FINAL REPORT TO

Alberta Crop Innovation and Development Fund, Alberta Pulse Growers, Saskatchewan Pulse Growers and Manitoba Pulse Growers Association

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1. Project Number: 2011F024R (ACIDF number)
2. Project Title: Determination of the Host Status of Field Pea and its Associated Rotations and Weeds to the Stem and Bulb Nematode in the Canadian Prairies
3. Abbreviations:

4. Project Start Date: (04/01/2011)	**5. Project Completion Date:** (08/31/2013)
6. This is a final report of this two **year project.**	

7. Research Team Information

The personal information being collected is subject to the provisions of the Freedom of Information and Protection of Privacy Act.

a) Research Team Leader:		
Name	***Institution***	***Expertise Added***
Dr. Mario Tenuta	Department of Soil Science, University of Manitoba	Nematology, Cropping Systems, Project Management

b) Research Team Members		
Name	***Institution***	***Expertise Added***
1. Dr. Mehrdad Madani	Department of Soil Science, University of Manitoba	Nematology, Molecular Biology
2. Abolfazl Hajihassani	Department of Soil Science, University of Manitoba	Nematology, Host Screening
3. Dr. Rob Gulden	Department of Plant Science, University of Manitoba	Weed Science
4. Dr. Sergei Subbotin	California Department of Agriculture	Molecular Analysis, Expert in *Ditylenchus*
5. Dr. Shabeg Briar	Department of Soil Science, University of Manitoba	Nematology

8. Abstract and Background

Abstract

The results for this project demonstrate that the stem and bulb nematode present in yellow pea fields in the Prairie Provinces to be *Ditylenchus weischeri* and not the quarantine stem and bulb nematode *Ditylenchus dipsaci*. Results of our previous SPG/APG/ACIDF project showed a very low frequency of occurrence of the stem and bulb nematode in farmer provided yellow pea grain samples from the Saskatchewan, Alberta and Manitoba. Further, the nematode was associated with the presence of creeping thistle (*Cirsium arvense*) seeds in the yellow pea grain samples. A combination of low levels of weed seeds and infestation primarily to this species of weed, resulted in the very low and extremely variable analysis results for farmer provided yellow pea grain samples found positive for the nematode. In 2011, a survey of creeping thistle plants from fields in Manitoba and Saskatchewan showed most were infested with the nematode. Molecular analysis of DNA as sequencing of whole ITS, 28s, and hsp 90, RFLP-ITS, species specific designed primers for whole ITS and hsp 90, single nucleotide polymorphism of whole ITS, and secondary structure of whole ITS all corroborated that *Ditylenchus* from yellow pea harvest grain samples, creeping thistle from pea fields and creeping thistle from roadsides was *D. weischeri*. Samples of garlic from Quebec were shown by the above methods to be infested with *D. dipsaci*. Recently two new stem and bulb nematode species have been removed from *Ditylenchus dipsaci*. Chizhov et al. (2010) showed molecular evidence that the nematode infesting creeping thistle in Russia to be a different species than *D. dipsaci* and gave it the name *D. weischeri*. Similarly, the stem and bulb nematode that primarily infests faba or broad bean was removed from *D. dipsaci* and given the name *D. gigas* (Vovlas et al., 2011). These reports and the results of this project are important to Canadian growers because they indicate the *D. dipsaci* species was not monophyletic (one species) but really a cluster of species. In light of the results, phytosanitary protocols used by government agencies for screening of stem and bulb nematode species has been updated and past positive pea grain export shipments have been reanalyzed.

Attempts at mass rearing of *D. weischeri* in the laboratory on carrot disks, carrot callus, and fungi from surface of creeping thistle failed. Rearing of the nematode on creeping thistle plants in growth chambers was thus done to carry out host preference studies. The host preference studies conducted in the greenhouse showed *D. weischeri* preferred creeping thistle of plant species examined. *D. weischeri* was able to reproduce on yellow pea but to a lower extent that on creeping thistle. Large green seed lentil, kabuli and desi chickpea, garlic, spring wheat and canola were not hosts for *D. weischeri*. *D. dipsaci* reproduced aggressively on garlic, and to a lesser extent on yellow. It survived on creeping thistle but spring wheat and canola were not hosts. The results of the host preference studies indicate the need to conduct field studies examining the reproduction of *D. weischeri* on yellow pea and possible effects on yield and quality. However, methods for mass rearing of the nematode in the laboratory are needed to obtain the large number required for field studies. Further, garlic fields close to yellow pea fields should be scrutinized for the presence of *D. dipsac*i to prevent this quarantine pest from establishing in Prairie Canada. Lastly the molecular and host preference analysis strengthen the justification for species distinction of *D. weischeri* from *D. dipsaci* and *D. gigas*.

Background.

The stem and bulb nematode *Ditylenchus dipsaci* (Kühn, 1857) Filipjev 1936 is migratory endoparasite that feeds within the parenchymatous tissues of more than 500 plants (Bridge and Star, 2007). About 30 host races of *D. dipsaci* have been described based on host preferences (Sturhan and Brezeski, 1991; Hooper, 1971). Some races of *D. dipsaci* cannot interbreed while those that can may have different host preferences than the parent races (Webster, 1967). Thus, not surprising, recent studies showed that *D. dipsaci* is the species complex containing several morphologically similar and phylogenetically related

species. It includes *D. dipsaci sensu stricto, D. gigas, D. weischeri* and at least four still undescribed *Ditylenchus* species named as species D, E, F and G (Subbotin *et al* 2005; Chizhov *et al* 2010; Vovlas *et al.*, 2011). Only *D. dipsaci* and *D. gigas* have presently considered as pests of agricultural crops.

Ditylenchus dipsaci occurs in most temperate areas of the world and is a parasite of many crops including onion, garlic, carrots, pea, lentil, sunflower, potato, strawberry, sugar beet, and alfalfa. The prevalence of *D. dipsaci* on Western Canadian crops is unknown though reported in alfalfa fields in British Columbia (Vrain and Lalik, 1983) and Alberta (Hawn 1973). Symptoms of *D. dipsaci* infestations on pea include swollen and distorted stems and petioles, with distinct lesions that are brown to black. Discoloration and distortion of pods and seeds is also observed (Bridge and Star, 2007). Infected crops can become stunted with infected tissues being spongy and the damage predisposing the plant to colonization by plant pathogens (Vrain, 1987; Hawn, 1963). In addition to being the infective stage, the fourth stage juvenile (J4) is the survival stage of *D. dipsaci* in plant tissue, seed and soil. Dry seeds of the host plants carrying this pest are an important means of dissemination from one region to another (Hooper, 1971). The juveniles enter a state of cryptobiosis and can survive 3-5 years and even decades of desiccation (Sturhan and Brzeski, 1991). The longevity and wide host range of *D. dipsaci* can make disinfestation of fields difficult. As a result *D. dipsaci* is considered a quarantine nematode of many countries preventing the introduction of infested soil, seed and plants.

In Western Canada, near Regina, in Saskatchewan, Watson and Shorthouse (1979) reported *D. dipsaci* infesting creeping thistle plants, *Cirsium arvense* (L.) Scop. Chizhov et al. (2010) suggested that this finding belonged not to *D. dipsaci sensu stricto*, but to *D. weischeri*. *Ditylenchus weischeri* is presently known as a highly specialized stem nematode parasitizing only *Cirsium* species. Differentiation of *Ditylenchus weischeri* from *D. dipsaci* is difficult even for experts and based on differences in some morphometrical characters and whole ITS-rRNA gene sequences. Because, creeping thistle is a persistent perennial widely distributed and an aggressive weed and nematode infected plants might be found in fields, the problem of diagnostics of agricultural important *D. dipsaci sensu stricto* from *D. weischeri* becomes important.

Canada is the world's largest producer and exporter of peas. Peas are Canada's largest pulse crop and are grown over a very large geographic area, including significant portions of the Provinces of Saskatchewan and Alberta, and some parts of Manitoba. The value of total pea (*Pisum sativum* L.) exports averaged CDM$ 890M yr^{-1} (2010-2012) with Saskatchewan (78%) and Alberta (18%) accounting for most of the exports (Statistics Canada 2013). India is the major importer of pea accepting 41% of annual Canadian production (CDM$ 391M yr^{-1}; Statistics Canada 2013). The majority (94%) of pea exported to India yellow varieties. Though congenial climatic conditions prevail and host crops are under regular production in some regions, *D. dipsaci* is stated not to occur in India (Lal and Lal, 2005). Therefore, India has a strict quarantine importation restriction of materials with *D. dipsaci*. Pea shipments have been subject to scrutiny since *D. dipsaci* is known to be present in shipments; there have been challenges and severe costs to exporters to certify shipments as being free from *D. dipsaci*. The incidence of nematodes identified as *D. dipsaci* in pea shipments analyzed by Canadian Food Inspection Agency (CFIA) is extremely small and rare indicating the occurrence of the pest in pea fields to be limited. Nevertheless, market access problems for yellow peas to India and fumigation measures to certify shipments free of the pest are costly to exporters and passed to processors and growers.

In 2009, a 2-yr project investigating the occurrence of *Ditylenchus* in pea harvest samples was initiated by the University of Manitoba supported by the Alberta Pulse Growers, Alberta Crop Innovation and

Development Fund, and Saskatchewan Pulse Growers. A total of 329 (254 SK, 64 AB, 10 MB) were analysed. Eleven (3.3%) samples were positive from no specific area. Very low nematode incidence confirmed CFIA findings. However, incidence was related to the weed seeds presence more specifically, creeping thistle. Although 18% of samples had weed seeds, the amount of weeds was below the Foreign Matter allowance for contracts to India making extra cleaning likely not feasible. The incidence in weed seeds was also very low (8.1%) but higher than that for harvest (pea grain and foreign matter including weed seeds) samples (3.3% of harvest samples). Infested seeds were identified as creeping thistle. A combination of low levels of weed seeds and infestation primarily creeping thistle resulted in the very low and extremely variable analysis results for farmer provided yellow pea grain samples found positive for the *Ditylenchus*. Later in 2010 a preliminary survey of weeds was conducted in MB pea fields. Analyses of creeping thistle samples revealed mean population of 7 and 139 of nematode individual's g^{-1} of dry plant tissues (Stems + leaves) and flower heads, respectively. Flower heads high infestations indicate it to be a reservoir of the stem and bulb nematode leading to contamination of pea harvest samples.

In light of the observation of extremely low occurrence of *Ditylenchus* in yellow pea grain samples, creeping thistle seeds in grain with the weed a host for *D. weischeri* and recent alteration to the *D. dipsaci* taxonomy the University of Manitoba undertook a new project to identify the stem and bulb nematode from Prairie Canada with more recent analytical methods than used by CFIA. The project focused on a) confirming that creeping thistle common in pea fields are a source of contamination, b) using more recent and methods developed in this project identify the stem and bulb nematode, c) determine if pea, lentil, and chickpea are a host to the nematode, d) determine if crops in rotation with pea such as canola and spring wheat hosts of the nematode, and e) transfer the findings and methods to CFIA for updating of their pea grain surveillance program. Overall the project aims at reducing the cost associated with assuring shipments are pest free.

