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Distribution, Population Dynamics and Sustainable Management of Plant-Parasitic Nematodes Associated with Cut-Flowers in Ethiopia

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SUMMARY

Plant-parasitic nematodes are a growing concern for the floriculture industry in Ethiopia. Two surveys were conducted during the wet season (August to September 2011) and dry season (April to May 2012) to assess the incidence of economically important plant-parasitic nematodes associated with major cut-flowers. Soil samples were collected from rose, carnation, gypsophila, freesia and statice. Thirteen nematode taxa viz. *Criconemella, Ditylenchus, Helicotylenchus, Hemicycliophora, Meloidogyne, Merlinius, Longidorus, Paratrichodorus, Paratylenchus, Pratylenchus, Rotylenchus, Rotylenchulus* and *Tylenchorhynchus* were detected. Roses harboured all of the genera. *Helicotylenchus* was found in all cut-flower species and *Meloidogyne* was found more frequently during the dry than the wet season.

Meloidogyne hapla was the most important root-knot nematode detected and reported here for the first time from Ethiopia. Pure cultures of this species were morphologically and molecularly characterized. For the latter, the D2-D3 expansion of the 28S ribosomal DNA and the region located between cytochrome oxidase unit II and the 16S rRNA gene of the mitochondria (mtDNA) were chosen. Phylogenetic comparison resulted in a highly supported clade containing the Ethiopian isolates of *M. hapla* together with *M. hapla* populations from other regions of the world. Female, male and second-stage juvenile (J2) morphology was in line with other descriptions of *M. hapla* with few exceptions. The female vulva slit was smaller than for the Hawaiian population and J2 body size was larger compared to previous descriptions from Hawaii and East Africa.

The host status of commonly grown cut-flowers in Ethiopia was assessed for *M*. *hapla* and *M*. *incognita* under greenhouse conditions. Overall, infected plants were less vigorous than non-infected plants. *Meloidogyne hapla* reproduced higher on roses than any other plant species assessed. Freesia appeared to be a poor host for *M. hapla* and *M. incognita* whiles all other cut-flower species were intermediate to good hosts for *M. hapla* and *M. incognita*.

The threshold level for *M. hapla* on *Rosa corymbifera* 'Laxa', *R. multiflora* and *R. canina* Inermis was assessed in pot experiments with increasing initial nematode densities from 0 to 128 J2 g^{-1} soil. *M. hapla* significantly reduced plant fresh weight of all three rose rootstock species independently of the initial nematode density. The minimum yield was below 0.73 and a tolerance limit of below 0.1 J2 g^{-1} soil. Nematode multiplication rate was highest on *R. multiflora* (24.39) followed by *R. corymbifera* 'Laxa' (4.34) and *R. canina* (3.62). Hence, all three rootstock species turned out to be sensitive to even low initial densities of *M. hapla*.

Ethanolic and aqueous extracts of native *Rumex abyssinicus, Plumbago dawei* and *Maesa lanceolata* significantly inhibited *M. hapla in vitro* and reduced nematode infection of tomato. Therefore, those botanical nematicides are a promising alternative for nematode control, especially since those plants are abundantly available throughout Ethiopia.

Key words: 28S rRNA, plant extracts, host status, *Meloidogyne hapla*, mtDNA, roses.

ZUSAMMENFASSUNG

Pflanzenparasitäre Nematoden entwickeln sich zunehmend zu einem wirtschaftlichen Problem in der Schnittblumenproduktion Äthiopiens. Im Rahmen eines Monitorings während der Regenzeit 2011 (August bis September) und Trockenzeit 2012 (April bis Mai) wurden an Rosen, Nelken, Schleierkraut, Freesien und Strandflieder 13 wirtschaftlich bedeutende Nematodengattungen erfasst: *Criconemella, Ditylenchus, Helicotylenchus, Hemicycliophora, Meloidogyne, Merlinius, Longidorus, Paratrichodorus, Paratylenchus, Pratylenchus, Rotylenchus, Rotylenchulus* und *Tylenchorhynchus.* Alle Gattungen traten an Rosen auf. *Helicotylenchus* wurde an allen untersuchten Schnittblumenarten nachgewiesen und *Meloidogyne* war häufiger in der Trockenzeit als in der Regenzeit anzutreffen.

Meloidogyne hapla war die mit Abstand wirtschaftlich bedeutendste Nematodenart an Rosen in Äthiopien. Da *M. hapla* erstmals in Äthiopien nachgewiesen wurde, erfolgte eine vergleichende Charakterisierung der Äthiopischen Population von *M. hapla* mit Populationen aus anderen Ländern. Mittels molekularer Verfahren wurde die D2-D3 Extension der 28S ribosomale DNA und die Region zwischen der Cytochrome Oxidase Unit II und dem 16S rRNA Gen der Mitochondrien verglichen. Demnach war die Äthiopische Population von *M. hapla* sehr eng mit anderen Populationen verwandt. Auch morphologisch zeigten sich bei Weibchen, Männchen und dam zweiten Juvenilstadium nur geringe Unterschiede zwischen der Äthiopischen Population und den anderen Populationen.

Die in Äthiopien angebauten Schnittblumen wurden hinsichtlich ihres Wirtsstatus für *M*. *hapla* und *M*. *incognita* untersucht. Befallene Pflanzen waren weniger wüchsig als nicht befallene Pflanzen. *Meloidogyne hapla* konnte sich an Rosen besser vermehren als an allen anderen Schnittblumenarten. Freesien erwiesen sich als schlechte Wirte für *M. hapla* and *M. incognita.* Sonstige Schnittblumenarten erwiesen sich als mittelmäßige bis gute Wirte für die beiden Nematodenarten.

Die Schadschwelle für *M. hapla* an *Rosa corymbifera* 'Laxa', *R. multiflora* und *R. canina* Inermis wurde in Topfversuchen mit kontinuierlich ansteigenden Nematodendichten von 0 auf 128 J2 g^{-1} Boden ermittelt. Der minimale Ertrag lag bei etwa 0,73 und die Toleranzschwelle für Ertrag bei 0,1 J2 g^{-1} Boden. Die höchste Vermehrungsrate von *M. hapla* trat an *R. multiflora* (24,39) auf, gefolgt von *R. corymbifera* 'Laxa' (4,34) und *R. canina* (3,62). Somit erwiesen sich alle drei Unterlagensorten als sehr anfällig für *M. hapla*.

Äthanolische und wässrige Extrakte der in Äthiopien einheimischen Pflanzen *Rumex abyssinicus, Plumbago dawei* und *Maesa lanceolata* führten zu einer signifikanten Unterdrückung von *M. hapla in vitro* und reduzierten den Nematodenbefall an Tomate. Solch pflanzliche Extrakte könnten eine vielversprechende Alternative zur chemischen Bekämpfung darstellen, zumal die Pflanzen in Äthiopien weit verbreitet sind.

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Chapter One

1. General Introduction

1.1 THE CUT-FLOWERS

Floriculture crops include cut flowers, foliage plants and pot plants that are either annual or perennial species, grown in the field and greenhouses for cut-flower production. The crop choice may depend on adaptability to the environmental conditions and market potential of specific species or cultivars. In 2003, the production value of floriculture world wide was estimated to 60 billion dollar. Europe is the largest market for cut flowers with an export value of $2660 million followed by North America which has about 108 million US dollar (Uffelen and Groot, 2012).

In recent years, cut flower production has emerged and growing at an alarming rate in Afrika (Uffelen and Groot, 2012). The low rate of annual operating cost particularly in Ethiopia, Malawi, Zimbabwe, Zambia, Uganda and Kenya had played a great motive in encouraging European investment in Afrika (Gebreyeesus and Lizuka, 2010;Rooyne *et al*., 2001). Ethiopia entered to a commercial cut flower production state in the late 1990s. Nevertheless, the rate at which Ethiopia grew to a known flower export industry is quite immense. To date, the floriculture industry is the most rapidly expanding segment of agriculture ever in the country. The Ethiopian cut flower industry comprises the production of a wide variety of flowers including roses, freesia, statice, gypsophila, hypericum, chrysanthemum and carnations. The negligible cost of labour coupled with ideal agro-climatic conditions has in particular attracted foreign investors from different countries particularly from the Netherlands, Germany and Israel (Anonymous, 2014; Willem, 2010, Gebreeyesus and Lizuka, 2010; Anonymous, 2005).

Despite being a young sector, it has demonstrated a promising foreign exchange of 200 million US dollar in the 2013 physical year (EPEHA, 2013). Roses make about 80% of the total annual cut flower production on about 2000 ha of land and are supplied mainly to European countries such as the Netherlands, Belgium and Germany. In recent years, Ethiopia’s share on rose export into European countries has accounted for 12% following Kenya (32%) and Ecuador (13%).Between 2008 and 2012, the annual production growth of cut flower production in Ethiopia was 20% compared to 1% in Kenya and 4% in Ecuador (Eurostat, 2012).

Although the floriculture industry is among the most successful sectors in the country, there are production challenges that may impair its future potential, such as damage caused by pests and diseases.

1.2 MAJOR PESTS OF CUT-FLOWERS IN ETHIOPIA

In Ethiopia, yield losses from diseases and insect pests are generally minimized using diverse types of chemicals, except for few greenhouses using biological control agents (DLV-Plant, 2011). Pest and disease control demands the application of high doses of pesticides, insecticides and nematicides. It is obvious that these chemicals not only increase the cost of production but also increase the risk of soil toxicity in the near future. Yet, with less attention given in the past plant-parasitic nematodes have become a major challenge for the industry (Meressa *et al*., 2014; Meressa *et al*., 2012).

1.2.1 *Insects and diseases*

In its widest sense, diseases alter the physiology of the plant and can be caused by various kinds of factors that include pathogens, parasites, nutrient deficiency and environmental entities. Root rot, damping-off, downey and powdery mildew are common diseases of cut flowers. In all cases, diseases are directly linked to a reduction in quality and quantity of the crop yield. Insects such as thrips, aphids and mites also cause serious plant damage in cut flowers. In Ethiopia, a combination of chemicals such as Abamectine, Tetradifon dicofol, Acephate, Thiamethoxam, Pirimiphos Methyl, Diafenthiuron, Potassium phosphite etc. are routinely applied to control insects and disease (DLV-Plant, 2011; Willem, 2010).

1.2.2 *Plant-parasitic nematodes*

Plant-parasitic nematodes are minute roundworms which live in the soil and attack plants. They cause high economic losses in both large and small-scale agriculture worldwide (Blaxter *et al*., 1998; Kleine *et al*., 1998). Mostly growers are unaware of the economic losses caused by plant-parasitic nematodes as the damage goes unnoticed, unless soil or plant samples are taken for nematode diagnostics.

Plant-parasitic nematodes have developed a particular relationship for nutrients achieved from their host plant that has evolved over thousands of years. They feed on the content of living cells by inserting and sucking the fluid with their stylet (Hussey *et al*., 2002). Nearly ten genera are considered to cause most of the economic loss in the world agriculture. Some genera have limited geographical and host range (Starr *et al*., 2002), while others are widespread with a broad host spectrum. In floriculture, species of the genera root-knot (e.g. *Meloidogyne hapla*, *M*. *incognita*, *M*. *arenaria*, *M*. *javanica*), reniform (*Rotylenchus* spp.), root-lesion (*Pratylenchus vulnus*), ring (*Criconemoides* spp.), and stunt (*Tylenchorhynchus* spp.) nematode are known to cause economic yield loss (McSorley and Fredrick, 1994;

Santamour and Riedel, 1993; Bernard and White. 1987; Nemec and Struble, 1968). Of these, *Meloidogyne* spp. creates complex interactions with the host root causing major morphological and developmental changes (Williamson and Hussey, 1996).

Nematode species such as *Xiphinema* index, *X. diversicaudatum*, *Meloidogyne hapla*, *Pratylenchus penetrans* and *P. vulnus* are known to cause serious damage on roses (Wang *et al*., 2004; Santo and Lear, 1976; Coolen and Hendricks, 1972). Carnation, gypsophila and statice are mainly damaged by *Meloidogyne incognita* (Nagesh and Reddy, 2005; Cho *et al*., 1996; McSorley and Frederick, 1994 Goff, 1936). Furthermore, carnation and freesia can get infected by *Meloidogyne arenaria* and M. *javanica* (Tyler, 1941; McSorley and Frederick, 1994). Among the spiral nematodes, *Helicotylenchus dihystera* and *H. varicaudatus* are important parasites of carnation (Khanna and Jyot, 2002).

1.2.3 *Damage and symptoms*

Plant-parasitic nematodes can kill particularly annual host plants when occurring at high population densities (Vrain, 1981). At lower densities, yield loss may also occur without notable changes on the plant. In Ethiopia's floriculture, plant-parasitic nematodes have not yet received considerable attention as a pest of economic importance (Meressa *et al*., 2012), because the above ground symptoms of nematodes root damages are nondescript resembling to other factors such as lack of water and nutriments (Chen *et al*., 2004). Damage symptoms vary depending on the type of nematode and the crop. In general, leaf chlorosis, twisting, wilting, early senescence, stunting and poor yield are among the symptoms that need to be considered when scouting nematode infestation (Lambert and Bekal, 2009).

Below-ground damage symptoms are mostly specific to a nematode group and are easier to recognize compared to above ground symptoms. For example, visual observation of root galling (*Meloidogyne* spp.), development of excessive lateral roots (*Meloidogyne hapla* and *Pratylenchus* spp.), root lesions (*Pratylenchus* spp., *Criconemella* spp.), stubby roots (*Paratrichodorus* spp.), rotting of bulbs (*Pratylenchus* spp., *Ditylenchus* spp.), root tip galls (*Xiphinema* spp.) and presence of cysts attached to roots (*Heterodera* spp. and *Globodera* spp.) indicate nematode damage. Generally, rose plants attacked by plant-parasitic nematodes show stunted growth and leave chlorosis (Olson, 1972).

1.2.4 *Plant-parasitic nematodes in Ethiopia*

Since the first nematological survey conducted in Ethiopia (O'Bannon, 1975), other surveys have been carried out. Subsequently, several species of major plant-parasitic genera such as *Pratylenchus*, *Rotylenchus*, *Rotylenchulus*, *Scutellonema*, *Helicotylenchus*, *Xiphinema*, *Longidorus*, *Paratrichodorus*, *Meloidogyne*, *Tylenchorhynchus*, *Ditylenchus*, *Hemicycliophora*, *Criconemella*, and *Heterodera* have been reported associated with vegetables, enset and coffee (Meressa *et al*., 2012; Van den Berg and Mekete, 2011; ; Mekete *et al*., 2008; Bogale *et al*., 2004; Wondirad and Mekete, 2002). All those studies focused on the identification of the nematode species and no reports are available on the extent of yield losses caused by those nematodes. For cut flowers, neither plant-parasitic nematodes associated with them nor plant injuries caused by them were yet reported.

