Host-parasite interaction between the potato tuber rot nematode (*Ditylenchus destructor*), stem nematode (*Ditylenchus dipsaci)* and potato

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SUMMARY

Nematodes are among important pest constraints influencing potato production worldwide. The tuber rot nematode, *Ditylenchus destructor* Thorne 1945, and the stem nematode, *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936, cause lesions on potato tubers degrading their quality and market value. These nematodes are difficult to control due to their wide host range and therefore are listed as quarantine nematodes in many countries. In this PhD thesis, experiments were conducted to investigate the interaction between potato and each of these nematode species.

Molecular and morphometric characterization of different populations of *D. destructor* and *D. dipsaci* were studied. Sequence analysis of the cytochrome oxidase subunit I (COI) gene located on the mitochondrial DNA (mtDNA) was used to develop a phylogenetic relationship of the studied populations. The results demonstrated two highly supported clades containing *D. destructor* populations and the other *D. dipsaci* populations. The discriminant function analysis (DFA) of the morphometric data of males and females of *D. destructor* and *D. dipsaci* populations revealed that these species could be separated using the ***a***-ratio and the highest body width. The combination of both methods molecular and morphometric methods complemented the identity of the species under study.

Two greenhouse experiments were performed to evaluate sources of resistance and tolerance to *D. destructor* and *D. dipsaci* in 25 cultivated potato varieties. A standard screening protocol for resistance and tolerance to *D. destructor* and *D. dipsaci* was developed. Resistance and tolerance was evaluated based on the current definition of these terms in nematology. Relative susceptibility (RS) and external potato tuber damage were found to be the best methods for resistance and tolerance evaluation respectively. Potato varieties tested were not resistant or tolerant against *D. destructor* or *D. dipsaci*. However, some varieties were more tolerant than others.

Pre-plant densities of *D. destructor* and *D. dipsaci* and their impact on yield loss were also assessed. Initial population densities of *D. destructor* and *D. dipsaci* had significant influence on potato tuber damage and nematodes reproduction factor under greenhouse conditions. Damage caused by *D. destructor* started at a lower initial population density compared to that caused by *D. dipsaci*.

Influence of temperature on *D. destructor* and *D. dipsaci* population density and their impact on tuber damage was studied under climate chambers. Temperature influenced the nematodes population dynamics and consequently levels of potato tuber damage caused by *D. destructor* and *D. dipsaci*. Temperature of 26°C was optimal for both *D. destructor* and *D. dipsaci* multiplication compared to 16°C. Although *D. destructor* and *D. dipsaci* are reported to have different temperature requirements, both species caused external potato tuber damage at similar temperature ranges.

Beauveria bassiana is a cosmopolitan fungus used mainly in the management of insect pests in potato production. Dual infestation of potatoes with spore suspensions of *B. bassiana* in the soil together with *D. destructor* or *D. dipsaci* benefited the nematodes, thus leading to increased nematodes reproduction and tuber damage. *B. bassiana* on its own was not harmful to potato. It was hypothesized that *B. bassiana* played an indirect role in the nematode-plant interaction. In

order to add value, experiments are suggested which might help to give detailed mechanisms involved during the nematode-*B. bassiana*-plant interactions

ZUSAMMENFASSUNG

Nematoden rufen weltweit erhebliche Verluste in der Kartoffelproduktion hervor. Der Knollenfäule-Nematode *Ditylenchus destructor* Thorne 1945 und das Stock-und Stangelälchen *Ditylenchus dipsaci* Kühn 1857 Filipjev, 1936, verursachen Läsionen an der Kartoffelknolle, was deren Qualität und Marktwert verringert. Diese Nematoden sind aufgrund ihres umfangreichen Wirtspflanzenspektrums schwierig zu kontrollieren und werden daher in vielen Ländern als Quarantäne-Nematoden geführt. In der hier vorliegenden Doktorarbeit wurden Experimente durchgeführt, um die Wechselwirkung von Kartoffel mit jeder dieser beiden Nematodenarten zu untersuchen.

Verschiedene Populationen von *D. destructor* und *D. dipsaci* wurden molekular und morphometrisch charakterisiert. Die Gensequenz kodierend für die Untereinheit I der Cytochrom Oxidase (COI) auf der mitochondrialen DNA (mtDNA) wurde analysiert, um eine phylogenetische Beziehung zwischen den untersuchten Populationen darzustellen. Die Ergebnisse zeigten zwei deutlich getrennte Cluster für die *D. destructor* und die *D. dipsaci* Populationen. Eine Diskriminanzanalyse der morphometrischen Daten von Männchen und Weibchen von *D. destructor* und *D. dipsaci* Populationen verdeutlichte, dass diese beiden Arten mittels **a**-ratio und ihrem Durchmesser unterschieden werden können. Die sich ergänzende Kombination von molekularen und morphometrischen Untersuchungen ermöglichte die Identifikation der untersuchten Arten.

Es wurden zwei Gewächshausexperimente mit 25 angebauten Kartoffelsorten durchgeführt, um diese auf Resistenz und Toleranz gegenüber Nematoden zu testen. Dazu wurde ein Standard-Screening Protokoll entwickelt. Resistenz und Toleranz wurden gemäß der derzeitigen Definition (Nematologie) bewertet. Die relative Anfälligkeit und äußere Verletzung der Kartoffelknolle stellten sich als beste Parameter für die Beurteilung der Resistenz respektive der Toleranz heraus. Alle 25 untersuchten Kartoffelsorten waren weder resistent noch tolerant gegenüber *D. destructor* oder *D. dipsaci*. Jedoch wurden Unterschiede in der Toleranz der Sorten festgestellt.

Im Vorfeld zum Pflanzenexperiment wurden die Populationsdichten von *D. destructor* und *D. dipsaci* und deren Einfluss auf Ertragsverlust untersucht. Die initiale Populationsdichte hatte einen signifikanten Effekt auf den Schaden an der Kartoffelknolle und den Fortpflanzungsfaktor der Nematoden unter Gewächshausbedingungen. *D. destructor* verursachte Schäden bereits bei einer geringeren initialen Populationsdichte als *D. dipsaci*.

Unter Klimakammerbedingungen wurde gezeigt, dass die Temperatur Einfluss auf die Schadensrate von *D. destructor* und *D. dipsaci* an Kartoffelknollen nimmt. Im Gegensatz zu einer Temperatur von 16°C waren 26°C sowohl optimal für die Vermehrung von *D. destructor* als auch für *D. dipsaci*. Obwohl für *D. destructor* und *D. dipsaci* unterschiedliche Temperaturanforderungen beschrieben wurden, verursachten beide Arten in ähnlichen Temperaturbereichen äußere Verletzungen an der Kartoffelknolle. *Beauveria bassiana* ist ein weltweit verbreiteter Pilz, der vor allem zur Bekämpfung von Insektenschädlingen in der

Kartoffelproduktion Anwendung findet. Doppelbefall von Kartoffeln mit *B. bassiana* Sporensuspensionen im Boden mit *D. destructor* oder *D. dipsaci* begünstigte die Nematoden, was zu einer erhöhten Nematodenfortpflanzung und Knollenbeschädigung führte. *B. bassiana* allein war nicht schädlich für die Kartoffel. Es wurde vermutet, dass *B. bassiana* eine indirekte Rolle in der Wechselwirkung Nematode-Kartoffel spielt. Vorschläge für zukünftige Experimente werden angebracht, die einen weiteren Beitrag zu den hier vorgestellten Studien leisten können.

Chapter 1:

General introduction and literature review

Importance of potatoes worldwide

Potato (*Solanum tuberosum* L.) is the world's fourth most important staple crop, after maize, rice and wheat (Manrique, 2000). It plays a very important role in global food security. In the year 2013, global annual potato production was estimated to be 365 million tons (FAOSTAT, 2014). The year 2008 was declared as the international year of potato by the United Nations' Food and Agricultural Organization (FAO) (FAO, 2009). During that year, potato was declared as a crop able to help fulfill the first millennium development goal aimed at eradicating extreme poverty and hunger in the world (FAO, 2009).

Currently, developing countries are steadily increasing their potato production, with countries such as China and India leading in quantities of potato produced annually (FAOSTAT, 2014). Increase in potato production over the years is attributed to continuous improvement of potato varieties, introduction of seed potato and better cultivation methods. Shifts in eating habits in many countries have lead to increased potato demand (FAO, 2009). As a result of increased and intensive cultivation, there is a greater possibility of potato infestation from existing pests and diseases. Consequently, there is also a higher potential for emergence of new threats to production.

Potato production constraints

Potato is host to over 40 different air or soil borne pathogens affecting all parts of the plant leading to reduction in quantity and quality of yield (Hooker, 1981). Soil borne pathogens cause damage to potato tubers and roots (Gudmestad *et al.*, 2007). Damage affecting tubers can be categorized into three categories: galls, blemishes and rots. Nematodes are among important pathogens influencing potato production, leading to qualitative and quantitative damage (Hooker, 1981). Worldwide yield losses on potatoes caused by nematodes are difficult to estimate since in some continents there is limited information regarding the impact of nematodes on cultivated crops (Gressel *et al.*, 2004). Nematodes caused a 12% reduction in the world potato harvest (Sasser & Freckman, 1987), a fact which could be different in the current year.

Potato nematodes

Potato is attacked by several nematodes belonging to different species, which are able to feed and reproduce on tubers causing direct and indirect losses (Mugniéry & Phillips, 2007). Major nematode species of potato include *Globodera* spp., *Meloidogyne* spp., *Nacobbus aberrans*, *Ditylenchus* spp and *Pratylenchus* spp. (Scurrah *et al.*, 2005). Several other nematodes species are also associated with potato, but their economic relevance has not been properly assessed (Scurrah *et al.*, 2005). Some nematodes species, previously regarded as non-damaging to crops, are continuously reported as a threat to crop production due to the effect of climate change (Hijmans, 2003) and varying cropping patterns (Nicol, 2002).

The genus *Ditylenchus*

The family *Anguinidae* Nicoll, 1935 (1926) contains mycophagous nematodes which attack plant tubers, bulbs and aerial parts (Fortuner & Maggenti, 1987). The genus *Ditylenchus* (Nematoda: Anguinidae) comprises many cosmopolitan species and is known to have the widest impact on agriculture (Fortuner, 1982). The genus has over 90 described species (Brzeski, 1991). Four species in this genus are known to be significant pests of crop plants (Sturhan & Brzeski, 1991). These include *Ditylenchus destructor* Thorne 1945, *Ditylenchus dipsaci* (Kuhn) Filipjev 1936, *Ditylenchus angustus* (Butler 1913) Filipjev 1936 and *Ditylenchus africanus* (Wendt *et al.*, 1995). *Ditylenchus destructor* commonly referred to as the potato tuber rot nematode, which is widespread and important in cool and humid environments (Thorne, 1945; Plowright *et al.*, 2002). On the other hand, the stem and bulb nematode, *D. dipsaci*, is composed of numerous biological races and is prevalent in a wide range of climatic conditions, including temperate, subtropical and tropical (Webster, 1967; Viglierchio, 1971; Brzeski, 1991; Janssen, 1994). *Ditylenchus destructor* and *D. dipsaci* are morphologically similar but pathogenetically different (Brodie *et al.*, 1993). *Ditylenchus dipsaci* was recovered from plant tissues after 23 years (Fielding, 1951), depicting its ability to survive desiccation. *D. destructor* cannot survive excessive desiccation (Sturhan & Brzeski, 1991). The damage caused by these nematodes on potatoes is reportedly different (Baker, 1947; Seinhorst, 1949; Kotthoff, 1950; Brodie, 1984; Cotten *et al.*, 1992; Janssen, 1994).

Damage caused by *Ditylenchus* on potatoes

The earliest record of potato tuber rot associated with nematodes was reported in Europe in 1888 by Kühn, who identified the causal agent as *Anguillulina dipsaci* Kühn, 1857. Kühn observed two distinct types of damage on potatoes, namely tuber rot associated with distorted top growth and tuber rot without above-ground symptoms. Most of the research and biology of this nematode was reported under the name *Anguillulina dipsaci* until it was defined as *D. dipsaci* almost 100 years later (Filipjev, 1936). Thorne (1945) separated *D. destructor* from *D. dipsaci* and described the first as a new species, which made earlier literature in relation to potato and *D. destructor* not entirely reliable since it was a mixture of two species. Following this description, *D. destructor* was continuously reported to be a troublesome nematode in the north-western USA and Prince Edward Island in Canada (Thorne, 1945). It was in Canada where the first *D. destructor* infested farm was quarantined (Baker, 1947). To-date, it is reported to occur in many parts of Europe and localised in some areas in North America (Canada, USA, Mexico), South America (Equador), Asia and Oceania (New Zealand) (EPPO, 2008).

Influence of *Ditylenchus destructor* on potatoes

Potato is the main host to *D. destructor*, but the nematode is occasionally found on over 70 crops and weeds including a similar number of fungal species (Baker *et al.*, 1954; Faulkner & Darling, 1961). *Ditylenchus destructor* is favoured by cool and moist soils which is favourable for development and movement of the nematode (Andersson, 1967). The nematode overwinters in the soil as adults, juveniles or eggs, and multiplies by feeding on host plants, weeds and fungal mycelium (Andersson, 1967; Hooper, 1973; Švilponis *et al.*, 2011). Shortly after juveniles hatch, the juveniles are immediately able to parasitize plants (Thorne, 1945). Data on optimal temperatures for hatching, development, and pathogenicity on potato is scarce. Development and reproduction of *D. destructor* occur in the range from 5 to 34°C, where 20-27°C is the optimum temperature as summarised by Decker (1969) from data collected mostly in former Soviet Union (USSR). Although this data may be relevant, there is evidence of adaptation of tuber rot nematode to different climatic conditions, even where potatoes are cultivated under irrigation (Sturhan & Brzeski, 1991). As such, current studies are needed to elucidate the impact of different temperature regimes and their impact on tuber damage levels.

Symptomology on potato tubers due to *Ditylenchus destructor*

Ditylenchus destructor enters potato tubers through lenticels, where the nematodes multiply and spread inside the tuber (Thorne, 1945). The first symptoms are white spots under the skin which progress into sunken areas and into cracks as the infection progresses (Thorne, 1945). In severely damaged potato tubers, the tissue becomes spongy or completely rotten. Symptoms progress after storage (Thorne, 1945). However, tubers damaged by the nematodes are usually invaded by fungi, bacteria and free living nematodes forming a complex and sometimes synergistic interaction (Baker *et al.*, 1954; Rojankovski & Ciurea, 1986; Janowicz, 1990). Damage on tubers is evident upon harvest since *D. destructor* hardly produces visible above-ground symptoms (Thorne, 1945).

Influence of *Ditylenchus dipsaci* on potatoes

On the other hand, *Ditylenchus dipsaci* is one of the earliest described nematode species, (Filipjev, 1936) and also of the most devastating plant parasitic nematodes especially in the temperate regions with an ability to colonize over 500 plant species (Hooper, 1972). This species has over 20 described biological races, making it a species complex (Seinhorst, 1949; Webster, 1967; Viglierchio, 1971; Janssen, 1994). As a result of the complex morphological similarities within the genus, in combination with high intraspecific variations, numerous taxonomic revisions have been published (Fortuner, 1982; Fortuner & Maggenti, 1987; Brzeski, 1991). There have been many additions and changes since then, with most recent updated taxonomy of nominal species of the genus compiled by Brzeski in 1991.

Symptomology on potato tubers due to *D. dipsaci*

Ditylenchus dipsaci also enters the tubers through the lenticels. The earliest symptoms of potato tuber damage caused by *D. dipsaci* were reported by Kühn in 1888. After differentiation of *D. destructor* from *D. dipsaci*, studies were conducted to investigate the differences in potato tuber damage characteristics caused by *D. destructor* and *D. dipsaci* (Seinhorst, 1949; Kotthoff, 1950). It was observed that damage caused by *D. dipsaci* frequently extended into a considerable depth inside the tuber as opposed to superficial lesions produced by *D. destructor* (Seinhorst, 1949; Kotthoff, 1950; Brodie, 1984; Cotten *et al.*, 1992). Another difference between the species was that *D. destructor* could live on a wide range of fungi and higher plants, while the host range of *D. dipsaci* was almost confined to higher plants (Winslow, 1978). Since then, there have been

only few studies focussing on interaction between *D. dipsaci* and potatoes. In the United Kingdom, *D. dipsaci* was recorded for the first time infecting warehouse potatoes causing a 10% yield loss (Cotten *et al.*, 1992). Despite earlier interest on *Ditylenchus* spp., and its impact on agricultural crops, to-date, there is limited current information on the economic damage of potato due to *D. dipsaci,* a subject that is relevant to international trade, as dry seeds and planting materials of host plants are traded daily on the international markets.

Factors influencing interaction between potato and *D. destructor* or *D. dipsaci*

Temperature

Nematodes are poikiothermic organisms, whose behaviour and physiological processes are largely regulated by temperature (Barbercheck & Duncan, 2004). *Ditylenchus destructor* and *D. dipsaci* rates of multiplication, sex determination, mortality and damage expression on host plants is determined by temperature (Sturhan & Brzeski, 1991). As such, temperature is a key element influencing global distribution of these nematodes and rates of development, since both species have different thermal requirements. *Ditylenchus destructor* cannot survive desiccation unlike *D. dipsaci* and therefore *D. dipsaci* could survive higher temperatures compared to *D. destructor* (Perry, 1977). Influence of temperature on the severity of damage on potato caused by these nematodes are scanty and reported close to 60 years ago (Seinhorst, 1950; Ladygina, 1957). Since then, numerous aspects have changed, including daily average temperatures and cultivated potato genotypes. Therefore, current investigations are required to evaluate impact of different temperature regimes on severity of tuber damage caused by these nematodes and also the impact on their population densities in the soil and tuber tissues.

Pre - planting population densities of *D. destructor* and *D. dipsaci*

Knowledge of nematode pre-plant densities influences the management strategies to be implemented based on predicted yield losses. The fact that both *D. destructor* and *D. dipsaci* can overwinter in the soil for over 23 years making susceptible crop losses potentially high. Additionally, both species have short life cycles and are able to complete over nine generations in one vegetative cycle (Decker, 1969; Sturhan & Brzeski, 1991). Impact of these nematodes on potato tuber damage is reported by few authors, while the relationship between pre-plant densities and their associated damage loss on potatoes are scanty (Hijink, 1963). Such knowledge

is important in establishing tolerance levels and damage potential of different varieties to these nematodes. Additionally the information may help improve management.

Management of *D. destructor* and *D. dipsaci*

Management of *D. destructor* and *D. dipsaci* once present in the field is a formidable task due to the wide host range and multiple generations per vegetative cycle of host crops (Decker, 1969; Sturhan & Brzeski, 1991). Several weed species are hosts to these nematodes making crop rotation a limited option (Hooper, 1972; 1973). Attempts to manage these nematodes using nematicides has not been adequate. Additionally, there is pressure to minimize nematicides use due to health risks and enviromental contaminations (Darling *et al.*, 1983). Infact, the restriction on the use of nematicides for nematode control has necessitated exploring other control strategies even in intensive agriculture. Resistance and tolerance to nematodes has proved to be an effective way of controlling nematodes. Trials for resistances in potato varieites against *D. destructor* and *D. dipsaci* were intiated in the early1950s shortly after *D. destructor* was separated from *D. dipsaci* (Goodey, 1956). Although most these trials focused more on *D. destructor*, little focus was given to *D. dipsaci*. Results from these trials demonstrated that none of the tested varieties were either resistant or tolerant to these nematodes (Kornobis, 1980). Trials for resistances have since been abandoned. Since new varieties come into cultivation every year, there is need to evaluate the presence of resitance and tolerance in modern cultivated potato varieties. Availability of such varieties would improve management of these nematodes in potato cultivation or form a basis for further potato variety improvements.

Lack of resistant and tolerant varieties and pressure to minimise the use of nematicides invoked research interest into alternative management strategies such as use of antagonistic organisms. Numerous fungal and bacterial antagonists have been explored in management of different nematodes species (Timper, 2011). However, management of *D. destructor* and *D. dipsaci* using fungal antagonists has received little success partly because these nematodes are fungal feeders (Yakimenko & Efremenko, 1973; Janowicz, 1990). *Beauveria bassiana*, an entomopathogenic fungus has been successfully been integrated in the management of Colorado potato beetle and therefore closely associated with potato plant as an endophyte (Jones, 1994). The spores of these fungus can survive in soil following a single application and have been shown to be effective in management of overwintering larve of colorado beetle (Watt & LeBrun, 1984). However, the

interaction between *D. destructor* or *D. dipsaci* with *B. bassiana* is less studied, and little is known on its influence on these nematodes and potential influence on damage levels on potato tubers. Therefore further studies were deemed necessary for the current study.

In an effort to reduce spread and thus increased crop damage arising from infestation by these two nematodes species, over 50 countries in the world have imposed phytosanitary regulation on trade of crop produce which are primary pathways for distribution (Anonymous, 2000; EPPO, 2008). The impact these nematodes have on trade, especially on seeds intended for planting is immense (Kruus, 2012). In the recent past, new cases of crop damage in garlic and sugarbeet arising from *D. destructor* and *D. dipsaci* have been reported (Kühnhold, 2011; Yu *et al.*, 2012). The interaction between tuber rot nematode, stem nematode and the potato plant remains relevant due to its phytosanitary importance, and the potential high potato damage these species have on potatoes. Understanding these interactions and factors influencing these interactions is vital towards the development of management strategies.

It is therefore the aim of this thesis to expand current knowledge on the influence of biotic and abiotic factors on interactions between potato tuber rot nematode (*D. destructor*), stem nematode (*D. dipsaci*) and potato. Several experiments were conducted in the laboratory, climate chambers and in temperature regulated greenhouse, with the following objectives:

1. To characterise *D. destructor* and *D. dipsaci* populations using morphometric and molecular parameters
2. To screen current potato varieties for resistance and tolerance to *D. destructor* and *D. dipsaci*
3. To evaluate the influence of initial population densities of *D. destructor* and *D. dipsaci* on potato tuber damage and reproduction potential
4. To investigate the influence of temperature on potato tuber damage caused by *D. destructor* and *D. dipsaci* in climate chambers
5. To assess the interaction between *D. destructor*, *D. dipsaci* and *Beauveria bassiana* and its influence on potato tuber damage and nematode reproduction.

THESIS OUTLINE

Chapter 1: This is the introductory chapter, highlighting the importance of potatoes in food security. Nematodes in general are discussed as part of major constraints to potato production. The genus *Ditylenchus* and subsequently, *D. destructor* and *D. dipsaci* are presented, including biology and impact each of these have on potato. Pathological differences between *D. destructor* and *D. dipsaci* on potatoes are presented. Challenges experienced during management of these nematodes is also summarised highlighting the research gaps which exists and the attention needed to address these gaps. At the end, with the research gaps identified, a link to these is linked to the objectives of this dissertation. Each objective is highlighted and presented as individual chapters.

Chapter 2: in this chapter, morphometric and molecular data is presented following characterization of different populations of *D. destructor* and *D. dipsaci*. Morphometric data which is obtained from three populations of *D. destructor* and five populations of *D. dipsaci* is analysed using the Principal Component Analysis (PCA) with an aim of finding suitable morphometric characters suitable for differentiating *D. destructor* and *D. dipsaci*. Sequence data obtained from different genes is also used in developing phylogenetic relationship among different populations of single species and also to identify reliable sources of genetic differences between *D. destructor* and *D. dipsaci*. The study links both the morphometric data and molecular data for identification of both species.

Chapter 3: Two greenhouse experiments were conducted to screen twenty five potato varieties for resistance and tolerance to *D. destructor* and *D. dipsaci.* One population of each nematode species was used during the screening. Results are presented based on the current definition of the terms resistance and tolerance in nematology and compared with those in literature. Reproduction factor and relative susceptibility are discussed as methods for resistance evaluation. None of the tested varieties were fully resistant or tolerant to *D. destructor* or *D. dipsaci*. Differences in resistance and tolerance levels of various potato varieties against *D. destructor* and *D. dipsaci* are also discussed. The study provides essential information on the status of resistance and tolerance in potato varieties against *D. destructor* and *D. dipsaci*.

Chapter 4: Two experiments were conducted in a temperature regulated greenhouse to test the effect of different pre-planting densities of *D. destructor* and *D. dipsaci* on potato tuber damage and nematode reproduction. Pre-planting densities if both nematodes species significantly influenced tuber damage and nematode reproduction. Potato tuber numbers and weight were influenced differently by both nematode species. *D. dipsaci* influenced tuber numbers and weights at a P*i* level of 14.29 g^{-1} of growing medium. Tolerance limit estimates according to the Seinhorst model were very low indicating both nematode species have a major impact on potato tuber weight. Damage caused by *D. destructor* started at a lower initial population density compared to that caused by *D. dipsaci*. External and internal tuber rot caused by both species increased with increasing P*i* levels. Reproduction rates of *D. destructor* were higher at all P*i* levels studied compared to *D. dipsaci*. Further studies considering Seinhorst research program and involving different potato varieties and different populations of each nematode species are needed to investigate further observed differences in reproduction between *D. destructor* and *D. destructor*.

Chapter 5: Two climate chamber experiments were conducted under different temperature regimes, to investigate the influence of temperature and the duration of the experiments on damage caused by *D. destructor* and *D. dipsaci* on potato tubers. Temperature and duration of the experiments significantly influenced potato tuber damage and nematode multiplication. Our study indicated that even at the lowest temperature settings studied (16°C and 13°C day and night temperature), both *D. destructor* and *D. dipsaci* caused significant potato tuber damage reducing tuber quality. *Ditylenchus destructor* and *D. dipsaci* damage and maximum population increase was attained when the temperature setting was at 20°C and 17°C day and night temperatures. Our findings agreed with the limited laboratory experiments on thermal temperature requirements of *D. destructor* on potatoes. Thermal temperature requirement for *D. dipsaci* and its relevance to potato tuber is to our knowledge reported for the first time in this study.

Chapter 6: In this chapter, the application of the entomophathogenic fungus (*B. bassiana*) to the soil together with either *D. destructor* or *D. dipsaci* and its impact on tuber damage and nematodes reproduction is presented. Results from these two experiments indicated that addition of *B. bassiana* into the growing medium together with the nematodes influenced external and internal potato tuber damage and nematodes reproduction. External and internal tuber damage and nematodes reproduction were higher in treatments where also *B. bassiana* was added,

compared to only where nematodes were added. Tuber numbers, tuber weight and weight of above ground plant parts were influenced by the interaction between the nematodes and *B. bassiana*. *Beauveria bassiana* did not establish itself as an endophyte in potato tuber tissues. Although *B. bassiana* is an effective bio-control agent against some nematodes, its occurrence together with *D. destructor* and *D. dipsaci* in the presence of potato plants results in complex interaction leading to higher potato tuber damage and higher nematodes population densities.

Chapter 7: This is a summary of the main findings. General conclusion is made here and future perspectives in respect to gaps which were identified in the current study and which were beyond the scope of the current research are stated.

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Chapter 2:

Morphometrics and molecular characterization of *Ditylenchus destructor* and *Ditylenchus dipsaci* populations

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Abstract

Ditylenchus destructor and *D. dipsaci* are economically important nematodes distributed and associated with the damage of diverse groups of plants in cultivated and uncultivated fields. Morphological identification of many species in the genus *Ditylenchus* is complicated because the species share very similar diagnostic characters. Additionally, the presence of a high intraspecific variation complicates identification. To verify species identification,geographically distant populations of *D. destructor* and *D. dipsaci* were tested for differences using classical morphometric features. Sequence analysis of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) was used to develop a phylogenetic relationship of the studied populations. The multivariate statistic of the populations revealed that body width, a' ratio, c'ratio and post uterine sack length are the most reliable morphometric characters in adult nematodes of *D. destructor* and *D. dipsaci*. Sequence analysis of the COI revealed that there were differences between species and within populations of each species. The combination of both methods complimented the identity of the species under study.

Keywords: Phylogeny, taxonomy, potato tuber rot nematode, stem nematode, cytochrome oxidase subunit I (COI), Multivariate analysis

1.0 Introduction

The genus *Ditylenchus* Filipjev, 1936 consists of over 90 described nematodes species, some of which are among the oldest described nematodes (Sturhan & Brzeski, 1991). Only few of the described species in this genus are parasites of higher plants, while the majority of the species are fungi feeding (mycophagous) (Sturhan & Brzeski, 1991). Among important plant parasitic nematodes in this genus are the tuber rot nematode, *Ditylenchus destructor* , (Thorne, 1945), and the stem nematode, *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936.

Morphologically, these two nematodes are very similar to each other but differ pathogenetically (Brodie *et al.*, 1993). *Ditylenchus dipsaci* has an extensive intraspecific variation which includes over 20 biological races, with different host ranges, and occurrence of different stages of speciation and reproductive isolation (Sturhan, 1969; Sturhan & Brzeski, 1991). As such, *Ditylenchus dipsaci* is considered as a species complex and has received considerable taxonomic revisions over time (Sturhan & Brzeski, 1991).

As a result, these nematode species are considered difficult to identify due to the limited number of distinguishable taxonomic characters and overlapping morphometric measurements (Barraclough & Blackith, 1962). Although *D. destructor* is mainly of relevance to temperate climates, *D. dipsaci* is a nematode of worldwide concern mainly found in temperate zones, including the Mediterranean basin (Hooper, 1972). In many countries in the world, these two species are of quarantine importance (EPPO, 2008).

Consequently, there is an increasing demand by nematode taxonomists to assess these nematodes with multiple aims. One of the aims is to develop new tools for agronomic management and to address the quarantine regulations requirements (Powers, 2004). Therefore, accurate detection and identification of both *D. destructor* and *D. dipsaci* is important due to the presence of variability in field populations. Additionally, accurate identification of *D. destructor* and *D. dipsaci* is important for the screening of plant germplasm with an intention of breeding and development of resistant cultivars. In contrast to morphometric data, nematode identification using diverse molecular tools provides accurate and fast identity of species under investigations. There are numerous molecular methods available for identification of specific nematodes species, but the choice of the methods must meet the need for the information required.

The European and Mediterranean Plant Protection Organization (EPPO) offers a diagnostic protocol for *D. destructor* and *D. dipsaci* (EPPO, 2008). The protocol advocates the use of both morphometrics and molecular techniques for the identification of both nematodes species. The use of the data obtained from these methods is of practical use in the management and risk assessment of these nematodes. To harmonize identification of European quarantine nematodes, the Q-bank nematodes database has been set up (www.q-bank.eu/nematodes/) which describes a detailed molecular decision scheme to be followed for the identification of these nematodes. The use of Mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) using the JB3 and JB5 pair of primers are recommended among other methods.

In our study, three populations of *D. destructor* and five populations of *D. dipsaci* were obtained from the Julius Kühn Institut (JKI) collection. These populations were extracted from different countries and different hosts. Since the intention was to use these populations in subsequent studies, identity was important. It was therefore the objective of the current experiment to:-

- characterize three populations of *D. destructor* and five populations of *D. dipsaci* populations using morphometric data
- perform sequence based characterization of the same populations and compare this data with sequence data deposited in the NCBI database
- reconstruct phylogenetic relationships between *D. destructor* and *D. dipsaci* populations of different geographical origin and other *Ditylenchus* species
- combine both morphometrics and molecular data for comprehensive analysis of these populations

2.0. Materials and Methods

Ditylenchus destructor and *D. dipsaci* populations used in this study (Table 1) were originally extracted from different host plants, sampled in Germany, Russia and Ukraine (JKI collection).

Table 1: Origin of *Ditylenchus destructor* and *D. dipsaci* populations used in this study and their host

Population	Species	Country and location of origin	Host plant
A	*D. destructor*	Germany	sugar beet
B	*D. destructor*	Russia	Potato
C	*D. destructor*	Ukraine	Potato
Pop 91	*D. dipsaci*	Frankenbach, Germany	sugar beet
pop 80	*D. dipsaci*	Schellerten,, Germany	sugar beet
Pop 79	*D. dipsaci*	Korschenbroich, Germany	Celery
Pop 60	*D. dipsaci*	Renningen, Germany	Maize
Pop 31	*D. dipsaci*	Netherlands	Onion

2.1. Nematode culture on carrot disks

These populations were maintained on a modified carrot disks culture method adopted from Speijer & De Waele (1997). Nematodes were sterilized using a streptomycin sulphate (AppliChem®, Darmstadt, Germany) solution at 0.06 mg/10 ml of sterile water for six hours. Thereafter, nematodes were rinsed three times using sterile water. Approximately 100 µl of water containing about 20 mixed development stages of nematodes were transferred to sterile carrot discs using a sterile pipette. The Petri dishes were sealed with Parafilm® and placed in an incubator (Heraeus®-model BK 5060 EL, Burladingen, Germany) set at 20±1°C for approximately eight weeks.

2.2. Collection of nematodes for morphometrics identification

After eight weeks, some nematodes had egressed onto the surface of the Petri dishes. These nematodes were collected by rinsing the petri-dishes with water using the wash bottle, into a collection bottle. The carrot discs were cut into small pieces using a scalpel blade and transferred to a Baermann funnel overnight to extract nematodes. The nematode suspension was tapped off into the glass bottle the after 12 hours. Nematodes were then used directly for morphometric identification.

2.3. Preparation of nematodes for morphometrics identification

A tipped pipette was prepared prior to nematodes identification for picking individual nematodes in a suspension. The sucking tipped pipette was prepared by burning the tips of two Pasteur pipettes pressed against each other. Then the pipettes were pulled apart after melting started,

resulting into a tiny syringe-like opening, which was used to suck the nematodes from the suspension by capillary action.

In order to perform morphometrics, fifteen individual males and female nematodes per population were handpicked using the tipped pipette and placed onto a glass slide (Menzel GmbH, Braunschweig, Germany), to make temporary slide mounts. Two drops of clean water was added into the glass slide, which was placed onto a hot plate set at 50^{o}C for 3 to 5 seconds. A cover slide was then placed onto the water droplet and sample placed under a camera equipped ZEISS Axioskop50® microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). *Ditylenchus destructor* and *D. dipsaci* nematodes remained straight when killed by heat, a typical character of *Ditylenchus* spp.

Morphometric data and light microscopic images were obtained from digital images on a computer screen with the aid of AxioVision® software version 4.8.2 (Carl Zeiss MicroImaging GmbH, Jena, Germany). Morphometrics measurements in micrometers (µm) (unless otherwise stated) were collected under different magnification depending on the feature of interest. Where necessary, references were made to the original description of *D. destructor* and *D. dipsaci* (Hooper, 1972; 1973).

2.4. Morphometric measurements

The morphometric data used to characterize the populations were: total nematode length (L), stylet length, stylet knobs diameter and height, body with at the vulva/anus, W = diameter of the body, OES= oesophagous length, PUS = post uterine sack, VBW= body width at vulva, VA= distance from vulva to anus. Nematode body ratios (a, b and c) were also estimated in our population following the De Manian formula as summarized by Siddiqi(2000). The ratios were calculated as follows:

$$a = \frac{\text{body length}}{\text{maximum body width}}, \quad b = \frac{\text{body length}}{\text{oesophageal length}}, \quad c = \frac{\text{body length}}{\text{tail length}},$$

$$c' = \frac{\text{tail length}}{\text{tail diameter at anus or cloaca}}, \text{ and } V' = \frac{\text{distance from head end to vulva}}{\text{distance from head end to anus}} \times 100$$

Nematodes images and measurements were recorded before data analyses were performed as shown for some examples in Fig. 1

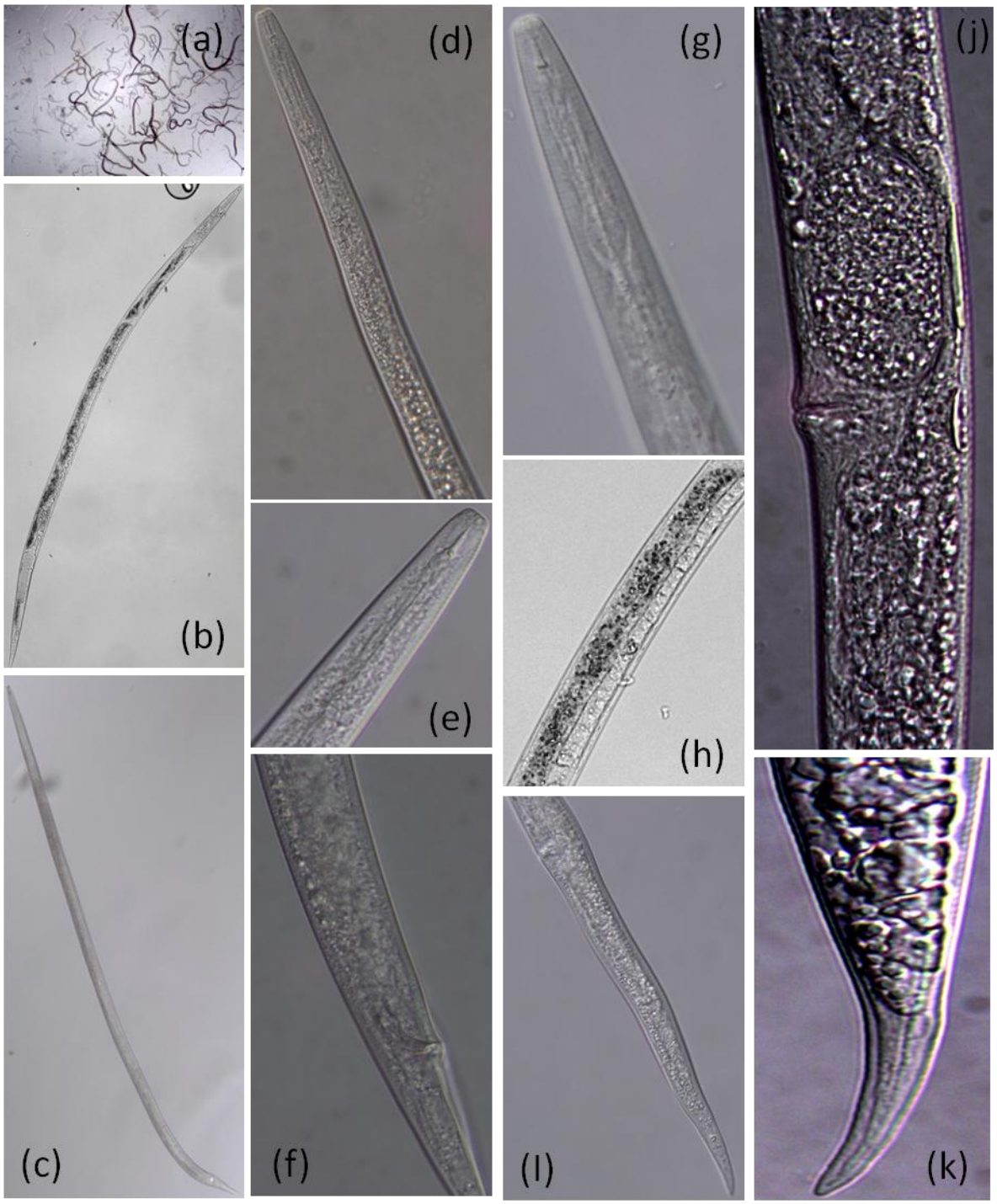

Fig. 1: Photo micrographs of *Ditylenchus destructor* and *D. dipsaci* (a) *D. destructor* in suspension; (b) body length of female *D. destructor*; (c) body length of male *D. destructor* (d) anterior body of female *D. destructor* in lateral view; (e) stylet view of female *D. dipsaci*; (f) spicule of *D. destructor* male and part of tail; (g) anterior body of female *D. dipsaci* in lateral view (h) Ovary germinal apex zone of *D. dipsaci*; (i) *D. dipsaci* vulva and tail, (j) vulva of *D. destructor* and egg inside the body; (k) Tail of *D. destructor*

2.5. Morphometric data analysis

Principal Component Analysis (PCA) was used in SAS statistical software version 9.3 (SAS Institute, Cary, NC, USA). A correlation structure estimate among the female and male morphometrical values of *D. destructor* and *D. dipsaci* were analyzed by means of component of variance using the Principal Component Analysis procedure referred to as PRINCOMP in SAS. The males and females characters used in the analysis are as described in morphometric measurements as stated in section (2.4).