Objectives of the Project

Our primary goal of this project was to build on the results of the previous project funded by ACIDF, and APG, SPG to provide the information base for development of a strategic approach to limit export access issues of peas to India. The original project objectives were to:

(A) Conduct a field survey to confirm the infestation of weeds in pea fields with *D. dipsaci*,

(B) Conduct a greenhouse study to confirm what weed species are a source of contamination of harvest samples and that field pea is not the primary host for the pest,

(C) Conduct a greenhouse study to determine if rotation crops of field pea in Western Canada are or are not a host for the pest,

(D) Develop a rapid molecular means to determine the race of *D. dipsaci* and its preferred hosts.

New developments in methodologies and our findings necessitated a revision of the original objectives to meet the goal of addressing the market access issue of yellow pea to India. The revised objectives are as follows;

(1) Conduct a field survey to confirm the infestation of weeds in pea fields with the stem and bulb nematode,

(2) Determine the species identity of the stem and bulb nematode from Prairie Canada,

(3) Conduct a greenhouse study to confirm what weed species are a source of contamination of harvest samples and that yellow field pea is not the primary host for the stem and bulb nematode from Prairie Canada,

(4) Conduct a greenhouse study to determine if rotation crops of yellow field pea in Western Canada are or are not a host for the stem and bulb nematode from Prairie Canada,

(5) Convey findings to the Canadian Food Inspection Agency that their analysis protocols for export pea shipments may be updated to differentiate *D. weischeri* and *D. dipsaci* and reanalyze past positive samples.

9. Results towards realization of objectives

(1) Conduct a field survey to confirm the infestation of weeds in pea fields with the stem and bulb nematode

This objective was met by having conducted weed surveys in three Provinces (Manitoba, Alberta and Saskatchewan) of Western Canada for the stem and bulb nematode.

Survey: In the summer of 2011 a total of 14, 5 and 4 fields were surveyed in Manitoba, Saskatchewan and Alberta, respectively. Mainly creeping thistle weed plant samples were collected from the different pea and other crop fields for the presence of stem and bulb nematode.

Nematode extraction and microscopic evaluation: We followed a standardized procedure for extracting the stem and bulb nematode from weed samples. The protocol used is the same used by CFIA for screening the stem and bulb nematode in export shipments. Extraction and enumeration protocols are described in Appendix-I. Nematodes were extracted separately from weed plant's flower-heads and, stem and leaves. Extracted samples were observed under the microscope for the presence of nematodes in them and quantified on dry weight basis.

Nematode preservation: Stem and bulb nematodes extracted from different samples have been preserved using nematode lysis buffer or in 0.1 M NaCl Appendix-II.

Results: Thirteen fields were positive from Manitoba, three from Saskatchewan and no field was positive from Alberta where only four fields were surveyed this year (Table 1). Pigweed (*Amaranthus retroflexus*) collected from one field in Manitoba was also observed to be positive with nematode.

Table 1: Occurrence of stem and bulb nematode in creeping thistle (CT) and pigweed (PW) plants

Field	Province	Crop	Weed	*Nematodes/10g dry weight	
				Stem + Leaves	Flower heads
Field 1	MB	Soybeans	CT	110	0
Field 2	MB	Soybeans	CT	238	12751
Field 3	MB	Pintobeans	PW	18	0
Field 4	MB	Soybeans	CT	24	-[µ]
Field 5	MB	Soybeans	CT	41	103
Field 6	MB	Soybeans	CT	45	11707
Field 7	MB	Alfalfa	CT	220	2662
Field 8	MB	Peas/Oats	CT	170	10
Field 9	MB	Peas	CT	1804	768
Field 10	MB	Peas	CT	12	364
Field 11	MB	Peas	CT	68	301
Field 12	MB	Peas	CT	22	760
Field 13	MB	Peas	CT	0	0
Field 14	MB	Peas	CT	0	17
Field 15	SK	Peas	CT	0	0
Field 16	SK	Peas	CT	0	0
Field 17	SK	Peas	CT	2	94
Field 18	SK	Peas	CT	1394	1930
Field 19	SK	Peas	CT	57	287
Field 20	AB	Peas	CT	0	0
Field 21	AB	Peas	CT	0	0
Field 22	AB	Peas	CT	0	0
Field 23	AB	Peas	CT	0	0

*: Nematode numbers are average of three replicates per field; [µ]: No flower-heads

2) Determine the species identity of the stem and bulb nematode from Prairie Canada

This objective was met by comparing DNA sequences of several regions of the the stem and bulb nematode from Prairie Canada to that of *D. dipsaci*. The Prairie Canada stem and bulb nematodes were obtained during survey of weeds fields in 2010 (MB only) and 2011 (AB, SK, and MB described above), and from pea harvest samples collected in 2009 (AB, SK, MB). DNA sequences of the nematodes were compared to the already published sequences of stem and bulb nematode species including *D. weischeri* and *D. dipsaci*. This work was done in collaboration with Dr. Sergei Subbotin, Plant Pest Diagnostics Center, California Department of Food and Agriculture, Sacramento, California, USA grain.

DNA extraction, PCR, RFLP, and sequencing

DNA extraction: DNA was obtained from both individual and bulk nematodes (Appendix III). Briefly: individual nematodes as well as bulk nematodes of 10 to 20 were transferred to a 250 µl PCR tube containing 10 µl distilled water. To this were added another 10 µl of Worm Lysis Buffer (WLB) (KCl, 500mM ;Tris pH 8.3,100mM ; $MgCl_2$, 100mM; NP40, 4.5%; Tween 20 4.5%) and nematodes were then crushed for 90 sec using a laboratory mixer (handy device homogenizer, Sigma Aldrich) fitted with a glass pipette with a tip blunted by flaming. The suspension was then centrifuged at 10,000 rpm for 45 sec and placed at -20°C for 10 minutes. After thawing, 2 µl of Proteinase K (600 µg/ml) was added to each tube and incubated for 60 min at 60°C. Following this step, Proteinase K was deactivated by placement for 10 min at 94°C. The quality and quantity of DNA in suspensions were confirm by spectrometry (NanoDrop, Thermo Scientific) of 1 µl of the extracted DNA at 260 nm. Tubes were then kept at -20°C until used.

PCR: Four gene fragments were used to identify the *Ditylenchus* nematodes from the DNA extractions. The genes were the whole Internally Transcribed Spacer (ITS) (ITS1, 5.8s and ITS2) of the rRNA gene, the 18S rRNA gene, the D2-D3 expansion region of the 28S rRNA gene, and the heat shock protein, hsp 90 gene. These genes were amplified using polymerized chain reaction (PCR) and sequenced using Sanger methods.

The primers used in PCR were TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'ATATGCTTAAGTTCAGCGGGT-3') or 5.8s forward (5'-CTCGGTTCATAGATCGATG-3') and 5.8sR (5'-CAGATGTGCCAAAGGATAGA-3') for the whole ITS gene. It is important to note that the whole ITS gene was amplified compared to only a partial region of the gene (only ITSI) used by CFIA in testing pea grain export samples. Primer 18sF (5'TGGATAACTGTGGTAATTCTAGAGC-3'), and 18sR (5'-TTACGACTTTTGCCCGGTTC-3') for 18S gene. D2A forward (5'-ACAAGTACCGTGAGGGAAAGTTG, and D3B reverse (5'-TCG GAAGGAACCAGCTACTA-3'), or D2AA forward (5'-GAAACGGATAGAGCCGACGTAT-3') and D2B reversed (5'-CCAAGTCAGACGATCGATT-3') for the 28s gene. U831 forward (5'AAYAARACMAAGCCNTYT GGAC, and L1110 reversed (5'-TCRCARTTVTCCATGATRAAVAC-3') for the hsp 90 gene.

All the PCR reactions were conducted in a total volume of 25 µl with 1 to 3 µl of DNA suspensions from each sample for 36 cycles. PCR reactions were performed in an Eppendorf Thermal Cycler using prepared premaster mix (Life science, US). Detail protocols for PCR and sequencing applied are described by Maafi et al. (2003) and Madani et al. (2011) (see Appendix IV).

RFLP: The whole ITS-PCR products amplified by primers TW81 and AB28 were restriction enzyme digested to create banding patterns to differentiate *D. weischeri* and *D. dipsaci*. The amplicons were isolated by gel electrophoresis, the band excised and were purified using the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON). Six to 7 µl of purified DNA product was then used to generate a restriction enzyme profile. One to 2 µl of five restriction enzymes in buffer was added to a middle size Eppendorf tube containing purified PCR product and incubated at 37°C for at least 2hr. Five restriction enzymes were used: Bsh123, HinfI, MspI, RsaI and TaqI. Then 2 µl of the restricted DNA samples were loaded onto a 2% agarose gel in TAE buffer and electrophoresis conducted at 80 V. A 100 bp molecular marker ladder (Promega 100 bp ladder, Madision, USA) was used to estimate fragment size. Gels were visualized after staining with ethidium bromide using a UV transilluminator (G-Box,

SynGen, Cambridge, UK). Restriction enzymes used were able to produce discriminative pattern that distinguished positive samples for *D. weischeri* from a sample of *D. dipsaci*, collected from Garlic, Quebec, used as control. This were including *Bsh*123, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I and *Pst*I, by having bands of approximate size: unrestricted, (280,220),(330,140), (480,310), (350,240), (480,320), respectively, compare to those of *D. dipsaci* by having bands of (520,480), (480,380), (480,260,120), (320,200,140), (390,220,150,140), and (620,400) for the same restriction enzymes, respectively Fig. 1A shows an example of the RFLP pattern obtained from DNA of the stem and bulb nematode retrieved from field yellow pea grain samples from our weed survey conducted in 2011 (Field 18 from SK in Table 1) with a pattern typical for *D. weischeri*. DNA extracted from *Ditylenchus* infested garlic from Quebec was also obtained and subjected to RFLP analysis. The garlic samples resulted in a different RFLP pattern than from pea harvest or creeping thistle samples being consistent for identification as *D. dipsaci* (Fig. 1B). In total a least 16 samples positive for stem and bulb nematode from Prairie Canada pea harvest grain and creeping thistle samples were analyzed for RFLP-whole ITS profiles. All samples showed a profile consistent for identification to *D. weischeri* (Table 2).