The three most economically important species *M*. *javanica*, *M*. *ethiopica* and *M*. *incognita* occur distributed mainly in the tropical climate regions (Jepson, 1987; Whitehead, 1968). These species have been also reported from Ethiopia (O'Bannon, 1975), although not yet from cut flowers. The occurrence of *M. hapla* on rose cut-flowers in Ethiopia was reported recently during this study (Meressa *et al*., 2014).

1.3 DETECTION AND IDENTIFICATION OF PLANT-PARASITIC NEMATODES

Growers might be able to diagnose the presence of root galls or cysts (Jepson, 1987; Whitehead, 1968) however; identification of plant-parasitic nematodes can only be done by specialists. Thus, it is recommended to collect soil or plant samples for nematode analysis before any new plantings are established in the field. Furthermore, nematodes recovered have to be identified when possible to a race level using a combination of morphological and molecular diagnostic techniques.

1.3.1 *Morphological analysis*

Accurate identification of plant-parasitic nematodes to species level is essential to initiate any nematode control strategy. In cases, where taxonomic characters are limited, unclear or overlapping morphometric values occur, identification solely based on morphology and morphometrics might not be possible (Stanton *et al*., 1997). Distinguishing species is mostly difficult because diagnostic characters are shared among species (Olivera *et al*., 2011). The influence of environmental and seasonal factors on the morphometric measurements of nematode population also limits the potential of such identification. For example, Handoo *et al* (2005) reported shorter second-stage juveniles of *M. hapla* during summer season than

during winter season. Nevertheless, classical nematode taxonomy is still essential in nematode identification. In cases of doubt, morphological-based identification needs to be combined with molecular diagnostics.

1.3.2 *Molecular analysis*

Compared with morphology, nematode identification using molecular techniques provides rapid and accurate identity of a species in a sample. Various molecular techniques have been developed for nematode identification allowing differentiation at species or even race level (Handoo *et al*., 2001; Zijlstra *et al*., 2000; Demek *et al*., 1992; Williamson 1991; Edward *et al*., 1992).

Protein electrophoresis involves the extraction and separation of soluble proteins on the basis of their molecular mass on polyacrylamide gels (Subbotin and Moens, 2006). This approach can be best used to identify *Meloidogyne* species (Esbenshade and Triantaphyllou, 1990). For some groups of plant-parasitic nematodes, isozyme analysis results in a wide variation within populations of the same species (Subbotin and Moens, 2006). In addition, it relies on specific developmental stage of adult female, which makes it practically difficult if only juveniles are available. Hence, its application in nematode identification is not much used compared to the polymerase chain reaction (PCR) techniques.

PCR-based molecular diagnostics demonstrated a simple, reliable and rapid application in nematode systematics (Olivera *et al*., 2011). For instance, the sensitivity of detection of specific species based on DNA extracted from single individual nematode is an exciting application of this technique (Harris *et al*., 1990).

A number of different genes have been used for nematode molecular phylogenetic (Blaxter, 2001). In root-knot nematodes, ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) have been extensively used to differentiate species (Block *et al*., 1997; Powers and Harris, 1993). For example, amplification of the region between cytochrome oxidase subunit II (COII) and 16S ribosomal RNA of the mtDNA was able to produce a typical fragment size of 1.7 kb for *M*. *incognita* and *M*. *javanica*; 1.1 kb for *M*. *arenaria* and 540 bp for *M*. *hapla* (Orui, 1998; Powers and Harries, 1993). Moreover, it has been revealed that PCR-restriction fragment length polymorphism (PCR-RFLP) of mtDNA has enabled to distinguish among *M. hapla*, *M*. *incognita*, *M*. *arenaria*, *M*. *javanica*, *M*. *mali*, *M*. *camelliae*, *M. marylandi* and *M. suginamiensis* (Orui, 1998). Use of ITS regions has also been used to differentiate between isolates of *M. hapla* and *M. chitwoodi* (Zijlstra *et al*., 1995).

1.4 NEMATODE MANAGEMENT AND CONTROL STRATEGIES IN CUT FLOWERS

When nematode damage is realized, it is important to take appropriate control measures to keep the population below the economic threshold level (Singh and Sitaramaiah, 1994). But cut-flower growers have to be aware that nematode symptoms can be similar with other anatomical and physiological disorders of the plant. Obviously, administering a possible control measure based on visual inspection can be misleading while it is important to take root and/or soil samples for confirmation.

In principle, nematode management strategies are not curative but are implemented to keep the soil nematode population below the damaging level. Moreover, a single management practice may not effectively control the nematode. Hence, a combination of one or more of the following strategies should be considered in cut flower greenhouses to reduce damage from plant-parasitic nematodes.

1.4.1 *Chemical nematicides*

Nematicides are chemicals that are lethal to nematodes or at least protect young seedlings from nematode parasitism within the first weeks (McKenry, 1994). In the past, chemical nematicides have been widely used in large scale agriculture particularly in high-value crops. There are several reports on a successful nematode control by treating nematode infested soils with fumigants or granular nematicides (Dale, 1973; Dale and Mespel, 1972). However, nematicides are highly toxic and thus their use is both ecologically and economically infeasible. In developed countries, applications of nematicides (e.g. methyl bromide) have been essentially banned. In developing countries including Ethiopia, there seem to be no indications if these nematicides are restricted from use. Hence, there seems to be still room that accommodates the use of nematicides to minimize the damage from nematodes on cut flowers.

From global perspectives, the increase in environmental concern, withdrawal of most nematicides from market and high cost of previously effective chemical nematicides has brought a move to alternatives in integrated pest management programs.

1.4.2 *Prevention of new introduction or spread of nematodes*

Nematodes commonly exist in a patchy pattern on the field and thus there will be spots where nematodes are severe and others where nematodes are almost absent (Singh and Sitaramaiah, 1994). Therefore, once a nematode is already present in some spots of the field

or greenhouse, anything that moves soil or infected plant roots will also move the nematodes. If a greenhouse/field with a severe problem is realized, further spread of the nematode has to be limited. Thus, infected plant materials or soil should not be moved from these spots into nematode free greenhouses/fields or even within plots (Viaene *et al*., 2005).

Farming machinery, muddy shoes, irrigation water and plant debris are mostly responsible for nematode spread. In Ethiopia, growers rent machineries particularly during soil preparation phase. In this case, nematodes might be moved from one greenhouse to another greenhouse or even from farm to farm thereby introducing nematodes to previously non-infested sites. Thus, limiting all possibilities of caring infested soil is important to reduce the risk of nematode spread.

Under Ethiopian situation, whenever new roses have to be planted, previous old plants are uprooted and left on the surface of the soil to dry. From a nematological point of view, this is indeed a bad practice in which nematodes hatched from lifted roots get back to the soil and infect the new plants. Therefore, proper disposal of plant debris improves sanitation thus reduce the density of nematode inoculums in the soil (Lamondia, 1997).

Some nematodes of cut-flowers are found in vegetative planting materials such as n bulbs of freesia. Although, it may possible to treat these planting materials with hot-water or chemicals (Towson and Lear, 1982), risk of nematode survival in the planting material is high and while complete exclusion of infected planting materials is recommended.

1.4.3 *Cultural management*

Brassica cultivars are known to be resistant for several *Meloidogyne* species such as *M. chitwoodi*, *M. fallax*, *M. hapla* and *M. incognita* (de Meijer, 1993). Hence, growing such resistant cultivars immediately before planting cut flowers would bring the initial nematode populations density below damaging levels. On the other hand, incorporation of the green manures releasing secondary metabolites toxic to the plant-parasitic nematodes might also help to reduce nematode densities in the soil (Pattison *et al*., 2006; Zasada and Ferris, 2004; Stirling and Stirling, 2003).

The increase demand for alternative control measures has brought emphasis on the application of plant derived products to control plant-parasitic nematodes (Chitwood, 2002). Many studies have already shown the suitablility of plant products as bioinsecticides (Boursier *et al*., 2011; Ayazpour *et al*., 2010). Plant derived extracts constitute active compounds such as isothiocyanates, glucosinolates, cyanogenic glycosides, alkaloids, terpenoids, diterpenoids, triterpenoids, steroids, tannins, plumbagin, and phenolics, are promising sources for nematode control (Oka *et al*., 2006; Chitwood, 2002). Plant extracts

exhibiting nematicidal properties against *Meloidogyne* species have been repeatedly reported (Katooli *et al*., 2011; Ononuju and Nzenwa, 2011; Ugwuoke *et al*., 2011; Nimbalkar and Rajurkar, 2009; Pakeerathan *et al*., 2009; Wiratno *et al*., 2009; Khan *et al*., 2008). Though most research is based on *in vitro* assays, Khan *et al*. (2008) found an increase in growth components of papaya grown in *M. incognita* infested field after plants were drenched with aqueous extracts of *Azadirachta indica* and *Tagetes erecta*.

On the other hand, leaves of such plant species can be directly incorporated into the soil. Species of *Brassicaceae* family are known for their glucosinolates content that hydrolyse to release isothiocyanates upon incorporation into the soil (Zasada and Ferris, 2004).

1.4.4 *Plant resistance*

Plant resistance to nematodes is a complex mechanism that determines the host-nematode relationship (Singh and Sitaramaiah, 1994). Once the nematode species present in the soil is known, the use of resistance cultivars provides the most economic and ecological means of nematode control (Bridge, 1996). In this case, a plant resistant confers the potential to supress the development and reproduction of the nematode (Roberts, 1995). Resistance against *M*. *incognita* race 2 was reported in carnation from South Korea, where the cultivar 'Kappa' developed no root galls (Cho *et al*., 1996). A partial resistance on two cultivars of *Rosa canina* 'Succes' and 'Hainsohn's Rekord' to *M. hapla* has been also reported (Coolen and Hendrickx, 1972). According to Voisin *et al*. (1996) and Pizetta at al. (2010) *Rosa manetti* inoculated with *M*. *hapla* showed gall free roots and harboured only low numbers of nematodes. Nevertheless, complete resistance to any nematode species in cut flowers is not yet available.

1.4.5 *Physica*l *control*

Soil disinfection with solar radiation can be considered as one of the most effective means to kill nematodes in the soil (Egunjobi and Larinde, 1975). The method is of practical importance in countries with warm temperatures and intensive solar radiation (Gaur and Perry, 1991; Stapleton and Devay, 1983). Using clear plastic to cover the soil surface, solar radiation penetrates the plastic stimulating water molecules to a higher energetic level that will warm the soil temperature up to 10°C above air temperature. If soil temperature above 45°C is reached, plant-parasitic nematodes will be killed. Although, the efficiency of solarization depends on the environmental conditions and the thermal damage threshold of the nematode in the soil, it generaly gives an excellent nematode control (Ciancio and Mukerji, 2008; Stapleton, 2000). For instance, the population of *Meloidogyne* spp. in

greenhouse tomatoes was effectively reduced in southern Italy (Lambardo *et al*., 2012). Moreover, combination of soil solarization with organic amendments has effectively reduced the nematode population in the soil and galling indices caused by *M. javanica* on tomato (Oka *et al*., 2007). In Ethiopia, most cut flower greenhouses are located in the rift valley where temperatures reach up to 35^{o}C. In that case, soil solarization can be a promising alternative to control plant-parasitic nematodes in cut flower greenhouses.

There are few farms in Ethiopia that grow rose plants in hydroponic system aimed at preventing soil-borne pathogens including plant-parasitic nematodes. Interestingly, D'Errico and Ingenito (2003) have intercepted *M. incognita* and *M*. *arenaria* from rose plants grown in soilless culture. Hallmann *et al*. (2005) indicated that plant-parasitic nematodes might enter soilless cultures with infected plant material or irrigation water taken from a nearby reservoir or stream. Thus growing crops in soilless culture system does not guarantee absence of plant-parasitic nematodes. Therefore, the irrigation water needs to be treated before entering the soilless system. Grech *et al*. (1989) demonstrated that citrus nematodes were eliminated from irrigation water using UV irradiation. UV irradiation at a dose of 14 mJ.cm^{-2} was reported to inhibit nematode infectivity when the water flow rate was 2.5 m^{-3}h (Moens and Hendrickx, 1989). *Meloidogyne javanica* was unable to reproduce on tomato plants following exposure to different levels of UV irradiation (Pieterse and Van Mieghem, 1987). High dosages of UV radiation (>200 mJ.cm-2) are even lethal for *M*. *incognita* (Moens and Hendrickx, 1989). A rose farm in Sululta district, Ethiopia, using a soilless culture system in combination with UV treated irrigation water seems so far still free of plant-parasitic nematode infestation.

1.5 RESEARCH NEED

Since recent years, the floriculture industry in Ethiopia is becoming one of the most important sectors of increasing foreign exchange. To keep the current production sustainable, management of pest control is of paramount importance. Growers are fully aware of insects as well as fungal and bacterial disease problems and thus optimal control options are being implemented. However, serious crop damage from plant-parasitic nematodes has become apparent in most greenhouses, in which case the type and abundance of nematode involved is unknown. Moreover, with few exceptions, the existing nematode problem was not clearly realized. Consequently, understanding the diversity, current distribution and population density of plant-parasitic nematodes, extent of interaction with the host plants (cut flower species) is very important to implement any future nematode management practices. More specific, accurate identification of the most abundant and damaging nematode species is needed using classical and molecular techniques. This information will basically leave the

industry to get fully aware of the nematode damage and look forward towards developing feasible strategies to reduce the nematode impact on yield loss.

1.6 RESEARCH OBJECTIVES

The main objective of this study was to identify the type and distribution of plant-parasitic nematodes in relation to cut flower crops and investigate the degree of host-parasite relationships of economically important crops and nematode species.