Discriminant Function Analysis (DFA) was performed using the CANDISC procedure in SAS in order to find a set of variables that best discriminate the different populations within one species and also differences between the two species (*D. destructor* and *D. dipsaci*) based on the pooled within variance-covariance matrix, and to test the hypothesis whether or not the species are significantly different from each other based on morphometric values.

2.6. Molecular analysis

2.6.1. DNA extraction

Total genomic DNA was obtained from 20 hand-picked nematodes from each population as described in Table 1. DNA was isolated using DNeasy Tissue Kit (Qiagen, Hilden, Germany). DNA was stored at -4°C until further use. Primer set JB3-forward (TTTTTTGGGCATCCTGAGGTTTAT) and JB5 –reverse (AGCACCTAAACTTAAAACATAATGAAAATG) were used to amplify the mitochondrial DNA (mtDNA) Cytochrome Oxidase subunit I (COI) gene.

2.6.2. Polymerase chain reaction (PCR)

All the Polymerase chain reactions were carried out using an Eppendorf Thermal cycler (Mastercycler® 5333, Eppendorf AG, Hamburg, Germany). Reactions were performed in 50 µl reaction volumes, containing 5 µl 10x PCR buffer, 3 µl 25 mM $MgCl_2$, 3µl 10 mM each primer, 3µl of 2mM dNTP's (dATP, dCTP, dGTP, and dTTP), 0.8 µl Taq DNA polymerase 1 U/µl (Fermentas Life Science GmbH, St. Leon-Rot, Germany), 27.2 µl distilled water and 5 µl template DNA. The thermal cycler was programmed for 1 cycle of 5 min at 94°C; and 35 cycles of 94°C for 1 min, respective annealing temperature for each primer for 1 min and 72°C for 2

min; followed by a final elongation step of 7 min at 72°C and a holding step for 4°C. PCR mixture without DNA template was always included as a negative control.

2.6.3. Gel electrophoresis

Five microlitres of the amplified PCR product were mixed with 1 µl of 6x loading buffer (Fermentas life science GmbH, St. Leon-Rot, Germany) and loaded onto a 1.0% agarose strength gel in 1x TBE buffer. Five microlitres of DNA ladder 100 bp plus (Fermentas Life Science) was loaded on the first and the last wells next to the samples. Gel electrophoresis was performed at 5 V/cm for 1 hr, stained for 15 minutes with 0.1 ug/ml ethidium bromide, and visualized under UV-light using a computer aided NTAS® gel imager machine (Intas Science Imaging Instrument GmbH, Göttingen, Germany), using the GDS Version 3.32 software.

2.6.4. Cloning and sequencing

PCR products from the COII were cloned using the vector pGEM®-T Easy and *Escherichia coli* JM109 high efficiency competent cells (Promega, Madison, WI, USA) which were used for transformation of the ligation product. The resultant plasmid DNA obtained from the *E. coli* culture was purified following the PureYield™ Plasmid Miniprep System (Promega GmbH, Mannheim, Germany) purification kit product guidelines. Samples were sequenced in both directions so as to obtain overlapping sequences for both DNA strands at Macrogen Europe (Amsterdam, The Netherlands). Relevant sequences to *D. destructor* and *D. dipsaci* in reference to the current study were obtained from the National Centre for Biotechnology Information (NCBI) gene database (http://blast.ncbi.nlm.nih.gov) and used for comparison purposes with sequences obtained from our populations. The accession numbers and of the sequences sourced from the gene bank and used for phylogenetic analysis are given in Fig. 7.

2.6.5. Phylogenetic analysis

Phylogenetic analyses of sequences obtained in our study and those retrieved from the NCBI-gene bank were conducted using *MEGA* version 6 software (Tamura *et al.*, 2013). Our sequences pre-assembly processing was carried out in PreGAP4 software (Staden®, Germany) before passing the assemblies to GAP 4 software for comprehensive assembly of the contigs. All sequences were blasted in the NCBI database for similarity search and the relevant sequences from the database obtained for alignment. Additional sequences were sourced from the NCBI,

which were to serve as outlier groups in phylogenetic analysis (Table 3). Multiple sequence alignments were constructed using ClustalW 1.4 (Thompson *et al.*, 1994) with our sequences using BioEdit Sequence Alignment Editor (BioEdit V7.2.7, Thomas Hall, USA) (Hall, 1999). Phylogenetic analysis was based on consensus tree built on the basis of multiple alignments using maximum likelihood (ML) and Maximum Parsimony (MP) as implemented in the software package MEGA6. Kimura 2 parameter model was used to manage missing data and gaps in the contigs (Kimura, 1980). Bootstrap (bs) method was used to determine statistical consistency of each branch using 1000 bootstrapped data set in both ML and MP analysis as obtained in MEGA 6 (Tamura *et al.*, 2013).

3.0. Results

Measurements of males and females of the three *Ditylenchus destructor* populations are reported in table 4. Similar data for *D. dipsaci* populations is summarized in table 5. The bodies of *D. destructor* and *D. dipsaci* remained straight or almost straight after heat fixing. The total body length (in µm) of *D. destructor* males (♂) in the three populations were in the range of 800-1300 µm as described in the original description (Thorne, 1945).

Table 2: Morphometric indices and measurements of *Ditylenchus destructor* (15 males and 15 females) (n = 30). Measurements are in micrometers (µm) and in the form: mean ± standard deviation (range). Sample size N= 15 per nematode sex (males and females).

Characters	***D. destructor* (German population)**		***D. destructor* (Russian population)**		***D. destructor* (Ukraine population)**	
	Male (15) (♂)	**Female (15) (♀)**	**Male (15) (♂)**	**Female (15) (♀)**	**Male (15) (♂)**	**Female (15) (♀)**
Body length (µ)	1085.9 ± 94.7 (1005.8-1310)	1213±119.8 (1029 -1439.7)	1116.3 ± 71.2 (996.9 – 1265.3)	1205.2 ± 101.4 (1041.2 – 1399.9)	1077.7 ± 64.8 (1007.2 – 1189.0)	1184.9 ± 65.5 (1056 – 1289.0)
Body width (µ)	35.2±5.4 (29.9-43.2)	35.8±6.3 (23.4 – 44.1)	35.3 ± 6.7 (26.4 – 51.3)	42.6 ± 4.2 (33.1- 49.0)	31.9 ± 2.2 (27.4 – 35.9)	32.4 ± 2.1 (27.44 + 34.7)
Stylet length (µ)	11.33 ± 1.7 (10-15)	11.2 ± 1.7 (7.6 – 13.1)	11.0 ± 0.9 (9.8 – 13.1)	11.5 ± 1.1 (9.6 – 13.1)	10.8 ± 0.6 (10.1 – 12.1)	11.5 ± 1.1 (9.6 – 13.1)
Post vulval sac length	.	77.9 ± 5.2 (72.2 – 88.3)	.	77.9 ± 5.2 (70.2 – 88.6)	.	73.3 ± 1.2 (70.6 – 75.1)
Body width at vulva (µ)	.	41.4 ±8.6 (37.7 – 50.0)	.	33.9 ± 4.1 (23.9 – 39.4)	.	31.3 ± 1.3 (29.3 – 34.2)
Tail length	65.6 ±13.9 (46.2-98.0)	73.0 ± 9.8 (60.0 -80.0)	73.0 ± 3.3 (70 – 79.4)	73.4 ± 4.8 (65.2 – 78.9)	72.9 ± 3.3 (64.4 – 77.0)	74.9 ± 2.3 (70.8 – 78.7)
Body width at anus	29.09 ±3.8 (20.0-32.6)	29.2 ± 6.1 (20.0 – 44.0)	28.5 ± 3.8 (20.0 – 32.5)	30.5 ± 3.1 (25.1 – 36.9)	25.0 ± 3.8 (20.4 – 30.9)	28.5 ± 2.4 (20.1 – 35.2)
a ratio	31.5± 5.1 (23.8 – 38.0)	35.5 ± 10.1 (24.4 – 41.6)	32.5 ± 5.5 (22.1 – 40.1)	28.5 ± 3.1 (25.1 – 36.9)	34.0 ± 3.2 (30 -42.4)	36.8 ± 3.9 (31.2 – 43.8)
b ratio	4.6 ± 2.2 (2.7-8.3)	5.6 ± 2.3 (2.5 – 8.8)	6.5 ± 1.5 (3.7 – 9.0)	6.8 ± 1.9 (4.1 – 10.1)	6.3 ± 1.4 (3.6 -7.9)	6.7 ± 1.8 (4.2 – 10.1)
c ratio	7.3 ± 1.3 (5.2- 9.2)	7.3 ± 1.0 (5.6 – 9.0)	7.3 ± 1.2 (4.9 – 9.2)	7.6 ± 1.5 (5.4 – 10.2)	6.2 ± 1.1 (4.2-7.9)	7.2 ± 1.1 (5 – 9.6)
c’ ratio	5.3 ± 0.8 (4.5- 6.8)	6.0 ± 1.3 (3.3 – 8.0)	5.7 ± 1.8 (4.3 – 10.0)	5.4 ± 0.9 (4.1 – 7.1)	7.2 ± 1.8 (5.1 – 9.8)	5.9 ± 1.9 (4.2 – 8.5)
V%	.	80.0 ± 4.4 (72.7 – 90.2)	.	70.8 ±27.3 (75.5 – 93.7)	.	75.4 ± 5.0 (64.7 – 83.1)

Table 3: Morphometric indices and measurements of *Ditylenchus dipsaci* (15 males (♂) and (15 females **(**♀**)**) from different host plants. All measurements are in μm and in the form: mean ± standard deviation (range). Sample size N= 15 per nematode sex (males and females)

Characters	***D. dipsaci (Pop_80)***		***D. dipsaci (Pop_79)***		***D. dipsaci (Pop_60)***		***D. dipsaci (Pop_31)***		***D. dipsaci (Pop_91)***	
	Male (♂) (15)	**Female (♀) (15)**	**Male (♂) (15)**	**Female (♀) (15)**	**Male(♂) (15)**	**Female (♀) (15)**	**Male(♂) (15)**	**Female (♀) (15)**	**Male (♂) (15)**	**Female (♀) (15)**
Body length (μ)	1196.2 ± 65.0 (1098.5 - 1360.1)	1355.3± 85.7 (1149.6 - 1437.8)	1111.9 ± 67.1 (986.0 - 1296)	1206.8 ± 84.3 (1086.1 - 1329.6)	1080.6 ± 61.3 (1007.1 - 203.3)	1138 ± 84.6 (1036.2 - 1282.7)	1098.1 ± 126.4 (810 - 1326.1)	1167.9 ± 70.7 (1086.3 - 1326.1)	1176.1 ± 102.6 (993.6 - 1411)	1180 ± 99.5 (959.4 - 1329.0)
Body width (μ)	29.5 ± 2.8 (24.2 - 34.1)	29.3 ± 3.3 (20.1 - 34.1)	32.6 ± 4.1 (25.3 - 38.4)	33.5± 3.1 (28.3 - 38.5)	30.8 ± 2.7 (28.3 - 39.0)	31.6 ± 3.4 (27.6 -37.5)	31.8 ± 3.1 (25.7 - 37.9)	31.5 ± 41.2 (25.1 - 38.1)	26.9 ± 2.0 (24.0 -30.3)	31.8 ± 3.4 (26.0 -38.1)
Stylet length (μ)	12.1 ± 2.8 (10.0 -21.4)	11.3 ± 1.1 (10.0 - 13.5)	11.5 ± 0.8 (9.3 - 12.4)	11.6 ± 0.8 (10.0 -12.3)	10.4 ± 0.6 (9.8 - 11.9)	11.1 ± 1.0 (10.1 - 13.2)	12.0 ± 1.0 (10.3 - 13.3)	12.5 ± 2.5 (10.7 - 21.3)	12.5 ± 2.6 (10.4 - 21.4)	11.8 ± 1.1 (9.3 - 13.7)
Post vulval sac length	.	61.3 ± 2.4 (59.9 - 65.8)	.	66.5 ± 4.6 (59.8 - 73.3)	.	35.7 ± 7.2 (23.4 - 52.1)	.	73.6 ± 12.7 (52.4 - 98.49)	.	66.1 ± 4.4 (60.1 - 76.5)
Body width at vulva (μ)	.	28.9 ± 2.4 (23.7 - 31.4)	.	27.8 ± 2.2 (24.0 - 30.4)	.	28.4 ± 2.0 (24.2 - 30.4)	.	27.3 ± 12.7 (21.3 - 35.1)	.	27.9 ± 1.8 (24.1 - 29.5)
Tail length	66.1 ± 4.4 (60.1 - 76.5)	62.5 ± 3.1 (57.3 - 69.1)	69.1 ± 4.5 (61.2 - 73.1)	66.5 ± 4.6 (59.8 - 73.3)	80.6 ± 4.3 (69.8 - 84.7)	50.2 ± 34.3 - 94.3)	77.7 ± 12.8 (58.44 - 98.4)	73.6 ± 3.4 (52.5 - 98.5)	81.3 ± 4.7 (72.5 - 89.8)	81.7 ± 5.2 (69.8 - 86.9)
Body width at anus	19.9 ± 3.3 (13.4 - 22.9)	18.4 ± 2.5 (13.9 - 22.8)	17.1 ± 1.5 (13.1- 18.9)	17.8 ± 1.2 (16.0 - 19.4)	17.4 ± 2.2 (11.3 - 19.9)	18.3 ± 1.2 (16.3 - 20.1)	21.3 ± 1.7 (19.7 - 24.9)	28.5 ± 5.6 (19.03 - 32.6)	17.5 ± 1.7 (12.4 -19.3)	15.5 ± 1.8 (12.1 - 17.9)
a ratio	40.9 ± 4.8 (33.9 - 52.3)	47.0 ± 7.0 (40.0 -69.1)	34.5 ± 4.1 (28.8 - 44.6)	36.4 ± 5.1 (28.2 - 46.9)	35.4 ± 3.5 (25.8 - 38.8)	36.3 ± 3.5 (29.1 - 41.8)	34.7 ± 3.7 (29.0 - 42.5)	37.8 ± 2.3 (29.6 - 47.3)	43.8 ± 4.4 (37.8 - 53.8)	37.6 ± 5.7 (29.3 - 50.1)
b ratio	15.9 ± 1.2 (14.4 - 18.2)	18.4 ± 1.9 (15.0 - 21.8)	15.1 ± 1.1 (13.4 - 17.7)	17.0 ± 2.0 (14.1 - 21.5)	16.3 ± 1.5 (13.8 - 19.0)	15.7 ± 2.6 (12.1 - 19.7)	18.5 ± 2.8 (14.7 - 26.1)	20.7 ± 0.9 (17.4 - 26.1)	16.4 ± 1.4 (14.9 - 19.8)	15.5 ± 1.7 (13.1 - 19.4)
c ratio	5.6 ± 0.7 (4.3 - 6.8)	6.3 ± 0.6 (5.3 - 7.4)	5.9 ± 0.7 (5.0 - 7.2)	6.1 ± 0.8 (5.0 - 8.6)	6.2 ± 1.0 (4.8 - 8.5)	5.2 ± 0.8 (4.5 - 6.8)	6.6 ± 0.8 (5.4 - 7.9)	6.4 ± 0.8 (4.7 - 7.9)	5.9 ± 0.7 (4.4 - 7.8)	6.2 ± 0.8 (5.3 - 8.1)
c' ratio	11.1 ± 2.0 (7.6 - 14.3)	12.0 ± 1.6 (9.5 - 15.5)	11.2 ± 1.4 (8.9 - 13.3)	11.3 ± 1.4 (8.6 - 13.3)	10.3 ± 1.4 (7.5 - 13.5)	12.1 ± 1.3 (9.1 - 14.0)	7.9 ± 1.1 (5.9 - 9.3)	6.6 ± 0.8 (4.9 - 8.0)	11.5 ± 2.0 (9.2 - 15.3)	12.4 ± 1.1 (10.5 - 14.2)
V%	.	67.6 ± 5.0 (62.2 - 80.3)	.	74.7 ± 67.0 (87.0 - 82.2)	.	72.4 ± 5.1 (64.8 - 80.8)	.	73.4 ± 3.9 (67.4 -79.3)	.	74.6 ± 4.7 (67.2 - 81.3)

3.1. Morphometric analysis result

3.1.1. Morphometric differentiation within *D. destructor* populations

The Principle Component Analysis (PCA) of *D. destructor* populations (isolated from different host plants in Germany, Russia and Ukraine) demonstrated that the populations were statistically different based ($P < 0.0001$) on some morphometric attributes. The first two principle components (eigenvalues 4.1 and 3.4) accounted for 42% of the total variance (Fig 2). Based on the discriminant function analysis, males and females of *D. destructor* differed significantly from each other ($P < 0.0001$). The pooled within-class canonical structure coefficients of the Discriminant Function Analysis (DFA) showed that, when males and females of *D. destructor* populations were considered, the first axis was best explained by the highest body width (eigenvalue 1.1) and body with at the vulval (eigenvalue 0.8).

Morphometric differentiation within *D. dipsaci* populations

Similarly, significant ($P < 0.001$) differences were observed between the different populations of *D. dipsaci*. The highest morphological differences between the different populations were contributed by the c' ratio (eigenvalue 3.4) and the Oesophagoal length (eigenvalue 2.1) which contributed to 46% of the total variance. Post uterine sack length was the third most important parameter. (eigenvalue 1.2).

3.1.2. Morphometric differentiation between *D. destructor* and *D. dipsaci*

Principal Component Analysis (PCA) of *D. destructor* and *D. dipsaci* male and female characters showed a separation between *D. destructor* and *D. dipsaci* species (Fig. 1). However, the first two principle components (Eigen values 6.1 and 3.2) accounted for only 52.2% of the total variance (Fig 2).

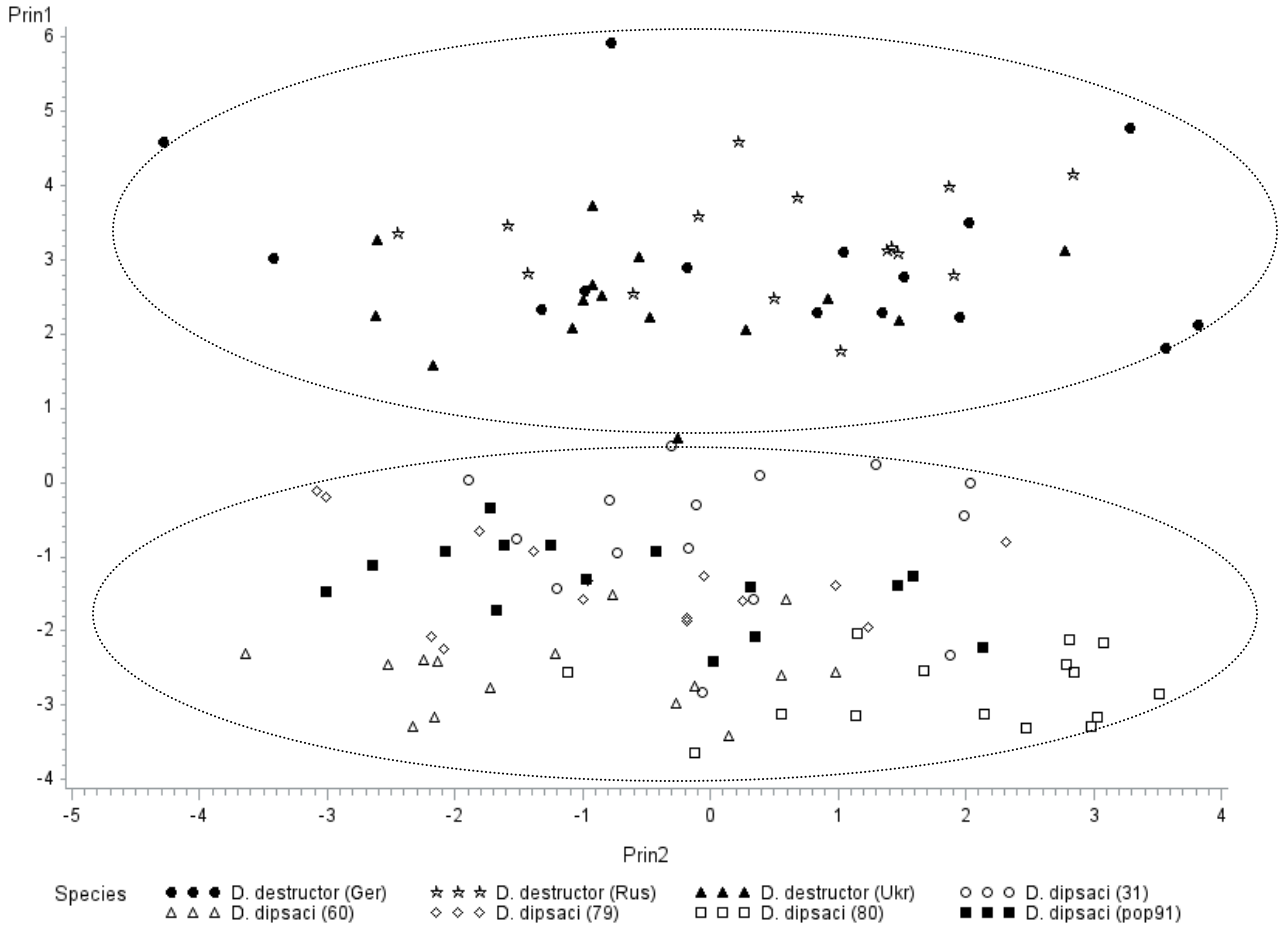

Fig. 2: Two dimensional correlation-based principle component analysis (PCA) of the selected male and female morphometrical data of *Ditylenchus destructor* and *D. dipsaci* populations. Morphometrical characters included total nematode length (L), stylet length, stylet knobs diameter and height, body width at the vulva/anus, the highest body width, total aesophagous length, post vulval sack length, body width at vulva, distance from vulva to anus, ratios a, b, c and V%.

The multivariate statistics of the Discriminant Function Analysis (DFA) analysis showed that the analyzed *D. destructor* and *D. dipsaci* species were significantly ($P < 0.0001$) separated based on the males and females morphometrical values. The pooled within-class canonical structure coefficients of the Discriminant Function Analysis (DFA) showed that the first axis was best explained by the ***a***-ratio (eigenvalue 0.7) (Fig. 3), the highest body width (eigenvalue of 0.6) (Fig. 4) and post uterine sac length (PUS) (eigenvalue 0.4) (Fig. 5). Therefore ***a***-ratio, highest body width and post uterine sac length have the highest morphometrical power to separate *D. destructor* from *D. dipsaci* populations. The principal component analysis demonstrated that, the

rest of the morphometric data collected did not show any differences ($P > 0.05$) between *D. destructor* and *D. dipsaci*.

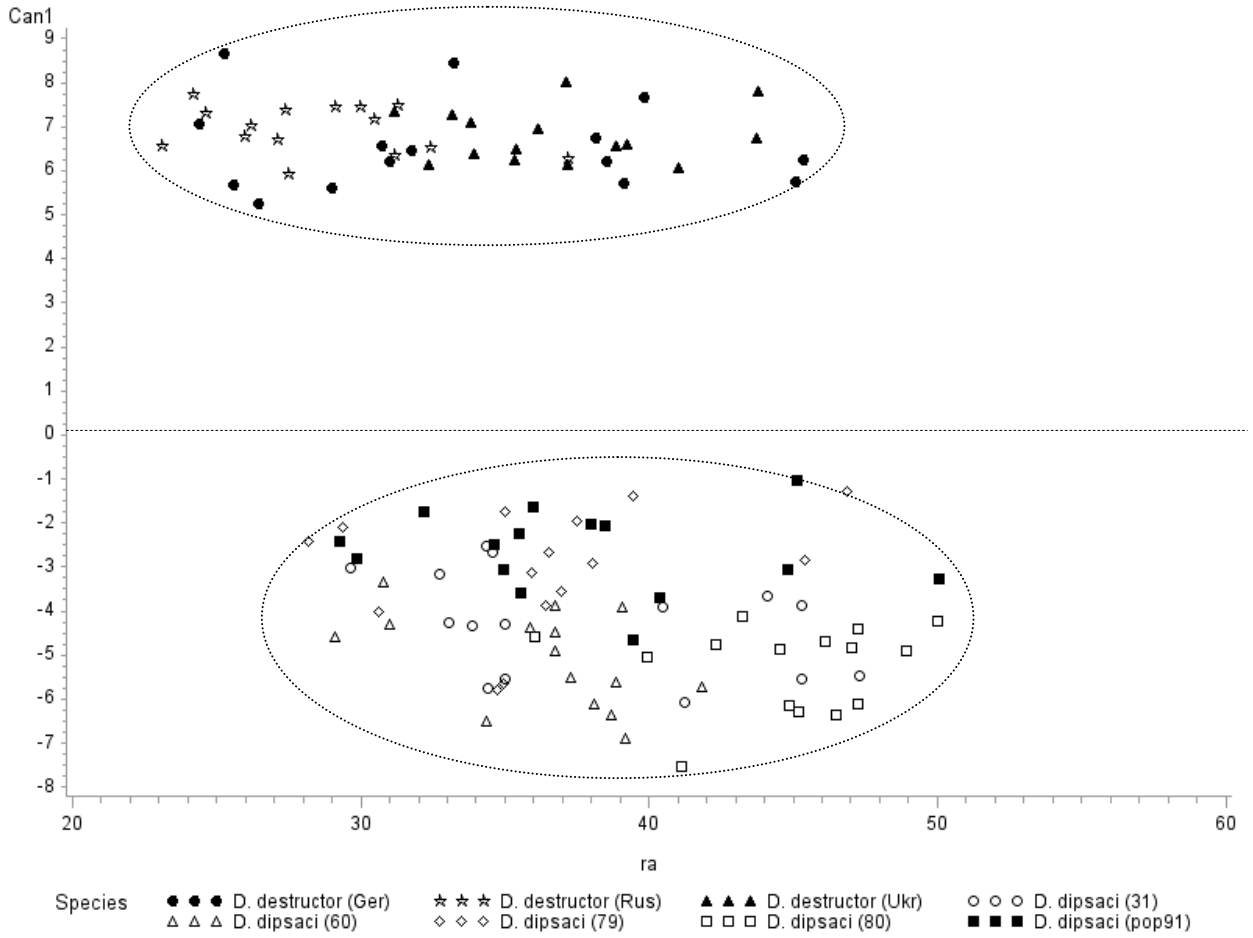

Fig. 3: Discriminant functions analysis (DFA) of the A ratio values (body length /highest body width) obtained from both *Ditylenchus destructor* and *D. dipsaci*. Non-overlapping ellipses indicate significantly different groups (*D. destructor* and *D. dipsaci*).

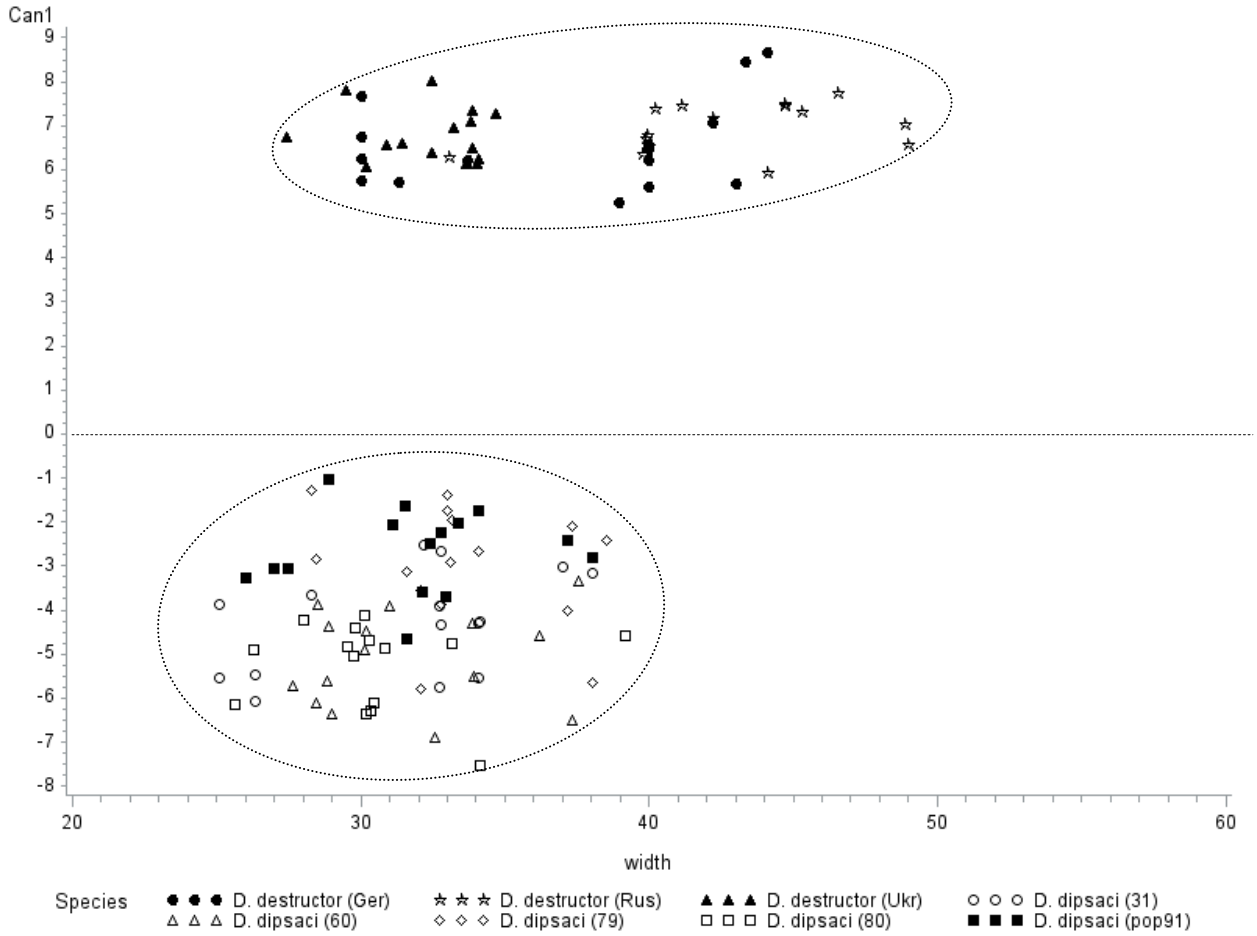

Fig. 4: Discriminant functions analysis (DFA) of the highest body width values obtained from both *Ditylenchus destructor* and *D. dipsaci*. Non-overlapping ellipses indicate significantly different groups (*D. destructor* and *D.dipsaci*).

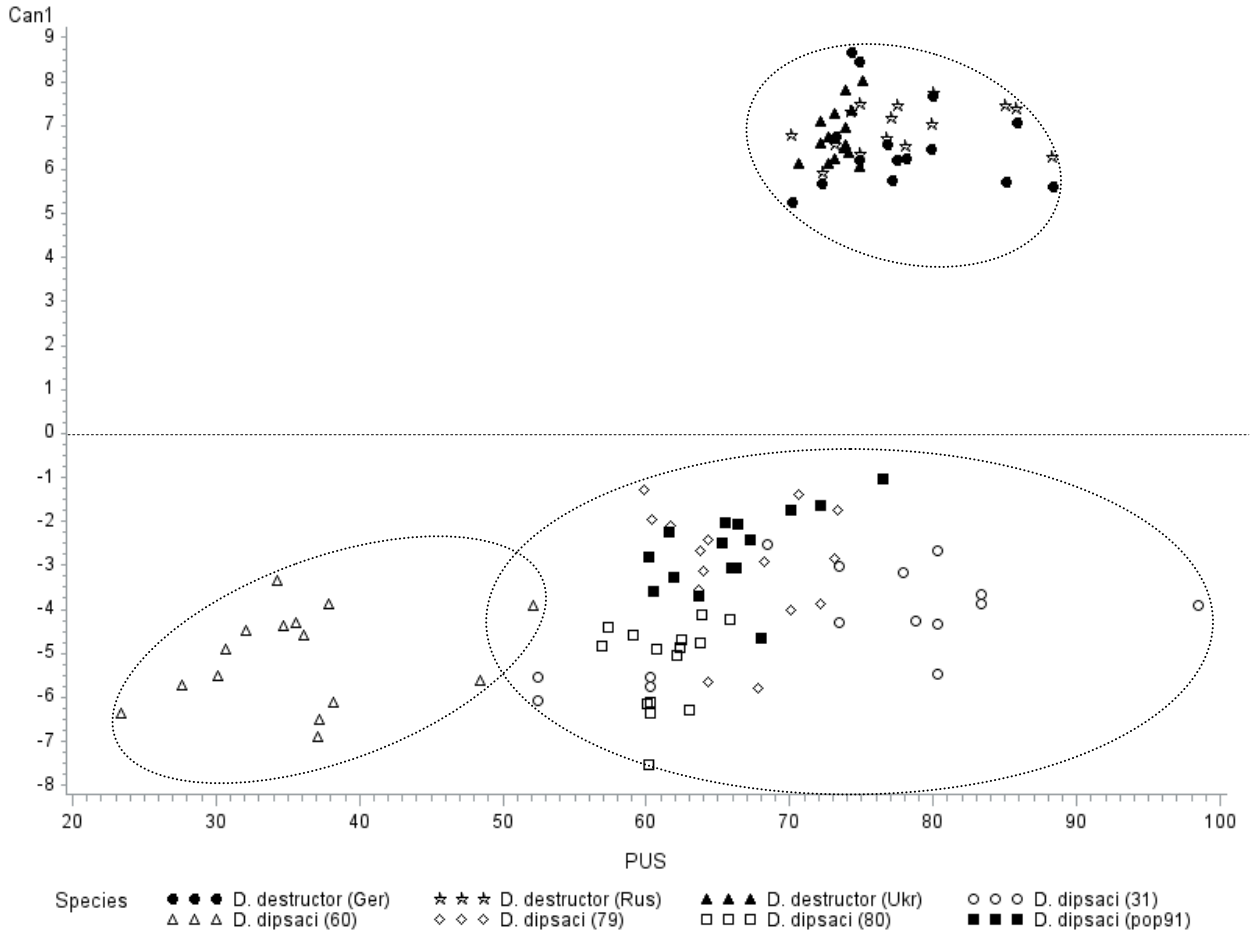

Fig. 5: Discriminant functions analysis (DFA) of the post uterine sac length (PUS) values obtained from both *Ditylenchus destructor* and *D. dipsaci*. Non-overlapping ellipses indicate significantly different groups (*D. destructor* and *D. dipsaci*), while overlapping ellipses indicate non-significance between the groups.

3.2.0. Polymerase chain reactions (PCR) results

The PCR amplification of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit I, using the COI gene resulted into a single DNA amplification of ~400 bp for both *D. destructor* and *D. dipsaci* (Fig. 5). DNA sequencing revealed the exact sizes of the amplified cytochrome oxidase subunit were 395 bp.

