STUDIES OF PLANT HOST PREFERENCES OF THE STEM NEMATODES, *DITYLENCHUS WEISCHERI* AND *D. DIPSACI*

BY

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ABSTRACT

Hajihassani, Abolfazl. Ph.D., The University of Manitoba, May 2016. Studies of plant host preferences of the stem nematodes, *Ditylenchus weischeri* and *D. dipsaci*. Major Professor: Dr. Mario Tenuta.

The occurrence of *D. weischeri* Chizhov, Borisov & Subbotin, a newly described stem nematode species of creeping thistle (*Cirsium arvense* L.), and *D. dipsaci* (Kühn) Filipjev, a pest of garlic and quarantine parasitic species of many crops, has been reported in Canada. This research was conducted to determine if *D. weischeri* is a pest of agricultural crops, especially yellow pea (*Pisum sativum* L.) in the Canadian Prairies. Significant ($P < 0.05$) slight reproduction ($1 <$ ratio of final to initial population < 2) of *D. weischeri* occurred on two (Agassiz and Golden) of five varieties of yellow pea examined. Other annual pulse and non-pulse crops, including common bean, chickpea, lentil, spring wheat, canola, and garlic were non-hosts for *D. weischeri*. Conversely, a range of reproduction responses to *D. dipsaci* was observed with all pulse crops being a host of the nematode. *Ditylenchus weischeri* was not a seed-borne parasite of yellow pea, unlike, *D. dipsaci* which was recovered from seed. Conversely, *D. weischeri* and not *D. dipsaci* was recovered from creeping thistle seeds. In callused carrot disks, with no addition of medium, an increase of 54 and 244 times the addition density of 80 nematodes was obtained for *D. weischeri* and *D. dipsaci*, respectively, after 90 days. Temperature had a significant influence on the development of *D. weischeri* and *D. dipsaci* in yellow pea. Development of *D. weischeri* did not proceed past adult stage at 17 and 22°C whereas a minimum generation time of 30 days was apparent at 27°C with the associated accumulated growing degree-days of 720 degree-days (above a base

temperature of 3°C). The minimum generation time for *D. dipsaci* was 24, 18 and 22 days with 336, 342 and 528 degree-days at 17, 22 and 27°C, respectively. In field microplots, grain yield of yellow pea were not significantly affected by addition density of *D. weischeri*. At harvest, the total number of recovered nematodes per plant was not significantly different than the added at the start. The results of these studies confirm that *D. weischeri* is unlikely to be a pest of yellow pea for weather conditions of the Canadian Prairies.

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LIST OF ABBREVIATIONS

C	Celsius
cm	Centimeter
var.	Variety
d	Day(s)
diam.	Diameter
DNA	Deoxyribonucleic Acid
e.g.	*Exempli Gratia*
ed.	Edition
F	Fahrenheit
Fig.	Figure
g	Gravity
g	Gram
h	Height
ha	Hectare(s)
hsp90	Heat shock protein
hr	Hour(s)
i.e.	*Id Est*
ITS	Internal Transcribed Spacer
J2	Second-stage nematode
J3	Third-stage nematode
J4	Fourth-stage nematode
kg	Kilogram

m	Meter
min	Minute(s)
mL	Milliliter
mon	Month(s)
µm	Micrometer
PCR	Ploymerase Chain Reaction
Rf	Reproductive Factor
RFLP	Restriction Fragment Length Polymorphism
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
t	Tonnes
sec	Second(s)
wk	Week(s)
2,4-D	2,4-Dichlorophenoxyacetic acid
5.8S gene	Non-coding component of the large subunit
18S gene	Small nuclear ribosomal subunit
28S gene	Large nuclear ribosomal subunit

FOREWORD

This thesis is organized in a "Sandwich" style as specified by the Faculty of Graduate Studies, and Department of Soil Science, University of Manitoba. The first chapter encompasses the general introduction follows a comprehensive literature review, three manuscripts and a last chapter relating the results of all three manuscripts and giving of recommendations. Two manuscripts are published, and the third one is submitted for peer-review. Each manuscript consists of an abstract, introduction, materials and methods, results, and discussion with the later manuscript combined (results and discussion) depending on the preference of the journal. The format of manuscript chapters is that of the journals they are intended to be published in. A single list of references is given at the end of the thesis. The manuscripts are presented as follows:

Hajihassani, A., and Tenuta, M., and Gulden R. H. 2016. Host preference and seed-borne transmission of *Ditylenchus weischeri* and *D. dipsaci* on select pulse and non-pulse crops grown in the Canadian Prairies. Plant Disease 100: 1087-1092.

Hajihassani, A., and Tenuta, M., and Gulden R. H. Influence of temperature on development and reproduction of *Ditylenchus weischeri* and *D. dipsaci* on yellow pea. Submitted to Plant Disease.

Hajihassani, A., and Tenuta, M., and Gulden R. H. Monoxenic rearing of *Ditylenchus weischeri* and microplot examination of the host suitability of yellow pea (*Pisum sativum* L.). It will be submitted to a journal soon.

1.0 GENERAL INTRODUCTION

Various diseases and pests can attack agricultural crops and cause a significant reduction in crop yields as well as economic output. Among four main plant pathogenic microorganisms, including plant-parasitic nematodes, fungi, viruses, and bacteria, the nematodes can affect cultivated crops and cause considerable yield loss (Agrios 2005). Plant-parasitic nematodes are obligate parasites that feed on plant cells and can complete their life cycle partially or completely in the soil or in plant tissues. All plant species may be affected by parasitic nematodes resulting in yield or quality damage. The annual yield loss of agricultural crops caused by nematode pests has been estimated to be approximately 5% of food production valued USD $125-157 billion worldwide (Chitwood 2003; Abad et al. 2008). However, Jones et al. (2013) mentioned that this amount of loss is expected to be even greater since many farmers, mainly in developing countries, are uninformed of the effect of these pests. Due to this fact, the mean yield loss in certain crops can reach up to 20% annually (Koenning et al. 1999).

The majority of plant-parasitic nematodes such as *Pratylenchus, Heterodera* and *Meloidogyne* attack and infest the belowground parts of plants, while there are a few nematode genera such as *Ditylenchus*, *Aphelenchus*, and *Anguina* that feed and develop on the aboveground parts (stems, leaves, seeds). The nematode genus *Ditylenchus* Filipjev, 1936 (Aunguinidae, Tylenchida) is comprised of several species and is widely distributed throughout the world. This genus comprises a large, variable (genetically and feeding behavior), and widespread (geographically) group of migratory endoparasitic nematodes. Species of *Ditylenchus* occur in many ecological niches with a great variety of feeding habits. Most of the *Ditylenchus* species are free-living nematodes inhabiting

soil and feeding on fungi while some are obligate parasites of higher plants (Sturhan and Brzeski 1990; Plowright et al. 2002).

The most important economic species include *D. dipsaci*, stem and bulb nematode; *D. destructor*, potato tuber nematode; *D. angustus*, rice stem nematode; *D. gigas*, common bean stem nematode; and *D. africanus* and *D. arachis*, peanut pod nematodes (Sturhan and Brzeski 1991; Plowright et al. 2002; EPPO 2008; Vovlas et al. 2011; Zhang et al. 2014; CABI 2015c). Amongst these species, *D. dipsaci* is the serious parasitic nematode of many plant species, mainly in temperate climates of the world. It is a quarantine nematode in many countries with a recognized host range of approximately 500 plant species in at least 40 plant families (Sturhan and Brzeski 1991). Most of these plant species are economically valuable ornamental and agricultural crops and cannot be traded if they are infested with *D. dipsaci* (Greco 1993). Susceptible crops include alfalfa, potato, garlic, onion, oat, clover, strawberry and various pulse crops such as peas or beans. *Ditylenchus dipsaci* lives within plant tissues mostly in the aboveground parts (stems, leaves, flowers), but it also invades bulbs, tubers and rhizomes (Subbotin et al. 2005). The life-cycle of *D. dipsaci* from egg through juvenile stages to egg laying adults varies from 3 to 4 weeks under suitable environmental conditions (Yuksel 1960). The nematode reproduces sexually and mating occurs within the tissues of the host plants and is not known to occur in the soil. *Ditylenchus dipsaci* reproduces quickly to have several generations per growing season. The reproduction can take place throughout the growing season, except in cold weather (Hooper 1972; Sturhan and Brzeski 1991).

When a plant hosting *D. dipsaci* dies or if the cold winter months are imminent, the nematode arrests its development at the fourth-stage juvenile (J4). The *D. dipsaci* J4s are

able to survive in soil for several years in the absence of host plants in a state of anhydrobiosis. As soon as favorable conditions arise, the juveniles resume their life cycle and attack new host plants (Moens and Perry 2009).

The stem and bulb nematode produces a variety of symptoms depending on the host and the type of tissue involved. Typical symptoms of *D. dipsaci* damage on plants include swellings, deformation and discoloration of aboveground plant parts, shortened internodes and an abundance of axillary buds, twisting and stunting of stems. The nematode can reduce seed vigor and cause blackening on seed pods (Sturhan and Brzeski 1991; EPPO 2008; Stoddard et al. 2010). Dry seeds of host plants carrying this pest nematode are considered as an important means of dissemination from one region to another (Hooper 1971). Information on the amount of yield loss in legume crops due to this nematode species is limited. However, it is reported that *D. dipsaci* is capable of causing yield reductions to broad bean, pea and perhaps lentil during moist seasons (Greco and Di Vito 1994: Bridge and Starr 2007). A yield loss of up to 70% has been reported in Vicia beans (*Vicia faba* L.) in the UK (Biddle and Cattlin 2007). Current management practices to control the stem and bulb nematode are dependent upon sanitation in fields, using nematode free seed, seed treatment, using fumigants and nematicides, rotation of non-host crops and use of resistant varieties/cultivars (Sikora and Greco 1990; Sturhan and Brzeski 1991; Jones et al. 2013).

With globalization, world trade of agricultural crops has increased. This has resulted in crucial importance of market access for Canada, as one of the main producers and exporters of pulse crops in the world. Preventing the introduction of new nematode pests into a state or country is an agricultural challenge which attracts growing attention.

Canada, compared to other countries, is rather free from many destructive parasitic nematode species. However, export of Canadian yellow peas to other countries, especially India, has been challenged with the detection of a new species of stem nematodes in grain shipments of pea in the past decade. In Canada, peas are grown over a very large geographic area, including significant portions of the prairie provinces of Saskatchewan, Alberta and Manitoba. Field pea (*Pisum sativum* L.) is one of the main pulse crops and Canada is the leading producer and exporter of this crop in the world.

Initial identification using morphological and morphometric characters showed that the nematode species found in the grain shipments is the quarantine species, *D. dipsaci*. However, this species has about 30 morphologically similar biological races (Sturhan and Brzeski 1991) and there are very few morphological differences among these races which make identification difficult (Vanstone and Russell 2011). The introduction of molecular techniques with the progress of polymerase chain reaction (PCR) has provided a convenient and reliable method to identify and distinguish nematode species (Powers and Fleming 1998). Therefore, further investigation on collected samples using comprehensive PCR-based molecular assays confirmed that the nematode species contaminating the grain shipments is in fact *D. weischeri,* a pest of creeping thistle, and not *D. dipsaci*. This finding was in agreement with a report which had already been revealed in Russia in which Chizhov et al. (2010) described a new species, *D. weischeri*, infesting creeping thistle (*Cirsium arvense* L.) in fields, roadsides, and ditches near Moscow. They suggested that *D. weischeri* is a non-agricultural pest; however, only onion and garden strawberry have been tested.

More recently, Madani et al. (2015) developed a conventional PCR and real-time PCR assay with species specific primers designed according to the nucleotide sequence of the heat shock protein (*hsp90*) gene for reliable and precise detection of both *D. weischeri* and *D. dipsaci*. Their procedure was also capable of detection of *D. weischeri* and *D. dipsaci* from samples containing different species of *Ditylenchus*. It is now confirmed that the contamination of the export grain shipments of pea with *D. weischeri* was actually related to the presence of creeping thistle seeds infested with this nematode species (Tenuta et al. 2014). Therefore, the issue with the stem nematode in export pea grain shipments was resolved by the identification of *D. weischeri*. However, no information is available on the nematode host preference, biology and damage potential.

1.1 Research objectives

In this thesis research the following objectives were examined:

i. Assess the host preference of pulse and non-pulse crops to *D. weischeri*, isolated from creeping thistle, and *D. dipsaci*, isolated from garlic in greenhouse.
ii. Determine if *D. weischeri* and *D. dipsaci* are seed-borne transmissible in yellow pea and creeping thistle under greenhouse conditions.
iii. Establish an efficient method to rear *D. weischeri* or *D. dipsaci* monoxenically using fungal cultures, callused carrot disks, and callus tissues of alfalfa and creeping thistle.
iv. Evaluate the influence of different initial densities of *D. weischeri* on the growth and yield of yellow pea in field microplots.
v. Examine the morphometric variability in *D. weischeri* and *D. dipsaci* specimens reared either on creeping thistle and garlic, respectively, or *in vitro* on callused carrot disks.

vi. Examine the influence of temperature on development, reproduction and generation time of *D. weischeri* and *D. dipsaci* on yellow pea.

2.0 LITERATURE REVIEW

2.1 Creeping thistle: *D. weischeri* host plant

2.1.1 Background

Creeping thistle (*Cirsium arvense* L.), also recognized as California thistle and Canada thistle, is a cool season, broadleaved weed. This weed species is the only known perennial dioecious (*i.e.* male and female flowers on different plants) thistle. This thistle is especially destructive because it is highly aggressive in its infestation, is very difficult to control and reduces forage yields since cattle avoid grazing near it and also crop yields as it removes potential moisture and nutrients (Beck 2013). It also has the potential to outcompete and displace wild grasses and plants from their natural habitats. It is so successful at colonization because of its extensive root and shoot growth (Donald 1990). As well, the decaying matter left from a dead thistle releases toxic chemicals into the soil, which can inhibit or reduce the germination of other plants (Scott and Robbins 1999). Creeping thistle is not only destructive, it is also widespread in north America including USA and Canada (Duncan and Jechetta 2005). The economic impacts of the creeping thistle are also extensive. For instance, alfalfa yield can be reduced by up to 48% due to competition from other plants such as thistles (Moyer et al. 1991).

2.1.2. Morphology of creeping thistle

Creeping thistle is a member of the Aster family – Compositae, and can be distinguished from other thistles by its green, spineless stems, spineless heads and creeping roots (Scott and Robbins 1999). There are four different varieties (morphotypes) of creeping thistle differing by the characteristics of their leaves. The four varieties are Vestitum, Integrifolium, Arvense, and Horridum. As well, creeping thistle plants can have varying morphology based on environmental conditions. For instance, in windy and dry environments, the leaves of the thistle produce higher amounts of lipids to reduce water loss (Hodgson 1973). Creeping thistle plants range from 0.3 to 1.5 m in height and are characterized by multiple branches, shiny, deep green leaves with spines and the leaves are arranged in an alternate fashion on the stem and leaves (Saskatchewan Ministry of Agriculture 2008). Its seedling growth form is a rosette. One thistle plant can colonize an area of up to 1.8 m in diameter in two years in a wide variety of soil types including those with high salt content. As well, the root structure of creeping thistles is extensive with roots being able to extend to as much as 4.5 m horizontally and 4.5 m vertically (Beck 2013). Creeping thistle has small, purple-white flowers and there is a difference between male and female flower heads such that male flowers are smaller and globular while females are larger and flask-shaped. The seeds produced by the female plant are tan in color, very small and have white hairs called pappus. Due to their size, the seeds are easily spread by wind as well as humans, animals, water and other vehicles (Amor and Harris 1975; Ontario Ministry of Agriculture, Food and Rural Affairs 2003; Beck 2013).

2.1.3 Distribution and ecology of creeping thistle

Creeping thistle is native to southeastern Europe and eastern Mediterranean regions, but can now be commonly found throughout much of Canada and the USA since its introduction to North America in 1777 by European settlers (Hodgson 1958). Anywhere in Canada or the USA that is between 37˚ and 59˚ N are suitable and creeping thistle has colonized these areas. It is found in every province and territory in Canada except Prince Edward Island (Rice 2008). The only US states without creeping thistle are Texas, Mississippi, Oklahoma, Georgia, South Carolina, Louisiana, Florida, and Alabama.

The reason why no thistles have been observed in these states is because all of these locations are south of the thistle's required latitude. As well, creeping thistle can survive at latitudes greater than 37˚ S (Amor and Harris 1974). Consequently, creeping thistle can be found throughout Europe, western Asia, northern Africa, Japan, northern India, China, New Zealand and southeastern Australia. The ideal habitat for creeping thistle is where temperature and rainfall amounts are moderate. It is commonly found to have infested roadsides, pasturelands, crops and disturbed ground such as ditches, overgrazed pastures, unused land and tilled fields (Beck 2013). This weed occurred frequently with green foxtail, wild oats, stinkweed, chickweed and wild buckwheat in the Canadian Prairies (Thomas et al. 1996).

Creeping thistle can reproduce either sexually or asexually with the latter coming in the form of vegetative propagation. For a successful reproduction, both male and female plants should grow closer to each other. Vegetative propagation can explain why creeping thistles are found in clusters of solely male or female plants that are genetically identical. The root system of thistles is highly designed toward reproduction and the

energy stores in the root promote the propagation of new plant buds (Moore 1975; Tiley 2010). The horizontal roots are not only responsible for the spread of the plant through vegetative propagation but also with the assistance of humans through cultivation, such that pieces of root as small as 3 mm can regenerate new plants.

Successful pollination is required for production of seed. Seed germinability of creeping thistle is very variable and a plant may produce approximately 700-1500 seeds per flowering stem per growing season (Moore 1975; Tiley 2010). Although germination rates of seeds have been found to be fairly high it has been shown that vegetative propagation has an even higher growth potential (Saskatchewan Ministry of Agriculture, 2008). Seeds can be transported by runoff water, in contaminated crop seed lots, or through attaching to animals (*e.g.* insects) or farm vehicles (Tiley 2010). The creeping thistle follows a life cycle that centers on its propagation. Though the majority of reproduction takes place from vegetative buds on the root system, creeping thistle can reproduce from seed (Donald 1990). As soil temperatures increase in spring, buds on the roots are stimulated to grow new shoots which emerge in late May or June. Seedlings slowly grow into the rosette shape with extensive sunshine promoting their growth. After around four weeks, lateral root growth begins as well as stalk formation and after these roots develop, vegetative propagation can begin (Moore 1975; Tiley 2010). Energy expenditure is focused mostly upon reproduction and this continues the seed production. As winter approaches, energy is moved to the roots where it is stored and the above ground parts of the plant die back until the next spring. In total, aboveground growth of a thistle occurs from late May until November (approximately 6 months) (Scott and Robbins 1999).

2.2.4 Control methods for creeping thistle

In terms of controlling creeping thistle, there are a number of ways in which this can be achieved although all require extensive effort (Hodgson 1958; Tiley 2010). Physically, creeping thistle cannot be removed by pulling out the plants due to the vast root systems but they can be controlled by aggressive mowing for years to deplete their root systems or shallow cultivation (1 cm) monthly (Scott and Robbins 1999). Creeping thistle can be controlled effectively via competition because the seedlings are very sensitive if grown in shade; therefore, by growing a competitor along with the thistles, this could deplete their colonies since this reduces the production of thistle photosynthates and root carbohydrates which are important for regrowth via perennation. Crops such as alfalfa, barley, rye and sudangrass have been shown to be effective competitors (Donald 1990; Bicksler and Masiunas 2009). Creeping thistle can also be controlled with chemical treatment with herbicides such as picloram, 2-4 D, dicamba and glyphosate for large infestations while seedlings can effectively be contained with 2-4 D Amine 500. 2-4 D is the most commonly used herbicide for most applications (Saskatchewan Ministry of Agriculture 2008; Beck 2013).

Biologically, thistles can be disrupted either through predation or disease that will attack the weed and minimize its impact (Larson et al. 2005). *Ceutorhyncus litura* is a weevil that is used to control creeping thistle. The female weevil lays her eggs on the axils of thistle leaves and when they hatch, the larvae enter into the main leaf vein which can kill the plant (Beck 2013). Another weevil, *Larinus planus* can consume the flower heads of thistles which thereby reduces the amount of seeds produced (Scott and Robbins 1999). Additionally, there is a tephritid fly known as *Urophora cardui* that is able to

burrow in stem tissue (Scott and Robbins 1999). There are also microorganisms that have been found to specifically attack only creeping thistle. One such example is the rust fungus, *Puccinia punctiformis* (Thomas et al. 1994). This fungus infects the shoots and leaves and it eventually leads to necrosis. Early signs of infection include orange coloured spermatogonia and a sweet fragrance. Bailey et al. (2000) also found that certain species of fungi belonging to the genus *Fusarium* were also able to infect and reduce root and shoot emergence of creeping thistle. It is a major issue in finding organisms that are suitable to be released to biologically control creeping thistles because they need to be specific such that they only attack thistles and no other unintended plants. They also need to be able to survive in the same harsh environments that thistles can tolerate.

2.2 Yellow pea: main study plant of thesis

2.2.1 Background

Pea (*Pisum sativum* L.) is a pulse crop and a member of the family Fabaceae (formerly known as Leguminosae) (Welbaum 2015). It is grown through much of Canada and the world. There are several types of peas with yellow and green peas as the two main types. Generally yellow peas have slightly higher yields than green peas but are otherwise very similar (Saskatchewan Pulse Growers 2000). As such, both will collectively be referred to as field peas from this point and on. Field peas are also legumes, which means that they are able to fix mineral nitrogen from diatomic nitrogen gas with the help of the symbiotic microbe, *Rhizobium.*

Seeds of pea have high amounts of essential amino acids and protein (21 to 25%) especially when compared to other crops such as cereal grains and this makes them

desirable both for human and livestock consumption (Shatz and Endres 2009). In fact, over half of the production of field peas is designated for the dry pea market or for the eventual use of the seeds to be planted as garden or canned peas (Oelke et al. 1991). In other instances, field pea can be grown as a forage, hay or silage cop. When field peas are grown in a mixture with either oats or barley for the purpose of hay or silage production there is an increase in the dry matter that is harvested per acre due in part to the fact that the added cereal creates competition with the pea and forces it to stand up straighter, facilitating harvest operation. As well, as a green manure crop, pea plants return nitrogen back into the soil (Oelke et al. 1991; Saskatchewan Ministry of Agriculture 2005). In terms of the characteristic features of field pea plants, field peas usually attain a stem height between 0.6 to 1.2 m tall and have a relatively shallow root system. The leaves of field peas are made up of as many as three leaflets that are connected to branched tendrils and the mature pea plant is a vine. The flowers pattern themselves as racemes that are purple or white in color and they are largely self-pollinated due to their floral structure. Seed pods are two to three inches long and house between six to eight seeds (Saskatchewan Pulse Growers 2000). For yellow peas, the seed coat color is yellow. Lastly, in terms of worldwide distribution, field peas are found throughout the world with the largest producers being Russia, China, Canada, Europe, Australia and the USA (Shatz and Endres 2009; Welbaum 2015).

2.2.2 Brief history of the pea

Since the field pea prefers cooler temperatures, it has been speculated that its area of origin is in southwest to central Asia and this is reinforced by the fact that wild relatives of the now domesticated field pea still exist in Iran, Turkmenistan, Turkey and

Afghanistan (Oelke et al. 1991; Welbaum 2015). Archaeologists have found evidence of wild pea consumption dating back to 9750 BC while exploring the Spirit Cave in Thailand (Solheim 1970). It is due to the domestication of field peas along with other plants and animals that spurred the transition of humans from their nomadic, hunter-gather lifestyle to a more settled, pastoralist style. As this idea of agriculture spread throughout the world, so did the seeds of field peas. Places such as China, Europe and eventually North America now are able to grow this crop. During the 1800s, some of the most important work in the field of science was performed by Austrian Monk and geneticist Gregor Mendel on his subjects of choice, the field pea. With them he was able to understand the idea of chromosomal alleles and their segregation during cell division. As well, in the 1800s and 1900s canning and freezing vegetables became possible and this led to more people having access to vegetables such as the pea since they no longer needed to be fresh to consume. Currently, over one thousand varieties of field pea, both green and yellow, exist and are grown worldwide (Zohary and Hopf 2000; Hirst 2015).

2.2.3 Economic importance of pea

Worldwide, field peas have proven to be a very important crop and this is reflected in the large amounts of peas produced in various countries (Kelley et al. 2000). China is by far the largest worldwide producer of peas, and they produced 11,500,000 metric tonnes in 2012 (FAO 2012). In second place is India who produced 3,650,000 metric tonnes and third is Canada, producing 3,340,800 t. The United States, Russia and parts of Africa are also significant producers of field peas. In Canada, field pea farming, production and consumption have become significant contributors to the Canadian economy. In Canada, the average harvested area of field peas is over 1,200,000 ha. In

total, 2,830,000 metric t of field peas were harvested in 2012 from 1,311,000 ha of land and these add up to a grand total of CAN $372,000,000 at market value (Wang 2012). As well, Canadians consumed 846,000 metric t of peas in 2007 of mostly Canadian grown field peas because the amount imported is rather small (Agriculture and Agri-Food Canada 2008).

In terms of distribution throughout Canada, Saskatchewan is by far the largest producer at 911,000 ha and 1,734,000 t of field peas while Alberta is a distant second at 378,000 ha and 1,037,000 t in 2012 (Wang 2012). Both the amount of land used and the tonnes of peas produced increased significantly in the 2012 survey as compared to the one in 2011 such that 714,000 more t were produced in 2012 on 397,000 more ha of land (Wang 2012). The value of the Canadian field pea exports has increased from CAN $550 million in 2008 to CAN $890 million in 2012 with Saskatchewan and Alberta leading for the total production with about 78% and 18%, respectively (Agriculture and Agri-Food Canada 2013).

2.2.4 Field pea in Canada

The pea plant was introduced into North America by early European explorers at the end of the 15th century. Field peas have been grown successfully in western Canada since the early 20th century because of suitable environmental conditions (Ali-khan and Slinkard 1995). The field pea is a cool weather plant that can adapt to different types of soil from sandy loam to soil that is high in clay content. However, field peas are very sensitive to soil that is high in salt or high in moisture content and will not grow in these soils (Ali-khan and Slinkard 1995). In terms of maturity, the field pea follows a similar timeline to wheat in that flowering occurs after 60 days from seeding on average and they

are ready to harvest after approximately 100 days in the Canadian prairies environment. The plant is seeded in spring and harvested in fall. As soon as the soil temperature reaches 4-7°C in the spring, pea plants can be seeded and expected to germinate effectively (Oelke et al. 1991). The best growing temperature range for field pea growth is between 18 to 23˚C. Field pea can tolerate some high temperatures during flowering, but yields may be reduced (Saskatchewan Pulse Growers 2015).

Pea crops currently rank the fourth or fifth largest field crop of Canada after wheat, barley, canola and oats. The production of peas in Canada has increased over recent years. Increasing production of peas in Canada in recent years has sparked great interest among farmers, livestock feed industry and pig producers. Yellow pea is the most widely seeded and produced pea type, with approximately 40 varieties (Ali-khan and Slinkard 1995). Oelke et al. (1991), the Agriculture and Agri-Food Canada (2012), and the Canadian Food Inspection Agency (2014) have listed varieties that were bred to be suitable to be grown in this region and they are compiled in Table 2.1 along with their respective relative yields and maturities.

Numerous spring types of field peas are currently grown in Canada including yellow, green, maple, and marrowfat peas (Saskatchewan Ministry of Agriculture 2012).

Table 2.1 Suitable field pea varieties for the Canadian Prairies according to Oelke et al. (1991), the Agriculture and Agri-Food Canada (2012), and the Canadian Food Inspection Agency (2015) along with their relative yields, maturities and other relevant information.

Variety Name	Yield	Maturity[X]	Other Information
Carneval	High	Late	Semi-leafless[Y]
Bronco	High	Medium	Semi-leafless and resistant to powdery mildew
Agassiz	Medium	Early	Semi-leafless and resistant to powdery mildew
Admiral	High	Early	Semi-leafless and resistant to powdery mildew
Meadow	High	Early	Semi-leafless and resistant to powdery mildew
Golden	Medium	Medium	Standard variety in which new varieties are compared to
Carrera	High	Early	Semi-leafless
Montanna	High	Early	Semi-leafless
Century	Medium	Medium	Large seeds
Lenca	High	Medium	Susceptible to powdery mildew
Miranda	High	Early	Large seeds and Susceptible to powdery mildew
Paloma	High	Early	Very short in height
Procon	High	Early	Very high in protein
Trapper	Low	Late	Small seeds that are used for birdfeed
Belinda	High	Early	Very short with large seeds
Tipu	High	Medium	Semi-leafless
Victoria	High	Early	Small seeds
Bellevue	High	Medium	Susceptible to *Ascochyta* and *Septoria* leaf blotch
Impala	Medium	Early	Resistant to *Ascochyta* race C
Kimo	Medium	Medium	Semi-leafless
Renata	Medium	Medium	Resistant to *Fusarium* wilt, *Ascochyta* race C and Downy mildew
Solara	Medium	Medium	Semi-leafless and resistant to *Fusarium* wilt and *Ascochyta* race C.

[X] Categorized as early, medium and late based on the average maturity time of 100 days where early signifies less than one hundred days and late as over one hundred days.
[Y] Semi-leafless refers to pea varieties that have their leaflets replaced by tendrils while still retaining stipules.

2.2.5 Diseases and nematode pests of pea

Diseases of pea plants are caused by several pathogenic microorganisms including fungi, bacteria, viruses, and plant-parasitic nematodes. Some of the most important diseases on peas are listed in Table 2.2 It has been suggested that pea crops should not be grown on the same field more than once every four years to avoid or reduce problems with soil-borne and foliar diseases (Agriculture and Agri-Food Canada 2013). Disease management in pea crops depends greatly on an integrated management system involving crop management and sanitation.

Table 2.2 Common diseases and nematode pests of field peas found throughout the world (Saskatchewan Pulse Growers 2000; Hannaway and Larson 2004; Hawthorne et al. 2012).