Therefore, the study encompassed:

- Survey on the occurrence, distribution, and abundance of major plant-parasitic nematodes associated with cut-flowers
- Molecular and morphological characterization of *Meloidogyne hapla* detected from rose greenhouses (New report for Ethiopia)
- Host suitability test of the cut-flower species currently growing in Ethiopia for *M. hapla* and *M. incognita*
- Evaluation of three plant extracts native to Ethiopia as a way to develop sustainable management tools for IPM of plant-parasitic nematodes
- Investigation of the threshold damage of *M. hapla* to three rose rootstocks (*Rosa corymbifera* Laxa, *R. multiflora* and *R. canina* Inermis)

1.7 REFERENCE

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Chapter Two

2. Occurrence and distribution of plant-parasitic nematodes associated with cut-flowers in Ethiopia*

* Meressa, Beira-H., Dehne, H-W. and Hallmann, J. 2004. Plant-parasitic nematodes associated with commercial cut-flowers in Ethiopia. *International Journal of Nematology* 24(1):1-10

ABSTRACT

Surveys were conducted from August to September 2011 (wet season) and April to May 2012 (dry season) to assess the incidence of economically important plant-parasitic nematodes associated with cut-flowers Ethiopia. Soil samples were collected from rose (*Rosa hybrida*), carnation (*Dianthus caryophyllus*), gypsophila (*Gypsophilia paniculata*), freesia (*Freesia laxa*) and statice (*Limonium sinuatum*) plants. A total of thirteen nematode taxa associated with these five cut-flower species were recorded viz. *Criconemella, Ditylenchus, Helicotylenchus, Hemicycliophora, Longidorus, Meloidogyne, Merlinius, Paratrichodorus, Paratylenchus, Pratylenchus, Rotylenchulus, Rotylenchus and Tylenchorhynchus.* Each cut-flower species harboured at least one nematode genus. Rose plants sampled at 11 farms harboured all of the genera detected and *Meloidogyne* was recovered exclusively from this plant species. Statice, carnation, gypsophila and freesia sampled from one farm each harboured 5, 2, 2 and one nematode genera, respectively. *Helicotylenchus* was recovered from all cut-flower species. The incidence, population density and genera composition varied between sampling seasons. Hence, only 61.5% of the genera recovered during the wet season were detected again during the dry season. During the wet season, *Helicotylenchus* was the most frequently encountered genus (77%) followed by *Meloidogyne* (46%). In the dry season, *Meloidogyne* was encountered in 78% of the samples followed by *Helicotylenchus* (33%). This survey showed the wide presence of plant-parasitic nematodes in cut-flower farms of Ethiopia emphasizing the need to initiate nematode management strategies.

2.1 INTRODUCTION

Floriculture industry is a rapidly expanding export business in Ethiopia. The export volume of major flowers such as freesia, statice, roses, gypsophila, hypericum, chrysanthemum and carnation is growing and particularly remains attractive for the growers in a country where labour cost is negligible (Willem, 2010). Cut-flowers like other crops harbour pests that cause potential economic losses. In most of the greenhouses, nematode pests are a major threat but their effect is hardly understood or recognized by the growers owing to the nematodes hidden way of life and the unspecific symptoms that they cause. In general, plant-parasitic nematodes have been long known as a threat in ornamental crop production (Benson and Barker, 1985) affecting the quality of marketable cut-flowers (Arbelaez, 1999). For instance, *Meloidogyne hapla* and *Pratylenchus penetrans* cause severe damage on greenhouse roses (Peng and Moens, 2002). Common symptoms of visible damage include decline in vigour, leaf chlorosis, shoot dwarfism, early leaf drop and reduction in yield (Epstein and Bravdo, 1973).

Earlier nematode surveys conducted in Ethiopia have documented the presence of plant-parasitic nematode genera associated with *Coffea arabica* such as *Helicotylenchus*, *Meloidogyne*, *Heterodera*, *Xiphinema*, *Rotylenchus*, *Scutellonema*, *Tylenchorhynchus,* and *Criconemella* (Mekete *et al*., 2008), with vegetable crops such as *Meloidogyne* spp. (Wondirad and Mekete, 2002), and with *Ensete ventricosum* such as *Pratylenchus* and *Aphelenchoides* (Bogale *et al*., 2004). The presence of plant-parasitic nematodes on ornamental crops has been documented for countries other than Ethiopia (Nagesh and Reddy, 2005; Peng *et al*. 2003; Khanna and Jyot, 2002; Peng and Moens, 2002; Cho *et al*., 1996). However, despite the presence of major plant-parasitic nematodes in Ethiopia, no record of plant-parasitic nematodes associated with cut-flowers has yet been published, perhaps because it is a newly raised industry or because of a lack of awareness of nematodes. Consequently, baseline information concerning the occurrence, population density and distribution of plant-parasitic nematodes on cut-flowers in Ethiopia is highly needed. If plant-parasitic nematodes turn out to become a major pest problem, sustainable control measures need to be developed in time. Within this respect, the objective of the present work was to study the incidence, distribution, and abundance of economically important plant-parasitic nematodes in cut-flower production in Ethiopia.

2.2 MATERIALS AND METHODS

2.2.1 *Sampling sites*

The survey was conducted in different localities of Ethiopia during the wet season from August to September, 2011 and the dry season from April to May, 2012. Sampled localities were randomly selected representing the different geographical directions from the capital city Addis Ababa and type of cut-flower species under production: Debre Zeit and Ziway in the South of Addis Ababa producing rose and gypsophila; Sululta, Holleta and Menagesha in the North producing rose, freesia and statice; Sebeta in the west producing rose and Sendafa in the East producing carnation (Fig. 1). All farms were located at a distance of 30-175 km from Addis Ababa allowing international air travel of cut-flowers within 24 hours after harvest. For sampling locations the following parameters were collected: GPS coordinates elevation, soil type and cropping history (Table 1).

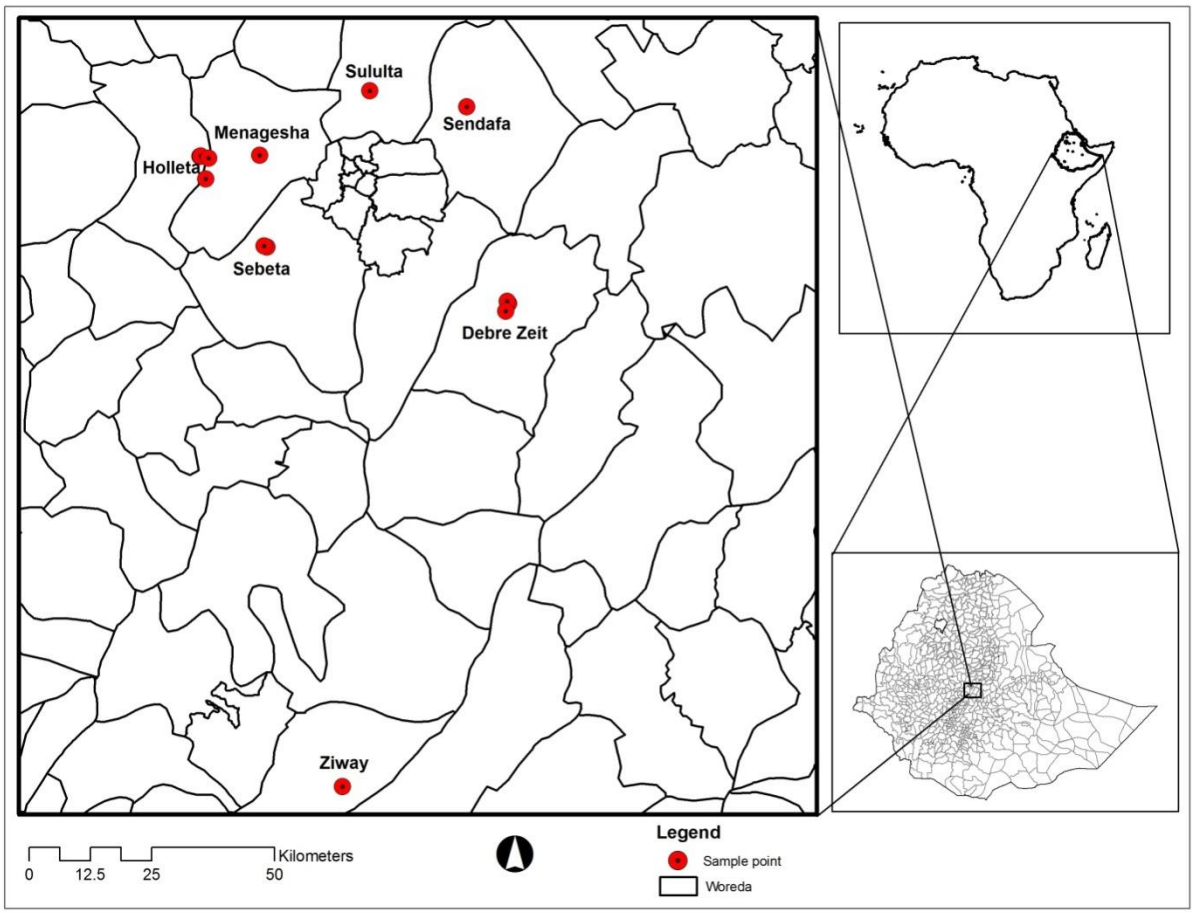

Figure 1. Map of Ethiopia showing the sampled cut-flower producing farms (Woreda=districts).

Table 1. Currently growing cut-flower species, cropping history, soil type and altitude of the sampling site.

Sampling district	Altitude (a.s.l)	Cropping History	Dominant soil type	Common name	Botanical Name
Ziway	1600	Tomato	Andosols	Rose	*Rosa hybrida* L.
Debre Zeit	1900	Vegetable	Vertisols	Gypsophila	*Gypsophila paniculata* L.
Debre Zeit	1900	Vegetables	Vertisols	Rose	*Rosa hybrida* L.
Sebeta	2100	Teff	Andosols	Rose	*Rosa hybrida* L.
Menagesha-	2500	Eucalyptus	Nitosols	Rose	*Rosa hybrida* L.
Holleta	2400	Cereals	Luvisols	Rose	*Rosa hybrida* L.
Sendafa	2500	Cereals	Vertisols	Carnation	*Dianthus caryophyllus* L.
Sululta	2500	Maze	Nitosols	Limonium	*Limonium sinuatum* L.
Sululta	2500	Maize	Nitosols	Freesia	*Freesia spp.*

2.2.2 *Soil sampling*

During the wet season of 2011 a total of 14 cut-flower producing farms were sampled. Eleven out of those 14 farms were resampled during the dry season in 2012. Except for gypsophila grown in the field, cut-flowers were cultivated in greenhouses (Fig. 2). For each farm and flower species, five greenhouses were sampled at two samples per greenhouse totalling in 10 samples per greenhouse and flower species. For gypsophila grown in the field 10 samples were collected from different plots in 2011 and 5 samples in 2012. In total 152 samples were taken in 2011 and 115 samples in 2012. For each sample, 40 soil cores of 2 cm in diameter were taken from the top 20-25 cm next to the plant to make a bulk of about 2 l soil per sample (Fig. 3A). For handling reasons, after thorough mixing by hand, a sub-sample of about 1 kg was packed in a 2 l labelled plastic bag and taken at the same day to the laboratory at Addis Ababa University, Plant Physiology Lab, for processing the following day (Fig. 3B).

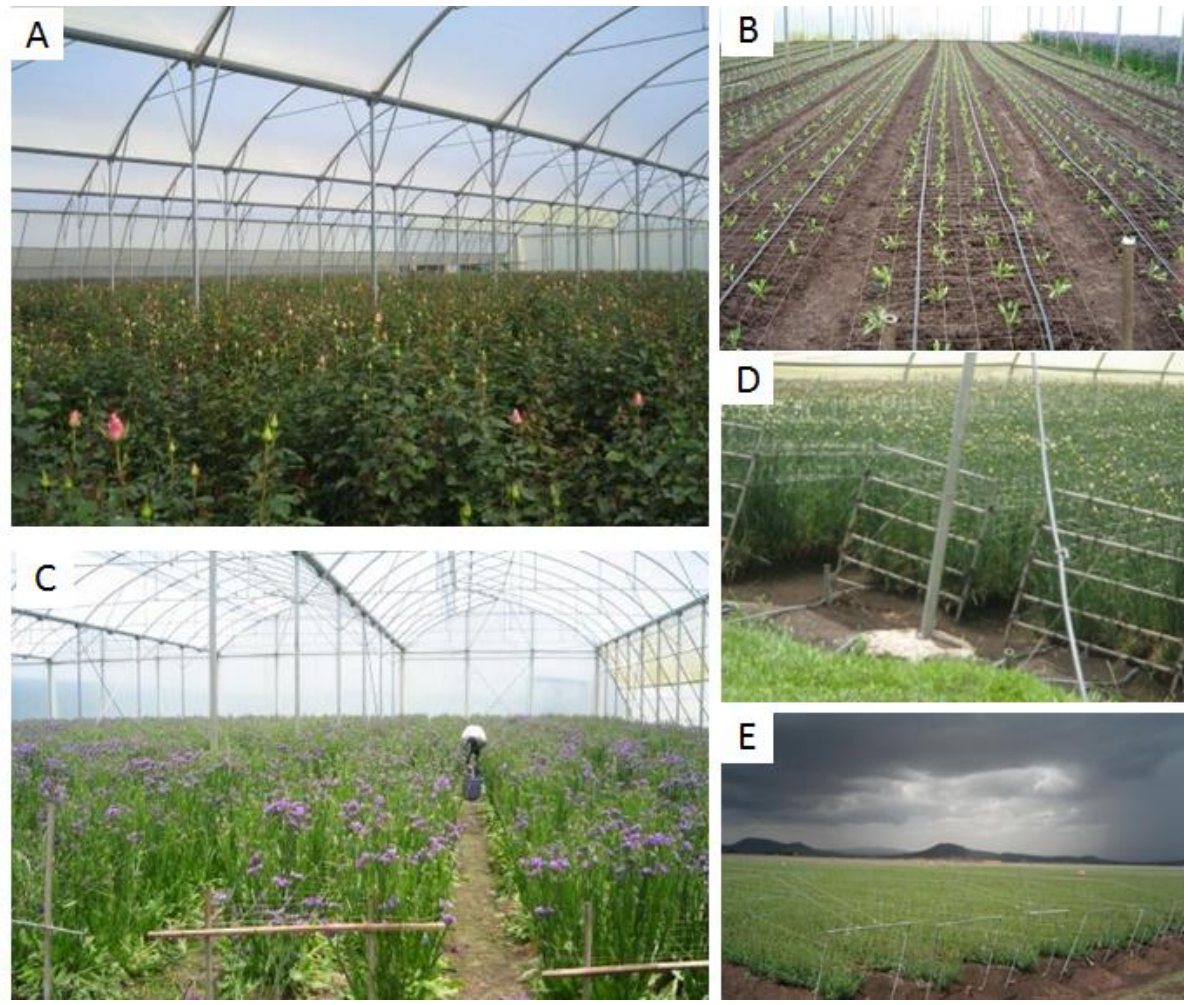

Figure 2. Cut flower crops of the sampling sites. Rose (A), statice (B), freesia (C), carnation (D) and gypsophila (E).

Figure 3. Soil sampling (A), handling (b) and nematode extraction from soil (C) in Ethiopia. Nematode extraction was performed at Addis Ababa University, Department of Biology.

2.2.3 *Nematode extraction*

Aliquots of 200 ml soil were taken from each sample to extract nematodes using the modified Baermann technique given in Fig. 3C (Hooper *et al.* 2005). Nematodes were collected after 24 hours on a 20 µm aperture stainless steel sieve and transferred in a 2 ml Eppendorf tube.

Nematodes were then heat killed by immersing the Eppendorf tubes in 60°C warm water for 10 min. Finally, nematodes were fixed in TAF solution (7 ml formalin, 2 ml triethanolamine, 91 ml distilled water) (Courtney *et al*. 1975). All fixed nematode samples were brought to the JKI in Münster, Germany, for nematode counts and identification.