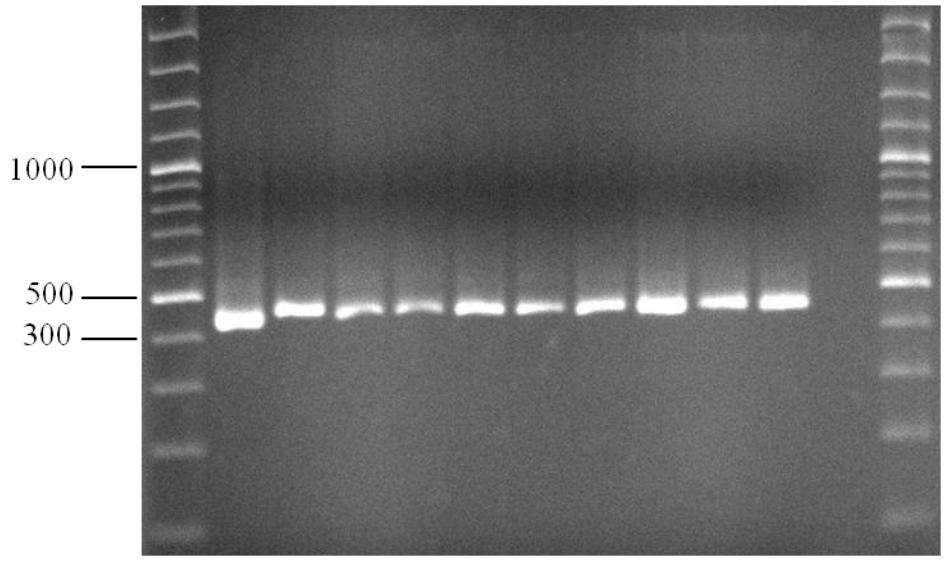

Fig. 6: Agarose gel of the amplification of cytochrome oxidase subunit I (COI) segment amplicons of the mitochondrial DNA (mtDNA) gene.

3.2.1. Sequence analysis of the different segments of DNA

The alignment of the sequences from our eight samples and those obtained from the gene bank (13 from gene bank) resulted into a length of 407 bp. The nucleotide compositions of the amplified cytochrome oxidase subunit COI were the following: 21.6% A, 12.9% C, 26.7% G and 38.8% T for *D. destructor* and 25.1% A, 13.6% C, 21.1% G, and 40.2% T for *D. dipsaci* populations. The cytochrome oxidase subunit I segment showed a GC content of ~40% for *D. destructor* species and 35% for *D. dipsaci* species.

There were no relevant sequences in the gene bank of the cytochrome oxidase subunit I (COI) for *D. destructor* and *D. dipsaci*. Therefore, the sequences obtained in the current experiment were the first for these two species. However, COI sequences from related species were available and were therefore used in the current phylogenetic analysis. Based on the maximum likelihood (ML) phylogentic analysis of the cytochrome oxidase subunit I (COI) segment two distinct clades were obtained from our population. The tuber rot nematodes (*D. destructor*) was in a distinct clade

supported by 99% bootstrap value, while the stem nematodes (*D. dipsaci*) populations were in a distinct clade. The bootstrap values for *D. dipsaci* were lower than 50%, meaning that there were high similarities within the populations based on the cytochrome oxidase subunit I (COI) sequences from these populations. However, it was clear that the sequences from cytochrome oxidase subunit I (COI) segment obtained from our samples were distinct from the out-groups obtained from the gene bank as indicated by a bootstrap value of 100% (Fig. 7).

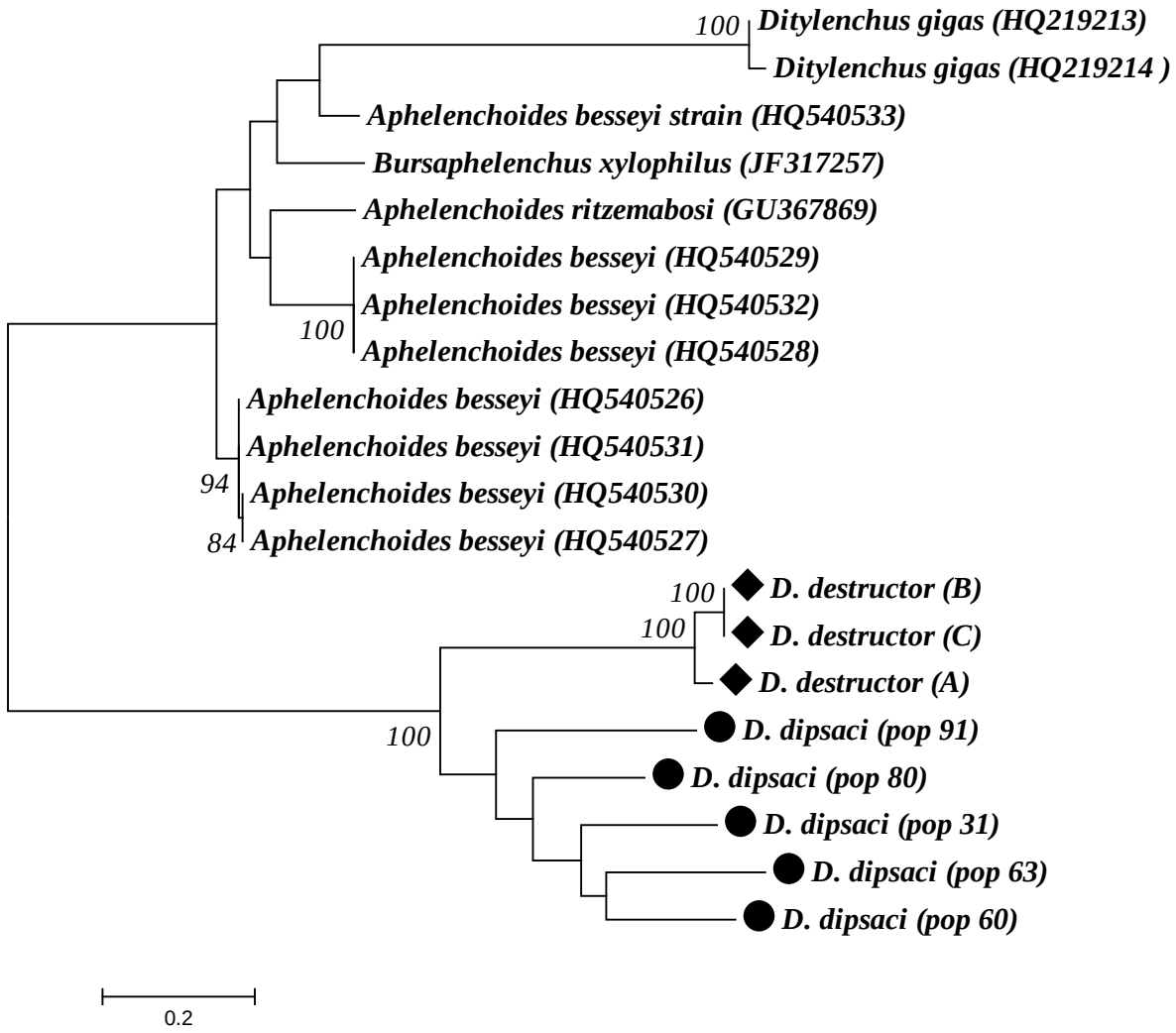

Fig: **7**. Maximum Likelihood tree inferred from *D. destructor* and *D. dipsaci* populations sequence data of the Cytochrome Oxidase subunit I (COI) of the mitochondrial DNA (mtDNA) gene. Newly sequenced species are indicated using display markers in two different shapes, while those from the gene bank are in normal bold font followed by accession number. Values at branches denote percentual bootstrap values (out of 1000 replicates).

4.0. Discussion

Morphometric analysis is considered the first step towards the identification *Ditylenchus destructor* and *D. dipsaci* (EPPO, 2008). In this study, the morphometric characters of *D. destructor* and *D. dipsaci* collected concurred with the original descriptions of these species (Hooper, 1972; 1973). However, due to limitations of the light microscope, ideal features such as the number of lateral lines were difficult to identify in our populations. *Ditylenchus destructor* and *D. dipsaci* are morphologically very similar and therefore very hard to differentiate using routine features. The intraspecific variations complicate morphological identification. In our morphometric study, the two most variable characters observed for each species accounted for less than 50% of the observed differences. This indicated that, the nematodes within the populations were morphologically very similar to each other and only finer characteristics could be used to separate the populations and species. Morphometric differences *in D. destructor* are contributed by the geographical distribution and ecophenotypic effects of the different hosts (Goodey, 1952). It could be possible that the differences between populations could be attributed to the geographical location or host plant where these populations were extracted from.

Ditylenchus destructor and *D. dipsaci* morphometric data obtained from adults is known to vary based on the host they were isolated from, stage of development and environment (Evans & Fisher, 1970; Hooper, 1972; 1973; Brzeski, 1991). In our study, variations within *D. destructor* populations were best discriminated based on body width, body width at the vulva for females, while *D. dipsaci* were best differentiated by the c' ratio and the esophageal length. Differences between *D. destructor* and *D. dipsaci* were additionally differentiated using the post uterine sack length in the current study. Although some morphometric features are known to vary under changing environments, the post uterine sac length has been demonstrated to be reliable for characterization of *Ditylenchus* species (Goodey, 1958; Evans & Fisher, 1970).

All the populations used in the current study were maintained on carrot disks at equal length of culture duration and ideal constant temperature of 20°C. Factors such as culture medium, temperature and nematode initial density have been demonstrated to cause morphometric variations within apopulation (Ludwig, 1938; Fisher, 1965). We can conclude that, in our study, any influence of culture medium, temperature and initial density in our populations were uniform across all cultures, since the standard treatments were distributed to all cultures. Therefore, it's

justified to conclude that in our study the differences observed within populations of similar species were due to genetic variations. Similar observations were made by (Thorne & Allen, 1959), who observed that, 10-35% of the mean variation in field populations of *D. destructor* were due to genetic variations.

Previously, most reports on morphometric data were only reported based on ratios and means of the various measurements studied. Studies including multivariate analysis provide better resolution on variations among and between populations and species. The use of the Principle Component Analysis and the Dicriminant Function Analysis made it easier for the identification of the most suitable character for separating the two species studied. The main goal was to use morphometrics to infer to species differences between *D. destructor* and *D. dipsaci*. However the advance in multivariate analysis was ideal to detect even slight differences in populations.

Although body width, a' ratio, c'ratio and post uterine sack length were suitable characters in separating the two *Ditylenchus* species studied, it was not possible to conclude experimentally that these characters could be stable under different environmental condition. It has been shown that *D. destructor* cultured in different hosts had a mean body length deviation of up to 64% (Goodey, 1952). Similarly, *D. dipsaci* races have also been observed to vary in different morphological characters depending on the host plant (Barraclough & Blackith, 1962). The use of de Manian ratios for the description of new species or for distinction between species has come under serious criticism due to its inability to stay constant even within the same sample size (Barraclough & Blackith, 1962). Therefore, it could be ideal to study the same populations after isolation from host plants and compare the results with those of the same populations reared on carrot disc cultures. Due to the variations of morphometric data, molecular data of the same populations was included in the current study for comparison and confirm the identity of the populations.

Molecular analyses have improved reliability and sensitivity of nematode identification, especially where morphometric data is compromised by the presence of morphologically similar characteristics such as *D. destructor* and *D. dipsaci* (Subbotin *et al.*, 2005). All the sequences obtained and blasted in the gene bank for species identity were consistent with morphometric evidence collected from the same populations. This indicated that our morphometrically based identification of *D. destructor* and *D. dipsaci* was congruent with molecular-based phylogenies.

To the best of our knowledge, sequences from amplified genes of COI from *D. destructor* and *D. dipsaci* based on JB3 and JB5 primers as suggested in Q-Bank, were obtained for the first time in the current study. Therefore comparisons could be only done with related species deposited in the gene bank. However, it was clear that *D. destructor* was separated from *D. dipsaci*.

5.0 Conclusion

Molecular phylogenies of *D. destructor* and *D. dipsaci* point to intriguing questions of morphological evolution and challenge us to employ emerging new tools in a comparative framework, in order to unravel these complex patterns in support of a refined and improved classification. This study demonstrated that some finer morphological characters are ideal in separating the two species studied. It could be that culturing the nematodes in the laboratory could have reduced or introduced new variations in morphometric and genetic characters. After such characterizations, studies on the same populations after they have been reared on a host plant could further evaluate morphometric or genetic variations. Although morphometric and molecular diagnostics methods could be used independently, it's suggested that both methods are combined to compliment identification.

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Chapter 3:

Resistance and tolerance of potato varieties to potato rot nematode (*Ditylenchus destructor*) and stem nematode (*Ditylenchus dipsaci)*

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Abstract

Ditylenchus destructor and *D. dipsaci* are economically important plant-parasitic nematodes, affecting potato production mostly in temperate climates. Management through crop rotation is not feasible due to their wide host range. These nematodes are listed as quarantine pests in many countries. Limited information exists on the resistance and tolerance of currently cultivated potatoes to *D. destructor* and *D. dipsaci*. Two greenhouse experiments were conducted to screen twenty-five potato varieties for resistance to and tolerance for *D. destructor* and *D. dipsaci* infections. Reproduction factor (RF) and relative susceptibility (RS) were used to evaluate resistance, while potato tuber damage and tuber weight reduction was used to evaluate tolerance. Based on the RF, sixteen varieties were evaluated as susceptible (S) while five varieties were evaluated as resistant (R) to *D. destructor*. Varieties "Innovator", "Aveka" and "Spunta" were resistant to *D. dipsaci* based on RF. "Désirée" was observed to be highly susceptible to *D. destructor* and *D. dipsaci* in both experiments and was used as the standard susceptible control variety for the calculation of relative susceptibility. A scale of 1 to 9 was used to classify relative susceptibility of the potato varieties to *D. destructor* and *D. dipsaci*, where 9 indicated the highest level of resistance. All classes of resistance to *D. destructor* and *D. dipsaci* were observed in the potato varieties tested in the experiments. Six varieties had significantly lower RS to *D. dipsaci* than the standard susceptible control variety. Tolerant to highly sensitive potato varieties to both nematodes were also observed. Relative susceptibility and external potato tuber damage were identified as suitable methods for resistance and tolerance determination, respectively. This study provides essential information on the status of resistance and tolerance in potato varieties against *D. destructor* and *D. dipsaci* but needs to be confirmed under field conditions.

Keywords – *Solanum tuberosum*, susceptibility, sensitivity, reproduction factor, relative susceptibility, damage, screening, *Ditylenchus* spp.

1.0 Introduction

Potato tuber rot nematode, *Ditylenchus destructor* Thorne, 1945, and the stem nematode, *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936, are among the major potato (*Solanum tuberosum* L.) nematodes causing serious economic losses especially in temperate climate zones (Plowright *et al.*, 2002). Potato is the main host to *D. destructor*, however, the nematode can be found feeding on over 70 crops and weeds and also on many fungal species (Henderson, 1951; Andersson, 1971; Ivanova, 1973; Winslow, 1978; De Waele *et al.*, 1991; Sturhan *et al.*, 2008). *Ditylenchus dipsaci* is a cosmopolitan nematode with an ability to colonize over 500 plant species (Viglierchio, 1971; Sturhan & Brzeski, 1991). Several biological pathotypes or races have been described for *D. dipsaci*, making it a complex nematode species (Seinhorst, 1957; Subbotin *et al.*, 2005).

Ditylenchus destructor and *D. dipsaci* cause qualitative damage to potato tubers by producing conical pits, often accompanied by skin splitting and rotting due to secondary invasion by bacteria and fungi (Jenkins & Taylor, 1967; Southey, 1971; Mai *et al.*, 1981; De Waele *et al.*, 1991; Cotten *et al.*, 1992). This type of damage makes the tubers unmarketable, while at the same time, infested but symptomless tubers facilitate dissemination of these nematodes. Symptoms caused by *D. destructor* and *D. dipsaci* on potato tubers differ in depth of damage inside the tuber tissues (Seinhorst & Dunlop, 1945; Jenkins & Taylor, 1967). Lesions caused by *D. destructor* are superficial, while those of *D. dipsaci* frequently extend into considerable depth inside the potato tuber (Seinhorst & Dunlop, 1945; Jenkins & Taylor, 1967).

Management of *D. destructor* and *D. dipsaci* through crop rotation is difficult due to their polyphagous nature. As a result these nematodes are listed within the European Union as quarantine nematodes to limit their spread (EPPO, 2008). Cultivation of resistant varieties often provides an effective alternative method for management of various plant parasitic nematodes (Cook & Starr, 2006). Host plant resistance and tolerance to pests and diseases are desirable characters for plant varieties to reduce yield losses (Peng & Moens, 2002; Cook & Starr, 2006).

In earlier reports, damage levels were often used as indicators for resistance whereas nowadays damage levels are used to quantify tolerance. Over the years, the definition of the terms resistance and tolerance in nematology have been under constant review to harmonise

communication among scientists (Trudgill, 1991; Barker, 1993). A resistant plant (antonym: susceptible plant) is a host plant which is able to prevent multiplication of the nematode (Trudgill, 1991; Cook & Starr, 2006). Tolerance (antonym: sensitivity) is measured as the amount of injury nematodes cause on a host plant, or the ability to withstand or recover from the injury caused by the nematode (Cook & Evans, 1987; Wallace, 1987; Roberts, 1992; Trudgill, 1992; Cook & Starr, 2006). Currently, both plant resistance and tolerance to plant parasitic nematodes have increased in importance in the management of nematodes due to economics of production and increasing concerns over environmental hazards caused by continuous use of pesticides (Peng & Moens, 2002).

Until *D. destructor* was described (Thorne, 1945), populations of this species were considered to belong to *D. dipsaci*. The first report on resistance in potato varieties to *D. destructor* was published by Seinhorst and Dunlop (1945). In 1956, 25 commercial potato varieties were tested and found to be all susceptible to *D. destructor* (Goodey, 1956). In Belarus, 29 varieties were reported to be susceptible to *D. destructor* (Guskova, 1966). In Poland, Kornobis, (1968) tested 92 potato varieties, and reported only three varieties with resistance to *D. destructor*. In Ireland, 15 commercial potato varieties were all susceptible to *D. destructor* (Moore, 1971). Similar tests in Sweden including 19 potato varieties reported that the variety "Bintje" was more susceptible compared to other varieties (Andersson, 1971). In 1972, 62 varieties were screened and only 11 were found to be resistant to *D. destructor* (German, 1972). Two years later, 111 varieties were screened by Kostina & Zholudeva, 1974, finding only 7 resistant varieties. In Belarus, most of the local varieties tested were all susceptible to *D. destructor*, while only a few foreign varieties were reported to be fully resistant (Ponin *et al.*, 1983).

Trials for resistance and tolerance of potato varieties to the stem nematode (*D. dipsaci*) have been published only in a few cases. Tests for resistance against *D. dipsaci* in potato varieties were first reported by Nikulina in 1970. In 1971, Shepshelev & Chernikova, found no case of complete resistance in 79 potato varieties and more than 100 hybrids. The same authors tested 57 potato varieties in 1975, and found 8 varieties resistant against *D. dipsaci*. Since then, the search for resistant potato varieties against *D. dipsaci* has remained unreported. Although earlier reports documented presence of resistant or tolerant potato varieties against *D. destructor* and *D. dipsaci*, most of these varieties are no longer available.

Evaluation of resistance and tolerance to *D. destructor* and *D. dipsaci* is complicated due to the lack of a standardized screening method. Resistance tests often use the nematode reproduction factor (RF) as a measure of resistance to nematodes (Oostenbrink, 1966). The use of the reproduction factor ($RF = P_f/P_i$ where P_f is the final population density and P_i is the initial population density) has its shortcomings, since it is density dependent (Oostenbrink, 1966). Also, nematode damage in tubers may influence nematode multiplication. As an alternative to RF, relative susceptibility (RS) (Phillips, 1984) is used. Relative susceptibility can be expressed as the ratio of final population density of a nematode population on a test variety compared to the final population density on a standard susceptible reference variety (EPPO, 2006). Until this study, no standard susceptible reference variety to both *D. destructor* and *D. dipsaci* has been reported.

Two greenhouse experiments were conducted with the following objectives:

- To assess resistance and tolerance of currently cultivated potato varieties against *D. destructor* and *D. dipsaci*.
- To compare the suitability of the reproduction factor and relative susceptibility for resistance evaluation in potato varieties.

2.0. Materials and methods

Planting material

Tubers from 25 potato varieties were pre-germinated in the dark at 20±3^{o}C until sprouts were observed after which they were placed in the light to harden the sprouts. Tubers or tuber pieces weighing 15±1g each and bearing a single sprout (about 1 cm long) were used as planting material.

Growing medium

Growing medium was prepared by mixing dry heat sterilised field soil and peat mix (Klasmann® Lithuanian peat moss medium, pH 3.5) at the ratio of 3:1, respectively. Heat sterilization was carried out using an electric sterilizer (Sterilo®), at 100±5^{o}C for 12 hours. Slow release fertiliser (Osmocote Exact®Standard® 15% N, 9% P_2O_5, 12% K_2O and 2% M_gO) was added to the

artificial growing medium mix at the rate of 1.5 g/kg growing medium. The final growing medium had a pH of 4.7, organic matter 26%. The texture was clay 7.5%, silt 19.1% and sand 73.4%. The minerals in the growing medium calculated in mg/100g of growing medium consisted of Potassium (K): 36 mg/100 g of growing medium, Phosphorus (P): 16 mg/100 g of growing medium and Magnesium (Mg): 10 mg/100 g of growing medium.

Nematode culture and nematode suspension preparation

Ditylenchus destructor and *D. dipsaci* populations used in this study were originally isolated from celery and sugar beet plants respectively sampled in Germany (Julius Kühn-Institut collection). Axenic cultures of these populations were maintained and multiplied on carrot discs in Petri dishes (10 mm Ø). The carrot disc culture method was a modification from a protocol developed by Speijer and De Waele in 1997. Nematodes were sterilized using a streptomycin sulphate (AppliChem®) solution at 0.06 mg/10 ml of sterile water for six hours. Nematodes were rinsed three times using sterile water. Approximately 100 µl of water containing about 20 mixed development stages of nematodes were transferred to sterile carrot discs using a sterile pipette. The Petri dishes were sealed, labeled and incubated (Heraeus®-model BK 5060 EL, Germany) at 20^{o}C for approximately eight weeks.

Nematodes were collected by rinsing the Petri dishes with tap water into a clean 500 ml glass bottle. Carrot discs were obtained from the Petri dishes, cut into small pieces using a scalpel blade and transferred to a Baermann funnel overnight to extract nematodes. The nematode suspension was tapped off into the glass bottle the following day. Nematode suspensions were stored at approximately 4^{o}C until further use. To estimate the population density, the stock solutions were mixed and total nematode numbers determined using a 1 ml sub-sample and counted at 40X magnification using an Axiovert 25 (Carl Zeiss®) inverted microscope. The counting was replicated three times and the mean calculated. Suspensions were adjusted to 500 nematodes/ml of water.

Growing conditions

Plants were cultivated in a greenhouse set at 20 ± 3^{o}C and a 13 hour photoperiod. Humidity was maintained at approximately 63-70%. All experiments were conducted in 1 litre pots filled with

700 ml growing medium. Pots were first half-filled with the growing medium and the pre-germinated tubers placed in the middle of the pot before being filled. The pots were placed on saucer plates before being completely randomised on greenhouse benches. Watering was done as needed.

Experimental setup

Experiment 1: In this experiment, 21 varieties were screened and each variety was replicated five times giving a total of 105 plants in the experiment. In this experiment, varieties were screened for resistance and tolerance against *D. destructor*. Two weeks after planting, growing medium was infested with *D. destructor*. Four holes of approximately 4 cm in depth were made in the growing medium around the plant. In each of the four holes, 1 ml tap water containing 500 nematodes of mixed life stages (males, females and juveniles) was added, giving a total of 2000 nematodes per pot. The holes were covered with growing medium immediately after infestation. Control pots were not infested with nematodes. The potato tubers were assessed 12 weeks after infestation with nematodes, giving a total duration of 14 weeks for the experiment from planting to harvest.

Experiment 2: In this experiment, ten varieties were screened. Varieties were screened for resistance and tolerance against *D. destructor* and *D. dipsaci*. Each treatment consisted of a single species of nematode replicated ten times (five control pots and five nematode treated pots per variety) giving a total of 200 pots. Infestation of growing medium with nematodes followed the same procedure as described in experiment 1. The potato tubers were assessed 14 weeks after infestation with nematodes, giving a total duration of 16 weeks for the experiment from planting to harvest.

Data collection

Potato tubers were harvested by passing the growing medium from each pot through a sieve. Adhering growing medium was washed using tap water and number of tubers and tuber weight recorded. External and internal damage were recorded prior to nematode extraction from tuber tissues as explained later in evaluation for tolerance. Potatoes from each replicate were completely peeled using a knife. Peels were of approximately 2 mm in thickness and

approximately 22% of tuber weight. From the total tuber peels per replicate, a composite 10 g of potato tuber peels was obtained and chopped into fine pieces and used for nematode extraction. Nematodes were extracted using the modified Baermann funnel method for 12 hours (Hooper, 1990). The nematodes extracted were used to determine nematode numbers and developmental stages.

Growing medium from each pot was thoroughly mixed and a subsample of 300 ml collected, and packed in polythene bags and stored at 5°C until further use. Nematodes were extracted from 250 ml of the growing medium subsample for 24 hour using an Oostenbrink dish with 24 cm inner diameter (Oostenbrink, 1960) and extrapolated to the total growing medium volume per replicate (700 ml). Nematode numbers (all developmental stages) from both total tuber peels and growing medium were determined under an inverted microscope (Axiovert25 CarlZeiss®) at 40X magnification using a nematode counting slide chamber of 1 ml capacity.

Assessment of resistance

Two methods were used to evaluate resistance of potato varieties to *D. destructor* and *D. dipsaci*. These methods were: i) reproduction factor (RF) and ii) relative susceptibility (RS).

i. Reproduction factor

Resistance to *D. destructor* and *D. dipsaci* was determined using the RF formula where RF = Pf/Pi (Oostenbrink, 1966). The Pf was the final nematode population in peels plus growing medium while the Pi = Initial population density (in these experiments 2000 nematodes). A variety was considered to be resistant (R) when the ratio was lower that initial population density (Pf/Pi < 1). On the other hand, a variety was considered resistant (R) when the ratio was lower than 1, and susceptible when the ratio was higher than 1.

ii. Relative susceptibility

Relative susceptibility (RS) of the potato varieties to *D. destructor* and *D. dipsaci* was calculated using the RS formula (EPPO, 2006): $Pf_{\text{test variety}}/Pf_{\text{standard susceptible control variety}} \times 100$, where $Pf_{\text{standard susceptible control variety}}$ was that of "Désirée" variety. A score scale between 1 and 9 was adopted from the EPPO protocol to classify the potato varieties for resistance to *Globodera* spp. into different

levels of RS (EPPO, 2006). The score scale and its corresponding RS scores in brackets are as follows: 1 (> 100), 2 (50.1-100%), 3 (25.1-50%), 4 (15.1-25%), 5 (10.1-15%), 6 (5.1-10%), 7 (3.1-5%), 8 (1.1-3%), 9 (<1%). The scores of 1 and 9, respectively, indicate the lowest and highest levels of resistance respectively.

Assessment of tolerance

Tuber damage and yield loss were used to evaluate tolerance of the potato varieties for *D. destructor* and *D. dipsaci*. Tuber damage was evaluated as follows: (i) External damage was assessed before tuber peeling. Whole tubers were visually assessed, and a completely damaged tuber with cracks and lesions all over was recorded as 100% damaged. A symptomless tuber with no nematodes symptoms was recorded as 0%. Intermediate damage was recorded based on extent of damage expressed as proportion of damaged surface. (ii) Internal damage: Tubers were sliced into half to determine internal damage. Only one half of tuber was used. The extent of damage of the tuber skin and cortex was estimated. A fully internally damaged tuber was that whose entire skin and part of the cortex was damaged.

Tuber weight loss was used to determine tolerance using the formula: Loss (% tuber weight reduction) = (control treatment tuber weight - treatment tuber weight)/control treatment tuber weight x 100. A scale was developed in this study to classify the potato varieties tuber weight loss into several classes of tolerance. The classes were as follows based on percentage tuber weight loss: 0-25% (tolerant), 25.1-50% (moderately tolerant), 50.1-75% (sensitive), >75.1% (highly sensitive).

Statistical analysis

Data were analysed using a one way ANOVA. When resistance and tolerance was analysed, nematodes were the dependent variables, while potato varieties were independent variables. Homogeneity of variance and assumption of normality of the residuals was tested using Levene's and Shapiro-Wilk's test, respectively in SAS software Version 9.2 (SAS Institute Inc., Cary, NC, USA). Prior to analysis of variance (ANOVA), percentage damage data was arcsine square root transformed, while nematode counts were log transformed $\log_{10}(x + 1)$. General linear model (GLM) procedure was used in SAS to analyse the data. Bonferroni adjustment was used for

multiple mean comparisons at $P = 0.05$ confidence levels. To determine significant differences in the RS of various potato varieties to *D. destructor* and *D. dipsaci*, means were separated by Bonferroni adjustment to the standard control variety "Désirée". Where means were compared to the standard control, Dunnett test was applied. The non-transformed means are presented in the figures and tables.

3.0. Results

Resistance of potato varieties to *D. destructor*

Experiment 1

Reproduction factor: The RF of *D. destructor* isolated from growing medium and tuber peels per replicate significantly differed ($DF = 20$, $F = 6.0$, $P < 0.0001$) among the 21 varieties screened during experiment 1 (Table 1). Highest RF was obtained from "Désirée", which differed significantly from all other varieties apart from "Amanda". Overall, 14 varieties were evaluated as susceptible (S) because Pf/Pi ratio was >1 while seven varieties were evaluated as resistant (R) to *D. destructor* since Pf/Pi was < 1 (Table 1).

Table 1: Reproduction factor (RF) of *D. destructor* calculated from total growing medium and tuber peels per replicate obtained during experiment 1 and 2 and assessment of resistance.

Variety	Reproduction Factor (RF)	Resistance/susceptible
Désirée	20.9 ± 6.5^{a}	S
Amanda	11.7 ± 4.4^{ab}	S
Amado	6.1 ± 2.1^{bc}	S
Bintje	5.1 ± 1.2^{bc}	S
Euroflora	3.8 ± 1.4^{bc}	S
Eurobola	3.8 ± 1.4^{bc}	S
Innovator	3.5 ± 0.5^{bc}	S
Aveka	3.3 ± 1.3^{bc}	S
Pallina	2.7 ± 2.1^{bc}	S
Sieglinde	2.1 ± 2.1^{bc}	S
Avano	2.0 ± 0.4^{bc}	S
Saturna	1.6 ± 0.1^{bc}	S
Seresta	1.4 ± 0.1^{bc}	S
Grata	1.4 ± 0.4^{bc}	S
Darwina	0.9 ± 0.4^{c}	R
Adretta	0.9 ± 0.6^{c}	R
Hela	0.6 ± 0.3^{c}	R
Achilles	0.5 ± 0.3^{c}	R
Laura	0.4 ± 0.3^{c}	R
Hansa	0.4 ± 0.2^{c}	R
Festien	0.3 ± 0.2^{c}	R
Pentland Crown•	27.0 ± 7.3^{a}	S
Désirée•	25.3 ± 5.6^{a}	S
Amanda•	21.5 ± 4.2^{a}	S
Bintje•	9.8 ± 5.3^{ab}	S
Hansa•	8.2 ± 1.3^{ab}	S
Belana•	5.3 ± 0.7^{dc}	S
Agria•	4.1 ± 2.5^{dc}	S
Innovator•	2.9 ± 0.9^{d}	S
Aveka•	2.4 ± 1.2^{d}	S
Spunta•	0.0 ± 0.0^{d}	R

Reproduction factors are means of five replicates followed by ± standard error. Means separated by the same letter are not significantly different at $P = 0.05$ according to Bonferroni adjustment multiple comparison test. A variety was considered resistant (R) when the reproduction factor (RF) was lower than 1 and susceptible (S) when the ratio was higher than 1. Varieties followed by • were screened during experiment 2. Data was analyzed separately from experiment 1.

Relative susceptibility: Since "Désirée" had the highest Pf (Table 1), this variety was used as a standard susceptible control in the determination of RS to *D. destructor* (Table 2). Relative susceptibility significantly differed among the potato varieties ($DF = 20$, $F = 12.9$, $P < 0.0001$). Varieties "Amanda", and "Amado" had similar RS to *D. destructor* as the standard susceptible control variety "Désirée" (Table 2). The other seventeen varieties had significantly ($P < 0.0001$) different RS to *D. destructor* compared to the standard susceptible control variety (Table 2). Based on the RS score, the 21 varieties were grouped into seven classes. "Amanda" and "Désirée" were grouped in score 2, whose RS to *D. destructor* was very high (score 2). Varieties

"Hela", "Achilles", "Laura", "Hansa" and "Festien" had high level of resistance (score 8) against *D. destructor* (Table 2). The other varieties belonged to intermediate RS classes ranging between 3 and 7 (Table 2).

Table 2: Final nematode population densities and relative susceptibility of potato varieties to *D. destructor* obtained during experiment 1 and 2.

Variety	Final nematode population (Pf)	Relative susceptibility	Relative susceptibility Score
Désirée	41863 ± 13051[a]	100.0	2
Amanda	23434 ± 8854[ab]	56.0	2
Amado	12212 ± 4121[bc]	29.2	3
Bintje	10095 ± 2392[bc]	24.1*	4
Euroflora	7594 ± 2728[bc]	18.1*	4
Eurobona	7509 ± 2863[bc]	17.9*	4
Innovator	6986 ± 4257[bc]	16.7*	4
Aveka	6669 ± 2601[bc]	15.9*	4
Pallina	5473 ± 1039[bc]	13.1*	5
Sieglinde	4193 ± 970[bc]	10.0*	5
Avano	3964 ± 886[bc]	9.5*	6
Saturna	3137 ± 200[bc]	7.5*	6
Seresta	2868 ± 207[bc]	6.9*	6
Grata	2709 ± 116[bc]	6.5*	6
Darwina	1752 ± 917[c]	4.2*	7
Adretta	1746 ±109[c]	4.2*	7
Hela	1143 ± 515[c]	2.7*	8
Achilles	946 ± 606[c]	2.3*	8
Laura	873 ± 566[c]	2.1*	8
Hansa	772 ± 529[c]	1.8*	8
Festien	496 ± 495[c]	1.2*	8
Belana•	53916 ± 14666[a]	125.4	1
Pentland Crown•	50682 ± 11169[ab]	117.9	1
Désirée•	42985 ± 8550[abc]	100.0	2
Aveka•	19627 ± 10752[abcd]	45.7	3
Bintje•	16334 ± 2627[bcd]	37.9	3
Amanda•	10650 ± 1477[cd]	24.8*	4
Agria•	8213 ± 5033[cd]	19.1*	4
Innovator•	5759 ± 1693[d]	13.4*	5
Hansa•	4850 ± 2453[d]	11.3*	5
Spunta•	71 ± 7[e]	0.2*	9

Final population densities followed by ± standard error and relative susceptibility means were obtained from five replicates (exp 1) and ten replicates (exp 2). Final nematodes population means separated by the same letter are not significantly different at $P = 0.05$ according to Bonferroni adjustment multiple comparison test. Relative susceptibility significant differences with the control (var. Désirée) using Dunnett test are indicated by asterisks (*).Varieties followed by • were screened during experiment 2. Data was analyzed separately from experiment 1.

Experiment 2

Reproduction factor of D. destructor: In experiment 2, the RF of *D. destructor* significantly differed among the ten tested varieties ($DF = 9$, $F = 10.8$, $P < 0.0001$) (Table 1). "Belana" had the highest RF. However, there were no significant differences ($P = 0.649$) in the RF of *D.*

destructor between “Belana”, “Pentland Crown”, “Désirée”, “Aveka” and “Bintje” (Table 1). Based on the RF, only variety “Spunta” was evaluated as resistant (R) since the Pf/Pi was = 0, while the other nine varieties were all susceptible to *D. destructor* because the Pf/Pi ratio was >1 (Table 1).

Relative susceptibility: Significant (DF = 9, F = 11.9, P < 0.0001) differences in RS to *D. destructor* among the ten potato varieties were observed during the experiment 2 (Table 2). Unlike experiment 1, where the highest RS of 100% was recorded from variety “Désirée”, two varieties (“Belana” and “Pentland Crown”) were more susceptible than the standard susceptible control variety (Table 2). Relative susceptibility of “Amanda”, “Agria”, “Innovator”, “Hansa” and “Spunta” was lower compared to the standard susceptible control (Table 2). Using the RS score, six classes of RS were observed. “Belana” and “Pentland Crown” were in score of 1, meaning that they had higher susceptibility to *D. destructor* than the susceptible control “Désirée”. Variety “Spunta” had a score of 9, which indicated the highest level of resistance to *D. destructor*.

Resistance of potato varieties to *D. dipsaci*

Reproduction factor of D. dipsaci: The RF for *D. dipsaci* in growing medium and tuber peels was significantly (DF = 9, F = 3.6, P < 0.0027) different among the ten potato varieties screened during experiment 2 (Table 3). “Spunta” varied significantly from Pentland Crown in its RF. Since varieties “Innovator”, “Aveka” and “Spunta” had RF of less than 1, they were all classified as resistant (R), while the rest of the varieties in experiment two were classified as susceptible to *D. dipsaci* since the Pf/Pi ratio was > 1 (Table 3).

Table 3: Reproduction factor (RF) of *D. dipsaci* calculated from total growing medium and tuber peels per replicate obtained during experiment 2.

Variety	Reproduction factor	Resistance
Pentland crown	20.8 ± 12.5[a]	S
Désirée	9.2 ± 5.2[ab]	S
Amanda	8.5 ± 1.7[ab]	S
Bintje	6.4 ± 3.2 [ab]	S
Hansa	3.8 ± 1.4 [ab]	S
Belana	3.1 ± 1.4 [ab]	S
Agria	1.4 ± 1.2 [ab]	S
Innovator	0.9 ± 0.4 [ab]	R
Aveka	0.5 ± 0.3 [ab]	R
Spunta	0.0 ± 0.0[b]	R

Reproduction factors are means of ten replicates followed by ± standard error. Means separated by the same letter are not significantly different at $P = 0.05$ according to Bonferroni adjustment multiple comparison test. A variety was considered resistant (R) when the reproduction factor (RF) was lower than 1 and susceptible (S) when the ratio was higher than 1.