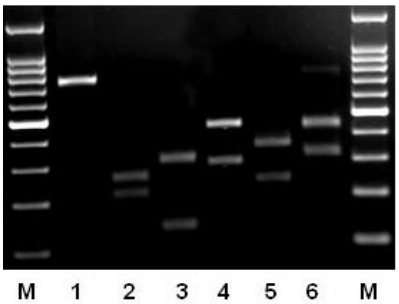

Fig.1A

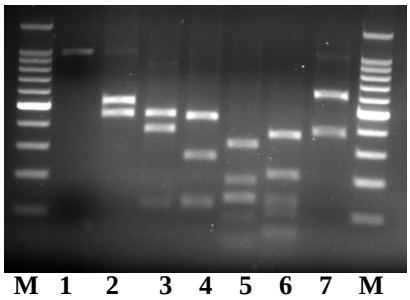

Fig 1B

Figure 1. RFLP pattern of DNA from *Ditylenchus weischeri* and *D. dipsaci*.(4A) *D. weischeri*, Field 18, in Saskatchewan, Lanes 1 to 6: Bsh123, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I and *Pst*I. (4B) *D. dipsaci*, garlic, Quebec. Line 1, unrestricted PCR product, Lanes 2 to 7: Bsh123, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I and *Pst*I. M, 100 bp ladder, (Promega 100 bp ladder, Madision, WI). The results clearly show a difference in DNA restriction pattern from that in 4A consistent with *D. weischeri* and 4B for *D. dipsaci*, respectively.

Table 2. Summary of stem and bulb nematode codes and province of origin, plant source material, and resulting identification of the nematode by RFLP-whole ITS and whole ITS sequence analysis.

Sample (province)	Determinations	Source material	Identification
Field 58(AB), 33(AB), 41 (SK)	RFLP- whole ITS and whole ITS sequence	Pea grain harvest samples from 2009 study	*D. weischeri*
Field 1,2,3,4, and Road side1 (all MB)	RFLP- whole ITS and whole ITS sequence	Creeping thistle samples in yellow pea fields (Fields 1-4) and nearby road side from 2010	*D. weischeri*
F7, F18, F19 (all SK) F8, F9, F10,F11(all MB)	RFLP- whole ITS and whole ITS sequence	Creeping thistle weed survey from 2011	*D. weischeri*
Garlic QB	RFLP-whole ITS and whole ITS sequence	Garlic samples from 2012	*D. dipsaci*
Creeping thistle-Glenlea (MB)	RFLP-whole ITS and whole ITS sequence	Creeping thistle samples from 2012	*D. weischeri*

Sequencing and design of *D. weischeri* specific primers

Each of the amplified genes of the whole ITS, 28s, 18s and hsp 90 were also subjected to direct sequencing of both DNA strands. Amplification products were isolated and purified by gel electrophoresis as described previously. The quality and concentration of purified DNA suspension was determined by absorption spectroscopy with NanoDrop (Thermo scientific) by measuring 1 µl of the extracted DNA at 260 nm. For sequencing 50 to 100 ng of DNA submitted for sequencing to Macrogen sequencing facility (Rockville, MD). The computer software programs: Chromas Lite version 2.01 (Technelylium Pty, Ltd, and GenDoc version 2.7 (Nicholas and Nicholas, 1997)) were used to visualize the sequence chromatogram and edit the DNA sequences, respectively. Clustal X software (Thompson et al., 1997) was then used for multiple sequence alignments of new sequence data from this study with sequences of *Ditylenchus* species in the NCBI GenBank.

The sample samples of pea harvest grain and creeping thistle samples identified as being *D. weischeri* by RFLP-whole ITS analysis were also subjected to whole ITS sequencing (Sanger methods at York University) and sequence alignment in GenBank. Not surprisingly all samples showed a profile consistent for identification to *D. weischeri* (Table 2).

Starting from the results of multiple sequence alignments, several sets of primers were designed for each of the whole ITS and hsp 90 genes to be specific to the *D. weischeri* obtained in this study. The whole ITS based primers developed specific to *D. weischeri*, named Weischeri_ITS, was forward primer (5'-GTGTCATGTTTTTGTGAAGTGGA-3') in combination with rDNA2 universal primer 5'-TTTCACTCGCCGTTACTAAGG-3') yielded a single PCR product around 260 bp for *D. weischeri* (Fig. 2). PCR reaction mixtures and denaturing thermal profiles are given in Appendix IV.

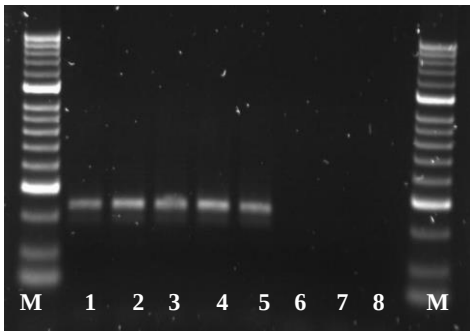

Figure 2. Result of PCR amplification with the developed whole ITS based specific primer set Weischeri ITS+rDNA2 of *Ditylenchus*. Lines: M – HyperladderII DNA ladder, VWR, Mississauga, ON); 1 = (Creeping Thistle SK-18), 2= Creeping Thistle MB-Field 1), 3= Creeping Thistle from SK-19), 4= pea harvest sample Field 58, 5= R1-4 (Creeping Thistle from MB roadside), 6 – 8 = *D. dipsaci* from Garlic from Quebec. Results of lanes 1 to 5 are consistent with *D. weischeri*.

The second designed primer set was based on the hsp 90 gene and named Weischeri hsp 90. The primer set was a combination of U831 forward primer (5'-AAYAARACMAAGCCNTYTGGAC-3') with Weischeri_hsp 90 reverse primer (5'-AGC ACTAAAATTAAGYGTAAAGG-3'). PCR using Weischeri hsp 90 yielded a single PCR product around 196 bp and 182 bp for *D. weischeri* (Fig. 3A) and *D. dipsaci* (Fig. 3B), respectively.

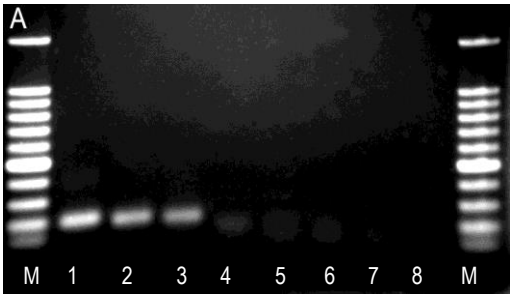

Figure 3A

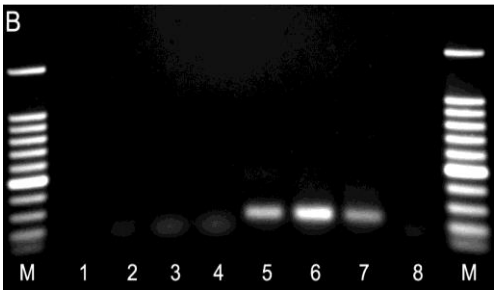

Figure 3B

Figure 3. Result of PCR amplification with the developed hsp 90 based specific primers sets. A: *D. weischeri* species specific primers (Weischeri_hsp 90 + U831); B: *D. dipsaci* species specific primers (Dipsaci_hsp 90 + U831). Lines: M- 100 bp DNA ladder (Promega, 100 bp ladder, Madision, USA); 1- *D. dipsaci* from Onion, MS, USA; 2 - *D. dipsaci* from Phlox, Russia; 3- *D. dipsaci* from Strawberry, Russia; 4- *D. gigas*, Morocco; 5- *D. weischeri*, Russia; 6, 7, *D. weischeri*, SK-1, MB-1, Canada; 8 - control without DNA.

Due to lack of DNA for most of the pea harvest grain and creeping thistle samples from 2009, 2010, and 2011 we were not able to run diagnostic PCR with specific primers for this samples, except for those indicated in Fig. 5 and 6. However, virtual PCR analysis on hsp 90 sequences of the samples obtained in this study listed in Table 2 indicate for stem and bulb nematode samples from Prairie Canada the presence of annealing sites for the hsp 90 based primers. For the hsp 90 gene specific primer

Weischeri_hsp 90 + U831virtual PCR was positive for the obtained sequences from samples identified as *D. weischeri* in Fields 58 (AB) and 81 (MB) from pea harvest grain samples 2009; and Field 11(MB) from creeping thistle in a yellow pea field in 2010, and Field 4 and 11 (MB) from creeping thistle samples obtain in 2011. No annealing site was observed in these samples with primer Dipsaci_hsp 90. Virtual PCR with Dipsaci_hsp 90 + U831primers was positive on sequences of DNA obtained from garlic isolates from QB.

Phylogenetic analysis

The sequences of the whole ITS, hsp 90 and 28s genes obtained in this study were aligned separately with sequences of the same genes from *Ditylenchus* available in GenBank using ClustalX 1.83 with default parameters used by Vovlas et al. (2011). Outgroup taxa for each dataset was selected according to the results of previously published data (Subbotin et al., 2005). Sequence datasets were analysed with Bayesian inference using the program MRBAYES 3.1.2. Phylogeny analysis of the 28S, whole ITS and hsp 90 genes are presented in Fig. 4 A, B and C, respectively. The results are similar for the three genes indicating *D. weischeri* being separate from the other species of stem and bulb nematode, *D. dipsaci* and *D. gigas*. Further, *D. weischeri* seems more closely genetically related to *D. gigas* than to *D. dipsaci*.

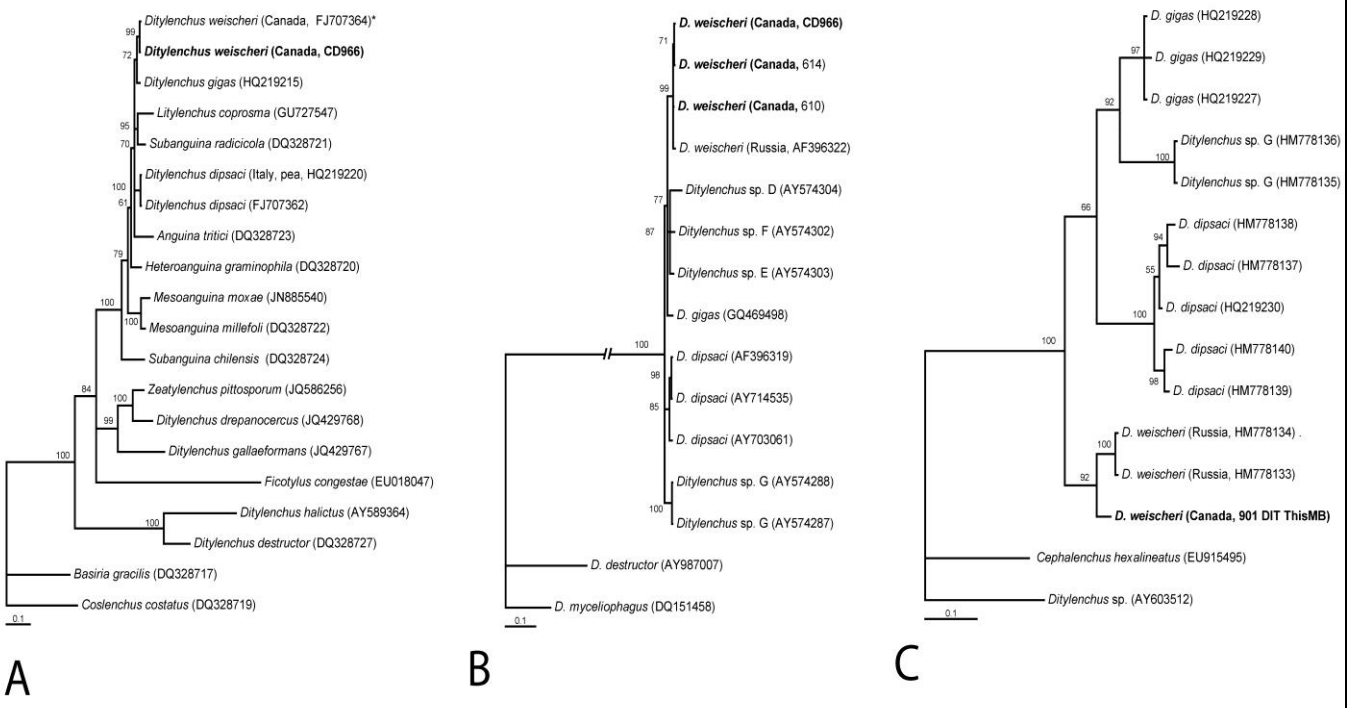

Figure 4A, B and C. Phylogenetic trees constructed based on 28s, whole ITS and hsp 90 gene sequences, respectively, of *D. weischeri*, *D. dipsaci* and *D. gigas* as well as other out group nematodes.