Disease or pest	Scientific Name	Treatments
Complex of diseases known as leaf and pod spot, blackspot foot rot and mycosphaerella bligh	*Mycosphaerella pinodes*, *Phoma medicaginis* var *pinodella* and *P. koolunga*, *Ascochyta pisi*, *Macrophomina phaseolina*	Use pathogen free seed or seed treatment, fungicide, crop rotation
Damping-off, root rot, seed rot , seedling blight, wilt	*Pythium* spp., *Rhizoctonia solani* and *Fusarium* spp. (e.g. *F. solani* f. sp. *pisi*, *F. oxysporum* f.sp. *pisi*), *Aphanomyces euteiches*	Legume-free rotation, seed treatment, resistant cultivars
Alternaria Leaf Spot.	*Alternaria alternata*	Fungicide
Sclerotinia Stem Rot	*Sclerotinia sclerotiorum, S. minor*	Crop rotation, deep plowing
Septoria leaf blotch.	*Septoria pisi*	Use pathogen free seed, crop rotation
Botrytis grey mould	*Botrytis cinerea*	Fungicide
Downy Mildew	*Peronospora viciae*	Seed treatment, crop rotation
Powdery Mildew	*Eryisphe pisi*	Crop rotation, resistant cultivars
Rust	*Uromyces fabae*	Crop rotation
Bacterial Blight	*Pseudomonas syringae pv. pisi, P. syringae pv. syringae*	Use pathogen free seed or resistant cultivars
Pea Seed-borne Mosaic Virus	Pea Seed-borne Mosaic Virus	Use virus-free seed or resistant cultivars, control weeds
Pea stunt	Red clover vein mosaic virus	Use virus-free seed, control weeds
Pea enation mosaic virus	Pea enation mosaic virus	Use virus-free seed or resistant cultivars, control weeds
Aster Yellows	Mycoplasma (Phytoplasma)	None
Root knot nematode	*Meloidogyne hapla, M. incognita*	Crop rotation, Fumigants or nematicides
Root lesion nematode	*Pratylenchus penetrans*	Crop rotation, resistant cultivars, Fumigants
Cyst nematode	*Heterodera goettingiana*	Crop rotation
Sting nematode	*Belonolaimus longicaudatus*	Crop rotation, Fumigants or nematicides
Stubby root nematode	*Trichodorus obtusus*	Crop rotation, resistant cultivars, fumigants
Reniform nematode	*Rotylenchulus reniformis*	Crop rotation, Fumigants or nematicides
Stem nematode	*Ditylenchus dipsaci*	Nematode free seed, seed treatment, crop rotation

2.3 Stem nematodes (*Ditylenchus* spp.)

Plants can be infected by various types of pests, including plant-parasitic nematodes. There are hundreds of nematodes that attack plants. Even though many of them are associated with many hosts, others can parasitize only one plant species. Plant-parasitic nematodes feed on plants using a special organ 'stylet' in their buccal cavity which enable them to suck plant cell contents (Southey 1978). In general, plant-parasitic nematodes feed either on aerial parts or on roots and other subterranean plant structures such as rhizomes, corms, and bulbs. These organisms differ in their feeding habits, with some being endoparasites (sedentary or migratory) entering plant tissues, and others being ectoparasites residing out of plant tissues (sedentary or migratory) to feed and develop (Southey 1978; Sasser 1989).

2.3.1 *Ditylenchus* genus

Ditylenchus Filipjev, 1936 is one of the most important genera of plant-parasitic nematodes and is known to have considerable impact on agricultural crops (Fortuner 1982). *Ditylenchus* nematodes are slender and transparent organisms, straight or somewhat curved when killed by heat with a head skeleton and stylet. Nematodes belonging to the genus *Ditylenchus* are migratory endoparasites. *Ditylenchus* genus comprises of more than 60 (Siddiqi 2000) to 80 species (Brzeski 1991) and is broadly spread throughout the world with a wide variety of hosts. This genus is closely related to two other important genera, *Tylenchus* and *Anguina* (Southey 1978; Plowright et al. 2002). The systematic position of this genus in Tylenchida has changed several times. A high number of species formerly included in this genus have been transferred to other

genera in Tylenchina and Hexatylina (Sturhan and Brzeski 1991). Differentiation between species is not easy due to morphological similarity of many of them, and there are limited features for the precise diagnosis of these species. Therefore, most species have not been well investigated. In addition, there are several races within species that can only be differentiated using advanced molecular identification techniques.

2.3.2 Taxonomic position of *Ditylenchus*

The taxonomic position of the genus *Ditylenchus* has been studied in many different ways over time. In order to classify, species with median bulb valves were first located in the genus *Ditylenchus* while species without valve were placed in *Nothotylenchus* genus. Andrássy (1976) placed *Ditylenchus* in the family of Anguinidae, and superfamily Tylenchoidea and *Nothotylenchus* in the family Nothotylenchidae, and superfamily Nothotylenchoidea and both of these were classified under the Order Tylenchina (Sturhan and Brzeski 1991). Siddiqi (1980) combined Anguinidae, Nothotylenchidae and Sychnotylenchidae and placed them in Anguinoidea in Tylenchina. Then he moved the superfamily to the suborder Hexatylina and placed *Ditylechus*, *Nothotylenchus*, *Diptenchus* and *Orrina* in the family of Anguinidae. In later classification, *Ditylenchus* was located in family Anguinidae and superfamily Sphaerulariodea (Siddiqi 2000). Generally, taxonomic position of the genus *Ditylenchus* is as follows (Fortuner and Maggenti 1987):

Order: Tylenchida

Suborder: Tylenchina

Family: Tylodoridae

Cephalenchus Goodey, 1962

Pleurotylenchus Szczygieł, 1969

Anguinidae

Anguina Scopoli, 1777

***Ditylenchus* Filipjev, 1936**

Halenchus N.A.Cobb in M.V. Cobb, 1933

Subanguina Paramonov, 1967

= *Heteroanguina* Chizhov, 1980

= *Mesoanguina* Chizhov and Subbotin, 1985

2.3.3 The most significant species of the genus *Ditylenchus*

Among mycophagous nematodes in the family Anguinidae, *Ditylenchus* comprises many cosmopolitan species (Fortuner 1982). *Ditylenchus* species have successfully adapted to many ecological habitats and have the greatest diversity in terms of feeding habits or behavior which is only comparable with the foliar nematodes, *Aphenenchus* and *Aphelenchoides*. Most species of parasitic nematodes live in the shoot and roots, tubers, rhizomes or stolons. Only six species of *Ditylenchus* are economically important and are considered a significant pest of agricultural crops: *D. dipsaci* (Kühn) Filipjev (stem and bulb nematode), *D. destructor* Thorne (potato tuber nematode), *D. angustus* (Butler) Filipjev (rice stem nematode), *D. gigas* Vovlas et al. (broad bean stem nematode), and *D. africanus* Wendt et al. (peanut pod nematode) and *D. arachis* Zhang et al. (peanut pod rot nematode) (Prowright et al. 2002; Zhang et al. 2014; CABI 2015a; CABI 2015b). Other species like *D. mycetophagus* Goodey is a pest of edible mushrooms and *D. phyllobius*

(Thorne) Filipjev may be used for biological control of weeds (Sturhan and Brzeski 1991). Most other species in *Ditylenchus* live in the soil and feed on fungi. Some species living in soils such as *D. weischeri* Chizhov et al. or *D. oncogenus* Vovlas et al. are seen in the tissues of wild weeds, but they cause no damage on the host plants (Chizhov et al. 2010; Vovlas et al. 2015).

2.4. Stem nematode of creeping thistle, *Ditylenchus weischeri*

2.4.1 Background and identification

Infection of creeping thistle by races of stem nematode (*D. dipsaci*) was reported about six decades ago (Salentiny 1957; Nolte 1959). Later in 1979, Watson and Shorthouse described *D. dipsaci* infesting creeping thistle in Western Canada, near Regina, Saskatchewan (Watson and Shorthouse 1979). *D. dipsaci* is considered a 'species complex' containing several morphologically similar and phylogenetically related species. This complex comprises of *D. dipsaci sensu stricto, D. gigas, D. weischeri* and at least five still undescribed *Ditylenchus* species (Sturhan and Brzeski 1991; Subbotin et al. 2005; Chizhov et al. 2010; Vovlas et al. 2011). There are few morphological and morphometrical differences between *D. dipsaci* races which makes identification difficult (Vanstone and Russell 2011). In the past, separation of *D. weischeri* from *D. dipsaci sensu stricto* was based on morphological and morphometrical characters. The introduction of molecular techniques with the progress of PCR has provided a convenient and reliable method to distinguish and identify nematode species (Powers and Fleming 1998).

Several studies revealed that species of *D. dipsaci sensu stricto* that naturally infest *Cirsium* plants are different in their morphology, karyology, biology (Ladygina and Barbashova 1976; 1980) as well as in the whole ITS-rRNA gene sequences (Subbotin et al. 2005). Due to this fact, Chizhov et al. (2010) stated that the stem nematode infesting creeping thistle should be considered as an independent species. They showed differences in ITS-rRNA and nucleotide sequences of the *hsp90* gene among *D. weischeri* with *D. dipsaci sensu stricto* and several other still undescribed *Ditylenchus* species, and described *D. weischeri* a new species parasitizing creeping thistle. Chizhov et al. (2010) found this new species on the infested plants in fields, road sides, and ditches near Moscow, Russia. They also reported that *D. weischeri* differs from *D. dipsaci sensu stricto* by larger 'c' index, shorter spicules, longer vulva-anus distance, larger vulva-anus distance to tail length ratio, longer posterior sac and shorter tails in adults.

More recently, *D. weischeri* parasitizing creeping thistle was reported in Canada (Tenuta et al. 2014). In 2009 and 2010, a survey to examine the occurrence and distribution of stem nematodes was conducted in Saskatchewan, Alberta and Manitoba. A total of 538 seed samples were collected from yellow pea grain harvest samples. Results showed that, over the two years, 2% of samples collected were infested by a species of *Ditylenchus* with a population density of 4 to 1,500 nematodes kg^{-1} pea harvest sample. Tenuta et al. (2014) reported that the creeping thistle samples collected from Manitoba and Saskatchewan were infested with *Ditylenchus*. The nematode was also observed in the soil below the creeping thistle shoots.

The Canadian population of *Ditylenchus* obtained from creeping thistle (Tenuta et al. 2014) is morphologically similar to the *D. weischeri* population from *C. arvense* in

Russia (Chizhov et al. 2010). However, Tenuta et al. (2014) reported that the adult individuals differed from those of the Russian population by smaller body sizes and other morphometrical characters (Table 2.3). They also pointed out that *D. weischeri* can be distinguished from populations of *D. dipsaci* on garlic morphometrically using shorter tail in adults, larger index 'c', and longer post vulval uterine sac. Using analysis of the rDNA ITS fragment with RFLP technique, Tenuta et al. (2014) indicated that *Ditylenchus* obtained from pea grain harvest and creeping thistle samples were similar to that for *D. weischeri* (Chizhov et al. 2010) from Russia. They also differentiated *D. weischeri* from *D. dipsaci* based on analysis of ITS-RFLP patterns using five restriction enzymes (*Bsh1236I, Hinf I, MspI, RsaI*, and *TaqI*). The use of PCR-ITS-RFLP technique for accurate separation of different species of *Ditylenchus* in a sample, however, is not appropriate. Due to this fact, Madani et al. (2015) developed conventional PCR and real-time PCR with species-specific primers arranged based on the nucleotide sequence of the *hsp90* gene for reliable and precise detection of both *D. weischeri* and *D. dipsaci*. Their protocol was also capable of detection of *D. dipsaci* and *D. weischeri* from samples containing mixes of both nematode species.

Table 2.3 Morphometrics of *Ditylenchus weischeri* isolated from creeping thistle in Canada (Tenuta et al. 2014) and in Russia (Chizhov et al. 2010) (all measurements in µm).

Character	***D. weischeri* (Canada)**		***D. weischeri* (Russia)**	
	Female	**Male**	**Female**	**Male**
L	[x]1,202 (1,049-1,355)	1,249 (1,244-1,254)	1,545 (1,371-1,619)	1,433 (1,281-1,578)
Body maximum width	30 (27-33)	23.9 (21.9-26.0)	37 (32-44)	26 (23-29)
Body width at anus	15.2 (14.4-16.0)	15.9 (14.8-17)	17 (14-19)	14 (13-16)
a	39.9 (38.8-41.0)	52 (48-56)	40.6 (35.5-44.8)	54.3 (50.1-60.4)
b	6.7 (6.2-7.2)	6.6 (6.4-6.8)	8.2 (7.0-9.0)	7.4 (6.3-8.2)
Tail length	58 (53-62)	62 (58-66)	65 (54-84)	61 (50-73)
c	20.8 (19.7-21.8)	20.2 (19.0-21.4)	23.2 (18.3-28.1)	23.4 (18.4-26.4)
c´	3.7 (3.6-3.8)	3.85 (3.8-3.9)	3.7 (2.9-4.8)	4.2 (3.5-5.0)
Stylet length	10.0 (8.5-11.5)	8.5 (8-9.1)	11.0 (9-13)	11 (9-13)
V%	81.2 (82.3-80.0)	NA	83 (81-85)	NA
Spicule length	NA	24.5 (23.0-26.0)	NA	21 (20-24)
Gubernaculum length	NA	6.35 (5.30-7.40)	NA	7 (6-9)
Esophagus length	177 (169-186)	187 (179-196)	184 (170-203)	194 (176-214)
Distance anterior terminus to:				
Median bulb	71 (69-72)	71 (69-72)	71 (65-75)	75 (67-86)
Hemizonid	130 (125-135)	130 (125-135)	137 (127-145)	139 (128-145)
Excretory pore	145 (140-150)	145 (140-150)	143 (133-150)	143 (130-150)
Vulva to anus distance	NA	NA	194 (172-240)	NA
Postvulval uterine sac length	NA	NA	121(101-150)	NA
Bursa length	58 (50-65)	58 (50-65)	NA	65 (48-78)
Tail length without bursa	19 (18-20)	19 (18-20)	NA	21(12-30)

[x]Mean (Minimum-Maximum). L= overall body length; a= overall body length/maximum body width; b= total body length/pharyngeal length; c= total body length/tail length; c´= tail length/body width at anus; V% = % distance of vulva from anterior.

2.4.2 Hosts and symptoms of *Ditylenchus weischeri*

In terms of host-parasite interaction, little is known about pathogenicity, host preference and life cycle of *D. weischeri*. This nematode species is presently known as a highly specialized stem nematode parasitizing only *Cirsium* species. Tenuta et al. (2014) reported that the occurrence of *D. weischeri* in yellow pea grain was associated with the presence of creeping thistle seeds and debris in pea harvest samples. They observed that infested flower heads of creeping thistle contained a high density of the nematode juveniles. *Ditylenchus weischeri* is suggested to have three to four generations during a vegetation season in the Moscow region (Chizhov et al. 2010). The nematode invades the aboveground portion of the creeping thistle plants.

Disease symptoms caused by *D. weischeri* on creeping thistle can be detected from the second half of the summer and become visible at the flowering stage. *Ditylenchus weischeri* causes symptoms of necrosis, twisting and stunting in the shoots (Chizhov et al. 2010; Madani et al. 2015). The symptoms of stem swelling and galls have already been reported by Watson and Shorthouse (1979) for *D. dipsaci* on *C. arvense*. The galls are induced by extensive hypertrophy and hyperplasia, differentiation of nutritive tissue, nuclear modification, and a central cavity containing nematodes (Watson and Shorthouse 1979). At the end of flowering, stem swellings crack and become necrotic. At this time, the majority of nematode individuals, mostly at the J4, leave the plant and move into the soil. These nematodes desiccate in soil or in dry plant materials and overwinter. *Ditylenchus weischeri* is believed not to parasitize agricultural crops since no reproduction of this nematode has been occurred on garden strawberry and onion crops (Chizhov et al. 2010).

2.5 Stem and bulb nematode (*Ditylenchus dipsaci*)

2.5.1 Background and distribution of *D. dipsaci*

Ditylenchus dipsaci (Kühn 1857) Filipjev 1936 commonly recognized as the stem and bulb nematode, the stem and bulb eelworm, or onion bloat, is one of the polyphagous and common species of plant-parasitic nematodes in agriculture systems worldwide. This species was observed for the first time by Schwertz on teasel, *Dipsacus fullonum*, in Germany in 1855. Two years later, Kühn formally reported the nematode and finally in 1936, Filipjev described it (Kühn 1857; Sturhan and Brzeski 1991). *Ditylenchus dipsaci* is one of the most important nematode species of economic importance in the world. Although it has a wide range in adaptation to various climatic conditions from temperate, subtropical to tropical (Sasser 1989; Brzeski 1991), *D. dipsaci* occurs in humid climates (northern and southern Africa, Asia, Europe and North and South America), where moisture regimes assist nematode invasion, development, reproduction and dispersal (Plowright et al. 2002). *Ditylenchus dipsaci* has been listed as an A2 quarantine pest in many European countries (EPPO 2015). This nematode is capable of extensive dissemination through infested plant materials and seeds of host plants, movement of soil, irrigation water, contaminated farm equipment and vehicles. Therefore, in many countries phytosanitary measures (*e.g.* certification systems) are regulated and used to lessen further dispersal of *D. dipsaci* (EPPO 2015; CABI 2015c).

2.5.2 *Ditylenchus dipsaci* in Canada

Ditylenchus dipsaci was found in Europe for the first time and is believed to have entered Canada by early European settlers (Gussow 1931). In fact, the economic

importance of plant-parasitic nematodes in Canada was first documented with the discovery of *D. dipsaci* parasitizing flower bulbs in Western Canada. The prevalence of *D. dipsaci* has been restricted to foliage (*e.g.* alfalfa) and vegetable (*e.g.* onion and carrot) crops and its occurrence has been reported from Alberta, British Columbia, Ontario, Quebec, Prince Edward Island, and Saskatchewan (Mountain 1957; Bocher 1960; Hawn 1963; Fushtey and Kelly 1975; Vrain and Lalik 1983; Hughes et al. 2012). *Ditylenchus dipsaci* was also reported from creeping thistle in Saskatchewan (Watson and Shorthouse 1979) and was considered as a new plant-specific race. Yu et al. (2010) pointed out that the Canadian population of *D. dipsaci* has some remarkable morphological variations from the classical description of the nomenclature types. However, they reported that the ITS sequence of the Canadian population has a 100% match to sequences of many populations from Europe and Asia.

The incidence of *D. dipsaci* has been first restricted to some isolated onion fields in Ontario. It was first reported from the Leamington Marsh, Essex County (Mountain 1957), and some years later in Kent (Johnson and Kayler 1972) and Simcoe Counties (Fushtey and Kelly 1975). Result of a recent survey from garlic growing fields in Ontario province revealed that 73% of the samples collected were infested with *D. dipsaci* (Hughes et al. 2013) indicating that the nematode has been distributed widely across the province over time. There are several ways by which this nematode pest can be introduced to nematode-free regions. The use of uncertified infested garlic bulbs by growers has been revealed as the main reason for the nematode dispersal (Hughes et al. 2013).

2.5.3 Morphological identification of *Ditylenchus dipsaci*

The *Dityenchus* species have generally been difficult to identify due to the limited number of distinguishable morphological characters and overlapping morphometric measurements. However, slight morphological changes exist between *Ditylenchus* species and diagnosis is based on the length of stylet and post-vulval sac, the position of the vulva to the length of the body, spicule length, the presence and size of the bursa in males, shape and size of the tail, and the number of cuticular lateral lines (Hooper 1972; Fortuner 1982; Brzeski 1991). These characters are usually difficult to identify in older individuals of adult males and females. Normally, these features can be influenced by environmental factors such as temperatures or host plant (Barraclough and Blackith 1962), and thus morphological and morphometric diagnosis alone is insufficient to reliably detect and distinguish *Dityenchus* species from each other. Furthermore, due to the presence of a great intra-specific biological variation within *D. dipsaci* species complex a consistent means is needed to identify these distinct races or populations. Principally the first step to diagnosing *D. dipsaci* is to examine and measure morphological and morphometrical characteristics. Morphometric measurements of male and female individuals of a garlic population of *D. dipsaci* occurred in Canada and Russia are listed in Table 2.4.

Table 2.4 Morphometrics of *Ditylenchus dipsaci* isolated from creeping thistle in Canada (Tenuta et al. 2014) and in Russia (Chizhov et al. 2010) (all measurements in µm).

Character	*D. dipsaci* (Canada)		*D. dipsaci* (Russia)	
	Female	Male	Female	Male
L	1,555 (1,532-1,578)	1,500 (1,400-1,625)	1,392 (1,250-1,708)	1,362 (1,201-1,473)
Body maximum width	33 (32-35)	33 (30-35)	35 (31-45)	28 (24-34)
Body width at anus	21 (20-22)	21 (20-21)	20 (17-23)	16 (14-18)
a	54 (45-63)	46.5 (46.4-46.6)	40.0 (31.3-45.5)	44.4 (39.7-53.5)
b	6.2 (6.1-6.3)	6.8 (6.5-7.1)	7.1 (6.4-8.2)	6.9 (6.3-7.8)
Tail length	89 (88-91)	86 (83-89)	93 (85-103)	87 (80- 97)
c	17.4 (17.3-17.4)	17.5 (16.8-18.2)	15.1 (13.5-19.5)	14.9 (13.9-16.3)
c’	4.3 (4.1-4.4)	4.2 (4.1-4.2)	4.8 (4.2-5.5)	5.3 (4.7-6.2)
Stylet length	8.7 (7.5-9.8)	9.7 (9.0-10.5)	11 (10-12)	11 (10-12)
V%	77 (75-79)	NA	82 (80-86)	NA
Spicule length	NA	29 (28-30)	NA	26 (22-28)
Gubernaculum length	NA	5.4 (5-5.8)	NA	9 (8-11)
Esophagus length	249 (240-258)	223 (195-250)	194 (175-208)	189 (159-207)
Distance anterior terminus to:				
Median bulb	76 (73-80)	76 (74-77)	73 (67-78)	73 (68-85)
Hemizonid	137 (137-138)	132 (129-135)	144 (127-158)	142 (131-151)
Excretory pore	165 (161-168)	159 (158-160)	153 (140-168)	151 (136-162)
Vulva to anus distance	169 (165-173)	NA	150 (132-175)	NA
Postvulval uterine sac length	82 (80-83)	NA	83 (70-100)	NA
Bursa length	NA	67 (61-73)	NA	82 (62- 97)
Tail length without bursa	NA	18 (15-20)	NA	29 (25-34)

[X]Mean (Minimum-Maximum). L= overall body length; a= overall body length/maximum body width; b= total body length/pharyngeal length; c= total body length/tail length; c´= tail length/body width at anus; V% = % distance of vulva from anterior.

2.5.4 Molecular identification of *Ditylenchus dipsaci*

Detection of *D. dipsaci* according to morphological features alone has been a difficult task over time. Since the biological races with different host range was identified for *D. dipsaci* and also morphological and morphometric features showed changes to environmental factors, the nematode classification has been difficult. This nematode species reveals significant variation and its chromosome number varies from n = 6 to 30 (Sturhan and Brzeski 1991).

Ditylenchus dipsaci has the highest number of synonyms (13 nominal species). There are about 30 morphologically indistinguishable races of *D. dipsaci* that differ principally in their host range. *Ditylenchus dipsaci* is considered a 'species complex' or a 'collective species' containing several morphologically similar and phylogenetically related species (Sturhan and Brzeski 1991). Ladygina and Barabashova (1980) hypothesized that two groups can be distinguished in the *D. dipsaci* complex. The first group is comprised of parasites of cultivated plants which form an impartially homologous group according to morphology and chromosome number (n = 12). However, an exception in this group is the giant race of *D. dipsaci* where n = 27. The second group contains parasites of wild plants that constitutes a heterogeneous group both in morphology and karyotype (n = 18 to 28). Various attempts have been made in developing methods for distinguishing *D. dipsaci* races and species synonymous with *D. dipsaci*, but the taxonomic status is still unclear, though differences in chromosome number have been found (Brzeski 1998).

With respect to plant-parasitic nematodes, the rDNA repeat units including the 18S gene, the 28S gene, and the 5.8S gene, the ITS1 and ITS2 of rDNA, and the mtDNA have

been used widely for nematode identification, phylogenetic studies and solving taxonomic problems (Subbotin and Moens 2006). Intraspecific variation within the *D. dipsaci* complex has been examined by several molecular methods including RFLP of the ITS region (Wendt et al. 1993; Kerkoud et al. 2007), genomic DNA (Palmer et al. 1991), specific primers for PCR (Subbotin et al. 2005; Kerkoud et al. 2007), ITS-rDNA gene sequence analysis (Subbotin et al. 2005), amplified fragment length polymorphism (AFLP) (Esquibet et al. 2003), and random amplified polymorphic DNA (RAPD) (Esquibet et al. 1998).

The ITS regions are perhaps the most widely used genetic markers for diagnostic purposes that revealed differences; however, in some cases the ITS region of nematode rDNA may have very similar sequences (Kaplan et al. 2000). In such situations, alternative sequences can be used for nematode identification. Using the ITS of rDNA and phylogenetic analysis of the ITS sequence arrangements, Subbotin et al. (2005) discovered evolutionary trees with two major clades in the *D. dipsaci* complex: first, *D. dipsaci sensu stricto* with diploid chromosome numbers ($2n = 24$) which includes most isolates from agricultural, ornamental, and numerous wild plants; and second, *Ditylenchus* spp. with polyploid chromosome numbers ($2n = 48–60$), isolated from diploid populations and subdivided into six subclades. Recent studies have shown that the *D. dipsaci* complex is comprised of *D. dipsaci sensu stricto*, *D. gigas*, *D. weischeri*, *D. oncogenes* and several other still undescribed *Ditylenchus* species (Subbotin et al. 2005; Chizhov et al. 2010; Vovlas et al. 2011; 2015). Amongst these species, only *D. dipsaci sensu stricto* and *D. gigas* are known as the parasites of cultivated crops with the latter being specific to broad bean (*Vicia faba* L.). Jeszke et al. (2014) showed *Ditylenchus*

from onion (*Allium cepa* L.), endive (*Cichorium endivia* L.), and garden phlox (*Phlox paniculata* L.) in Poland belonged to *D. dipsaci sensu stricto* and that from *V. faba* to be *D. gigas*.

2.5.5 Hosts of *Ditylenchus dipsaci*

At least 450 to 500 different plant species including agricultural, horticultural, ornamental and nursery crops as well as weed species are known to host *D. dipsaci*; however, it represents a broad intraspecific variation in host preference. In fact, various biological races of *D. dipsaci* vary in their host specificity. For example, strawberry, sugar beet, and oat races have wide host ranges, while red and white clover races have low range of host specificity. The presence of variability in host range between *D. dipsaci* populations from different regions was discovered more than a century ago. Since then several biological races were differentiated and named according to the original host or plant species on which they had been isolated for the first time (Sturhan and Brzeski 1991). Seinhorst (1957) first discovered 11 distinct races of *D. dipsaci*, but other researchers reported 15, 21 or 30 races based on host range studies using different nematode races and plant species (Hooper 1971; Sturhan and Brezeski 1991). The use of differential hosts for distinguishing biological race of *D. dipsaci* has been rejected by some nematologists, but many researchers have considered it the best way to identify and differentiate the races. The *D. dipsaci* races are limited to particular hosts upon which they feed, grow and reproduce. They may also infest and feed on alternative hosts but have no ability to reproduce. For example, the alfalfa race of *D. dipsaci* can invade onion seedlings where it may severely damage the plants; however, reproduction does not occur in the secondary host.

The suitability of a plant species for a parasitic nematode is defined as the capacity of a nematode species to develop and reproduce on a host plant. Principally, host suitability is determined as an index, reproduction factor, by enumerating the number of nematodes recovered from a plant and dividing it by the number of nematodes added to the plant (Lewis 1987). In the case of *D. dipsaci*, determination of host range is difficult to some extent because many studies reported significant variability in the nematode reproduction rate on host crops (Whitehead et al. 1987; Moussart et al. 2007). Variability in the nematode reproduction may be due to the presence of dissimilar races or biotypes in different populations.

2.5.6 Host-parasite relationship of *Ditylenchus dipsaci*

Ditylenchus dipsaci is a soil- and seed-borne nematode attacking and infesting various below- and above-ground parts of host crops. The stem nematode is a migratory endoparasite with the fourth-stage juveniles (J4) known as the most important infective stage. *Ditylenchus dipsaci* initially attacks plant tissues while the seedling is still below the soil surface. A thin film of water on the surface of host plants is required for the nematodes to move to the invasion site. Penetration of young seedlings by the nematode individuals proceeds through several entry points such as buds, stomata, lenticels or other breaks in the cortex of the plant. In the case of alfalfa, the nematodes gain entry into the plant tissue directly from the epidermal cells of the shoot apex or leaf axil (Krusberg 1961).

Once inside plant tissues, the nematodes do not move very far into the tissues, but are migrated up inactively with plant growth and are able to infest upper organs of the

plant including flowers, pods or seeds. Hooper (1971) reported that infested seeds are an important means of spreading this nematode pest. Within plant tissues, the nematode movement may be intercellular and intracellular depending upon dissolution of cell walls and middle lamellae which is a process essential for the nematode development (Seinhorst 1957; Krusberg 1961; Southey 1978). It is believed that *D. dipsaci* causes lysis of cells by secretion of pectinase enzyme in plant tissues during the early stages of the infestation (Krusberg 1967; Riedel and Mai 1971). Hussey and Krusberg (1968) found that *D. dipsaci* invades the embryonic leaves of shoot apices of pea seedlings and penetrates directly through the epidermis and then causes formation of cavities in the parenchyma tissue 48 hours after nematode inoculation. Chitwood and Krusberg (1977) reported that an onion population of *D. dipsaci* can induce gall formation in seedlings of a resistant variety of pea.

2.5.7 Crop loss caused by *Ditylenchus dipsaci*

Ditylenchus dipsaci occurs worldwide with the greatest impact on cultivated crops mainly in temperate regions with fairly high rainfall. The nematode problem is most severe during humid cool weather in foggy coastal regions and fields watered with sprinkler-irrigation systems. Main host crops for the different races include onion, garlic, alfalfa, clover, oats, rye, field beans, peas, strawberry and flower bulbs (*e.g.* narcissi, tulips). In alfalfa fields, *D. dipsaci* damage normally occurs in the first and second cuttings of the crop but warm and dry climate occurring later in the growing season reduces the nematode activity. Very low population densities of *D. dipsaci* can build up high densities which results in severe loss to the host plant (Sturhan and Brzeski 1991; Plowright et al. 2002).

The relationship between initial population density of *D. dipsaci* and the reaction of host plants has been studied in the past. Damage threshold level of *D. dipsaci* on onion has been determined to be two nematodes per gram of soil (Bridge and Starr 2007). Yield losses attributed to *D. dipsaci* have been reported to occur in onion field at population densities of ten or more nematode per 500 cm^3 soil (Seinhorst 1956). Palo (1962) sited that an initial density of 10 nematodes in an area of 120 cm^2 can result in high infestation of the alfalfa plants. Information on the amount of yield loss and economic importance of *D. dipsaci* are limited. Whitehead et al. (1983) found a yield loss of up to 37% due to *D. dipsaci* infestation on oat in England. Nickle (1991) suggested crop losses of 60 to 80% in soils heavily infested by this nematode species.

With regard to legume crops, it is reported that *D. dipsaci* is capable of causing yield reductions to broad bean, pea and possibly lentil during humid seasons (Greco and Di Vito 1994; Bridge and Starr 2007). In severe cases, yield loss of up to 70% has been reported to Vicia beans in the UK (Biddle and Cattlin 2007). Furthermore, severe infestation and damage of *D. dipsaci* has been occasionally stated to be associated with the presence of pathogenic fungi (e.g *Fusarium* spp., *Verticillium* spp. and *Rhizoctonia solani*) and bacteria (Hawn, 1963; Griffin, 1990; 1992; Hillnhutter et al. 2011). Bergeson (1972) hypothesized that the fungal pathogens opportunistically enter the plant through wounds caused by plant-parasitic nematodes. This theory was supported by Hillnhutter et al. (2011) as they observed that the injuries or wounds caused by *D. dipsaci* on the petioles and crown of beet plants are appropriate entry sites for *R. solani*. A disease complex between *D. dipsaci* and *F. oxysporum* f. sp. *medicaginis* on alfalfa has been found by Griffin (1990). He reported that these two organisms synergistically affect the

growth of susceptible and resistant cultivars of alfalfa plants. In addition, Griffin (1992) observed an increase in the incidence of plant mortality and Fusarium wilt in soil infested with *D. dipsaci* and *F. oxysporum* when the level of moisture in the top 10 cm of soil at the time of cutting of alfalfa was high. A synergistic interaction was also found when associations of *D. dipsaci* and crown rot fungus (*R. solani*) occurred on potato plants which consequently lead to severe crop loss (Hillnhutter et al. 2011).

