera rostochiensis* and four *Heterodera* species (Nematoda: *Heteroderidae*) by PCR-RFLP analysis. Jpn J Nematol. 27:67-75.
- Parks Canada. 2007. Riding Mountain National Park of Canada and Riding Mountain Park East Gate registration complex national historic site of Canada: management plan. Ottawa (ON): Parks Canada.
- Perry RN, Moens M, Jones JT. eds. 2018. Cyst nematodes: life cycle and economic importance. Wallingford UK: CAB International.
- Powers T. 2004. Nematode molecular diagnostics: from bands to barcodes. Ann Rev Phytopathol. 42:367-383.
- Powers TO, Todd TC, Burnell AM, Murray PCB, Fleming CC, Szalanski AL, Adams BA, Harris TS. 1997. The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. J Nematol. 29(4):441-450.
- Reinking D. 2003. Fire regimes and avian responses in the central tall-grass prairie. Stud Avian Biol. 30:116-126.
- Roach BA, Spears JF, Kulik MM, Gingrich SF. 1968. The golden nematode handbook: survey, laboratory, control, and quarantine procedures. US Agricultural Research Service; p. 351-355.

- Sabo A, Reis LGL, Krall E, Mundo-Ocampo M., Ferris VR. 2002. Phylogenetic relationships of a distinct species of *Globodera* from Portugal and two *Punctodera* species. J Nematol. 34:263-266.
- Samson FB, Knopf FL. 1994. Prairie Conservation in North America. Bioscience. 44:418-421.
- Schacht H. 1859. Über einige Feinde der Rübenfelder. Zeitschrift Vereines Rübenzucker-Industrie Zollverein. 21:1-19.
- Schmidt A. 1871. Über einige Feinde der Rübenfelder. Zeitschrift Vereines Rübenzucker-Industrie Zollverein. 9:175-179.
- Schmitt DP, Norton DC. 1972. Relationships of plant parasitic nematodes to sites in native Iowa prairies. J Nematol. 4(3):200-206.
- Seinhorst JW. 1974. Separation of *Heterodera* cysts from dry organic debris using ethanol. Nematologica. 20:367-369.
- Sharma SB. 1998. The cyst nematodes. Dordrecht, The Netherlands: Kulwer Academic Publisher.
- Sirca S, Urek G. 2004. Morphometrical and ribosomal DNA sequence analysis of *Globodera rostochiensis* and *Globodera achilleae* from Slovenia. Russ J Nematol. 12:161-168.
- Skantar AM, Handoo ZA, Carta LK, Chitwood DJ. 2007. Morphological and molecular identification of *Globodera pallida* associated with potato in Idaho. J Nematol. 39:133-144.
- Solovjeva GI, Vasiljeva AP. 1973. *Heterodera punctata* a parasite of wild grass in south Karelia. Parazitologiya. 7:521525.
- Stone AR. 1985. Co-evolution of potato cyst nematodes and their hosts: implications for pathotypes and resistance1. EPPO Bull. 15:131-137.
- Sturhan D. 2006. Zystenbildende Nematoden und verwandte Heteroderiden in Deutschland. In: Hallmann, J. and B. Niere (Eds.). Aktuelle Beiträge zur Nematodenforschung. Mitteilungen

- aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft Berlin-Dahlem. [Cyst-forming nematodes and related Heteroderids in Germany]. 404:18-30.
- Sturhan D, Lišková M. 2004. Cyst nematodes in the Slovak Republic. *Helminthologia*. 41:217-219.
- Subbotin SA. 2015. *Heterodera sturhani* sp. n. from China, a new species of the *Heterodera avenae* species complex (*Tylenchida: Heteroderidae*). *Russ J Nematol*. 23(2):145-152.
- Subbotin SA, Franco J, Knoetze R, Roubtsova TV, Bostock RM, Cid Del Prado Vera I. 2020. DNA barcoding, phylogeny and phylogeography of the cyst nematode species from the genus *Globodera* (*Tylenchida: Heteroderidae*). *Nematology*. 22:269–297.
- Subbotin SA, Halford PD, Warry A, Perry RN. 2000a. Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitising Solanaceae. *Nematology*. 2:591-604.
- Subbotin SA, Mundo-Ocampo M, Baldwin JG. 2010. Systematics of cyst nematodes (Nematoda: *Heteroderinae*): *Nematology Monographs and Perspectives*. In: Hunt DJ, Perry RN, editors. Leiden, The Netherlands: Brill. 8:1-351.
- Subbotin SA, Sturhan D, Rumpenhorst HJ, Moens M. 2003. Molecular and morphological characterisation of the *Heterodera avenae* complex species (*Tylenchida: Heteroderidae*). *Nematology*. 5:515-538.
- Subbotin SA, Vierstraete A, De Ley P, Rowe J, Waeyenberge L, Moens M, et al. 2001. Phylogenetic relationships within the cyst-forming nematodes (Nematoda, *Heteroderidae*) based on analysis of sequences from the ITS regions of ribosomal DNA. *Mol Phylogenet Evol*. 21:1–16.

- Subbotin SA, Vera ICDP, Mundo-Ocampo M, Baldwin JG. 2011. Identification, phylogeny and phylogeography of circumfenestrate cyst nematodes (Nematoda: *Heteroderidae*) as inferred from analysis of ITS-rDNA. *Nematology*. 13(7):805-824.
- Subbotin SA, Waeyenberge L, Molokanova IA, Moens M. 1999. Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs. *Nematology*. 1:195–207.
- Subbotin S, Waeyenberge L, Moens M. 2000b. Identification of cyst forming nematodes of the genus *Heterodera* (Nematoda: *Heteroderidae*) based on the ribosomal DNA RFLP. *Nematology*. 2(2):153-164.
- Sveinson JMA, Brook RK, Thompson BA. 2001. Yellow quill mixed-grass prairie vegetation inventory and mapping. Nature Conservancy of Canada, Manitoba region.
- Thiery M, Mugnie'ry D. 1996. Interspecific rDNA restriction fragment length polymorphism in *Globodera* species, parasites of *Solanaceous* plants. *Fundam Appl Nematol*. 19:471–479.
- Thorne G. 1928. *Heterodera punctata* n. sp. A Nematode Parasitic on Wheat Roots from Saskatchewan. *Sci Agric*. 8(11):707-710.
- Thorpe J, Baldwin K, Allen L. 2015. Great Plains Rough Fescue Prairie. Sault Ste. Marie, Ontario (ON): Canadian National Vegetation Classification; Macrogroup: CM332. [Accessed 2023 May 8].
- <http://cnvc-cnvc.ca>. ISSN 1916-3266
- Uehara T, Kushida A, Itou K, Narabu T, Momota Y. 2005. Discrimination of three cyst-forming nematodes of the genus *Globodera* (Nematode: *Heteroderidae*) from Japan based on PCR-RFLP of ribosomal DNA. *Appl Entomol Zool*. 40:537-543.

- Vallejo D, Rojas DA, Martinez JA, Marchant S, Holguin CM, Pérez OY. 2021. Occurrence and molecular characterization of cyst nematode species (*Globodera* spp.) associated with potato crops in Colombia. Plos One. 16(7): e0241256.
- Widenmaier KJ, Strong WL. 2010. Tree and forest encroachment into fescue grasslands on the Cypress Hills plateau, Southeast Alberta, Canada. Ecol Manag. 259:1870-1879.
- Wouts WM, Sturhan D. 1995. *Heterodera aucklandica* sp. n. (Nematoda: *Heteroderidae*) from a New Zealand native grass, with notes on the species of the *H. avenae* group. N Z J Zool. 22(2):199–207.
- Wouts WM, Weischer B. 1977. Eine klassifizierung von fünfzehn in Westeuropa häufigen arten der *Heteroderinae* auf grund von larvenmerkmalen. Nematologica. 23:289–310.
- Wouts WM, Weischer B, Triggs CM. 1986. On the identity of European populations of *Punctodera punctata* (Nematoda: *Heteroderidae*). Nematologica. 32(1):79-88.
- Zheng J, Subbotin SA, Waeyenberge L, Moens M. 2000. Molecular characterisation of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. Russ J Nematol. 8(2):109-114.