Sequence analysis and secondary structure

Sequence comparisons of 43 whole ITS sequences from the samples which were identified as *D. weischeri*, mainly from creeping thistle samples from 2011 and 2012, and *D. dipsaci* from garlic

samples from QB. In addition, sequences for *D. weischeri* from the original type speciment from Russia were included in this study. In addition deposited sequences of *D. dipsaci* and *D. gigas* in Genbank were included in the analysis for a total of 67 sequences over the three species. Sequence multiple alignments were performed using the program Clustal X (Thompson et al., 1997). Intra species analysis in the creeping thistle samples from MB showed the presence of at least 2 haplotypes in whole ITS of *D. weischeri* (Table 3). Inter species sequence variation revealed present of 11 SNP's (Single Nucleotide Polymorphism) that were specific to *D. weischeri* (Table 4). These results provide further evidence to consider the three species of *D. wiescheri*, *D. gigas* and *D. dipsaci* to be genetically different. These variations are useful information for study of different isolate or population of *D. weischeri* and for purpose of identification and diagnosis as a basis of development of species specific primer sets and real time PCR probes.

Table 3. Nucleotide position of the SNP's in ITS1 and ITS2 of the studied nematodes. Inter and Intra nucleotide sequence variation among *D. weischeri* from Manitoba, Saskatchewan isolates (MB, SK), *D. weischeri*, holotype originated from Russia (HR), *D. weischeri* sample from Russia processed in Manitoba (RM); *D. gigas* (D.g) and *D. dipsaci* (D.d). Positions showing haplotypes of *D. weischeri* are bold underlined (W=A or T; Y= A or C).

sample	***ITS1***																***ITS2***														
MB/ SK	A	A	G	T	G	T	**W**	C	A	T C	G	G	T	G	T	-	A	T	A	G	AA C	A	T A	T A	T	G A	**AG Y-**	G	C R-	TA T	A
HR	A	T	G	T	G	T	**W**	C	A	T C	G	G	T	G	T	-	A	T	A	G	M AC	A	T A	T A	T	G A	**AG Y-**	G	C R-	TA T	A
RM	A	T	G	T	G	T	**W**	C	A	T C	G	G	T	G	T	-	A	T	A	G	AA C	A	T A	T A	T	G A	**AG Y-**	G	C R-	TA T	A
D. g	T	T	A	T	G	T	T	T	A	T C	A	G	T	A	A	A	A	A	A	G	AA A	A	C -	T G	G	A C	GA CT	A	C G-	TA G	T
D. d	A	T	G	C	A	C	T	C	G	C T	G	C	G	G	T	A	T	A	T	A	GG T	G	T T	C A	G	A C	GA CT	A	T G T	C G T	-

Table 4. Nucleotide positions and single nucleotide polymorphism (SNP) of whole ITS after multiple sequence alignment between *D. weischeri* from MB and SK, haplotype of *D. weischeri* from Russia (HR), *D. weischeri* originated from Russia and processed in Manitoba (RM), *D. gigas* (D.g) and *D. dipsaci* (D.d). SNP's specific to *D. weischeri* which differs in both *D. gigas* and *D. dipsaci* are at position: 67 (a), 218 (C) 320 (deletion), 502 (T), 557(T) 576-7 (GA), 634 (AG-) and 635(G). No polymorphism and SNP's in 5.8s gene. (W=A or T; Y= A or C).

Nematode sample	***ITS1***				***5.8s***	***ITS2***				
Nucleotide Position	67	122	218	320	502	557	574	576-7	634-7	643
MB/ SK	**A**	**W**	**C**	**-**	**T**	**T**	**T**	**GA**	**AGY-**	**G**
HR	**A**	**W**	**T**	**-**	**T**	**C**	**T**	**GA**	**AGY-**	**G**
RM	**A**	**W**	**C**	**-**	**T**	**T**	**T**	**GA**	**AGY-**	**G**
D. g	**T**	**T**	**C**	**A**	**A**	**T**	**G**	**AC**	**GACT**	**A**
D. d	**A**	**W**	**C**	**-**	**T**	**T**	**T**	**GA**	**AGY-**	**G**

Analysis of whole ITS secondary structure

Secondary structure of the DNA by analysis of whole ITS sequences was computed based on a energy minimization approach using the program Mfold version 3 (Zuker, 2003). The approach relies upon examination of the whole ITS 2-D structure by considering interactions between pairs of nucleotide bases in terms of nucleotide composition and energy. The outcome structure has the general core structure built in loop and helix forms (Fig. 8). The %GC content, nucleotide similarity and differences in structure are presented for *D. weischeri* and its holotype, and *D. gigas* and *D. dipsaci* in 2-D figures of the folding of the whole ITS sequences. Differences were observed for *D. dipsaci* and *D. gigas* to D. *dipsaci* because of %GC content being slightly higher in the later. The 2-D structure was more conservative and consistant among D. weischeri samples for the length of two helixes being much longer than for *D. gigas* and *D. dipsaci* (Fig. 5).

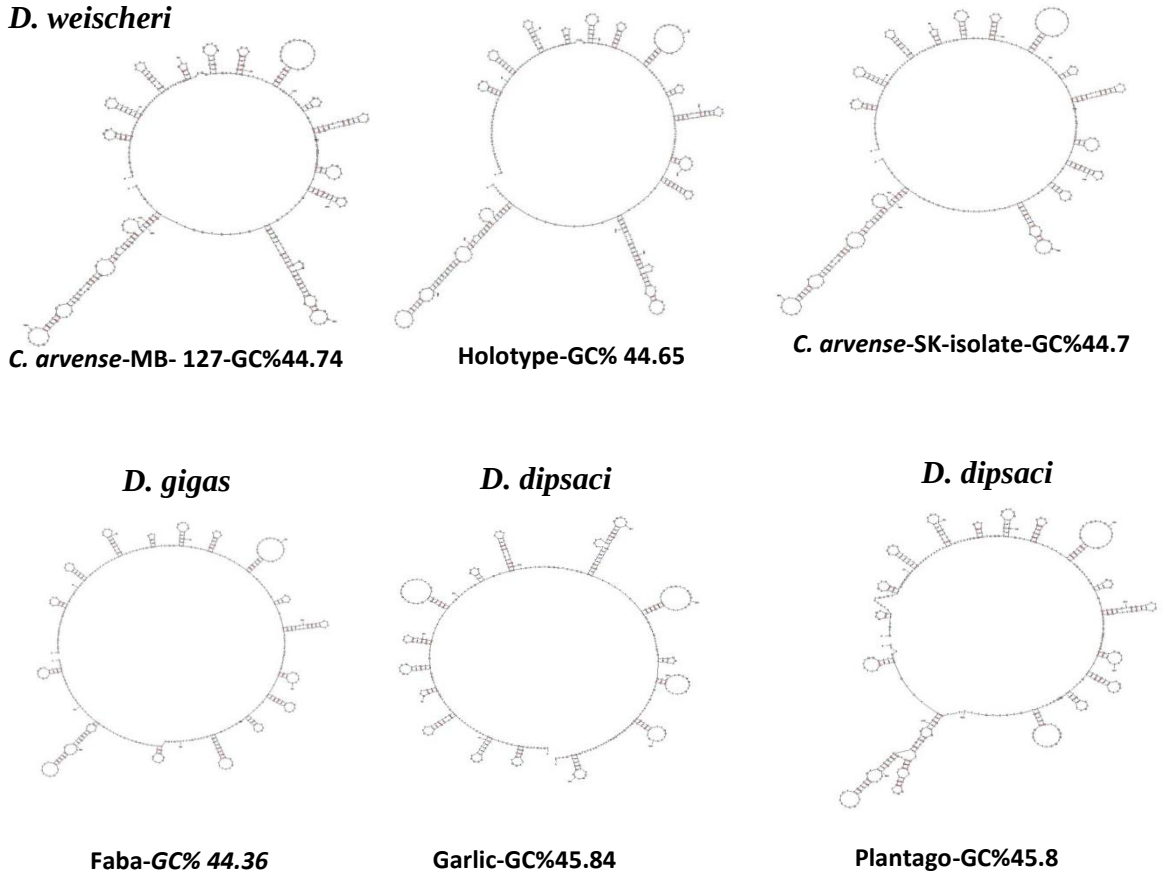

Figure 5. Secondary structure of whole ITS and the %GC content for *D. weischeri* from Creeping thistle from MB; holotype *D. weischeri* isolated from creeping thistle in Russia; D. weischeri from creeping thistle in SK, , *D. gigas* from Faba, and *D. dipsaci* from garlic and *Plantago*.

The results here of multiple lines of molecular DNA evidence show the Prairie Canada stem and bulb nematode to definitively be *Ditylenchus weischeri* and not the quarantine stem and bulb nematode *Ditylenchus dipsaci*. *D. weischeri* and *D. gigas* had recently been separated from *Ditylenchus dipsaci*. Chizhov et al. (2010) showed molecular evidence that the nematode infesting creeping thistle in Russia to be a different species than *D. dipsaci* and had named it *D. weischeri*. Similarly, the stem and bulb nematode that primarily infests faba or broad bean was removed from *D. dipsaci* and given the name *D. gigas* (Vovlas et al. 2011). These reports are important because they indicate the *D. dipsaci* species was not monophyletic (one species) but really a cluster of species. The results here are further evidence validating species separation of *D. weischeri* and *D. dipsaci*. Establishing quarantine restrictions to trade for species across all taxa within *D. dipsaci* may not have been needed. Further it seems it is weed seeds and particularly, creeping thistle seeds, that are the source of the very rare occurrence of *D. weischeri* in yellow pea export grain. The nematode can be commonly found on creeping thistle plant parts.