Transmission of *Corynebacterium insidiosum* (McCulloch) Jensen, by *D. dipsaci* was detected by Hawn (1963). Later he found that *C. insidiosum* was carried on cuticles of the nematode and cause an increase in the rate of bacterial wilting in a susceptible variety of alfalfa. Generally, *D. dipsaci* alone can considerably increase disease development and crop loss, and is often able to enhance the destructive impact of secondary pathogens even at very low population densities. However, the impact of the stem nematode is very variable and depends on some biotic and abiotic factors, including host species, nematode population level, nematode race or biotype, soil type, and environmental conditions.

2.5.8 Disease symptoms caused by *Ditylenchus dipsaci*

Generally, *D. dipsaci* produce swellings and distortion on stems and leaves of different susceptible crops in which the parenchyma cells are hypertrophied or destroyed (Sturhan and Brezeski 1991). However, the symptoms of nematode damage on various crops differ depending on the invaded plant species.

On legume crops (*e.g.* peas or faba beans), *D. dipsaci* injuries appear as swelling and deformation of stems and petioles. Depending on environmental factors and plant variety, the nematode produces reddish-brown to black wounds on the stems and as

the disease progresses these lesions increase in length. Heavy infestations induce total chlorosis and local necrosis of leaves and petioles that are very similar to fungal infection symptoms. Infected seeds are small and distorted with speckle-like spots on the surface (Bridge and Starr 2007; Sikora and Greco 1990; CABI 2015c).

On onion and garlic, early symptoms include irregular swelling and malformed and twisted leaves. Blister-like areas on the surface of leaves can be detected. As the disease progresses, the base of seedlings become swollen and leaf dieback usually begins in the tips. In severe nematode infestations, scales of bulbs often split and may become spongy. The roots may be missing and stunting and discoloration of the plants can be observed. Eventually, plants turn yellow and die. Infested bulbs are very prone to be infected by pathogenic fungi or bacteria and thus, symptoms often look similar to damage caused by these secondary pathogens such as *Fusarium* basal plate rot (Celetti 2004; Hajihassani and Tenuta, *in press*).

On alfalfa, *D. dipsaci* attacks the young tissue of buds. A very typical symptom of the early stage of infestation is that the base of an infested stem becomes enlarged, discolored with shortened internodes and swollen nodes (Thorne 1961). Later in the season, young plants tend to be stunted with very small “mouse eared” leaves. Infected plants can appear yellow or even white in color. The infected leaves often have a crinkled appearance. Infected stems are brittle and spongy and tend to break off from the crown. In the field, patches of stunted plants can be seen.

On carrot, leaves first become distorted and by the end of the growing season the infested crowns become spongy and brittle. Infested carrot plants are very sensitive to infection by crown rot fungi and soft rot bacteria (Celetti 2004).

2.5.9 Biology and survival of *Ditylenchus dipsaci*

The biology of *D. dipsaci* has previously been studied on some plants such as teasel (*Dipsacus fullonum*), hydrangea (*Hydrangea macrophylla*) and onion (*Allium cepa* L.). *Ditylenchus dipsaci* reproduces sexually after four molts of developmental stages (Hooper 1972). In onion, the egg-laying stage lasts in 7 days, J2s emerge from eggs in 2-$2^1/_2$ days, J3 in 3-$3^1/_2$ days, and J4 in 4 to 5 days (Yuksel 1960). The various juvenile stages of the nematode can be separated by their body length, the presence of vulva and spicule in adults and by the position of gonads (Anderson and Darling 1964; Perry 1976). Mating is required for reproduction of the nematode and a single male is able to fertilize more than one female (Sturhan and Brzeski 1991).

The infective juvenile stage of *D. dipsaci* is the J4 and not the second stage, as in most other plant-parasitic nematodes. Conditions unsuitable to continued growth on the plant (ex. plant senescence) results in arrested development stage of J4. A typical life cycle of *D. dipsaci* takes between 19 and 23 days at 15°C (Yuksel 1960). When mature, the nematode lives for 45 to 73 days, individual females being capable of producing 200 to 500 eggs (Yuksel 1960). The reproduction of *D. dipsaci* is highly impacted by some factors including the growth rate of the host plants (Blake l962a), and also environmental conditions such as temperature and moisture. Minimal temperature for *D. dipsaci* activity is between 3 to 5˚C (Wallace 1958; Griffith et al. 1997) while the optimum temperature for the nematode development has been determined to be 15˚C (Yuksel 1960) and 21˚C on onion (Sayre and Mountain 1962); 15°C on oat (Blake 1962a), and 20˚C on alfalfa (Griffin 1968). This nematode species can produce several generations per growing season, thereby nematode populations can increase to damaging levels.

At the end of the growth season, *D. dipsaci* produces a dormant structure referred to as "nematode wool" in dry plant material, which is an aggregation of nematode juveniles (mostly J4) that can survive adverse climatic conditions. The nematode aggregation can be found on the surface of tissues and seeds of highly infested plants (Hooper 1972; Hajihassani and Tenuta, *in press*). The *D. dipsaci* individuals move out of dry plant tissues into the soil only after the tissues become degraded. This nematode species represents anhydrobiosis capacity, a state of development that allows the nematode to withstand and survive under unfavorable environmental conditions such as water stress or low temperature.

The dispersal of a plant-parasitic nematode requires, in part, the ability of its free-living juveniles to survive in variable environmental conditions. For *D. dipsaci*, the free-living stage is the J4s that can tolerate desiccation for many years. Miyagawa and Lear (1970) found that the optimum temperature for *D. dipsaci* to survive in soil and maintain their pathogenicity on onion is 15˚C. Wallace (1962) found that the *D. dipsaci* J4s dehydrated in an atmosphere of 50% relative humidity can readily regain activity when immersed in water. It has been stated that garlic and bean races of *D. dipsaci* can survive 2 and 9 years, respectively in soil, representing the nematode ability to survive desiccation (Tenente 1996).

2.5.10 Effect of environmental conditions on *Ditylenchus dipsaci*

Temperature. Nematodes, like other invertebrates, are poikilothermic organisms and their metabolic rates and physiological processes such as movement, development, reproduction and sex determination are influenced by ambient temperature (Freckman and Caswell, 1985; Tzortzakakis and Trudgill 2005). Many plant-parasitic nematodes are

adapted to specific temperature limits and temperature is a significant environmental factor in regulating the time required for completing the life cycle of a nematode. Temperature can have an influence on different aspects of the life cycle and development of any plant-parasitic nematodes. Various nematode species can have different ideal temperatures for feeding, survival, development and reproduction (Singh and Sharma 1994.; Mizukubo and Adachi 1997; Thompson et al. 2015).

Ditylenchus dipsaci is sensitive to environmental conditions and can adapt to different climatic conditions. It is reported that *D. dipsaci* can tolerate sub-zero weather temperatures ranging from -5 to -20°C for a period of four months (Thorne 1961). The development and multiplication of *D. dipsaci* in white clover and alfalfa plants is directly associated with temperature (Griffin 1968; Griffith et al. 1997). As well, the duration of the nematode life cycle is influenced by temperature and seems to change among isolates from different regions. For example, Tenente and Evans (1998) reported that the teasel race of *D. dipsaci* complete its life-cycle (J4 to J4) in 21 to 28 days at 20°C and between 28 to 34 days at 15°C. They also found that a prior chilling at 5°C for one month required for completion of each life cycle at 10 and 26°C. This may indicate that a temperature between 15 and 20°C is optimum for activity and development of teasel race of *D. dipsaci.*

Williams-Woodward and Gray (1999) reported the number of *D. dipsaci* in stem tissue of alfalfa is associated with mean air temperature. Tseng et al. (1968) stated that the number of nematodes in soil at various depths in an alfalfa field is noticeably affected by the temperature deviation from 15˚C. Tenente and Evans (1998) reported the generation time of the teasel (*Dipsacus* spp.) race of *D. dipsaci* was 21 to 28 days at 20°C

and 28 to 34 days at 15°C. The temperatures optimal for *D. dipsaci* reproduction, activity and mobility have been reported to be 18˚C (Barker and Sasser 1959), and 15 to 20˚C (Wallace 1961), respectively. It seems that the optimum temperature for the nematode development is around 15 to 20°C. However, it is important to notice that *D. dipsaci* reproduction is related not only to the temperature but also to the growth of host plant (Blake l962b).

Moisture. The nematode activities require water. It is well accepted that the movement of plant-parasitic nematodes in soil and their attacks to plants are favored when the amount of soil moisture is high. In the case of foliar nematodes (*e.g. Ditylenchus* spp., *Aphelenchoides* spp.), the nematodes move in a thin film of water covering the surface of plant tissues to reach different organs of a host plant (Thorne 1961; Jagdale and Grewal 2006). The *D. dipsaci* movement, invasion, parasitism, and the amount of disease severity caused by the nematode are related to cool and humid environmental conditions that usually occur in early spring. Williams-Woodward and Gray (1999) found that the only environmental factor that was associated with an increase in the numbers of *D. dipsaci* in alfalfa stem tissue was total precipitation. Wallace (1962) found that the number of *D. dipsaci* in an oat field increased significantly after rain but decreased after a dry period. The reproduction of *D. dipsaci* is directly associated with relative humidity levels (Wallace 1958). It is also reported that reproduction and damage of *D. dipsaci* in alfalfa is influenced greatly by the increase of moisture in soil (Barker and Sasser 1959).

Aeration. The degrees of respiration of many species of plant-parasitic nematodes have been revealed to be greater in ambient air (0.03% CO_2) than in either a CO_2-free

atmosphere or in higher concentrations of CO_2 (Rohde 1960). *Ditylenchus dipsaci* is well adapted to persist inside dry organs of plants, where osmotic pressure and carbon dioxide concentrations are high. The nematode respires in the range of osmotic pressures from 0 to 44.8 atmospheres. It has the ability to stay alive under anaerobic conditions to some extent. But it is reported that recovery of *D. dipsaci* individuals declines with increasing depth of water in a test tube (Wallace 1963). Consequently, aeration is one of the environmental factors that affect activity and survival of *D. dipsaci*.

Soil type. Gerasimow (1954) found that the activity and mobility of *D. dipsaci* juveniles is dependent on the soil texture. Normally, the movement of *D. dipsaci* in soil may impact the dispersal of the nematode. Many studies (Seinhorst 1956; Wallace 1962; Miyagawa and Lear 1970; Elgin et al. 1975) showed that movement of the nematode is greater in sandy soils than in clay soils. However, *D. dipsaci* can be distributed in both light and heavy soils with a higher degree of disease severity being observed in heavy soils. Seinhorst (1950) reported that the nematode infestations are more frequent in clay soils. He also found that *D. dipsaci* reproduces more rapidly in heavier soils during cold and wet periods and that during the winter populations declined more quickly in sandy than in clayey soils (Seinhorst 1956). Consequently, there is sophisticated interactions between soil type and stem nematode and some physical (*e.g.* pore size) and chemical (*e.g.* pH) factors can affect the activity of *D. dipsaci* in the soil. However, it should be noted that nematode development and reproduction may also be influenced by the growth rate of host plants (Blake l962a).

2.5.11 Nematode management strategies

Management methods of plant-parasitic nematodes include cultural control, physical control, plant resistance and biological and chemical controls. The population of plant-parasitic nematodes in the field and the impact of the disease can be minimized by means of a combination of multiple practices (*i.e.* integrated pest management) or may reliance only on a single control method. A suitable control technique should be chosen based on the nematode species, biotype or race, nematode-host interaction, and nematode biology (Nicol 2002).

In the case of *D. dipsaci*, the presence of intraspecific diversity in this nematode species (based on host specificity and the degree of pathogenicity to particular crops) should be considered an essential factor in selecting an effective control method. Thus, the control of *D. dipsaci* is rather a difficult task because of the ability of the nematode to reproduce on the same hosts, leading to rapid build-up in nematode population, and that the infested dry plant organs such as bulbs or seeds can contribute to dissemination of the desiccated nematode (Sikora and Greco 1990; Tenente 1996). Hughes et al. (2013) stated that no effective control method is available neither to prevent the spread of the nematode nor to control the nematode damage. However, there are different control practices for nematode management that growers can use as describe bellow.

2.5.11.1 Cultural control

Use of nematode-free seeds. The management strategies of *D. dipsaci* should be primarily preventative. Since *D. dipsaci* is a seed-borne parasite, one of the strategies effective for nematode prevention is the use of un-infested certified seeds and cultivation

in nematode-free fields. This practice has been suggested by several researchers, particularly in garlic or onion growing fields. For example, Hughes et al. (2013) reported that widespread distribution of *D. dispaci* in Ontario is due to using infested garlic bulbs by growers.

Crop rotation. One of the most effective cultural methods to control plant-parasitic nematodes is rotation using non-host crops. This method helps to reduce nematode population in the soil through growing host plants unsuitable for *D. dipsaci* reproduction. Rotation with non-host crops for the control of this nematode species is limited due to polyphagous feeding behavior and wide host ranges of *D. dipsaci*. Accurate identification of the nematode races or biotypes, determination of nematode host range, and control of weed species in the infested fields which may act as source of the nematode inoculum should be highly considered when designing a rotation system (Nusbaum and Ferris 1973). Rotation with non-host plants for 3 to 4 years is often effective to prevent *D. dipsaci* crop losses and to decrease soil population of the nematode (Sturhan and Brzeski 1991; Lorbeer et al. 1997). In California, growers use a rotation of 4 years of non-host crops between garlic cultivations to control *D. dipsaci* (Roberts and Matthews 1995).

Antagonistic plants and cover crops. Antagonistic plants and cover crops are effective for decreasing populations of plant-parasitic nematodes in soil, improving soil texture and protecting soil from erosion (Nusbaum and Ferris, 1973; Trivedi and Barker, 1986). Extracts from some plants have nematicidal natural substances (*e.g*. alkaloids, dithioacetylenes terpenes) that can suppress the nematodes growth and reduce their populations in soil (Tando et al. 1989). Therefore, growing and incorporation of these plants into the soil can be used to manage plant-parasitic nematodes infestation. It has

been reported that fresh extracts from leaves of *Chenopodium ambrosioides* L., *Aristotelia chilensis* L., and *Ruta graveolens* L. successfully reduced disease severity and final population densities of *D. dipsaci* on garlic (Insunza and Valenzuela 1995). In addition, mulching the soil with *Artemisia dracunculus* L. has been reported to be effective against *D. dipsaci* (Chen et al. 2004).

2.5.11.2 Physical control

Soil solarisation. Soil solarization is a simple but effective and safe method. It has been used as a non-chemical alternative to the toxic nematicides and fumigants in order to control several soil-borne pests including plant-parasitic nematodes. Normally in warm climates, transparent polyethylene sheets or plastics can be used prior to planting to trap solar heat in soil and increase temperature to levels (40 to 50˚C) lethal for nematodes (Katan 1987; Whitehead 1998). The effect of soil solarization on *D. dipsaci* has been studied in Italy (Greco et al. 1985) and Israel (Siti et al. 1982), and it considerably reduced the population of the nematode.

Hot water treatment. Hot water treatment is suggested as a control means to disinfest plant material to kill nematodes. It is often used with different combinations of temperature and time to disinfect seeds or bulbs (Gratwick and Southey 1972). A hot-water treatment at 43 to 44˚C for 2 h can be used to control *D. dipsaci* (Decker 1969). This method was also successful for the disinfestation of onion bulbs infested by the stem and bulb nematode. However, treating with hot water alone resulted in reduction of seed or bulb germination. Furthermore, when infested garlic bulbs were treated with hot water for one hour, the treatment did not lead to 100% nematode mortality (Tenente 1996). Due

to these facts, some researchers in the USA used a combination of hot water treatment and formaldehyde to control *D. dipsaci* and obtained very good results (Courtney and Gould 1951; Qiu et al. 1993). But with regard to concerns over the safety of formaldehyde, other chemicals such as abamectin, mercury and sodium hypochlorite have been examined in combination with hot water. For example, Roberts and Matthews (1995) reported that abamectin cool-dip treatment following hot-water treatment of infested garlic bulbs can effectively control *D. dipsaci*.

2.5.11.3 Plant resistance to *Ditylenchus dipsaci*

Some plants harbor specific resistance genes that protect them against plant-parasitic nematode damage. Normally, resistance can be defined as the ability of a plant to prevent nematode reproduction. Plant resistance offers the most effective and economical practice of controlling nematodes since it is a sustainable, cost effective and environmentally safe strategy (Trudgill 1991; Cook and Starr 2006). In the case of *D. dipsaci*, however, special attention should be given to the race of the nematode. Some varieties of clover (*Trifolium* spp. L.), lucerne (*Medicago sativa* L.), oat (*Avena sativa* L.), and rye (*Secale cereal* L.) are recorded to be resistant to *D. dipsaci*. However, some crops (*e.g.* garlic, onion, field bean) with different levels of susceptibility have been found (Sturhan and Brzeski 1991). Although earlier studies recognized the presence of resistant varieties/cultivars to *D. dipsaci*, most of these plants are no longer obtainable.

2.5.11.4 Chemical control of *Ditylenchus dipsaci*

Fumigant and non-fumigant (nematicide) chemicals are effective constituents of management programs for plant-parasitic nematodes. The main aim of using these

chemicals is to alleviate disease severity and damage while increasing yield quantity and quality (Haydock et al. 2006). Soil fumigation with chemical nematicides is effective, but very expensive and poses risks to animal and human health as well as the environment. In addition, they are probably not cost effective in most fields unless high populations of the nematode are present in soil.

Chemical disinfestation of both soil and plant material (*e.g.* seed or bulb) with fumigants and non-fumigants can be applied for the control of *D. dipsaci*, thereby considerably increasing the production of healthy crops. However, fumigating soil to control this pest is not often economical because more than 99% of the nematodes in the soil must be killed; otherwise, surviving nematodes in soil or plant material may infest and reproduce on host or non-host plants (Whitehead 1998; Sturhan and Brzeski 1991). Soil fumigation with dichloropropene-dichloropropane mixture prior to planting has been determined to effectively control *D. dipsaci* (Vernon et al. 1965). Systemic nematicides are also rather effective to control *D. dipsaci* in some cultivated and ornamental crops. For example, Westerdahl et al. (1991) reported that foliar applications with oxamyl on narcissus plants resulted in reduction in the number of nematodes in leaves and bulbs. Lewis (1979) examined the effect of aldicarb, oxamyl, ethoprophos, tirpate and thionazin on severely damaged strawberry plants in pot trials and found that all these chemicals decreased the number of infested plants. He also reported that under field conditions the application of aldicarb at planting and during plant growth reduced the number of plants infested by *D. dipsaci* and improved the fruit yield of strawberry.

Controlling the spread of *D. dipsaci* through infested plant materials, bulbs and seeds is also critical for managing the nematode infestations. Fumigation of seeds with

methyl bromide to control *D. dipsaci* has been examined on several crop species (*e.g.* onion, red clover, lucerne) and satisfactory controls have been obtained in seed or plant lots (Powell 1974; 1975). Recently methyl iodide (Ciesla et al. 2010) and hydrogen cyanide gas (Manasová et al. 2012) have been introduced as alternative fumigants to methyl bromide to control *D. dipsaci*. Ciesla et al. (2010) reported that treating alfalfa seeds with methyl iodide at concentrations between 20 to 80 g/m^3 for 24 h can eliminate the nematodes from the seeds.

Non-fumigant nematicides or insecticides can be used as a seed treatment in controlling plant-parasitic nematodes (Schiffers et al. 1984; Whitehead and Tite 1987). The potential use of carbofuran, phenamiphos, and oxamyl for seed treatment of alfalfa in controlling *D. dipsaci* has been recorded by Gray and Soh (1989), as they reported that all three treatments not only reduced crop loss by the nematode but increased survival of susceptible and resistant cultivars of alfalfa. Treatment with abamectin can kill about 93% of nematodes in infected garlic bulbs (Becker 1999). In addition, treating narcissus plants with a combination of formaldehyde and thiabendazole in the UK gave the best results against *D. dipsaci* (Hanks and Linfield 1999).

2.5.11.5 Biological control of *Ditylenchus dipsaci*

In recent years, attention to biological practices as a control practice for plant-parasitic nematodes has increased to improve integrated pest management systems and as alternatives to fumigants and non-fumigants nematicides. Many biological control agents such as nematophagous fungi or antagonistic bacteria have been screened and suggested to be antagonistic to plant-parasitic nematodes (Sikora 1992; Stirling 2005). The biological control offers an environmentally friendly practice to managing nematodes;

however, there are a few commercially available biocontrol products to use under field conditions. For example, Cayrol and Frankowski (1986) demonstrated that endoparasitic fungus of *Hirsutella rhossiliensis* has the potential to control *D. dipsaci*. Spores of *H. rhossiliensis* adhere to the cuticle of the nematode, and penetrate through the cuticle and kill the nematode (Cayrol and Frankowski 1986). In Australia, pot experiments showed that the nematophagous fungus of *Verticillium balanoides* is a suitable biological agent for *D. dipsaci* control (Hay and Bateson, 1997) because it significantly affected the number of the nematode in the foliage of white clover.

In addition, antagonistic bacteria have been shown to be important biocontrol agents for plant-parasitic nematodes (Sikora 1992). For example, Mendoza et al. (2008) reported that the bioactive metabolites produced by *Bacillus firmus* can cause mortality of *D. dipsaci* juveniles under *in vitro* conditions and may play a significant role in the control of this nematode.

MANUSCRIPT 1

3.0 HOST PREFERENCE AND SEED-BORNE TRANSMISSION OF *DITYLENCHUS WEISCHERI* AND *D. DIPSACI* ON SELECT PULSE AND NON-PULSE CROPS GROWN IN THE CANADIAN PRAIRIES

3.1 Abstract

The stem nematode, *Ditylenchus weischeri*, was recently reported on creeping thistle (*Cirsium arvense* L.) in Canada. Two greenhouse studies examined host suitability of crops commonly grown in the Canadian Prairies for *D. weischeri* and the closely related parasite of many crops, *D. dipsaci*. In the first study, common pulse crops (yellow pea, chickpea, common bean, and lentil), spring wheat, canola, creeping thistle and garlic were evaluated. Plant biomass and reproductive factor (R_f = nematode recovered/inoculated) eight weeks post-inoculation were used to determine host suitability. Creeping thistle biomass was reduced by *D. weischeri* whereas, *D. dipsaci* reduced biomass of four of five pea and two of three bean varieties. Two pea varieties were week hosts for *D. weischeri* with Rf slightly > 1. *Ditylenchus weischeri* aggressively reproduced on creeping thistle (R_f = 5.4). *Ditylenchus dipsaci* reproduced aggressively on garlic (R_f = 6.4), a known host, moderately on pea varieties (R_f > 2), and weakly on chickpea and bean (R_f > 1). In the second study, using creeping thistle and yellow pea, *D. weischeri* was recovered from above-ground parts of the plants and seeds of the former and *D. dipsaci* from the later. The results show *D. weischeri* parasitizes creeping thistle but not other crops and that *D. weischeri* host preference is different from that of *D. dipsaci*.

3.2 Introduction

Cool season pulse and non-pulse crops can be parasitized by some plant-parasitic nematodes of the genus *Ditylenchus* (Sikora and Greco 1990; Stoddard et al. 2010). Among the many species in the genus, *D. dipsaci*, *D. destructor*, *D. gigas*, *D. angustus*, *D. africanus*, and *D. arachis* are major pests of crops such as potato (*Solanum tuberosum* L.), broad bean (*Vicia faba* L.), pea (*Pisum sativum* L.), common bean (*Phaseolus vulgaris*), lucerne (*Medicago sativa* L.), onion (*Allium cepa* L.), garlic (*A. sativum* L.), rice (*Oryza sativa* L.) and peanut (*Arachis hypogaea* L.), while the majority of species feed on some soil fungi (Plowright et al. 2002; Sturhan and Brzeski 1991; Vovlas et al. 2011; Zhang et al. 2014).

The stem and bulb nematode, *D. dipsaci* (Kühn) Filipjev is almost globally distributed with significant potential to reduce production of many crops. It is an obligate parasite that feeds and reproduces in the parenchymatous tissues of 450 to 500 plant species causing the middle lamellae of cell walls to break down (Sturhan and Brzeski 1991; Bridge and Star 2007). This nematode has been the subject of external quarantine regulations of many countries and almost all countries have strict quarantine regulations to prevent the probability of its importation through infested soil, seed, and plant materials. Both the above- and below-ground parts of host plants are infested by the nematode (CABI 2015c; Plowright et al. 2002).

In Canada, the occurrence and prevalence of *D. dipsaci* has been confined to garlic, alfalfa, onion, and carrot crops in Alberta, British Columbia, Ontario, Quebec, and Prince Edward Island (Fushtey and Kelly 1975; Hawn 1963; Mountain 1957; Vrain and Lalik 1983; Yu et al. 2010). Watson and Shorthouse (1979) reported *D. dipsaci* infesting

creeping thistle, *Cirsium arvense* near Regina, Saskatchewan. However, Chizhov et al. (2010) described a new species *D. weischeri* on creeping thistle in Russia based on morphological and PCR-RFLP analysis. Recently we reported *D. weischeri* and not *D. dipsaci* commonly associated with creeping thistle in Saskatchewan and Manitoba (Tenuta et al. 2014). Species specific primers have been developed to quickly differentiate *D. weischeri* and *D. dipsaci* (Madani et al. 2015). *Ditylenchus weischeri* has been found on creeping thistle and is believed not to parasitize crops; however, only onion and strawberry have been examined (Chizhov et al. 2010).

Various procedures for determining the host suitability of plant-parasitic nematodes have been used previously. The most common approach is the Reproductive Factor (R_f) value; the ratio of recovered to added nematode numbers. In some host-suitability studies, the comparison between a reference host of known R_f value and the R_f value of the test host was used (Inomoto and Asmus 2010; Vanstone and Russ 2001). Other studies have used assessment of host suitability of test plants as non-host ($R_f < 1$, nematode penetrates into plant with no reproduction occurs), poor host ($R_f = 1$, little reproduction or nematode survival occurs), and increasing host suitability with R_f into qualitative categories such as intermediate host (medium increase in the number of nematode occurs) and good or excellent host (large increase in the number of nematode occurs) (Forge et al. 2012; MacGuidwin et al. 1992).

Although *D. weischeri* is presently known as a highly specialized stem nematode parasitizing only *Cirsium* species in the Canadian Prairies, there is a lack of knowledge about host preference of this nematode species to commonly grown pulse and non-pulse crops in the Canadian Prairies, and if it can be transmitted by infected seeds of the crops.

Therefore, the objectives of this study were to (i) assess the host status of four pulse and two non-pulse crops for *D. weischeri* and *D. dipsaci*, (ii) evaluate the reproduction capacity of these nematodes on different varieties of yellow pea, common bean and chickpea, and (iii) determine if these nematodes are seed-borne transmissible in yellow pea and creeping thistle.

3.3 Materials and Methods

3.3.1 Preparation of *Ditylenchus* species inoculum

A *D. weischeri* population was obtained from an infected creeping thistle plant collected at the University of Manitoba Glenlea Research Station, Glenlea, Manitoba. Additionally, a population of *D. dipsaci* from Ontario, isolated from infected garlic, was obtained. To prepare nematode inoculum, *D. weischeri* and *D. dipsaci* fourth-stage juveniles (J4) were reared on creeping thistle and garlic plants, respectively. Four creeping thistle rhizomes and garlic cloves (artichoke garlic), were planted in 24-cm-diameter polyethylene pots filled with nematode free clay:sand:peat mix (1:1:2 v/v). Young plants were inoculated by pipetting approximately 2,000 J4 into three holes in the soil around each shoot. The plants were covered with transparent polyethylene bags and then grown for three to four months in a growth chamber at 23 ± 2°C and a 14-hr photoperiod of fluorescent light. Active nematodes were extracted using a Whitehead tray method (Whithead and Hemming 1965) from the infested plants and stored in water at 4°C until use. The nematodes were identified morphologically and morphometrically (Tenuta et al. 2014) and confirmed by conventional PCR using species-specific *hsp*90 and ITS gene primer sets (Madani et al. 2015).

3.3.2 Host preference study

Two experiments were conducted in a greenhouse from Nov 2012 to Feb 2014 to examine the host suitability of pulse and non-pulse crops to *D. weischeri* and *D. dipsaci* and to assess the reproduction variability of these nematodes on yellow pea, common bean and chickpea varieties. Seeds of yellow pea (*P. sativum*) vars. Agassiz, Admiral, Meadow, Golden and Bronco, chickpea (*Cicer arietinum* L.) vars. CDC Corinne (Desi) and CDC Frontier (Kabuli), large green seed lentil (*Lens culinaris* L.) var. CDC Greenland, common bean (*P. vulgaris* L.) vars. Windbreaker (Pinto), Pink panther (Kidney), and Envoy (Navy), spring wheat (*Triticum aestivum* L.) cv. AC Barrie, and canola (*Brassica napus* L.) var. Invigor were planted (one per pot) to 12-cm-diameter polyethylene pots containing the same soil mixture as described previously. Prior to inoculation, the nematode suspension was microscopically examined to confirm all nematode individuals were J4. Suspensions of approximately 5,000 J4 were transferred to a glass centrifuge tube and concentrated at 1,500 × *g* for 3 to 4 min. Then the supernatant was removed and replaced with 1.5% carboxymethyl cellulose (CMC; Sigma-Aldrich, St Louis, MO). Carboxymethyl cellulose was added to the nematode suspension to reduce nematode death due to drying on the leaf axil surface and to increase potential of penetration of J4 individuals into the seedlings. The nematode inoculum was adjusted to 5,000 fresh individuals per 1.5 mL and then aliquots of 15 µl containing 50 J4 pipetted onto leaf axils of seedlings at second true leaf stage (except for garlic which was inoculated at first-leaf stage), and then again five days later. Therefore, the total number of nematodes inoculated was 100 J4 per plant. Plants inoculated with CMC alone (no nematode) were used as a negative control. In addition to the negative control, in each

trial a positive host control was included by inoculating creeping thistle and garlic, grown in 12-cm-diameter polyethylene pots, with *D. weischeri* and *D. dipsaci*, respectively. Because high humidity is required to favor nematode penetration and infestation under greenhouse conditions and also to increase relative humidity (~80 to 90%) and prevent inoculum droplets from drying quickly, plants were incubated for total of six days in a chamber comprised of a translucent plastic canopy and external humidifier (Herrmidifier 707U, Effingham, IL) introducing water vapor to the former via a PVC pipe. Relative humidity in the chamber was not determined. However, condensation was avoided to prevent washing added nematodes off seedling by using the PVC pipe with perforation to introduce humidified air into the chamber.