Relative susceptibility of potato varieties to D. dipsaci: Although "Pentland Crown" had the highest final population density, it was not significantly different from that of "Désirée". Therefore, for consistency, "Désirée", was used as a standard susceptible control variety for the calculation of the RS to *D. dipsaci* (Table 4).

Table 4: Final nematode population densities and relative susceptibility of potato varieties to *D. dipsaci* obtained during experiment 2

Variety	Final nematode population (Pf)	Relative susceptibility	Relative susceptibility Score
Pentland Crown	41553 ± 24933[a]	227.0	1
Désirée	18309 ± 1718[ab]	100.0	2
Amanda	17000 ± 3459[ab]	92.9	2
Bintje	12789 ± 6589[b]	69.8	2
Hansa	7617 ± 2863[b]	41.6*	2
Belana	6193 ± 4934[b]	33.8*	3
Agria	2713 ± 213[b]	14.8*	4
Innovator	1886 ± 833[b]	10.3*	5
Aveka	1072 ± 103[b]	5.9*	6
Spunta	0 ± 0.0[c]	0.0*	9

Final population densities followed by ± standard error and relative susceptibility means were obtained from ten replicates. Final nematodes population means separated by the same letter are not significantly different at $P = 0.05$ according to Bonferroni adjustment multiple comparison test. Relative susceptibility significant differences with the control (var. Désirée) using Dunnett test are indicated by asterisks (*).

The RS of six potato varieties significantly differed ($DF = 9$, $F = 3.6$, $P < 0.0032$) from that of the standard susceptible control variety (Table 4). "Pentland Crown" was more susceptible to *D. dipsaci* than the standard control variety "Désirée". When the RS score was applied, seven classes were observed. "Spunta" had the highest resistance index to *D. dipsaci*.

Tolerance of potato varieties to *D. destructor*

Ditylenchus destructor caused significant (*DF* = 20, *F* = 6.0, *P* < 0.0001) potato tuber damage, both externally and internally during experiment 1 **(**Fig. 1**)**. Percentage external and internal damage ranged between 7.2 - 44.5% and 0 - 22% **(**Fig. 1**)**. Among the most severely damaged potato varieties included "Amado" and "Bintje" while "Désirée" and "Saturna" were the least damaged varieties **(**Fig. 1**)**. Internal potato tuber damage varied significantly among 21 varieties **(**Fig. 1**)**. Varieties "Amando" and "Bintje" had the highest internal damage, significantly differing from the rest of the varieties **(**Fig. 1**)**.

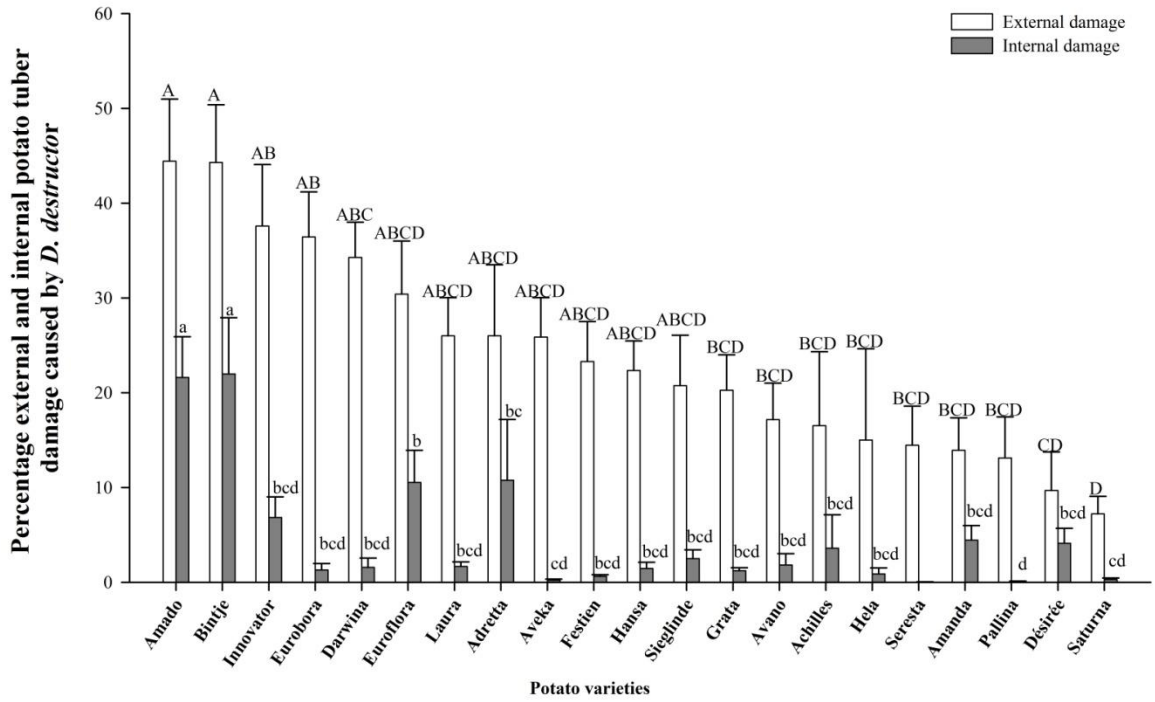

Fig. 1: Mean external and internal potato tuber damage obtained from experiment 1 (expressed as percentage of means of all tubers per replicate) caused by *Ditylenchus destructor* on twenty one potato varieties. Means separated by the same letter are not significantly different at *P* = 0.05 according to Bonferroni adjustment multiple comparison test.

In experiment 2, external tuber damage varied significantly (*DF* = 9, *F* = 16.7, *P* < 0.0001) among the ten varieties tested (Fig. 2). "Bintje" was the most externally and internally damaged variety (80%), (Fig. 2). Variety "Spunta" was observed as symptomless after damage evaluation (Fig. 2).

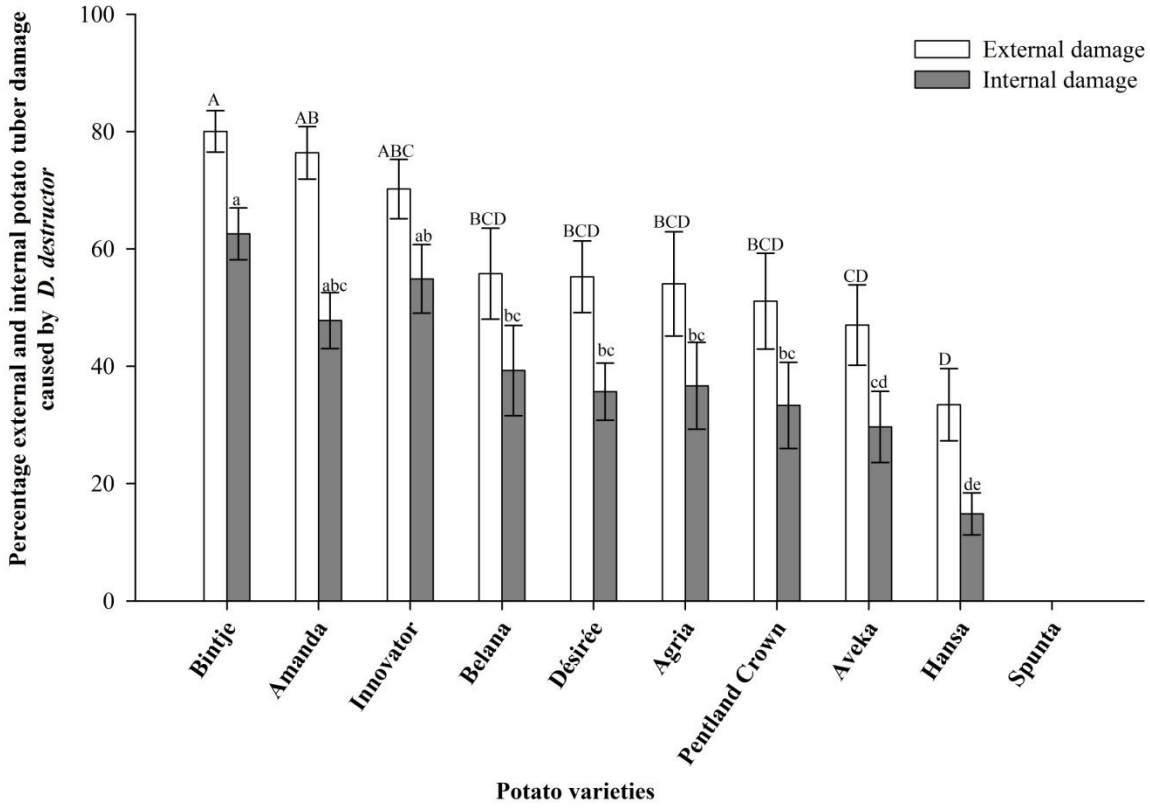

Fig. 2: Mean external and internal potato tuber damage caused by *Ditylenchus destructor* on ten potato varieties obtained from experiment 2 (expressed as percentage of means of all tubers per replicate). Means separated by the same letter are not significantly different at $P = 0.05$ according to Bonferroni adjustment multiple comparison test.

Comparison between experiments on tolerance to *D. destructor*

Increase in experiment duration from 14 weeks to 16 weeks lead to an increased tuber damage of potato varieties to *D. destructor* in experiment 2 (Fig. 1 and 2).

Tuber weight reduction in the presence of *D. destructor*

Significant tuber weight reduction was observed in all ten potato varieties tested when *D. destructor* was present during experiment 2 (Table 5). “Spunta” had no external or internal damage symptoms but a yield loss of 31% was recorded (Table 5).

Table 5: Influence of *Ditylenchus destructor* and *D. dipsaci* on potato tuber weight (g) and percentage yield reduction across ten potato varieties

Variety	Treatment	Tuber numbers	Tuber weight (g)	% weight loss	Tolerance Score
Agria	Control	4 ± 0.7[a]	123.0 ± 20.1[a]	0	
	D. destructor	2 ± 0.4[a]	27.2 ± 4.9[c]	69.2	sensitive
	D. dipsaci	3 ± 0.4[a]	45.8 ± 5.4[b]	57.88	sensitive
Amanda	Control	3 ± 0.2[a]	136.6 ± 3.8[a]	0	
	D. destructor	2 ± 0.6[a]	18.0 ± 5.2[b]	86.8	highly sensitive
	D. dipsaci	2 ± 0.5[a]	32.0 ± 3.2[b]	76.64	highly sensitive
Aveka	Control	2 ± 0.2[a]	128.4 ± 5.2[a]	0	
	D. destructor	3 ± 0.7[a]	31.8 ± 10.2[c]	48.08	moderately tolerant
	D. dipsaci	3 ± 0.4[a]	66.0 ± 6.2[b]	75.34	highly sensitive
Belana	Control	2 ± 0.5[a]	47 .0 ± 3.4[a]	0	
	D. destructor	4 ± 1.2[a]	24.8 ± 3.6[b]	47	moderately tolerant
	D. dipsaci	2 ± 0.5[a]	35.2 ± 3.3[ab]	24.8	moderately tolerant
Bintje	Control	3 ± 0.8[a]	58.6 ± 12.3[a]	0	
	D. destructor	4 ± 0.5[a]	30.8 ± 3.6[b]	43.64	moderately tolerant
	D. dipsaci	4 ± 0.7[a]	52.0 ± 8.6[a]	0.68	tolerant
Desiree	Control	3 ± 0.4[a]	101.2 ± 24.3[a]	0	
	D. destructor	5 ± 0.7[a]	28.4 ± 4.4[b]	65.44	sensitive
	D. dipsaci	3 ± 0.6[a]	34.2 ± 9.7[b]	55.65	sensitive
Hansa	Control	3 ± 1.0[a]	138.6 ± 1.6[a]	0	
	D. destructor	4 ± 0.7[a]	40.8 ± 7.8[b]	69.87	sensitive
	D. dipsaci	4 ± 0.4[a]	42.0 ± 1.5[b]	70.58	sensitive
Innovator	Control	3 ± 0.7[a]	66.6 ± 16.7[a]	0	
	D. destructor	4 ± 0.2[a]	25.0 ± 1.9[b]	49.23	moderately tolerant
	D. dipsaci	3 ± 0.5[a]	38.0 ± 3.0[ab]	18.2	tolerant
Pentland crown	Control	3 ± 0.6[a]	135.4 ± 9.3[a]	0	
	D. destructor	2 ± 0.3[a]	34.4 ± 3.2[b]	73.64	sensitive
	D. dipsaci	3 ± 0.4[a]	38.2 ± 10.5[b]	72.05	sensitive
Spunta	Control	3 ± 0.8[a]	55.6 ± 6.2[a]	0	
	D. destructor	4 ± 0.5[a]	35.8 ± 4.6[b]	28.2	moderately tolerant
	D. dipsaci	3 ± 0.6[a]	38.2 ± 4.4[ab]	30.68	moderately tolerant

Means were separated using Dunnett test (P = 0.05) with the control treatments per variety. Means within a column and per variety separated by the same letter are not significantly different from each other.

Tolerance of potato varieties to *D. dipsaci*

Damage of potato varieties due to *D. dipsaci* differed significantly (DF = 9, F = 13.9, P < 0.0001) among the varieties (Fig. 3). “Amanda” was the most externally damaged at 66.9%, differing significantly (P < 0.0001) from “Belana”, “Désirée” “Hansa” and “Spunta” (Fig. 3). “Belana”, “Hansa”, and “Spunta” did not suffer any external and internal tuber damages (Fig. 3).

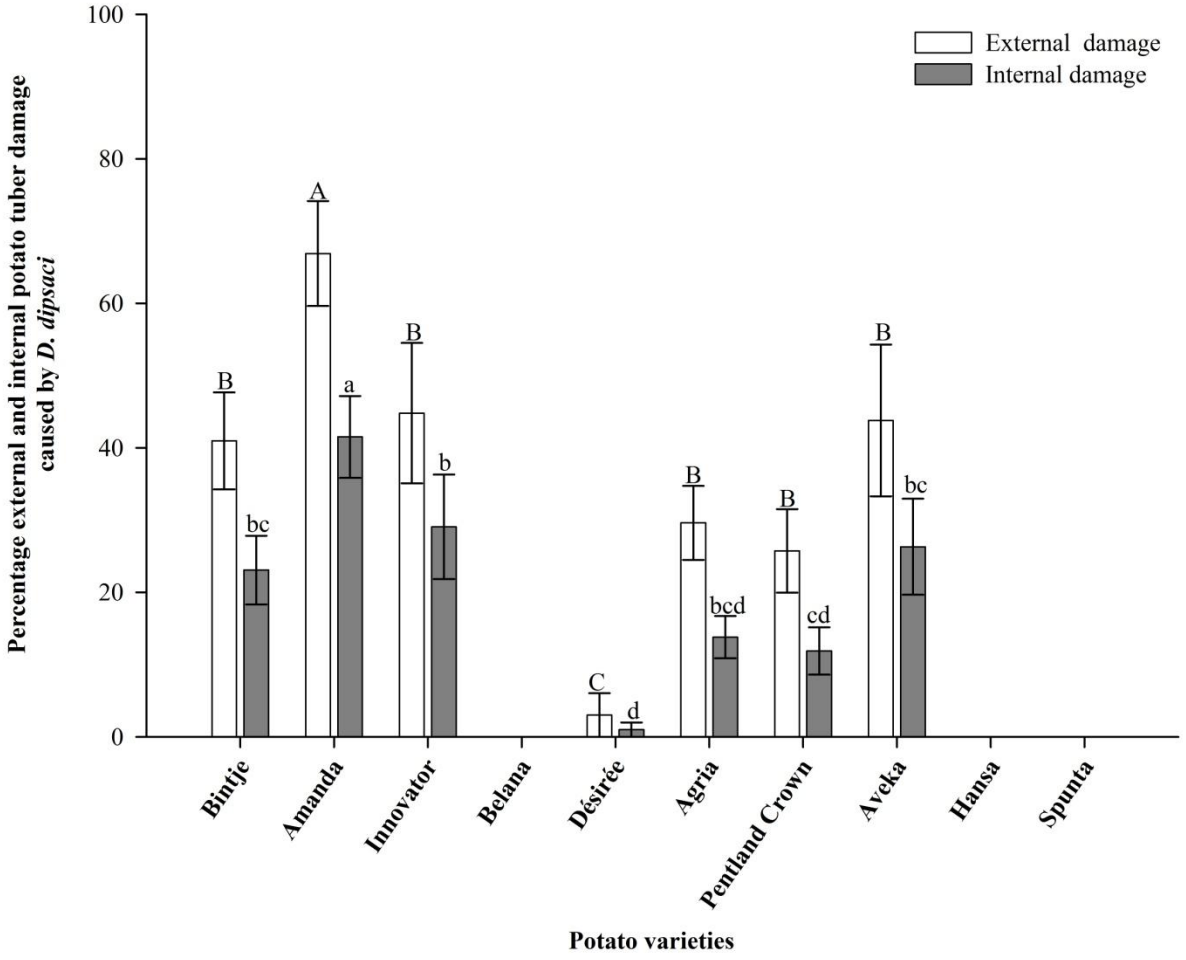

Fig. 3: Mean percentage external and internal potato tuber damage obtained from experiment 2 caused by *Ditylenchus dipsaci* (expressed as percentage of means of all tubers per replicate) on ten potato varieties. Means separated by the same letter are not significantly different at $P = 0.05$ according to Bonferroni adjustment multiple comparison test.

Ditylenchus dipsaci caused tuber weight loss in all potato varieties tested, except "Belana", "Bintje", "Innovator", and "Spunta" (Table 5).

4.0. Discussion

Screening plant germplasm for resistance requires availability of an axenic, viable and infective nematode population. The ability of the *D. destructor* and *D. dipsaci* populations used during the current experiments to reproduce on potato varieties indicated that the populations were viable and infective.

The definitions of both resistance and tolerance of crops to nematodes has been under regular review (Trudgill, 1991; Barker, 1993). Previously, external and internal potato tuber damage was used as a measure for resistance (Kornobis, 1968; Nikulina, 1970; Moore, 1971; Shepshelev & Chernikova, 1971; Moore, 1978). Tolerance was defined as the ability of a plant to support nematode reproduction without being damaged significantly (Dropkin & Nelson, 1960). As a result, most of earlier published results on resistance are reports on tolerance (Roberts, 1992). Nowadays nematode reproduction levels on plant tissues are used as a measure for resistance while damage levels are used to quantify tolerance (Trudgill, 1991). Since resistance and tolerance are genetically independent characters, they should be evaluated separately (Trudgill, 1991).

Evaluation of resistance

The classification of potato into resistant and susceptible varieties based on a RF greater or less than 1 (Pf/Pi value) was not supported by statistical analysis. Varieties which had no statistically different RFs were classified as either resistant or susceptible which made it impossible to detect different levels of resistance. This demonstrated the difficulties of using RF as a measure of resistance and also problems of combining two methods to evaluate the resistance.

Relative susceptibility has been proposed as a suitable measure for nematode resistance evaluation in crops (Phillips, 1984; J.W., 1984; Seinhorst *et al.*, 1995). The variety "Désirée" was selected as the standard susceptible control variety in the current screening experiments because of its high susceptibility to *D. destructor* and *D. dipsaci*. This variety is also susceptible to other nematodes and is also used as a susceptible control variety in the screening protocol for

potato cysts nematodes (EPPO, 2006). Statistical differences in RS of potato varieties to *D. destructor* and *D. dipsaci* did not support varieties grouping into different resistance levels. However the RS classes ranked the varieties into nine different resistance groups.

Relative susceptibility of potato varieties to *D. destructor*

The use of RS classes made it possible to quantify levels of resistance of the potato varieties to *D. destructor*. During experiment 1, some varieties had high resistance (score 8), while some varieties were as susceptible (score 2) as the control variety. During experiment 1, "Désirée" was the most susceptible variety. However, during experiment 2, "Belana" and "Pentland Crown" were observed to be more susceptible than Désirée. These two varieties were not included in experiment 1. Similar observations have been made in evaluations for resistance of potato varieties to cysts nematodes, where more susceptible varieties were observed than the standard susceptible control variety (Niere, 2006). All classes of resistance to *D. destructor* were observed ranging from the highly susceptible varieties such as "Pentland Crown" and "Belana" (score 1) to highly resistant varieties such as "Spunta" (score 9).

Relative susceptibility of potato varieties to *D. dipsaci*

This study supports the fact that *D. dipsaci* is as viable on the potatoes as *D. destructor*. Variety "Désirée" was also susceptible to *D. dipsaci*. Similar to *D. destructor*, "Pentland Crown" was more susceptible to *D. dipsaci* when compared to "Désirée". "Spunta" was also resistant to *D. dipsaci* as observed for *D. destructor*. Resistance screening of potato against *D. dipsaci* was only performed once. To ascertain the results obtained during this experiment, it would be important to repeat these experiments.

Ditylenchus dipsaci is among harmful plant parasitic nematodes listed in Annex IIAII of European Council Directive 2000/29/EC. This means that this species must not be present on seeds, bulbs and corms intended for planting (European union, 2000). Whereas *D. destructor* is regulated on potato, *D. dipsaci* is not. The findings from the current experiments demonstrate the importance of *D. dipsaci* on potato. This finding confirms observations by Seinhorst, 1957, who considered it a serious pest of potato in Germany and the Netherlands. Recently, concerns over *D. dipsaci* re-emerging as a major threat to other crops in Europe has been raised (Mouttet *et al.*, 2014). Our study offers information which may be important in regulating pathways for *D.*

dipsaci. Whereas some of the varieties studied were highly susceptible to *D. destructor* and *D. dipsaci*, some varieties were moderately resistant to highly resistant. This offers new control options in cases where either one or both nematodes species may be present in the field.

Tolerance of potato varieties to *D. destructor*

Ditylenchus destructor affects potato tubers by reducing their marketable quality. External and internal tuber damage was used to evaluate potato tolerance to *D. destructor*. The potato varieties screened during experiment 1 were all sensitive to *D. destructor* since they all expressed characteristic cracking of the skin and rotting at varying percentages. During experiment 2, tuber damage was observed in all varieties except for "Spunta" which expressed neither external nor internal tuber damage. During experiment 1 and 2, external potato tuber damage was always higher than internal damage. It was difficult to evaluate tolerance to *D. destructor* using the combination of external and internal tuber damage. Similar observations were made by Moore, 1978, who noted that the use of external tuber damage as an indicator for tolerance to *D. destructor*, ranked variety "Golden Wonder" as more sensitive than variety "King Edward". In contrast, use of internal tuber damage, on the other hand, categorized variety "King Edward" as more susceptible to *D. destructor* (Moore, 1978). Based on our experiments, external damage was found to be consistent and suitable for tolerance evaluation.

In addition to damage, yield loss expressed as tuber weight reduction was assessed. "Spunta" did not express external or internal damage symptoms but was found to have lost 28% of tuber weight. Although some varieties such as "Bintje" and "Innovator" were classified as tolerant varieties since they expressed insignificant tuber weight loss, their external damage was very high. Such varieties are not suitable for cultivation on tuber rot and stem nematode infested fields. *Ditylenchus destructor* did not influence tuber numbers of all the varieties tested during experiment 2 when each variety was compared to their respective control plants. In respect to tuber rot and stem nematodes, an ideal tolerant variety would be a variety which is symptomless, and expresses no reduction in tuber numbers or tuber weight loss

Tolerance of potato varieties to *D. dipsaci*

Ditylenchus dipsaci caused both external and internal damage to seven out of ten varieties screened during experiment 2. Similar to *D. destructor*, external damage was higher in all cases than the internal damage. Earlier reports documented that *D. dipsaci* caused higher internal damage than *D. destructor* (Seinhorst & Dunlop, 1945; Jenkins & Taylor, 1967). However, current experiments contradicted those finding and revealed that internal damage caused by *D. dipsaci* were lower than damage caused by *D. destructor* in the same potato varieties. Varieties "Belana", "Hansa" and "Spunta" were not damaged by *D. dipsaci* at the end of the experiment. However, when tuber weight loss was considered, "Belana" and Spunta had lost 25% and 31% of tuber weights, respectively, which ranked them as moderately tolerant varieties. "Hansa" on the other hand was classified as sensitive variety due to a high percentage (71%) tuber weight loss.

Tolerance of 25 potato varieties to *D. destructor* and *D. dipsaci* obtained in the current experiments were performed under pot experiments. Although such greenhouse screening experiments offer many advantages, the pots used could have constrained potato tubers into limited space, exposing them to the inoculum, which could have enhanced infection rates .It should be considered that indication for resistance and tolerance need to be verified under open field conditions in several environments.

Effect of extended experiment period on experiments

Potato varieties are either early maturing, intermediate or late maturing (Van Eck, 2007). The duration of cropping determines the length of time the plants are predisposed to pathogens. Varieties used during the current experiments were from all the maturity index groups. To take into consideration the cropping period, the harvest time was prolonged between experiments. It was observed that increasing the duration of the experiment led to reduced resistance and tolerance in potato varieties. *Ditylenchus destructor* and *D. dipsaci* have short life cycle and under optimal conditions, they can be able to complete several generation in one cropping season (Anderson, 1964; Hooper, 1972; Hooper, 1973; Sturhan & Brzeski, 1991). As a result of short life cycles, the rapid population growth of these nematodes could have lead to severe potato tuber damage and higher nematodes numbers at week 16 compared to week 14. Nematode

population densities and damage to crops are known to increase with time in presence of host (Seinhorst, 1956; Seinhorst, 1965). Detailed experiments on effect of experiment duration on potato resistance to *D. destructor* and *D. dipsaci* are needed.

Possible influence of growing medium pH and soil moisture content

Soil abiotic factors such as texture, organic matter content, soil pH, temperature and moisture influences the distribution and behavior of nematodes, subsequently determining the incidence and severity of potato damage (Norton & Hoffmann, 1974; Robertson & Freckman, 1995; Fiers *et al.*, 2012). Optimal soil pH for nematodes attacking potatoes vary depending on nematode species (Fiers *et al.*, 2012). The soil pH obtained in the growing medium used during our experiments was low (pH of 4.6). However, results from tuber damage and nematode RF demonstrated that both *D. destructor* and *D. dipsaci* survived and reproduced well even at low soil pH. Similar observations were made by Ivanyuk & Ilyashenko, (2008) who recorded highest potato tuber damage caused by *D. destructor* at soil pH in the range between 4.0-5.5. Influence of soil pH on *D. dipsaci* on potato damage has not been investigated before. Our experiments indicate that *D. dipsaci* can reproduce and cause damage to potato even at low soil pH.

Moisture and soil type influences nematodes movement in soil (Wallace, 1958). Sandy soil has been demonstrated to be optimal for *D. dipsaci* locomotion while heavy clay soil reduced locomotion and nematode activity (Seinhorst, 1950; Seinhorst, 1956). Our growing medium had adequate sand and nutrients to support both the nematode movement and plant growth. It's evident from the damage levels that after infesting the soil with the nematodes they were able to locate the host plant. Plants were watered on a daily basis to keep the soil moist. The subject of soil properties and their influence on potato soil borne diseases especially in relation to *D. destructor* and *D. dipsaci* has been poorly addressed as summarized in a review by Fiers *et al.*, (2012). Further research on the influence of soil properties on *D. destructor* and *D. dipsaci* and subsequently its impact on resistance and tolerance of potato varieties to these nematodes is needed.

5.0. Conclusion

This study provides information on resistance and tolerance of potato varieties to *D. destructor* and *D. dipsaci*. Resistance was best evaluated using RS instead of the RF. The RS method was based on more classes which separated varieties into more resistant classes. Additionally, inclusion of a susceptible variety as an internal standard in both screening experiments helped normalize variations in the screening conditions. External damage was found more suitable as a measure for tolerance than internal damage. Although some varieties did not show substantial tuber weight loss, tuber damage was in most cases high. Although our experiments offer important information, future experiments in *D. destructor* and *D. dipsaci* infested micro-plots and fields are necessary to assess tolerance under outdoor conditions. The study also demonstrates the importance of *D. dipsaci* as a serious nematode pest of potato. Since both are regulated through phytosanitary measures, it may be important to regulate potato as a pathway for *D. dipsaci*.

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Chapter 4:

Effect of initial population densities of *Ditylenchus destructor* and *Ditylenchus dipsaci* on potato tuber damage and nematode reproduction

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Abstract

Two greenhouse experiments were conducted to evaluate the effect of varying initial population densities of *Ditylenchus destructor* and *D. dipsaci* on potato tuber damage and nematode reproduction. *D. destructor* did not influence tuber numbers but influenced tuber weight at high P*i* levels of 2.85 and 14.29 nematodes g^{-1} of growing medium. *D. dipsaci* influenced tuber numbers and weights at a P*i* level of 14.29 nematodes g^{-1} of growing medium. Tolerance limit estimates according to the Seinhorst model were very low indicating that both nematode species have a major impact on potato tuber weight. Damage expressed as percentage external and internal tuber rot caused by both species increased with P*i* levels. *D. destructor* was more damaging to potato tubers than *D. dipsaci* at all P*i* levels. Damage caused by *D. destructor* was already observed at P*i* levels of 0.01 and 0.14 nematodes g^{-1} of growing medium. Reproduction rates of *D. destructor* were higher at all P*i* levels studied compared to *D. dipsaci*. The equilibrium density of 1.3 and 0.6 for *D. destructor* and *D. dipsaci* respectively was observed at P*i* level of 14.29 g^{-1} of growing medium.

Keywords*:* *Solanum tuberosum* L., potato tuber rot nematode, stem nematode, Seinhorst model

1.0. Introduction

The potato tuber rot nematode, *Ditylenchus destructor* Thorne, 1945 and the stem nematode *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936, are nematodes affecting potato production especially in temperate regions (Hooper, 1972; 1973). *D. destructor* and *D. dipsaci* are morphologically similar but differ in pathogenicity (Brodie *et al.*, 1993). They are both polyphagous nematodes feeding on numerous plant species (Hooper, 1972; 1973). Damage by these nematodes reduce potato tuber quality through cracking of the skin and eventual tuber rot due to secondary invasion by opportunistic pathogens such as fungi (Baker *et al.*, 1954). Such tubers are not marketable, thus leading to direct yield loss (Ilyashenka & Ivaniuk, 2008).

Both nematodes are quarantine pests in many countries (Lehman, 2004). In the European Union (EU), both nematode species are regulated on certain plants (Anonymous, 2000). The European and Mediterranean Plant Protection Organization (EPPO) provides a diagnostic protocol (PM 7/87(1)) and lists *D. dipsaci* as a quarantine pest for the EPPO region (EPPO, 2008).

The main dissemination pathway for both *D. destructor* and *D. dipsaci* is passive through infested planting materials. Other dissemination pathways include contaminated equipments (Seinhorst, 1950). Once introduced in the field, these nematodes are difficult to control through crop rotation due to their wide host range including several weed species, which serve as a potential source of inoculum (Andersson, 1967).

Ditylenchus destructor and *D. dipsaci* have short life cycles (Hooper, 1972; 1973). As a result of their short life cycles, population growth is rapid and often leads to severe damage (Mennan, 2005). Initial population densities (P*i*) (or pre-plant nematode densities) are important for nematode population development and yield losses in crops (Seinhorst, 1965). Studies on the impact of initial population densities of *D. destructor* and *D. dipsaci* on associated yield losses on potatoes are rare (Hijink, 1963; Butorina *et al.*, 2006). Although damage on potato tubers caused by *D. destructor* and *D. dipsaci* has been published (Goodey, 1956; Cotten *et al.*, 1992), data on the effect of varying initial population densities of *D. destructor* and *D. dipsaci* on tuber damage, final population densities and their reproduction factor in potatoes was not addressed by these authors.

We therefore conducted two greenhouse experiments with the objectives of i) determining the effect of varying initial population densities (P*i*) of *D. destructor* and *D. dipsaci* on potato tuber numbers, tuber weight and tuber damage and ii) assessing the effect of initial population densities (P*i*) of *D. destructor* and *D. dipsaci* on reproduction of these nematode species.

2.0. Materials and methods

2.1 Planting material preparation

Potato tubers (*Solanum tuberosum* L, 'Désirée') were pre-germinated in the dark at 20±3°C until sprouts were observed. Thereafter, the tubers were exposed to light for a week to harden the sprouts. Where one tuber had more than one sprout, the redundant were removed and only one single sprout was retained. Single-sprout seed tubers of approximately 15 g each were used as planting material.

2.2 Growing medium

Field growing medium was sieved to remove growing medium particles larger than 1 cm^2 and then dry sterilized for 12 hours using an electric growing medium pasteurizer (Sterilo®, Schenkenzell, Germany) set at 100±5°C. After growing medium cooled down, it was mixed with peat (Klasmann® Lithuanian peat moss medium, pH 3.5) at a ratio of 3:1. Slow release fertiliser (Osmocote Exact® Standard® 15% N, 9% P_2O_5, 12% K_2O and 2% M_gO) was added to the growing medium mix at the rate of 1.5 g/kg. The final growing medium had a pH of 4.7 and 2.6% organic matter. The texture consisted of 7.5% clay, 19.1% silt and 73.4% sand. The mineral content of the growing medium was: Potassium (K): 36 mg/100 g, Phosphorus (P): 16 mg/100 g, and Magnesium (Mg): 10 mg/100 g. All experiments were conducted in one litre pots filled with 700 ml of the growing medium.

2.3. *Ditylenchus destructor and D. dipsaci populations*

D. destructor and *D. dipsaci* populations used in this study were originally extracted from celery and sugar beet plants, respectively, sampled in Germany (Julius Kühn-Institut collection). Axenic cultures of these populations were maintained and multiplied on carrot discs in Petri dishes (10 mm Ø). The carrot disc culture method was a modification from a protocol developed by Speijer & De Waele, (1997) as follows. Nematodes were sterilized using a streptomycin sulphate

(AppliChem®, Darmstadt, Germany) solution at 0.06 mg/10 ml of sterile water for six hours. Thereafter, nematodes were rinsed three times using sterile water. Approximately 100 µl of water containing about 20 mixed development stages of nematodes were transferred to sterile carrot discs using a sterile pipette. The Petri dishes were sealed with Parafilm® and placed in an incubator (Heraeus®-model BK 5060 EL, Burladingen, Germany) set at 20±1°C for approximately eight weeks.

Nematodes were collected by rinsing the Petri dishes with water into a clean 500 ml glass bottle. Carrot discs were cut into small pieces using a scalpel blade and transferred to a Baermann funnel overnight to extract nematodes. The nematode suspension was tapped off into the glass bottle the following day. Nematode suspensions were stored at approximately 4°C until further use for a maximum period of one week. To establish the population density, the stock solutions were mixed and total nematode numbers determined. A 1 ml subsample of the nematode suspension was pipetted onto a counting slide and nematodes counted at 40X magnification using an inverted microscope (Axiovert 25 CarlZeiss®, Göttingen, Germany). Counting was replicated three times and the mean value calculated. Nematode stock suspensions were adjusted to 10, 100, 500 nematodes per ml of water. Appropriate volumes from each nematode stock suspension were used to infest the growing medium (700 ml) in pots at seven population densities of 0, 10, 100, 500, 1,000, 2,000 and 10,000 nematodes. These initial population densities were equivalent to 0, 0.01, 0.14, 0.71, 1.42, 2.85, and 14.29 nematodes $g^{-1}$of growing medium.

2.4. Potato growing conditions and experimental setup

Two experiments were conducted in a greenhouse maintained at 20±3°C with a 12 hour photoperiod. Humidity was maintained at approximately 63-70%. Pots were first half-filled with the growing medium and the pre-germinated tuber placed in the middle of the pot before being finally filled. The pots were completely randomised on greenhouse benches. Each pot was placed on a saucer and plants watered once per day as required.

2.5 Infesting growing medium with *D. destructor* and *D. dipsaci*

In both experiments, the growing medium was infested with suspension of either *D. destructor* or *D. dipsaci* two weeks after planting when potato stems and leaves were visible. Four holes of approximately 4 cm deep were made in the growing medium around the potato plant. The

nematodes suspensions were evenly inoculated into these holes. Finally, the holes were covered with the growing medium.

Experiment 1 consisted of six initial population densities (P*i*), i.e. 0, 0.01, 0.14, 0.71, 1.42, and 2.85 nematodes g^{-1} of growing medium. Each treatment was replicated ten times. The assessment of plant data and nematode numbers, tuber damage, and nematode densities was done 12 weeks after infestation.

Experiment 2 consisted of seven initial population densities (P*i*), i.e. 0, 0.01, 0.14, 0.71, 1.42, 2.85, and 14.29 nematodes $g^{-1}$of growing medium (equivalent to 0, 10, 100, 500, 1,000, 2,000 and 10,000 mixed stages of *D. destructor* and *D. dipsaci*, respectively). All treatments were replicated ten times. Assessment of plant data and nematode numbers was done 14 weeks after infestation.

3.0 Data collection

3.1 Damage assessment

Potato tubers were collected by passing the growing medium from each pot through a 1 cm x 1 cm sieve into a collection container. The growing medium was thoroughly mixed and a sample of 300 ml packed into polythene bags. Growing medium adhering onto tubers was gently washed off with water. Tuber numbers and fresh weight were recorded followed by evaluation of the external and internal tuber damage. External potato tuber damage was visually assessed on a whole tuber and expressed as percentage damage per tuber (Fig. 1). Internal damage was evaluated after slicing each tuber into two equal halves. One half of the tuber was used for internal damage calculation. Damaged skin and cortex was necrotic and darker than healthy tissues. The extent of damage from the skin into the cortex of the tuber was calculated by dividing the tuber into four sections of 25% each (Fig. 1). Internal damage per tuber (n%) was the sum of all the four sections. Total internal potato tuber damage per replicate was calculated using the same formula as for external damage.