3) Conduct a greenhouse study to confirm what weed species are a source of contamination of harvest samples and that yellow field pea is not the primary host for the stem and bulb nematode from Prairie Canada, and (4) Conduct a greenhouse study to determine if rotation crops of yellow field pea in Western Canada are or are not a host for the stem and bulb nematode from Prairie Canada.

These objectives were met by conducting green-house trials for screening of creeping thistle, yellow pea, hard red spring wheat, canola, lentil, and chickpea as hosts for *D. weischeri* and *D. dipsaci*. *D. dipsaci* had been used to possibly contrast the host preference profile to *D. weischeri*. Garlic was included as a test plant because *D. dipsaci* was obtained from infested bulbs. In addition, plant parasitic nematodes are easily spread by garlic bulbs and thus we needed to check if *D. weischeri* could damage garlic and be spread.

Nematode rearing: Culturing attempts to rear *D. weischeri* in the laboratory were performed (See Appendix-V for details related to nematode culturing methods). We took four approaches to culturing the nematode. The first was rearing the nematode on sterile carrot disks (Figure 6), second on the callus tissue (Figure 7), the third rearing on agar plates colonized by fungi from surfaces of creeping thistle, and lastly rearing the nematodes on live creeping thistle plants in the greenhouse/growth chambers (Figure 8). The first three methods of culturing showed limited success as the inoculated nematodes failed to multiply in large numbers. However, creeping thistle plants inoculated with 2000-3000 individuals per plant in the green house yielded high numbers (>100/g) of nematode after 90 days of inoculations. For the host screening studies, nematodes were thus reared and extracted from creeping thistle maintained in the growth chambers.

Figure 6

Figure 7

Figure 8

Host Screening Trials: The objectives of these trials were to determine if the stem nematode, *Ditylenchus weischeri*, can survive and reproduce on creeping thistle (*Cirsium arvense*) and yellow field pea (*Pisum sativum*). In addition, the host status of two other pulse crops, chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*), and non-legume crops, spring wheat (*Triticum aestivum*) and canola (*Brassica napus*) to parasitism by the nematode was assessed. In these studies, a population of *D. dipsaci* from infested garlic (*Allium sativum*) in Quebec was used as a reference species. Nematode juveniles of *D. weischeri* and *D. dipsaci* were reared on creeping thistle and garlic plants, respectively, in a growth chamber and used to infest the test plants (Appendix-VI).

The trial procedure was as follows. Creeping thistle from the Glenlea Field Station (University of Manitoba), garlic (artichoke garlic), field pea var. Agassiz, chickpea var. CDC Corinne (a desi variety) and CDC Frontier (a kabuli variety), lentil var. CDC Greenland (large sized), hard red spring wheat cv. AC Barrie, and canola var. Invigor were grown in small polyethylene pots filled with clay-peat mix. The plants were inoculated twice (first at two-leaf stage and then five days later) with *D. weischeri* or *D. dipsaci* by placing 15µl of 1.5% carboxymethyl cellulose containing 50 nematodes in the leaf axils of recently emerged seedlings. Each treatment for *D. dipsaci* and *D. weischeri* was replicated seven and nine times, respectively, per plant species. Plants inoculated with water alone (no nematode) were used as a control.

After inoculation and to increase humidity and prevent inoculum droplets from drying, plants were placed for 72 hours in a small humidity chamber comprised of a translucent plastic canopy and humidifier. Afterwards, the pots were maintained in a completely randomized design (CRD) on benches in greenhouse. Treatments (plant species x nematode challenge) were separated on benches using plastic boards to prevent touching of foliage and water splash which may have transferred nematodes. Plants were observed during the trials for any symptoms associated with infestation. The plants were grown for eight weeks prior to extraction for the nematodes. Extraction for the nematodes was done using the Whitehead tray method (Whitehead and Hemming, 1965) with all above ground plant tissue chopped an extracted on Baermann pans. The reproduction factor (R_f) of the nematodes was determined by dividing the recovered population by the added population with a $R_f > 1$ showing the plant was capable of being a host and $R_f < 1$ not being a host.

Many preliminary trials were done to establish the experimental systems described above. Thereafter, five trials were conducted with the following treatments:
Trial 1 – Creeping thistle and yellow field pea challenged with *Ditylenchus weischeri*
Trial 2 – Creeping thistle and yellow field pea challenged with *Ditylenchus dipsaci*
Trial 3 – Garlic, spring wheat and canola challenged with *Ditylenchus weischeri*
Trial 4 – Garlic, spring wheat and canola challenged with *Ditylenchus dipsaci*
Trial 5 – Chickpea and lentil challenged with *Ditylenchus weischeri*

In addition to a control (no nematode challenge), a secondary control of creeping thistle and garlic were included when the trial included challenge with *D. weischeri* and *D. dipsaci*, respectively. Either nematode was not recovered from the control treatments.

The preferred host of *D. weischeri* was creeping thistle having an R_f of 3.3 (Table 5). The other host for the nematode was yellow pea with an R_f of 1.7. The preferred host of *D. dipsaci* was garlic with an R_f of 6.2 (Table 5). Yellow pea was a host for *D. dipsaci* with an R_f of 3.4. It is ambigious if creeping thistle is a host of *D. dipsaci* because of having an R_f of 1.0.

Table 5. Summary of host screening results performed in the greenhouse for creeping thistle, yellow pea, garlic, hard red spring wheat and canola as hosts of the nematodes *D. weischeri* and *D. dipsaci*.

Nematode	Plant[ab]							
	Creeping Thistle	Yellow Pea	Lentil	Chickpea Corinne	Chickpea Frontier	Garlic	Spring Wheat	Canola
D. weischeri	3.3 (0.3)	1.7 (0.2)	0.4 (0.1)	0.4 (0.1)	0.5 (0.1)	0.6 (0.0)	0.5 (0.1)	0.4 (0.1)
D. dipsaci	1.0 (0.1)	3.4 (0.2)	NT	NT	NT	6.2 (0.6)	0.1 (0.0)	0.0 (0.0)

[a] Values are the mean of nine replicates (± standard error) of each treatment
[b] >1 = host; <1 = non-host; R_f = Pf/Pi. NT = not tested

The results of the host screening trials confirm *D. weischeri* to parasitize creeping thistle. The nematode had a relatively high R_f value which was not surprising to find considering it was often present on the weed in the field surveys given above and being reared on creeping thistle. Yellow pea needs to be considered a possible host of *D. weischeri* because the nematode was able to reproduce, though not to the extent as on creeping thistle. Field experiments challenging yellow pea with *D. weischeri* are warranted based on these greenhouse results. The R_f value for the nematode on yellow pea though greater than one was relatively low compared to that for creeping thistle, a known host. Producers have not observed yellow pea damage expected to be caused by a foliar parasitic nematode. This indicates that the nematode should not be damaging in field experiments. Nevertheless, confirmation with field experiments is needed in light of the greenhouse trials results here. Methods for mass rearing of the nematode in the laboratory are needed to obtain the large number required for field studies. The other pulse plants tested here, lentil and chickpea, were not a host for *D. weischeri*.

D. dipsaci was confirmed to strongly parasitize garlic with a relatively very high R_f value. This is not surprising since the nematode is causing large yield losses of garlic in northeastern North America; the isolate used was isolated and reared on garlic. *D. dipsaci* was able to parasitize yellow pea though not with same preference as for garlic but more so than *D. weischeri* to yellow pea. The results indicate potential for *D. dipsaci* to parasitize yellow pea. Thus there is potential for concern for the nematode to

move from garlic fields to yellow pea. Fortunately most commercial garlic in Canada is not grown on the Prairies. Nevertheless growers should in future avoid the possibility of yellow pea and garlic being grown near each other.

5) Convey findings to the Canadian Food Inspection Agency that their analysis protocols for export pea shipments may be updated to differentiate D. weischeri and D. dipsaci and reanalyze past positive samples.

In early 2013, Dr. Tenuta met with Pulse Canada to summarize the finding of the project to date. It was agreed that a sufficient body of information and confidence in the results existed to contact CFIA and request meetings. Between March and July 2013 a total of four conference calls and two in person meetings in Ottawa were held with the participation of Dr. Tenuta. The results, developed methodologies, sequences, and creeping thistle material colonized with *D. weischeri* from this project were transferred to CFIA. Since our initial meeting, much progress by CFIA has been made to advance addressing the market access issue of yellow pea with India. For further information contact Pulse Canada.

Acknowledgments

We thank APG, ACIDF, SPG, and MPGA for financial support to carry out this project. The technical assistance of Jehn Francisco and Sue Yi is appreciated. Thank you to Dr. Linda Hall, and Eric Johnson for graciously providing creeping thistle samples. Receipt of garlic samples from Dr. Michael Celletti (Ontario Ministry of Agriculture, Food and Rural Affairs) and Nancy Shallow (Ministere de l'Agriculture, des Pecheries et de l'Alimentation du Quebec) is greatly appreciated. Gord Kurbis and Gordon Bacon of Pulse Canada were tremendous assets to relay the project findings to CFIA. We thank CFIA for providing nematode extraction protocols and the original ITSI molecular identification method used in this study (results not presented here because method did not discriminate *D. weischeri* and *D. dipsaci*), and for a very professional and cooperative collaboration to move the market access issue forward.

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10. Targets Achieved Compared to Those Contemplated

Details are provided on page four of this report comparing the original objectives of the project with those realized and justification for alteration of the objectives.

11. Communications

Briar S, O Molina, and M Tenuta. Very Low Occurrence of the Stem Nematode, *Ditylenchus dipsaci*, in Field Pea Grain Samples in Western Canada. Society of Nematologists Meeting, July, 2011, Corvallis, Oregon. (Poster)

Madani M, M Tenuta, S Briar, and SA Subbotin. Determining the Stem and Bulb Nematode Present on Canada Thistle in the Canadian Prairie Provinces and Implication to Phytosanitary Measures of Export Pea Grain. Society of Nematologists Meeting, July, 2012, Savannah, Georgia. (Poster)

Madani M, M Tenuta, S Briar, and SA Subbotin. Determining the Stem and Bulb Nematode Present on Canada Thistle in the Canadian Prairie Provinces and Implication to Phytosanitary Measures of Export Pea Grain. Canadian Phytopathology Meeting, August, 2012, Niagara, Ontario. (Poster)

Tenuta M, S Briar, M Madani, and SA Subbotin. Determining the Presence of the Stem and Bulb Nematode (*Ditylenchus*) on Grain Pea and Canada Thistle in the Canadian Prairie Provinces. Canadian Pulse Research Workshop, November, 2012, Niagara, Ontario. (Oral)

Madani M, M. Tenuta, and SA Subbotin. Analysis of Internal Transcribed Spacer (ITS) of *Ditylenchus weischeri* parasitizing *Cersium arvense*. Canadian Phytopathology Meeting, June, 2013, Edmonton, Alberta. (Oral)

Hajihassani A, M Tenuta, and RH Gulden. A preliminary greenhouse study for the host status of spring wheat and canola to parasitism by the stem nematodes, *Ditylenchus weischeri* and *D. dipsaci*. Canadian Phytopathology Meeting, June, 2013, Edmonton, Alberta. (Poster)

Stem and Bulb Nematode Project Yields Surprising Results. Alberta Pulse Crop News – Summer Edition. pg. 30-31.