Thereafter, the pots were placed on two benches in a greenhouse at 25 ± 2^{o}C and watered every other day. Because of the risk of cross-contamination, the treated plants were not placed directly beside the control plants; however, treatment and control plants for each nematode species were placed on two separate benches with inoculated treatment groups (nine plants of a single plant/variety) separated from other groups by polyethylene sheets. Control and treatment groups (nine plants within each) were randomized on the bench. Therefore, individual pots served as pseudo-replicates/sub-samples and the two repeats of the experiment were considered the true replicates.

The treated plants were grown for eight weeks prior to extraction of nematodes. At eight weeks, each plant was cut off at the soil surface and the fresh weight of aboveground biomass was recorded. The leaves of the seedling were detached from stems and the stems split in half. Then the leaves and stems were cut into small segments of 0.5 to 1 cm in length and nematodes were extracted for three days using the Whitehead tray

method. In a previous test (unpublished data), we determined that 99% of added *D. weischeri* and *D. dipsaci* where recovered three days after inoculation. If there was more than 15 g fresh weight plant material in a pot, the material in excess of 15 g was extracted using a another tray. The trays (18-cm-diameter) were plastic containing one layer of paper towel on a wire mesh (700 µm screen size) supported by 3 mm thick plastic rings. Tap water was added to the trays to just cover the plant material on the paper towel. After nematode extraction, the volume of the nematode suspension obtained from each plant was concentrated to 3 mL by centrifugation and then the population density of the nematodes was enumerated under a microscope using a counting slide. The reproductive factor (R_f) of the nematodes was determined by dividing the total number of nematode recovered from a plant by the initial number of nematodes added (100 J4). In this study, we categorized R_f as < 1 being non-host, 1 to 2 as a poor host, 2 to 4 as a good host, and > 4 being an excellent host.

3.3.3 Seed-borne transmission study

This study was conducted twice during Feb to May 2014 to determine feeding or reproduction location preference of *D. weischeri* and *D. dipsaci* within below- and above-ground parts of yellow pea and creeping thistle plants including roots, stems, leaves, seed-pods, flower heads and seeds. Seeds of yellow pea var. Agassiz and segmented rhizomes of creeping thistle were germinated after surface-sterilization with sodium hypochlorite (5%) on damp filter paper to produce sprouts. To supply sufficient inoculum of *D. weischeri* and *D. dipsaci*, the nematodes were reared on callused carrot disks (Hajihassani et al, Manauscript 3). For this culturing method, *D. weischeri* and *D. dipsaci* were isolated from creeping thistle and garlic stems, respectively and placed in

streptomycin sulphate solution (4,000 mgL^{-1}, Sigma-Aldrich, St Louis, MO) overnight and then washed several times in sterile distilled water. Individuals of each nematode species were then pipetted onto surface sterilized callused carrot disks and incubated in dark at 23°C.

As soon as emergence of coleoptile, one pre-germinated seed of yellow pea or segmented root of creeping thistle was sown in each pot and inoculated with 1,000 mixed life stages (8% eggs, 78% juveniles and 14% adults) of either *D. weischeri* or *D. dipsaci* in 20 µl of a 1.5% CMC suspension. The difference in nematode life stage used in the transmission study compared to host preference study (only J4) was due to using nematodes recovered from callused carrot disks rather than from creeping thistle and garlic plants. Each pot was considered a replication and treatments were set on a bench in greenhouse. The design of the experiment was similar to the host preference study with six replications (pots) per plant species. Inoculated plants were checked carefully during the tests to record any symptoms of disease or injury by the nematode species.

Plants infested by *D. weischeri* and *D. dipsaci* were harvested when yellowing and senescence of plants occurred, about 67 to 69 days after emergence. Extraction of nematodes from roots, stems, leaves and seed-pods, flower heads and seeds was done as described previously. The roots were cut at the point of attachment to the base of the stems and washed free of soil. The leaves were first detached from the stems and then each plant stem was separated into two parts (bottom and top parts). With regard to pea seed, seed coats were removed and retained. Nematode individuls were extracted from seed coats, storage cotyledons, and germ portion (hypocotyl and radicle) by soaking for 48 h in tap water. The total number of the nematodes recovered from each plant part

(roots, stems, leaves, seed-pods, flower heads, and seeds) was determined as described previously. For pea seeds, however, the nematode numbers were sum of the individulas recovered from storage cotyledons and seed coats.

3.3.4 Statistical analysis

A mixed model ANOVA was used to analyze nematode recovery data generated in the host preference and transmission studies. Prior to each analysis, the assumptions for normality of residuals was tested and corrected when necessary. To meet the assumption of normality, response variable data were $\log_{10}$ transformed when necessary and inspected for outliers. For the host preference study, host species/variety was considered the fixed effect while experimental repeat, the true replicate, and plant repeat, the pseudo-replicate, were considered random effects. The pseudo-replicate (plant) was nested within the true replicate (experiment). Due to the unbalanced nature of plant species and varieties, the ANOVA was conducted using a one-way treatment structure.

All tests were conducted at $\alpha = 0.05$. When necessary, heterogeneity of variance was corrected by using the Repeated/Group statement in Proc Mixed. Means comparison of the nematode R_f in the host preference study and recovered number of nematodes in the seed borne transmission study was done using Tukey's Honestly Significant Difference test and the pdmix 800 macro (Saxton 1998). Individual least squares means (LSmeans) t-tests indicating whether the mean was significantly different from zero were used to determine if the nematode R_f was different from 1; the base 10 logarithm of 1 is zero. Thus, individual t-tests that showed that the treatment mean was significantly different from 0 indicated that the R_f deviated significantly from 1.

To determine whether the nematodes affected plant aboveground biomass in the host preference study, a one-way ANOVA using the same model as for the R_f response variable was conducted. For this analysis, biomass of a plant species inoculated with a species of nematode was expressed as a ratio of the average of all non-inoculated plants of the same species. The ratio data were then log-transformed to meet the assumption of ANOVA. The log-transformation also allowed for testing which of these values were significantly different from their respective non-inoculated controls by using the LSmeans t-test that determines when a mean is different from 0 (similar to t-test used above for nematode R_f).

A similar mixed model approach was used to examine the data (nematode count) from the transmission study. A separate one-way ANOVA was conducted for each plant and nematode species combination. Plant organ was considered the fixed effect while experimental repeat and plant within experimental repeat were considered random effects. Assumption testing and means separation were conducted as outlined above. All statistical analyses were done using SAS (v. 9.3, SAS Institute, Cary, NC).

3.4 Results

3.4.1 Host preference of *Ditylenchus weischeri* and *D. dipsaci*

Ditylenchus weischeri reproduced best on creeping thistle with this plant being the most preferred host of the plant species examined here, having a significantly higher R_f than that for other plant species and varieties examined (Figure 3.1). The R_f of *D. weischeri* on yellow pea varieties Agassiz, Golden, Meadow, and Admiral was > 1. The variety Bronco had an R_f value < 1 (Figure 3.1). However, our statistical analysis showed

that the nematode R_f of varieties Meadow, Admiral and Bronco was not different than 1. The R_f of *D. weischeri* was slightly greater than 1 (R_f = 1.17, $P < 0.0001$), when data across all five yellow pea varieties were pooled over the two experiments.

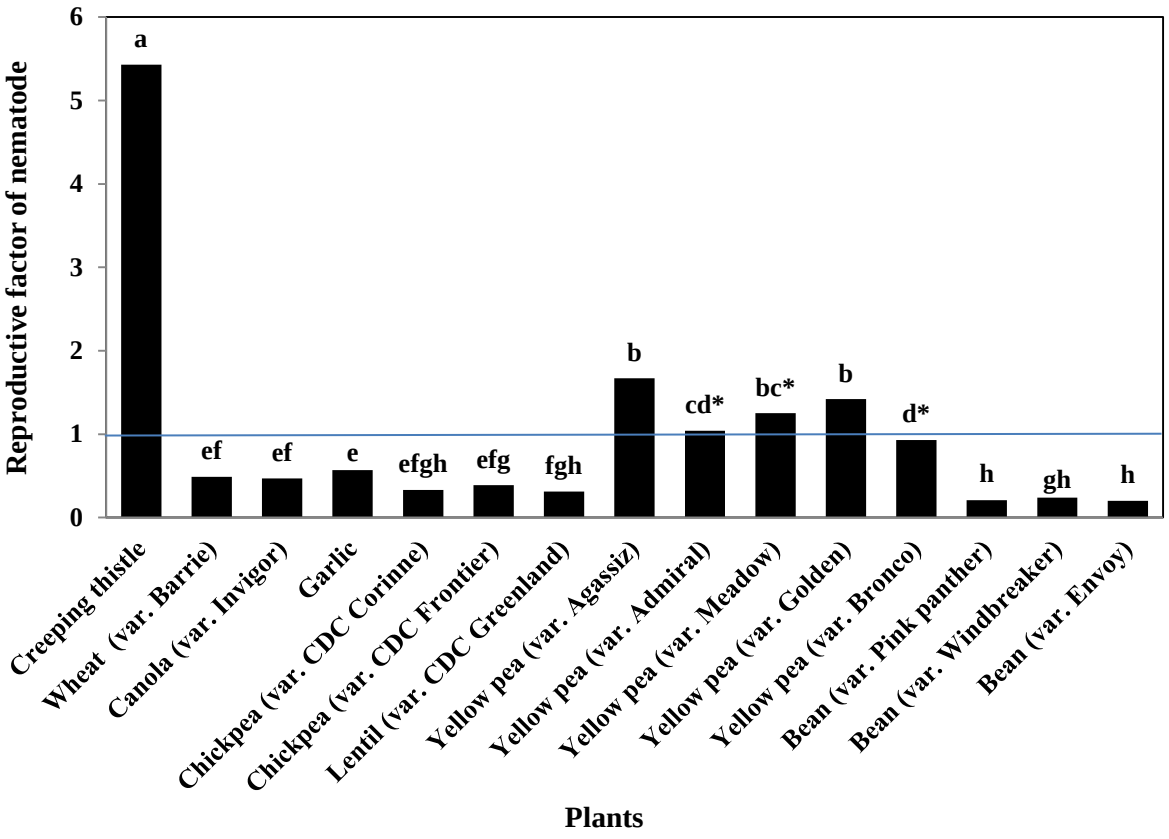

Figure 3.1 Reproductive factor values (R_f: final density/initial density ratio) of *Ditylenchus weischeri* on pulse and non-pulse crops and creeping thistle grown in pots inoculated with an initial density of 100 fourth-stage juveniles/plant in host preference study. Values are the mean of two trials with nine pots per trial (n = 18). Mean values with same letter are not significantly different according to Tukey's Honestly Significant Difference test (P = 0.05). Asterisks indicate R_f values are not different from 1 using the LSmeans t-test.

The other pulse and non-pulse crops tested, lentil, chickpea, common bean, spring wheat, canola, and garlic had R_f values < 1 ($P < 0.05$; Figure 3.1) for *D. weischeri*. Eight weeks after nematode inoculation and compared with the noninoculated control, *D. weischeri* did not affect aboveground biomass of any of the plants evaluated except creeping thistle (Table 3.1). Acid fuchsin stained eggs, juveniles and adults of *D. weischeri* were observed in the stem of creeping thistle (Figure 3.2). In the current study, symptoms of the nematode injury on creeping thistle were chlorosis, slight swelling of stems and twisting and malformation of leaves.

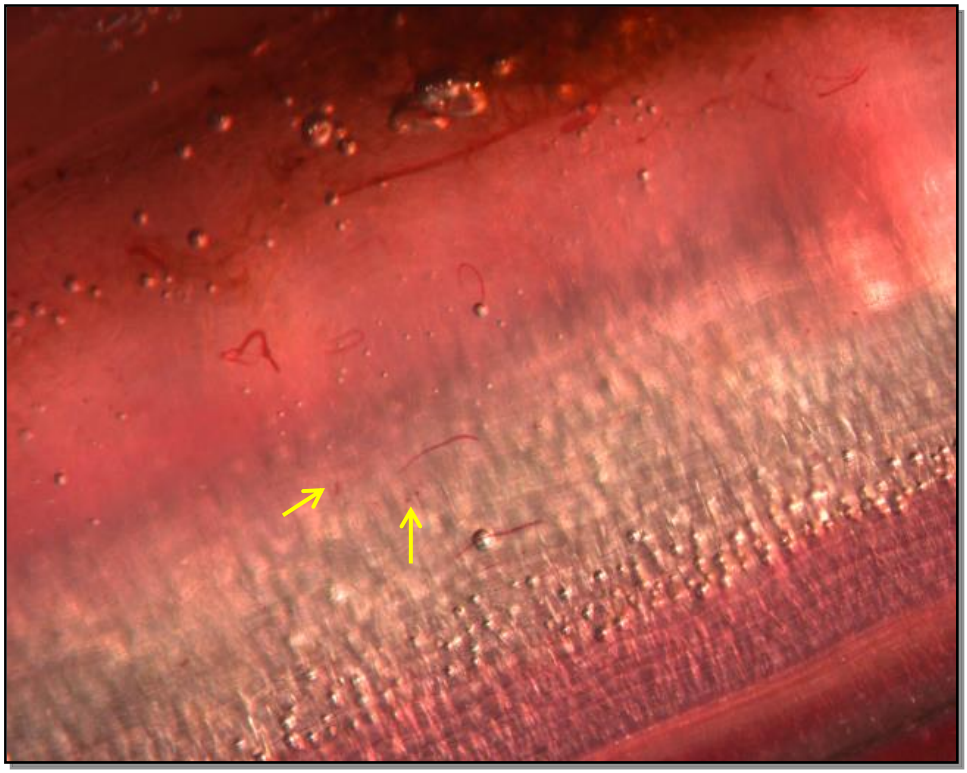

Figure 3.2 Eggs (arrows) and different life stages of *D. weischeri* in creeping thistle stem stained with acid fuchsin in host preference study.

Table 3.1 Percentage reduction compared to the control in aboveground biomass of pulse and non-pulse crops with inoculation of *Ditylenchus weischeri* and *D. dipsaci* and biomass of corresponding uninoculated controls.

Plant	Biomass of treated plants (% of control plants)		Biomass of control plants (g fresh / plant)
	D. weischeri	***D. dipsaci***	
Creeping thistle	84*[a]	94	13.9
Wheat (var. Barrie)	96	84	9.1
Canola (var. Invigor)	90	84	17.4
Garlic	92	85	19.8
Chickpea (var. CDC Corinne)	88	97	9.2
Chickpea (var. CDC Frontier)	100	78	11.3
Lentil (var. CDC Greenland)	77	76	3.1
Yellow pea (var. Agassiz)	92	88	16.4
Yellow pea (var. Admiral)	90	78*	13.7
Yellow pea (var. Meadow)	82	73*	14.1
Yellow pea (var. Golden)	90	78*	16.1
Yellow pea (var. Bronco)	93	85*	17.5
Bean (var. Pink panther)	102	82	23.4
Bean (var. Windbreaker)	92	87*	24.6
Bean (var. Envoy)	87	86*	23.8

[a] Asterisks indicate which treatments had significantly ($P < 0.05$) lower aboveground biomass weight according to Tukey's Honestly Significant Difference test compared to control.

Among the hosts examined in our study, garlic was most preferred by *D. dipsaci* with an R_f value > 6 (Figure 3.3). Yellow pea was also an excellent host for *D. dipsaci* with R_f values ranging from 2.3 to 6.9 among the varieties tested (Figure 3.3). Our results indicated that the nematode reproduction varied among yellow pea varieties (Figure 3.3). Among yellow pea varieties tested, Bronco was less susceptible to *D. dipsaci* followed by Admiral, Agassiz, Golden and Meadow in which the nematode reproduced aggressively. Under the experimental conditions, chickpea vars. CDC Corinne (desi) and CDC Frontier (Kabuli) and common bean vars. Windbreaker (Pinto), Pink panther (Kidney) and Envoy

(Navy) were rated as poor to good hosts for *D. dipsaci* (R_f > 1). However, the R_f value on Kabuli chickpea was not statistically different from 1. Although *D. dipsaci* R_f was lower than 1 in large green seed lentil var. Greenland (R_f = 0.92) and creeping thistle (R_f = 0.93), the R_f on these crops did not differ significantly from 1. The results showed that *D. dipsaci* failed to survive and reproduce in spring wheat and canola having R_f values of 0.17 and 0.18, respectively (Figure 3.3).

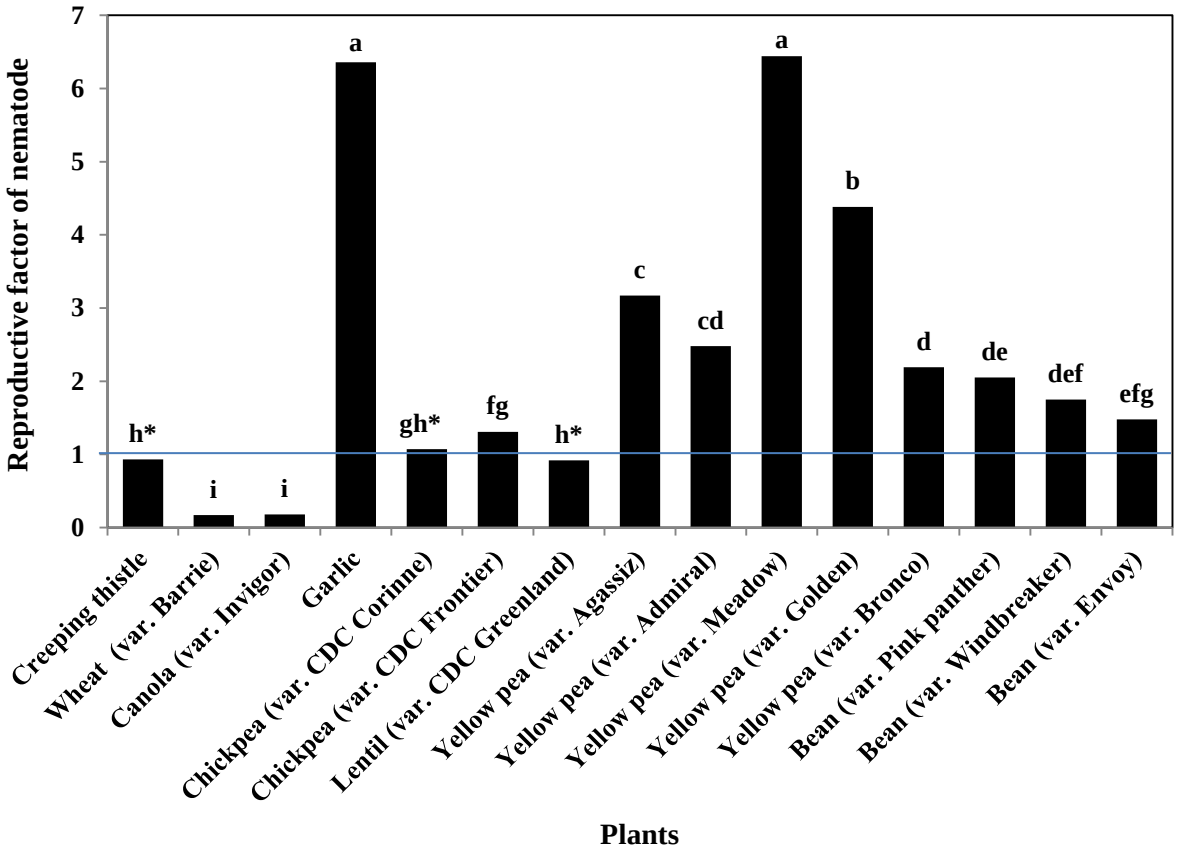

Figure 3.3 Reproductive factor values (R_f: final density/initial density ratio) of *Ditylenchus dipsaci* on pulse and non-pulse crops and creeping thistle grown in pots inoculated with an initial density of 100 fourth-stage juveniles/plant in host preference study. Values are the mean of two repeat trials with nine replicates per trial (n = 18). Mean values with same letter are not significantly different according to Tukey's Honestly Significant Difference test (P = 0.05). Asterisks indicate R_f values are not different from 1 using the LSmeans t-test.

Ditylenchus dipsaci reduced the biomass of four yellow pea (Admiral, Meadow, Golden and Bronco) and two common bean (Windbreaker and Envoy) varieties compared to the corresponding noninoculated controls (Table 3.1). No reduction in the aboveground biomass of spring wheat, canola, lentil or chickpea was observed, indicating that *D. dipsaci* was not able to affect plant performance in the current study. With regard to garlic, aboveground biomass weight was not reduced despite *D. dipsaci* reproducing well on the plant (Table 3.1). In the current study, different life stages of *D. dipsaci* stained with acid fuchsin were observed in the stem of garlic (Appendix I.1). The nematode reproduced and completed its life cycle successfully on garlic.

3.4.2 Seed borne transmission studies on *D. weischeri* and *D. dipsaci*

Ditylenchus weischeri individuals were recovered from the stems (both bottom and top half), leaves and roots of yellow pea. Densities of *D. weischeri* were significantly ($P < 0.001$; Table 3.2) greater in stem than leaves with only a few juveniles recovered from the roots of yellow pea. *Ditylenchus weischeri* failed to establish in seed-pods and seed of yellow pea var. Agassiz when the plant was inoculated with 1,000 nematodes (Table 3.2) indicating that the nematode is not a seed-borne parasite of yellow pea. Stems of creeping thistle supported the highest number of *D. weischeri* (Table 3.2). Juveniles and adults were also recovered from the roots, leaves and flower heads of creeping thistle. There was a significant difference between the number of nematode individuals in stem and leaves of creeping thistle ($P < 0.001$; Table 3.2). *Ditylenchus weischeri* was also recovered from flower heads and seed of creeping thistle. *Ditylencnus weischeri* parasitism on creeping thistle resulted in slight yellowing of leaves as well as swelling, twisting and malformation of the stems and leaves (Appendix I.2). Occasionally, some

necrotic lesions were seen on the stem of a few plants tested with many nematode juveniles recovered from these lesions when cut and soaked in tap water (Appendix I.2).

Ditylenchus dipsaci was recovered from throughout yellow pea tissues. Stems of yellow pea supported the highest number of the nematode (Table 3.2). There were significant differences in the number of the nematode between roots, stems and leaves ($P < 0.001$). More *D. dipsaci* were recovered from the bottom portion of the stem compared to top half of stem and leaves (Table 3.2). A few nematodes were recovered from roots of yellow pea. *Ditylenchus dipsaci* was recovered from seed-pods and seeds (Table 3.2). Very few *D. dipsaci* were recovered from the germ portion (7 ± 1.1 standard deviation) and storage cotyledons (11 ± 0.9) of yellow pea seeds. With regard to creeping thistle, no *D. dipsaci* individual was recovered from the seeds of the plant; however, a few nematodes were observed in the flower heads of creeping thistle (Table 3.2). In the present study, pea plants infested with *D. dipsaci* showed discoloration and deformation of leaf surfaces and margins as well as, swelling of the stems and nodes (Appendix I.2).

Table 3.2 Recovery of *Ditylenchus weischeri* and *D. dipsaci* from yellow pea (var. Agassiz) and creeping thistle in seed transmission studies.

Plant parts	Recovered nematode numbers	
	D. weischeri	***D. dipsaci***
Yellow pea		
Roots	16 d[a]	40 c
Stems (Bottom half)	544 a	981 a
Stems (Top half)	269 b	476 b
Leaves	173 c	232 b
Pods	0.0 e	20 c
Seeds	0.0 e	18 c
Total	1002	1767
Creeping thistle		
Root	53 e	24 c
Stem (Bottom half)	1854 a	367 a
Stem (Top half)	1263 b	261 ab
Leaves	516 c	171 b
Flower head	383 cd	7 c
Seed	284 d	0.0 d
Total	4353	830

[a] Values in the each column for a plant and nematode species combination followed by the same letter are not different significantly according to Tukey's Honestly Significant Difference test (P = 0.05) using log-transformed data.

3.5. Discussion

We report the first detailed examination of the host preference for the recently described stem nematode of creeping thistle, *D. weischeri*. These results provide further evidence for species distinction of *D. weischeri* from *D. dipsaci* based on very differing host preferences. This study confirmed that *D. weischeri* is capable of parasitizing creeping thistle which was expected considering the test population used in the current study was obtained from naturally infested creeping thistle in Saskatchewan and

Manitoba (Tenuta et al. 2014). Limited host preference screening of *D. weischeri* has previously been done. Chizhov et al. (2010) reported that the nematode was not able to parasitize another member of the *Allium* genus, onion. They also reported that the nematode did not reproduce on garden strawberry. Recently, the result of a host-suitability test showed that *D. oncogene*, a new stem nematode parasitizing sow thistle, does not infest broad bean (Vovlas et al. 2015). Based on the R_f values in our greenhouse study, two of five yellow pea varieties examined were very weak hosts for *D. weischeri*. Also, the range of nematode reproduction was narrow among the varieties of yellow pea tested. Yellow pea is clearly not a preferred host and it seems the nematode is capable of surviving on yellow pea and reproducing poorly on some varieties. There are no reports of *D. weischeri* or any other nematodes causing damage to yellow pea in the Canadian Prairies. This seems to be in agreement with the results of the current study of no reduction in biomass, symptoms and poor reproductive success on yellow pea varieties. However, further examination of *D. weischeri* development on yellow pea is required.

The seed-borne transmission studies showed that *D. weischeri* was unable to infest seed-pod and seed of yellow pea indicating that the nematode is not likely be dispersed through movement of harvested pea grain clean of creeping thistle seeds. In contrast, *D. weischeri* was a seed-borne pest of creeping thistle confirming previous observation (Tenuta et al. 2014). *Ditylenchus weischeri* was able to develop and increase in density in creeping thistle, causing swelling and malformation on the plant. Extensive stem swelling or galls on naturally infected creeping thistle by *D. weischeri* has been observed in Russia (Chizhov et al. 2010) and in Saskatchewan and Manitoba (our personal observations). We observed no symptom of *D. weischeri* injury on yellow pea in our seed borne

transmission study. This suggests that symptoms failed to become apparent due to very low development and reproduction of *D. weischeri* within the plant foliar tissue. However, lack of symptoms on yellow pea does not necessarily mean that nematode penetration and reproduction into plant tissues failed. Whitehead et al. (1987) observed symptomless plants such as onion, bean, potato and alfalfa though colonized by *D. dipsaci*.

The current study also reports the first detailed examination of host preference of the main crops grown on the Canadian Prairies for *D. dipsaci*. Our host preference study confirmed that *D. dipsaci* has a wide host range causing severe growth and yield reduction in many different plant species. Sturhan and Brzeski (1991) revealed the presence of several races of *D. dipsaci* which were distinguished on the basis of host response and parasite reproduction in a group of differential host plant tests. Some of these populations were later described as different *Ditylenchus* species based on molecular phylogenetic analyses. For example, a large-bodied stem and bulb nematode known as the giant race of *D. dipsaci* infecting broad bean was described as a new species, *D. gigas* (Vovlas et al. 2011).

In the present study, *D. dipsaci* was confirmed to strongly parasitize garlic with a relatively very high R_f value. This nematode has recently been reported to infest garlic fields in Québec, Ontario and the northeastern USA (Fushtey and Kelly 1975; Testen et al. 2014; Yu et al. 2010), and more recently Manitoba from seed pieces imported from Ontario (Hajihassani and Tenuta, *in press*). *Ditylenchus dipsaci* has also been reported in some alfalfa fields in British Columbia (Vrain and Lalik 1983). Our results revealed that all pulse crops tested can serve as hosts for *D. dipsaci*. In particular, yellow pea was an

excellent host for *D. dipsaci*. These results agree with other reports of yellow pea being a good host for *D. dipsaci* (Aftalion and Cohn 1990; Janssen 1994; McBurney 1981). Additionally, our results indicate the great potential for *D. dipsaci* to parasitize yellow pea and that the response of different varieties of yellow pea to this nematode species seems to be variable in terms of susceptibility suggesting that there are significant differences in the ability of the parasite to establish and reproduce in the plant tissues of yellow pea varieties. Thus, there is potential for concern for *D. dipsaci* to move from garlic fields to pulse crops, especially yellow pea and dry beans. The likely invasion of the stem nematodes would favor the mild and moist conditions on the Canadian prairies. Fortunately, except for a few market garden fields under cultivation of garlic in Alberta, Manitoba and Saskatchewan commercial production of garlic is limited on the Prairies. Nevertheless, growers should in the future avoid the possibility of yellow pea and garlic being grown near each other.

The results from the seed-borne transmission studies demonstrate that *D. dipsaci* is a seed-borne parasite of yellow pea indicating the ability of the nematode to be transmitted in harvested grain. *Ditylenchus dipsaci* has been reported to be a seed-borne nematode of many plant species including broad bean and pea (Green and Sime 1979; Hooper 1971). Therefore, surveillance for the nematode in pea and bean fields as well as outreach to garlic and pulse growers to prevent dispersal of *D. dipsaci* are recommended. We found that the amount of disease development and severity was closely related to the number of *D. dipsaci* juveniles infesting the plant. Production of symptoms due to nematode injury varies due to environmental conditions and also structures of the plant foliage (French and John 1971). Typical symptoms induced by *D. dipsaci* in legume

crops such as peas or beans have been observed as distortion and swelling of stem tissues as well as discoloration and deformation of leaves. Similar symptoms on yellow pea due to *D. dipsaci* were seen in the present study. In heavy infestations, the nematode may reduce seed viability and cause malformation and blackening of the seed coat and embryo (Bridge and Star 2007; McBurney 1981; EPPO 2008; Stoddard et al. 2010).

In conclusion, the current study provides further evidence for species differentiation of the recently described *D. weischeri* from *D. dipsaci*. The former is a specific parasite of creeping thistle and not the major pulse crops, as well as wheat and canola grown on the Canadian Prairies. However, further examination of *D. weischeri* is warranted considering the results shown here that the nematode survived or slightly reproduced, depending on yellow pea variety. *Ditylenchus dipsaci* was confirmed a parasite of varieties of yellow pea and bean grown on the Canadian Prairies. In regard to *D. dipsaci*, it is important that the recent spread of the nematode to Manitoba garlic fields because of seed pieces imported from Ontario (Hajihassani and Tentua, *in press*) be kept in check to prevent distribution to field peas.

MANUSCRIPT 2

4.0 INFLUENCE OF TEMPERATURE ON DEVELOPMENT AND REPRODUCTION OF *DITYLENCHUS WEISCHERI* AND *D. DIPSACI* ON YELLOW PEA

4.1 Abstract

The ability of the recently described stem nematode of creeping thistle (*Cirsium arvense* L.), *Ditylenchus weischeri*, to develop on and parasitize yellow pea (*Pisum sativum* L.) is uncertain. The current study examined nematode life-stage progression and generation time on yellow pea as affected by temperature with the related pest, *D. dipsaci* used as a positive control. Relationships for body length of the two nematode species and life-stage was unaffected by rearing on plant hosts compared to callused carrot disks. Plant-reared J4 individuals of both nematode species were used to determine the effect of temperature (17, 22, and 27ºC) on life-stage progression and minimum generation time with yellow pea. At 17 and 22ºC, *D. weischeri* J4 individuals progressed to only the adult stage, whereas at 27ºC, the minimum generation time from J4 to J4 was 30 days or 720 growing degree-days. The minimum generation time from J4 to J4 for *D. dipsaci* was 24, 18 and 22 days or 336, 342 and 528 growing degree-days at 17, 22 and 27°C, respectively. The results indicate development of *D. weischeri* is temperature-dependent and reproduction is unlikely on yellow pea in the Canadian Prairies where mean daily air temperatures of 27°C are rare and not sustained.