5.0 Overall Synthesis

5.1. Introduction

Heterodera glycines, a major soybean pest worldwide, is responsible for substantial yield losses in fields. In Canada, the presence of Soybean Cyst Nematode (SCN) has been documented in the provinces of Ontario and Quebec (Anderson et al. 1988; Mimee et al. 2014). The nematode is anticipated to soon establish itself in Manitoba, given its rapid spread from neighbouring United States counties, namely North Dakota and Minnesota.

Due to the considerable yield loss caused by SCN, it is essential to conduct surveys of soybean fields in Manitoba to detect the presence of this pest. Additionally, developing precise identification and quantification methods for SCN in soil is crucial for the development of management strategies. However, the current methods of microscopic identification and counting of the nematode are labour-intensive, time-consuming and lack specificity to distinguish among nematode species.

To address these challenges, this thesis conducted a survey of soybean fields in Manitoba to detect SCN presence. Furthermore, a real-time quantitative polymerase chain reaction (qPCR) assay based on SYBR Green I-based was developed to directly detect and quantify *H. glycines* in soil DNA extracts from fields. The research extends to the examination of cyst nematodes in three Prairie Preserves in Manitoba, as their distribution within native prairies, especially in Manitoba, had not been thoroughly examined. Both morphological and molecular identification methods, including PCR amplification and sequencing of four regions of the genome, were employed to study the cyst nematode species. This research contributes valuable insights into agricultural and environmental challenges by examining the native prairie ecosystems.

5.2. Important Findings

Three studies were conducted to examine cyst nematodes in Manitoba. The first study (Chapter Two) examined the presence of soybean cyst nematode in soybean fields across the province. Samples were collected from 31 soybean fields across different municipalities, predominantly along the MB/U.S. border. Through morphological cyst measurements, PCR using species-specific primers for *H. glycines* (COXIII and SCAR), and DNA sequencing of ITS and 18S regions (GenBank accession: OR265515-21 and OR265522-28) as well as COXIII and SCAR regions (OR291423-24 and OR291425-28), it was revealed that five fields in Manitoba tested positive for SCN. This study marked the first finding of *H. glycines* and SCN disease in Manitoba, and notably, these positive fields were dispersed across five out of the 18 municipalities surveyed.

It is important to highlight that the positive regions were geographically distant from each other, indicating the potential presence of SCN in other fields not covered by this study. Three rural municipalities that tested positive for SCN were located adjacent to the North Dakota or Minnesota border along the floodplain of the Red River, suggesting a potential northward dispersal of SCN through flooding. The remaining two positive areas, located away from the border and river waters flowing north from the U.S., indicate the likely dispersal of SCN through the introduction of infested soil. Moreover, the existence of the affected area at a machinery entrance, coupled with using used farm equipment purchased from the U.S. on the symptomatic field during the 2021 growing season, increases the probability of SCN introduction to the field.

The nematode's ability to reproduce on soybean plants was confirmed through growth chamber experiments, demonstrating robust reproduction on soybean plants of Manitoba SCN population on a susceptible soybean variety. HG-type analysis of the nematode population collected from the symptomatic field in south-central Manitoba identified the population of SCN as HG type 7 and

race 3. The findings from this survey offer valuable insights into the current status of soybean cyst nematode in Manitoba and will contribute to determining optimal practices for pest management.

Considering the current low to moderate population densities of SCN in Manitoba, it is recommended that farmers employ SCN-resistant soybean varieties and apply crop rotation practices with non-host crops such as barley, oats, corn, wheat, and canola.

The second study (Chapter Three) developed a molecular technique for directly quantifying *H. glycines* in field soils. Samples were collected from naturally infested fields with *H. glycines*, covering diverse soil types and SCN levels, in southern Ontario and Manitoba. The extraction methods for cysts, eggs, and DNA from soil were optimized. A SYBR Green I-based real-time PCR assay, using species-specific primers for *H. glycines* (COXIII and SCAR), was developed to quantify the number of *H. glycines* eggs in naturally infested field soils.