2.2.4 *Biotest assay*

An additional 200 ml soil from each sampling site was directly shipped to Germany for nematode detection in a biotest using *Rosa multiflora* 'Laxa' as bait plant. Therefore, the 200 ml sample was split into two samples of 100 ml each. Each 100 ml subsample was then thoroughly mixed with 1.9 litre steam-treated field soil shown to be free of plant-parasitic nematodes to fill up 2 litre pots into which one rooted rose cutting was planted. Plants were grown in a quarantine greenhouse. After two, three and four months, 100 ml soil was taken from each pot to check for nematode establishment. Nematodes were identified as described below. Plants confirmed positive were finally transplanted into 5 litre pots and maintained to serve as a nematode pool until identification was completed.

2.2.5 *Nematode identification*

Fixed plant-parasitic nematodes were identified to genus level using a compound microscope. Population density was expressed in nematode numbers per 100 ml of soil. Mean population density of nematode counts received per flower species, farm and sampling season were calculated. Moreover, total mean population density, frequency of occurrence and prominence value of each genus were calculated. The frequency of occurrence of each genus was determined based on Norton (1978). Prominence value (PV) that indicates the order of importance of each genus was calculated as: $\mathrm{PV} = \text{population density}\sqrt{\text{frequency of occurence}/10}$ (De Waele *et al*., 1998). The total mean nematode density was then plotted against the frequency of occurrence to evaluate the relative importance of each nematode genus in each sampling season.

Species identification was done on permanent slides after transferring the nematodes into glycerol following the method developed by Hooper *et al*. (2005). Identification was based on the morphology and morphometrics of females and in the case of root-knot nematodes also on second-stage juveniles (J2) and males. For the latter, identification was confirmed by mtDNA sequences of the cytochrome oxidase subunit II gene and D2-D3 expansion segment of 28S rDNA following the methods described by Adam *et al*. (2007), Handoo *et al*. (2005) and Powers and Harris (1993). Unfortunately, not all samples yielded sufficient numbers of females for a proper identification.

2.3 RESULTS

2.3.1 *Effect of sampling season*

During wet season sampling, a total of thirteen plant-parasitic nematode genera were recorded: *Helicotylenchus, Meloidogyne, Longidorus, Paratylenchus, Pratylenchus, Ditylenchus, Criconemella, Rotylenchulus, Tylenchorhynchus, Paratrichodorus*, *Hemicycliophora, Rotylenchus,* and *Merlinius* (Table 2). Species identified included *Tylenchorhynchus vulgaris*, *Meloidogyne hapla*, *M. ethiopica*, *Pratylenchus vulnus*, *Paratylenchus c.f. obtusicapitatus*, *Rotylenchulus parvus*, *Helicotylenchus californicus, H. psudorobustus, H. falcatus, H. egyptensis, H. multicinctus, H. dihystera,* and *H. digitatus, Paratrichodorus minor* and *Longidorus laevicapitatus.*

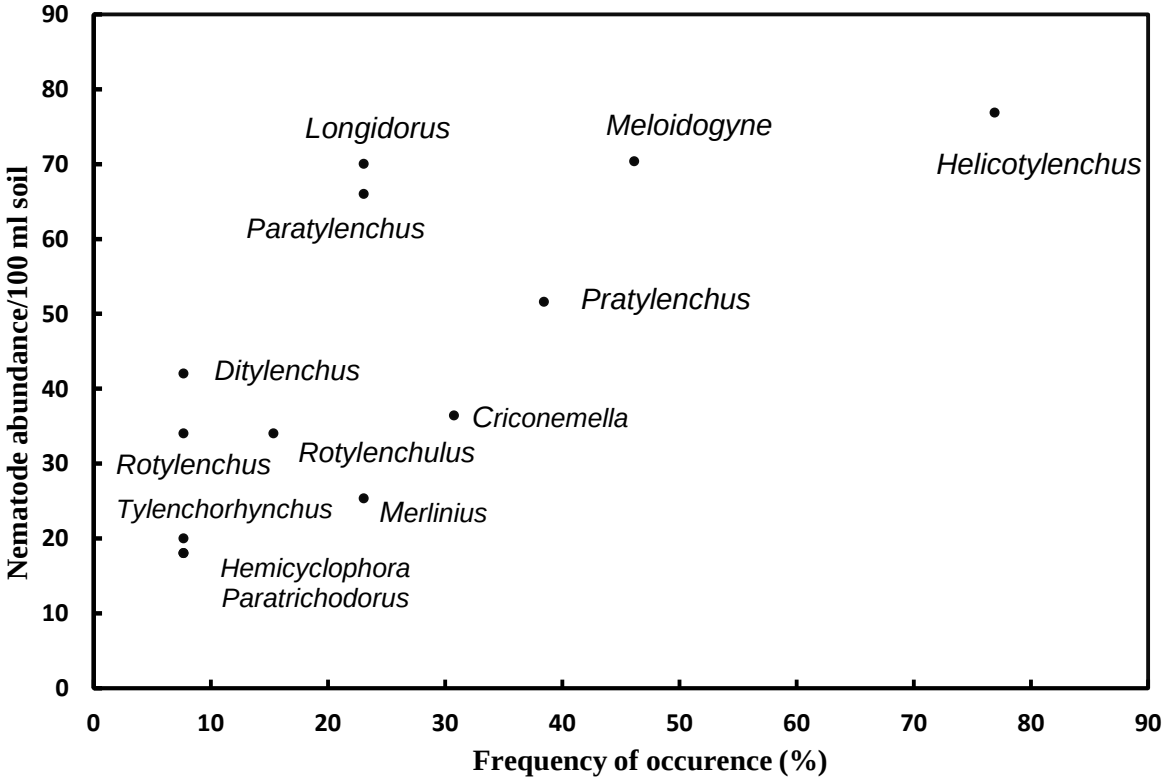

Figure 4. Frequency and abundance of major nematode genera recovered from cut-flowers during the wet season (August-September, 2011) from Ethiopia.

The highest frequency of occurrence was for *Helicotylenchus* with 77% followed by *Meloidogyne* with 46% and *Pratylenchus* with 38%, respectively (Fig 4; Table 2). Nematode taxa least frequently found were *Ditylenchus, Hemicycliophora, Rotylenchus, Paratrichodorus* and *Tylenchorhynchus*, all at about 8% frequency of occurrence. The highest population density per 100 ml soil was 389 for *Helicotylenchus* from carnation followed by 178 for *Meloidogyne* from rose (Table 2). The relative importance of each genus expressed in terms of their prominence value (PV) was highest for *Helicotylenchus* with 225

followed by *Meloidogyne* with 151 (Table 2). The genera *Hemicycliophora* and *Paratrichodorus* showed the lowest PV of 8.

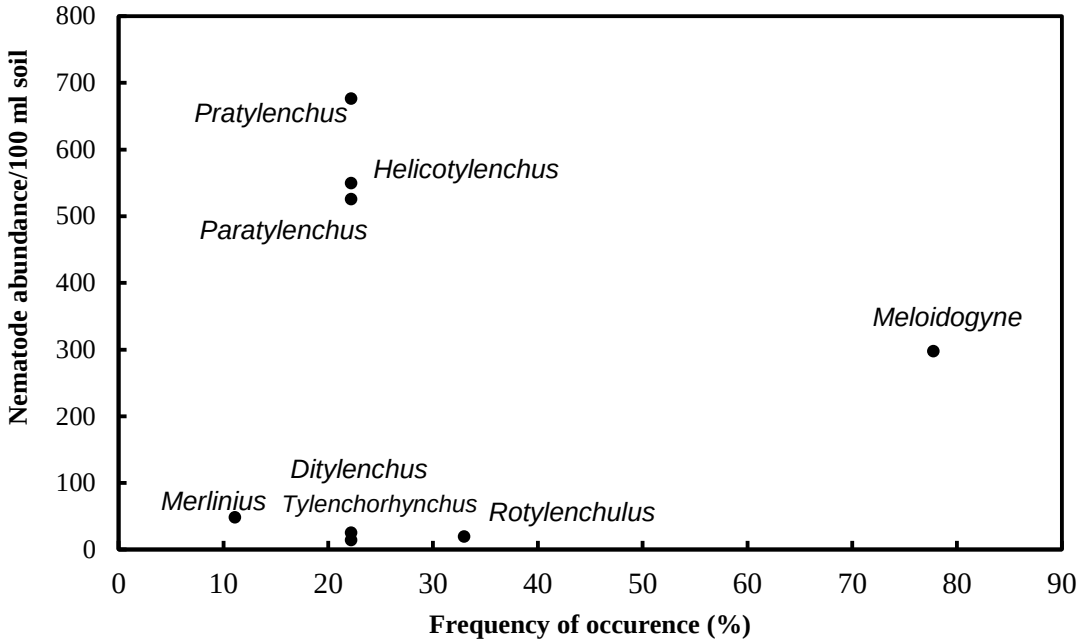

Figure 5. Frequency and abundance of major nematode genera recovered from cut-flowers during the dry season (April-May, 2012) from Ethiopia.

During dry season sampling, five out of the 13 nematode taxa found during the wet season were not detected at all, i.e. *Longidorus, Criconemella, Paratrichodorus, Hemicycliophora* and *Rotylenchus*. Overall, nematode frequency of occurrence was lower during the dry season compared with the wet season, except for *Meloidogyne* which occurred in 78% of all farms during the dry season but only in 46% of the farms during the wet season (Fig 5; Table 3). Frequencies of occurrence for the other genera during the dry season were 33% for *Rotylenchulus* and *Helicotylenchus* followed by 22% for *Ditylenchus, Paratylenchus, Pratylenchus* and *Tylenchorhynchus*, respectively. However, the mean and maximum population densities were much higher in the dry seasons than in the wet season (Fig 5; Table 3). The highest population density was achieved by *Pratylenchus* with 1090 nematodes/100 ml soil followed by *Helicotylenchus* with 1082 nematodes/100 ml soil and *Paratylenchus* with 918 nematodes/100 ml soils (Table 3). The highest prominence values were achieved for *Pratylenchus*, *Paratylenchus* and *Meloidogyne* with PV values of 1008, 862 and 829, respectively.

2.3.2 *Effect of crop species on nematode taxa*

For both surveys all five cut-flowers were found to host at least one nematode genus (Table 2 and 3). Rose plants hosted all nematode genera found, while statice hosted five genera, i.e. *Helicotylenchus*, *Paratylenchus*, *Pratylenchus, Rotylenchulus* and *Merlinius*. Gypsophila and carnation were hosts for *Helicotylenchus*, *Criconemella* and *Pratylenchus,* respectively. In freesia, only *Helicotylenchus* was found.

2.3.3 *Effect of altitude on nematode occurrence*

Altitude did seem to affect nematode distribution (Table 4). During the wet season 69.2% of all recorded genera were found at an altitude of 1900-2100 m, followed by 53.8% at altitudes ≤ 1600 m and 38.4% at altitudes ≥ 2300 m. For the dry season sampling all recorded nematode genera (= 100%) were detected at 1900-2100 m, compared with 87.5% of the genera detected ≥ 2300 m and 25.0% of the genera detected ≤ 1600 m. *Rotylenchulus* and *Meloidogyne* were the only two genera recovered from all altitudinal ranges during both sampling seasons, while *Rotylenchus* and *Paratrichodorus* were only recovered from the lowest altitude range (≤ 1600 m). Other genera such as *Pratylenchus*, *Paratylenchus* and *Tylenchorhynchus* only occurred at altitudes above 1900 m.

2.3.4 *The biotest*

The biotest using rose as bait plants detected *Meloidogyne* and *Tylenchorhynchus* in various soil samples. *Meloidogyne* occurred exclusively in soil samples collected from roses and was even detected in soils from three farms (JTC, DGD and AGF) during the wet season sampling where they had not been detected initially by direct extraction.

Table 2. Frequency of occurrence, population density per 100 ml soil and prominence value of major nematode genera recovered during wet season from the soil of cut-flowers in Ethiopia.

Sampling district	Altitude	Farm code	Host plant	Average number of nematodes per 100 ml soil												
				Criconemella	*Ditylenchus*	*Helicotylenchus*	*Hemicycliophora*	*Longidorus*	*Meloidogyne*	*Merlinius*	*Paratrichodorus*	*Paratylenchus*	*Pratylenchus*	*Rotylenchulus*	*Rotylenchus*	*Tylenchorhynchus*
Ziway	1646	HRB	Rose	36	–	58		44	178	–	18	–	–	42	34	–
Debre Zeit	1883	YSN	Rose	24	–	38	18	92	–	–	–	92	66	26	–	–
	1887	DGD	Rose	46	–	64	–	74	+	–	–	72	34	–	–	20
	1888	JTC*	Rose	38	–	82	–	–	+	–	–	–	–	–	–	–
			Gypsophila	38	–	82	–	–	–	–	–	–	–	–	–	–
Sebeta	2097	GUN	Rose	–	–	–	–	–	26	–	–	–	–	–	–	–
	2092	ETH	Rose	–	–	26	–	–	–	–	–	34	16	–	–	–
Sendafa	2545	EMF	Carnation	–	–	389	–	–	–	–	–	–	94	–	–	–
Menagesha	2557	FLM	Rose	–	–	46	–	–	44	–	–	–	–	–	–	–
Holleta	2395	ETD	Rose	–	42	34	–	–	56	18	–	–	48	–	–	–
	2425	AGF	Rose	–	–	46	–	–	+	–	–	–	–	–	–	–
	2251	FYR	Rose	–	–	–	–	–	66	–	–	–	–	–	–	–
	2205	RET	Rose	–	–	–	–	–	52	36	–	–	–	–	–	–
Sululta	2611	FRS*	Statice	–	–	26	–	–	–	22	–	–	–	–	–	–
			Freesia	–	–	31	–	–	–	–	–	–	–	–	–	–
Frequency of occurrence (FO %)				31	8	77	8	23	46	23	8	23	38	15	8	8
Mean population density/100 ml soil				36	42	77	18	70	70	25	18	66	52	34	34	20
Maximum population density				46	42	389	18	92	178	36	18	92	94	42	34	20
Prominence value (PV)				63	37	225	16	106	151	38	16	100	101	42	30	18

*Farms with two cut-flower species were treated as one farm to calculate frequency of occurrence.