4.2 Introduction

The nematode genus *Ditylenchus* (Anguinidae, Tylenchida) has about 80 recognized species with the greatest variety of feeding habits and behaviors. Among these species, migratory endoparasites such as *D. dipsaci*, *D. destructor*, *D. gigas*, and *D. weischeri* invade either the upper (stems, leaves, flowers, seeds) or lower (roots, bulbs) parts of many species of wild and cultivated plants worldwide (Sturhan and Brzeski 1991; Plowright et al. 2002; Chizhov et al. 2010; Vovlas et al. 2011). These nematodes live in soil or dry infested plant material and penetrate plant organs via stomata, petioles, buds or epidermal fractures/wounds. Once in the host plant, the nematode individuals break down the middle lamella, cause separation of the cells, and colonize parenchymal tissues where they feed by puncturing and sucking cell contents resulting in compromised growth and secondary pathogen damage (Krusberg, 1961; Jones and De Waele 1990; Sturhan and Brzeski 1991; Duncan and Moens 2013).

The occurrence of the stem nematode *D. weischeri* Chizhov, Borisov & Subbotin parasitizing creeping thistle, *Cirsium arvense* L., on the Canadian Prairies was recently reported (Tenuta et al. 2014). Additionally, species-specific conventional and real-time PCR methods for differentiation of *D. weischeri* from the related pest, *D. dipsaci* Kuhn were established (Madani et al. 2015). Most recently, *D. weischeri* was shown to develop prolifically on creeping thistle, but not on lentil (*Lens culinaris* Medikus), chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.), garlic (*Allium sativum* L.), spring wheat (*Triticum aestivum* L.), and canola (*Brassica napus* L.) under greenhouse conditions (Hajihassani et al. 2016). However, two of five yellow pea (*Pisum sativum* L.) varieties examined were poor hosts, allowing survival but not reproduction of the

nematode. Resolving if *D. weischeri* can parasitize yellow pea is important because creeping thistle is commonly present in yellow pea fields in the Canadian Prairies. Additionally, understanding potential concern for *D. weischeri* parasitizing yellow pea under warming climate conditions is important.

Chizhov et al. (2010) reported that *D. weischeri* likely produced three or four generations on creeping thistle per season near Moscow, Russia. More is known about life-stage development and generation production of the closely related pest nematode, *D. dipsaci* (Hooper 1972; Yuksel 1960). Egg production for *D. dipsaci* occurs over seven days, second-stage juveniles (J2) emerge from eggs after two days, then molting to third-stage juveniles (J3) occurs three days later, molting to fourth-stage juveniles (J4) after four days and adults present three days later at 15°C (Yuksel 1960).

Temperature affects life-stage development and behavior of plant-parasitic nematodes with different species/races having optimum temperature for penetration (Griffith et al. 1997), feeding (Boag 1980), survival (Miyagawa and Lear 1970), and reproduction (Acosta and Malek 1979; Thompson et al. 2015). Thus, not surprising, temperature is also an important factor in controlling generation time of a nematode (Trudgill et al. 2005). For example, Mizukubo and Adachi (1997) examined the effect of temperature on egg production and generation time of *Pratylenchus penetrans* on clover roots. They reported generation times of 46, 38, 28, 26, and 22 days at 17, 20, 25, 27, and 30°C, respectively. Tenente and Evans (1998) reported the generation time of the teasel (*Dipsacus* spp.) race of *D. dipsaci* was 21 to 28 days at 20°C and 28 to 34 days at 15°C. The development and multiplication of *D. dipsaci* in white clover (*Trifolium repens* L.) and alfalfa (*Medicago sativa* L.) plants was directly related to temperature (Griffin 1968;

Griffith et al. 1997). De Waele and Wilken (1990) reported an optimal temperature of 28°C for hatching, development and egg production of the potato rot nematode, *D. destructor*. However, as temperature decreased from 28 to 16°C, generation time increased from 6 to 12 days.

Determination of juvenile life-stages of plant-parasitic nematodes is often done using morphometric measures. For example, with many nematode species, progression from J2 to J4 stages results in increasing body length (Barraclough and Blackith 1962). However, variation in morphometric measures of nematodes can vary with geographical location (Brown and Topham 1985; Townshend 1991; Khan et al. 2012), and differing hosts (Tarte and Mai 1976; Foot and Wood 1982). For instance, Foot and Wood (1982) reported that males and females of a field population of *D. destructor*, were shorter than those cultivated on fungi, *Alternaria tenuis* and *A. solani*. Therefore, relation of body length to nematode life-stages should be determined for specific rearing condition and population of plant-parasitic nematode.

In the current study, production of pure inoculums of *D. weischeri* and *D. dipsaci* J4 were required. To distinguish different juvenile stages of the nematodes it was also necessary to establish relations of the body length to juvenile life-stages. Because host or conditions under which plant-parasitic nematodes are reared can affect body size and thus juvenile life-stage identification, we tested the hypothesis that rearing *D. weischeri* and *D. dipsaci* on the live plants of creeping thistle and garlic, respectively, and callused carrot disks had no effect on life-stage morphometric measures. The purposes of the current study were therefore to determine (i) the effect of rearing conditions on morphometrics of *D. weischeri* and *D. dipsaci* development life-stages, (ii) the effect of

17, 22 and 27°C temperature on the ability of *D. weischeri* to penetrate and reproduce on yellow pea var. Agassiz, and (iii) the effect of temperature on the generation time of the nematodes on yellow pea. *Ditylenchus dipsaci* which is known to reproduce on yellow pea was used as a positive control.

4.3 Materials and Methods

4.3.1 Nematode sources and species determination

Ditylenchus weischeri was obtained from naturally infested creeping thistle in a field at the University of Manitoba Glenlea Research Station south of Winnipeg, Manitoba, Canada. *Ditylenchus dipsaci* was obtained from garlic cloves from a commercial field in southern Ontario. The nematodes were identified morphologically and morphometrically (Tenuta et al. 2014) and confirmed by conventional PCR using species-specific *hsp*90 and ITS gene primer sets (Madani et al. 2015).

4.3.2 Nematode rearing

Ditylenchus weischeri and *D. dipsaci* were reared monoxenically on callused carrot disks (Hajihassani et al. Manuscript 3). Briefly, eighty J4 nematodes were first surface sterilized in sequential solutions of streptomycin sulphate (4,000 mgL^{-1}), mercuric chloride (1,000 mgL^{-1}) and sterile distilled water. They were then pipetted to 5 mm thick callused carrot disks sterilized in 95% ethanol in Petri dishes and incubated for three months at 23 ± 1°C. To prepare uniform populations of J4 individual, *D. weischeri* and *D. dipsaci* raised on callused carrot disks were then transferred to creeping thistle and garlic plants, respectively, in a growth chamber at 23 ± 1°C and 22% relative humidity

with 16 hr light and 8 hr darkness. For this purpose, four segmented rhizomes of creeping thistle and four garlic cloves (artichoke garlic) were planted to pots (12.5-cm-diameter × 12-cm-height) filled with equal parts of autoclaved nursery soil and growing mix (Sungro Horticulture, Seba Beach, AB). About one week after planting, emerged plants were inoculated with about 1,000 fresh mixed developmental stages of *D. weischeri* or *D. dipsaci* in 40 µL 1.5% carboxymethyl cellulose (Sigma-Aldrich Co., Oakville, ON) suspension distributed to three locations 5 mm from stems and deep in soil. Plants were watered every two days until natural senescence occurred about 65 to 75 days after planting.

4.3.3 Determination of nematode life-stages and effect of rearing conditions

Ditylenchus weischeri and *D. dipsaci* were reared on callused carrot disks or on creeping thistle and garlic, respectively, as described previously. The plant pots and callused carrot disk plates were arranged in a completely randomized design with six replications, and then incubated in a growth chamber at 23 ± 1°C. The nematodes were recovered from callused carrot disks and plants using a Baermann extraction pan method (Whitehead and Hemming 1965). The technique employed pans (18-cm-diameter) with one layer of laboratory tissue (Kimwipe, Kimberly-Clark, Roswell, GA) on a steel mesh (2-mm-grid) supported by 5 mm thick plastic rings. Callused carrot disks or plant stems and leaves were chopped to approximately 0.5 to 1 cm segment lengths and placed on an extraction pan filled with tap water to just cover the plant material on the laboratory tissue. After 72 hr incubation at room temperature, the water was passed through a 500-mesh (25 µm-pore) sieve to trap vermiform development stages. Eggs were recovered by blending material remaining on the extraction pan with sucrose (30% w/v) for 30 s and

centrifugation at 1,500 x *g* for 4 min and then collected on a 625-mesh (20 µm-pore) sieve (Eisenback 2000). For each rearing treatment, 20 J2 and 30 individuals each for egg, J3, J4, male and female (170 overall individuals) of each nematode species were hand-picked from the recovered nematodes and then transferred to a drop of water on a glass slide and gently heat killed. Individuals were observed using a compound microscope (BX-51, Olympus, Tokyo, Japan). Body length, width and ratio 'a' (body length by width; Hooper 1972) of vermiform stages were determined using a digital camera (Qcolor3, Olympus) and image processing software (Image-Pro Plus 6.2, Media Cybernetics, Rockville, MD). In addition, genital primordial development (size and position of gonad, and also number of germinal nuclei) of juveniles were examined to separate nematode juvenile stages (Anderson and Darling 1964; Perry 1976). Adult individuals were differentiated by presence of a spicule or vulva. The experiment was conducted twice.

4.3.4 Effect of temperature on nematode penetration and development

Polyethylene pots (10-cm-diameter × 9.5-cm-height) filled with equal parts of autoclaved nursery soil of loam texture and growing mix of peat moss, perlite and vermicullite were prepared. Seeds of yellow pea var. Agassiz (previously shown to allow slight reproduction of *D. werscheri*; Hajihassani et al. 2016) were surface-sterilized for 5 min in 20 mL 1.0% sodium hypochlorite, rinsed in sterile distilled water and germinated on damp sterile filter paper in Petri dishes at 24°C for up to three days. When hypocotyls and roots of 2 cm in length emerged, each seed was placed 2 cm below the soil surface of one pot and 300 J4 of *D. weischeri* or *D. dipsaci* in 40 µL 1.5% carboxymethyl cellulose suspension added to the base of each hypocotyl and all covered with moistened soil. The

D. weischeri and *D. dipsaci* J4 were obtained from growth chamber grown creeping thistle and garlic plants, respectively, as described previously. The creeping thistle and garlic plants matured and senesced naturally resulting in nematode arrested development to the J4 stage which was confirmed by microscopy. Pots were then placed in three separate growth chambers (Conviron CMP3244, Convrion Products Company, Winnipeg, MB) continuously at 17, 22 and 27°C respectively, with 16 hr light and 8 hr darkness. The lower temperature was selected being similar to mean daily air temperature mid to late summer in areas where yellow pea is grown on the Canadian Prairies. The high temperature was selected because we were interested in whether *D. weischeri* could reproduce at a temperature higher than 25°C in which it was unable to previously (Hajihassani et al. 2016). In each growth chamber, groups of two inoculated plants were placed next to each other. Each pot was covered with a transparent polyethylene bag for first three days to maintain soil surface moisture. Enough pots were setup at each temperature to allow destructive sampling of six plants inoculated with either nematode every two days up to 35 days, when a new generation of J4 individuals appeared.

Light intensity at the top of plants was between 1,300 to 1,400 microeinsteins per square meter per sec in each chamber using a photometer (LI-185B, Li-Cor, Lincoln, NE) and a photosynthetic active radiation sensor (Quantum Q2597, Li-Cor, Lincoln, NE), and adjustment of lamp height. The temperature of each chamber was verified using a humidity and temperature data logger (Extech RHT10 USB Data logger, Waltham, MA) placed 20 cm above the pots and set to record every two hours throughout the experiment. The mean temperature in each of the growth chambers was 17 ± 0.6, 22 ± 0.5 and 27 ± 0.6˚C; however, the humidity was different between the growth chambers

ranging from 22 to 45%. Pots were watered lightly every other day with care to avoid drainage from pots. Aerial plant parts were misted daily with tap water to not restrict nematode migration on plant surfaces. Misting was done with care to prevent washing the nematodes off the plant surfaces. On each sampling day, six plants of each treatment group was randomly taken, three of which were harvested destructively to recover the root system; however, only the proximal 5 cm of the root system were retained. Roots and stems were rinsed with running tap water to remove soil and plant surface nematodes, and then extracted for eggs and vermiform life stages as described previously. Adults were differentiated as described previously and males and females were not counted separately. Body length of nematodes was used to determine juvenile life-stages; however, if there was any doubt about stage identification, position and state of gonads was used (Anderson and Darling 1964; Perry 1976). Penetration by the nematodes to initiate development in the host was assessed from recovery of nematodes one and five days after inoculation. We acknowledge time to complete development stages varies with individuals in a population. Further, age of J4 individuals added to yellow peas likely differed. Thus, in the current study, we report a minimum development time for progression of development stages between the first occurrence of a stage and that of the subsequent stage. Sampling for recovery of life stages occurred every two days; thus first occurrence of a stage could have happened on a day not sampled. Therefore, when two or more nematodes occurred for the first time, we assumed at least one individual would have been recovered the previous day.

The three other plants obtained on each sampling day were used to visualize nematode development stages in plant tissues. Roots were washed free of soil using tap

water, and then soaked in 1% NaOCl (w/v) for four min. with occasional hand-agitation with a final rinse of tap water. The nematodes in roots were stained by boiling for 30 sec. in 20 mL distilled water containing 300 µL of 3.5% (w/v) acid fuchsin. Sectioned stems and leaves were cleared in lactophenol (equal parts glycerol, lactic acid, phenol, and water):ethanol (1:2 v/v) for 48 hr with one change of the solution to remove pigments from the tissues. Depending on the amount of tissue, cleared stems and leaves were then boiled for 30 to 40 sec. in 20 to 30 mL distilled water respectively, containing 1 mL of 3.5% (w/v) acid fuchsin to stain nematodes. After staining, all materials were rinsed in distilled water and destained in lactophenol for 24 hr with one change of the solution. Visualization of nematode development stages in the plant tissues was done with a stereo microscope (SZ 61, Olympus, Tokyo, Japan) at 20 to 90 X magnification and images captured using a digital camera (Qcolor3, Olympus, Tokyo, Japan). Enumeration of all nematodes in plant tissues and their body length measurments presented challenges and thus visualization was for qualitative purpose to confirm penetration of tissues with the nematodes. The experiment was conducted twice.

4.3.5 Statistical analysis

To examine if the measurement values of specimens reared on callused carrot disks differed from those reared on plants, a two sample t-test assuming equal variances was performed using SAS 9.3 (SAS Institute Inc.; Cary, NC). An $\alpha \leq 0.05$ was used to infer statistical differences between comparisons.

In the temperature development study, data on the number of penetrated J4s were analyzed using a two-way ANOVA mixed model approach in SAS, where temperature, repeat of the experiment, and their interaction were considered fixed effects and

replication nested within experimental repeat, was considered a random effect. Prior to each analysis and to meet the assumption for normality of residuals, data sets were examined using the Shapiro-Wilk test and inspected for outliers. Raw data sets were also checked for homogeneity of variances based on AIC. Significant differences between treatment means were compared using Fisher's protected least significant difference (LSD) test and the pdmix 800 macro (Saxton 1998). Accumulated growing degree days was determined using the formula Degree-Days = (T- t) × DAI, where T = growth chamber temperature (°C), t = base temperature (°C), and DAI = days after inoculation (Singh and Sharma 1994). A base temperature of 3°C was used for *D. dipsaci* according to Griffith et al. (1997). The base temperature of *D. weischeri* has not been reported but lacking information to the contrary we assumed it to be similar to that of *D. dipsaci*.

4.4 Results

4.4.1 Effect of rearing conditions on nematodes body size

Juvenile stages of the nematodes obtained from creeping thistle, garlic and carrot disk were placed in respectively successive groupings of longer body size of individuals obtained from creeping thistle, garlic and callused carrot disk. Generally, the body length of juvenile stages was slightly greater for the nematodes reared on plants than callused carrot disk (Table 4.1). However, body length, except for some J2 and J3 individuals of *D. weischeri* reared on callused carrot disk and also *D. dipsaci* reared on garlic, did not overlap in measures but were distinct and easily placed into development life-stage. With regard to separation of J2 and J3 individuals, on the few occasions when body length of the stages overlapped, J2 stage was assumed to have a shorter genital perimordium than

J3 stage and contained fewer of germinal nuclei. In addition, J3 individuals were identified as having anterior and posterior gonad primordia. The body length discrimination of development life-stages obtained in this study was used in the subsequent temperature development study to assign juvenile life-stage of recovered individuals.

Table 4.1 Mean morphometric characters of *Ditylenchus weischeri* and *D. dipsaci* development stages reared on creeping thistle and garlic respectively, and both on callused carrot disks.

Assigned stage		*D. weischeri*					*D. dipsaci*				
	Measure (µm)	**Creeping thistle**		**Callused carrot disk**		**t-test[z]**	**Garlic**		**Callused carrot disk**		**t-test**
J2[v]		**mean**	**range**	**mean**	**range**		**mean**	**range**	**mean**	**range**	
	Body length	457±66[x]	309-545[y]	446±75	325-549	ns	477±63	321-568	434±68	325-537	*
	Body width	14±1	12-16	14±2	11.6-16.9	ns	15±2	12-18	15±2	12-18	ns
	Ratio 'a'[w]	32.5±3	26.1-36.9	31.3±3	26.0-25.9	ns	30.9±3	24.5-35.9	28.5±3	22.6-32.5	*
J3											
	Body length	651±56	558-750	636±51	542-755	ns	660±62	558-745	650±68	553-760	ns
	Body width	19±1	16-20	18±2	16-23	*	21±3	18-26	19±2	17-26	**
	Ratio 'a'	36.1±3	31.6-46.2	35.0±2	27.7-39.5	ns	31±2	27-36	34.1±3	29-40.1	***
J4											
	Body length	875±83	771-1025	812±40	758-971	***	900±99	768-1091	874±62	791-1034	ns
	Body width	20±2	17-23	21±2	17-24	ns	26±2	21-30	25±2	20-29	*
	Ratio 'a'	43.5±6	34.8-53.8	39.8±4	32.3-47.3	**	34±3	29.1-40.2	35.5±2	31.4-40.5	*
Male											
	Body length	1224±82	1059-1374	1139±126	946-1384	**	1331±112	1142-1521	1173±100	994-1367	**
	Body width	26.3±1	23-28	25±2	21-27	*	31±2	28-34	30±3	24-35	ns
	Ratio 'a'	47.8±2	44.3-54.0	45.4±4	36.8-52.7	*	43.1±3	36.5-50.7	39.1±4	32.6-49.9	***
Female											
	Body length	1259±74	1139-1425	1178±141	938-1410	**	1379±109	1116-1580	1218±135	1035-1490	**
	Body width	31±1	29-33	29±3	21-34	**	32±2	29-39	31±2	25-35	*
	Ratio 'a'	40.2±2	37.4-45.5	40.6±4	33.9-49.8	ns	42.7±3	37.0-47.6	39.6±4	31.8-45.6	***

[v] J2, second-stage juvenile; J3, third-stage juvenile; J4, fourth-stage juvenile.
[w] Body length to body width ratio.
[x] Each value represents the mean ± standard error (n = 30).
[y] Range (minimum-maximum) of values.
[z] Comparison of means of morphometric variables between rearing hosts using *t*-tests. ns: no significant difference; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

The resulting average body lengths of the J2, J3 and J4 life-stages of recovered *D. weischeri* were 457 (309 to 545), 651 (558 to 750) and 875 (771 to 1,025) µm, respectively, on creeping thistle and 446 (325 to 549), 636 (542 to 755), and 812 (325 to 971) µm, respectively, on callused carrot disk. Body lengths were significantly longer by 8% for J4 and 7% for adult individuals from creeping thistle than callused carrot disk. Body width of *D. weischeri* from creeping thistle was significantly and slightly greater than from callused carrot disk. Only the ratio 'a' of *D. weischeri* J4 and male individuals from callused carrot disks was less than those from creeping thistle (Table 4.1).

The resulting average body lengths of the J2, J3 and J4 life-stages of *D. dipsaci* recovered were 477 (range 321 to 568), 660 (558 to 745), and 900 (768 to 1,091) µm, respectively, on garlic and 434 (325 to 537), 650 (553 to 760), and 847 (791 to 1,034) µm, respectively, on callused carrot disk. Body lengths were significantly longer by 10% for J2 and 13% for male and female individuals from garlic than callused carrot disk. Body width was significantly wider for J3, J4, and female individuals from garlic than callused carrot disk. There wasn't a consistent effect of rearing conditions on the ratio 'a' of juvenile stages with the value being greater for J2 and smaller for J3 and J4 individuals. For adults, the ratio 'a' was significantly greater for individuals from garlic than callused carrot disk (Table 4.1).

4.4.2 Effect of temperature on nematode penetration

***Ditylenchus weischeri*.** One day after inoculation of yellow pea plants, no significant ($P < 0.05$) difference in the nematode recovery was observed among the three temperatures tested (Table 4.2). However, the nematode penetration, as apparent from the number of J4 recovered one day after inoculation was greater ($P = 0.0452$) for the repeat

trial than in the initial trial (Table 4.2). Differences between repeats may have been due to differences in soil moisture and humidity of growth chambers that was not controlled for. Five days after inoculation, only the effect of temperature ($P < 0.0044$) was significant with nematode recovery greater at 22 (36% recovery) and 27°C (31%) than 17°C (23%). Microscopic examination one day after inoculation showed that the *D. weischeri* J4s were first detected in the base of the stems (Figure 4.1A) including hypocotyl and epicotyl of infested seedlings, migrating upward (Figure 4.1B).

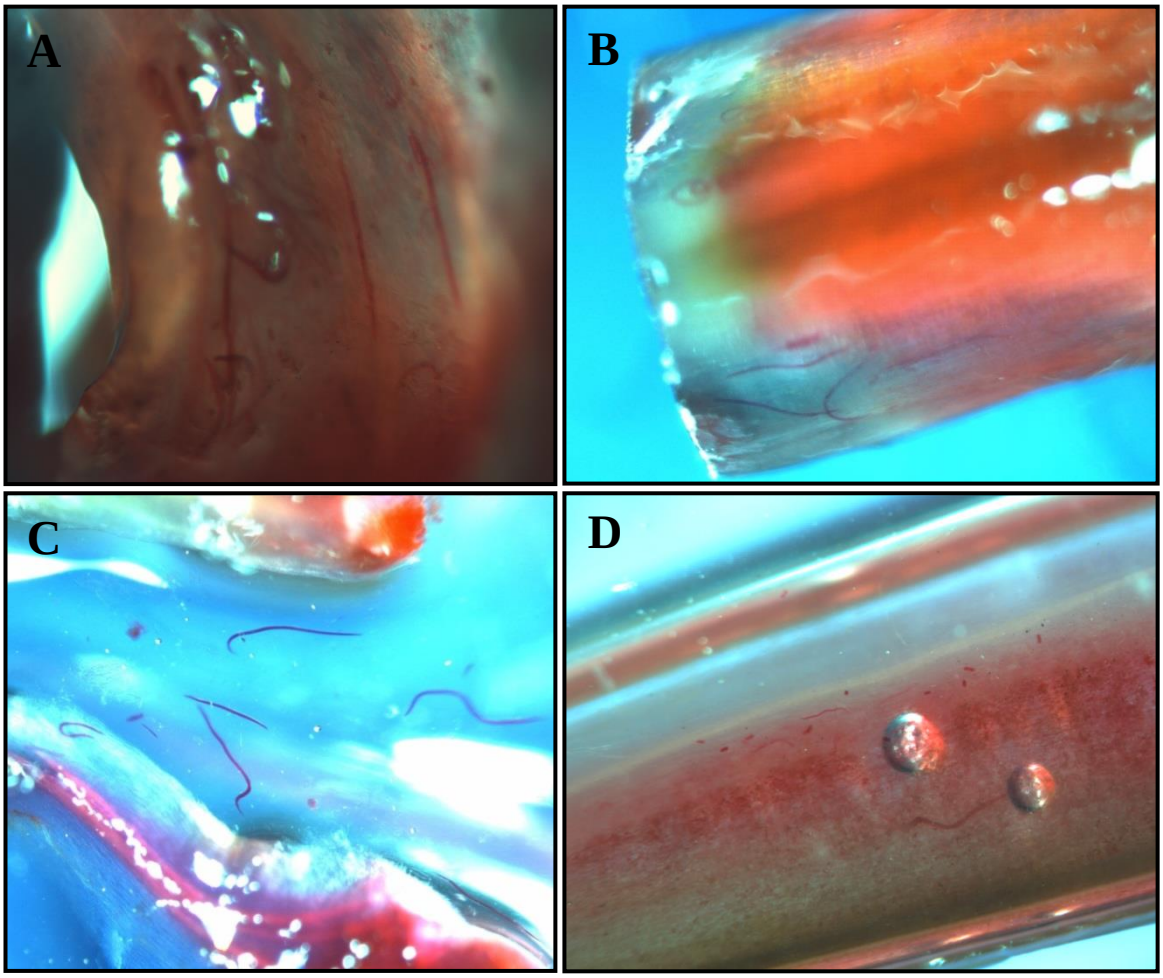

Figure 4.1 Magnified (20X magnification) view of *Ditylenchus weischeri* stained with acid fuchsin in yellow pea grown at 27˚C. Various developmental stages, including eggs (e), juveniles (j) and adults (a), of the nematode in A, base of stem, B, C, and D, in stems.

Table 4.2 Effect of temperature (ºC) on recovery of yellow pea seedling var. Agassiz by *Ditylenchus weischeri* and *D. dipsaci* fourth-stage juveniles (J4) one and five days after inoculation.

			Number of J4/Plant	
	Trial	**Temperature**	***D. weischeri***	***D. dipsaci***
1 DAI[x]				
	Both	17	42±6[y] a[z]	56±5 b
		22	50±3 a	72±4 a
		27	38±4 a	48±3 b
	1	17	35±8	53±3
		22	45±4	74±5
		27	33±5	48±6
		mean	38±5 B	58±4
	2	17	49±3	59±5
		22	56±4	71±3
		27	42±3	48±4
		mean	49±4 A	59±3
			P < F	
	Temp.[x]		0.0504	0.0103
	Trial		0.0452	0.9142
	Temp. × Trial		0.9283	0.7488
5 DAI				
	Both	17	69±3 b	120±5
		22	108±6 a	162±12
		27	94±7 a	113±3
	1	17	78±4	114±7 b
		22	108±7	192±10 a
		27	84±3	117±6 b
		mean	90±4	141±10 A
	2	17	61±3	127±10 b
		22	107±5	131±8 b
		27	105±6	110±7 b
		mean	91±4	123±12 B
			P < F	
	Temp.		0.0044	0.0002
	Trial		0.8945	0.0126
	Temp. × Trial		0.1243	0.0016

[x] DAI, day after inoculation; Temp, Temperature

[y] Each value represents the mean ± standard error of three replicates for either trial 1 and 2, and six replicates for both trials and mean.

[z] Any two means in the same column with a letter in common are not significantly different according to Fisher's protected least significant difference test ($P = 0.05$). Lowercase letters refer to temperature or trial effects but uppercase letters refer to temperature by trial interaction effect.

Ditylenchus dipsaci. Recovery of individuals one day after inoculation was higher at 22°C than the other temperatures (Table 4.2). Five days after inoculation, there was a significant temperature by repeat trial interaction. The interaction resulted because recovery increase for 22°C compared to 17 and 27°C was greater in the first trial than in the repeat trial. The interaction effect precluded establishing an effect of temperature on recovery five days after inoculation. A similar pattern was found for the effect of repeat trial as the number of recovered nematodes was numerically higher at 22 than 17 and 27°C. Microscopic examination showed that the J4s were first observed in the base of yellow pea stems and embryonic first root (hypocotyl) one day after inoculation at all three temperatures tested. Individuals did not position just beneath the epidermis of the stem, rather they resided in the stem cortex tissue closer to the vascular bundles (Appendix I.4).

4.4.3 Effect of temperature on nematode development

Ditylenchus weischeri. The number of recovered J4 individuals peaked seven and nine days after inoculation, respectively at 17 and 22°C and then declined with time. The decline coincided with a rise in adult individuals but these did not result in recovery of egg, J2 and J3 individuals from plants (Figure 4.2). At 27°C, the recovered J4 numbers peaked five days after inoculation with a rise in the recovery of adults. The peak recovery and subsequent decline of successive development stages coincided with rise in recovery of the next life-stage. Egg, J2 and J3 individuals were recovered 12, 20 and 25 days after inoculation and J4 individuals were again recovered 30 days after inoculation (Figure 4.2; Table 4.3) indicating that the minimum generation time required for *D. weischeri* to

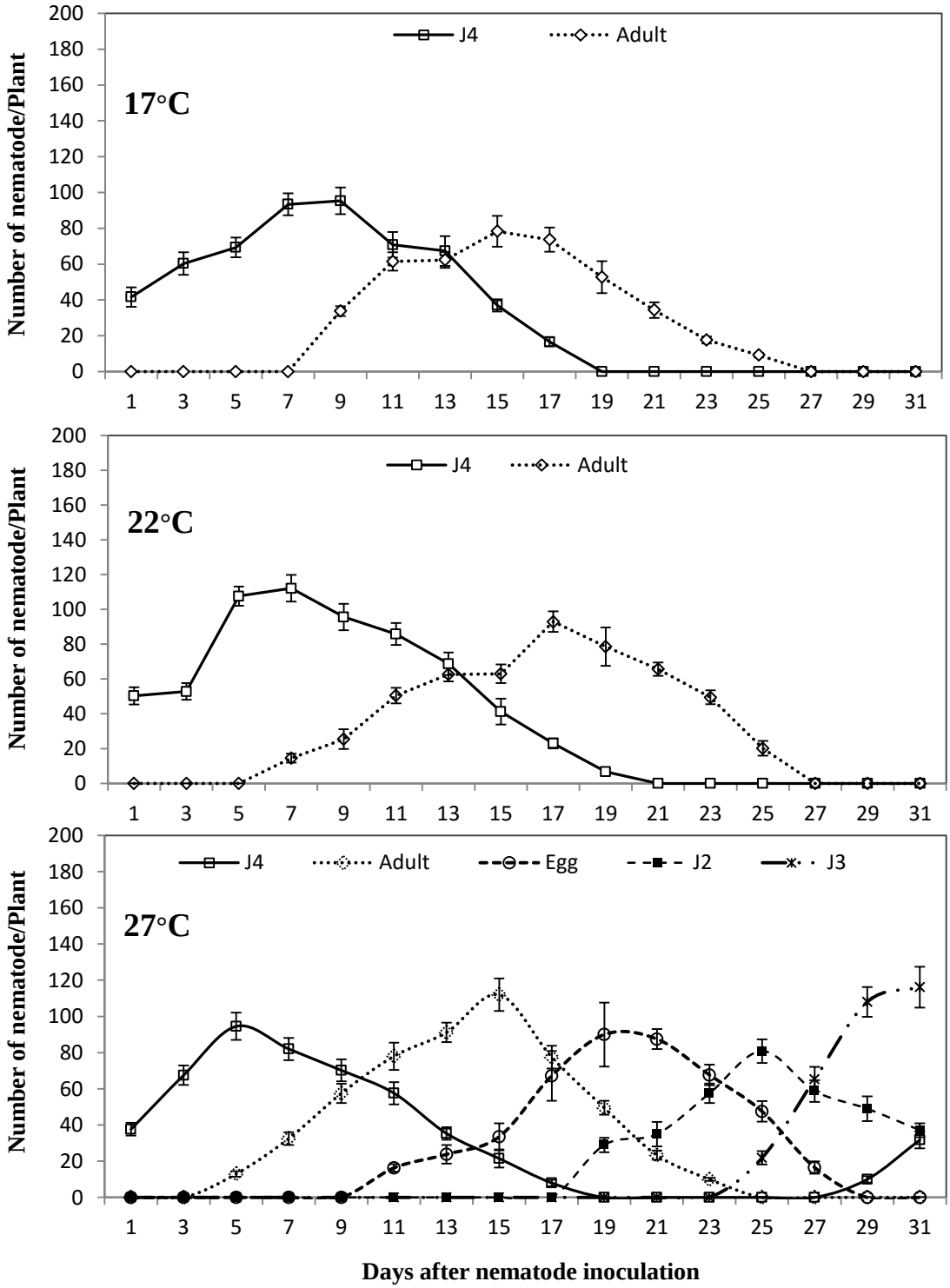

Figure 4.2 Mean number of *Ditylenchus weischeri* recovered by development stage over 31 days at three constsnt temperatures of 17, 22 and 27˚C in yellow pea var. Agassiz. J2, second-stage juvenile; J3, third-stage juvenile; J4, fourth-stage juvenile. Values are mean ± one standard error (n = 6) of the mean of temperature treatments and life stage.

complete a generation from the first observation of J4s in the plant to the J4 of the second generation was 30 days.