The total percentage external tuber damage per replicate was expressed using the formula:

$$P = \frac{\sum(n_1 + n_2 + n_3 + n...)}{N},$$

Where, P = is the percentage (%) potato tuber damage per replicate and

N = Total number of tubers per replicate.

n = percentage of potato tuber with lesions caused by *D. destructor* or *D. dipsaci*.

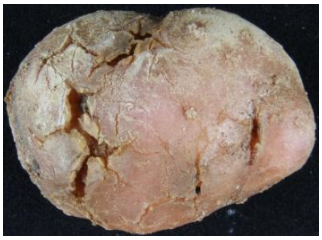
External damage level (%)	External tuber damage	Internal damage level (%)	Internal tuber damage
0 %		0%	
50%		30%	
100%		80%	

Fig 1: Examples of percentage external and internal tuber damage levels caused by *D. destructor* or *D. dipsaci.* External damage was assessed as the percentage of the whole tuber with damage, while internal damage was assessed on one half of the tuber.

3.2 Nematode extraction from potato tuber peels

Potatoes from each replicate were peeled using a knife. Peels were approximately 2 mm thick and made up approximately 22% of the tuber weight. The complete tuber peel per replicate was mixed and a 10 g sub-sample was then chopped into fine pieces of approximately 5 mm x 5 mm and used for nematode extraction. Nematodes were extracted for 12 hours using the modified Baermann funnel method (Hooper, 1990). Nematode numbers at different developmental stages (eggs, juveniles J2-J4, females and males) were determined under an inverted microscope (Axiovert25 CarlZeiss®) at 40X magnification using a 1 ml capacity nematode counting slide

chamber. Nematodes extracted from 10 g of tuber peels were used to calculate the total number of nematodes in tuber peels per replicate.

3.3 Nematode extraction from growing medium

After collecting growing medium as described in step 3.1, it was stored at 5°C up to a maximum of 5 days before nematode extraction. Nematodes were extracted from 250 ml sub-samples of the growing medium for 24 hour using a modified Oostenbrink dish with 24 cm inner diameter and milk filter paper (27cm Ø) (EPPO, 2013). Nematode numbers (all developmental stages) extracted from the growing medium were determined as described in step 3.2 and total number of nematodes per pot was calculated.

3.4. Determination of final population densities and reproduction factor of *D. destructor* and *D. dipsaci*

The final nematode population density was the sum of the total number of nematode from tuber peels and from the growing medium. The reproduction factor (Rf) of *D. destructor* and *D. dipsaci* was determined according to the formula Rf = P*f*/P*i* where P*f* was the final nematode population density and P*i* was the initial population density.

4.0. Data analysis

Prior to analysis of variance (ANOVA), data were tested for homogeneity of variance and assumption of normality in residuals using Levene's and Shapiro-Wilk's test, respectively, in SAS statistical software version 9.3 (SAS institute Inc., Cary, NC, USA). Percentage potato tuber damage data were arcsine square root transformed while nematodes counts were log transformed [log10(x+1)]. Effects of initial population densities on tuber damage (external and internal), tuber weight, tuber number, and influence on the nematodes reproduction factor were evaluated using a one way ANOVA. The General linear model (GLM) procedure was used to analyse the data. Where comparison of means was based on reference to the controls in the experiments, Dunnett's test was used. Where multiple means comparison tests were needed, Tukey's Studentized range test was performed. In all cases untransformed means of each variable studied are presented in tables or graphs.

Estimating the minimum yield loss (*m*) and nematodes tolerance limit using the Seinhorst model

Non linear regression analyses for estimating yield loss (tuber weight) and its relation to population densities of *D. destructor* and *D. dipsaci* were carried out in a script written in Tinn-R version 3.0.3.6 and run in R-Statistical software Version 3.1.2 (The R Foundation for statistical Computing). The script on damage function on tuber weight in relation to the different P*i* levels of *D. destructor* and *D. dipsaci* was described based on the Seinhorst exponential model (Schomaker & Been, 2006). The non linear regression function was used to estimate its coefficients and data were fitted to the equation $y = Y_{max}*(m+ (1-m)*0.95\text{^}(Pi-T/Pi))$ when $P \geq T$, and $y = Y_{max}$ when $P \leq T$ (Schomaker & Been, 2006). In this equation, y is the relative average value of potato tuber weight; Y_{max} is the tuber weight at densities lower than T; m is the minimum value of y at a very large initial density; Pi is the initial nematode population density; T is the tolerance limit for yield loss; Z is a constant < 1 indicating nematode damage; and z^{-T}= 0.95. The coefficient of determination (R^2) and the residual sum of squares were used to assess the goodness-of-fit of data to the model.

Tolerance limit (T) is the nematode population density at which damage becomes apparent due to reduction in plant growth and therefore yield loss, while, minimum yield (*m*) is the yield that remains unaffected by the nematodes even at the highest population densities (Seinhorst, 1965).

5.0. Results

5.1. Influence of varying initial population densities of *D. destructor* and *D. dipsaci* on potato tuber numbers and tuber weight

Initial population densities (P*i*) of *D. destructor* did not significantly influence potato tuber numbers during experiment 1 (P = 0.73) and experiment 2 (P = 0.07) (data not shown). Potato tuber weight was not influenced by *D. destructor* during experiment 1 (P = 0.09). During experiment 2, *D. destructor* caused significant tuber weight reduction (P < 0.0001) at high initial densities of 2.85 and 14.29 nematodes g^{-1} of growing medium.

Only a very weak relationship between P*i* of *D. destructor* and yield reduction (tuber weight) was described by the Seinhorst model in experiment 1 (R^2 = 0.16). In this experiment a minimum

yield (*m*) of 0.83 and a tolerance limit (T) estimate of 0.14 of *D. destructor* g^{-1} of growing medium was estimated by the Seinhorst model (Fig. 2).

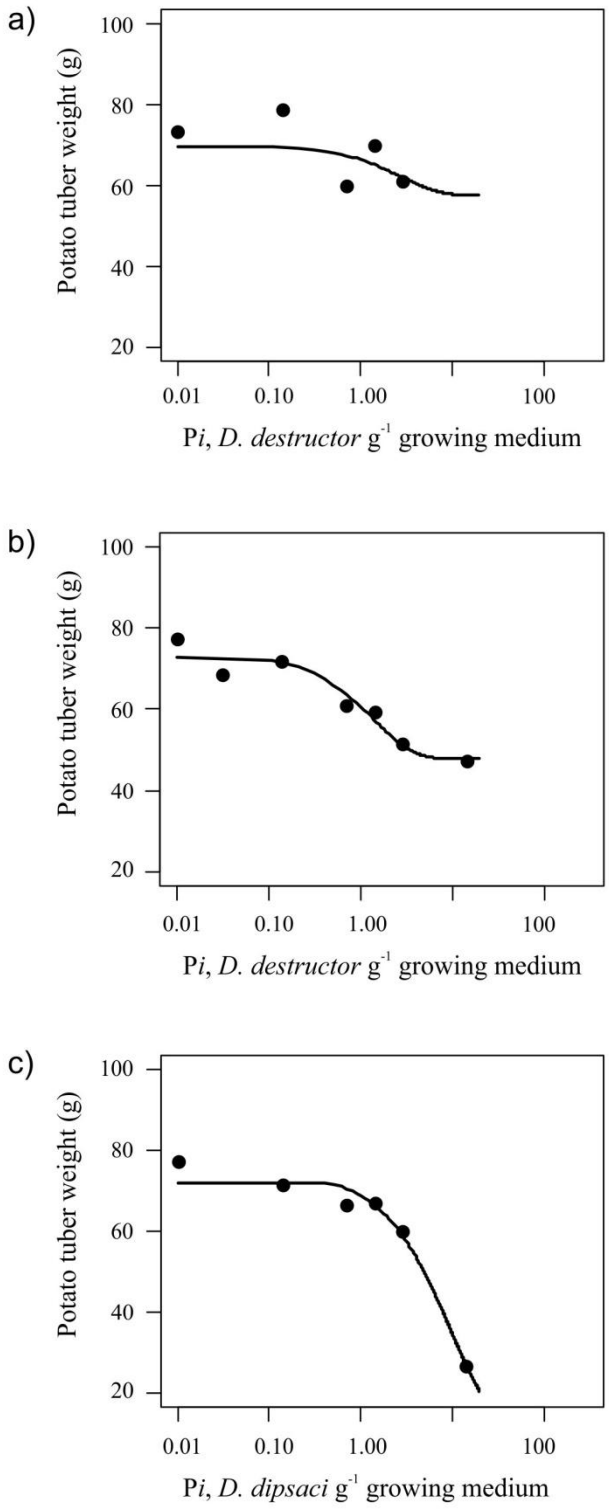


Fig. 2: The relation between initial population density of *D. destructor* (**a** and **b**), *D. dipsaci* (**c**) and potato tuber weight of 'Désirée'. Data shown in (**a**) were generated during experiment 1, while data in (**b)** and (**c)** were generated in experiment 2. Lines were fitted according to the Seinhorst model for yield loss (Schomaker & Been, 2006)

During experiment 2, the relationship between P*i* of *D. destructor* and yield reduction was stronger (R^2 = 0.92). The minimum yield (*m*) and tolerance limit (T) estimates were 0.66 and 0.72 of *D. destructor* g^{-1} of growing medium, respectively (Fig. 2).

In contrast to *D. destructor*, infestation with *D. dipsaci* led to a reduction in tuber numbers (P < 0.016) and tuber weight (P < 0.0016) in treatments infested with 14.3 *D. dipsaci* g^{-1} of growing medium (data not shown). The other initial population densities used in the experiments did not influence tuber numbers or weight. The relationship between P*i* of *D. dipsaci* and tuber damage was explained by R^2 = 0.96. The relation between *D. dipsaci* P*i* and tuber weight reduction revealed estimates of minimum yield (*m*) of 0.17 and tolerance limit (T) estimates of 0.49 *D. dipsaci* g^{-1} of growing medium (Fig. 2).

5.2. Influence of initial population densities of *D. destructor* and *D. dipsaci* on potato tuber damage

Significant differences in external (P < 0.001) and internal (P < 0.001) tuber damage caused by *D. destructor* were observed among the different (P*i*) treatments during experiment 1 (Fig. 3). External and internal potato tuber damage ranged from 0-78%, and 0-62%, respectively (Fig. 3). The highest external and internal potato tuber damage was observed in tubers infested with the highest initial population densities of 2,000 nematodes (Fig. 3). The lowest external and internal potato tuber damage was observed in treatments with initial population density of 14 nematodes (Fig. 3).

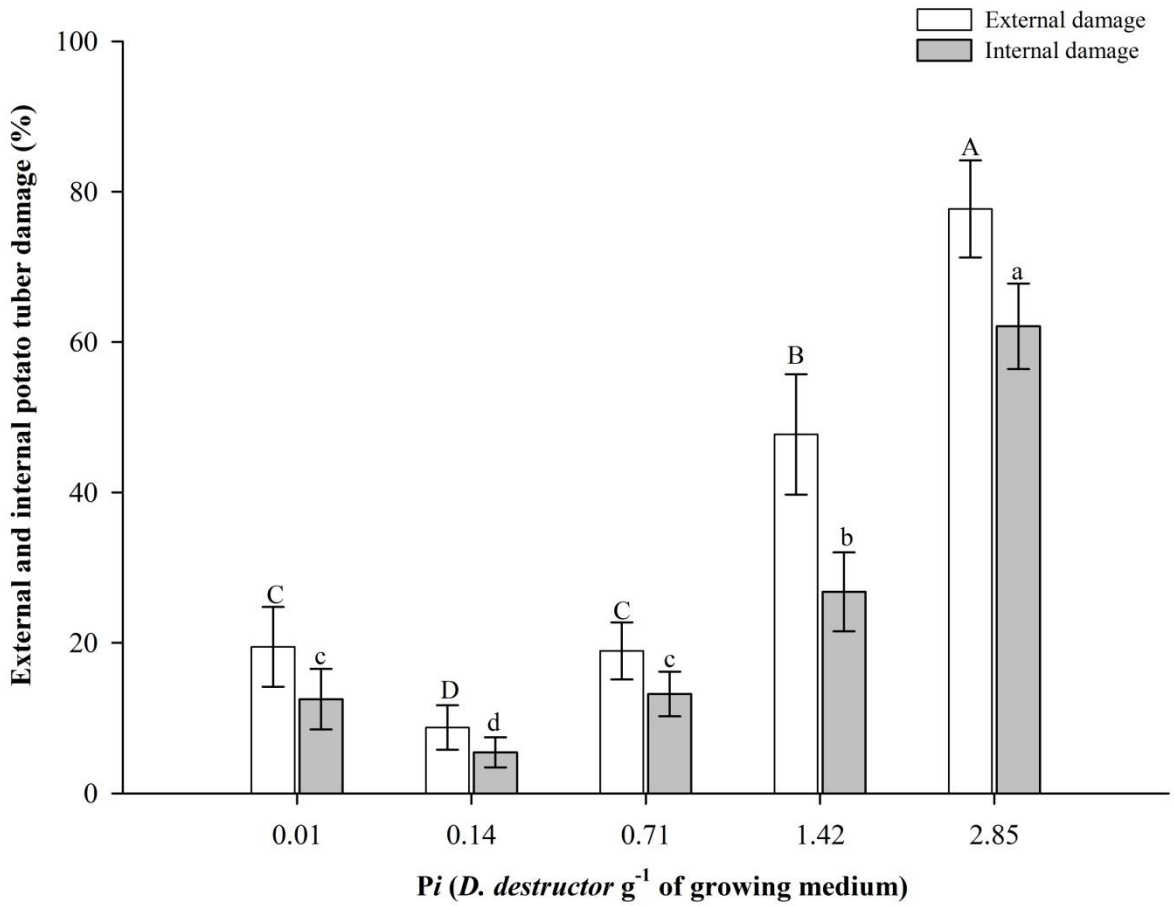


Fig 3: Mean external and internal potato tuber damage (*S. tuberosum* L., 'Désirée') caused by *Ditylenchus destructor* at varying initial population densities (P*i*) 12 weeks after infestation with nematodes. Bars with the same letters are not significantly different according to Tukey's studentized test (at $P < 0$ 05.) (uppercase letters for external damage; lowercase letters for internal tuber damage).

During experiment 2, potato tuber damage was significantly influenced by the varying initial population densities *of D. destructor* ($P < 0.0001$) (Fig. 4). Tuber damage increased with increasing initial population densities of *D. destructor* (Fig. 4). The highest percentage of external tuber damage of 84.4% was recorded in treatments infested with an initial population density of 14.29 nematodes g^{-1} of growing medium. Initial population densities of 0.71, 1.42 and 2.85 nematodes g^{-1} of growing medium resulted in similar damage intensity ($P > 0.05$) (Fig. 4). Mean external tuber damage of 10% was observed in treatments infested with the lowest initial population density of 0.01 nematode (*D. destructor*) g^{-1} of growing medium (Fig. 4). Internal tuber damage significantly varied ($P < 0.0001$) among the varying initial population densities of *D. destructor* (Fig. 4). The highest mean internal tuber damage of 57% was recorded in

treatments with an initial population density of 14.29 of *D. destructor* g^{-1} of growing medium (Fig. 4).

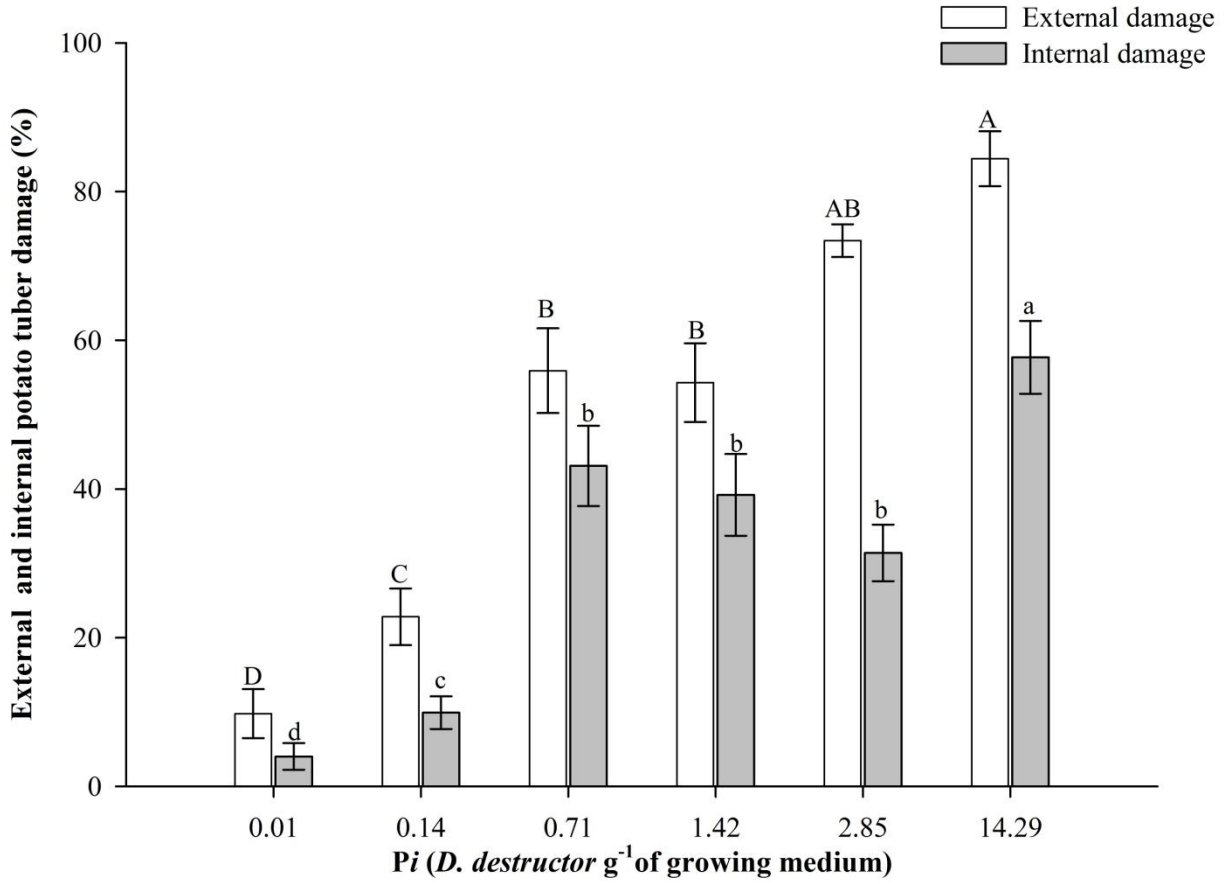


Fig. 4: Mean external and internal potato tuber damage (*S. tuberosum* L., 'Désirée') caused by *Ditylenchus destructor* at varying initial population densities (P*i*) 14 weeks after infestation with nematodes during experiment 2. Bars with the same letters are not significantly different according to Tukey's studentized test (at $P < 0$ 05.) (Uppercase letters for external damage; lowercase letters for internal tuber damage).

Mean tuber damage caused by *D. dipsaci* was only detectable when the initial population density (P*i*) was 0.14 nematodes g^{-1} of growing medium (Fig. 5). There were no significant differences in damage when potato plants were infested with 0.14 or 0.71 ($P > 0.05$) nematodes g^{-1} of growing medium (Fig. 5). Compared to the control plants (P*i* = 0), significant tuber damage was recorded when *D. dipsaci* initial population densities increased from 1.42 to 14.29 nematodes g^{-1} of growing medium (Fig. 5). The highest external percentage potato tuber damage (64.3%) caused by *D. dipsaci* was recorded when the initial population density (P*i*) was at 14.29 nematodes g^{-1} of growing medium. Similarly, the highest internal damage (55%) was also recorded at the highest infestation density (Fig. 5).

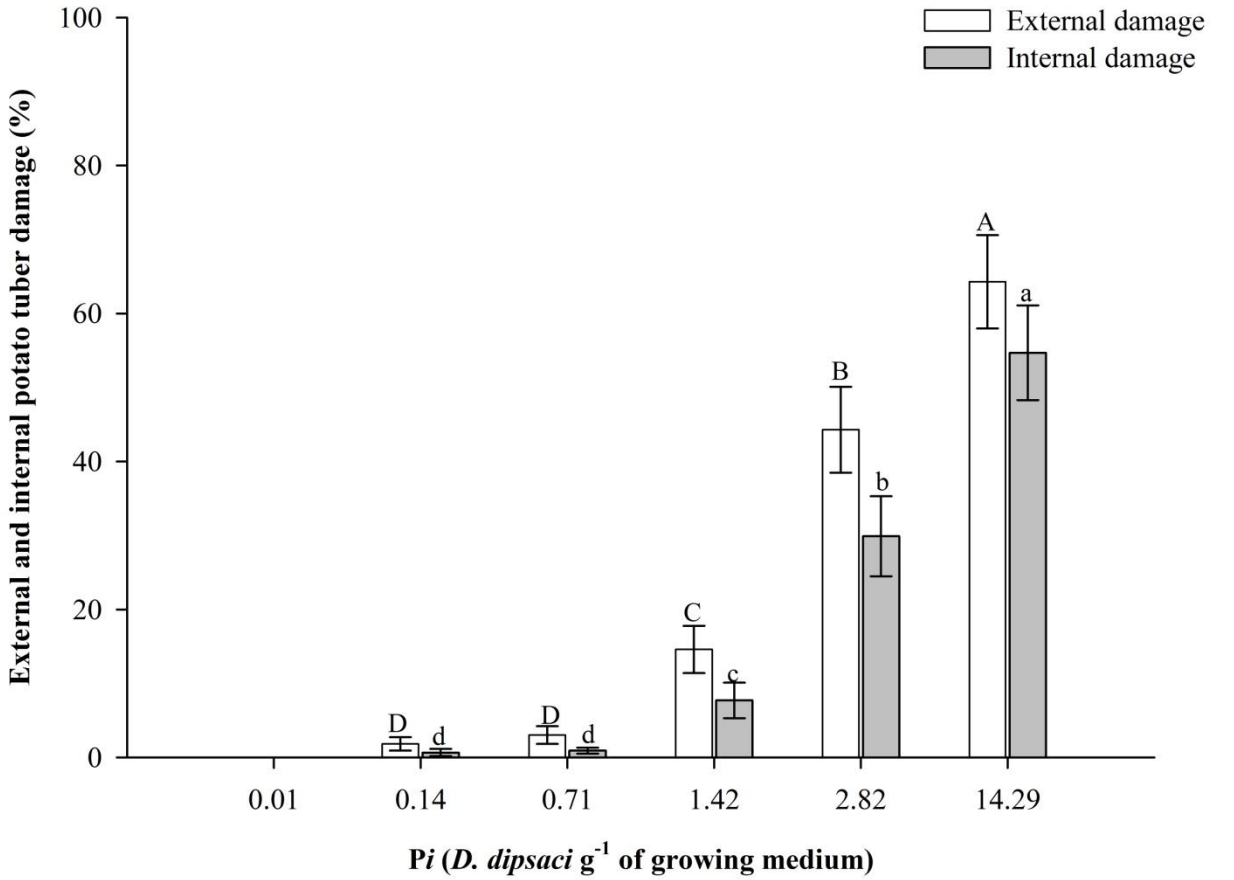


Fig 5: Mean external and internal potato tuber damage (*S. tuberosum* L., 'Désirée') caused by *Ditylenchus dipsaci* at varying initial population densities (P*i*) 14 weeks after infestation with nematodes during experiment 2. Bars with same letters are not significantly different according to Tukey's studentized test (at $P < 0\ 05$.) (Uppercase letters for external damage; lowercase letters for internal tuber damage).

5.3. Influence of *D. destructor* and *D. dipsaci* on initial population densities on nematode reproduction

D. destructor and *D. dipsaci* reproduced well on 'Désirée' confirming that this nematode population was virulent on 'Désirée'. *D. destructor* final population densities (P*f*) extracted from both the tuber peels and growing medium significantly varied ($P < 0.001$) among the different initial densities. During experiment 1, the numbers of *D. destructor* were higher in tuber peels at all initial densities compared to numbers extracted from the growing medium (data not shown). Lowest reproduction was observed in the treatment with the highest initial population density (2.85 nematodes g^{-1} of growing medium).

During experiment 2, the final population densities of *D. destructor* extracted from tuber peels and the total growing medium and varied significantly ($P < 0.001$) among the various initial population densities (Fig. 6). The highest number of *D. destructor* and *D. dipsaci* extracted from tuber peels was obtained from treatments which were infested with P*i* of 1.42 and 2.85 nematodes g^{-1} of growing medium (Fig. 6). The numbers of *D. destructor* extracted $g^{-1}$of tuber peels were significantly higher than those of *D. dipsaci* at all initial population densities apart from treatments with P*i* of 0.01 nematodes g^{-1} of tuber peels (Fig. 6).

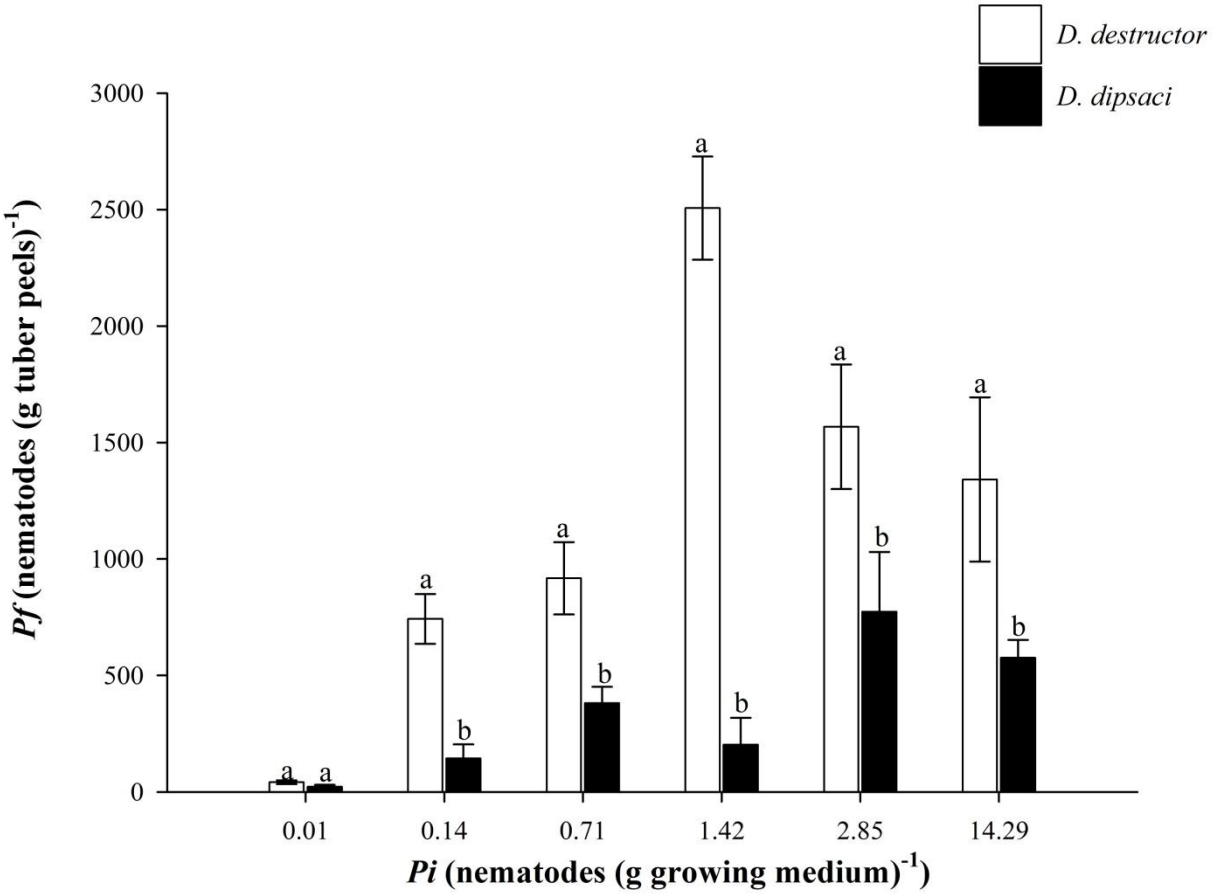


Fig. 6: *Ditylenchus destructor* and *D. dipsaci* nematode numbers extracted from potato tuber peels (g^{-1}) at different initial population densities. Bars with same letters are not significantly different according to Tukey's studentized test (at $P < 0$ 05.).

D. destructor attained the highest reproduction factor of 74.3 at a P*i* of 0.14 nematodes g^{-1} growing medium, while *D. dipsaci* attained highest reproduction factor of 21.8 at a P*i* of 0.01 g^{-1} of growing medium. The lowest reproduction factors form both *D. destructor* and *D. dipsaci* extracted from potato tuber peels were observed at the highest initial population density of 14.29 nematodes g^{-1} of growing medium.

Significant differences were observed between *D. destructor* and *D. dipsaci* extracted from growing medium (Fig. 7). *D. dipsaci* numbers were significantly higher than *D. destructor* at P*i* level of 0.14 nematodes g^{-1} of growing medium, while it was vice versa at P*i* of 0.71 and 14.29 nematodes g^{-1} of growing medium (Fig. 7).

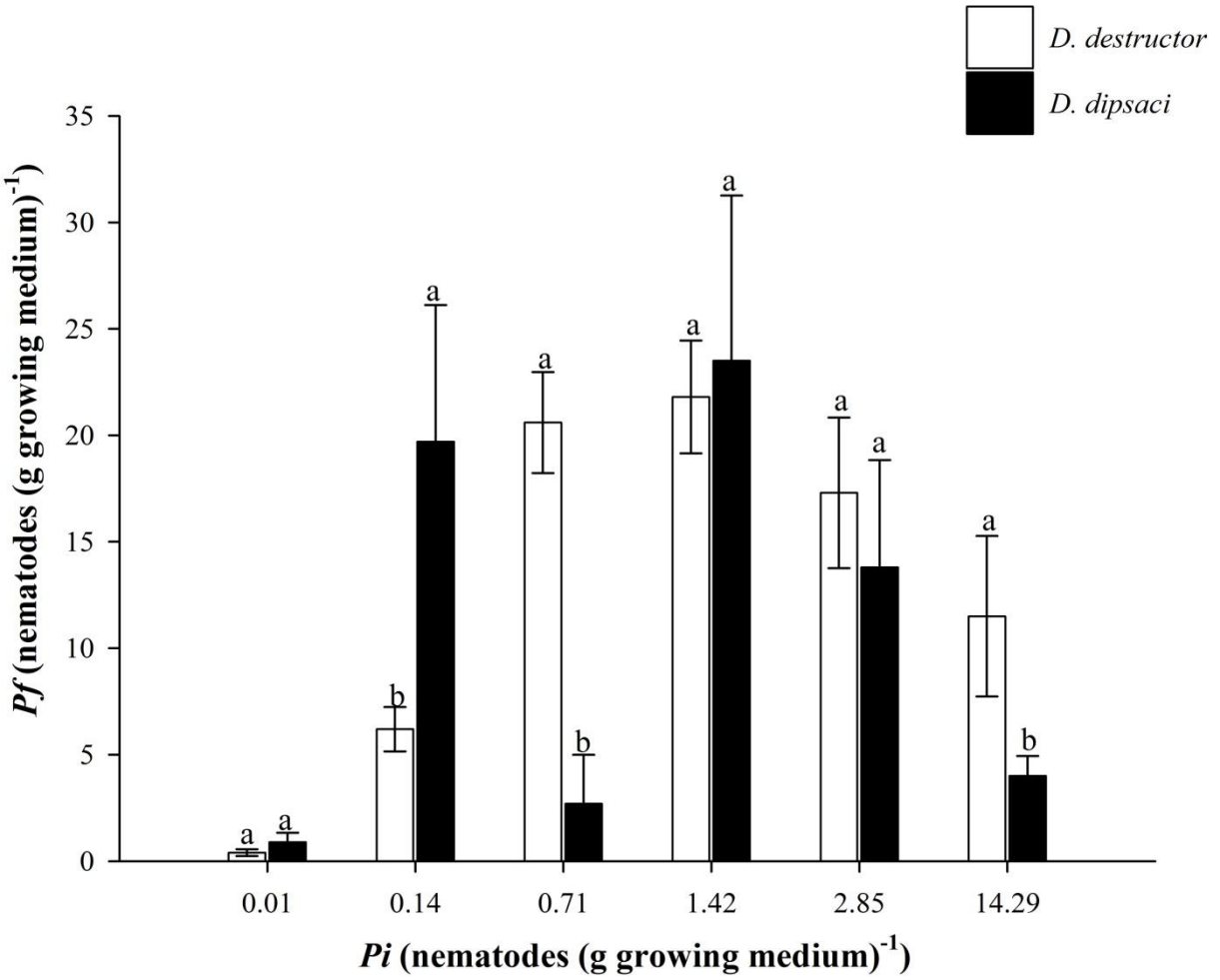


Fig. 7: *Ditylenchus destructor* and *D. dipsaci* nematode numbers extracted from growing (g^{-1}) at different initial population densities. Bars with same letters are not significantly different according to Tukey's studentized test (at $P < 0\ 05.$).

6.0 Discussion

The impact of *D. destructor* and *D. dipsaci* on potato plants in our experiments was measured as tuber number and weight reduction and external and internal tuber damage. Numbers of potato tuber were not affected by *D. destructor* at any P*i* level and *D. dipsaci* caused a reduction in tuber numbers only at the highest P*i* level investigated. The causes for reduction of tuber numbers were not further investigated.

Tuber weight reductions were observed at high initial population densities of *D. destructor* (2.85 and 14.29 nematodes g^{-1} of growing medium) and the highest P*i* level of *D. dipsaci*. There are no reports on tuber weight reduction due to *D. destructor*. Trials on the influence of different P*i* levels of *D. dipsaci* on potato are rare and only reported by Hijink (1963) in a field experiment. In the field, Hijink (1963) found that *D. dipsaci* reduced potato tuber weight and that this reduction was dependent on initial population densities. The highest tuber weight reduction as observed by Hijink (1963) when the initial population density was 0.6 nematodes $g^{-1}$of soil. Hijink (1963) hypothesized that potato tuber weight loss was caused by *D. dipsaci* damage on the stalks of the potato leading to an early die-back of the plants and deficient tuber formation. During our experiment, stem infestation or die back was not observed.

The Seinhorst model described a weak pathogenic relationship of *D. destructor* on potato during experiment 1 (R^2 = 0.16). Using the model, the estimated tolerance limit of 'Désirée' was low as 0.14 *D. destructor* g^{-1} of growing medium for tuber weight. An initial population density of *D. destructor* exceeding 0.14 g^{-1} of growing medium may decrease tuber weight by only 17% compared to non- infested controls. During experiment 2, a stronger pathogenic relationship was observed after inclusion of one higher initial population density treatment (R^2 = 0.92). Using the model, the estimated tolerance limit of 'Désirée' was 0.72 *D. destructor* g^{-1} of growing medium for tuber weight. Any population exceeding this limit may decrease weight by a maximum of 34%. The model adequately described pathogenic relationship of *D. dipsaci* P*i* levels during experiment 2 (R^2 = 0.96). The tolerance limit on 'Désirée' was 0.49 *D. dipsaci* g^{-1} of growing medium. An initial population density exceeding 0.49 *D. dipsaci* g^{-1} of growing medium may decrease tuber weight by 83%. Comparisons between species could only rely on the second experiment where both species were used. Based on the tolerance limits of 0.49 *D. dipsaci* g^{-1} of growing medium compared with 0.72 *D. destructor* g^{-1} of growing medium for potato tuber weight, it can be concluded that *D. dipsaci* influences tuber weight more than *D. destructor*.

However, when the tolerance limit levels for both nematodes species from the Seinhorst model were compared to the P*i* levels at which external tuber damage was observed, it was noted that damage (necrotic tuber tissue) occurred much earlier than the estimated tolerance limit levels at which nematodes started to reduce tuber weight. Based on our data and the absence of literature reporting on yield losses due to reduced tuber weight, apart from Hijink (1963), it is justified to

conclude that a reduction of tuber weight contributes little to overall yield loss and that the main damage is rotting of tubers.

D. destructor caused higher external tuber damage compared to *D. dipsaci*. The P*i* levels at which *D. destructor* caused damage concurs with findings of Butorina *et al.*, (2006) who observed damage at P*i* level of 0.02-0.05 nematodes $g^{-1}$of growing medium. The P*i* level at which *D. dipsaci* caused damage in our experiment was higher compared with results reported from field experiments, suggesting influence of other factors under field conditions (Hijink, 1963).

Potato tuber lesions caused by *D. destructor* are reported to be different from those caused by *D. dipsaci*. According to Cotten *et al*. (1992), *D. dipsaci* produces deeper lesions inside potato tubers. In our study, the depths of internal potato tuber lesions caused by *D. dipsaci* were similar to those caused by *D. destructor*. At all P*i* levels *D. destructor* had a higher reproduction compared to that of *D. dipsaci*, which could have led to higher tuber infestation and consequently higher mean tuber damage.

The rotting of potato tubers was measured as external and internal damage. Potato tuber damage assessments were done by scoring the percentage external damage from the entire tuber and internal lesions from one half of a sliced tuber. External and internal potato tuber damage caused by *D. destructor* and *D. dipsaci* were previously determined by counting individual feeding pockets and the numbers of coalesced lesions on the potato tuber surface (Moore, 1971). The method was not applicable in our case since at the end of the experiments after 12 and 14 weeks, respectively, most of the feeding pockets on tubers had already coalesced into lesions making it impossible to detect individual feeding pockets and count them. Our method was suitable in determining damage and could be applied to large numbers of potato tubers that needed to be assessed in a short time.

D. destructor and *D. dipsaci* numbers isolated from the tuber tissues were higher compared to nematodes isolated from the growing medium. Apart from the lowest P*i* level, *D. destructor* numbers extracted from potato tuber peels were significantly higher than those of *D. dipsaci*. There were minimal differences in total numbers of *D. destructor* and *D. dipsaci* in the growing medium. Both nematodes are known to leave the host plant tissues when conditions are

unfavorable and survive in soil until the next host plant, explaining why there was limited or no reproduction in our growing medium (Sturhan & Brzeski, 1991; Brodie *et al.*, 1993).