+ = frarms that were found positive after biotest

Table 3. Frequency of occurrence, population density per 100 ml soil and prominence value of major nematode genera recovered during dry season from the soil of cut-flowers in Ethiopia

Sampling district	Altitude	Farm code	Host plant	Average number of nematodes per 100 ml soil							
				Ditylenchus	*Helicotylenchus*	*Meloidogyne*	*Merlinius*	*Paratylenchus*	*Pratylenchus*	*Rotylenchulus*	*Tylenchorhynchus*
Ziway	1146	HRB	Rose	–	–	654	–	–	–	36	–
	1888	JTC*	Rose	–	–	22	–	–	–	8	–
Debre Zeit			Gypsophila	–	22	–	–	–	–	–	–
	1887	DGD	Rose	–	16	48	–	–	–	–	–
Sebeta	2097	GUN	Rose	34	–	–	–	66	–	–	–
	2092	ETH	Rose	–	–	42	–	–	–	–	22
Menagesha	2557	FLM	Rose	–	–	744	–	–	–	–	–
Holleta	2395	ETD	Rose	16	–	302	–	–	262	–	–
	2205	RET	Rose	–	–	280	48	–	–	–	–
Sululta	2611	SLT*	Statice	–	1082	–	–	918	1090	12	–
			Freesia	–	–	–	–	–	–	–	–
Frequency of occurrence (FO %)				22	33	78	11	22	22	33	11
Mean population density/100 ml soil				25	373	299	48	492	676	19	22
Maximum population density				34	1082	744	48	918	1090	36	22
Prominence value (PV)				37	818	829	51	862	1008	46	21

*Farms with two cut-flower species were treated as one farm to calculate frequency of occurrence

Table 4. Occurrence and distribution of plant-parasitic nematodes based on altitude

	≤ 1650 m[y]		1900 - 2100 m		≥ 2300 m	
Nematode genera	Wet	Dry	Wet	Dry	Wet	Dry
Ditylenchus	–	–	–	+	+	+
Criconemella	+	–	+ + +	–	–	–
Helicotylenchus	+	–	+ + + +	+	+ + + + +	+
Hemicycliophora	–	–	+	–	–	–
Longidorus	+	–	+ +	–	–	–
Meloidogyne	+	+	+	+ + +	++++	+ + +
Merlinius	–	–	–	+	–	+ + +
Paratylenchus	–	–	+ + +	+	–	+
Pratylenchus	–	–	+ + +	+ +	+ +	–
Rotylenchulus	+	+	+	+	+	+
Rotylenchus	+	–	–	–	–	–
Paratrichodorus	+	–	–	–	–	–
Tylenchorhynchus	–	–	+	+	–	+
Distribution (%)[z]	53.8	25	69.2	100	38.4	87.5

[y] **'–'** =Absent; + = positive detection. Each '**–**' or '+' represents individual farms).
[z]% distribution is given as the number of genera per altitudinal range to the total genera recovered in each respective sampling seasons.

2.4 DISCUSSION

The primary goal of this study was to investigate and develop baseline information on the incidence, population density and distribution of major plant-parasitic nematodes associated with cut-flowers in Ethiopia. Soil samples were taken from five cut-flower species grown at different geographical directions around Addis Ababa. Overall, thirteen plant-parasitic nematode genera were found. In general, distribution of nematode taxa does not seem to be in agreement with the previous cropping history.

All 13 genera of plant-parasitic nematodes occurred on roses. This can partly be attributed to the fact that 71.5% of the sampled greenhouses grew roses whereas all other cut-flower species were only grown at a single farm. The spectrum of nematode genera found resembled those reported from other cut-flower producing areas in tropical regions. Nematode surveys conducted on roses in West and Central Java (Anwar *et al*., 1991) and in Rawalpindi (Marwoto, 1999) confirmed *Meloidogyne*, *Rotylenchulus*, *Helicotylenchus, Pratylenchus* and *Rotylenchus* as important parasites of cut-flowers.

Within this survey, the genus *Meloidogyne* predominantly represented by the species *M. hapla* seemed to be restricted to rose plants as it was not found on any of the other cut flower species. However, in preliminary greenhouse experiments we were able to show that all five cut-flower species included in our survey were hosts for *M. hapla* and *M. incognita*

(unpublished data). *M. hapla* is known to infect most commercial rose rootstocks (Pizetta *et al*., 2010). Regarding nematode control the use of resistance rose rootstocks is of great importance to the rose growers to keep the population below damaging level (Coolen and Hendrickx, 1972). Wang *et al*. (2004) reported the existence of variable host reaction of *Rosa multiflora* depending on the isolates of *M. hapla* used. On the other hand, *Rosa manetti* revealed no galling while *Rosa indica* was heavily galled by a single isolate of *M. hapla* (Voisin *et al*., 1996). In Ethiopia, breeding programs of roses focus on developing high yielding cultivars. However, the search for resistance cultivars to control root-knot nematodes, particularly *M. hapla*, might become a crucial issue in the near future.

Of the other cut-flower species, carnation and gypsophila are well known to be highly susceptible to tropical *Meloidogyne* species such as *M. javanica* and *M. incognita* (McSorley, 1994). Those tropical species are particularly found in the Ethiopian rift valley (O'Bannon, 1975) but have not yet been reported from the higher altitudes where cut-flowers are cultivated. However, as tropical *Meloidogyne* species most likely can establish in greenhouses even at higher altitudes, special care should be taken to avoid any introduction of those tropical *Meloidogyne* species into the cut-flower greenhouses by means of infected planting material and soil attached to machinery.

Besides *Meloidogyne*, *Pratylenchus* is the next most important plant-parasitic nematode causing severe losses in rose production (Peng and Moens, 2002). One such species is *P. vulnus* (Santo and Lear, 1976) which was also detected in this study. Under more temperate conditions, *P. penetrans* can be very damaging, too. Although not yet found in cut-flower producing greenhouses in Ethiopia, *P. penetrans* can easily be imported with contaminated planting material and can likely become established as well. This bears a major threat for rose production in Ethiopia as root-stock genotypes and rose accessions in general seem to be susceptible for *P. penetrans* (Peng *et al*., 2003; Coolen and Hendrickx, 1972). Besides roses, *P. penetrans* can also damage carnations (Arbelaez, 1999).

For all other plant-parasitic nematodes found in this survey, very little is known about their damage potential on roses. For example, *Paratylenchus* has been reported to parasitize cut-flowers in California (Braun and Lownsbery, 1975), but no information is provided on its economic damage. Within this study, *Paratylenchus parvus* was found to be associated with rose but at too low levels to cause damage. *Longidorus* was detected only during the wet season, again at low levels without causing damage. In general, *Longidorus* has long been known as a parasite of garden roses (Sher and Munnecke, 1958), but its damage potential is not clearly revealed.

Next to roses, statice was associated with five genera of plant-parasitic nematodes *vis*. *Helicotylenchus, Pratylenchus, Paratylenchus*, *Rotylenchulus* and *Merlinius*. However, none

of these nematode genera have been mentioned so far causing economic damage on statice. *Meloidogyne*, which has been reported as a parasite on statice (Otipa *et al*., 2003), was not encountered in our survey on this crop.

Comparison of nematode communities between the wet and dry seasons indicated some interesting differences. First of all, all 13 recorded nematode genera were detected during the wet season, but only eight out of those 13 genera during the dry season. Namely *Longidorus*, *Criconemella*, *Rotylenchus*, *Hemicycliophora* and *Paratrichodorus* did not occur during the dry season. These genera might be less adapted to the warmer temperature associated with the dry season. Another reason might be that they had been outcompeted by *Helicotylenchus*, *Meloidogyne*, *Paratylenchus* and *Pratylenchus* which all developed very well under the dry season conditions resulting in high nematode densities up to > 1000 specimen/100 ml soil. The fact that environmental conditions can form taxa composition is well documented (Abd-Elgawad *et al*., 2007).

Overall, mean nematode densities were higher in the dry season than in the wet season. This was especially the case for *Helicotylenchus*, *Meloidogyne*, *Paratylenchus* and *Pratylenchus*. Contrary to our results, Mekete *et al*. (2008) reported a lower population density of *Helicotylenchus* during the dry season compared with the wet season for coffee growing areas in Ethiopia. Similarly, low soil moisture was argued by Talwana *et al*. (2008) causing reduced nematode densities in cereals in Uganda. Presumably, those references refer to field studies where dry season conditions resemble low soil water content. However, for crops grown in greenhouses such as in the current study, soil water content is usually not limiting as plants get regularly watered. Therefore, the reason for higher nematode densities during the dry season compared with the wet season might be attributed to the higher soil temperatures which could have sped up nematode development resulting in more generations per year and overall higher population densities (Termohlen, 1967).

During the dry season, *Meloidogyne* and *Rotylenchulus* were the only two genera recovered from Ziway area which had the lowest altitude of all sampling sites. The region is characterized by mean temperatures between 20°C and 30°C and sandy soil texture, both providing favourable conditions for these two genera (Chirchir *et al*., 2008). At the same time, those warm temperatures are less conducive for roses making them more susceptible for nematode damage (Goff, 1936).

In conclusion, this study reports 13 genera of plant-parasitic nematodes to be associated with cut-flowers in Ethiopia. Most of these genera have also been reported from Ethiopia in association with other host plants (Bogale *et al*., 2004, Mekete *et al*., 2008, Wondirad and Mekete, 2002). However, the species *M.hapla*, *P*. *vulnus* and *H*. *digitatus* are reported here as first findings for Ethiopia. Out of those, *M. hapla* is considered the most important species

already causing economic damage on roses in terms of reduced plant growth and poor quality of the stem. Although the cut-flower production industry in Ethiopia is still young and production rates at most sites are still good, awareness for nematode problems needs to be strengthened to avoid damaging nematode levels in the near future. Besides, control strategies should be implemented where needed such as growing green manure crops between two cut-flower crops, screening for resistant genotypes or applying biocontrol agents.

2.5 REFERENCE

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Chapter Three

3. Molecular and morphological characterisation of *Meloidogyne hapla* populations from Ethiopia*

*Meressa, Beira-H., Heuer , H., Dehne H-W. and Hallmann, J. (2014). Molecular and morphological characterisation of *Meloidogyne hapla* populations (Nematoda: Heteroderidae) from rose greenhouses in Ethiopia. Nematology (submitted).

ABSTRACT

Meloidogyne hapla, considered mainly a temperate nematode species, was recently detected parasitizing rose plants in greenhouses in Ethiopia. Since the last ten years the floriculture industry of Ethiopia is rapidly growing and became a major investment area attracting many foreign investors. Roses are grown in more than 80% of the existing cut-flower producing farms. Rose production is increasingly facing serious nematode problems. Consequently, a nematological survey was carried out in 2011 and 2012. Soil samples were collected from greenhouses randomly selected from 12 farms distributed in six districts around the capital Addis Ababa. Pure nematode cultures of *M. hapla* were established for each farm rearing single egg masses on tomato cv. Moneymaker. Molecular identification of *M. hapla* was based on the 28S D2-D3 expansion segments within the ribosomal DNA and the region located between cytochrome oxidase unit II and the 16S rRNA gene of the mitochondria (mtDNA). In addition, light and scanning electron microscope images together with morphometric measurements of females, males and second-stage juveniles (J2) were compared with populations of *M. hapla* from different countries. In general, morphological characters of females, males and J2 were in line with descriptions of other *M. hapla* populations but few exceptions in morphometric measurements occurred. The female perennial pattern of the Ethiopian populations fitted the original description of the species with punctuations but shorter of vulva slit length. J2 body size was larger compared to previous descriptions from Hawaii and East Africa. Values of hyaline tail length were similar to the description of Jepson but higher than the Hawaiian population, and the *a* ratio value was much greater than for the East African population but similar to the Hawaiian population. The male spear length was typical of the species. Phylogenetic relationships of the Ethiopian *M. hapla* population with other related *Meloidogyne* species on the bases of both mtDNA and D2-D3 expansion segment sequence analysis revealed highly supported clades containing the Ethiopian isolates and other published isolates of *M. hapla* from different countries. The Ethiopian isolates shared 83-100% sequence similarity values of the D2-D3 region with isolates retrieved from the GenBank and newly obtained sequences from Germany and the Netherlands.

3.1 INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are endoparasites that are spread worldwide causing severe damage on many economically important crops. They are particularly a problem on greenhouse crops including ornamentals (Nagesh and Reddy, 2005). Currently, the genus comprises more than 90 species (Hunt *et al*., 2005). Out of these, the three known tropical species *M*. *arenaria*, *M*. *javanica* and *M*. *incognita* have been previously reported from open fields in Ethiopia (O'Bannon, 1975). These species are responsible for most nematode damage on most agricultural crops in the tropics (De Waele and Elsen, 2007). Information regarding nematode problems in greenhouses in Ethiopia is almost non-existent. Nevertheless, cut-flower growers in Ethiopia repeatedly reported plant damages most likely caused by plant-parasitic nematodes, but this was never followed-up. For this reason a survey was conducted in cut-flower greenhouses in 2011 and 2012 to monitor the occurrence and distribution of plant-parasitic nematodes (Meressa *et al*., 2012). This survey indicated among others increased densities of root-knot nematodes with *M. hapla* being the prominent species. Regardless of some optional strategies (Zijlstra *et al*., 2000; Orui, 1988), accurate species identification is required before any control measures can be started. Hence, the use of both morphological and molecular analysis is important for reliable diagnostics of the species (Onkendi and Moleleki, 2013; Birithia *et al*., 2012; Luca *et al*., 2011; Blok *et al*., 2002). Furthermore, since *M. hapla* has only recently reported from Ethiopia (Meressa *et al.,* 2014), a comparison of the Ethiopian *M. hapla* population with *M. hapla* populations from other regions of the world might facilitate future diagnostics and control programs.

Consequently, the objectives of this study were (i) to characterize the Ethiopian *M*. *hapla* population based on morphological and molecular data; (ii) to compare the Ethiopian isolates of different localities and with populations from other countries based on morphology and morphometry; and (iii) to reconstruct phylogenetic relationships with *M. hapla* populations of different geographical origin and other *Meloidogyne* species.

3.2 MATERIALS AND METHODS

3.2.1 *Nematode isolates and pure culturing*

All Ethiopian *M. hapla* isolates were obtained from soils associated with rose plants cultivated in greenhouses in different parts of Ethiopia. A list of the farm codes and localities is given in Table 1. From each farm, ten soil samples of 40 soil cores each were collected in August 2011. After thorough mixing, 200 ml soil per farm was shipped to Germany in order to establish pure nematode cultures for further analysis. To sustain such pure cultures,

nematodes were first propagated on roses as their original host and then on tomato cv. Moneymaker for experimental ease and mass production. All propagation was done in the greenhouse at 20 ± 3°C and 14 hour photoperiod. For each farm, the 200 ml soil sample was thoroughly mixed with 3.8 l steam treated field soil to fill up two 2 l pots into which a two months old rose (*Rosa corymbifera*) seedling per pot was transplanted.

Two months later, rose plants were assessed for the development of root galls to confirm *Meloidogyne* establishment. From plants that developed root galls, about 100 ml of soil was drawn and mixed with steam sterilized field soil and silver sand (2:1, v:v) and filled into 1 l pots into which a one month old tomato seedling cv. Moneymaker was transplanted.