Upon validating the developed qPCR method to estimate the number of *H. glycines* eggs in naturally infested fields in Manitoba and Ontario, a strong relationship was observed between the number of eggs counted conventionally and those quantified using the soil calibration curve and qPCR for both primer sets regardless of SCN populations. The assay demonstrated that DNA from *H. glycines* could be efficiently extracted directly from nematode suspensions and soil samples using a commercial DNA extraction kit. The sensitivity of the assay was notable, specifically detecting the DNA equivalent of as little as one SCN egg in solution and quantifying as few as 10 *H. glycines* eggs /100cm³ soil.

Considering the variability in damage thresholds for SCN based on soil and environmental factors, applying a confident threshold to individual fields proves challenging. However, the minimum detection sensitivity achieved in this study was significantly lower than the threshold levels employed in commercial laboratories (3 and 1 cyst/100cm³ soil, respectively, according to

Niblack 2005). The developed methodology in this chapter offers a reliable, sensitive and specific means to detect and quantify *H. glycines* eggs directly in DNA extracts of soils across a wide range of population densities. Importantly, this method eliminates the need for time-consuming microscopic identification and egg counting in various soil types, allowing for efficient processing of multiple nematode samples in a single day, including DNA extraction, sensitive identification, and quantification.

Early and accurate identification and quantification of nematodes are crucial steps in implementing effective nematode management strategies. The findings from these two data chapters presented in this study hold significance for soybean growers in Manitoba, providing valuable insights into the management of this pest. It is now evident that SCN is not merely a suspected pest but has indeed established a population in Manitoba farms. The implications of these studies extend beyond confirmation, as they also provide soybean growers with details on the virulence of the SCN population in Manitoba. With the annual fluctuations in nematode populations, these findings empower growers to make well-informed decisions regarding crop selection and cultivars. Additionally, the awareness that biosecurity measures on certain farms are ineffective in controlling the distribution of *H. glycines*, which easily spreads across farms due to the unavoidable use of farm machinery and tools, is a crucial realization for farmers.

The introduction of molecular methods, replacing the traditional methods for identifying *H. glycines* and quantifying its egg numbers in the soil, emerges as a preventive measure against the potential spread and substantial economic losses in the future.

The third study (Chapter Four) investigated the presence of cyst nematodes in three native prairies of Manitoba. Among the sampled prairies, only one was positive for cyst nematodes and it was from the rhizosphere soil of prairie cordgrass located in the Manitoba Tall Grass Prairie

Preserve (MTGPP). Upon resampling the positive prairie, the following year, cyst nematodes were once again recovered from the root zone of the same native plant. However, it remains unclear whether this nematode is parasitizing prairie cordgrass, necessitating further research.

Through morphological and molecular analysis, including PCR amplification and sequencing of four regions of the genome (ITS rDNA, D2-D3 expansion segments of 28S rDNA; 18S rDNA, and a fragment of Mt-DNA (COI)), the cyst nematodes recovered were identified as *Punctodera stonei*. The genus *Punctodera* Mulvey and Stone, 1976, comprises cyst-forming nematodes that parasitize plants within the *Gramineae* family, both cultivated and wild-growing. This study represents the first report of *P. stonei* in Manitoba and only the second case in Canada.

The prairies sampled in our study, designated as preserves and untouched by cultivation, minimized the likelihood of introduced nematode populations in these areas. Consequently, any nematode genus discovered in this region was likely native, suggesting that the species may be more widespread than currently acknowledged.

Research findings, as presented in this study, significantly contribute to our understanding of the geographical distribution of cyst nematode species and the potential host they can inhabit. This information underscores that many nematodes found in agricultural crop fields in Manitoba are, in fact, native to the local soil.

5.3. Challenges and Surprises

The initial step in detecting and quantifying *H. glycines* through morphological and molecular methods involves isolating cysts and eggs from the soil. This process presents challenges, particularly when the target nematode density is anticipated to be very low, as observed in the sampled Manitoba soils during our research, presenting a significant challenge in my research. Our laboratory's prior surveys of Manitoba fields had not found SCN. Anticipating a low SCN

presence, the sampling process involved taking as large a volume of soil as possible from the field, ensuring comprehensive processing for cyst extraction in the laboratory. Adequate field sample points were also essential to address the heterogeneity of the nematode distribution.