Tenuta M, M Madani, S Briar, O Molina, R Gulden, and S Subbotin.Occurrence of the Stem Nematode, *Ditylenchus weischeri* in Field Pea Harvest Samples and Creeping Thistle Plants in Western Canada. (Manuscript in preparation)

Appendix-I

NEMATODE EXTRACTION AND ENUMERATION METHODS

I.1 Extraction and enumeration protocol for stem and bulb nematode from weed plants (Adapted from CFIA protocols: CL-DIA-NEM-001.03 external)

Materials

- Four litre plastic buckets
- Sieves in sizes 710µm, 250µm and 25 µm.
- 500 mL wash bottle.
- Inverted microscope.
- Stereo microscope
- Petri dishes with grided bottom
- Balance
- Centrifuge tubes (50mL)
- Aeration pump system
- Fridge or incubator (4 ˚C)

Sample preparation, weighing, soaking and aeration

Weigh a sample size of 20 g of the mix of stem and leaves and about 2-3 g separately for flower-heads. Label the appropriate sample number or sample ID on the 4 L buckets. Cut the plant tissues into small pieces preferably 2-3 cm length. Place the cut samples in the each corresponding bucket. Fill each bucket to approximately an inch from the top with RO water (300 mL). Place one hose into each bucket from the aeration manifold and open each valve to produce a slow stream of air bubbles. Aerate the samples for 24 hours at 10˚C to prevent any bacterial growth.

Sieving aerated samples

Assemble three sieves in order of size (Largest to smallest). Wet the sieves before use. Remove the soaking samples from the aeration and pour each sample through a clean sieve assembly. Rinse the sample three times using approximately 2 L of tap water each time. During every rinse agitate the sample with hand or using a clean plastic stick.

Wash the debris retained on the 25 µm sieve into a 50 mL centrifuge tubes using a squirt bottle containing tap water. Keep the sample volume to a maximum of 40mL. Make sure to clean sieves between samples using the hose attached to the tap.

Microscopic evaluation

Let the sample settle for about two hours and remove the supernatant. Samples tubes can also be stored in the fridge at 5˚C until microscopic evaluation. Decant the samples and leave about 5 to 10 mL in the tube, mix the sample gently by swirling, and pour into a dish with grid scribed on the bottom. Wait for about 2-3 minutes to let the sample settle down. Scan the dish completely under the stereoscope and record number of stylet bearing stem and bulb nematodes and the other free-living nematodes (without stylet). Confirm nematode identification preparing a slide at high power under the compound microscope or in a drop of water under the inverted microscope. Nematode numbers are expressed on dry or wet weight basis.

Appendix-II

NEMATODE PRESERVATION METHODS

2.1 Preservation in lysis buffer

Procedure

- Transfer a single suspect nematode to a slide in 3µl of sample water.
- Add 5µl of nematode lysis buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA, 10% Triton X-100).
- Using a dissecting microscope to view the specimen, cut, or crush the nematode with a scalpel.
- Transfer the macerated nematode to a 0.5 ml sterile microfuge tube containing 20µl of nematode lysis buffer.
- Place the sample on ice or freeze at -20 °C for later analysis.

Preparation of Lyis buffer

- Add 5 ml Tris HCL to 20 ml of dH2O in a beaker (100 ml volume) and stir.
- Add 1 ml EDTA and stir.
- Add 5 ml triton and stir.
- Make the final volume to 50 ml by adding dH2O.

2.2 Preservation in NaCl

Procedure

- Pick up nematodes from the clean suspension and transfer them to small eppendorf tubes containing 100 µl of ster. H_2O. pick upto 50 specimens one by one with the clean pick. If the nematodes are not in clean suspension transfer the nematodes to the ster. H_2O for clearing any debris attached to the nematodes and then transfer them to the eppendorf tubes.
- Add double strength 0.2 m NaCl to get 0.1 m NaCl strength solution.

Preparation of NaCl:

To prepare 0.1M NaCl solution, weigh 5.84g of NaCl and dissolve it in 1 litre of water; or 0.5844g of NaCl in 100mL of water.

Appendix-III

DNA ISOLATION FROM NEMATODES

Material:

- Proteinase K, (P.K) 60mg/100 ml
- Extraction buffer, commonly used Worm Lysis Buffer (WLB):

1 ml	KCl	500mM
1ml	Tris pH8.3	100mM
250µl	MgCl2	100mM
1ml	NP40	4.5%
1ml	Tween 20	4.5%

- Laboratory mixer, handy device homogenizer, (Sigma Aldrich).
- Glass pipette and table centrifuged up to 10,000 rpm.
- Stereoscope, glass slid, fishing niddle,dH2o water
- Eppendorf tube, PCR sized 0.25 ml.
- Flame powerd by gas

Procedure

- Make blunted glass piped by hold glass pipet tip on flame to shape tip blunted, for each sample separate pipet tip needed. Attach the prepared pipette to the homogenizer device.
- Under stereoscope transfer individual or several nematode to a drop of distilled water on middle of glass slide
- Cleaning nematode using fishing needle by removing any debris attached to nematodes if there is. Passage nematode to a new drop of water.
- Add 12 µl of WLB to PCR tube, fishing nematode from drop of water and transfer them to tube.
- Crush body nematode using homogenizer equipped with blunted glass tip, for 90 sec.
- Wash glass tip with 8 µl water to the tube and centrifuge briefly. Store tube at -20°C for at least 1 hr.
- After thawing, add 2µl P.K to each tube and incubate them for 60 min at 60°C follow by 10 min at 94°C. Centrifuge at 10,000 rpm for 45sec.
- Keep tubes at -20°C until used.

Appendix-IV

PCR AMPLIFICATION OF WHOLE ITS, hsp 90, 28s, GENES AND SPECIES SPECIFIC

5.1-Preparation of PCR reaction

Materials:

PCR premixed master solution (Life science, US), 12.5 µl
Primers, each 25 mM
DNA 1 to 3 µl
DH_2O, add to the final volume of 25 µl

Procedure, (adjusted for 9 PCR reaction)

- Let thaw the master mix while keeping on ice.
- Label PCR tubes with date and sample name.
- Making PCR master mix by adding 120 µl of dH2o to the middle size eppendorf tube contained 125 µl of PCR premixed master solution; to this add 3 µl of each primer.
- Vortex 5 second and aliquot the prepared solution to PCR tubes each 27 µl.
- Add 1-3 µl of DNA sample, and place in PCR machine.

5.2- Run the PCR

Thermal profile, set thermal profile as below:

- Step 1: Initial DNA denaturation for 4 min at 94°C;
- Step 2: (30 sec at 94°C, 60 sec at 57°C and 60 sec at 72°C) for 35 cycle
  Annealing temperature of 53, 55 and 57 was used for hsp 90, whole ITS and 28s, respectively.
  For species specific PCR annealing temperature of 55 and 57 was used for hsp 90 and whole ITS, respectively.
- Step 3: 10 min at 72°C.

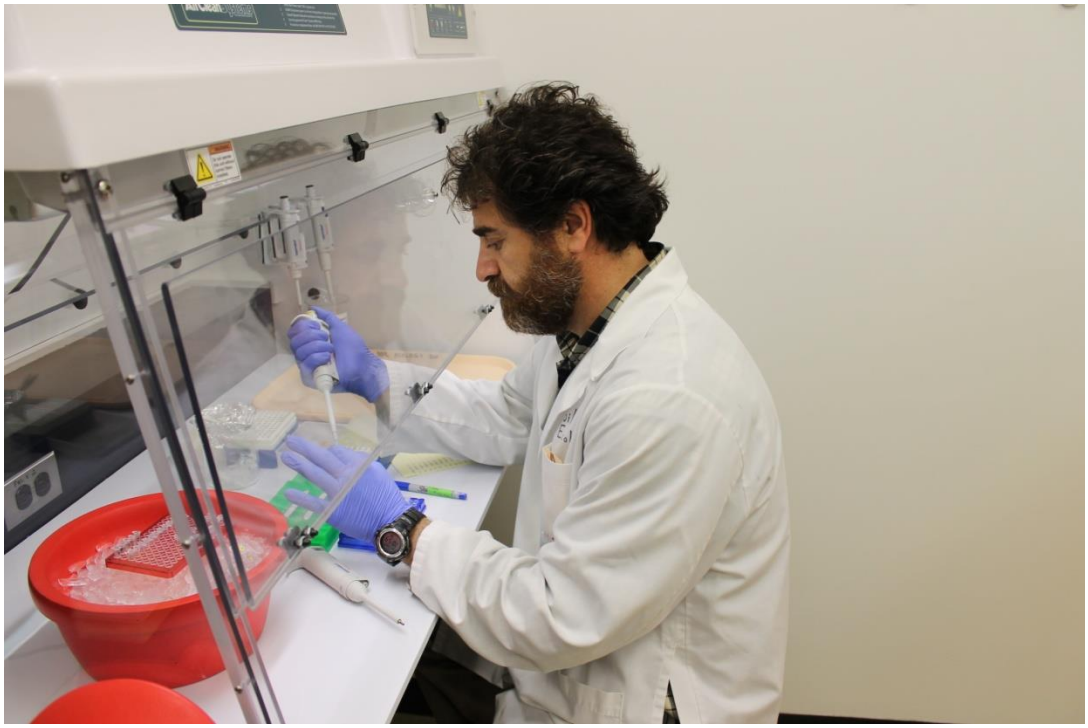
5.3- Gel electrophoresis

Materials:

- Agarose (ultra pure, Invitrogen, CA, USA)
- TAE buffer 0.5%, prepared from 10X stock solution
- power supply apparatus, comb and gel cast

Procedure:

- Prepare 1% agarose gel, by adding 0.3 gr of agarose, to a100 ml beaker contained 30 ml TAE buffer.
- Heat in microwave for 30 sec, continue heating on hotplate by stirring until gel dissolved cool down to 60°C, add 3 µl GelRed, and cast the gel.
- Mix 2 µl of PCR product with 1 µl of loading dye (Blue/Orange 6x, Promega, WI, USA), and run the prepared sample under 80 volt for at least 45 minutes.
- Visualize gel picture under UV transluminator at 260, in G-box automated imaging (SynGen, Cambridge, UK).



Research Associate Dr. Mehrdad Madani conducting species specific identification by PCR of nematode samples from creeping thistle.