At 27°C, the first presence of male and female individuals in the stems (Fig. 4.1A to C) was observed about five days after inoculation (Table 4.3). Eggs were first observed in the base of the stems (Fig. 4.1A) 12 days after inoculation (Table 4.3), and then throughout stems (Figure 4.1D). The J2 and J3 life-stages were observed in plant tissues 20 and 25 days after inoculation, respectively, and the J3 developed to the J4 in about five days (Table 4.3). Total minimum accumulated degree days above the base temperature of 3°C required to obtain adult, egg, J2, J3, and J4 was 120, 1168, 192, 120 and 120 degree-days, respectively. The minimum accumulated growing degree days for *D. weischeri* to complete one generation was about 720 degree-days (Table 4.3).

***Ditylenchus dipsaci*.** The recovery of all development stages of *D. dipsaci* from yellow pea plants varied with temperature (Figure 4.3). Recovered J4 individuals peaked seven, five and seven days after inoculation, respectively at 17, 22, and 27°C and then declined with time. The greatest recovery of any development stage was for 22°C followed by 17 and then 27°C. At all three temperatures examined, greatest recovery of adults occurred 15, 9 and 13 days after inoculation (Figure 4.3). The J4 individuals from the second generation were again recovered at earliest, 24, 18 and 22 days after inoculation, respectively at 17, 22 and 27°C.

At all temperatures tested, J4 individuals were observed inside the root and base of stems; however, the number of nematodes in the stems appeared to be much greater than that in the roots. As time progressed, *D. dipsaci* migrated up from the initial inoculation point at the base of stem and infested upper regions of the stem (Appendix I.4**)**.

Table 4.3 Mean minimum development time and accumulated degree-days of nematode development stages of *Ditylenchus weischeri* and *D. dipsaci* on yellow pea grown at three temperatures.

	Temperature (˚C)					
	Minimum development time (Day)			Growing degree-days (above 3˚C)		
Developmental stage	**17**	**22**	**27**	**17**	**22**	**27**
Ditylenchus weischeri						
J4[x] to adult	8±1.5[y]	6±1.2	5±1.1	112	114	120
Adult to egg laying	-[z]	-	7±1.2	-	-	168
Egg laying to J2	-	-	8±1.2	-	-	192
J2 to J3	-	-	5±1.1	-	-	120
J3 to second generation J4	-	-	5±1.1	-	-	120
Total			30±1.1			720
Ditylenchus dipsaci						
J4 to adult	6±1.0	4±0.5	5±0.4	84	76	120
Adult to egg laying	6±1.2	4±1.2	6±1.2	84	76	144
Egg laying to J2	6±1.0	4±1.1	5±0.3	84	76	120
J2 to J3	4±1.2	3±1.2	3±1.1	56	57	72
J3 to second generation J4	2±0.9	3±0.5	3±1.1	28	57	72
Total	24±1.2	18±1.1	22±1.2	336	342	528

[x] J2, second-stage juvenile; J3, third-stage juvenile; J4, fourth-stage juvenile.
[y] Each value signifies the mean (± standard error; $n = 6$).
[z] Development did not proceed to the indicated stage.

The nematode development (progression from J4 to adults to second generation of J4) was faster at 22°C than 17 and 27°C. Similar findings were observed for the minimum time requirements for completion of each life stage (Table 4.3). *Ditylenchus dipsaci* of different developmental stages were present in various plant tissues including leaves (Appendix I.4**)**. The minimum accumulated growing degree days required for the nematode to complete a generation was 336, 342, and 528 degree-days at 17, 22, and 27°C, respectively (Table 4.3).

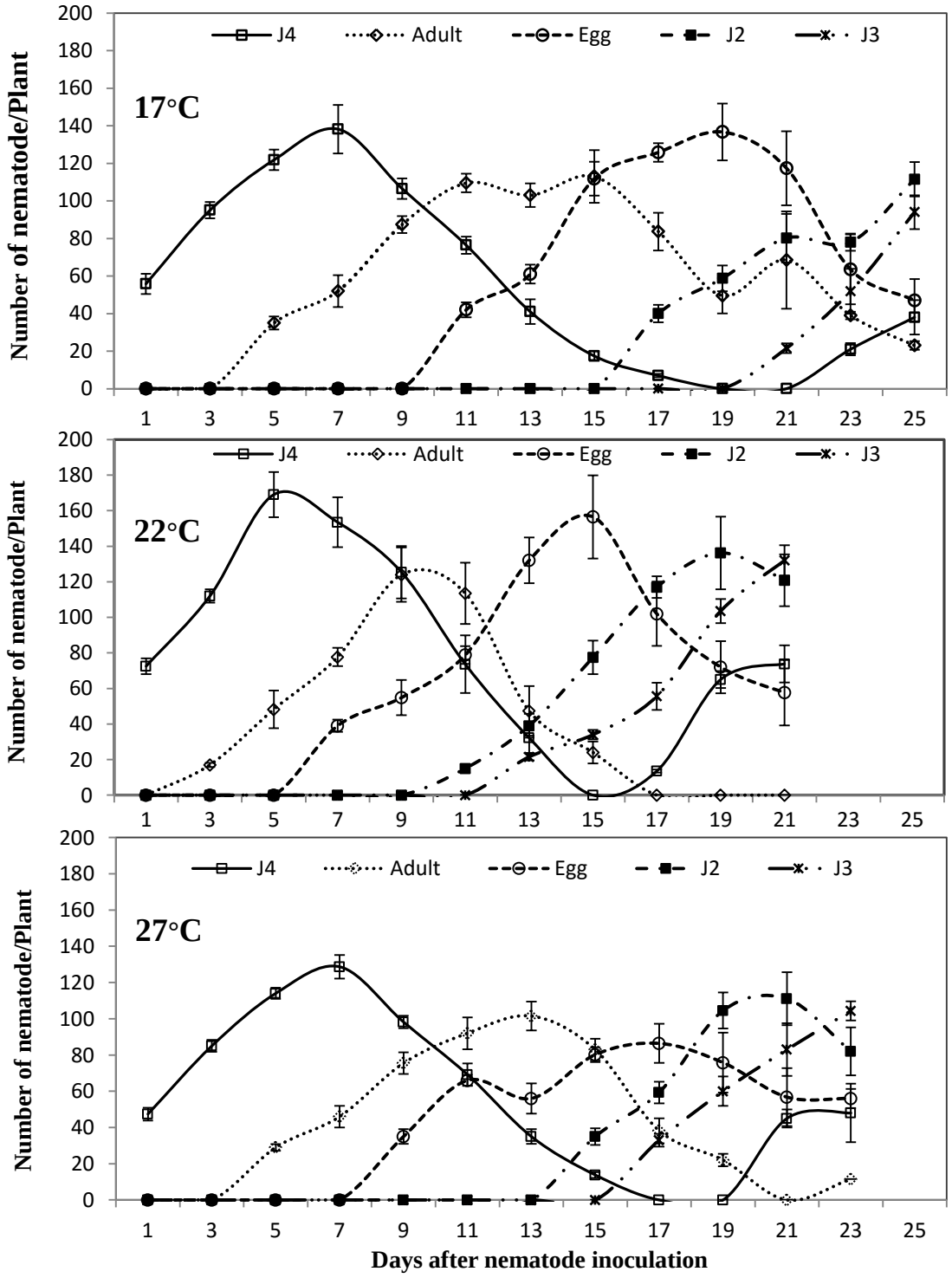

Figure 4.3 Mean number of *Ditylenchus dipsaci* recovered over 25 days at three constant temperatures of 17, 22 and 27˚C in yellow pea var. Agassiz. J2, second-stage juvenile; J3, third-stage juvenile; J4, fourth-stage juvenile. Values are the mean ± one standard error (n = 6) of the mean of temperature treatments and life stage.

4.5 Discussion

The current study demonstrated the ability to discriminate juvenile development stages of *D. weischeri* and *D. dipsaci* based on body length. This finding is similar to the observations of Barraclough and Blackith (1962) and Perry (1976) who stated that juvenile stages of *D. dipsaci* from a plant species can be separated by length alone. Also, the current study is the first examination of the effect of temperature on penetration and *in planta* development of the recently described stem nematode, *D. weischeri*.

4.5.1 Effect of rearing conditions on *D. weischeri* and *D. dipsaci* body size

In the current study, the juvenile life-stages were clearly separated by their body length, though overlap in body lengths of the J2 and J3 stages for *D. weischeri* reared on callused carrot disk and *D. dipsaci* reared on garlic were observed. Body length, body width and ratio 'a' of males and females reported here are nearly similar to those given by Tenuta et al. (2014). They reported *D. weischeri* obtained from creeping thistle to have average body length of 1,249 (range 1,244 to 1,254) µm for males and 1,202 (1,049 to 1,355) µm for females compared to 1,224 (1,059 to 1,374) µm and 1,259 (1,139 to 1,425) µm, respectively, in the current study. The adult body length difference between studies was less than 5%.

In the current study, juvenile and adult individuals of *D. weischeri* and *D. dipsaci* reared on callused carrot disks were slightly shorter and thinner than those from creeping thistle and garlic for the respective nematode. Tarte and Mai (1976) reported that *P. penetrans* from pea plants was shorter and wider than from callus tissue of alfalfa. We observed that the ratio 'a' of both nematodes was slightly less from callused carrot disks

than in plants. Others have reported variation in the body size of nematode developmental stages to be a consequence of host induced differences (Tarte and Mai 1976; Fortuner and Quénéhervé 1980; Foot and Wood 1982; Doucet et al. 2001). In the current study, host induced differences were small as body length of stages reared on plant or callused carrot disk were approximately similar. Thus, body length of the nematodes can be used with care to separate juvenile life-stages regardless of rearing condition.

4.5.2 Effect of temperature on *D. weischeri* development

Temperature plays a significant role in regulating the life-cycle of plant-parasitic nematodes, including *Ditylenchus* spp. (Griffin et al. 1997). In the current study, temperature affected the penetration of the J4, five days after inoculation. At 22 and 27°C, the increase in the J4 penetration in the first five days was likely due to migration of added J4 from the point of inoculation in soil to harvested plant components. Our finding is comparable with observations of Castillo et al. (1996) who reported that *P. thornei* penetration into chickpea roots was greater at 20 and 25°C than that at 15°C. We found *D. weischeri* adults failed to produce eggs within plant tissues at 17°C and 22°C. In contrast, nematode reproduction was observed at 27°C. In a previous host preference study, *D. weischeri* reproduced slightly on yellow pea but prolifically on creeping thistle at 25°C (Hajihassani et al. 2016). To our knowledge, this is the first report that a much higher temperature was required for reproduction of a plant parasitic nematode on one plant species compared to its preferred host. Perhaps physiological changes in yellow pea itself occurred at 27°C allowing for reproduction of *D. weischeri*. The *D. weischeri* minimum generation time was 30 days at 27°C.

The influence of temperature on nematode reproduction was not surprising as temperature is known to impact plant-parasitic nematode penetration, reproduction and survival (Griffith et al. 1997; Miyagawa and Lear 1970; Thompson et al. 2015). The results of the current study further differentiate the stem nematode species *D. weischeri* and *D. dipsaci* based on temperature requirement to parasitize yellow pea, the former requiring higher mean daily temperatures for development.

When calculating the growing degree days, knowledge of the base temperature is important (Trudgill 1995; Trudgill et al. 2005). The accumulated growing degree days are based on physiologically effective temperatures and can be helpful in estimating the occurrence of each developmental stage (Kakaire et al. 2015). In the current study, *D. weischeri* required a minimum of 720 growing degree-days at 27°C assuming a base temperature of 3°C, to complete a life cycle. Base temperatures of 3 to 10°C have been reported for several plant-parasitic nematode species (Umesh and Ferris 1992; Griffith et al. 1997; Mizukubo and Adachi 1997; Ploeg and Maris 1999). Previously, we observed *D. weischeri* on creeping thistle collected in late summer from fields in Manitoba and Saskatchewan (Tenuta et al. 2014) and reproduction by 90 days on callused carrot disks at 23°C. Further, our laboratory routinely rears *D. weischeri* on creeping thistle in growth chambers at 20 to 22°C with reproduction evident one month after inoculation. However, we have not recovered *D. weischeri* from yellow pea plants in late summer from fields (unpublished) and reproduction at 25°C on yellow pea in growth chambers is slight to none (Hajihassani et al. 2016).

The average for accumulated growing degree days, above a base temperature of 3°C (May to August) for the City of Regina, Saskatchewan, near the geographical centre

where field peas are grown in the Canadian Prairies, is 1367 degree days (using daily mean temperatures from Environment Canada 2015a). This amount is greater than the growing degree days (720) required for *D. weischeri* to complete a minimum generation time at 27°C on yellow pea obtained in this study. However, *D. weischeri* has not been recovered from yellow pea under field conditions (Tenuta et al. 2014). If the discrepancy in observations is due to selection of 3°C as the base temperature, increasing that value to match the degree days required for a minimum of one complete generation observed in our study would require an apparent base temperature of 15°C. With a base temperature of 15°C, growing degree days (May through August) for the City of Regina would be 330 which is lower than the 360 determined in this study at 27°C. However, the nematode reproduction on creeping thistle occurs readily under field conditions and in growth chamber at temperatures lower than 27°C where a base temperature of 15°C is not justified. Having such a difference in the base temperatures between two plant species suggests the hypothesis that high temperatures may effect yellow pea growth and remove the barrier to *D. weischeri* reproduction which warrants further examination. It can also be hypothesized that at high temperatures the number of male nematodes would increase allowing for higher rates of *D. weischeri* development and reproduction. Based on our visual observation, numbers of *D. weischeri* males were usually much lower than females in nematode recovery from creeping thistle or pea plants grown in field or in greenhouse. Whereas in the present study, numbers of males were considerably greater and detected earlier at 27°C than 17 and 22°C indicating that a rise in temperature may influence on males development.

Ditylenchus weischeri is in pea fields parasitizing creeping thistle in Saskatchewan and Manitoba (Tenuta et al. 2014). It may be that high sustained mean daily temperature of 27°C is required to initiate development of *D. weischeri* on yellow pea. The duration of sustained high temperatures to allow development of the nematode requires further examination. Average daily air temperatures in the pea-growing regions in the Canadian Prairies during the growing season in May, June, July, and August is 12, 17, 20 and 19°C (for City of Regina, SK; Environment Canada 2015a) but not 27°C. Therefore, it seems that mean daily temperatures on the Canadian Prairies are not conducive for *D. weischeri* development on pea crops. Indeed, there are no reports of field damage of yellow peas by *D. weischeri* as well as lack of presence on yellow pea grain from the Canadian Prairies (Tenuta et al. 2014). The Canadian Prairies are experiencing warming over recent decades and it is projected that the mean annual temperature will warm between 1 to 4°C from 2020 to 2050 (Blair 2015; Warner 2012). Even if the most pessimistic increase in temperature is achieved, monthly summer mean air temperatures are not expected to reach 27°C in this century.

4.5.3 Effect of temperature on *D. dipsaci* development

We observed that temperature affected the penetration of J4 and subsequent nematode growth and minimum generation time in yellow pea plant. Over the range of temperatures used, penetration of the seedlings by *D. dipsaci* J4 increased with increasing temperature from 17 to 22°C, but decreased from 22 to 27°C. Significantly higher penetration occurred at 22°C, with 56% penetration within five days after inoculation. Comparable observations have been reported for red clover, where the penetration rate of

D. dipsaci females increased two days after inoculation from 4% at 4°C to 12% at 20°C (Griffith et al. 1997).

In the current study, *D. dipsaci* completed its life cycle (Appendix I.3**)** on yellow pea 24, 18 and 22 days after inoculation at 17, 22 and 27°C, respectively. The development stages of *D. dipsaci* recovered from infested yellow pea at various times corresponded well to the life-cycle observations made by Yuksel (1960) and Tenente and Evans (1998). The generation time of *D. dipsaci* on onion takes between 19 and 23 days at 15°C (Yuksel 1960). The optimum temperature, as defined by Trudgill et al. (2005), is the temperature at which the generation time is shortest. Our results showed that the optimum temperature for *D. dipsaci* development on yellow pea is 22°C. At this temperature, most J4 penetration occurred; the egg production began eight days after inoculation; the maximum number of eggs recovered; and the minimum generation time was 18 days. Results of the present study on optimal temperature for *D. dipsaci* development and reproduction on yellow pea generally paralleled those found for *D. dipsaci* on onion and alfalfa. Optimum temperature for *D. dipsaci* has been reported to be 20°C on alfalfa (Griffin 1968) and 21°C on onion (Sayre and Mountain 1962). In the current study, as temperature increased, the accumulated heat units required to complete the life cycle of *D. dipsaci* increased. This may be related to greater respiration without feeding of the *D. dipsaci* (a temperate crop nematode) at higher temperature that may result in lower energy available for growth. The accumulated growing units, above a base temperature of 3°C, were 336, 342 and, 528 degree-days at 17, 22 and 27°C, respectively. These degree-day requirements are comparable to those observed with other *D. dipsaci*

populations. Yuksel (1960) reported that an onion race of *D. dipsaci* required 312 degree-days above a base temperature of 3°C to complete one generation at 15°C.

In conclusion, the current study is the first to report that body length can be used to differentiate juvenile life-stages of *D. weischeri* and that rearing on plant host or callused carrot disk did not affect life-stage differentiation of *D. weischeri* and *D. dipsaci*. The development and minimum generation time of *D. weischeri* on yellow pea differed from that of *D. dipsaci*. *Ditylenchus weischeri* completed a minimum generation in 30 days at 27°C but not 22 and 17°C, whereas *D. dipsaci* minimum generation time was more optimal at 22 than 17 and 27°C. Development of *D. weischeri* was arrested to adults with no egg production at the temperatures below 27°C. We hypothesize that growing degree days does not seem to be the reason for lack of parasitism of *D. weischeri* on yellow pea, but rather a lack of sustained high mean daily temperature near 27°C. Even with worse case scenarios for warming of the Canadian Prairies in the next decades, sustained mean daily temperatures approaching 27°C will not be achieved making development on yellow pea unlikely.

MANUSRIPT 3

5.0 MONOXENIC REARING OF *DITYLENCHUS WEISCHERI* AND MICROPLOT EXAMINATION OF THE HOST SUITABILITY OF YELLOW PEA (*PISUM SATIVUM* L.)

5.1 Abstract

Ditylenchus weischeri was recently reported in the provinces of Manitoba and Saskatchewan, Canada. Populations of *D. weischeri* from creeping thistle (*Cirsium arvense* L.) in Manitoba and *D. dipsaci* from garlic (*Allium cepa* L.) in Ontario were examined in the current study for potential to be reared on callused carrot (*Daucus carota* subsp. *sativus*) disk, alfalfa (*Medicago sativa* L.) and creeping thistle callus tissues, and pure cultures of eight fungal species. *Ditylenchus weischeri* and *D. dipsaci* could not be reared on the fungal isolates as well as the callus tissues of creeping thistle examined. In contrast to *D. weischeri*, *D. dipsaci* was successfully reared on the alfalfa callus tissue. In callused carrot disks, with no media used, an increase of 54 and 244 times the addition density of 80 nematodes was obtained for *D. weischeri* and *D. dipsaci*, respectively. Monoxenic rearing was performed using callused carrot disks to provide sufficient *D. weischeri* inoculum for the microplot study. The effect of *D. weischeri* addition on yellow pea var. Agassiz and Bronco was determined in a microplot trial using addition densities of 0, 100, 200, 400, 800, 1600, and 3200 nematodes/plant. Pea plant height and grain yield were not significantly affected by any addition densities of *D. weischeri*. However, slight reduction in above-ground biomass and pod length was observed at addition densities greater than 400 nematodes/plant, seemingly related to occurrence of

foliar fungal diseases. Recovery densities of the nematode at harvest were not significantly different than initial addition densities indicating lack of reproduction of the nematode. The results of the current study indicate that even under growth conditions similar to commercial fields, *D. weischeri* is not a parasite of yellow pea. However, further examination of the possible stimulation of foliar fungal diseases of pea by high densities of the nematode is required.

5.2 Introduction

The genus *Ditylenchus* Filipjev, 1936 is a large and geographically widespread group of migratory endoparasitic nematodes with many species affecting both agricultural crops and weeds. This group has the greatest diversity of feeding habitats in comparison with other groups of plant-parasitic nematodes. Most members are soil free-living nematodes feeding on fungi, while some are obligate parasites of plants (Sturhan and Brzeski 1991; Plowright et al. 2002; CABI 2015c). *Ditylenchus dipsaci* (Kühn 1857) Filipjev 1936 is the most prevalent plant-parasite within the genus being a destructive pest of many crops. Recently, a new species was described, *D. weischeri* Chizhov, formerly recognized as *D. dipsaci*, parasitizing creeping thistle, *Cirsium arvense* L. Scop. in Russia (Chizhov et al. 2010). More recently Tenuta et al. (2014) reported this nematode species associated with creeping thistle seeds and above-ground tissues from the provinces of Saskatchewan and Manitoba in Canada.

The understanding of the plant host range of *D. weischeri* is limited. Results of greenhouse assays showed creeping thistle to be a host (Hajihassani et al. 2016) and lentil (*Lens culinaris* L.), chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.), garlic (*Allium sativum* L.), spring wheat (*Triticum aestivum* L.), canola (*Brassica napus*

L.), onion (*Allium cepa* L.) and strawberry (*Fragaria* × *ananassa*) not to be hosts (Chizov et al. 2010; Hajihassani et al. 2016). Recently, under greenhouse conditions, we reported that *D. weischeri* reproduced slightly (1 < reproduction factor < 2) on two (Agassiz and Golden) of five yellow pea (*Pisum sativum* L.) varieties without producing any symptoms on the plants (Hajihassani et al., 2016). We recovered *D. weischeri* from stems and leaves of plants, indicating that the nematode was not a seed-borne parasite of yellow pea. In another study, *D. weischeri* failed to complete its life cycle on yellow pea and its development arrested at the adult stage with no eggs produced at 17 and 22°C (Hajihassani et al. Manuscript 2). Host screening studies with *D. weischeri* have been done to date under greenhouse conditions. Under commercial field grown conditions, canopy closure can increase humidity and inter-plant contact possibly increasing parasitism success (Sun et al. 2009). Thus, where a plant is a weak host under greenhouse conditions it cannot be ruled out to not be a good host under field conditions. In a previous greenhouse screening study, we examined the effect of only one density (100 nematodes/plant) of *D. weischeri* addition on yellow pea. It is unknown if addition density (Mwaura et al. 2015; Yavuzaslanoğlu et al. 2015) can determine the ability of *D. weischeri* to reproduce on yellow pea. Additionally, it is also important to examine the effect of increasing nematode density under field conditions where plant growth could be impacted by several biotic (such as soil and foliar fungal pathogens) and abiotic (such as temperature) factors that may affect both the nematode development and plant performance indicators.

Host screening studies of *D. weischeri* to date have also used nematodes reared on greenhouse-grown creeping thistle (Hajihassani et al. 2016). Rearing on creeping thistle

requires a great deal of planning, labour and resources for mass production and extraction from a lot of plant tissues. Further, nematodes other than *D. weischeri* are also recovered, complicating the ability to challenge potential hosts with the single species. Mass rearing of *D. weischeri* under monoxenic conditions may perhaps provide an easier, cheaper and reliable means of producing *D. weischeri* for host screening studies than rearing on creeping thistle.

There are many different techniques to rear plant-parasitic nematodes monoxenically which include *in vitro* procedures such as fungal cultures as well as a variety of callus tissues derived from carrot (*Daucus carota* subsp. *Sativus* (Hoffm.) Schübl. & G. Martens), alfalfa (*Medicago sativa* L.), onion (*Allium cepa* L.), clovers (*Trifolium repens* L.), and corn (*Zea mays* subsp. *mays* L.). A number of studies have reported species of *Diytylenchus* such as *D. destructor*, *D. angustus* and *D. triformis* feeding and reproducing on fungal hyphae in culture (Faulkner and Darling 1961; Hussey and Krusberg 1971; Latif and Mian 1995). Callus induction from carrot disks has been used to rear migratory endoparasitic nematodes such as *Pratylenchus vulnus* (Moody 1973), *P. thornei* (Hajihassani et al. 2013), *Radopholus similis* and *Zygotylenchus guevarai* (Verdejo-Lucas and Pinochet 1992), and *D. dipsaci* (Kühnhold et al. 2006). Alfalfa callus tissue culture has been used for mass production of many plant-parasitic nematodes including *D. dipsaci*, *P. penetrans* and *P. zeae*, *Bursaphelenchus lignicolus*, and *Aphelenchoides ritzemabosi* (Krusberg 1961; Riedel et al. 1973; Tamura and Mamiya 1975; Verdejo-Lucas and Pinochet 1992).

The current study examined the host suitability of yellow pea to *D. weischeri* under field grown conditions using microplots. In order to produce enough inoculum to

challenge seedlings with *D. weischeri* alone, methods for monoxenic rearing (fungal cultures, callus of alfalfa and creeping thistle, callused carrot disk) of the nematode was first explored. As greenhouse host screening studies used the closely related *D. dipsaci* for comparison, the rearing methods were also examined using this nematode. Then, host suitability of yellow pea under field conditions was examined in a microplot study using different addition densities of monoxenic reared *D. weischeri* individuals. In the host challenge study, *D. dipsaci* was not used to avoid spreading an economically important nematode not prevalent in Manitoba.

5.3 Materials and Methods

5.3.1 Nematode source and species determination

Ditylenchus weischeri was collected from stems of naturally infested creeping thistle plants growing in fields at the University of Manitoba Glenlea Research Station, Winnipeg, Manitoba. *Ditylenchus dipsaci* was obtained from infested garlic bulbs obtained from commercial fields in southwestern Ontario. Species identification was confirmed using morphological and morphometric characters of adult individuals (Tenuta et al. 2014) and species-specific primers (Madani et al. 2015).

5.3.2 Nematode rearing study

5.3.2.1 Preparation of nematodes

Vermiform individuals of *D. weischeri* and *D. dipsaci* were extracted from infested plant tissues using a Baermann pan method (Whitehead and Hemming 1965). The plant parts were cut into small pieces and spread onto laboratory tissue papers (Kimwipe,

Kimberly-Clark, Roswell, GA) on a metal mesh (700-µm-screen size) in a plastic pan supported by 5 mm thick plastic rings. Tap water was added to pans to just cover the plant material and then pans were incubated for 74 hr at room temperature. Thereafter, the nematode suspensions were passed through a 25-µm- mesh size screen, collected in glass conical tubes with tap water and then centrifuged at 1500 x *g* for 3 to 4 min. The supernatant was carefully discarded and nematodes transferred to sterilized micro-centrifuge 2 mL tubes containing disinfectant solutions; streptomycin sulphate (Sigma-Aldrich, St Louis, MO; 4000 mg/liter) overnight then mercuric chloride (Fisher Scientific, Fair Lawn, NJ; 1000 mg/liter) for 10 min at 4°C and then rinsed with sterile distilled water three times. Nematodes were kept in the water at 4°C until use.

5.3.2.2 Fungal cultures

Pure cultures of the plant pathogens, *Botrytis cinerea, Fusarium solani*, *Rhizoctonia solani, Verticillium dahliae, Sclerotinia sclerotiorum, Cladosporium cucumerinum, Colletotrichum gloeosporioides*, and *Chaetomium* spp. (isolated from creeping thistle leaves) were examined for ability to rear *D. weischeri* and *D. dipsaci*. A plug of each fungal culture was transferred onto potato-dextrose agar (BD Difco, Becton Dickinson and Company Canada, Mississauga, ON) in 9 cm diam. petri dishes and kept in the dark at 24°C for 7 to 10 d. When mycelium covered the petri plates, 80 surface-sterilized J4 individuals of *D. weischeri* or *D. dipsaci* in 100 µl sterile water were pipetted onto plates of fungal culture and lids sealed with laboratory film (Parafilm; Bemis, Oshkosh, WI). Prior to addition, the nematode suspension was microscopically examined to confirm all nematode individuals were J4. The petri plates were then placed in an incubator at 23 ± 1°C in the dark for 45 d (Figure 5.1A).

5.3.2.3 Callus of creeping thistle

Creeping thistle seeds are difficult to germinate, being largely non-viable. Thus, roots of creeping thistle were used to obtain plant material to establish callus tissue. Small pieces of creeping thistle rhizome were surface sterilized in 1% (v/v) NaOCl solution and planted in polyethylene pots containing equal parts of sterilised clay and peat. After four weeks incubation in a growth chamber at 23°C, the young plants were harvested, leaves were detached and stems chopped to 2 to 3 cm sections and surface-sterilized in 200 mL NaOCl solution supplemented with three drops of surfactant (Tween-20, Fisher Scientific Canada, Ottawa, ON) while agitated using an orbital shaker for 10 min. The stem pieces were then dipped in 95% ethanol for 15 to 30 sec, rinsed several times with sterile distilled water, and dried on sterile paper towel. Stem pieces were then placed on solid Murashige and Skoog basal salt mixture with Gamborg′s vitamin powder (MS, 4.4%, Sigma-Aldrich Company Canada, Oakville, ON), 1 mL/liter 2,4-Dichlorophenoxyacetic acid (2,4-D; Nufarm, Calgary, AB), 0.5 g/liter 2-(N-Morpholino)ethanesulfonic acid (MES, Sigma-Aldrich Company Canada), 1 mg/liter kinetin (Sigma-Aldrich Company Canada), 30 g/liter sucrose (Sigma-Aldrich Company Canada), and 8 g/liter micropropagation agar (pH 5.8, Caisson Laboratories Inc., Smithfield, UT) in deep Petri dishes (6 cm x 2 cm diam.). The cultures were incubated at 24°C for 20 to 30 d. Some cultures of creeping thistle callus were contaminated with bacteria and fungi thus discarded. Eighty surface-sterilized J4 individuals of *D. weischeri* or *D. dipsaci* in 100 µl sterile water were pipetted onto plates of callus tissue (Figure 5.1B), and the lids were sealed with laboratory film and incubated at 23 ± 1°C in the dark for three months.