Increasing P*i* levels resulted in lower reproduction rates for *D. destructor* and *D. dipsaci* in potato tuber peels. The reproduction factors of both species declined at comparable levels. Although the equilibrium density was not the focus of these experiments, *D. dipsaci* had a reproduction rate of 0.6 in tuber peels at the highest initial population density investigated. Equilibrium density is the nematodes population density which can be sustained by a host plant and is expressed as the P*i* for which P*f*/P*i* = 1.0 (Seinhorst, 1966). The reproduction rate of 0.6 indicates that this species reached it equilibrium densities under our experimental conditions. At such high initial population density, *D. destructor* had also only a reproduction rate of 1.3 in tuber peels suggesting that the equilibrium density is also similar as for *D. dipsaci*.

7.0. Conclusion

The impact of *D. destructor* and *D. dipsaci* on tuber numbers and weight was minor and therefore the best estimate of yield loss was observed to be potato tuber damage. *D. destructor* was demonstrated to be more damaging compared to *D. dipsaci* even at the lowest initial densities. Depth of internal tuber damage caused by *D. destructor* and *D. dipsaci* were similar, contrasting previous observations that *D. dipsaci* causes deeper lesions into the potato tubers. Although the method adopted in our study was suitable, a more refined method for internal tuber damage assessment my improve damage evaluation. Damage was observed to be closely related to nematode reproduction. The reason for reproduction factor differences between *D. destructor* and *D. dipsaci* was not investigated, but it was attributed to the reproduction fitness of *D. destructor* on 'Désirée'. Further studies considering Seinhorst research program and involving different potato varieties and different populations of each nematode species are needed to investigate further observed differences in reproduction between *D. destructor* and *D. destructor*. Additionally, micro plot studies could offer better tolerance limit estimates and minimum yield losses as opposed to pot experiments.

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Chapter 5:

Influence of soil temperature on *Ditylenchus destructor* and *Ditylenchus dipsaci* population density and their impact on potato tuber damage

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Abstract

Temperature influences nematodes activities and interaction with host plants which determine severity of infestation or damage. Two experiments were conducted in two climate chambers set at different day and night temperatures, to investigate the influence of soil temperatures and duration of the experiments on *Ditylenchus destructor* and *D. dipsaci* population increase, and potato tuber damage. During experiment 1, the first climate chamber was set at 22°C and 13°C day and night temperatures, while the second chamber was set at 26°C and 17°C day and night temperatures respectively. During the second experiment, the first chamber was set at 16°C and 13 °C during the day while the second chamber was set at 20°C and 17°C day and night temperatures respectively. The total duration of the experiments were 16 weeks, with monthly harvest conducted to evaluate potato tuber damage and nematode multiplication rates. Temperature and duration of the experiments significantly influenced potato tuber damage and nematodes multiplication. Our study indicated that even at the lowest temperatures settings studied (16°C and 13°C day and night temperature), both *D. destructor* and *D. dipsaci* caused significant potatoes tuber damage reducing tuber quality. *Ditylenchus destructor* and *D. dipsaci* damage and optimal population increase was attained when the temperature setting was at 20°C and 17°C day and night temperatures. *D. destructor* and *D. dipsaci* did not have influence on above ground fresh and dry weight, potato tuber numbers and tuber weight. However, interaction between the duration of the experiment and temperature had influence on these parameters. This study indicates that *D. destructor* and *D. dipsaci* populations used in the current study have similar optimal temperature range of 20 and 17°C day for multiplication and for causing high potato tuber damage on potato tubers. However significant damage also is reported in other temperature settings during the current experiment. Our findings agree with the limited laboratory experiments on thermal temperature requirements of *D. destructor* on potatoes. Thermal temperature requirement for *D. dipsaci* and its relevance to potato tuber is to our knowledge reported for the first time in this study.

Keywords: Tuber rot nematode, stem nematode, thermal optimum optimal temperature, *Solanum tuberorum*

1.0. Introduction

Nematodes are poikilothermic organisms and therefore temperature is an important abiotic factor known to modulate their behavior and physiological processes (Barbercheck & Duncan, 2004). Most of the important life history traits of nematodes, such as their rate of reproduction and population growth, sex determination, motility and expression of damage to host plants, respectively, are regulated by temperature regimes (Wallace, 1973). Under field conditions soil temperatures fluctuate diurnally, depending on the soil depth and season, directly influencing host plants and nematodes interactions (Jones, 1978).

Potato is a host to several nematodes species, which often results in yield reductions, both quantitatively and qualitatively (Hooker, 1981). The potato tuber rot nematode, *Ditylenchus destructor* Thorne 1945, and the stem nematode *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936, are regarded important nematode species influencing potato production (Hooper, 1972; Hooper, 1973).

Ditylenchus destructor and *D. dipsaci* can cause serious damage to potatoes leading to severe lesions cracking and eventual rotting of potato tubers in the field and in storage (Thorne, 1945; Cotten *et al.*, 1992). Within one potatoes vegetative period, *D. destructor* is able to complete 6 to 9 generations (Saf'yanov, 1964). Optimal temperature at which *D. destructor* causes major potato tuber damage caused by *D. destructor* is reported to occur within the temperature range of 15 and 20°C (Sturhan & Brzeski, 1991). Temperature is reported to have no influence on the severity of potato tuber damage caused by *D. dipsaci* (Kotthoff, 1950). Generally, *D. dipsaci* maximum activity and highest invasive ability has been reported to range from 10 to 20°C (Sturhan & Brzeski, 1991). Temperature is also an important factor influencing the survival and distribution of *D. destructor* and *D. dipsaci* in the soil and host plants such as potatoes (Miyagawa & Lear, 1970; Švilponis *et al.*, 2011a).

Most of the data collected about thermal optimal requirements of most nematode species are obtained through studies using constant soil temperatures normally in greenhouse pot experiments. However, soil temperature fluctuates with ambient temperature conditions (Jacobs *et al.*, 2011), showing diurnal and seasonal variation under field conditions, depending on the

climatic zones and the depth of the soil studied (Dao, 1970; Jacobs *et al.*, 2011). As such, these fluctuations are expected to increase or decrease certain nematode activities (Wallace, 1963).

To date, thermal optimum for *D. destructor* damage and reproduction on potato has been reported in a few papers dating back 40-50 years ago, as reviewed by Decker (1969). Temperature was observed to have no correlation with the severity of potato tuber attack by *D. dipsaci* (Kotthoff, 1950). Recent study suggests that soil temperature has gradually increased over time (Jacobs *et al.*, 2011), a fact which could impact on the ecology and biology of soil and plant inhibiting nematodes. It was therefore hypothesized that, since *D. destructor* and *D. dipsaci* attack and damage potato tubers, then both nematodes species have similar thermal optimum on potatoes. Secondly, it was hypothesized that temperature has an impact on their population development which in turn influences severity of damage on potatoes. To answer these hypotheses two climate chamber experiments were set up using different temperature regimes to (i) quantify the influence of soil temperature on the reproduction rates of *D. destructor* and *D. dipsaci* and (ii) to evaluate the influence of these population numbers of nematodes on potato external tuber damage.

2.0. Materials and methods

2.1. Potting substrate

Field soil was dry sterilized for 12 hours using an electric soil pasteurizer (Sterilo®, Schenkenzell, Germany), set at 100 ± 5°C. The soil was then sieved with a mesh size of 1 cm by 1 cm to remove particles larger than 1 cm^2. Sieved field soil was mixed with peat (Klasmann® Lithuanian peat moss medium, pH 3.5) at the ratio of 3:1. Slow release fertiliser (Osmocote Exact®Standard® 15% N, 9% P_2O_5, 12% K_2O and 2% M_gO) was added to the artificial growing medium mix at the rate of 1.5 g/kg growing medium. The final growing medium had a pH of 4.7, and an organic matter content of 2.6%. The texture consisted of 7.5% clay, 19.1% silt, and 73.4% sand. The mineral content of the growing medium was analysed as: Potassium (K): 36 mg/100 g, Phosphorus (P): 16 mg/100 g, and Magnesium (Mg): 10 mg/100 g. All experiments were conducted in one litre pots filled with 700 ml of the growing medium.

2.2. Planting material

Potato tubers, variety '*Bintje*', were pre-germinated in the dark at 20 ± 3^{o} C until sprouts were observed. Prior to planting, the sprouted tubers were placed at daylight for one week to harden the sprouts. Redundant sprouts were removed to retain only one single sprout. Single-sprout seed tubers of approximately15 ± 1 g each were used as planting material.

2.3. Multiplication of *Ditylenchus destructor* and *D. dipsaci* populations on carrot discs

Ditylenchus destructor and *D. dipsaci* populations used in this study were originally extracted from celery and sugar beet plants, respectively, sampled in Germany (Julius Kühn-Institut collection). A modified carrot disk culture method adopted from Speijer & De Waele (1997) was used to maintain an axenic culture of both nematode populations.

Nematodes were sterilized using a streptomycin sulphate (AppliChem®, Darmstadt, Germany) solution at 0.06 mg/10 ml of sterile water for six hours. Thereafter, nematodes were rinsed three times using sterile water. Approximately 100 µl of water containing about 20 mixed development stages of nematodes were transferred to sterile carrot discs using a sterile pipette. The Petri dishes were sealed with Parafilm® and placed in an incubator (Heraeus®-model BK 5060 EL, Burladingen, Germany) set at 20±1°C for approximately eight weeks.

Nematodes were collected by rinsing the Petri dishes with water into a clean 500 ml glass bottle. Carrot discs were cut into small pieces using a scalpel blade and transferred to a Baermann funnel overnight to extract nematodes. The nematode suspension was tapped off into the glass bottle the following day. Nematode suspensions were stored at approximately 4°C until further use for a maximum period of one week. To establish the population density in a given solution, stock solutions were homogenised and a 1 ml sub-sample of the suspension was pipetted onto a counting slide. Nematodes were counted at 40X magnification using an inverted microscope (Axiovert 25 CarlZeiss®, Göttingen, Germany). Counting was replicated three times and the mean value calculated. Nematode stock suspensions were adjusted to 500 nematodes per ml of water.

2.4. Climate chambers settings

Two climate chambers each with an area of 13 m^2 (Weiss Klimatechnik GmbH, Gießen, Germany), were set at different day and night (d/n) ambient temperature regimes during the two

experiments. During experiment 1, the first climate chamber was set at an ambient temperature of 22°C during the day and 13°C during the night with a photoperiod of 13 hours. The second climate chamber was set at an ambient day temperature of 26°C and a night temperature of 17°C. During experiment 2, the first climate chamber was set at 16°C during the day and 13°C during the night, while the second chamber was set at 20°C during the day and 17°C during the night. The photoperiod remained the same as in experiment 1. The relative humidity in both experiments was approximately 70%. Soil and air temperatures in both climate chambers were monitored continuously throughout the entire duration of the experiments using Testo® 175-T3 data loggers.

2.5. Experimental setup

Pre-germinated potato tubers were planted singly per pot, which were later placed on saucer plates before complete randomization on benches. Watering was done on a daily basis to keep the growing medium moist throughout the experiment. Two weeks after planting when potato stems and leaves were visible, the growing medium was infested with nematodes suspensions of either *D. destructor* or *D. dipsaci*. Four holes (~4 cm deep) were drilled into the growing medium around the stem and nematode suspensions of approximately 2000 mixed developmental stages were inoculated into these holes. Control plants were infested with an equal volume of water. The holes were covered with growing medium immediately after nematode application. The duration of the experiments was 16 weeks from planting to final harvest.

The two experiments were laid out in a split-plot design with a whole plot factor and sub-plot factors, where the whole plots were the climate chambers (Temperature), sub-plots were the treatments, with repeated measures within each plot (plants which were sampled per month). There were three treatments in each chamber (*D. destructor*, *D. dipsaci* and their controls) each replicated twenty times. Within each treatment, fifteen pots (five per treatment) were randomly harvested at fortnight intervals at weeks 4, 8, 12 and 16 after planting.

3.0 Data collection

3.1. Assessment of potato tuber numbers, weight, external and internal tuber damage

A total of 30 pots (15 per climate chamber, 5 per treatment) were harvested per month from both climate chambers. Potato tubers were collected by passing the growing medium from each pot through a sieve with mesh size of 1 cm x 1 cm into a collection container. The growing medium collected was packed into polythene bags and transferred to the laboratory for nematode extraction. Growing medium adhering on tubers was washed off using water. Tuber numbers and fresh weight were recorded followed by evaluation of external potato tuber damage. External potato tuber damage was evaluated as the percentage of the entire tuber with lesions and cracks caused by *D. destructor* or *D. dipsaci* infestation. The total external percentage tuber damage per replicate was calculated using the formula:

$$P = \frac{\sum(n_1 + n_2 + n_3 + n...)}{N},$$

where, P = is the percentage (%) potato tuber damage per replicate,

N = Total number of tubers per replicate, and

n = percentage of potato tuber with lesions caused by *D. destructor* or *D. dipsaci*, respectively.

3.2 Nematodes extraction from potato tuber peels and growing medium

Potatoes harvested per replicate were completely peeled using a knife. Peels were approximately 2 mm thick and made up approximately 22% of the total tuber weight. The complete tuber peels per replicate were mixed and a 10 g subsample was then picked and chopped further into fine pieces of approximately 5 mm x 5 mm and used for nematodes extraction. Nematodes were extracted using the modified Baermann funnel method for 12 hours (EPPO, 2013). Nematode numbers at different developmental stages (eggs, juveniles J2-J4, females, and males, respectively) were determined under an inverted microscope (Axiovert25 CarlZeiss®) at 40X magnification using a 1 ml capacity nematode counting slide chamber. Nematodes were extracted from 10 g of tuber peels and used to calculate the total number of nematodes in tuber peels per replicate. The final counts were presented per gram of tuber peels.

3.3. Nematodes extraction from growing medium

After collecting growing medium as described in step 3.1, the soil was stored at 5°C up to a maximum of 5 days before nematode extraction. Nematodes were extracted from 250 ml sub-samples of the growing medium for 24 hour using a modified Oostenbrink dish with 24 cm inner diameter and milk filter paper (27 cm Ø) (EPPO, 2013). Nematode numbers (all developmental stages) extracted from the growing medium were determined as described in section 3.2 and total numbers of nematodes per gram of soil calculated.

3.4. Determination of final population densities and reproduction factor of *D. destructor* and *D. dipsaci*

The final nematode population density was calculated as the sum of the total number of nematodes from tuber peels and from the growing medium. The reproduction factor (Rf) of *D. destructor* and *D. dipsaci* was determined according to the formula Rf = P*f*/P*i* where P*f* is the final nematode population density and P*i* is the initial population density (Oostenbrink, 1966).

3.0. Data analyses

Prior to repeated measures analysis of variance (RM-ANOVA), data were checked for homogeneity of variance and assumption of normality in residuals using Levene's and Shapiro-Wilk's tests. Data analyses were performed in PROC Mixed model in SAS software Version 9.3 (SAS Institute Inc., Cary, NC, USA). Where necessary, percentage data were arcsine square root transformed using the formula: y = arcsin(sqrt(x/100)), while nematode counts were log transformed [$\log_{10}(x+1)$] to standardise variances. Plant yield data was square root transformed using the formula: y = sqrt(x). All three factor interactions between temperature, nematode treatments and duration were assessed. Where factor interactions were significant, effects of one factor were analysed at each level of the other factor. Where significant differences in means were observed, Tukey studentized (HSD) mean separation method was used at 5% confidence levels. The non-transformed means ± SE are presented in figures and tables below.

4.0. Results

5.1. Influence of temperature on external damage caused by *D. destructor* and *D. dipsaci*

Experiment 1: The development of potato external tuber damage caused by *D. destructor* was influenced by the interaction between temperature and the duration of the experiment (*DF* = 3, *F*

= 32, *P* = 0.0253). Tuber lesions caused by *D. destructor* were significantly higher during week 8 in the climate chamber set at 22°C and 13°C compared to the same week at 26°C and 17°C day and night (d/n) temperatures (Fig. 1). External potato tuber damage caused by *D. destructor* observed on tubers at week 12 and 16 were not significantly (*P* > 0.05) different in the two temperature settings (Fig. 1a).

Potato tuber damage caused by *D. dipsaci* during experiment 1 were only influenced by the duration of the experiment (*DF* = 3, *F* = 30, *P* < 0.0001), while the interaction between temperature and duration was insignificant in influencing tuber lesions development (*DF* = 3, *F* = 0.70, *P* = 0.5584) (Fig. 1b). External tuber damage caused by *D. dipsaci* was significantly higher (*DF* = 3, *F* = 42, *P* < 0.0001) at week 16 in the climate chamber set at 22°C and 13°C compared to the same week at 26°C and 16°C d/n temperatures (Fig 1. b).

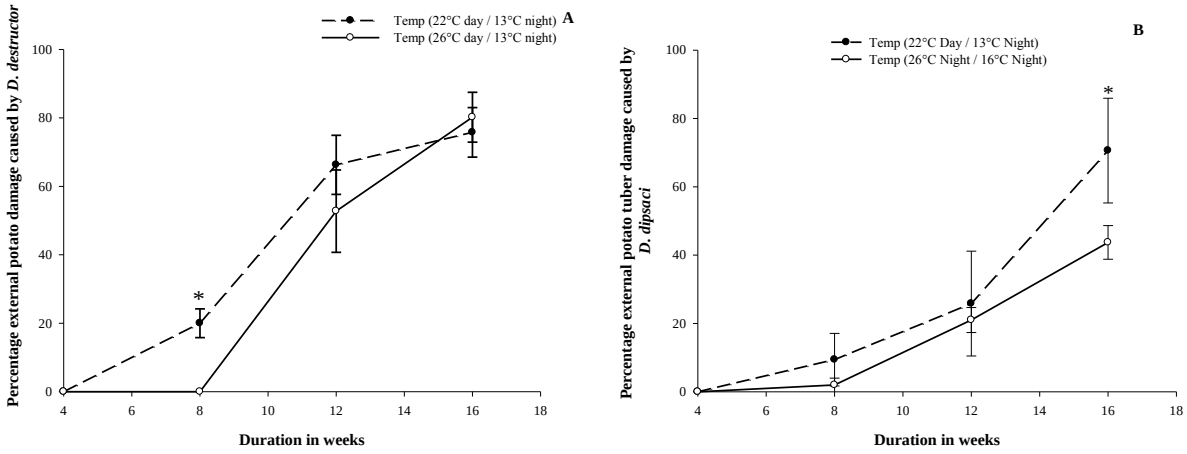

Fig. 1 (a and b): Percentage external potato tuber damage caused by *D. destructor* and *D. dipsaci* at two different temperature regimes during experiment 1.

Experiment 2: External potato tuber damage caused by *D. destructor* during experiment 2 was influenced by both temperature (*DF* = 1, *F* = 9.62, *P* = 0.004) and the duration of the experiment (*DF* = 1, *F* = 6.81, *P* = 0.0011). The interaction between temperatures and duration of the experiment was not significantly (*DF* = 1, *F* = 2.40, *P* = 0.0865) influencing tuber damage caused by *D. destructo*r. External potato tuber damage caused by *D. destructor* at 20°C and 17°C was higher compared to potato tuber damage at temperatures at16 and 13°C d/n, respectively (Fig. 2 a).

External potato tuber damage caused by *D. dipsaci* during experiment 2 was significantly influenced by temperature ($DF = 1$, $F = 8.83$, $P < 0.0056$) and the duration of the experiment ($DF = 1$, $F = 8.83$, $P < 0.0056$) while the interaction between temperature and duration was significantly in influencing tuber lesions development ($DF = 3$, $F = 4.16$, $P = 0.0135$) (Fig. 1b).

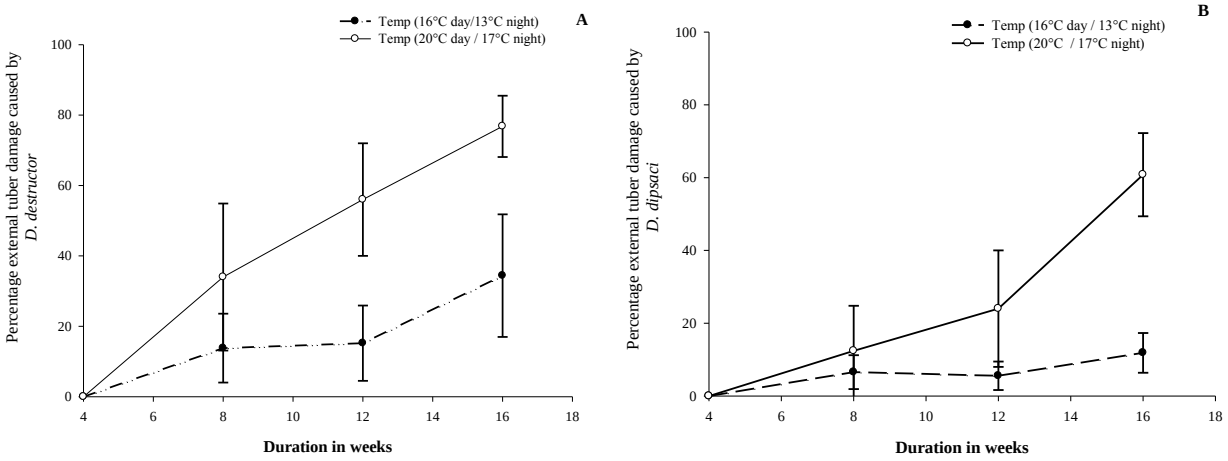

Fig. 2 (a and b): Percentage external potato tuber damage caused by *D. destructor* and *D. dipsaci* under two different temperatures during experiment 2.

5.2. Influence of temperature and duration of the experiment on nematode numbers isolated from soil and tuber tissues.

Experiment 1: Nematode numbers were significantly influenced by the duration of the experiment ($DF = 3$, $F = 88.57$, $P < 0.0001$) and temperature ($DF = 1$, $F = 43.72$, $P < 0.0001$). The interaction between temperature and duration of the experiments significantly influenced the total *D. destructor* and *D. dipsaci* numbers isolated from both the growing medium and potato tuber peels ($DF = 3$, $F = 31.56$, $P < 0.0001$).

Numbers of *D. destructor* isolated from the growing medium and potato tuber peels increased over time, but differences were found with regard to the different temperature regimes (Fig. 3). Highest *D. destructor* numbers were observed after 16 weeks in both climate chambers. After 16 weeks, the total *D. destructor* numbers were significantly higher ($DF = 28$, $F = 6.62$, $P < 0.0001$) in potato tuber peels and growing medium obtained from climate chamber set at 22°C and 13°C

d/n temperatures compared to the respective data from the 26°C and 17°C d/n temperature regime (Fig. 3).

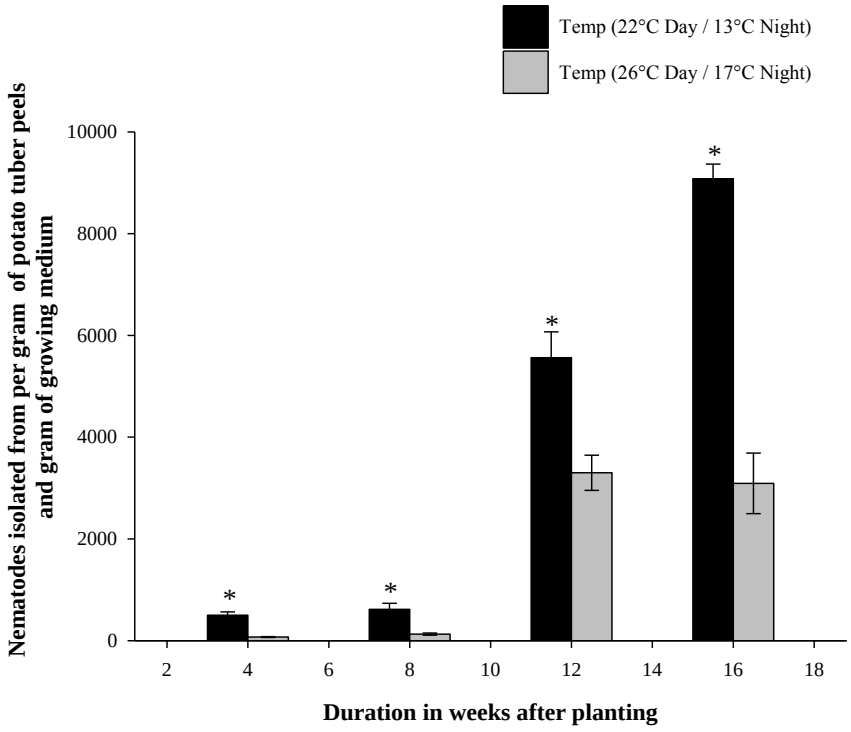

Fig 3: *Ditylenchus destructor* numbers isolated per gram of potato tuber peels and gram of the growing medium in climate chambers set at temperatures of 22°C/13°C day and night for the first climate chamber and 26°/17°C day and night for the second climate chamber during experiment 1. Standard error bars are followed by an asterisk according to Tukey studentized (HSD) mean separation method was used at 5% confidence levels.

D. dipsaci numbers isolated from tuber peels and growing medium were significantly influenced by temperature ($DF = 1$, $F = 100.83$, $P < 0.0001$) and the duration of the experiment ($DF = 3$, $F = 164.32$, $P < 0.0001$). At week sixteen, the highest nematodes numbers of *D. dipsaci* were recovered from plants cultivated under a 22°C and 13°C day and night temperature regime, respectively, compared to those from the 26°C and 17°C d/n temperature regime (Fig 4).

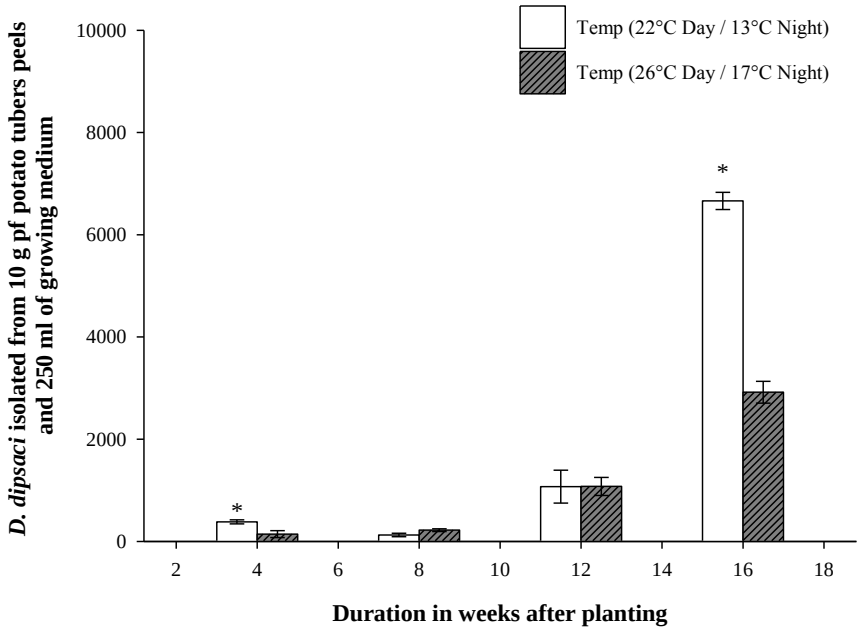

Fig 4: *Ditylenchus dipsaci* numbers isolated per gram of potato tuber peels and gram of the growing medium in climate chambers set at temperatures of 22°C/13°C day and night for the first climate chamber and 26°/17°C day and night for the second climate chamber during experiment 1. Standard error bars are followed by an asterisk according to Tukey studentized (HSD) mean separation method was used at 5% confidence levels.

Percentage external potato tuber damage caused by *D. dipsaci* at temperature setting of 22°C/13°C and 26°/16°C day and night temperatures in two climate chambers respectively during experiment 1. Means were separated using Tukey studentized Range test at 5% confidence level and are represented in the graph as standard error bars.

Experiment 2: D. destructor numbers isolated from 10 g of potato tuber peels and 250 ml of the growing medium were significantly influenced by temperature ($DF = 1$, $F = 100.83$, $P < 0.0001$), duration of the experiment in weeks ($DF = 3$, $F = 164.32$, $P < 0.0001$) and the interaction between temperature and duration ($DF = 3$, $F = 32.62$, $P < 0.0001$), respectively. Significantly higher numbers of *D. destructor* were isolated from potato tuber peels and growing medium obtained at all sampling dates when temperature regimes were set at 20°C and 17°C d/n temperatures compared to numbers isolated from the respective treatment at 16°C and 13°C d/n temperature regime (Fig. 5).

The highest numbers of *D. destructor* were observed when potatoes were grown for 16 weeks at 26°C (Fig. 3 and 4). Duration of the experiment had a significant influence on total nematode

numbers of both *D. destructor* and *D. dipsaci* isolated from the growing medium and tuber tissues ($P < 0.0001$).

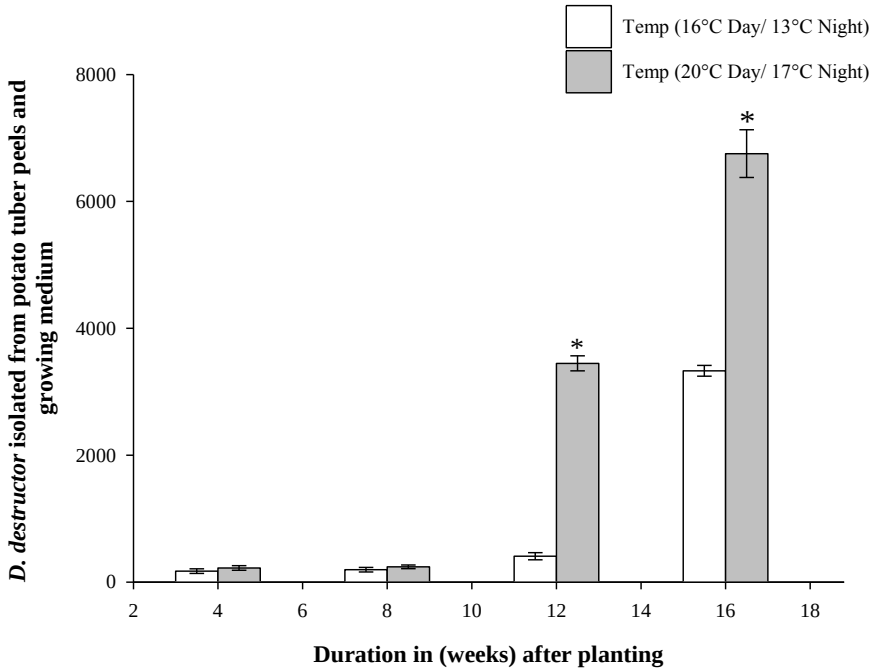

Fig 5: *Ditylenchus destructor* numbers isolated per gram of potato tuber peels and gram of the growing medium in climate chambers set at temperatures of 16°C and 13°C day and night for the first climate chamber and 20°C and 17°C day and night for the second climate chamber during experiment 1. Standard error bars are followed by an asterisk according to Tukey studentized (HSD) mean separation method was used at 5% confidence levels.

Percentage external potato tuber damage caused by *D. destructor* at temperature setting of 16°C/13°C and 20°/17°C day and night temperatures in two climate chambers respectively during experiment 2. Means were separated using Tukey studentized Range test at 5% confidence level and are represented in the graph as standard error bars.

D. dipsaci numbers were significantly influenced by temperature ($DF = 1$, $F = 69.47$, $P < 0.0001$), duration of the experiment in weeks ($DF = 3$, $F = 224.52$, $P < 0.0001$) and the interaction between these two factors ($DF = 3$, $F = 190.15$, $P < 0.0001$). At weeks 8, 12 and 16, the total numbers isolated from potato tuber peels and growing medium were significantly higher ($DF = 1$, $F = 100.83$, $P < 0.0001$), in climate chamber set at 20 and 17°C d/n temperature, compared to the chamber set at d/n temperatures of 16°C and 13°C (Fig. 6).

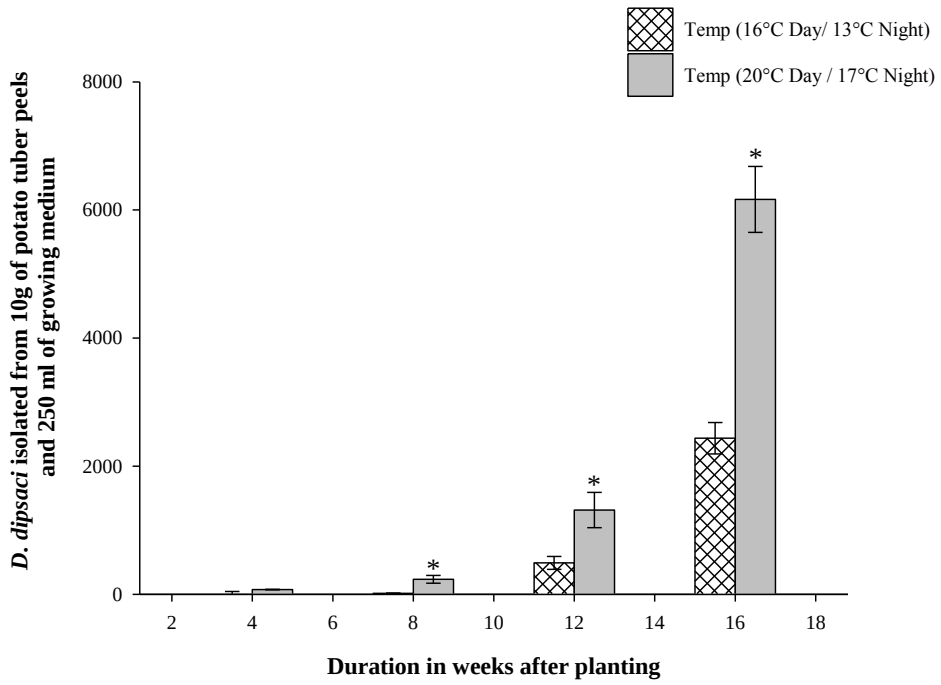

Fig 6: *Ditylenchus dipsaci* numbers isolated per gram of potato tuber peels and gram of the growing medium in climate chambers set at temperatures of 16°C and 13°C day and night for the first climate chamber and 20°C and 17°C day and night for the second climate chamber during experiment 1. Standard error bars are followed by an asterisk according to Tukey studentized (HSD) mean separation method was used at 5% confidence levels.

Percentage external potato tuber damage caused by *D. dipsaci* at temperature setting of 22°C/13°C and 26°/16°C day and night temperatures in two climate chambers respectively during experiment 2. Means were separated using Tukey studentized Range test at 5% confidence level and are represented in the graph as standard error bars.

The total numbers of *D. dipsaci* isolated from both the growing medium and potato tuber peels were significantly influenced by temperature ($DF = 1$, $F = 53.41$, $P < 0.0001$), duration of experiment in weeks ($DF = 3$, $F = 155.67$, $P < 0.0001$) and the interaction between temperature and duration of the experiment ($DF = 3$, $F = 29.19$, $P < 0.0001$).

5.3. Influence of temperature and duration of the experiment on different developmental stages of *D. destructor* and *D. dipsaci.*

Experiment 1: Numbers of *D. destructor* males, females, juveniles and eggs recovered from the growing medium and the potato tuber peels were significantly influenced by temperature ($P <$

0.0001), duration of the experiment ($P < 0.0001$) and the interaction between temperature and duration ($P < 0.0001$). *Ditylenchus destructor* numbers at different developmental stages were higher at a temperature regime of 22°C and 13°C d/n temperature compared to a 26°C and 16°C d/n temperature regime (Table 1 - 4).

D. dipsaci males, females juveniles and eggs were also significantly influenced by temperature ($P < 0.0001$), the duration ($P < 0.0001$) and the interaction between temperature and duration ($P < 0.0001$) (Table 2). All the developmental stages were at the highest during week 16 in climate chamber set at 22°C and 13°C day and night temperatures compared to chamber set at 26°C and 16°C day and night temperature respectively (Table 1).

Experiment 2: The numbers of *D. destructor* and *D. dipsaci* were significantly influenced by temperature ($P < 0.0001$), duration of the experiment ($P < 0.0001$) and also the interaction between temperature and duration ($P < 0.0001$). The different developmental stages of *D. destructor* and *D. dipsaci* varied between the two temperature settings of 16°C and 13° day and night in chamber one and 20°C and 17°C day and night temperatures in chamber 2 (Tables 3 and 4).

Table 1: Influence of temperature on different developmental stages of *D. destructor* under different durations in weeks during experiment 1.

Developmental stage	Temp (°C)	Week 4	Week 8	Week 12	Week 16
Males	22	69 ± 12[a]	147 ± 31[a]	1140 ± 183[a]	2243 ± 88[a]
	26	10 ± 3[b]	12 ± 2[b]	615 ± 107[b]	425 ± 93[b]
Females					
	22	107 ± 16[a]	277 ± 75[a]	1880 ± 188[a]	3120 ± 126[a]
	26	14 ± 3[b]	30 ± 7[b]	1144 ± 169[b]	865 ± 187[b]
Juveniles (J2- J4)					
	22	324 ± 51[a]	193 ± 29[a]	2541 ± 188[a]	3715 ±152[a]
	26	49 ± 6[b]	88 ± 13[b]	1541 ± 215[b]	3616 ± 343[b]
Eggs					
	22	324 ± 50[a]	49 ± 13[a]	513 ± 50[a]	936 ± 32[a]
	26	49 ± 6[b]	7 ± 2[b]	206 ± 46[b]	172 ± 25[b]

Numbers are mean ±standard error. Means in columns followed by the same letter are not statistically different (P > 0.05) according to tukey studentised test

Table 2: Influence of temperature on different developmental stages of *D. dipsaci* under different durations in weeks during experiment 1.