Appendix-V

CULTURING METHODS FOR STEM AND BULB NEMATODE

3.1. Carrot disc method

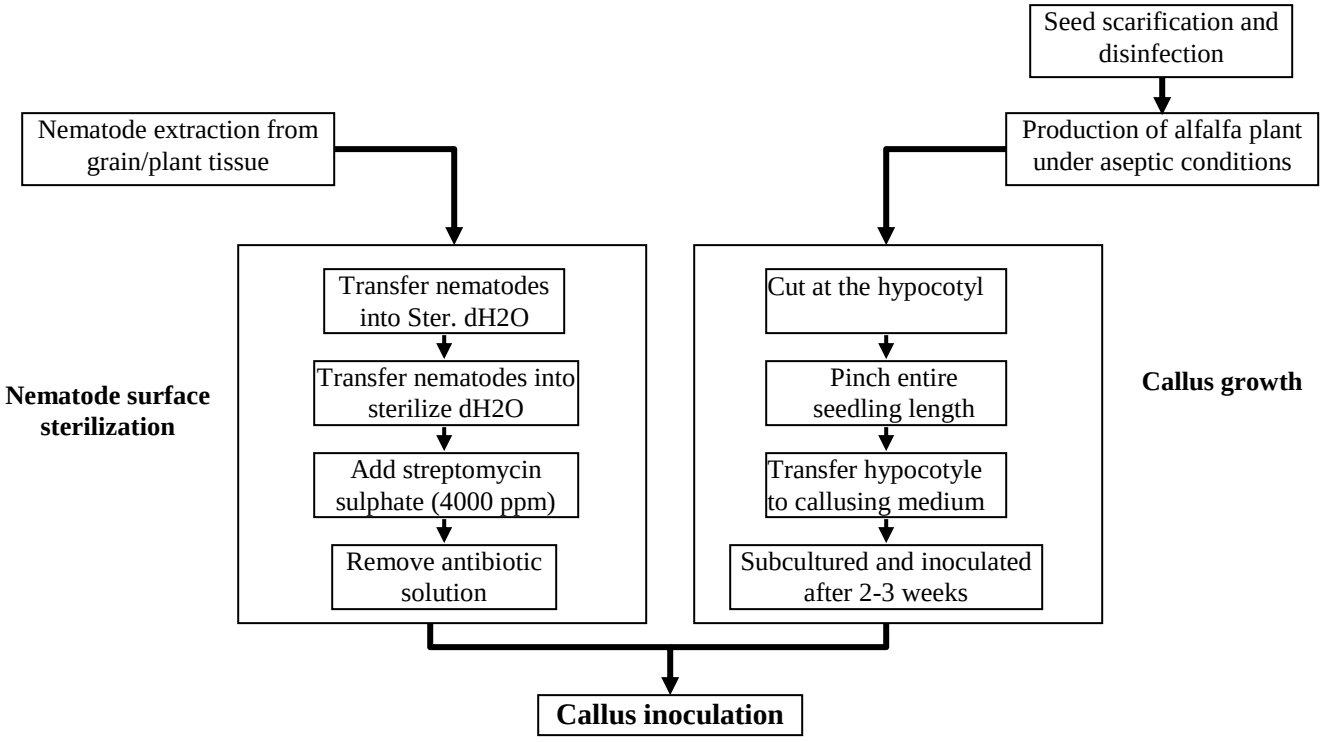
Materials

- Carrots
- Lukewarm soapy water
- Nylon brush
- 10% household bleach solution to volume of sterile dH_2O
- Clean tub
- Vegetable peeler
- Agar
- Sterilized paper towels
- Trays
- Laminar airflow chamber
- 95% alcohol
- Knife
- Flame
- Disposable petri plate
- Forceps
- Streptomycin sulphate (to prepare a 10% agar media with 50 ppm).

Procedure

- Place carrots in lukewarm soapy water and scrub thoroughly with nylon brush.
- Rinse carrots with cold sterile dH_2O.
- Leave carrots in bleach solution for 30 minutes. Rinse with autoclaved dH_2O.
- Place carrots on paper towels on trays and cover with paper towels. Transfer carrots on tray to a laminar airflow chamber. Remaining operations are to be done in chamber.
- In the airflow chamber, sterilize a knife by dipping it in 95% alcohol and passing it over a flame. Cut off the crown end of the carrot. Cutting operations may be done over layer of paper towels on the chamber counter. Hold carrot at the tapered end and using an alcohol-dipper and flamed vegetable peeler, peel the carrot downwards in slightly overlapping longitudinal strips. Sterilize peeler after every two strips.
- Using an alcohol-dipped and flamed sharp knife, cut the pealed carrot portion into 0.4-0.6 cm long, and not less than 2 cm wide cylindrical pieces (disc). Sterilize the knife after every cut. Cutting may be done over a sterile disposable Petri plate so that cut pieces fall into the plate.
- Transfer cut carrot cylinders to Petri dishes plates using a set of sterilized forceps. Sterilize the forceps after every cylinder. Put at least 2-3 cylinders per dish
- Seal with SARAM, label the plate and store in dark at 20 C.
- After 1-2 weeks or as soon as white specks of callus are visible on the carrots, inoculate with nematodes.

3.2. Callus method



Seed scarification, sterilization and germination

- Place non-scarified seed, enough to cover the base of a 100 ml sterile glass beaker.
- Add concentrated sulphuric acid to cover seeds. (Make sure the seeds are completely dry).
- Stir with glass rod and leave to stand for 5-10 min depending on seed size and hardness.
- Pour off excess H_2SO_4.
- Rinse seed with excess sterile d-H_2O (to counteract heating effect of dilution of the acid).
- Repeat rinse with sterile d-H_2O four times.
- Fill beaker with 1000 ppm. $HgCl_2$ in 30 % ethanol.
- Allow to stand in fume hood for 15 min.
- Pour off $HgCl_2$/ethanol.
- Rinse with excess sterile d-H_2O.
- Repeat rinse with sterile d-H_2O four times.
- Allow seed to stand in sterile d-H_2O in laminar flow hood for 2-3 h to imbibe.
- Pour off water and rinse with sterile d-H_2O to remove leached tannins.
- Transfer seed to Petri dishes of nutrient agar and germinate for 2 days at 15-20°C.
- Discard any contaminated seedlings or plates and transfer healthy seedlings to 30-ml tubes of Gamborg's media without hormones, and grow on at 24°C and 16 h photoperiod.

Alternatively, we successfully use alcohol (95%) and home-bleach (15%) to sterilize seeds. Seeds were placed into a 100 mL beaker. 50 mL of alcohol 95% was added for 15 minutes. Seeds were rinsed with sterile d-H_2O three times. Then, 50 mL of bleach 15% was added to cover seeds for 15

minutes. Seeds were rinsed three times with sterile d-H_2O. Excess of water was removed with a sterile paper towel. Finally, seeds were spread onto Gamborg's media without hormones in a Petri dish.

Callus production

- Select seedlings of alfalfa (Lucerne) or Creeping thistle with 3 or 4 trifoliate leaves, cut at the hypocotyl and transfer them to callusing medium (B51, that is. Gamborgs B5 medium, pH 5.8, supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (2 mg I-1), kinetin (0.5 mg L^{-1}), sucrose (2 w/v), agar (8 g L^{-1})). Perform all steps in laminar flow.
- Pinch entire seedling length including laminae lightly between serrated forceps points to create multiple wounds. Insert cut end of hypocotyl into the agar with the rest of the seedling laid on the agar surface, 3 to 4 seedlings per dish and store in the dark for about 7 days.
- Primary callus will begin to form from each wound point and can be subcultured and inoculated with after 2-3 weeks to new plates.

Nematode surface sterilization

Batches of 1-10 nematodes are quickly transferred, one at a time using an entomological micro-pin or mounted eyelash, to a few drops of sterile distilled water. Once sufficient nematodes (30-50) have been collected in an Eppendorf microtube (1.5mL volumen) they can be surface sterilized as described below.

- Leave nematodes settle in 0.5 mL of sterile dH_2O.
- Add an amount of streptomycin sulphate (4000 ppm) to an equal amount of nematode suspension. This reduces the streptomycin concentration to 2000 ppm.
- Shake gently and store overnight at 4°C.
- Remove the supernatant above the settled nematodes with a pipette. Fill half of the tube with sterile water and shake gently. Allow the nematodes to settle and remove the supernatant. Repeat this washing 3 times, leaving 40-45 minutes between washings.

Note: Make sure you work in a laminar flow chamber and use aseptic techniques through-out. Avoid using big tubes (15 mL) you will get more chances of contamination. You must have to use new tips when removing or adding sterile dH_2O.

Alternate method of nematode sterilization: Alternatively, Hibitine 1% (Chlorohexidine gluconate 20% v/v) for up to 3 h at ambient temperature or overnight at at 2-5°C can also be used. Nematode washing is performed as described above.

Nematode inoculation

After washing, nematodes are left in a small volume of sterile d-H2O. The nematodes are then quickly transferred to the callus in a drop (5-10 µL) of sterile dH_2O. The drop of water is adding around the callus not on top. This procedure must be carried out in a laminar flow chamber. Close the Petri dish with SARAM and incubate in dark at 20C.

Always inoculate young actively growing callus by selecting those which have increased in volume. Establishing an initial culture can prove difficult, in which case sterilized seedlings can be inoculated

between the cotyledons or in a leaf axil 1-2 days after transfer to the callusing medium. The nematodes then invade and develop at the same time as the primary callus is initiated.

Established cultures are maintained in the dark, preferably in an incubator at 20°C. Depending on the size of the initial inoculum and the nematode multiplication rate, callus will require subculture after 40-90 days.

Nematode extraction from callus

Nematodes are harvested by breaking up callus and agar with sterile blades and placing the entire culture with any nematodes washed from the dish on a Baermann funnel or similar device to select active worms. Nematodes should be collected regularly to sterile Petri dishes. Residual 2,4-D can be removed from the nematode suspension in three or four of the rinsing cycles described above, and the clean nematodes stored at 2-4 °C until use.

Preparation 1 L of Gamborg's media.

- Add 1000ml of dH2O to an Erlenmeyer (2 L volume). Make sure the Erlenmeyer is properly clean.
- Add 23.08 g of Gamborg's B-5 Caison Labs. (Cat. No. GBP07-10LT).
- Add 0.8 mL of 2,4D stock solution.
- Add 0.5 mL of Kinetin stock solution.
- Add 8 gr of agar powder micropropagation Type I.
- Autoclave for 15 min.

3.3 CULTURING ON LIVE-PLANTS IN GREENHOUSE

Preparation of pots

Pot soil can be used to grow plants in the growth chamber. There is no need of adding more nutriments, at least not for the first 30 to 60 first days. Alternatively, 2:1:1 mixture (Soil: Sand: peat moss) can be used with nutrient supplements for active growth of plants. One or two kg pots have been used to grow plants. However, the plants growing in 1 kg pot need to be moved to bigger pots later on. If needed soil can be sterilized by putting it into metallic trays and adding dH_2O until the soil is wet, but not too wet. However, if no plant parasitic nematodes are present in the soil soil sterilization is not required to grow Creeping thistle plants.