Adding to the complexity, most sampled Manitoba fields had clay content, intensifying the difficulty of extracting cysts from the substantial soil volume. Extracting nematodes from clay is generally more challenging than from sandy soils, as fine clay particles settle at a pace similar to nematodes. To address this, our extraction method included repeating nested sieving in the laboratory and performing ethanol floatation on soil debris retained on the sieve to facilitate cyst isolation under the microscope. The methodology described in this thesis is aimed at optimizing cyst extraction efficiency.

Another significant challenge was performing identification and quantification of the nematode using traditional microscopic methods. The morphological measurements of a large number of nematodes can be time-consuming even for experienced nematologists. The morphological and morphometric similarities among closely related cyst nematode species further complicate accurate identifications, demanding additional time for species assignment.

The next challenge in this study involved conducting a growth chamber experiment to assess the reproductive ability of the Manitoba SCN population on a susceptible soybean variety planted directly in naturally infested Manitoba field soil, which tested positive in our study. Additionally, we aimed to culture the nematode in pots to multiply its numbers for subsequent analyses. Despite several attempts and repeated growth cycles using resampled positive fields in subsequent years, no reproduction was observed on soybean plants due to the initially low density of the Manitoba SCN population in soils and inoculum.

This series of unsuccessful attempts continued until the summer of 2021 when we repeated the experiments, this time employing naturally infested soil from a symptomatic field in south-central Manitoba with a high number of *H. glycines* eggs. Surprisingly this field had only five years of soybean cultivation and one year of edible beans. It marked the first occurrence of above-ground symptoms of SCN in the province. It was interesting that, despite starting the experiment with extremely low initial densities of *H. glycines* in Manitoba field soils, there was a notable and significant increase in populations observed in one soybean field.

Adjustments in growth chamber conditions, soil preparation for planting, and the timing of inoculation played crucial roles in facilitating *H. glycines* reproduction during the growth chamber studies. Furthermore, we recognized the significance of preparing the nematode suspension, excluding the number of eggs and juveniles. Utilizing eggs and juveniles as inoculum from cysts extracted from fresh soils, as opposed to air-dried samples, along with a few days of inoculum incubation on a heat-shaker, enhanced the readiness of eggs to hatch after inoculating soybean seedlings. The viability of eggs and juveniles in fresh samples significantly influenced the reproductive process. However, the current study did not assess female counts on infested roots, prompting the need for further research to confirm this hypothesis by comparing *H. glycines* female counts under different inoculum preparations.

The symptomatic soybean field was unexpectedly situated in south-central Manitoba, farther from the Manitoba/U.S. border and other previously identified positive fields. The presence of above-ground symptoms of SCN and cysts on roots at machinery entrance, coupled with their distribution throughout the entire field in alignment with tillage practices, suggested the potential spread of cysts through contaminated farm machinery.

During the 2021 revisiting and sampling of this symptomatic soybean field to investigate the density and distribution of *H. glycines* in an affected patch, an interesting observation was made. In several areas of the affected patch where soybean plants displayed yellowing and stunting, soil taken from the root zones revealed egg counts that were not sufficiently high to cause above-ground symptoms. This could be attributed to the exceptionally dry year experienced by this area and all of Manitoba, which accelerated the visual symptoms of SCN infection.

Furthermore, it was not surprising to find that the symptomatic field had a sandy loam soil texture. In periods of dry weather, especially in sandy soils, there is a significant increase in the potential for yield reduction due to nematode infestation, making sandy soils in dry years prime candidates for displaying symptoms first. Interestingly, during our subsequent visit the following month, the SCN-affected area was heavily infested with weeds. However, it was surprising to observe that the soybean plants in this area were green and growing, while the rest of the field was progressing towards maturity. Adequate rainfall prior to the revisit relieved water stress, allowing the plants to recover and exhibit growth due to improved moisture availability.