5.3.2.4 Callus tissue of alfalfa

Seeds of alfalfa var. Algonquin were surface-sterilized in 100 mL 1% NaOCl solution containing three drops of Tween-20 placed on an orbital shaker for 30 to 40 min. and then in 95% ethanol for 15 to 30 sec. Thereafter, the seeds were rinsed with sterile distilled water and placed aseptically in petri dishes containing MS medium supplemented with 1 g/liter sucrose and 8 g/liter micropropagation agar, then incubated at 24°C. After 3 to 5 d, when the seed radicles were 2 cm long, seeds were transferred to magenta boxes (Sigma-Aldrich Company Canada) containing shoot elongation media of MS medium supplemented with 0.5 g/liter MES, 3 g/liter sucrose, 7 g/liter micropropagation agar, and 50 mg/liter kanamycin (Caisson laboratory, Inc. North Logan, UT). The boxes were incubated for approximately 10 d at 24°C. Thereafter, alfalfa seedlings free of microbial contamination were removed from the media, the root system detached and seedlings chopped to 2 to 3 cm sections under sterile conditions. Stem and leaf pieces were placed on MS medium as described previously and then incubated for 20 to 30 d. Eighty surface-sterilized J4 individuals of *D. weischeri* or *D. dipsaci* in 100 µl sterile water was pipetted onto callus and lids sealed with laboratory film (Figure 5.1C). Cultures were incubated in the dark at 23 ± 1°C for three months.

5.3.2.5 Callused carrot disk

Callused carrot disks were prepared as described by Kaplan and Davis (1990), but with some modifications. The modifications included substitution of gentamycin, tetracycline, and chlorhexidine digluconate by streptomycin sulphate and mercuric chloride, and that no nutrient agar was used in this procedure for inducing callus and

maintaining moisture in petri dishes. Our previous attempt to rear *D. weischeri* on callused carrot disks placed on nutrition agar in petri dishes with 20 to 30 nematode individuals was unsuccessful, perhaps because of contamination of callused carrot disks with bacteria that grew on nutrient agar and ruined the carrot disks. Fresh grocery store carrots were thoroughly washed with tap water, surface sterilized in a 6% (v/v) NaOCl solution for 2 min, peeled, and soaked in 95% ethanol for 15 min. Thereafter, the outer surfaces were burned thoroughly on a flame, peeled, and sliced into 4 to 5-mm-thick disks. One disk was placed with forceps into 35-mm-diam. petri dishes and then incubated in the dark at 23 ± 1°C for 12 to 18 d until the formation of callus on the surface of disks. During this time, disks contaminated with bacteria and fungi were discarded. Eighty fresh surface-sterilized J4 individuals of *D. weischeri* and *D. dipsaci* in 100 µl sterile water were transferred to the margins of callus produced on the carrot disks (Figure 5.1D), and lids sealed with laboratory film to prevent moisture loss. The disks were incubated at 23 ± 1°C in the dark for three months before extracting the nematodes as described previously. For each rearing technique, two experiments were conducted in a completely randomized design with 10 replications.

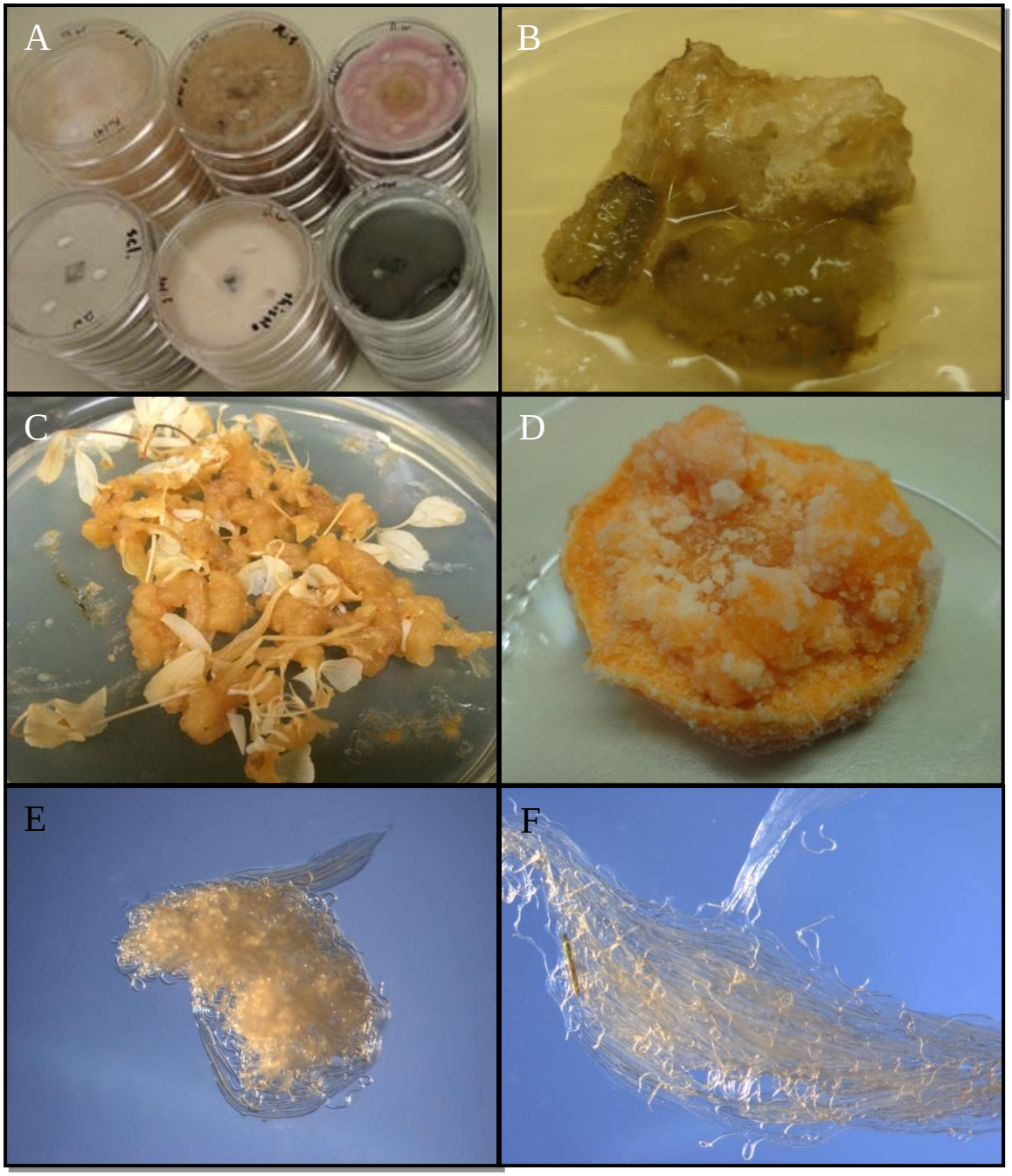

Figure 5.1 Fungal cultures (A), callus tissues of creeping thistle (B) and alfalfa (C), and callused carrot disk (D) added with *Ditylenchus weischeri* or *D. dipsaci*, aggregation of *D. weischeri* (E) and *D. dipsaci* (F) on surface of inside lids of callused carrot disk cultures (85x magnification).

5.3.3 Effect of *D. weischeri* on growth and yield of yellow pea in microplots

A microplot study was conducted at the University of Manitoba Point Research Station in Winnipeg, Manitoba to determine the effect of different population densities of *D. weischeri* on growth and yield of field-grown yellow pea. Two varieties of yellow pea, Agassiz and Bronco, were examined having previously the greatest (1.6) and lowest (0.9) Rf value for *D. weischeri*, respectively, of five varieties examined under greenhouse conditions (Hajihassani et al., 2016). A 4 x 8 m plot was used in the current study. Soil was of the Blacklake series of pH 7.4, electrical conductivity 0.40 (dSm^{-1}), 6.7% organic matter and silt loam in texture (Manitoba Agriculture, Food and Rural Development 2010). Five soil samples were taken from the section and were negative for *D. weischeri* using a Baermann pan extraction method.

The study was arranged as an RCBD split-plot experiment where pea variety was the main plot and nematode addition densities were the sub-plots. Soil was tilled on June 1, 2015 to 20 cm depth using a small rotary tiller. Each plot was 1 x 1 m. Seed surfaces were sterilized using 1% (v/v) NaOCl for 5 min and were planted by hand to a 3 cm depth at a rate of 80 seeds per square meter on June 1 2015, equivalent to a commercial seeding density of approximately 65 kg seeds per hectare. Seed spacing within each row was 7 cm and row spacing was 20 cm, being typical for commercial fields in Prairie Canada (Saskatchewan Pulse Growers 2015). Commercial inoculant of N-fixing bacteria, *Rhizobium leguminosarum* (BASF AgSolutions, Mississauga, ON) was added to each planted seed to ensure proper nodule formation and optimum growth of peas. Randomly selected plants for addition with the nematode had 7 cm diam. x 12 cm long PVC tubes inserted around a seed with the tube protruding 2 cm above the soil surface (Figure

5.2A). Tubes were used to prevent movement of the added nematodes away from root zone of target seedlings, for example by rainfall.

Ditylenchus weischeri added to emerged yellow pea seedlings were obtained from callused carrot disks as described previously. The plots were hand watered one d before nematode addition and then selected seedlings were treated with one of seven population densities; 0, 100, 200, 400, 800, 1600, and 3200 nematodes/plant mixed vermiform life-stages of the nematode/plant in 50 µl distilled water. The nematode suspensions were pipetted into the soil just beside the stem of each seedling. After nematode addition, the top part of a slightly transparent 2 L soft-drink bottle was placed on each PVC collar for 2 d to prevent soil drying and sunlight stressing the nematodes as well as increase humidity to encourage association with the seedling. The experimental area was enclosed in a 2.5 m mesh screen and 1.5 meter snow fence to prevent deer grazing of the yellow pea (Figure 5.2A).

Rosettes of creeping thistle naturally growing around the experimental site were added with *D. weischeri* as positive controls. Some of the plants had been examined for the nematode and were negative. Three creeping thistle plants were tested with either of three population densities of the nematode; 0, 100, and 3200 per plant as described previously. Mean daily air temperature and total daily precipitation was monitored 200 m from the study site at a weather station using a shielded temperature/relative humidity probe (HMP50, Vaisala, Vantaa, Finland) and tipping bucket rain gage (TE525M, Texas Electronics, Dallas, TX).

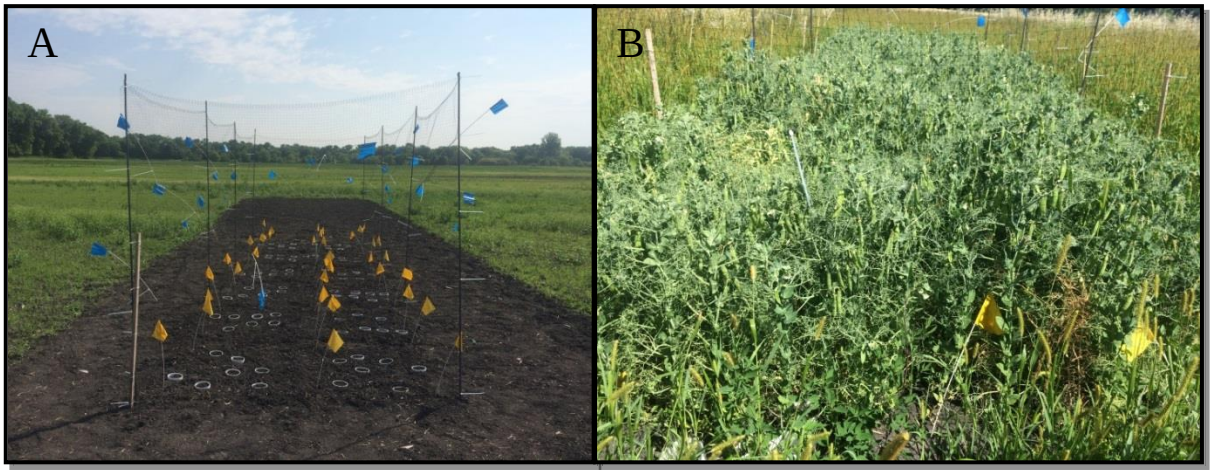

Figure 5.2 The experimental field site of yellow pea seeds planted in microplots and added with different addition densities of *Ditylenchus weischeri* (A) and plants at pod-filling stage (B).

5.3.4 Assessment procedure

At the end of each experiment, the fungal, callus tissue and callused carrot disk cultures were chopped into small pieces and vermiform life stages of the nematodes were recovered by using the Baermann pan method described previously. Nematode eggs were recovered by sucrose flotation (Eisenback 2000). The cultures were blended with 30% (w/v) sucrose solution for 10 to 15 sec and centrifuged at 1500 x *g* and the supernatant was passed through a 149 µm-pore sieve over a 20 µm-pore sieve. The number of eggs, juveniles, and adults of either *D. weischeri* or *D. dipsaci* were counted using a compound microscope at 40x and either the whole suspension examined in a gridded petri dish or for large populations, using aliquots of the suspension with 1 mL nematode counting slide.

In the microplot study, plant height (cm), plant aboveground biomass (g), seed pod length (cm), and grain yield (g), were measured and recorded 88 d after planting (end of

August 2015) when plants senesced naturally (Figure 5.2B). Plant height was measured from the soil surface to the plant apex. At harvest, plants were cut at the soil surface and above-ground dried biomass was determined. Average seed pod number in each microplot was recorded and seed weigh determined. The final population of *D. weischeri* was determined by counting the number of nematode individuals extracted from chopped aerial parts (stems and leaves), as well as the seed-pods and seeds of each plant using the Baermann pan method, as described previously. Similarly, three to four non-treated plants around each treated plant were examined for transmission of the nematode from infested plants. The final number of recovered nematodes was counted and then the reproduction factor (Rf) of *D. weischeri* in each treatment was determined by dividing the number of nematode recovered at the end of the experiment by the addition density at the experiment start.

5.3.5 Statistical analysis

Statistical assessments were performed using a mixed model analysis of variance in SAS 9.3 (SAS Institute Institute, Cary, NC). Prior to analysis, the assumptions for normality of residuals were tested using the Shapiro-Wilk test and homogeneity of variance was examined based on Akaike's information criterion and corrected when necessary. To meet the assumption of normality, nematode counts were log-transformed before analysis. For the rearing study, data for the two repeat trials for each rearing technique were pooled as there was no difference between means of repeats and no interaction effect with nematode addition ($P > F = 1.0$). Effect of rearing method on nematode recovery was done with rearing technique considered as a fixed effect while replication and repeat trial as random effects. In the microplot field study, pea varieties

(main plot) and addition density (subplot) were fixed effects, while block (replication) was a random effect. When treatment means were significant at $P < 0.05$, Tukey's Honestly Significant Difference test and the pdmix 800 macro (Saxton 1998), were used for means comparison.

In the microplot field study, the relationships between plant performance indicators and final number of recovered nematodes were checked using regression analysis. Since the regression analysis did not yield significant slopes between the recovered nematode densities and plant performance indicators, least squares means (LSmeans) *t*-tests were used to examine if any Rf means were significantly different from 1. For this purpose, the data were log-transformed and individual LSmeans *t*-tests indicating whether a mean was significantly different from zero were used; zero was used because being equal to the base 10 logarithm of 1. Thus, significant *t*-tests that treatments mean was significantly different from 0 indicated that the Rf deviated significantly from 1.

5.4 Results and Discussion

5.4.1 Nematode rearing study

5.4.1.1 Rearing *D. weischeri* and *D. dipsaci* on fungi

Neither nematode species increased their numbers on *F. solani, B. cinerea, R. solani, V. dahliae, S. sclerotiorum, C. gloeosporioides, C. cucumerinum,* and *Chaetomium* spp. compared to addition density used. The individuals recovered from fungal cultures were J4 and adult stages indicating that nematodes had no reproduction by the time of nematode extraction (Tables 5.1,2). Furthermore, no live *D. weischeri* were recovered from *V. dahliae* and *S. sclerotiorum* cultures, as well as *D. dipsaci* from

Chaetomium spp. cultures (Tables 5.1,2). Although slight multiplication of a garlic population of *D. dipsaci* on *V. theobromae* and *Caldosporium* spp. has been reported (Viglierchio 1971), other studies noted that fungi are unsuitable to rear *D. dipsaci* (Hooper and Southey 1978; Tenente et al. 1995). In general, our results indicated that the feeding behaviour and multiplication of *D. dipsaci* and *D. weischeri* are different from other *Ditylenchus* species which are capable of being reared on fungi.

5.4.1.2 Rearing *D. weischeri* and *D. dipsaci* on callus tissue of creeping thistle

Most of the callus tissue cultures of creeping thistle were contaminated with bacteria and fungi. The cultures that were free of microbial contamination did not result in an increase in recovered numbers of *D. dipsaci* or *D. weischeri* (Tables 5.1,2).

TABLE 5.1 Mean number (± standard error) of *Ditylenchus weischeri* recovered from callused carrot disk, callus tissues of alfalfa and creeping thistle after 90 d as well as fungal cultures after 45 d having been inoculated with 80 individuals.

Procedure	Eggs/ culture	Juveniles[a]/ culture	Adults/ culture	Total number/ culture
Callused carrot disk	616±20	3,294±41	439±16	4,351±66a
Alfalfa callus tissue	0	25±3	17±2	42±5b
Creeping thistle callus tissue	0	18±3	15±1	33±5b
Fungal cultures				
Fusarium solani	0	8±3	11±1	19±5b
Botrytis cinerea	0	18±3	20±2	38±6b
Rhizoctonia solani	0	15±2	8±1	23±4b
Verticillium dahliae	0	0	0	0x
Sclerotinia sclerotiorum	0	0	0	0[y]
Cladosporium cucumerinum	0	27±3	14±2	41±7b
Colletotrichum gloeosporioides	0	14±3	8±1	22±5b
Chaetomium spp.	0	12±2	5±1	17±4b

[x,y] Nematode recoveries of zero were excluded from statistical analysis. Means (n = 20) with the same letter are not different significantly according to Tukey's Honestly Significant Difference test ($P = 0.05$).

5.4.1.3 Rearing *D. weischeri* and *D. dipsaci* on alfalfa callus tissue

No increase in the recovery of *D. weischeri* was observed when the nematode was raised on alfalfa callus tissues. Some live individuals of J4 were recovered from the callus tissues indicating that *D. weischeri* was capable of surviving after three mon of incubation (Table 5.1).

Alfalfa callus tissue added with *D. dipsaci* resulted in recovery of 67 times the number initially added. The proportion of eggs, juveniles (second, third and fourth-stages), and adults (males and females) was 23%, 62%, and 15%, respectively (Table 5.2). Mass production of *D. dipsaci* on alfalfa callus tissues has been reported with variable rates of success (Krusberg 1961; Krusberg and Blickenstaff 1964; Riedel and Foster 1970; Riedel et al. 1973). For instance, alfalfa callus tissue on a nutrient agar medium with 50 individuals of *D. dipsaci* added resulted in recovery of 40,000 to 80,000 nematodes. Similarly, *D. dipsaci* reproduced very well on callus tissue of onion on Krusberg's medium (Riedel and Foster 1970; Riedel et al. 1973). Addition of 2,4-D to growth media can make callus tissue incompatible with plant parasitic nematodes to being compatible (Webster and Lowe 1966). Krusberg and Blickenstaff (1964) reported alfalfa callus tissue grown on medium containing 2,4-D and kinetin considerably increased reproduction of *D. dipsaci*. In the current study, kinetin and 2,4-D were included in the growth media.

TABLE 5.2 Mean number (± standard error) of *Ditylenchus dipsaci* recovered from callused carrot disk, callus tissues of alfalfa and creeping thistle after 90 d as well as fungal cultures after 45 d having been inoculated with 80 individuals.

Procedure	Eggs/ culture	Juveniles/ culture	Adults/ culture	Total number/ culture
Callused carrot disk	2,495±38	13,872±132	3,189±41	19,556±150a
Alfalfa callus tissue	1,229±29	3,310±40	814±13	5,353±101b
Creeping thistle callus tissue	0	13±3	8±1	21±5c
Fungal cultures				
Fusarium solani	0	25±3	13±1	38±5c
Botrytis cinerea	0	30±3	11±2	41±6c
Rhizoctonia solani	0	12±1	7±1	19±3c
Verticillium dahliae	0	14±2	6±2	20±4c
Sclerotinia sclerotiorum	0	0	0	0[x]
Cladosporium cucumerinum	0	24±2	12±2	36±7c
Colletotrichum gloeosporioides	0	16±3	14±1	30±6c
Chaetomium spp.	0	0	0	0[y]

[x,y] Nematode recoveries of zero were excluded from statistical analysis. Means (n = 20) with the same letter are not different significantly according to Tukey's Honestly Significant Difference test ($P = 0.05$).

5.4.1.4 Rearing *D. weischeri* and *D. dipsaci* on callused carrot disks

This study showed that both *D. dipsaci* and *D. weischeri* were capable of reproducing on calloused carrot discs (Tables 5.1,2). In the present study, masses of dormant individuals of both *D. weischeri* and *D. dipsaci* were noticed on the inside surface of lids of some petri dishes of callused carrot disks (Figures 5.1E,F). The number of nematodes recovered from calloused carrot discs varied considerably for the two species examined. The addition of 80 individuals of *D. weischeri* resulted in a recovery increase of 54 times the number nematodes after three mon of incubation. The proportion of eggs, juveniles, and adults was 14%, 76%, and 10%, respectively (Table 5.1). A

recovery increase of 244 times the addition density was obtained for *D. dipsaci* with a proportion of 13%, 71% and 16% for eggs, juveniles and adults, respectively (Table 5.2). The multiplication of *D. weischeri* on calloused carrot discs was significantly ($P < 0.05$) lower than that of *D. dipsaci* according to *t*-test.

No agrose or nutrient agar as a basal medium for inducing callus tissues of carrot disks or maintaining moisture in the petri dishes was used. The callused carrot disks were not dried, even after three months of incubation. Rearing nematodes on callused carrot disks without adding nutrient agar has previously been used for other migratory endoparasites such as *Pratylenchus* spp. and *Radopholus* spp. (Moody 1973; Verdejo-Lucas and Pinochet 1992). Rearing nematodes on calloused carrot discs without growth medium is simple and inexpensive.

5.4.2 Effect of *D. weischeri* on growth and yield of yellow pea in microplots

In the microplot study, the average daily air temperature generally fluctuated between 13 to 26°C during the experiment. The mean daily air temperature in June, July, and August was 19, 21, and 19°C. About 259 mm of rainfall occurred between June 1 and August 26, 2015 (Fig. 3). In Winnipeg over the past 20 years (1996 to 2015), the average daily air temperature in June, July, and August was 17, 20, and 20°C with 230 mm of rainfall (using daily mean temperatures and precipitations from Environment Canada 2015b). This shows that the average temperature and total precipitation of the study was fairly similar to the climate normal for the area.

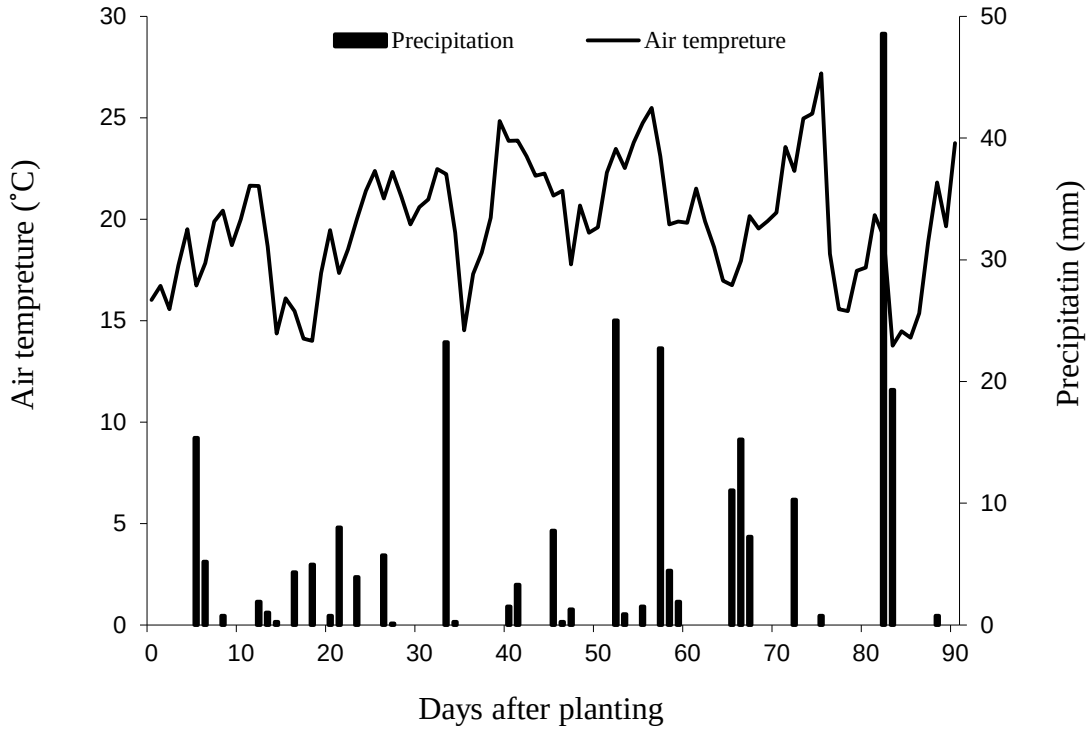

Figure 5.3 Mean daily air temperature and total daily rainfall at the University of Manitoba Point Research Station during the microplot study from June 1 (day of planting) through end of August, 2015 after harvest.

In the present study, *D. weischeri* reproduced well (Table 5.4) on the creeping thistle plants used as a positive control. The effect of addition density was significant for abouveground biomass and pod length but not for plant height and grain yield (Table 5.3). The addition density, variety, and variety by density interaction tested did not significantly affect plant height and grain yield (Table 5.3) indicating that *D. weischeri* had no effect on plant height and grain yield even at the highest density of nematode addition. At the addition density of 400 and greater there were significant differences ($P < 0.05$) between plant aboveground biomass and the non-treated control. A similar pattern was observed for pod length at the addition density of 800 and greater (Table 5.3).

TABLE 5.3 Effect of addition density of *Ditylenchus weischeri* on plant growth indicators and recovered number of nematodes per plant of two yellow pea varieties in the microplot study.

Variety	Addition density (#/plant)	Plant height (cm)	Aboveground biomass (g dry/plant)	Pod length (cm)	Grain yield (g dry/plant)	Recovered nematode #/Plant
Agassiz	…	64.7	8.5	6.0	4.9	1011
Bronco	…	63.3	8.5	5.8	5.1	878
	0	67.7	8.9a	6.0a	5.1	…
	100	66.4	9.0a	6.0a	5.1	109
	200	66.0	8.7ab	6.0a	5.1	220
	400	64.5	8.4bc	5.9ab	5.1	387
	800	62.8	8.3bc	5.8bc	5.0	768
	1600	62.3	8.2c	5.8bc	5.0	1493
	3200	61.9	8.2c	5.7c	5.0	2687
				P > *F*		
Variety		0.2198	0.9702	0.0001	0.2986	<0.0001
Density		0.0501	0.0005	0.0001	0.8862	<0.0001
Variety×Density		0.7520	0.9004	0.9885	0.9997	<0.0001

Means (n = 12) with the same letter are not different significantly according to Tukey's Honestly Significant Difference test (P = 0.05).

Reduction in aboveground biomass and pod length of varieties Agassiz and Bronco, at the density of 400 to 3200 nematodes/plant, could be due to two factors. Firstly, the nematode feeding as Griffin (1975) stated that stem nematode of *D. dipsaci* is able to attack nonhost plants following by feeding and development without the occurrence of reproduction. Secondly, we observed some irregular and dark lesions on the plant foliage and seed-pods from early August to harvest which resembled symptoms of Mycosphaerella blight or Ascochyta leaf and pod spot, common foliar diseases of yellow pea grown in the Canadian Prairies. Both yellow pea varieties (Agassiz and Bronco), tested in this study, have been previously reported to be moderately susceptible to

Mycosphaerella (Bing et al. 2006; Warkentin et al. 2005). Foliar fungicides were not used in the current study in case they compromised *D. weischeri* development and reproduction. Symptoms of damage of *D. weischeri* on the stems, leaves and pods of plants were not apparent in the current study.

It seems that increasing the density of *D. weischeri* had no significant impact on the yellow pea growth performances even at the highest nematode densities and the reductions in the aboveground biomass and pod lenght observed were possibly due to fungal diseases that may have been aggravated by the nematode penetration and development. However, to our knowledge, there have not been reports of associations between stem nematodes and foliar fungal pathogens. In the present study, *D. weischeri* was not recovered from seed-pods and seeds of either variety tested but recovered only from stems and leaves. Similarly, we reported recently that *D. weischeri* was unable to infest seed-pods and seed of yellow pea plant var. Agassiz grown under greenhouse conditions (Hajihassani et al. 2016). Additionally, migration of the nematode from infested yellow pea plants to adjacent non-infested plants was not observed in all plots with overlapping plant canopies.

A significant variety by density interaction ($F = 3.57$, $P < 0.0072$) was found for recovery of nematodes by the end of the microplot study. The interaction resulted because recovery for the nematode densities of 800, 1600, and 3200 was greater for Agassiz than Bronco (Figure 5.4). The reproduction factor decreased with increasing nematode density possibly due to lack of suitable environmental conditions specially temperature for *D. weischeri* development and reproduction (Hajihassani et al. Manuscript 2). The *D. weischeri* Rf was numerically slightly greater than 1 at densities of

100, 200, and 800 individuals per plant on var. Agassiz, and for 200 per plant on var. Bronco (Table 5.4); however, Rf values were not different than 1 (Table 5.4). This result is in agreement with the results from a previous greenhouse study (Hajihassani et al. 2016), which demonstrated that though *D. weischeri* is capable of invading yellow peas, but the ability of the nematode to reproduce on the plant is negligible.