Developmental stage	Temp (°C)	Week 4	Week 8	Week 12	Week 16
Males	22	65 ± 15[a]	36 ± 12[a]	158 ± 33[b]	1163 ± 79[a]
	26	13 ± 4[b]	35 ± 6.2[a]	638 ± 92[a]	150 ± 29[b]
Females					
	22	85 ± 13[a]	55 ± 14[a]	224 ± 29[b]	2156 ± 90[a]
	26	37 ± 8.4[b]	64 ± 12[a]	733 ± 55[a]	1266 ± 52[ab]
Juveniles (J2 - J4)					
	22	231 ± 52[a]	35 ± 9.2[b]	688 ±167[b]	3343 ± 144[a]
	26	93 ± 28[b]	123 ± 19[a]	1548 ± 202[a]	2609 ± 102[a]
Eggs					
	22	32 ± 8.2[a]	29 ± 11[a]	88 ±34[b]	985 ±140[a]
	26	12 ± 3.7[b]	28 ± 11[a]	207 ± 38[a]	481 ± 29[b]

Table 3: Influence of temperature on different developmental stages of *D. destructor* under different durations in weeks during experiment 2.

Developmental stage	Temp (°C)	Week 4	Week 8	Week 12	Week 16
Males	16	55 ± 12^{a}	62 ± 11^{a}	108 ± 16^{a}	782 ± 36^{a}
	20	43 ± 6^{ab}	42 ± 7^{b}	474 ± 22^{a}	355 ± 32^{b}
Females	**Temp (°C)**	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	16	100 ± 25^{a}	117 ± 24^{a}	180 ± 27^{b}	1989 ± 90^{b}
	20	74 ± 15^{ab}	45 ± 8^{b}	752 ± 34^{a}	2783 ± 58^{a}
Juveniles (J1 - J4)	**Temp (°C)**	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	16	17 ± 3^{b}	16 ± 3^{b}	120 ± 23^{b}	559 ± 33^{b}
	20	104 ± 25^{a}	155 ± 22^{a}	2222 ± 108^{a}	1802 ± 340^{a}
Eggs	**Temp (°C)**	**Week 4**	**Week 8**	**Week 12**	**Week 16**
	16	15 ± 2^{b}	18 ± 3^{ab}	103 ± 24^{b}	390 ± 29^{b}
	20	116 ± 23^{a}	27 ± 7^{a}	488 ± 48^{a}	915 ± 149^{a}

Table 4: Influence of temperature on different developmental stages of *D. dipsaci* under different durations in weeks during experiment 2.

Developmental stage	Temp (°C)	Week 4	Week 8	Week 12	Week 16
Males	16	7 ± 4^{b}	31 ± 10^{a}	104 ± 14^{a}	803 ± 91^{b}
	20	21 ± 11^{a}	5 ± 2.6^{b}	159 ± 34^{a}	1029 ± 133^{a}
Females					
	16	51 ± 25^{a}	4 ± 1.4^{b}	281 ± 63^{a}	1030 ± 164^{b}
	20	1 ± 0.2^{b}	56 ± 14^{a}	138 ± 35^{b}	2256 ± 431^{a}
Juveniles (J1 - J4)					
	16	2 ± 0.6^{a}	6 ± 1.6^{b}	80 ± 20^{b}	377 ± 47^{b}
	20	2 ± 0.8^{a}	147 ± 41^{a}	1073 ± 261^{a}	3107 ± 145^{a}
Eggs					
	16	1 ± 0.4^{a}	3 ± 1.3^{b}	76 ± 20^{a}	351 ± 47^{b}
	20	5 ± 3.5^{a}	31 ± 11^{a}	79 ± 30^{a}	697 ± 42^{a}

5.4. Effect of temperature, nematode species and duration of the experiment in influencing potato tuber numbers

Experiment 1: The inoculation of potato plants with *D. destructor* or *D. dipsaci* did not have a significant influence on potato tuber numbers in experiment 1 in both temperature settings (*DF* = 2, *F* = 0.10, *P* = 0.0769) nor were any significant interactions found (*DF* = 1, *F* = 0.14, *P* = 0.7043) and interaction between temperature and nematodes (*D. destructor* and *D. dipsaci*) (*DF* = 4, *F* = 1.67, *P* = 0.1622) did not have influence on tuber numbers. However tuber numbers were significantly influenced by the duration of the experiment (*DF* = 3, *F* = 21.59, *P* < 0.0001) and the interaction between duration of the experiment and temperature (*DF* = 3, *F* = 0.14, *P* < 0.0001).

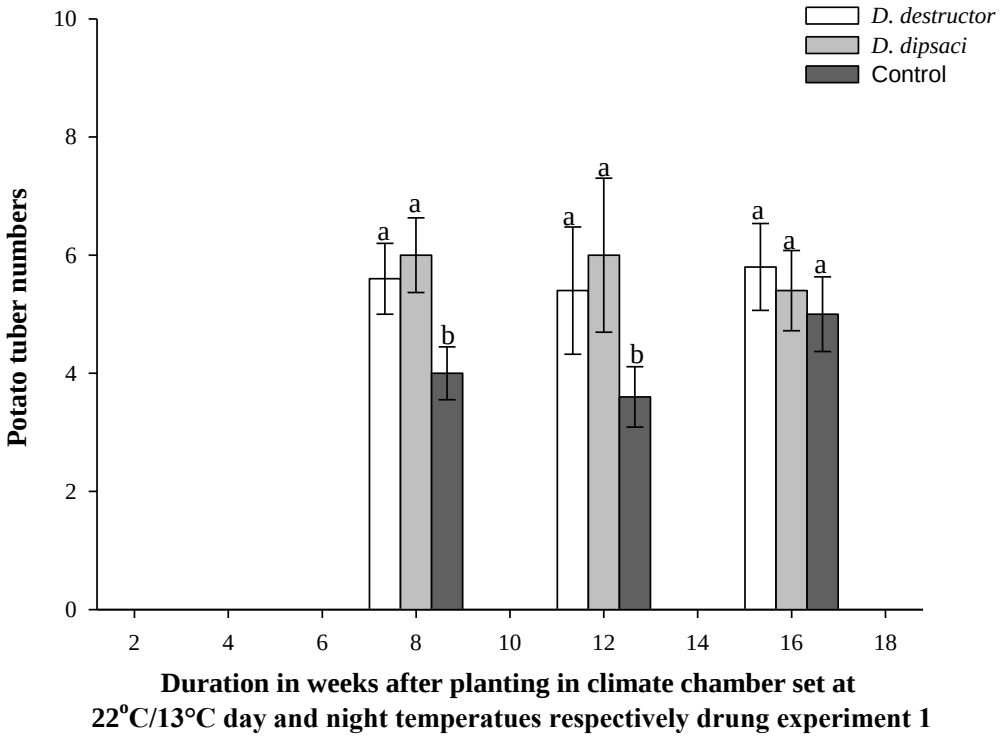

Fig 7 (a): Influence of nematodes *D. destructor* and *D. dipsaci*), duration of the experiment and temperature (22°C day and 13°C night) on potato tuber numbers during experiment 1. Means were separated using Tukey studentized Range test at 5% confidence level and are represented in the graph as standard error bars.

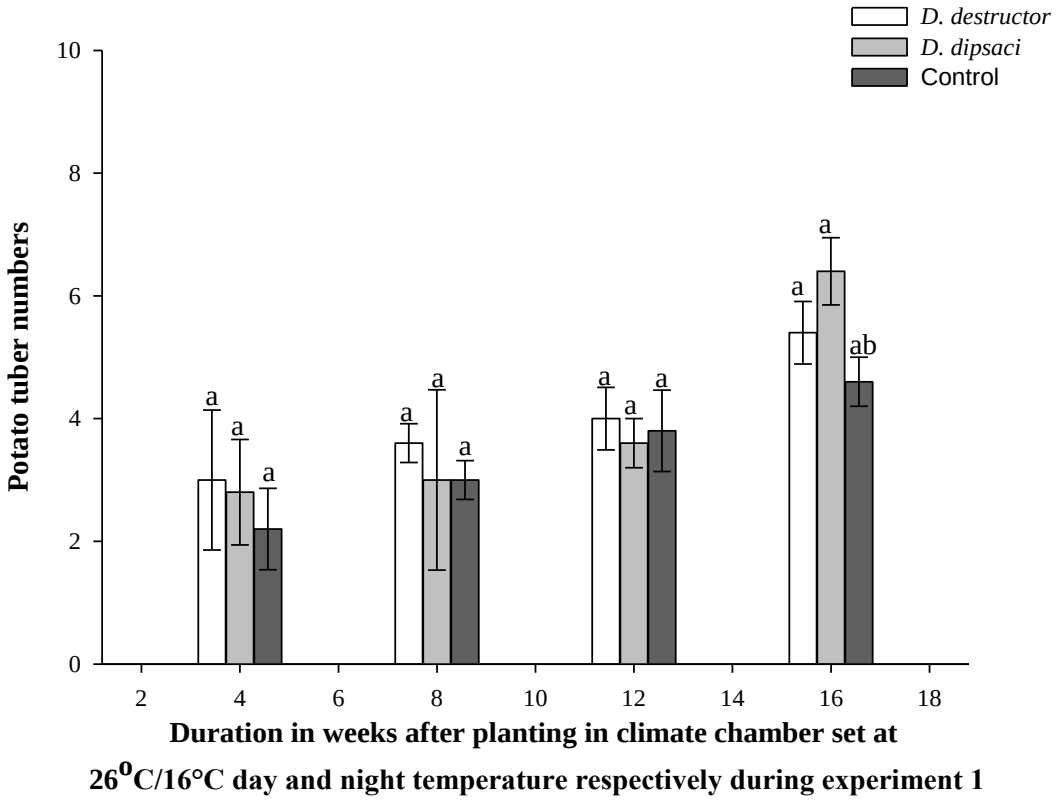

Fig 7 (b): Influence of nematodes *D. destructor* and *D. dipsaci*), duration of the experiment and temperature (26°C day and 17°C night) on potato tuber numbers during experiment 1. Means were separated using Tukey studentized Range test at 5% confidence level and are represented in the graph as standard error bars.

Experiment 2: Temperature had significant (*DF* = 1, *F* = 6.11, *P* < 0.0158) influence on tuber numbers. *Ditylenchus destructor* and *D. dipsaci* did not influence tuber numbers (*DF* = 2, *F* = 0.95, *P* = 0.3917) and neither were tuber numbers influenced by the interaction between temperature and nematodes (*DF* = 2, *F* = 2.17, *P* = 0.1195). Duration had a significant influence on tuber numbers (*DF* = 2, *F* = 2.17, *P* = 0.1195) and the interaction between all the three factors i.e. temperature, duration and nematodes had a significant influence on tuber numbers (*DF* = 17, *F* = 7.96, *P* = 0.0009).

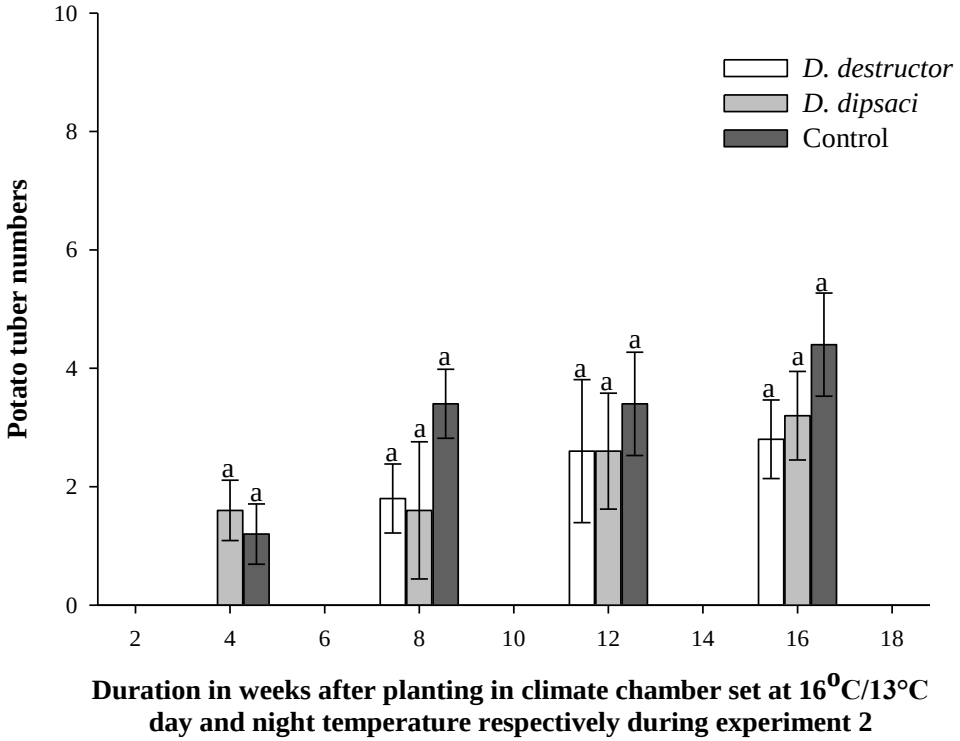

Fig. 8 (a): Influence of nematodes (*D. destructor* and *D. dipsaci*), duration of the experiment and temperature (16°C day and 13°c night) on potato tuber numbers during experiment 2. Means were separated using Tukey studentized Range test at 5% confidence level and are represented in the graph as standard error bars.

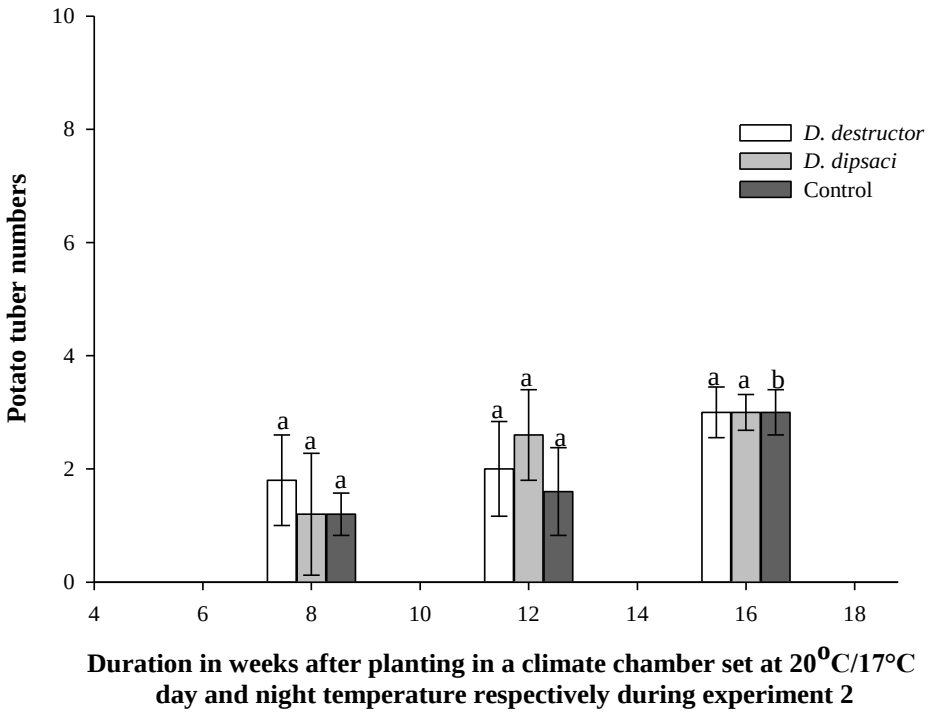

Fig. 8 (b): Influence of nematodes (*D. destructor* and *D. dipsaci*), duration of the experiment and temperature (20°C day and 13°C night) on potato tuber numbers during experiment 2. Means were separated using Tukey studentized Range test at 5% confidence level and are represented in the graph as standard error bars.

Ditylenchus destructor and *D. dipsaci* did not influence the potato tuber weight ($P = 0.2121$). However, the interaction between nematodes, the duration of the experiment, and temperature resulted in significant differences in tuber weight between the treatments ($P < 0.001$) during the experiments 1 and 2.

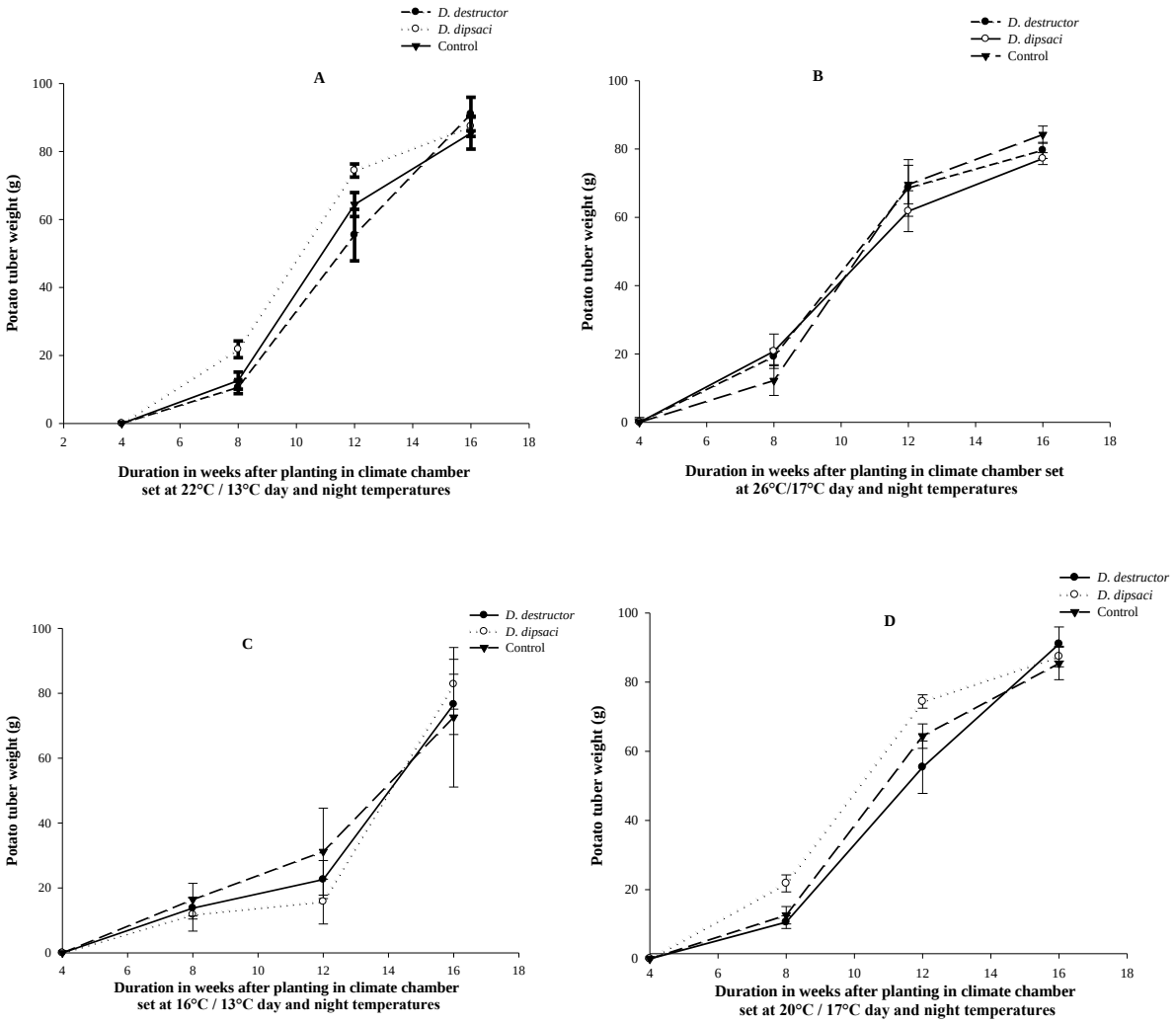

Fig. 9 a, b c, and d: Influence of *Ditylenchus destructor*, *D. dipsaci* and temperature on potato tuber weight at different duration in weeks. Bars represent standard errors.

6.0 Discussion

Soil temperature fluctuates seasonally with ambient temperature conditions affecting the nematode numbers, distribution and survival of *D. destructor* and *D. dipsaci* in soil (Seinhorst, 1956; Walker, 1962; Švilponis *et al.*, 2011b). Previous studies investigating thermal requirements for *D. destructor* and *D. dipsaci* were conducted mainly in laboratories under conditions of artificial medium or on plant callus (Ladygina, 1957; Sturhan & Brzeski, 1991). Studies investigating thermal requirements of nematodes including the host plants are difficult due to complex interaction between host plants and nematodes, and lack of advanced climate control units (Freckman & Caswell, 1985). In our experiment, the use of climate chambers was opted to closely mimic the natural day and night ambient temperature fluctuations experienced under natural situations. Unlike experiments in the greenhouses, it was possible to set day and night temperature fluctuation as well as manage photoperiod and relative humidity in the climate chambers. The intervals of harvest were designed to monitor the development of host plants, nematode species and the extent of external tuber damage caused by the nematodes.

Development of potato tuber damage caused by *D. destructor* and *D. dipsaci* was influenced by the temperature and the duration of the experiment. In both experiments, mean external tuber damage caused by *D. destructor* and *D. dipsaci* increased with the duration of the experiment. Mean external tuber damage ranging from 12-80% was recorded at all temperatures ranging from 16°C to 26°C day temperatures and 13°C to 17°C night temperatures in both experiments. This indicated that *D. destructor* and *D. dipsaci* were infective at these temperatures and caused significant potato tuber damage. The optimal temperature range for damage on crops including potatoes caused by *D. destructor* and *D. dipsaci* is reported to range between 10°C and 20°C (Sturhan & Brzeski, 1991).

Our findings however showed that at 26°C, *D. destructor* and *D. dipsaci* was able to cause significant tuber damage, suggesting that, *D. destructor* and *D. dipsaci* could have a wider optimal temperature range for damage on potatoes than previously reported (Ladygina, 1957; Sturhan & Brzeski, 1991). Thermal optimum for damage on host crops caused by *D. destructor* and *D. dipsaci* is dependent on the populations tested and the temperature they have been acclimatized to, and as such, the thermal temperature requirement may vary from populations to populations of the same species (Croll, 1967; Sturhan & Brzeski, 1991). In fact, *D. destructor*

and *D. dipsaci* has been demonstrated to be present in some warmer countries such as Iran and Saudi Arabia (Al Hazmi *et al.*, 1993; Moafi *et al.*, 2005), causing significant tuber damage, suggesting that *D. destructor* can adapt to temperatures and regions where potato plants are able to grow. On the other hand, *D. dipsaci* is more cosmopolitan and is found infesting plants in many parts of the world.

Potato tuber external damage caused by *D. destructor* and *D. dipsaci* was comparable only at week 16, when the climate chamber was at 22 and 13°C day and night temperatures. During these temperature settings the reproduction factors for *D. destructor* and *D. dipsaci* was 4.5 and 3.5 respectively at week 16 of the experiment. *Ditylenchus destructor* and *D. dipsaci* nematode numbers are closely related to the levels of damage caused on potato tubers (Baker, 1947; Southey & Staniland, 1950; Hijink, 1963) and is shown to be influenced by temperature under *in vitro* conditions (Doncaster, 1966; Evans, 1970). It was therefore evident that, both *D. destructor* and *D. dipsaci* could be a common problem at similar temperature exposures. At this optimal temperature for both species, nematodes numbers were closely related to damage levels observed.

Our results demonstrated that the longer the duration of the experiments from 4 to 16 weeks, led to increased *D. destructor* and *D. dipsaci* numbers. At week 16, the nematode population density was at the highest in most temperature settings. Nematodes activities are known to increase with increase in temperature (Wallace, 1973). However, at the highest temperature setting during our experiment of 26°C and 16°C day and night temperatures, *D. destructor* and *D. dipsaci* population density was lower compared to other temperature settings. The predominant developmental stage observed in both *D. destructor* and *D. dipsaci* were the juveniles, which are regarded as the most infective stage in both species (Hooper, 1972; Hooper, 1973). As such, their higher numbers observed during the last week of the experiment could be related to increased tuber damage observed at the same duration. Optimal temperature for *D. destructor* development in our experiment was observed to be temperatures of 20°C and 17°C day and night respectively, which concurs with the observations by Ladygina (1957), who observed that the optimum temperature for development of *D. destructor* on potatoes was between 20°C to 27°C. During this study, it was observed that the optimal temperature for development of *D. dipsaci* on potatoes was in the same range as that of *D. dipsaci*, agreeing with previous observations made by Sturharn and Brezeski, 1991 that the maximum activity and the highest invasive ability for *D.*

dipsaci is generally between 10 and 20°C. Studies to determine the influence of temperature on the nematodes reproduction in potatoes are complicated, since temperature has influence on potatoes tuber development, root, shoot and stolon development (Struik *et al.*, 1989c; Struik *et al.*, 1989a; Struik *et al.*, 1989b).

Ditylenchus destructor and *D. dipsaci* rarely influences the above ground vegetative plant part (haulm) of potatoes, and damage is only evident upon harvest on tubers (Thorne, 1945; Southey & Staniland, 1950). Potato tuber population densities were not influenced by the *D. destructor* or *D. dipsaci* alone, but with an interaction of temperature. It was not possible to clearly separate when such an influence occurred since potato is sensitive to temperature changes. Similarly, the nematodes did not influence tuber weight, but interaction with temperature led to some fluctuation in weight between treatments.

7.0 Conclusion

Our study revealed that *D. destructor* and *D. dipsaci* populations used in the current study have similar optimal temperature requirement of 20 and 17°C day for multiplication and for causing potato tuber damage on potato tubers. Duration of exposure of potato tubers to nematodes was important in determining the ideal thermal optimum for nematodes activity. This study demonstrates that temperature has a significant effect on the rates of development of both *D. destructor* and *D. dipsaci*, which governs the population dynamics of the nematodes in potatoes. Further studies are needed to evaluate the impact of different acclimatization temperature of different populations of *D. destructor* and *D. dipsaci* and their impact on thermal optimum requirements for potato damage. Studies on the numbers of generations completed during one vegetative cropping period and under different temperature settings and moisture could also be valuable in improving *D. destructor* and *D. dipsaci* management strategies.

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Chapter 6:

The entomopathogenic fungus *Beauveria bassiana* benefits *Ditylenchus destructor* and *D. dipsaci* on potatoes

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Abstract

Beauveria bassiana is a cosmopolitan fungus, occurring in soils and occasionally also as an endophyte in plants. Commercial biological insecticides based on specific isolates of *B. bassiana* have been developed for the control of pests including the potato Colorado beetle. The potato tuber rot nematode (*Ditylenchus destructor*) and the stem nematode (*Ditylenchus dipsaci*), cause damage to potato tubers resulting in economic losses. These two nematode species are polyphagous feeders on many fungal species. Although it was previously reported that an application of a commercial spore suspension of *B. bassiana* resulted into higher nematodes numbers, relationship between increase in numbers and crop damage was not investigated. In this study we therefore hypothesized that *B. bassiana* would be beneficial to *D. destructor* and *D. dipsaci* population dynamics resulting in increased damage levels on potato tubers. To test these hypothesis two greenhouse experiments were conducted to investigate the influence of *B. bassiana* on *D. destructor* and *D. dipsaci* reproduction and consequently damage caused on potato tubers. In both experiments, *B. bassiana* was inoculated singly, or in combination with *D. destructor* or *D. dipsaci* in the growing medium. *B. bassiana* alone did not negatively influence potato growth or tubers. However, where *B. bassiana* was inoculated together with nematodes, higher nematode reproduction and tuber damage was observed at the end of the experiments. Tuber weight was significantly reduced when nematodes were present together with *B. bassiana*. Tuber numbers and above ground fresh weight were not influenced by nematodes nor combination of *B. bassiana* and nematodes. Although *B. bassiana* is an effective bio-control agent against some nematodes, its occurrence together with *D. destructor* and *D. dipsaci* results in a detrimental interaction leading to higher nematode population densities and higher potato tuber damage

Keywords: *Beauveria bassina* naturalis, tuber rot nematode, stem nematode, entomopathogenic fungi, *Solanum tuberosum* L,

1.0 Introduction

The genus *Ditylenchus* (Nematoda: Anguinidae) comprises more than 90 described nematode species (Brzeski, 1991). Two species from this genus, namely, the potato tuber rot nematode *Ditylenchus destructor* (Thorne, 1945) and the stem nematode *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 are of importance in potato production systems (Thorne, 1945; Brzeski, 1991). The main host plant for *D. destructor* is potato (*Solanum tuberosum* L.), however it is also found feeding on more than 70 different plant species and a similar number of fungal species (Hooper, 1972; 1973). *D. dipsaci* comprises about ten described biological races, feeding on more than 500 different plant species and several fungal species as well (Viglierchio, 1971). Since both *D. destructor* and *D. dipsaci* are polyphagous, they are difficult to manage by crop rotation. Chemical control by nematicides is not feasible in many countries because registered compounds have been phased out due to health and environmental concerns. In most countries in the world, *D. destructor* and *D. dipsaci* are listed as quarantine nematodes regulated through phytosanitary measures (Lehman, 2004). Apart from quarantine regulations, nematodes are managed using other different approaches, including the use of fungal antagonists such as *B. bassiana* (Balsamo) Vuillemin (Ekanayake & Jayasundara, 1994; Liu *et al.*, 2008).

Beauveria bassiana is a cosmopolitan fungus, occurring in soils throughout the world, and is also a fungal antagonist to a wide range of insects (Zimmermann, 2007). *Beauveria bassiana* is also able to endophytically colonize plants including potato, following application of conidia as foliar spray or when drenched into the soil (Bing & Lewis, 1991; Jones, 1994; Tefera & Vidal, 2009). This endophytic relationship between plants and *B. bassiana* has been reported to have adverse effect on crop pests, either directly or indirectly (Vidal, 2015). Biological insecticides based on *B. bassiana* isolates have been commercialised for the control of several pest species, including potato pests (Butt *et al.*, 2001).

Beauveria bassiana persists in the soil for more than two years following application of conidia into the soil or when applied as foliar sprays to crop canopies (Inglis *et al.*, 1997). The persistence of conidia in the soil is utilized for the management of overwintering adults of Colorado potato beetles in the soil (Watt & LeBrun, 1984). There are only few reports on the management potential of *B. bassiana* targeting plant parasitic nematodes. Ekanayake and Jaysundura, 1994 reported that root gall index on tomato plant was significantly reduced where

B. bassiana was drenched into the soil together with *Meloidogyne* spp. Liu *et al*., (2008) reported that soil drenching with culture filtrate of *B. bassiana* significantly reduced *Meloidogyne hapla* population densities in soil and in the roots and subsequent gall formation and egg-mass production under glasshouse conditions. When 'Beverol', a commercial *B. bassiana* product, was applied at a concentration of 18 x 10^{-10} live spores per 1 g of *B. bassiana*, was applied in the field at the rate of 7 kg per hectare , population densities of plant parasitic nematodes in the soil were reported to increase four months following application (Hanel, 1994). Since the field was a fallow site, the author did not investigate the influence of nematodes increase on crop damage. In another study, a fermentation product (Juaxianke) arising from *B. bassiana* is reported to have lethal effect to *D. destructor* (Liu *et al.*, 2007). We are not aware of any studies reporting on the effects of *B. bassiana* conidial suspension application into the soil on *D. destructor* or *D. dipsaci* population numbers and subsequent influences on damage levels on potatoes.

In the field, interactions between different fungal species and *D. destructor* or *D. dipsaci* have been observed resulting in higher nematode populations and potato stem or tuber damage (Baker, 1947; Baker *et al.*, 1954; Hijink, 1963; Rojankovski & Ciurea, 1986). Since so far there are no reports published demonstrating an antagonistic effect of *B. bassiana* on *D. destructor* and *D. dipsaci* following soil drenching with conidial suspensions of *B. bassiana*, we hypothesized that, according to Hanel (1994), *B. bassiana* would be beneficial to *D. destructor* and *D. dipsaci* population dynamics and damage levels caused on potato tubers.

We tested this hypothesis by (i) assessing the direct impact of a *B. bassiana* application on developmental stages and final populations of the nematode species isolated from both growing medium and potato tuber peels, (ii) by assessing the impact of a *B. bassiana* application on *D. destructor* and *D. dipsaci* external and internal potato tuber damage and (iii) assessing the impact of an *B. bassiana* application on potato yields in the presence and absence of the nematodes.

2.0. Materials and methods

Planting material

Tubers of the potato variety "Innovator" were selected and pre-germinated in the dark at 20±3^{o}C until sprouts were observed. After sprouting, tubers were kept at daylight for one week to harden

the sprouts. Redundant sprouts were removed to retain only one sprout per tuber. Tubers used for planting weighed approximately 15±1g each.

Growing substrate

Field soil was sieved to remove soil particles larger than 1 cm^2 and then dry sterilized for 12 hours using a Sterilo electric soil pasteurizer (Harter Electrotechnik GmbH, Schenkenzell, Germany) set at 100±5°C. After soil had cooled down, it was mixed at a ratio of 3:1 with Klasmann Lithuanian peat moss medium, pH 3.5 (Klassmann-Deilmann GmbH, Geeste, Germany). Slow release fertiliser-Osmocote Exact® Standard® 15% N, 9% P_2O_5, 12% K_2O and 2% M_gO) (Hermann Meyer KG, Rellingen, Germany) was added to the growing medium mix at the rate of 1.5 g/kg. The final growing medium had a pH of 4.7 and 2.6% organic matter. The texture consisted of 7.5% clay, 19.1% silt and 73.4% sand. The mineral content of the growing medium was: Potassium (K): 36 mg/100 g, Phosphorus (P): 16 mg/100 g, and Magnesium (Mg): 10 mg/100 g.

Greenhouse conditions

All experiments were conducted in a temperature controlled greenhouse maintained at 20±3°C and a 12 hour photoperiod. Humidity was maintained at approximately 65%. Experiments were conducted in 1litre pots (Meyer GmBH, Germany) filled with 700 ml planting substrate. Pots were placed on saucer plates to avoid contamination by water running off adjacent pots.

Ditylenchus destructor* and *D. dipsaci populations

The *Ditylenchus destructor* population used in this study was originally isolated from celery while *D. dipsaci* originated from sugar beet plants sampled in Germany (Julius Kühn-Institut collection). Axenic cultures of these populations were maintained and multiplied on carrot discs in Petri dishes (10 mm Ø). The carrot disc culture method was a modification from a protocol developed by Speijer & De Waele, (1997) as described in details below.

Culturing of *Ditylenchus dipsaci* and *D. destructor* on carrot disks

Fresh carrots bearing foliage were sourced from local supermarket. Foliage was removed in the laboratory and carrots washed with water to remove any adhering soil particles. Under a clean bench, the carrots were held with long forceps, sprayed with 96% ethanol and flamed until all the

ethanol had burnt out. Using a flame sterilized carrot peeler, the carrots were peeled once before another flame sterilization was done. Following the second peeling, the carrots were sliced into approximately 1 cm thick carrot disks using a flame sterilized scalpel blade. Thereafter, the disks were placed in sterilized glass Petri dishes (10 mm Ø) and left to cool under the laminar flow for at least one hour before they were used for nematodes culture.

Nematodes were collected from previously cultured carrot discs and used in subsequent cultures. Suspensions of nematodes in water were left to settle at the bottom of a 25 ml Duran® bottle. Excess water was siphoned off after nematodes had settled down. Nematodes were sterilized using a streptomycin sulphate (AppliChem®, Darmstadt, Germany) solution at 0.06 mg/10 ml of sterile water for six hours. Thereafter, excess streptomycin was siphoned off and nematodes rinsed three times using sterile water. Approximately 100 µl of water containing about 20 mixed development stages of nematodes were transferred to freshly prepared sterile carrot discs using a sterile pipette. The Petri dishes were sealed with Parafilm® and placed in a Heraeus incubator (Labexchange GmbH, Burladingen-Hausen, Germany) set at 20±1°C for approximately eight weeks with regular checks for contamination and nematode multiplication.

Preparation of nematodes suspensions for soil inoculation

Petri dishes whose nematodes had egressed outside the carrot discs were rinsed using distilled water into a clean 500 ml glass bottle. Nematodes were further isolated from the carrot discs after chopping them into small pieces using a sterile scalpel blade and transferring them into a Baermann funnel overnight. To estimate the population density, the nematode suspension was stirred and a 1 ml sub-sample was pipetted and placed into a nematode counting chamber. The nematodes were counted under an Axiovert25 inverted microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) at 40X magnification. The procedure was repeated three times and the mean number of nematodes estimated for the entire volume. The final population density was adjusted to 500 nematodes/ml of water.

Preparation of *Beauveria bassiana* spore suspension

Beauveria bassiana (Strain Naturalise ATCC740040-based bio-insecticide USA) was originally isolated from cotton boll weevil (*Anthonomus grandis*). The strain was multiplied on Petri dishes containing potato dextrose agar (PDA) (39 g/l). The Petri dishes were incubated (Heraeus BK

5060 EL) in dark at 25^{o}C for 20 days for conidia production. Conidia were harvested by scrapping the surface of the culture with a sterile wire loop into a 1litre glass beaker containing sterile water plus tween 80 (0.1% v/v; Applichem®). The conidia suspension was then mixed using a magnetic stirrer for 5 min. Using a Neubauer® improved hymocytometer, the conidia concentration was determined under a microscope (Axioovert25®) at 40X magnification. The conidia suspension was adjusted to 5x10^{7} conidia ml^{-1} in the final volume of spore suspension.

Planting potato and drenching planting substrate with *B. bassiana*

Experiment 1: The experiment consisted of six treatments replicated six times. One litre plastic pots were half filled with the planting substrate and one pre-germinated tuber placed in the middle of the pot. 10 ml of a *B. bassiana* spore suspension at a concentration of 5x10^{7}was drenched on the planting substrate around the pre-germinated tuber. Control plants were drenched with 10 ml distilled water per replicate. The pots were then filled with the planting substrate and completely randomised on greenhouse benches.