Several challenges were encountered in the molecular analysis of nematodes in this study. One notable issue was the low DNA concentration in some samples, falling below the recommended 20 ng μl^{-1} for sequencing by MacroGen Inc. (now Psomagen, Rockville, MD, USA). This inadequacy affected the generation of high-quality sequence results necessary for confirming the identification of nematode species.

The low DNA concentration observed in the purified PCR products could be due to either the low yield of the PCR reaction or the loss of DNA during the purification process. Considering that PCR reactions are primarily designed for diagnostic purposes rather than producing large amounts of DNA, an analysis of the results indicated the need for modifying PCR protocols to enhance

concentration. Adjustments to specific PCR parameters, such as PCR cycling conditions, annealing temperatures, increased total reaction volume, and higher initial DNA amounts, proved effective in increasing yield and minimizing non-specific amplifications.

Another adjustment for samples with DNA concentrations below 20 ng μl^{-1} , involved using purified PCR products as a DNA template in a re-amplification procedure. The resulting PCR product was subsequently re-purified using the QIAquick PCR Purification Kit to achieve a concentration exceeding 20 ng μl^{-1} .

During experiments, another important aspect that required careful consideration was the potential alterations in the quantity of target nematode DNA resulting from changes in the number of nematodes over the storage period (van Bezooijen 2006). To prevent the impact of these changes on our analysis, soil extraction is required to be performed in a short time after sampling (Bridge and Starr 2007). The necessity of simultaneously performing both traditional and molecular quantifications complicated the challenges associated with storage time.

Regarding the conventional process of *H. glycines* quantification, a crucial challenge was manually counting SCN eggs under a microscope. This task, taking approximately 10 minutes for an experienced nematologist to complete on one slide, required processing three slides per sample resulting in a total of 30 minutes per sample, excluding the time needed for staining and heating the eggs before transferring them to the counting slide. Moreover, achieving a reliable count demanded extensive training, given the potential for confusion between nematode eggs and cysts and the surrounding debris. These laborious and time-consuming conventional counting steps were required at every developmental stage of this study, including cysts, eggs, and DNA extractions, as well as conducting calibration curves along with consideration of biological replicates to enhance quantification accuracy.

Developing a quantitative DNA extraction method from soil posed another challenge in conducting this study. Ensuring the proper crushing of all nematode cysts and extracting a sufficient amount of DNA for molecular studies was crucial. In this current optimized protocol, I combined grinding soil debris containing cysts on a sieve with an extended bead beating time to achieve this. I ensured the integrity of the extracted DNA by measuring its concentration and purity using a Nanodrop spectrophotometer, followed by performing qPCR to amplify the DNA and checking the amplification efficiency. I made adjustments to filtration, centrifugation, and purification steps, aiming to obtain high-yield DNA. The final optimized extraction method retained all original steps but included small changes and additions for enhanced extraction capabilities. Both the quality and quantity of DNA were analyzed to confirm improved extraction efficiency.

I also made optimizations to the qPCR protocol to increase amplification efficiency. Challenges included selecting the best DNA template for conducting the standard curve, covering a wide range of test sample concentrations. Further optimizations involved determining the optimal annealing temperature through gradient PCR, selecting effective concentrations of primers and DNA templates, and adjusting the number of cycles to prevent non-specific PCR products and primer dimers. Regardless of nematode numbers, I ensured efficient, quantitative, and specific amplification.

In this study, relatively strong positive relationships were detected in the quantification of egg numbers using qPCR and traditional microscopic counting when employing both SCAR and COXIII primer sets. However, some variations emerged between the numbers estimated by qPCR and those conventionally counted. It was surprising that, for two very highly infested soil samples (exceeding 10,000 SCN eggs/100 cm³ soil), a more pronounced variability in egg number

estimations was observed between the two quantification methods, when using the COXIII primer set. This variability might be associated with a reduction in DNA extraction or recovery efficiency and the variation in the nematode DNA at various developmental stages existing in naturally infested field soils (Yan et al. 2013; Baidoo et al. 2017). Moreover, the COXIII primer set targeting the Mt-DNA gene is distinguished by having multiple copies of the mitochondrial genome per cell, and this copy number could potentially vary across different developmental stages of *H. glycines*, offering a possible explanation for this observed variability.