Propagation of Creeping thistle plants

Creeping thistle plants grow well from the underground roots. Collect roots from healthy growing plants preferably form newly established plants. Clean the roots under running tap water and may be left in 2% bleach for 15 minutes and further washing with clean water to romove belach residuals form the roots. Cut roots into small pieces of 2-3 cm length. Roots should be free of nematodes. Check randomly selected root for nematode infestation using the method described in Appendix-I. If roots are free of nematodes, place the 3-4 root pieces in the center of pot and bury them in the soil. Regularly water the pots as needed and inoculate the germinating plants as described below.

Inoculation

Nematodes don't need to be surface sterilized after being extracted from the plant tissues. However, nematodes should be extracted under aseptic conditions and placed in sterilized dH_2O. Nematodes should be used for inoculation in short time. If needed, nematodes can be kept at 4° C for 1-2 days. Only plants with 2-4 leaves and healthy appearance should be selected for inoculation. Nematodes in sterile-dH_2O are placed on the top of the plant with a pipette. After inoculation cover the plants with a clear plastic bag for 48 hours in order to keep good moisture around the inoculated plant.

Harvest and extraction from the plant tissues

Nematodes are harvested from plants once the flower heads appears to be dry. Leaves, stems and flowers are cut in small pieces (2-3 cm).

Appendix – VI

REARING THE STEM AND BULB NEMATODES ON CREEPING THISTLE AND GARLIC FOR HOST PREFERENCE SCREENING TESTS

Materials

4-1.

- 10 polyethylen pots
- Sieves in size 37µm (400 mesh).
- 500 mL squeeze bottle.
- Polyethylene plates (22 cm diam.)
- Large mesh sieves (20 cm diam.)
- Centrifuge tubes (15mL)
- Tissue paper
- Sterilized plastic petri dishes (9 cm diam.)
- Special pipette tip to pull nematode
- Microscope
- Stereo microscope
- Microliter pipettes (200µl and 1mL)
- Counting slide for nematode
- Small beakers
- Centrifuge
- Humidifier
- Growth chamber
- Fridge (4 ˚C)

Rearing *Ditylenchus weischeri* population on Creeping thistle plant

To prepare sufficient population of *D. weischeri* for conducting host screening trails, the nematode can be reared on Creeping thistle live-plants in a growth chamber according to method described in Appendix-III (section 3.3).

4-2. Rearing *Ditylenchus dipsaci* population on garlic plant

Growing Plants: Fresh garlic bulbs prepared from groceries can be used for growing plants. 5-7 kg Polyethylene pots have been used to grow plants. Fill pot with a mixture of nursary soil and potting mix (1:2) after sterilizing in an autoclave in plastic bags for 1 h at 121 ºC. Separate garlic bulb into cloves right before planting. Plant each clove 3-4 cm deep into soil with the pointy tip facing up and the basal/root end facing down. Four to five cloves can be planted in each pot. Keep seedlings in a growth chamber at 22-24°C and 70% relative humidity, under light supplied by white fluorescent lamps with a photoperiod of 16 h of light/8 h of darkness. Water plants every three days for optimal plant growth seedlings. Fertilize seedlings at least two times during the plant developmental stage with 400-500 ml water-soluble NPK (20:20:20) fertilizer, first application being one week post emergence to compensate for nutrient loss due to using sterilized soil.

Nematode inoculation:

Nematodes can be either inoculated into pot soil at the time of planting or one week after planting time of garlic cloves. Inoculate 2000-3000 nematode juveniles in 1 ml distilled water suspension using a microliter pipette by delivering around each clove at the time of planting or make three holes in the pot around each recently emerged seedling one week after planting time. Afterwards, cover the holes with additional soil. Before inoculating, make sure that the soil is moist enough but not too wet. There is no need to sterilize nematode juveniles before injection into soil. After inoculation, do not water pots for at least three days to allow establishment of juveniles in soil. Take a look at plants at least every other day to prevent unexpected problems.

Harvesting plants and nematode extraction:
Two months after inoculation plants can be removed from pot soil. Nematode juveniles can be extracted from stem, leaves and bulbs of infected garlic plants. A modification of the protocol described by Whitehead and Hemming (1965) can be used in extracting nematode juveniles.
In our modified technique, a wire mesh (one rings of PVC pipe with a window screen material glued on one side) is placed on a plate or dish. Add water to the dish until it slightly covers the wire mesh. One layer of tissue paper is superimposed in the bottom of wire sieve. A single plant sample can be chopped into small pieces (1 cm long) with scissors. Put and distribute the chopped foliar plant pieces evenly on wire mesh and add some tap water using squeeze bottle to slightly immerse the chopped plants pieces. Incubate plates at room temperature for three days and do not turn off the room light for optimal extraction.
Before collecting nematodes, choose two 100 and 400 mesh sieves separately for either *Ditylenchus weischeri* or *D. dipsaci*. Wash the work area thoroughly with tap water to avoid any contamination. Stack the sieves from top to bottom in the following order: 100 and 400 meshes. Slowly and gently remove wire mesh from the plate and pour water suspension containing nematode through the sieves. Wash three times plate into the sieves and collect the nematode into a clean beaker or centrifuge tube with minimum amount of water. Observe the nematodes using a stereo microscope to check for viability before incubating in a freeze and also later use. Discard the chopped plant pieces used for the extraction after autoclaving.

HOST SCREENING TRIALS IN GREENHOUSE

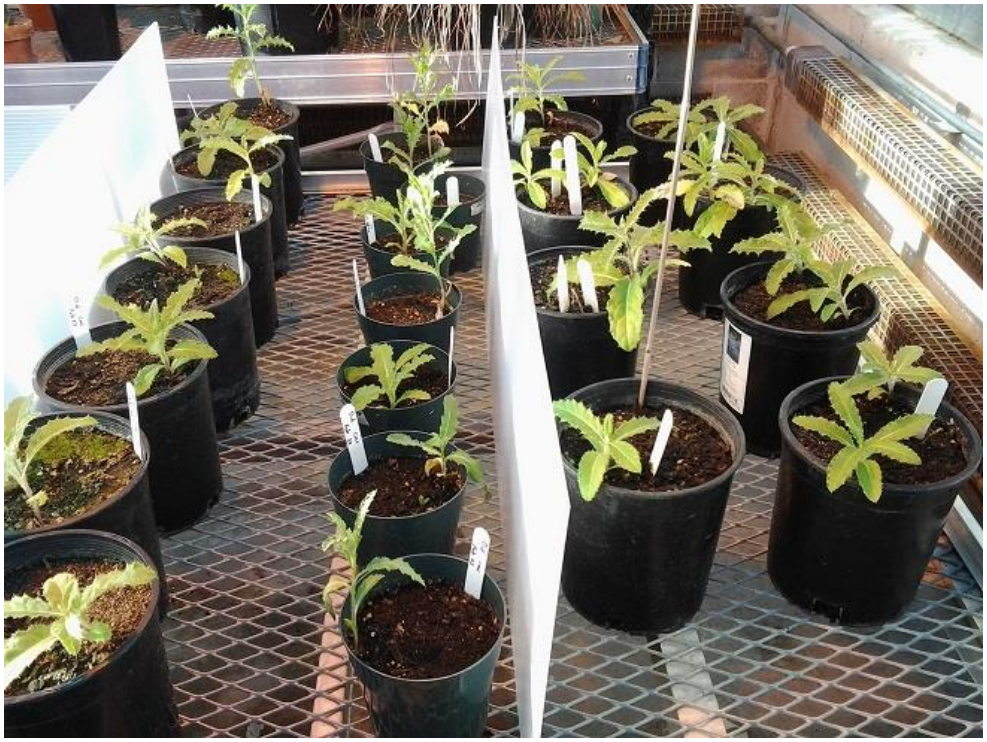
Growing plants: Fill Polyethylene pots (12 cm diam.) with clay-peat mix (1:2). If no plant-parasitic nematodes are present in the soil, soil pasteurization is not required to grow crop seedlings. Otherwise, soil should be pasteurized. Seeds of test crops can be either germinated for 2-3 days in a Petri dish (90 mm diameter), lined with two layers of filter paper incubated in room temperature to produce roots approximately 1-2 cm long, and transferred singly to each polyethylene pot or placed directly into the pot soil and germinated. In later, plant three seeds in each pot but thin to one plant seedling per pot. Surface sterilize all seeds with 0.5% sodium hypochlorite solution (NaOCl) for 3 min followed by washing three times with sterile water prior to germinating in Petri dish and or planting directly in pot soil. Maintaining optimal plant growth condition in greenhouse is critical for host screening tests against plant-parasitic nematodes.
Water plants every other day to keep the pot soil only moist. Avoid over-watering plants in pots. Over-watering as indicated by standing water in the bottom of the pot can promote fungal growth and diminish root health. Protect plants against insects such as aphid and trips by spraying insecticide before starting trials.

Nematode infestation on test plants:
Nematode inoculum collected in water suspension in centrifuge tube can be used as a source of nematode inoculom. To adjust a known number of nematode individuals, deposit the nematode in 15 mL tube by centrifuging at 1550 g for 3 min. Then, reach the water volume to 10 mL in the tube by pulling the additional water using a microlitter pipette. Use three aliquots of nematode suspension to count the number of juveniles in a counting slide, calculate the average and determine the desired volume for inoculation. Deposit the nematodes at the bottom of the tube again and pull all water and

leave only 0.5 mL water above the deposited nematode. Instead of water, add 1.5% Carboxymethyl cellulose to tube to reach the volume to 10 mL. Pull the desired volume and pour in small glass tubes for application to test plants. Adequate volume of initial inoculom for applying to plant seedlings is between 10-15 µl. Agitate the nematode suspension repeatedly using a microlitter pipette during this last step to prepare equal condition.
At two-leaf stage of seedlings, test plants are ready for inoculation with nematode. Apply 50 nematode individuals to the leaf axils of leaves twice (first at two-leaf stage and then 5 days later). It is advisable to use at least 7-10 plants per crop species/cultivar for nematode inoculation to determine the average of reproduction factor of nematode. Variation in nematode multiplication between plant species can be reduced by using uniform plant size, accurate amount of initial inoculum and also, accurate application of nematode inoculum to test plant.
After inoculation and to increase humidity and prevent inoculum droplets from drying, incubate the inoculated plants in a small humidity chamber comprised of a translucent plastic canopy and presence of a humidifier for 72 hours. Afterwards, maintain the pots in a completely randomized design (CRD) on a bench in greenhouse.

Harvesting plants and evaluating plants:
Observe the plants during the test to record any symptom associated with infestation. After 8 weeks of inoculation of nematode, cut each single plant from the soil surface of pot soil, put in a plastic bag and keep in freeze (4°C) for later extraction of nematode. Extraction for juveniles and adults can be done using the Whitehead tray method according to method described above. Count the number of the nematode juveniles (final population) for a single plant by using three aliquots of nematode suspension in a counting slide under stereo microscope. Calculate the average of the three aliquots and determine the reproduction factor of nematode by dividing final population by initial population.

Graduate student, Abolfazl Hajihassani inoculating seedlings in the greenhouse with *Ditylenchus weischeri*.



Creeping thistle plants inoculated with *D. weischeri* and *D. dipsaci* in the greenhouse.