After tomato plants were grown for 2 months, roots were checked for galling. Six out of the nine populations (hereafter each population representing one farm) formed galls and were further processed. For each population 20-60 egg masses were randomly handpicked. Individual egg masses were then placed in 1.5 ml distilled water into wells of a 24 well cell culture plate (Greiner Cellstar®, Frickenhausen, Germany) and kept at room temperature to stimulate juvenile hatch. Each well was daily observed under the stereomicroscope to evaluate the hatching level. Egg masses that gave hatch to more than 50 juveniles within 3 days received a farm code followed by consecutive isolate number. Hence, each of the six farms was represented by 7 to 51 isolates to make a total of 125 isolates. Juvenile suspensions from each well were then individually pipetted with a micropipette (Eppendorf GmbH, Hamburg, Germany) and injected into two holes made around the stem of two weeks old tomato seedlings grown in 100 ml pots filled with a mix of steam sterilized field soil and silver sand (1:1, v:v).

Table 1. *Meloidogyne hapla* isolates used for molecular analysis in this study

Locality	Altitude m a.s.l	Farm code	Isolate code	Sequence	
				mtDNA	28S D2-D3
Holleta	2300	STR	STRB4	√	√
			STRB6	√	√
		FYR	FYRB60	√	√
			FYRB62	√	-
			FYRB66	√	√
			FYRB67	√	√
		ETD	ETDB44	√	√
			ETDB47	√	√
			ETDB54	√	√
Menagesha	2300	FLM	FLMB18	√	√
			FLMB24	√	√
Sebeta	2100	GUN	GUNB26	√	√
			GUNB42	√	√
Ziway	1600	HRB	HRBB82	√	√
			HRBB95	√	√
			HRBB98	√	√
			HRBB100	√	√
			HRBB103	√	√
			HRBB107	√	√
			HRBB112	√	√
			HRBB115	√	√
Germany	–	JKI	JKI	√	√
Netherlands	–	PPS	11	–	√
	–	PPS	C3093	–	√

Seventy-five days later, six egg masses per isolate were handpicked with forceps from each tomato root system using a stereomicroscope and placed in 1.5 ml double deionised water into wells of a 24 well cell culture plate to stimulate juvenile hatch. J2s that hatched within 24 hours were then pipetted into 1.5 ml Eppendorf tubes and stored at 4^{o}C until needed for DNA extraction.

3.2.2 *Morphology and morphometric analysis*

Following the result of molecular analysis, nine *M. hapla* isolates *viz*. STRB6, FYRB60, FYRB62, ETDB47, FLMB18, GUNB26, HRBB82, HRBB100 and HRBB107 representing the different farms and regions were randomly selected for morphological and morphometrical studies. Males and J2s were killed with gentile heat, fixed in a solution containing 7 ml formalin (40% formaldehyde), 2 ml triethanolamine and 91 ml distilled water (Courtney *et al*., 1955) and processed to anhydrous glycerol over a period of 12 days through slow evaporation technique at 38-40^{o}C (Hooper, 1970). Permanent slide mounts were then made

after transferring the nematodes into anhydrous glycerol following the method developed by Hooper (1990).

For females, perennial patterns were prepared from eight individuals per isolate. Each female was handpicked from galled roots under a stereomicroscope and placed in a drop of water sitting on a microscope slide. The posterior end was cut off with a surgical blade and transferred to 45% lactic acid for 25 min before the inner surface was gently cleaned using a curved fine needle. The cuticle with the perennial pattern was then carefully trimmed. For each isolate, four perineal patterns were transferred to a drop of glycerine for permanent slide mounts. In addition, intact females were prepared for morphometric measurements. Therefore, galled roots were fixed in 3% formaldehyde for 48 hrs. At least eight females per isolate were handpicked from the root galls and transferred to a drop of water to make temporary slide mounts.

For light microscopy, all specimens were examined under a Leitz Diaplan compound microscope (Leitz, Wetzlar, Germany) at 630-1000× magnification. Morphometric measurements were recorded from digital images taken with Leica DC 180 camera equipped to the microscope and analysed with Leica IM500 image measurement software (Leica Microsystems AG, Germany). Light microscopic images were taken with the same camera. All measurements were in micrometers (µm) unless otherwise specified. All morphometric data were subjected to analysis of variance (ANOVA) using SPSS version 20 (SPSS, Inc., Chicago IL). Tukey's HSD (honest significant difference) test was used to compare the means at the 0.05 level.

Nematodes of isolate HRBB100 were processed for SEM. Specimens were fixed in Trump's fixative and dehydrated in a graded ethanol series, critical point dried (Balzers union, CPD 020), mounted on stubs, sputter-coated with gold (Balzers union, SCD 040) and examined with a Jeol JSM-840 scanning electron microscope.

3.2.3 *Molecular analysis*

3.2.3.1 DNA extraction and purification

Total DNA was extracted from the 125 isolates of root-knot nematodes in which each farm was represented by varying numbers of isolates *viz*. STR=7, FYR=18, ETD=13, FLM=18, GUN=18 and HRB=51. Nematode suspensions of 100 µl were pipetted onto a cover slip under a stereomicroscope. A minimum of 25 J2s were then handpicked into 10 µl double deionized water in 0.2 ml Eppendorf microtubes. The tubes were then spinned for 5 s to get nematodes fully covered with water. For DNA extraction, tubes were left open overnight to let the water evaporate. Next, 10 µl worm lysis buffer (500 mM KCl, 100 mM Tris–HCl pH 8.2,

15 mM $MgCl_2.6H_2O$, 10 mM dithiothreitol (DTT); 4.5% Tween-20) and 2 µl 20 mg/ml Proteinase K was added. Sterilized tooth sticks were used to crush the nematodes and mix the lysate gently. The lysate was then freezed at -80^oC for 20 minute and incubated at 63^oC for 1 hr followed by 95^oC for 15 min to stop the activity of Proteinase K.

Total DNA was precipitated from the final aqueous phase by addition of 40 µl isopropanol which was pre cooled to -20^oC by centrifugation at 13,200 g for 30 min at 4^oC. The DNA was then washed by centrifugation at 13,200 g for 15 min with 30 µl of 70% ethanol, air-dried and re-suspended in 35 µl of 1x TE buffer (10 mM Tris and 1 mM EDTA at pH 8).

3.2.3.2 Polymerase Chain Reaction (PCR) assay

The first primer set used here were those described in Powers and Harris (1993). Primers C2F3 (5'-GGTCAATGTTCAGAAATTTGTGG-3') and 1108 (5'-TACCTTTGACCAATCAC GCT-3') amplify the region between the mitochondrial cytochrome oxidase subunit II gene and the large (16S) ribosomal gene. The other set of primers D2A (5'-ACAAGTACCG TGAGGGAAAGTT-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') used were to amplify the D2-D3 expansion region of the 28S rRNA gene (Baldwin *et al*., 1997). All primers were supplied by Eurofins Genomics, Ebersberg, Germany. The overall PCR mix (25 µl) contained 3 µl DNA template, 2.5 mM each dNTPs, 1 µl 10 µM each primer, 2.5 µl 10x Dream Taq™ buffer and 1 U of Dream Taq™ DNA polymerase (Fisher Scientific Inc., Schwerte, Germany). The COII-16S PCR reaction was set for heating at 94^oC for 3 min followed by 35 cycles of amplification at 94^oC for 2 min, 54^oC for 90 s, and 72^oC for 1 min with a final incubation for 5 min at 72^oC. For the D2-D3, all conditions were the same as above except that the annealing temperature was 56^oC and the timing for the extension phase was extended to 2 min. All PCR reactions were run in Applied Biosystem®2720 Thermal cycler (Foster City, California, USA). The amplified products were separated on 1.0% agarose gels in 1x TBE buffer at 5 V/cm for 1 hr, stained with 0.0001% ethidium bromide, and visualized at UV-light (Bio-Rad Laboratories GmbH, Munich, Germany).

3.2.3.3 COII-16S PCR-RFLP

The PCR product was first purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instruction. Standard restriction digestions were carried out on 5 µl of the PCR product, 1.5 µl 10x Tango buffer and 1 µl of either *DraI*, *AluI* or *HinfI* restriction enzyme (Fisher Scientific Inc.) in a final volume of 15 µl for 16 hours at 37^oC in Thermomixer Comfort Eppendorf (Eppendorf GmbH, Hamburg, Germany). The digested product was then separated on a 2.0% 1xTBE buffered agarose gel at 3.5 V/cm for 90 min

and visualized under UV-light and photographed. The literal lengths of each restriction fragment of the PCR products were further determined by a virtual digestion of the sequences using WebCutter 2.0 (www.firstmarket.com/cutter/ cut2.html).

3.2.3.4 Cloning and sequencing

The PCR products of both genes amplified by using COII-16S and D2-D3 primer sets were either sequenced directly or of cloned products. Prior to sequencing or cloning, PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instruction. For direct sequencing, 5 µl of the PCR products and 5 µl of 10 pmole/µl of the respective forward primers were mixed. Cloned PCR products on the other hand were obtained by cloning the PCR products into pGEM®-T Easy vector. JM109 high efficiency competent cells were used for transformation of the ligation product. Plasmid DNA from the bacteria culture was purified using PureYield™ Plasmid Miniprep System (Promega GmbH, Mannheim, Germany). At least two representative isolates from each rose farm were randomly selected for sequencing in one direction. Sequencing was performed at the Macrogene sequencing facility service (Amsterdam, The Netherlands).

Table 2. GenBank accession numbers for reference sequences used for phylogenetic analysis

Nematode species	COII-16S of mtDNA	Reference and origin of country	28S D2-D3	Reference and origin of country
Meloidogyne arenaria	FJ159610	Fargette *et al*., 2010; Australia	KF112873	Zeng and Huang, 2013; China
	AY942848	Tigano *et al*., 2006; Brazil	KF482372	Carneiro *et al*.,2013; Brazil
M. ethiopica	JN673275	Maleita *et al*.2012; Portugal	–	–
M. enterolobii	–	–	JN005866	–
M. graminis	–	–	JN019339	McClure *et al*., 2012; USA
	AY539839	Handoo *et al*., 2005; Hawaii	DQ145641	Nadler *et al*.,2006; Netherlands
	AY757902	Powers *et al*.,2005; USA	DQ328685	Subbotin *et al*., 2005; Moldova
	AY757903	Powers *et al*.,2005; USA	JN005873	Hu *et al*., 2011; China
	FJ159608	Fargette *et al*.,2010; Australia	GQ130139	Wang et.al.,2009;China
	AY757889	Powers *et al*.,2005; USA	–	–
	AY757887	Powers *et al*.,2005; USA	–	–
	AY757901	Powers *et al*.,2005; USA	–	–
	AY757899	Powers *et al*.,2005; USA	–	–
	L76262	Hugall *et al*.,1997; Australia	–	–
	AY757900	Powers *et al*.,2005; USA	–	–
M. hapla	AY942850	Tigano *et al*.,2006; Brazil	–	–
M. hispanica	JN673274	Maleita *et al*.2012; Portugal	EU443606	Landa *et al*.,2008; Spain
M. incognita	FJ159628	Fargette *et al*., 2008; Australia	JX100424	Wu *et al*.,2012; Taiwan
M. javanica	JX100439	Wu *et al*., 2012; Taiwan	KC953092	Wang *et al*.,2013; China
M. marylandi	–	–	JN019363	McClure *et al*.,2012; USA
M. partityla	AY672412	Thomas *et al*.,2004; USA	–	–
	AY757908	Powers *et al*.,2005; USA	–	–
M. thailandica	–	–	EU364890	Skantar *et al*.,2007; USA
Bursaphlenchus xylophilus	JQ423194	Valadas *et al*.,2012; Portugal	HM623784	Gu and Wang, 2010; China
Pratylenchus coffeae	–	–	KC857663	Tuyet *et al*., 2013; Vietnam

3.2.4 *Phylogenetic analysis*

Newly obtained sequences of both mtDNA (COII-16S) and D2-D3 expansion segment of 28S rRNA genes plus related published sequences from GenBank were used to reconstruct the phylogenetic trees. *Bursaphlenchus xylophilus* (JQ423203) for mtDNA (COII-16S) and *B. africanus* (HM623784) and *Pratylenchus coffeae* (KC857663) for D2-D3 were chosen as outgroup taxa. Raw sequences obtained were first proofread in Chromas Lite version 2.1 (Technelysium Pty Ltd., 2012) to exclude incorrect base calls before multiple alignment was performed. Consensus quality sequences were then subjected to BLAST engine (Altschul *et al*., 1990) for sequence similarity search in GenBank, NCBI database as given in Table 2. All the new sequences and sequences from the GenBank were aligned using ClustalX version 2.1 (Larkin *et al*., 2007) with default parameters. All sequences were trimmed to equal length using alignment editor in MEGA5 (Tamura *et al*., 2011).

Pairwise distance between *M. hapla* isolates from Ethiopia and isolates from the Netherlands (PPSC3093; PPS11), Germany (JKI-de) and sequences in GenBank from China (JN005873; GQ130139) and Moldova (DQ328685) of D2-D3 sequences was analysed by MEGA5 (Tamura, *et al*., 2011).

The variability in nucleotide position was compared. The alignment was manually edited by GeneDoc multiple sequence alignment editor (Nicholas and Nicholas, 1997), in which all sequences were trimmed to equal length.

The phylogenetic analysis was carried out with Maximum Likelihood (ML) and Maximum Parsimony (MP). ML analysis was performed using heuristics searches with Nearest-Neighbor-Interchange (NNI) branch swapping filter. The MP analysis was performed using heuristics searches with Tree-Bisection-Reconnection (TBR) with random addition of 10 sequence replicates. In each case, both gaps and missing data were also considered in the Tamura-Nie Model. The support for each branch was estimated using the bootstrap (bs) method with heuristics search and 1000 replicates in both ML and MP analysis in MEGA5 (Tamura *et al*., 2011). Values of the relative base frequencies, gamma distribution shape parameter, and base substitution rates were also estimated in MEGA5.

3.3 RESULTS

3.3.1 *Morphology and morphometric analysis*

3.3.1.1 Comparisons between Ethiopian isolates

Meloidogyne hapla from Ethiopia was determined by morphological and morphometrical analysis of females, males and second-stage juveniles (Table 3-5; Fig. 1 and 2). For

females, the perineal pattern had generally ovoidal shape with fine striae forming a wavy pattern on one side. Subcuticular punctuation round the tail terminus appeared from more dispersed to well concentrate manner (Fig. 1B). However, no punctuation was observed on SEM micrographs (Fig. 2G, H). The males had apparent set off head region and set off rounded but with varying size of stylet knob (Fig. 1F; 2A, B). In J2, most qualitative characters were similar to previous descriptions (Jepson, 1987) (Fig. 1E; 2J-M) with various tail shapes. Lateral lines were four in number (Fig. 1D).