In the study of native prairies presented in Chapter Four of this thesis, a challenge encountered was the identification of *Punctodera* cyst nematodes at the species level. This proved challenging because of the limited range of available morphological observations, comprehensive molecular studies, and the number of sequences available in GenBank representing *Punctodera* populations. These limitations further complicated the analysis of the relatively few intact cysts and juveniles recovered in this study. Further research is essential to obtain precise explanations for these unexpected findings and address the challenges linked to them.

5.4. Recommendations for Future Research

Determining the occurrence, abundance and distribution of SCN in fields is a vital aspect of effective management strategies. In soybean-producing regions such as Manitoba, where SCN prevalence is not yet widespread, there exists a unique opportunity to identify infested fields at an early stage, when nematode population densities are relatively low. Implementing management practices during this phase is essential to prevent population growth. Although the level of SCN infestation in Manitoba is currently less severe than in neighbouring provinces, it underscores the need for continuous surveys and monitoring of soybean fields to mitigate and prevent the spread of the pest into new areas.

To prevent a decline in the effectiveness of management strategies, it is crucial not only to expand surveys to areas that have not been previously examined but also to thoroughly investigate and confirm the distribution of SCN in regions that have already been surveyed. Once SCN establishes itself in a field, the protective nature of the cyst wall decreases the efficacy of various management approaches. Therefore, the emphasis should be on both broadening the geographical scope of surveys and conducting detailed assessments to control SCN distribution.

Monitoring virulence phenotypes of SCN is another effective management strategy for this pest. In Chapter Two of this thesis, the SCN populations from only one of the infested Manitoba fields have been HG-typed. Still little is known about the SCN virulent phenotypes in the province. Consequently, there is limited knowledge about the virulent phenotypes of SCN in the province. Understanding the prevailing virulence phenotypes of *H. glycines* populations within a province is essential for soybean breeders to develop cultivars effectively and for growers to select crops and cultivar varieties wisely. Therefore, we advocate for extending HG-type testing to other SCN-infested field soils across Manitoba.

In the growth chamber study, we assessed the reproduction of SCN on a susceptible variety of soybean (S007-Y4, Syngenta, Guelph, ON). Subsequent research could concentrate on evaluating the susceptibility of various commonly cultivated soybean varieties in Manitoba and examining the resistance of SCN-resistant soybean varieties available in the Manitoba market under both controlled and field conditions.

Future research should prioritize determining the economic/damage thresholds of SCN in Manitoba. The establishment of SCN thresholds is important for the successful production of soybeans. It offers growers a clear benchmark for timely action that needs to be taken.

According to findings from prior studies (Poromarto et al. 2009; Yan et al. 2017; Zhang 2018; Katsande 2019), dry bean growers should be aware of the presence of *H. glycines* to avoid potential crop damage, as dry beans are recognized as suitable hosts for SCN. It is important to note that this specific aspect was not investigated in our current study. Considering the significance of dry bean production in Manitoba, further research on the presence of *H. glycines* in dry bean fields in the region can provide insights to develop efficient management strategies.

In Chapter Three, our focus was on improving existing methods for extracting cysts, eggs, and DNA from soil, and skipping the time-consuming step in the conventional *H. glycines* quantification by developing a specific and reliable qPCR method. Despite these advancements, there is still room for improvement in this process. Future research building on this thesis should aim to improve the accuracy of quantifying *H. glycines* directly from the soils.

Future research should continue to explore the use of real-time PCR in combination with species-specific primers targeting different regions of the genome, particularly various mitochondrial markers known for their efficiency in distinguishing closely related cyst nematode species.

The wet-sieving method and the manual grinding cysts process, integral components of our developed quantification method, can be partially automated to enhance the pace of the quantification process.

Due to the potential variation in PCR reaction inhibition or DNA binding to soil particles or humic substances based on soil type, it is advisable to assess the impact of different soil types on the sensitivity of the real-time PCR assay employed in this study. To achieve this, it is recommended to validate the effectiveness and reliability of the qPCR method and DNA extraction approach developed in this study across a broader range of soil types and populations for the

detection and quantification of *H. glycines* eggs. Furthermore, additional research is encouraged to create different calibration curves for different soil types and evaluate variations in these curves in the subsequent steps to enhance overall accuracy, precision and reliability.