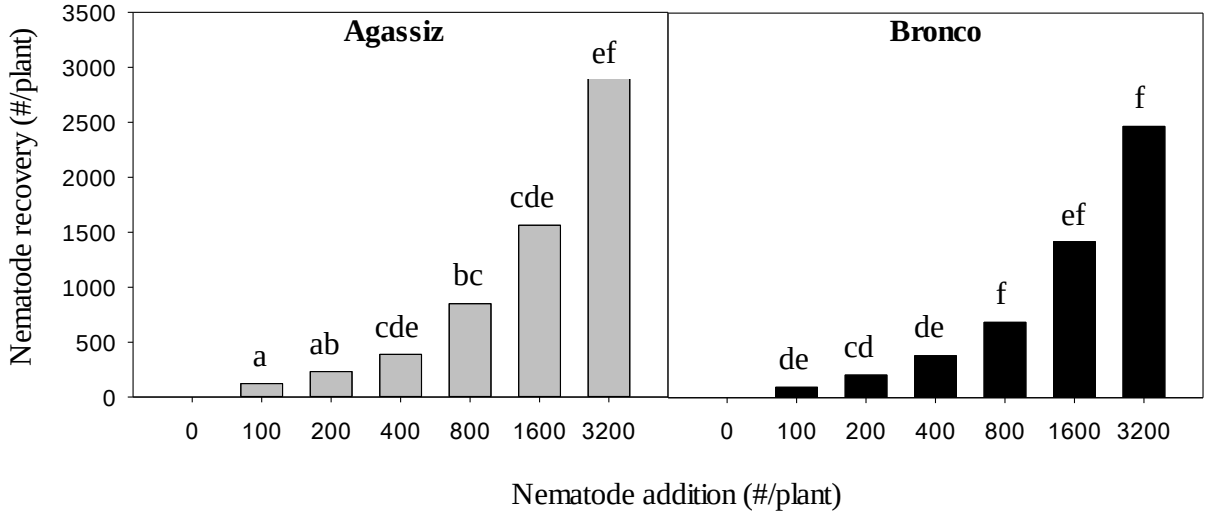

Figure 5.4 Recovery of *Ditylenchus weischeri* from two yellow pea varieties at harvest and density of addition of the nematode to seedlings. Values are means (n = 6) and those with the same letter are not different significantly from each other according to Tukey's Honestly Significant Difference test (P = 0.05).

TABLE 5.4 Recovery and reproduction factor (Rf) of *Ditylenchus weischeri* added at increasing density to two varieties of yellow pea and also creeping thistle in the microplot study.

Plant	Nematode addition/Plant	Nematode Rf [x]
Yellow pea var.		
Agassiz	100	1.21*
Agassiz	200	1.16*
Agassiz	400	0.97*
Agassiz	800	1.06*
Agassiz	1600	0.97*
Agassiz	3200	0.90*
Bronco	100	0.96*
Bronco	200	1.04*
Bronco	400	0.95*
Bronco	800	0.86*
Bronco	1600	0.89*
Bronco	3200	0.82*
Creeping thistle [y]		
	100	3.9±0.5
	3200	2.2±0.6

[x] Rf: recovered/addition ratio of nematodes.
[y] No statistical analysis was performed on data (n = 3).
Rf values are means (n = 6) of combinations of variety and nematode addition. For yellow pea varieties, asterisks indicate Rf values are not different from 1 using the LSmeans *t*-test.

The minimum generation time of *D. weischeri* on yellow pea var. Agassiz was 30 d under growth chamber conditions at a constant temperature of 27ºC (Hajihassani et al. Manuscript 2). During the current microplot study, mean daily air temperature during the growth of the crop reached 27°C once (75 d after planting). Thus, temperature likely restricted *D. weischeri* reproduction on yellow pea but not creeping thistle. The results of

the microplot study confirm under conditions similar to that of field situation that *D. weischeri* was unable to parasitize yellow pea.

5.5 Conclusions

The rearing of *D. weischeri* using a monoxenic culture procedure based on callused carrot disks was demonstrated for the first time. The culture procedure was capable of providing large quantities of nematode individuals for use in a host suitability microplot study for two yellow pea varieties. The culture procedure also was successful, with *D. dipsaci* reproducing more rapidly than the closely related *D. weischeri*. Callused carrot disk cultures are less costly, laborious and complicated to undertake compared to other monoxenic rearing procedures.

In the microplot study, similar to conditions (*e.g.* plant density, soil and environmental conditions) of commercial pea fields on the Canadian Prairies, *D. weischeri* was unable to reproduce on two varieties of yellow pea or affect grain yield. Further, the failure to reproduce on plants was not due to addition density. Increasing *D. weischeri* density had no impact on the plant height and grain yield of yellow pea at any addition density used. However, slight reduction observed in plant aboveground biomass and pod length at some addition densities were possibly due to foliar fungal diseases that may have been aggravated by the nematode penetration and development. Not surprisingly, *D. wiescheri* failed to migrate to pea plants adjacent to infested plants despite plant contact within a canopy. The results of the current microplot study confirm a previous greenhouse based study (Hajihassani et al. 2016) that yellow pea is unlikely a host for *D. wiescheri*.

6.0 GENERAL DISCUSSION

6.1 Important findings of this thesis research

The findings from this research showed that pulse crops, except yellow pea, and non-pulse crops are not hosts for one population of *D. weischeri* collected from Manitoba. Under greenhouse conditions, *D. weischeri* reproduced poorly on two yellow pea varieties (Agassiz and Golden), whease in field microplots the nematode failed to reproduce on var. Agasizz. This finding is of great importance for growers in the Canadian Prairies indicating that *D. weischeri* is unlikely to be an nematode pest of agricultural crops, in particular, yellow pea. In contrast to *D. weischeri*, *D. dipsaci* reproduced well on all pulse crops tested. This finding is not surprising as *D. dipsaci* has been a known parasite of many cultivated crops, including peas. In addition to differences in their genetic sequences (Madani et al. 2015), this study clearly confirms that *D. weischeri* and *D. dispaci* differ in their host status.

In recent years, biosecurity and border screening is considered as a key global issue for controlling economically important plant-parasitic nematodes because the introduction of a risky nematode pest to a country can cause a negative impact on market access and international trade. If a grain shipment is contaminated with a seed-borne nematode, the risk of distribution of nematode from one region to another within a province or across an international border will increase (Thorne 1961). The present work also confirmed previous observations by Tenuta et al. (2014) that the creeping thistle seeds are the source of *D. weischeri* infestation in yellow pea grain shipments. The data from this study also showed that *D. weischeri* cannot move to seeds of yellow pea plants, indicating that it cannot reasonably be expected to cause an issue with trade and market

access. This finding is of great importance for pea exporters and industry, as it shows that the expensive treatment of export grain shipments with fumigants such as methyl bromide to remove the nematode is not necessary. The absence of *D. weischeri* from seed also confirms that the seed is not a source of nematode and thus cannot infest pea crops in the following season.

Our results showed that *D. weischeri* is able to reproduce and complete its generation time on yellow pea at 27°C. Due to this fact, there are concerns about *D. weischeri* reproduction in high temperatures which warrant further investigation. Data from this research also indicates that it might be possible to differentiate *D. weischeri* and *D. dipsaci* based on temperature requirement to infest yellow pea, the former requiring higher mean daily temperatures for development.

6.2 Host suitability of crops to *D. weischeri* and *D. dipsaci*

In Canada, the occurrence of *D. dipsaci* on creeping thistle was reported in Regina, Saskatchewan in 1979 (Watson and Shorthouse 1979). More recently, molecular studies using species specific primers have revealed that the nematode parasitizing creeping thistle in Manitoba and Saskatchewan is *D. weischeri* and not *D. dipsaci* (Tenuta et al. 2014; Madani et al. 2015). We first hypothesized that the nematode population found on creeping thistle in Saskatchewan in 1979 could possibly be *D. weischeri*. This hypothesis could be acceptable considering that no damage by the creeping thistle population of *D. dipsaci*, to agricultural crops has been reported since 1979. In addition, because creeping thistle serves as an alternative host for the nematode we do not expect that *D. dipsaci* population has been eradicated from infested areas in Saskatchewan. A small number of *D. dipsaci* individuals present in soil could result in a large population over time when a

suitable host is present. In contrast to the nematode population found on creeping thistle, the occurrence of *D. dipsaci* on onion was first reported in Ontario in 1957 (Mountain 1957). Recent reports (Hughes et al. 2013; Ministre de l'Agriculture, des Pêcheries et de l'Alimentation du Québec 2013) showed the prevalence of the nematode in most of the garlic fields in Ontario and also some fields in Quebec indicating that *D. dipsaci* has been distributed widely over time with significant damage to garlic crop.

With this background, we then hypothesized that *D. weischeri* on creeping thistle is not a pest of agricultural crops. This was confirmed in our greenhouse and field studies when the nematode reproduced aggressively on creeping thistle and not on other pulse and non-pulse crops tested. This important finding points to the fact that *D. weischeri* is a host specialized nematode species parasitizing only creeping thistle. *Ditylenchus weischeri* has not been the focus of nematode management in pulse crops in western Canada, and the results from this work confirms that this nematode species is not a major concern for pulse growers, exporters and industry. However, further investigation is needed to better recognize why some yellow pea varieties are preferred by *D. weischeri* over others and whether the nematode feeding behavior is based only on the survival requirements where creeping thistle plant is absent, or on other factors, such as the nutritional features of the plants for the nematode. This information will result in better forecasting of the suitability of host plants.

It is also interesting to note that the different densities of *D. weischeri* added to plants neither increased on yellow pea plants nor reduced the seed yield in the field. However, slight reductions in the plant aboveground biomass and pod lenght at densities greater than 400 nematodes/plant were observed that could be due to occurrence of foliar

fungi of Mycosphaerella blight or Ascochyta leaf and pod spot on the plant foliages at the end of growing season. This research confirmed our hypothesis that grain yield of yellow pea wouldn't be impacted by *D. weischeri*. Several factors could explain the finding that *D. weischeri* is not a pest of yellow pea. Possibly, environmental conditions in the field, in particular temperature, were not favorable for nematode development. Another additional explanation is that yellow pea might not be a good host in terms of physical or physiological characteristics for *D. weischeri*.

6.3 Important findings on the reproduction of *D. weischeri* on yellow pea at 27°C

Based on the data from this research, the complete generation time of *D. weischeri* on yellow pea took 30 days, in comparison with 22 days for *D. dipsaci*, at 27˚C. It is reasonable to hypothesize that high temperatures might remove physiological barriers of yellow pea plant to *D. weischeri* reproduction, which warrants further investigation. Our finding clearly suggests that the generation time varies significantly depending on nematode species, host plant, and temperature of the habitat. It was also hypothesized that increasing temperature can shorten the generation time of *D. weischeri* because at higher temperatures the development of different nematode species is faster. The finding from this work, however, rejects this hypothesis.

It seems that the development of *D. weischeri* is not a linear function of temperature and various life stages, e.g. penetration, egg production or hatching, have slightly different optimum temperatures. The results from this research are, therefore, important since it signifies that *D. weischeri* and *D. dispaci* differ in their response to temperature. By assuming 3°C as the minimum temperature for *D. wiescheri* development, we calculated that the nematode needs 720 degree-days to complete one generation time on

yellow pea. This high degree-day requirement, in comparison with *D. dipsaci*, probably results from the fact that yellow pea is not a particularly good host for *D. weischeri*. Furthermore, our studies showed that *D. weischeri* reproduced well on creeping thistle in growth chamber, greenhouse, and field conditions and also on callused carrot disks at temperatures below 27°C. Therefore, reproduction of *D. weischeri* on only yellow pea at temperatures above 22°C is remarkable, indicating that *D. weischeri* development seems to be dependent on the host-nematode relationship, and not only on the nematode. This information may be useful in forecasting the development of *D. weischeri* in the future since the current environmental conditions in the Canadian Prairies are not suitable for the nematode's reproduction on yellow pea.

We also found that *D. dipsaci* can complete its generation time at all temperatures of 17, 22 and 27ºC. These finding can be useful in estimating the possible number of nematode generations in a growing season in the Canadian Prairies. Assuming 100 days required for yellow pea to reach maturity, *D. dipsaci* can probably produce between 4 to 5 generations per season. According to this research, the optimum temperature for *D. dipsaci* development and reproduction was 22˚C. In contrast, no optimum temperature for *D. weischeri* development was found. Therefore, further research might involve lower and higher temperatures than used in the present research to determine both the minimum and optimum temperatures for the *D. weischeri* development and reproduction.

Completion of the life cycle of *D. weischeri* on yellow pea at 27˚C, although is difficult to explain but we hypothesis that high temperatures may effect the plant growth resulting in removal of physiological barriers to the nematode reproduction. It can also be hypothesized that at high temperatures the number of male nematodes would increase

allowing for higher rates of *D. weischeri* development and reproduction. Our visual observations showed that numbers of males were considerably greater than females and detected earlier at 27°C indicating that a rise in temperature may influence on males development. Simmilar studies have demonstrated the importance of temperature on proportion of females to males in a mating process. Laughlin et al. (1969) reported that temperature significantly affected both the development rate and sex ratio of *Meloidogyne graminis*. They found that the percentage of the nematode males varied between 4% at 27°C and 80% at 32°C. Tenente and Evans (1998) reported that greater numbers of *D. dipsaci* males than females were obtained in onion tissues at 26°C four weeks after nematode inoculation and proposed that the number of male individuals was greater when conditions for plant growth were not suitable.

6.4 Occurrence of *D. dipsaci* on garlic in Manitoba and its relation to our findings

In 2015, we reported the occurrence of *D. dipsaci* on garlic in Manitoba (Hajihassani and Tenuta, *in press*). We also noticed that the nematode was first introduced into Manitoba in infested garlic seeds imported from Ontario. During the last sixty years, the increasing incidence of *D. dipsaci* in the eastern parts of Canada (Ontario and Quebec) was largely due to the exchange of infested seeds among local growers (Johnson and Kayler 1972; Fushtey and Kelly 1975; Hughes et al. 2013). The prevalence of *D. dipsaci* in Manitoba can increase dramatically even if a small amount of infested planting materials of garlic is imported from infested areas to nematode free areas. The situation could be worse if farmers continue to grow other susceptible host plants such as peas, common beans and chickpeas, as we found that *D. dipsaci* can infest and reproduce aggressively on these pulse crops.

The occurrence of *D. dipsaci*, with wide host ranges, can significantly limit the probability of using suitable non-host plants for rotation. Therefore, it is crucial to examine the host susceptibility of potential host crops before their widespread growing in the Canadian Prairies. It is also important to determine the host suitability of plant species or varieties for a local population of *D. dipsaci* before a rotation system is suggested for a particular field. The results from this research and previous studies (Greco and Di Vito 1994) with the use of other plant species revealed that finding non-host crops to be included in rotation programs is difficult for *D. dipsaci* due to the polyphagous behavior of this species. Crop rotations have economic costs for growers because the value of each crop in the rotation should provide a minimum profit. Given that spring wheat and canola crops did support the reproduction of *D. dipsaci* suggests that these non-pulse crops might be good choices for management of this nematode in a rotational system.

It is also necessary to take all precautionary actions to limit the spread of *D. dipsaci* from known infested areas to non-infested areas, particularly in Manitoba where the nematode is already established. Further research is required to examine the reproductive fitness of *D. dipsaci* by using new plant species or different varieties of an individual plant. The information will then be used to characterize potential non-hosts that could be grown in areas where crops highly susceptible to *D. dipsaci* are currently grown.

The results of this research thesis confirmed that *D. dipsaci* is a seed-borne parasite of yellow pea. In *D. dipsaci* management, it is important to recall that the nematode can move long distances in infested plant materials such as seeds and bulbs. Once *D. dipsaci* is introduced into a field, it cannot be eradicated easily, because it will persist in soil. Therefore, we recommand that infested material should not be planted, to prevent spread

of *D. dipsaci* to un-infested fields. Additionally, in order to limit the build-up of *D. dipsaci* population in soil, planting equipment and tools should be properly cleaned and sterilized. For *D. dipsaci,* survival occurs in the form of fourth-stage juveniles in infested seeds or in plant tissues remaining from the previous crop. Therefore, infested plants should be destroyed after harvest to prevent the build-up of nematode numbers on crop residues and therefore in the soil.

6.5 Summary of conclusions and recommendations

In summary, this research provides new understanding of the status of *D. weischeri* parasitism on yellow pea in order to limit risks in export shipments to other countries and address possible market access issues. The issue with the stem nematode in export pea grain shipments is now resolved by the identification of *D. weischeri* and also the results from this thesis reseach. The findings from this research are important because they provide important measures for differentiating *D. weischeri* and *D. dipsaci* based on their host preference. We believe that *D. weischeri* does not seemingly pose a threat to crop establishment and production in the Canadian Prairies in this century. The findings from this research along with future research on the effect of temperature on the nematode might enable an estimate of the possible influence of global warming on *D. weischeri* development. The information can also be used to create a simulation model of the nematode development in infested host crops.

Because of the occurrence of *D. dipsaci* in Manitoba, garlic fields close to pea fields should be scrutinized for the presence of the nematode to prevent this quarantine pest from establishing and further spreading in agricultural fields in the Canadian Prairies.

Given that the effect of increasing density of *D. weischeri* on growth and yield of yellow pea was examined for one growing season, testing over additional growing seasons using different geographically originated *D. weischeri* populations may lead to a broader understanding of the nematode develpment.

Future research should also be focused to analysis genetic variation within geographically separate populations of *D. weischeri*. The use of molecular markers displaying appropriate genetic polymorphism can help understand the etiology of this nematode species. Accordingly, the potential of different variants characterized on pulse crops can be examined.

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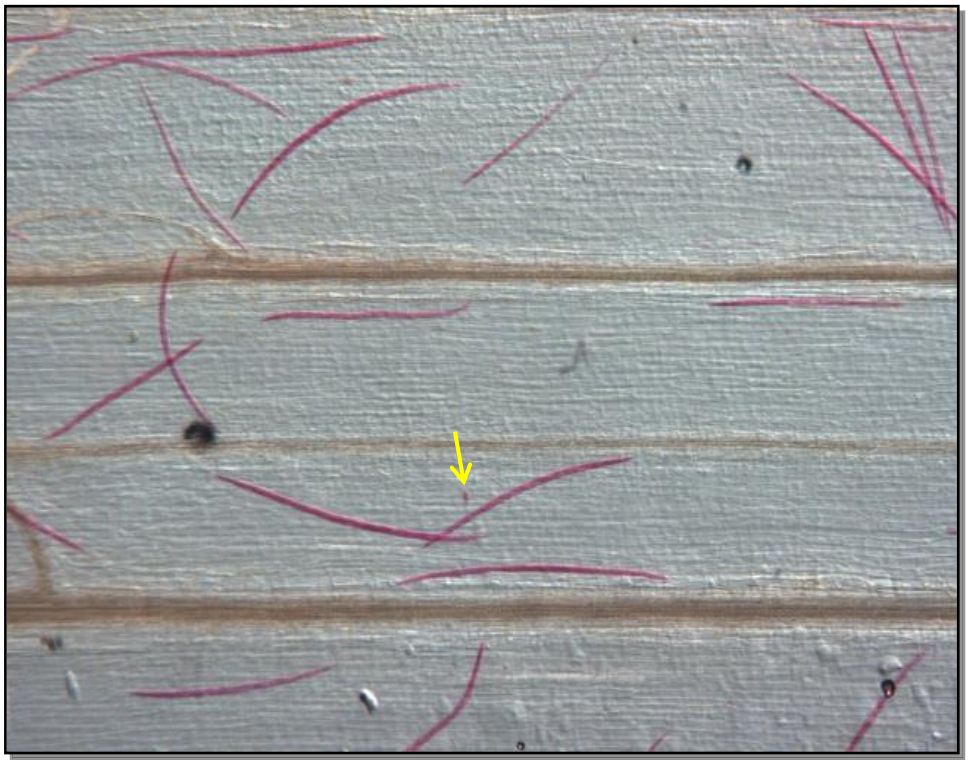
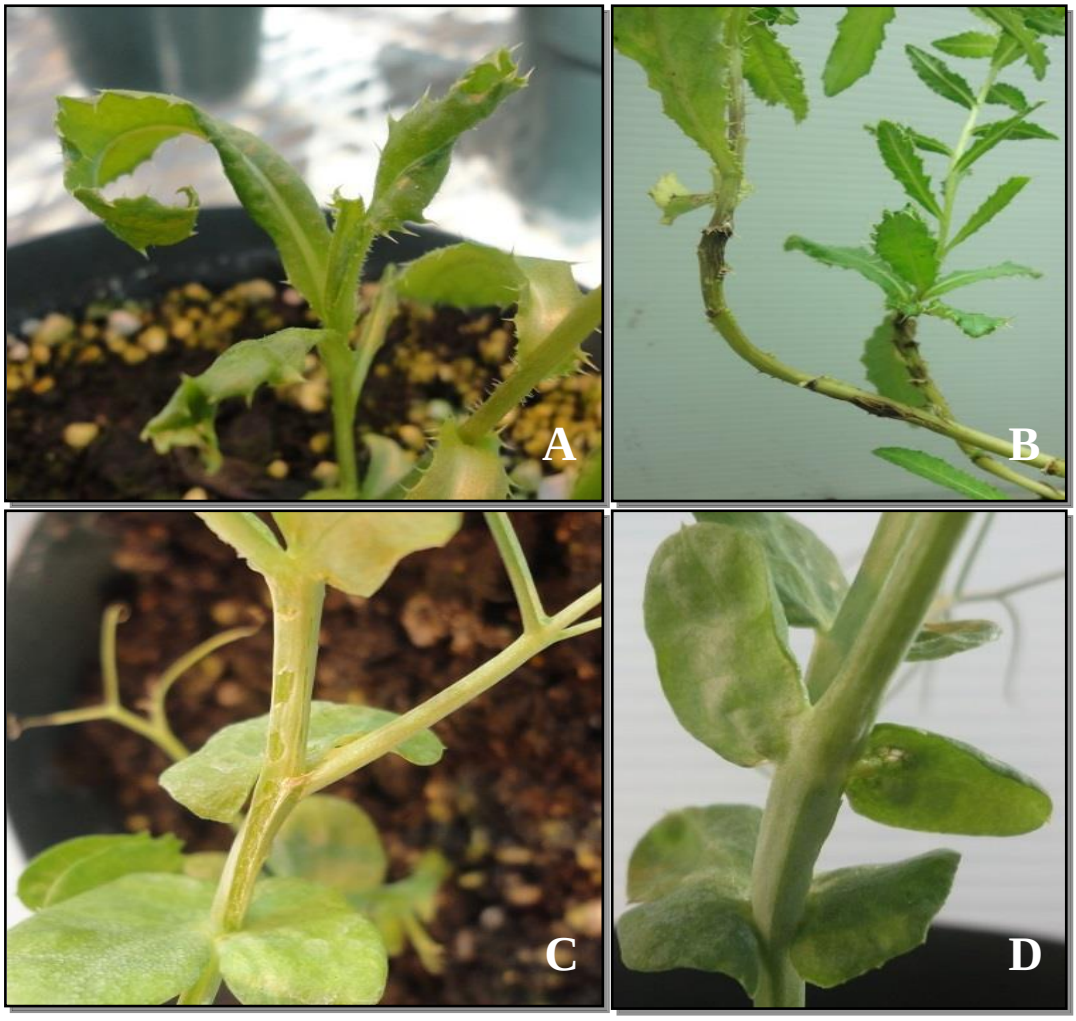
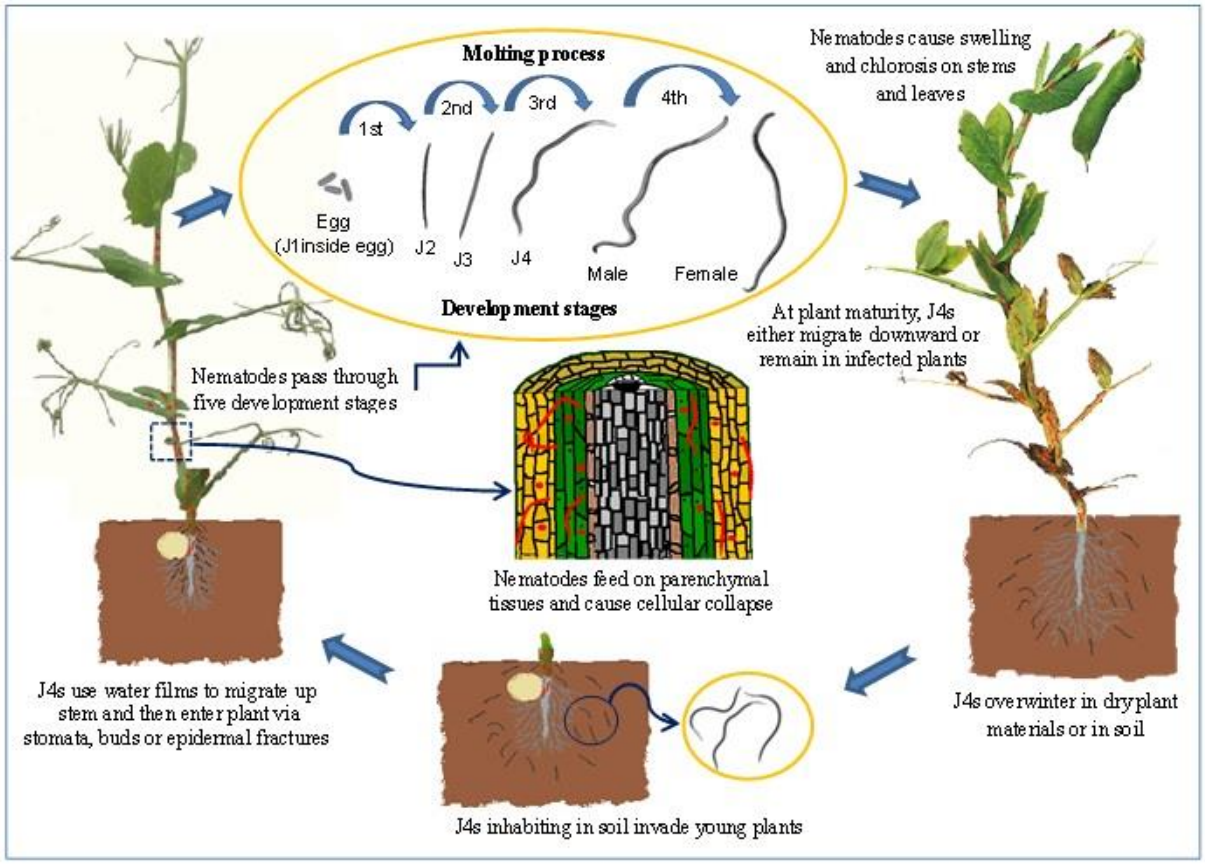
APPENDIX I



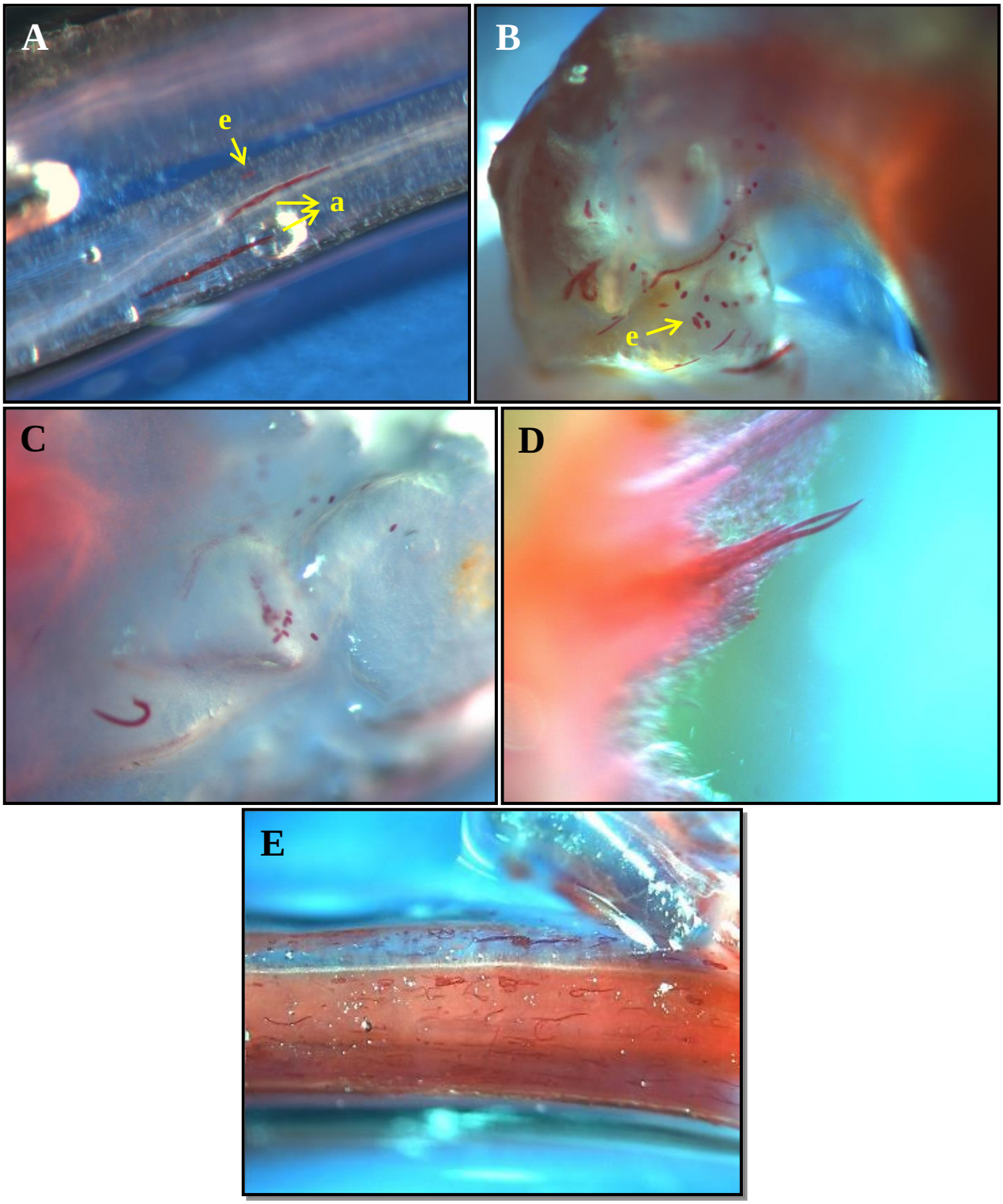
Appendix I.1 Eggs (arrows) and different life stages of *D. dipsaci* in garlic stem stained with acid fuchsin (Manuscript 1, host preference study).



Appendix I.2 Symptoms of twisting and deformation of stems and leaves as well as necrotic lesions on stems of creeping thistle caused by *D. weischeri* (A and B) and stem swelling, deformation and discolorations of leaves and internode swelling of yellow pea caused by *D. dipsaci* (C and D) when plants grown under greenhouse conditions and inoculated with 1000 nematodes (Manuscript 1, seed borne transmission study).



Appendix I.3 Schematic representation of life cycle of stem nematode, *Ditylenchus dipsaci* on yellow pea. J1, first-stage juvenile; J2, second-stage juvenile; J3, third-stage juvenile and J4, fourth-stage juvenile (Manuscript 2).



Appendix I.4 Magnified (20X magnification) view of *Ditylenchus dipsaci* stained with acid fuchsin in yellow pea. Various developmental stages, including eggs (e), juveniles (j) and adults (a), of the nematode in A, root, B and C, base of stem, D, leaf, and E, in stem (Manuscript 2).

Appendix I.5 Yellow pea plants with chlorosis symptoms on the foliage (A) and seed-pods (B) caused by fungal agents of Mycosphaerella blight or Ascochyta leaf and pod spot at the end of growing season (mid-August) when inoculated with different addition density of *Ditylenchus weischeri* at the experiment start (Microplot field study, Maniuscript 3).