Morphometrics for females, males and J2 of nine selected isolates representing six farms (populations) are presented in Table 3-5. Comparisons of morphometrics revealed intra-population variations in some characters. Female body length (P= 0.001), body width (P= 0.02), stylet length (P= 0.04) and distance between DGO and stylet base (P= 0.01) significantly varied between the Ethiopian isolates. Isolate ETD47 showed the greatest body length (706.9 µm) and body width (456.2 µm) of all the isolates but on the other hand the shortest DGO length from stylet base (4.1 µm). On the contrary, isolate HRBB100 had the smallest body length (528.6 µm) and body width (341.4 µm) but the highest DGO length froms the stylet base (5.2 µm).

Males of different isolates also showed differences, particularly in body length (P=0.015), head height (P=0.01), stylet length (P=0.001), body width at anus (P<0.001) and spicule length (P=0.04). The highest variation in male body length was found between isolates from closely located farms, e.g. FYRB60 had a significantly greater mean body length (1355.6 µm) than ETDB47 (1112.2 µm) and STRB6 (1143.4 µm). Generally, stylet length seems to be greater for those isolates obtained from the warmer than those from the cooler region. Isolate HRBB100 had the lowest spicule length (20.1 µm) and body width at anus level (10.9 µm) of all isolates. However, another isolate of the same farm, HRBB107, had greatest spicule length (27 µm) but a lower head height than HRBB100 (3.8 µm vs. 5 µm). No males were found from isolate FYRB62.

Second-stage juveniles showed significant differences in body length (P<0.001), head height (P=0.03), center of median bulb from anterior end (P<0.001), stylet length (P= 0.01), distance of DGO from stylet the base (P=0.002), tail length (P= 0.02), tail width at hyaline portion (P=0.01), *a* ratio (P<0.01) and caudal ratio A (P=0.01). Isolate STRB6 from the highland region had a lower average body length, center of median bulb from anterior end, excretory pore to anterior end, DGO from base of stylet and tail length than the rest of the isolates. On the contrary, HRBB107 of the lowland region had the greatest average body length, center of median bulb from anterior end, excretory pore to anterior end, DGO from base of stylet, tail length and *a* ratio of all isolates. Isolate HRBB107 and STRB6 were basically collected from regions of 1600 and 2300 m a.s.l., respectively.

The Ethiopian isolates were generally collected from localities located between altitudes ranging from 1600-2500 m a.s.l. and varying mean annual temperatures. However, lack of consistency for variation within isolates from the same farm may explain that the differences seen are not strong enough to explain the influence of geographical deference

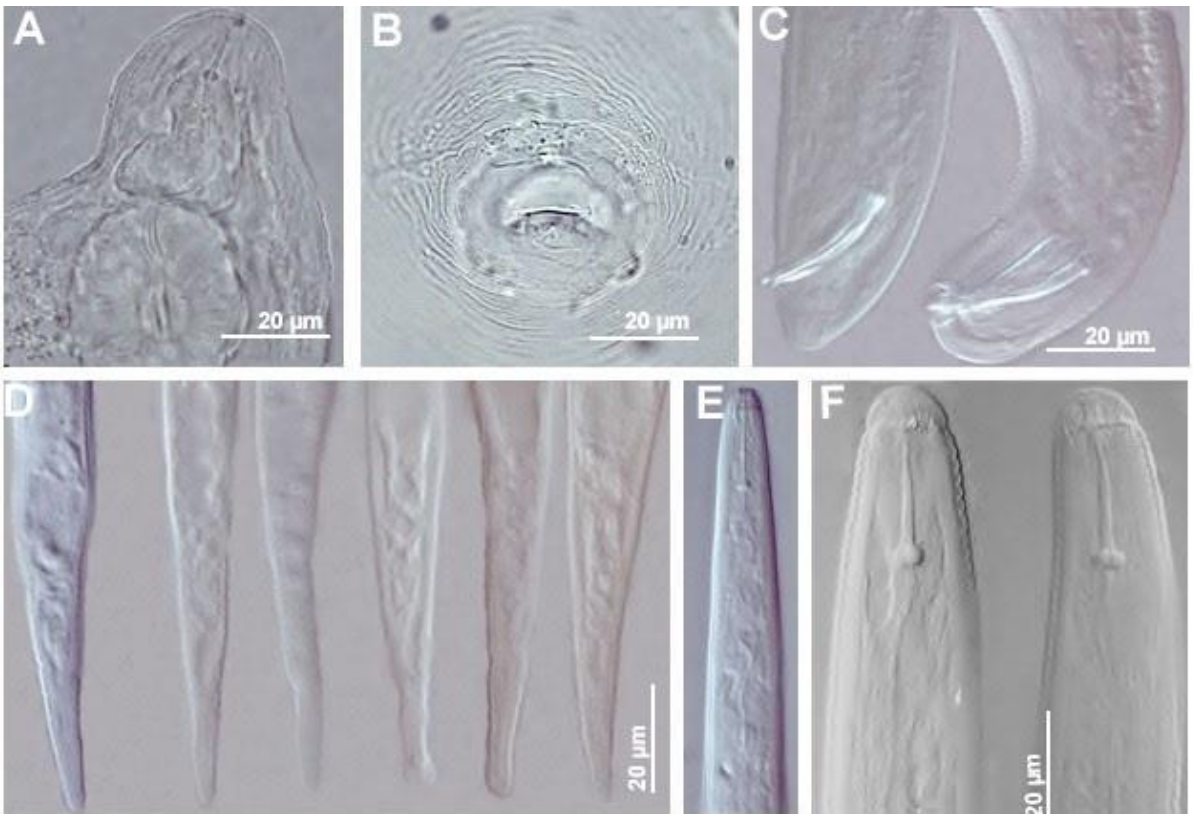

Figure 1. Light micrographs of *Meloidogyne hapla* isolate isolate HRBB100*.* Female anterior body part (A) and perineal pattern (B), male tail and spicule (C), J2 tail types (D), anterior part of J2 (E) and male (F).

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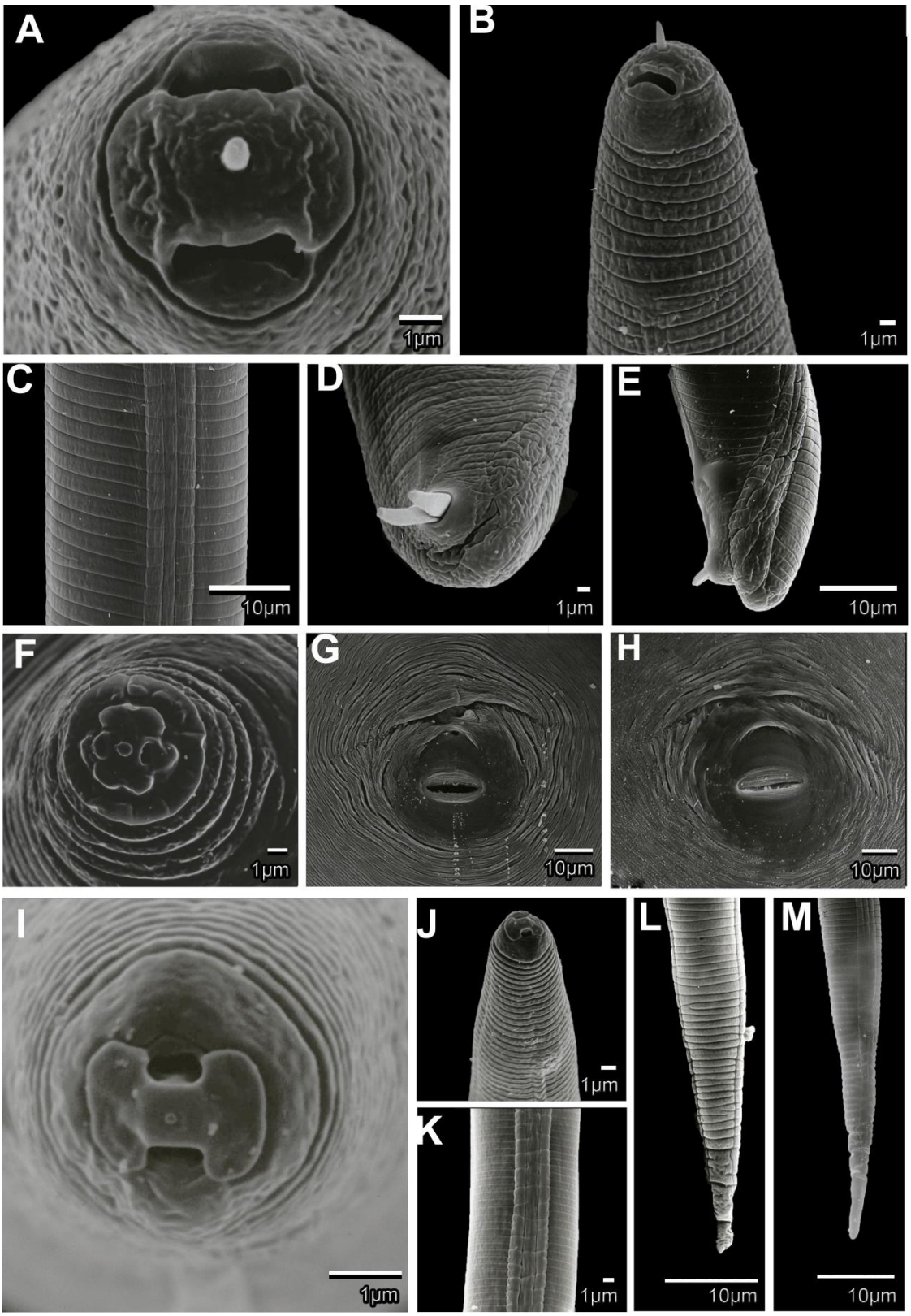

Figure 2. Scanning electron micrographs of *Meloidogyne hapla* isolate HRBB100. Male face view (A) head lateral view (B), lateral field (C) and tail, spicule (D, E). Female face view (F) and typical perineal pattern (G, H). Juvenile face view (I), head lateral view (J), lateral field (K) and tail (L, M).

Table 3. Morphometrics comparison of females of Ethiopian population of *Meloidogyne hapla* (n=45).

Morphological character[z]	*M. hapla* isolates								
	HRBB100	**HRBB107**	**FYRB60**	**FYRB62**	**HRBB82**	**ETDB47**	**FLMB18**	**GUNB26**	**STRB6**
Body length	528.6 ± 42.4^{c} (486–574)	549.8 ± 74.8^{bc} (442–640)	551 ± 40.8^{bc} (512–605)	596 ± 83.2^{bc} (463.8–685.8)	638.6 ± 54.1^{ab} (582.5–704)	706.9 ± 101.3^{a} (606–877.6)	579.8 ± 99.2^{bc} (482.8 –718)	520.2 ± 53^{c} (468–587)	523.1 ± 31.7^{c} (481.6–551)
Body width	341.4 ± 46^{b} (284–412)	397.8 ± 55^{ab} (344–485)	410 ± 56.3^{ab} (330–478)	423.4 ± 59.7^{a} (362.6–510.2)	443 ± 42.5^{a} (378.4–485)	456.2 ± 47.3^{a} (376–493)	407.2 ± 52.7^{ab} (369–498)	399.6 ± 59.5^{ab} (356–500.5)	410.9 ± 23.2^{ab} (380–434.9)
Neck width	101.2 ± 21.6 (68.4–124)	102.8 ± 36.8 (53.2–148)	98.2 ± 15.8 (71.4–112)	109.1 ± 21.1 (79.5–138)	78.1 ± 7.8 (65.7–84.9)	113.9 ± 20.1 (94–141.5)	76.2 ± 18.2 (51–99.7)	75.1 ± 7.7 (64–85.5)	90.9 ± 10.1 (75.8–104.3)
Stylet length	14.7 ± 0.9^{ab} (13.8–16)	16.1 ± 1.5^{a} (14–17.6)	14.7 ± 1.7^{ab} (13–17.4)	15.1 ± 2.3^{ab} (12.4–17.7)	13.4 ± 0.5^{b} (12.8–14)	14.3 ± 1.2^{ab} (13.2–16.2)	13.5 ± 0.6^{b} (12.9–14.2)	14.7 ± 1^{ab} (13.8–16.2)	13.6 ± 1^{b} (12.8–15)
Stylet knob height	3 ± 0.1 (2.8–3.2)	2.7 ± 0.4 (2.2–3)	2.4 ± 0.3 (2.2–3)	2.7 ± 0.2 (2.4–3)	2.4 ± 0.5 (2–3)	2.4 ± 0.1 (2.2–2.5)	2.8 ± 0.2 (2.5–3)	2.8 ± 0.3 (2.4–3)	2.2 ± 0.2 (2–2.5)
Stylet knob width	3.5 ± 0.5^{a} (3–4)	3 ± 0.1^{c} (2.8–3.2)	3.2 ± 0.3^{abc} (3–3.6)	3.4 ± 0.4^{ab} (3–4.1)	3 ± 0.1^{bc} (3–3.2)	3 ± 0.1^{bc} (3–3.2)	3 ± 0.1^{bc} (3–3.2)	3.1 ± 0.3^{bc} (2.9–3.6)	2.9 ± 0.3^{c} (2.5–3.2)
DGO from stylet base	5.2 ± 0.4^{a} (5–6)	5.1 ± 0.2^{a} (4.8–5.4)	5.4 ± 0.6^{a} (5–6.2)	5.1 ± 0.2^{a} (5–5.4)	4.9 ± 0.2^{ab} (4.6–5)	4.1 ± 0.6^{c} (3.4–5)	4.8 ± 0.4^{ab} (4.1–5)	4.4 ± 0.5^{bc} (4–5)	5.4 ± 0.5^{a} (5–6)
Excretory pore from anterior end	33.4 ± 5.8) (28–41)	31.4 ± 4.6 (26–37)	29.4 ± 5.2 (23–35)	34.2 ± 3 (31.4–38.4)	30.4 ± 4.4 (25–36)	34 ± 4.4 (29.7–41.3)	33.5 ± 5.8 (27–42)	30.8 ± 5.2 (26.3–39.4)	30.1 ± 4.9 (23–36.4)
Distance between phasmids	21.3 ± 1.8 (20–24)	20.7 ± 3.7 (14.5–23.2)	18.7 ± 1.2 (17.4–20)	21.8 ± 2.2 (19.8–24.4)	20.6 ± 3.1 (18–26)	19.3 ± 2.5 (16.4–23)	18.6 ± 1.8 (16.8–21.6)	20.6 ± 1.3 (18.6–21.8)	20 ± 1.2 (18.6–21.6)
Vulva slit length	19 ± 2 (17–22)	18.2 ± 1.6 (16–20)	18.9 ± 0.4 (18.4–19.2)	20.2± 2.4 (17.6–24)	18.4 ± 2.1 (16.3–21)	18 ± 3.7 (13–21)	19.9 ± 2.5 (16.8–23.7)	19.2 ± 0.6 (18.2–20)	18.8 ± 0.4 (18.4–19.2)
Vulva-anus distance	16.6 ± 1.3 (15–18.4)	15.6 ± 1.9 (14–18.4)	16.5 ± 1 (15.8–18.2)	17.6 ± 3 (14.8–22.4)	15.8 ± 0.6 (15–16.6)	16.4 ± 2.1 (14–19)	18.6 ± 3.4 (14.4–23.6)	16.1 ± 1.5 (13.6–17.6)	17.1 ± 1.3 (15–18.4)
Distance from anus to tail terminus	18.9 ± 3.3 (14.9–22.8)	16.5 ± 2 (13.3–18.6)	15.3 ± 2 (12.8–18.4)	17 ± 1.5 (14.6–18.4)	16.5 ± 1 (14.9–17.4)	16.5 ± 2.7 (13.8–20.8)	17 ± 1.4 (15.2–18.4)	16.8 ± 1.1 (15.2–18.4)	16 ± 1.9 (13.8–18.4)
a ration	1.6 ± 0.1 (1.4–1.7)	1.4 ± 0.3 (1.1–1.7)	1.4 ± 0.1 (1.2–1.6)	1.4 ± 0.2 (1.2–1.6)	1.5 ± 0.3 (1.2–1.9)	1.6 ± 0.2 (1.4–1.8)	1.4 ± 0.1 (1.3 – 1.5)	1.3 ± 0.1 (1.2–1.4)	1.3 ± 0.1 (1.1–1.4)
Excretory pore/stylet length	2.3 ± 0.3 (1.9–2.6)	2 ± 0.3 (1.6–2.3)	2 ± 0.3 (1.6–2.4)	2.3 ± 0.5 (1.8–2.7)	2.3 ± 0.4 (1.9–2.8)	2.4 ± 0.4 (1.8–2.9)	2.5 ± 0.4 (2.1–3.2)	2.1± 0.5 (1.6–2.9)	2.2± 0.4 (1.5–2.6)

[z]Means in a row followed by the same letter are not significantly different at *P*=0.05, according to Tukey HSD Multiple comparison test

Table 4. Morphometrics comparison of **second-stage juvenile (J2)** of Ethiopian population of *Meloidogyne hapla* (n=45). All measurements are in µm as mean ± s.d. (range).