Chapter Four discusses the discovery of the Stone cyst nematode (*P. stonei*) in a native prairie preserve in Manitoba. The chapter outlines the methods used to identify this nematode both morphologically and molecularly. However, more research is necessary to determine the prevalence of this nematode in other native prairies in Manitoba and throughout Canada. It is essential to investigate populations of this nematode from different locations, subjecting them to detailed molecular and morphological analyses to assess the reliability of diagnostic features and relationships among geographically diverse groups. Conducting similar studies in other native prairies can offer valuable insights into soil community dynamics, helping anticipate potential nematode-related challenges in Manitoba's agriculture. Furthermore, understanding how the presence of cyst nematodes is affected by host plants and soil characteristics in prairie preserves remains an unanswered question.

The methodologies developed in this study provide a valuable foundation for the future application of molecular methods in soil nematology, facilitating the identification and quantification of cyst nematodes for future monitoring and biodiversity surveys. While this thesis reveals fresh insights, it also raises unanswered questions for researchers and growers. Therefore, upcoming studies should set their sights on answering these questions, taking into account the gaps and challenges highlighted in this research.

5.5. References

- Anderson TR, Welacky TW, Olechowski HT, Ablett G, Ebsary BA. 1988. First report of *Heterodera glycines* on soybeans in Ontario, Canada. Plant Dis. 72:453.
- Baidoo R, Yan G. 2021. Developing a real-time PCR assay for direct identification and quantification of soybean cyst nematode, *Heterodera glycines*, in soil and its discrimination from sugar beet cyst nematode, *Heterodera schachtii*. Plant Dis. 105(12):3848-3857.
- Baidoo R, Yan G, Nelson B, Skantar AM, Chen S. 2017. Use of chemical flocculation and nested PCR for *Heterodera glycines* detection in DNA extracts from field soils with low population densities. Plant Dis. 101(7):1153-1161.
- Bridge J, Starr JL. 2007. Plant nematodes of agricultural importance: a colour handbook. London (UK): Manson publishing.
- Katsande TT. 2019. Evaluating seed treatments for the management of soybean cyst nematode (*Heterodera glycines* Ichinohe) in dry bean (*Phaseolus vulgaris* L.) [dissertation]. Guelph (ON): University of Guelph.
- Mimee B, Peng H, Popovic V, Yu Q, Duceppe MO, Tétreault MP, Belair G. 2014. First report of soybean cyst nematode (*Heterodera glycines* Ichinohe) on soybean in the province of Quebec, Canada. Plant Dis. 98(3):429-429.
- Niblack TL. 2005. Soybean cyst nematode management reconsidered. Plant Dis. 89(10):1020-1026.
- Poromarto SH, Nelson BD. 2009. Reproduction of soybean cyst nematode on dry bean cultivars adapted to North Dakota and northern Minnesota. Plant Dis. 93(5):507-511.
- Van Bezooijen J. 2006. Methods and techniques for nematology. Wageningen, The Netherlands: Wageningen University.

- Yan GP, Plaisance A, Chowdhury I, Baidoo R, Upadhaya A, Pasche J, Markell S, Nelson B, Chen S. 2017. First report of the soybean cyst nematode *Heterodera glycines* infecting dry bean (*Phaseolus vulgaris* L.) in a commercial field in Minnesota. Plant Dis. 101(2):391-391.
- Yan G, Smiley RW, Okubara PA, Skantar AM, Reardon CL. 2013. Developing a real-time PCR assay for detection and quantification of *Pratylenchus neglectus* in soil. Plant Dis. 97(6):757-764.
- Zhang K. 2018. Assessment of dry bean (*Phaseolus vulgaris* L.) Susceptibility to Soybean Cyst Nematode (*Heterodera glycines* Ichinohe) and the effects of biological and chemical control in a controlled environment [dissertation]. Guelph (ON): University of Guelph.