Two weeks after planting, the planting substrate was inoculated separately with *D. destructor* and *D. dipsaci*. Holes of approximately 4 cm in depth were drilled into the growing medium around the plant. In each of the four holes, 1 ml tap water containing 500 nematodes of mixed life stages (males, females and juveniles) was added, resulting in a total of 2000 nematodes per pot. The holes were covered with growing medium immediately after inoculation. Control pots and six *B. bassiana* pots were not inoculated with nematodes. Potato tuber damage was evaluated 12 weeks after inoculation with the nematodes. The total duration of the experiment was 14 weeks. Experiment 2 followed the same procedure as experiment 1. However, in this experiment the number of replicates per treatment was 10 and the total duration of the experiment was 16 weeks.

Data collection

Plant top fresh and dry weight

At termination of the experiments, potato above ground plant parts were chopped using a pair of scissors. Above ground fresh weight were recorded and packed into paper bags and oven dried (Memmert®, GmbH & Co. KG, Schwabach, Germany) for one week at 80^{o}C.

External and internal tuber damage assessment

Potato tubers were harvested by passing the planting substrate through a sieve whose mesh size measured 1 cm by 1 cm into a collection container. The growing medium was thoroughly mixed and a sample of 300 ml transferred into polythene bags. Growing medium adhering on the tubers was gently washed off with tap water. Tuber numbers, tuber weight, external and internal potato tuber damage were recorded immediately after harvest. External potato tuber damage was visually assessed on a whole tuber and expressed in percentage damage per tuber. Internal damage was evaluated after slicing each tuber into two equal halves. One half of the tuber was used for internal damage calculation. Damaged skin and cortices were necrotic and darker than healthy tissues. The extent of damage from the skin into the cortex of the tuber was calculated by dividing the tuber into four sections of 25% each. Internal damage per tuber was calculated as the sum of all the four sections. Total internal potato tuber damage per replicate was calculated using the same formula as for external damage.

The total percentage external tuber damage per replicate was expressed using the formula:

$$P = \frac{\sum(n_1 + n_2 + n_3 + n...)}{N},$$

Where, P = is the percentage (%) potato tuber damage per replicate and

N = Total number of tubers per replicate.

n = percentage of potato tuber with lesions caused by *D. destructor* or *D. dipsaci*.

Nematodes extraction from potato tubers

Potatoes from each replicate were peeled using a knife. Peels were approximately 2 mm thick and made up approximately 22% of the tuber weight. The complete tuber peel per replicate was mixed and a 10 g sub-sample was then chopped into fine pieces of approximately 5 mm x 5 mm and used for nematode extraction. Nematodes were extracted using the modified Baermann funnel method for 12 hours (Hooper, 1990). Nematode numbers at different developmental stages (males, females, juveniles (J2-J4), and eggs) were determined under an Axiovert25 inverted microscope at 40X magnification using a 1 ml capacity nematode counting slide chamber. Nematodes isolated from 10 g of tuber peels were used to calculate the total number of nematodes in tuber peels per replicate.

Nematodes extraction from growing medium

Following the collection of the growing medium, it was stored at 5°C up to a maximum of 5 days before nematode extraction. Nematodes were extracted from 250 ml sub-samples of the growing medium for 24 hour using a modified Oostenbrink dish with 24 cm inner diameter and milk filter paper (27cm Ø) (EPPO, 2013). Nematode numbers (all developmental stages) extracted from the growing medium were determined. Nematodes extracted were extrapolated to the total growing medium volume per replicate (700 ml).

Determination of final population densities and the reproduction factor of *D. destructor* and *D. dipsaci*

The final nematode population was the sum of extrapolated nematode numbers from total tuber peels and the growing medium (700 ml) per replicate. The reproduction factor (R*f*) of *D. destructor* and *D. dipsaci* in both experiments was determined according to the formula R*f* = P*f*/P*i* where P*f* was the estimated final nematode population per gram of potatoes tuber peels plus the total number of nematodes per gram of growing medium (700 ml), and P*i* was the initial population density (Oostenbrink, 1966).

3.0. Data analyses

Data on the influence of *B. bassiana* and nematodes on potato tuber numbers and weights were analysed using one way ANOVA. Means were compared to the control treatments using Dunnett's test. On the other hand, a T-test was used to compare external and internal potato tuber damage and nematodes final population densities in treatments with *B. bassiana* and nematodes in comparison with treatments where only nematodes were inoculated. Prior to data analysis, data was tested for homogeneity of variance and assumption of normality of the residuals using Levene's and Shapiro-Wilk's test, respectively, in SAS software Version 9.3 (SAS Institute Inc., Cary, NC, USA). Where necessary, percentage damage data were arcsine square root transformed, while nematode counts were $\log_{10}(x + 1)$-transformed. The General Linear Model (GLM) procedure was used in SAS to analyse the influence of *B. bassiana*. The non-transformed means are presented in the figures and tables.

4.0 Results

External and internal potato tuber damage caused by *D. destructor* and *D. dipsaci*

During experiment 1, inoculating the growing medium with *B. bassiana* and *D. destructor* led to significantly higher external (t (14) = 9.51, $P < 0.001$) and internal (t (14) = 2.44, $P < 0.03$) tuber damage compared to tuber damage caused by *D. destructor* only (Fig. 1). Similarly, inoculating the growing medium with *B. bassiana* and *D. dipsaci* led to significantly higher external (t (14) = 2.62, $P < 0.02$) and internal (t (14) = 2.45, $P < 0.02$) damage compared to damage levels caused on tubers when growing medium was inoculated with only *D. dipsaci* (Fig. 1).

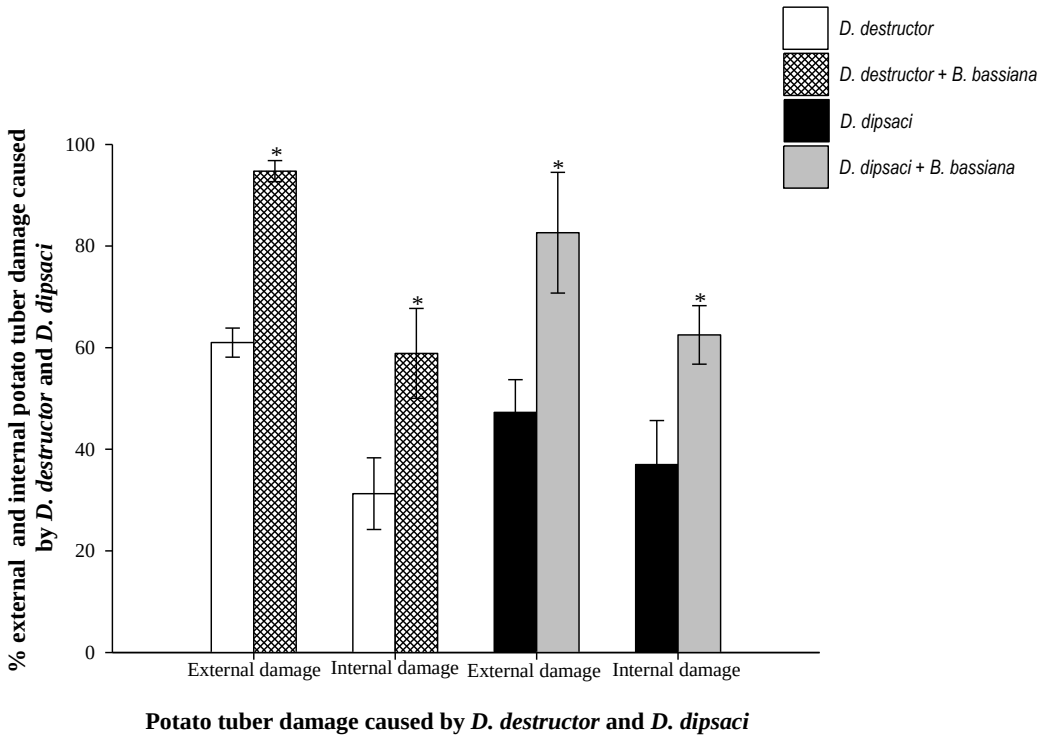

Fig 1: Influence of *B. bassiana* on potato tubers external and internal damage caused by *Ditylenchus destructor* and *D. dipsaci* during experiment 1. Asterisks above standard error bars indicate significant differences ($P < 0.05$) between treatments according to T-tests.

During experiment 2, significantly higher external (t (14) = 9.51, $P < 0.001$) and internal (t (14) = 2.44, $P < 0.03$) potato tuber damage was observed from tubers where both *B. bassiana* and *D. destructor* were inoculated together into the growing medium compared to tubers where only *D. destructor* was inoculated (Fig. 2). External potato tuber damage caused on tubers when *D.*

dipsaci was inoculated into the growing medium was not significantly different from damage on tubers where both *B. bassiana* and *D. dipsaci* were inoculated into the growing medium (Fig. 2).

Inoculation of the growing medium with both *B. bassiana* and *D. dipsaci* led to higher internal potato tuber damage (t (14) = 2.45, $P < 0.02$), compared to damage levels caused on tubers when only *D. dipsaci* was inoculated into the growing medium (Fig. 2).

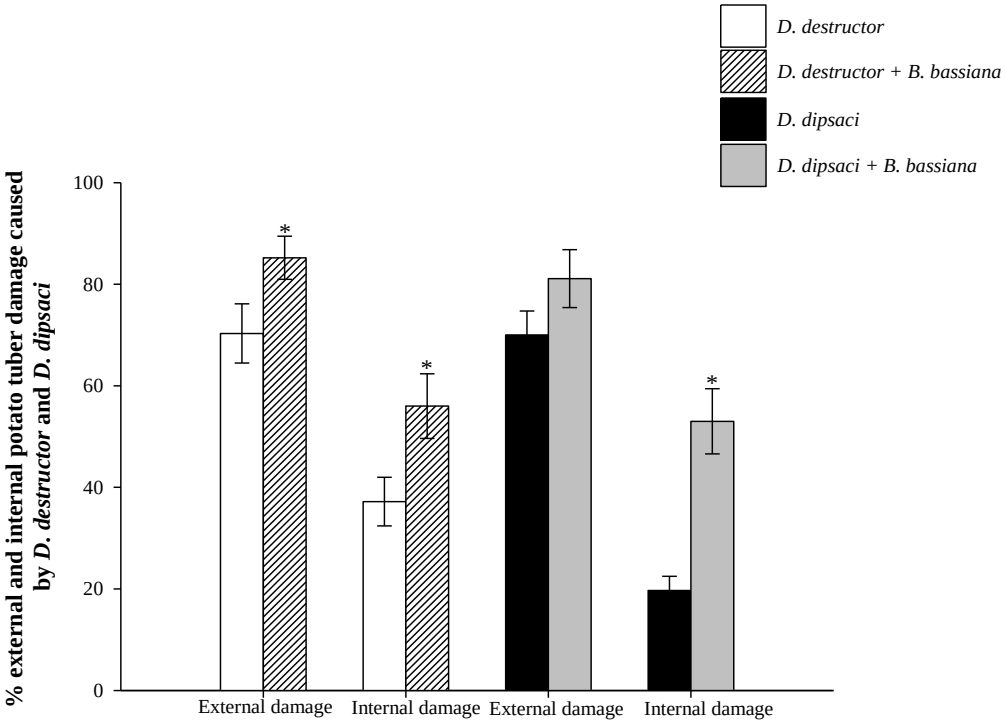

Fig 2: Influence of *B. bassiana* on potato tubers external and internal damage caused by *Ditylenchus destructor* and *D. dipsaci* during experiment 2. Asterisks above standard error bars indicate significant differences ($P < 0.05$) between treatments according to T-tests.

Influence of *B. bassiana* on *D. destructor* and *D. dipsaci* final population densities

During experiment 1, the final population density of *D. destructor* isolated from tuber peels and growing medium inoculated with *B. bassiana* were significantly higher (t (14) = 3.67, $P = 0.003$, compared to final nematodes density isolated from treatments which were inoculated with *D. destructor* only (Table 1). *D. dipsaci* final population densities were higher (t (14) = 3.14, $P = 0.007$) in treatments where *D. dipsaci* and *B. bassiana* were inoculated together into the growing medium compared to treatment where only *D. dipsaci* was inoculated alone (Table 1)

During experiment 2, final population densities of *D. destructor* varied significantly (t (18) = 2. 76, P = 0.0134) between treatments where *B. bassiana* and *D. destructor* were inoculated together in the growing medium compared to *D. destructor* inoculation alone (Table 1). Similarly, *D. dipsaci* final population densities extracted from potato tuber peels and growing medium in treatments where *B. bassiana* was simultaneously inoculated, were significantly different (t (18) = 0.98, P = 0.003) to final densities isolated from treatments with *D. dipsaci* inoculation alone (Table 1).

Table 1: Influence of *B. bassiana* on final population densities of *D. destructor* and *D. dipsaci* isolated per gram of potato tuber peels and growing medium during experiment 1 and 2.

	Experiment 1		**Experiment 2**	
Treatment	**Nematodes final population densities (P*f*)**	**Reproduction factor (RF)**	**Nematodes final population densities (P*f*)**	**Reproduction factor (RF)**
D .destructor + B. bassiana	7596 ± 727*	3.8	8976 ± 839*	4.6
D. destructor	4833 ± 289[c]	2.4	6110 ± 635	3.2
D. dipsaci + B. bassiana	6516 ± 721*	3.3	7734 ± 1102*	3.9
D. dipsaci	4029 ± 327	2.0	4274 ± 228	3.1

Nematodes final population densities (P*f*) are followed by ± standard error (SE). Means were compared between two treatments (one nematode species and *B. bassiana).* Asterisks denote significant differences between treatments based on T-test.

Influence of *B. bassiana, D. destructor* and *D. dipsaci* developmental stages

Inoculating *D. destructor* into the growing medium together with *B. bassiana* led to significantly higher numbers of males (t (14) = 6.11, P < 0.0001), females (t (14) = 2.98, P = 0.0100) and juveniles (t (14) = 2.16, P = 0.0485) in comparison to treatments where only nematodes were inoculated during experiment 1 (Fig. 3). *D. destructor* egg numbers were not significantly influenced by this treatment (Fig. 3).

D. dipsaci male (t (14) = 5.22, P = 0.0001) and female (t (14) = 3.17, P = 0.007) numbers were significantly higher where *B. bassiana* was added into the growing medium compared to treatments with *D. dipsaci* alone (Fig 3). *D. dipsaci* juveniles (t (14) = 1.22, P = 0.242) and eggs (t (14) = 0.13, P = 0.900) were not significantly affected by the *B. bassiana* treatment (Fig. 3).

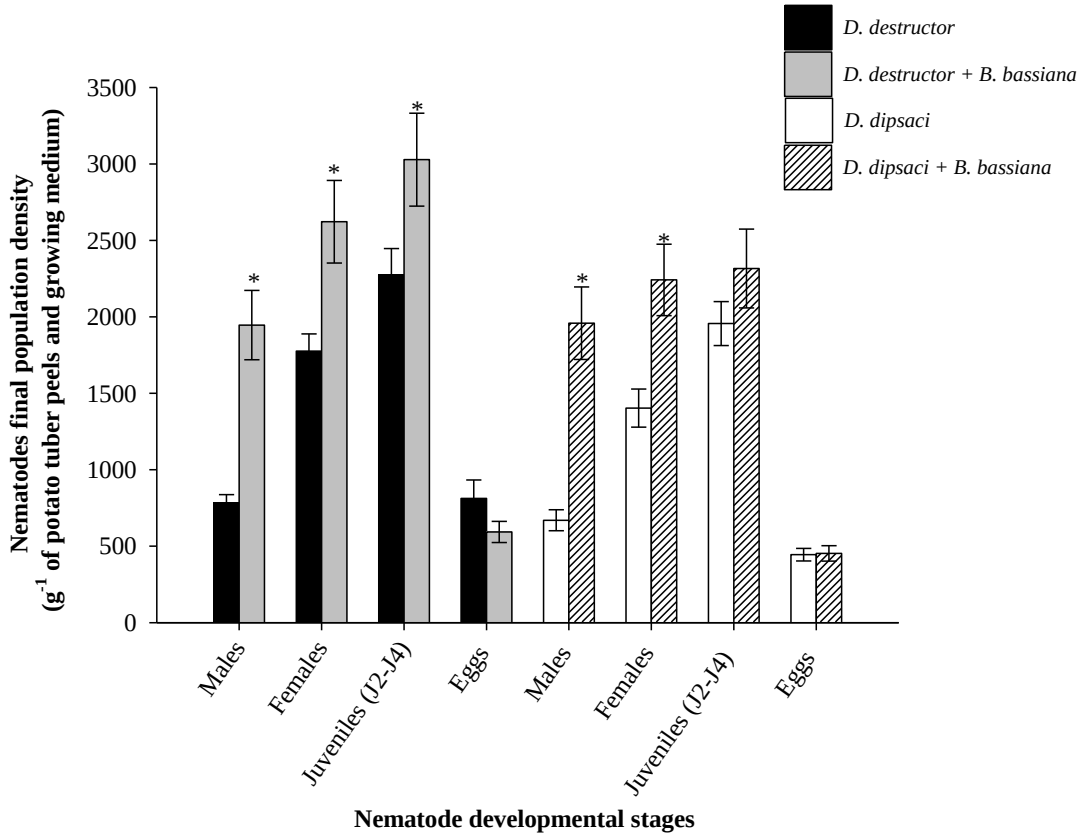

Fig 3: Influence of *Beauveria bassiana* on different *Ditylenchus destructor and D. dipsaci* developmental stages during experiment 1. Significant differences ($P< 0.05$) for nematode treatments according to T-tests are indicated by asteriks.

During experiment 2, numbers of *D. destructor* females (t (18) = 2.98, P = 0.011) and juveniles (t (18) = 2.54, P = 0.021) were significantly higher where *B. bassiana* was added into the growing medium compared to the nematodes alone treatment (Fig. 4). *D. destructor* males and egg numbers by the *B. bassiana* inoculation (Fig. 4).

During experiment 2, the different developmental stages of *D. dipsaci* varied with the treatments. *D. destructor* males (t (18) = 2.12, P = 0.0485) and females (t (18) = 3.03, P = 0.0072; Fig. 4) were significantly influenced when *B. bassiana* was drenched into the growing medium. While juvenile numbers were also significantly higher (t (18) = 2.77, P = 0.0126), egg numbers were not influenced by the *B. bassiana* treatment (t (18) = 0.78, P = 0.4458; Fig. 4).

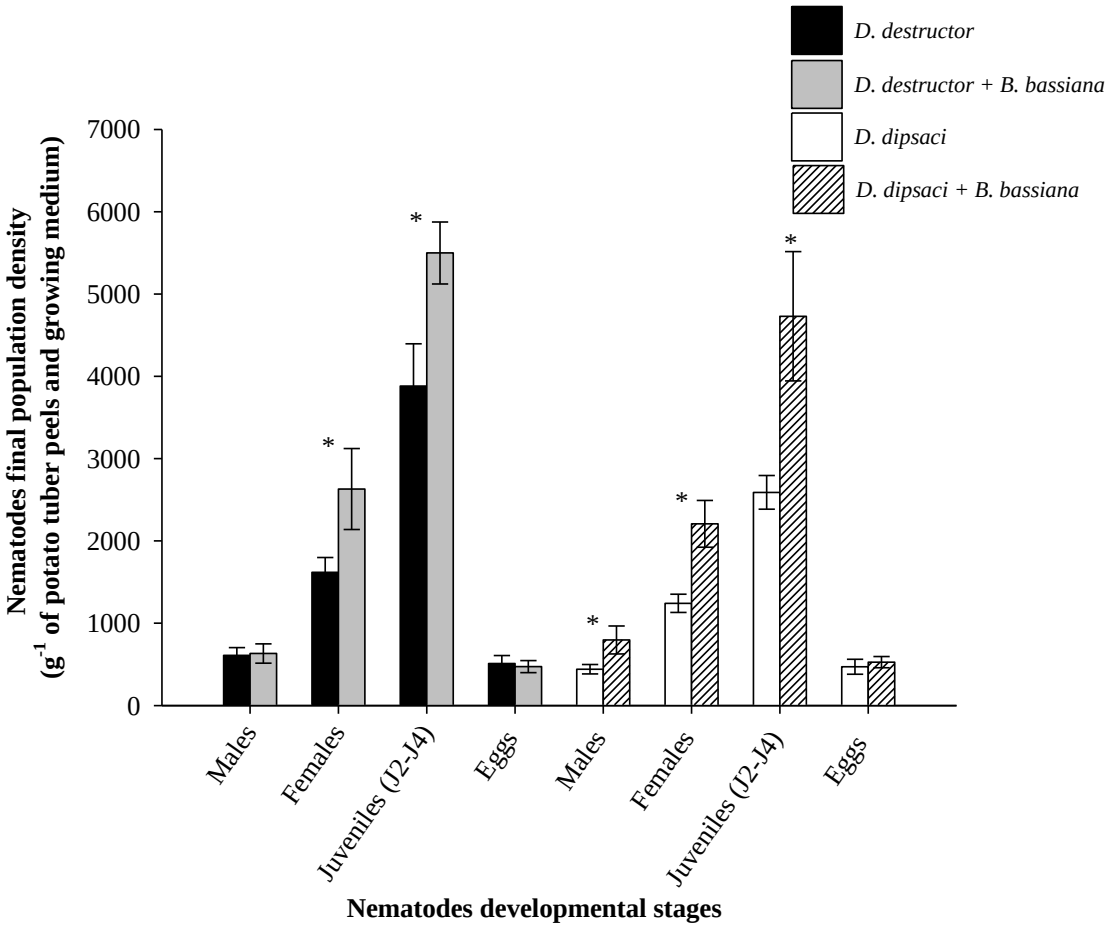

Fig 4: Influence of *Beauveria bassiana* on *Ditylenchus dipsaci* different developmental stages. Bars followed by an asterisk indicate significant difference ($P< 0.05$) between treatments according to T-test.

Influence of inoculation of *Beauveria bassiana* and nematodes on potato tuber numbers and weight

Tuber numbers were not significantly influenced by either the nematode species (*D. destructor* or *D. dipsaci*), by *B. bassiana* or combination of both *B. bassiana* and nematodes in the growing medium during experiments 1 and 2 (Table 2).

Table 2: Influence of *Ditylenchus destructor, D. dipsaci* and *B. bassiana* on potato tuber weight (g) during experiment 1 and 2

	Experiment 1		**Experiment 2**	
Treatments	**Tuber numbers**	$P < 0.05$	**Tuber No.**	$P < 0.05$
Control	5		5	
Beauveria bassiana	3	0.749	6	0.955
D. destructor	5	0.999	7	0.984
D. destructor + B. bassiana	5	0.999	6	0.875
D. dipsaci	4	0.999	7	0.998
D. dipsaci + B. bassiana	3	0.571	5	0.756

Mean tuber numbers are followed by respective p value ($P < 0.05$) according to Dunnett statistical test

In experiment 1, inoculating the growing medium with *B. bassiana*, *D. destructor*, *D. dipsaci* or dual inoculation with *B. bassiana* and *D. destructor* did not significantly influence potato tuber weight. Dual inoculation of growing medium with *B. bassiana* and *D. dipsaci* reduced tuber weight significantly (Table 3). However, during experiment 2, potato tuber weight was significantly influenced by nematodes (*D. destructor* and *D. dipsaci*) and a combination of both *B. bassiana* and *D. destructor* (Table 3).

Table 3: Influence of *Ditylenchus destructor*, *Ditylenchus dipsaci* and *B. bassiana* on potato tuber weight (g) during experiment 1 and 2

	Experiment 1		**Experiment 2**	
Treatments	Tuber weight (g)	$P < 0.05$	Tuber weight (g)	$P < 0.05$
Control	82.0		204.9	
Beauveria bassiana	63.0	0.4188	179.4	0.1706
D. destructor	65.0	0.5373	153.3	0.0034
D. destructor + B. bassiana	49.4	0.0502	118.0	<.0001
D. dipsaci	72.4	0.9024	164.4	0.0231
D. dipsaci + B. bassiana	48.4	0.0415	185.2	0.2988

Mean tuber weight (g) are followed by respective p value ($P < 0.05$) according to Dunnett statistical test

5.0. Discussion

Soil inoculation with *D. destructor* or *D. dipsaci* in combination with an application of a *B. bassiana* spore suspension increased the final population densities of both nematodes species when compared to treatments where only the nematode species was inoculated. Our findings corroborate with the results of Hanel (1994), who found that plant parasitic and bacteriophagous nematodes number increased after application of a *B. bassiana* commercial product to the soil. According to this author the increase in nematode numbers was according to a provision of larger amount of food for phytophagous nematodes, changes in the soil microflora followed by an increase in bacterivorous nematodes, and enhanced nutrient availability to plants. An influence of chitinolytic microflora (including *B. bassiana*) on nematode egg hatch was also hypothesised as another indirect factor leading to increased nematode numbers. *D. destructor* and *D. dipsaci* are polyphagous nematodes known to feed and reproduce on different fungal species (Baker *et al.*, 1954; Anderson, 1964; Janssen, 1994). *D. destructor* numbers are reported to increase inside potato tubers when fungal mycelia were heavily abundant and to decrease immediately following a complete consumption of the mycelia (Baker *et al.*, 1954). In line with these findings, *D. dipsaci* numbers were higher where fungal colonisation by *Phoma solanica* was present, in comparison to stems where only *D. dipsaci* was present (Hijink, 1963). These limited reports indicate that fungal mycelia offer alternative food sources to this nematode species, thus resulting in accelerated population growth. We therefore speculate that in our experiment, *D. destructor* and *D. dipsaci* fed on mycelia of *B. bassiana* either in the soil or in tuber tissues thus increasing their final population densities. However, the nature of this interaction is complex, and it's possible that other factors beyond the scope of the current study could have also played a role in influencing nematode numbers.

Soil drenching with spores or fermented products of *B. bassiana* have previously been demonstrated to have potential bio-control influence against plant parasitic nematodes thus leading to reduction in plant damage (Ekanayake & Jayasundara, 1994; Liu *et al.*, 2008). This study demonstrated that dual inoculation of *B. bassiana* and *D. destructor* or *D. dipsaci* into the growing medium led to significantly higher external and internal potato tuber damage. The cause of increased damage could be attributed to the increased nematodes numbers as observed in treatments where *B. bassiana* was present. Although Hanel (1994), indicated that nematodes

numbers increased in the soil after application of *B. bassiana*, the experiment did not test the influence of increased nematodes numbers on yield loss. In the current study, it can be concluded that, increase in nematodes numbers in treatments where *B. bassiana* was added, intensified tuber damage. The specific role played by *B. bassiana* in the intensification of tuber damage was not investigated. However, it can be hypothesised that *D. destructor* and *D. dipsaci* fed on the mycelia of *B. bassiana* and consequently increased in numbers, which had a direct impact on tuber damage. Although *B. bassiana* has not been previously reported to interact with *D. destructor* or *D. dipsaci* in potato, leading to increased nematodes numbers and consequently damage, other fungal species are reported to interact with *D. destructor* leading to increased nematodes numbers (Baker, 1947; Baker *et al.*, 1954; Rojankovski & Ciurea, 1986). Interaction of *D. dipsaci* and *Phoma solanica* on potato is the only reported incidence where *D. dipsaci* numbers were observed to increase rapidly, leading to intensified stem damage and consequently stem die back (Hijink, 1963). Although *D. dipsaci* is recorded to interact with several fungal species, its interaction with *B. bassiana* is reported in this manuscript for the first time to the best of our knowledge.

Potato tuber weight was reduced in treatments with dual inoculation of *B. bassiana* and a nematode species. Reports on the influence of *D. destructor* on tuber weight are rare, probably because the main parameter for yield loss is tuber damage. On the other hand, interaction of *D. dipsaci* and *Phoma solanica* is reported to indirectly cause reduction in weight of potato above ground parts and indirectly influence tuber numbers and weights due to early stem die back (Hijink, 1963). In our study, stem die back was not observed, and therefore cannot be associated with the observed reduction in tuber weight. Additionally, the above ground fresh weight and tuber numbers were not negatively influenced by any of the treatments, an indication that plant growth was not hampered. However, due to the complexity of factors involved in the reduction of tuber weight, further experiments are recommended to evaluate the individual role played by each factor studied.

6.0. Conclusion

Beauveria bassiana is documented to offer control of some plant parasitic nematodes, but our study demonstrated that not all nematodes species are similarly negatively affected by *B. bassiana*. Our results are significant in demonstrating that the interactions between *B. bassiana*

and *D. destructor* or *D. dipsaci* under greenhouse conditions results in increased nematodes numbers and tuber damage. *B. bassiana* alone is shown to have no negative influence on plant growth or tuber rotting. Potatoes are usually not cultivated under greenhouse conditions and therefore similar trials in micro-plots could offer more insight into the role of additional factors in the interactions between *B. bassiana* and the two nematodes species studied. However, the few data so far published from field studies involving *D. destructor*, *D. dipsaci* and different fungal species support our findings on a beneficial interaction between the plant, *B. bassiana* and nematodes (*D. destructor* and *D. dipsaci*), leading to higher nematode numbers and consequently tuber damage. The fate of *B. bassiana* in the soil was not investigated under the current study. Since *B. bassiana* is known to persist in the soil for over two years after a single application, studies on the fate of *D. destructor* and *D. dipsaci* in such a soil in the presence and absence of the potato could offer more insight on the survival of these nematodes on *B. bassiana*. It could be interesting to investigate varying dosages *B. bassiana* and a different timing of the applications of either *B. bassiana* or nematodes. Additionally, *in-planta* detection and monitoring of *B. bassiana* in tuber tissues could advance our knowledge of endophytic colonization on potato tuber tissues.

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Chapter 7: General discussion and outlook

Extensive knowledge exists in literature on nematodes taxonomy, biology and their interaction with plants. This knowledge has in many ways contributed towards development of species-specific nematode management strategies. Nematode-induced changes in host plants including damage on marketable plant parts such as potato tubers, are often used as measure for yield loss. In nature, host plants to nematodes are usually infested by one or several species at the same time, making it difficult to estimate the impact and mechanism of individual nematode species. Therefore, studies involving individual nematode species and specific host plants are common aimed at establishing the nature of host-nematode interactions.

In our study, maintaining a pure culture of the populations was critical for the success of subsequent experiments. The carrot disk culture method used in this PhD thesis was ideal for the multiplication of *D. destructor* and *D. dipsaci* at 20°C. Although not reported in this thesis, other methods of culture, including fungal cultures of *Botrytis cinerea* and *Beauveria bassiana* were explored. It was observed that some nematode populations could be reared on these fungal substrates. Particularly, the ability of *Ditylenchus destructor* and *Ditylenchus dipsaci* to multiply on *B. bassiana* could form interesting topic to explore.

Accurate identification of *D. destructor* and *D. dipsaci* is crucial for the purposes of studies and for the development of appropriate management methods. Morphometric and molecular identification of *D. destructor* and *D. dipsaci* populations used in the current study was an integral part to the subsequent studies. Contaminations of individual nematodes species by other similar species or populations may limit the outcome of experiments. In our case, morphometric and molecular data proved that axenic cultures had been maintained throughout the experiment. Therefore, the observed responses in potato varieties tested in experiments were attributed to either *D. destructor* or *D. dipsaci* with certainty. Only one population from each species was used for host plant response in greenhouse and climate chamber experiments. As populations of these species can vary greatly and hence their virulence on susceptible cultivars, or due to presence of different races of *D. dipsaci*, further differentiation is needed using different host plants. In our studies, differences in host responses was evaluated as tuber damage, which demonstrated differences between *D. destructor* and *D. dipsaci*. However, the main cause of this difference was attributed to *D. destructor* population fitness on certain potato varieties. This

indicates that, damage levels and reproduction potential on different varieties of potatoes could be a useful tool to differentiate *D. destructor* and *D. dipsaci* populations/races on potato.

Host resistance is a management tool that has much potential in management of *D. destructor* and *D. dipsaci*. Although screening potato varieties for resistance to *D. destructor* and *D. dipsaci* were initiated in the early 1940s and later abandoned in late 1960s, none of the earlier screened potato varieties were completely resistant or tolerant to these nematode species. In our study, evaluation of resistance and tolerance of different potato varieties to *D. destructor* and *D. dipsaci* was reported based on the current definitions of the terms resistance and tolerance in nematology. Our study demonstrated that there are no completely resistant or tolerant potato varieties to *D. destructor* and *D. dipsaci*, thus agreeing with previous findings. In this experiment, relative susceptibility (RS) and external tuber damage were presented as suitable methods for resistance and tolerance determination, respectively. As explained above, only one population from each species was used. To enable detection of broad resistance more populations of each species could be included in future screening experiments. It was also observed that, extended experimental period could influence host resistance and tolerance responses especially in pot experiments. Further screening should also consider the possible influence of the growing medium such as its pH and soil moisture content on resistance and tolerance levels of different potato varieties.

Although resistance is deemed important in the management of *D. destructor* and *D. dipsaci*, it may lack durability in case of variability in population composition or due to variations in nematodes initial population densities. Our study with different initial populations of *D. destructor* and *D. dipsaci* demonstrated that yield loss assessment was best evaluated based on tuber damage as opposed to tuber weight. Potato tuber damage increased with initial population densities. Depth of internal tuber damage caused by *D. destructor* and *D. dipsaci* were similar, contrasting previous observations that *D. dipsaci* causes deeper lesions into the potato tubers. The damage assessment method used in our study may need to be refined to better assess internal tuber damage. Population fitness of *D. destructor* on ‘Désirée’ was deemed as the main reason for differences in reproduction factor with *D. dipsaci*. However, future experiments should consider the use of more potato varieties and additional populations of each species. Further experiments considering Seinhorst research program integrating factors such as larger pots size

could compliment the result presented in our study. Moreover, tolerance limits estimates and minimum yield losses estimates could be better evaluated in micro plot experiments.

Temperature is one of the most important abiotic factors influencing nematode activities such as reproduction and consequently severity of damage on host plants. Our study with *D. destructor* and *D. dipsaci* under different temperature regimes revealed that, temperature and duration of the experiments significantly influenced potato tuber damage and nematode multiplication. Our findings agree with the limited laboratory experiments on thermal temperature requirements of *D. destructor* on potatoes, but demonstrated under *in vivo* conditions that *D. destructor* reproduction and damage potential is regulated by temperature. Influence of temperature on *D. dipsaci* reproduction and its relevance to potato tuber damage is to our knowledge reported for the first time in this study. With growing concern of global warming and its impact on nematodes, future studies are recommended. Further studies are needed to evaluate the impact of different acclimatization temperature of different populations of *D. destructor* and *D. dipsaci* and their impact on thermal optimum requirements for potato damage. Studies on the numbers of generations completed during one vegetative cropping period and under different temperature settings and moisture could also be valuable in improving *D. destructor* and *D. dipsaci* management strategies.

D. destructor and *D. dipsaci* are both fungal feeders and therefore form complex interrelationships with fungal pathogens. In our experiments, interactions between *Beauveria bassiana* and *D. destructor* or *D. dipsaci* led to increased nematode reproduction and consequently higher tuber damage. *B. bassiana* on its own did not have negative influence on potato. Although the application of *B. bassiana* had been reported to increase plant parasitic nematodes in the soil, the study did not link the increase of nematodes to crops yield losses. To the best of our knowledge, relationship between *B. bassiana*, *D. destructor* and *D. dipsaci* and yield loss on potatoes is reported for the first time in this thesis. The active role played by *B. bassiana* in the interaction could only be hypothesized and therefore further experiments exploring its fate in the soil or in the plant tissues are recommended. The use of quantitative polymerase chain reaction (qPCR) could improve knowledge on endophytic colonization of potato plants.

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RESEARCH GRANTS AWARDED

2011 **PhD scholarship from** DAAD - Deutscher Akademischer Austauschdienst (German Academic Exchange Service) for three and a half years study at Julius Kühn-Institut - Bundesforschungsinstitut für Kulturpflanzen, Braunschweing and Georg-August-University Department of Crop Sciences / Agricultural Entomology, Göttingen.

2010 **Rothamstead International African Fellows programme.** This was a six months capacity building and institutional strengthening to empower me on molecular biology. The **funding amounted to £ 13,200** to cover the cost of research carried out at Wageningen University (at Biointeractions and Plant Health Plant Research International section).

2009 **Funding amounting to -€2,000.** Funds were obtained from **Forum for Research in Agriculture (FARA)** and **Young Professionals' Platform for Agricultural Research for Development (YPARD)** for hosting and facilitating a two days workshop on ***"Writing Successful Grant Proposals in Agricultural Research"***. Was involved in initiation of the idea, programme implementation, facilitation and hosting of the workshop.

2005 **Scholarship for masters degree amounting to -€100,000. German Ministry of Foreign Affairs (BMZ)** for a collaborative project on the study of better understanding of banana endophytic.

COMPUTER SKILLS

- **Programming:** C++,
- **Statistics:** SAS, SPSS, Genstat
- **Office:** Word, Excel, PowerPoint, Adobe Photoshop, illustrator, In design, Office publisher.
- Can work on both Windows and Mac platforms

POSITION OF RESPONSIBILITIES

2011-2013: Steering Committee Member of the Young Professionals' Platform for Agricultural Research for Development (YPARD), FAO, Italy.

2001-To date: **Volunteer and Navigator-** World School Network – NGO based in *Japan* and has environmental activities in Kenyan schools**.**

MEMBERSHIP

- American Society for Microbiology (ASM)
- Young Professionals' Platform for Agricultural Research for Development (YPARD)
- Horticultural Association of Kenya (HAK)
- Word School Network (WSN) – Japanese NGO located in Tokyo, Japan (http://chie.worldschool.net)

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DECLARATIONS

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form. Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen, 11th Dec 2014

(Signature)

PETER MWAURA MUTUA

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen, 11th Dec 2014

PETER MWAURA MUTUA