Morphological character[Z]	*M. hapla* isolates								
	STRB6	FYRB62	FLMB18	HRBB82	HRBB100	FYRB60	HRBB107	ETDB47	GUNB26
Body length	335.8 ± 16.5^{d} (315– 351)	373.24 ± 11.1^{bc} (355–385)	380.2 ± 18.8b^{c} (350–402)	361.8 ± 17.3^{bc} (340–380	360.7 ± 14.7^{bc} (345–375.4)	364.8 ± 9b^{b} (352.7–378.1)	407.4 ± 6.8^{a} (399–417)	368.1 ± 8.2^{b} (354–373)	374.7 ± 8.4^{c} (362.5–384.8)
Body diameter	11.6 ± 0.5 (11– 12)	12.6 ± 0.8 (12–14)	134 ± 0.4 (12.5–13.6)	11.8 ± 0.6 (11.2–12.6	11.8 ± 0.7 (10.8–12.8)	12.6 ± 1.5 (11–15)	11.7 ± 0.9 (10.4–12.6)	11.4 ± 0.8 10.2–12)	11.4 ± 1.6 (9.6–13.7)
Head width	4.3 ± 0.5 (3.8–5)	3.92 ± 0.1 (3.8–4)	4.36 ± 0.6 (3.8–5)	4 ± 0 (4–4.1)	4.3 ± 0.3 (3.9–4.6)	4.4 ± 0.5 (4–5)	4.3 ± 0.4 (4–5)	3.8 ± 0.3 (3.4–4)	4.6 ± 0.5 (4–5)
Head height	2.1 ± 0.1^{abc} (2–2.2)	1.96 ± 0.3^{bc} (1.4–2.2)	2.3 ± 0.4^{bc} (2–3)	2.1 ± 0.1^{abc} (2. 0–2.3	2.5 ± 0.2^{abc} (2.3–2.8)	1.76 ± 0.3^{ab} (1.4–2)	2.1 ± 0.3^{ab} (1.6–2.3)	2.1 ± 0.1^{c} (2–2.2)	2.2 ± 0.5^{a} (1.8–3)
Center of median bulb from anterior end	43.7 ± 4.7^{d} (40–51)	49.64 ± 2.6^{c} (45.6–52)	52.3 ± 3.5^{a} (47.6–55.7)	49.8 ± 2.2^{bc} (46.4–52	54.3 ± 2.1^{abc} (51.3–56.2)	53.1 ± 1.8^{abc} (51.2–55)	55 ± 2^{ab} (52–57)	52.3 ± 3^{abc} (48.8–57)	53.8 ± 3.1^{a} (48.8–56.4)
Excretory pore to anterior end	63.2 ± 3.5^{d} (60–68.8)	70.76 ± 1.8^{c} (68.8–73)	76.88 ± 2.5^{ab} (74.8–79.8)	71.6 ± 1.1^{ab} (70.4–72.8	71.1 ± 2.1^{bc} (68.8–74	75.3 ± 1.7^{ab} (73.6–78)	78.6 ± 3.7^{a} (76–84)	73.7 ± 3.9^{ab} (68.4–79.1)	75.5 ± 2.2^{c} (73.2–79)
Length of hyaline tail terminus	13.2 ± 2.6 (10–17)	138 ± 1.5 (11.2–15)	14 ± 1.5 (12–15.4)	13.8 ± 0.2 (13.6–14.1	14.6 ± 2.2 (12.8–18)	16.7 ± 2.9 (12.8–19.2)	13.3 ± 0.3 (13–13.6)	14.1 ± 1.9 (12–17)	13.1 ± 2.3 (11.2–17)
Stylet length	12.8 ± 0.3^{c} (12.4–13)	13.48 ± 0.7^{abc} (12.8–14. 4)	13.4 ± 0.9^{ab} (12–14)	13.3 ± 0.5^{abc} (12.8–14	12.9 ± 0.4^{c} (12.2–13.3)	14.2 ± 0.4^{abc} (14–15)	14.2 ± 0.4^{a} (14–15)	12.7 ± 1.7^{c} (10.4–14)	13.9 ± 0.2^{bc} (13.6–14)
DGO from stylet base	2.3 ± 0.3^{c} (1.9–2.5)	2.34 ± 0.2^{bc} (2.1–2.5)	2.64 ± 0.1^{abc} (2.5–2.7)	2.6 ± 0.1^{abc} (2.4–2.7	2.5 ± 0.2^{a} (2.2–2.7)	2.5 ± 0^{ab} (2.4–2.5)	2.6 ± 0.4^{a} (2.1–3.2)	2.84 ± 0.2^{bc} (2.5–3.1)	2.8 ± 0.2^{bc} (2.5–3.1)
Tail length	45.8 ± 2.5^{cd} (42–48.8)	47.28 ± 3^{abcd} (44–51)	46.92 ± 2.1^{abc} (44.4–49.6)	47.4 ± 1.7^{abcd} (44.8–49.6	47.4 ± 3.1^{d} (42.4–50.4)	50.4 ± 5.8^{bcd} (44.8–59.2)	51.5 ± 0.5^{a} (51–52)	44.8 ± 1.3^{ab} (43.2–46.4)	49.9 ± 3.9^{abcd} (45.2–55)

(Table 4 *Continued*)

Morphological character[Z]	***M. hapla* isolates**								
	STRB6	FYRB62	FLMB18	HRBB82	HRBB100	FYRB60	HRBB107	ETDB47	GUNB26
Body diameter at anus level	9.8 ± 1.2 (9–11.8)	9.24 ± 1.6 (8–12)	8.2 ± 1.5 (6–10)	9.5 ± 0.6 (9–10.2	8.6 ± 0.5 (8–9)	9.8 ± 0.5 (9–10.2)	9.5 ± 0.6 (8.8–10)	8.8 ± 0.8 (8–10)	9.5 ± 0.5 (9–10)
Tail width at hyaline portion	3.9 ± 0.7^{ab} (3.2–5)	3.92 ± 0.2^{ab} (3.6–4)	3.44 ± 0.5^{a} (3–4)	3.9 ± 0.3^{ab} (3.6–4.3	4 ± 0.1^{ab} (4–4.2)	3.6 ± 0.5^{ab} (3–4)	3.1 ± 0^{b} (3–3.1)	3.4 ± 0.5^{ab} (3–4)	4 ± 0.7^{a} (3–5)
Tail width 5µm from tail terminus	2.6 ± 0.5 (2–3)	2.6 ± 0.5 (2–3)	2.22 ± 0.4 (2–3)	3.1 ± 0.1 (3–3.2	3 ± 0.1 (2.9–3.2)	2.7 ± 0.3 (2.4–3)	2.6 ± 0.4 (2.1-3)	2.1 ± 0 (2–2.1)	2.2 ± 0.4 (2–3)
a ratio	29 ± 2^{c} (26.3–31.8)	29.7 ± 1.9^{c} (26.9 -31.6)	29.2 ± 2.3^{ab} (25.7–32.2)	30.6 ± 1.8^{bc} (28.3–33.3	30.7 ± 1.1^{ab} (29.3–31.9)	29.2 ± 3^{abc} (24.3–32.1)	35 ± 2.7^{a} (32.1–38.9)	32.3 ± 1.8^{c} (30.8–34.7)	33.3 ± 4.3^{bc} (27.7–37.8)
c ratio	7.4 ± 0.5 (6.5–7.7)	7.9 ± 0.7 (7.2–8.8)	8.1 ± 0.7 (7.4–9.1)	7.6 ± 0.2 (7.4–7.8	7.6 ± 0.5 (6.9–8.1)	7.3 ± 0.8 (6.4–8.1)	7.9 ± 0.1 (7.8–8)	8.2 ± 0.2 (8–8.5)	7.5 ± 0.7 (6.6–8.3)
c' ratio	4.7 ± 0.4 (4.1–5.1)	5.3 ± 1 (3.7–6.4)	5.9 ± 1.1 (4.5–7.4)	5 ± 0.4 (4.4–5.3	5.6 ± 0.5 (4.7–6.1)	5.1 ± 0.7 (4.5–5.9)	5.4 ± 0.3 (5.1–5.8)	5.1 ± 0.6 (4.5–5.8)	5.3 ± 0.3 (4.9–5.7)
Head width/height	2.1 ± 0.3^{ab} (1.8–2.5)	2.1 ± 0.5^{ab} (1.7–2.9)	2.2 ± 0.7^{ab} (1.7–3.3)	1.9 ± 0.1^{ab} (1.8–2	1.7 ± 0.2^{ab} (1.5–1.9)	2.6 ± 0.6^{ab} (2–3.6)	2.1 ± 0.3^{ab} (1.7–2.5)	1.8 ± 0.2^{b} (1.5–2)	2.2 ± 0.4^{a} (1.7–2.5)
Caudal ratio A	3.4 ± 0.7^{c} (2.6–4.3)	3.3 ± 0.4^{c} (2.8–3.8)	4.2 ± 1^{ab} (3–5.1)	3.5 ± 0.2^{bc} (3.2–3.8	3.6 ± 0.6^{abc} (3.2–4.5)	4.6 ± 1.1^{abc} (3.6–6.4)	4.4 ± 0.1^{c} (4.2–4.5)	4.2 ± 0.5^{a} (3.6–4.8)	3.3 ± 0.3^{bc} (2.9–3.7)
Caudal ratio B	5.3 ± 1.4 (3.3–7)	5.3 ± 1.8 (3.7–7.5)	6.4 ± 0.9 (5.1–7.4)	4.4 ± 0.2 (4.3–4.7	4.8 ± 0.7 (4.3–6)	6.1 ± 1.3 (4.3–7.9)	4.1 ± 2 (0.6–5.7)	7 ± 1 (5.7–8.5	6 ± 0.4 (5.6–6.4)

[Z]Means in a row followed by the same letter are not significantly different at *P*=0.05, according to Tukey's HSD Multiple comparison test

Table 5. Morphometrics comparison of male of Ethiopian population of *Meloidogyne hapla* (n=40). All measurements are in µm as mean ± s.d. (range).

Morphological character [Z]	***M. hapla* isolates**							
	HRBB100	HRBB107	HRBB82	ETDB47	FLMB18	GUNB26	FYRB60	STRB6
Body length	1189.6 ± 111.6^{b}	1148.4 ± 65.8^{b}	1258.4 ± 128.1^{ab}	1112.2 ± 66^{b}	1159.8 ± 135.1^{b}	1233.4 ± 80.8^{ab}	1355.6 ± 128.7^{a}	1143.4 ± 71.2^{b}
	(1056–1356)	(1070–1221)	(1053–1372)	(1032–1197)	(990–1307)	(1132–1308)	(1260–1568)	(1070–1234)
Body width	26.1 ± 5	26 ± 3.5	26.2 ± 1	26.3 ± 2.1	26.4 ± 4.9	24.9 ± 2.2	27.4 ± 2.6	27.8 ± 4
	(23–34.5)	(20–29)	(25–27.1)	(24–29)	(22–33.6)	(21–26.4)	(23.2–30)	(24–32.8)
Stylet length	21 ± 1^{a}	20.6 ± 1 ^{a}	20.8 ± 0.4 ^{a}	19 ± 1.1^{bc}	20 1.4^{bc}	20.6 ± 0.4^{a}	20.6 ± 0.7^{a}	18.6 ± 0.7^{c}
	(20–22)	(19.4–22)	(20–21)	(18–20.8)	(17.6–21)	(20–21)	(20–21.6)	(17.6–19.2)
Center of median bulb from head	85.7 ± 8.3	75.3 ± 10.4	80.5 ± 4.7	74.5 ± 6.9	75.7 ± 6.2	86.5 ± 7.2	92 ± 21.8	72.5 ± 2.7
	(71.2–91.4)	(58–84.8)	(75.2–87)	(64.8–84)	(69.6–84.8)	(79.2–96)	(75.2–128)	(68–74.4)
DGO from stylet base	4 ± 0.6	4.1 ± 0.4	4.2 ± 0.4	4.6 ± 0.4	4.3 ± 0.5	4.2 ± 0.5	4.3 ± 0.7	4.7 ± 0.5
	(3–4.8)	(3.4–4.6)	(4–5)	(4–5)	(3.8–5)	(3.4–4.8)	(3.6–5.2)	(4–5.2)
S-E pore to head end	130.4 ± 20.2	120.7 ± 14.3	124.8 ± 12.4	125.8 ± 11.2	130.9 ± 6.8	143. 4 ± 18.4	149.6 ± 14.2	134.7 ± 23.4
	(96–147)	(101–138.7)	(103.5–135.1)	(112–138.7)	(120.8–137.6)	(129.6–175)	(129–164)	(108–154.4)
Body width at anus level	10.9 ± 1.1^{b}	11.5 ± 2.4^{ab}	18.4 ± 2.1^{a}	18.8 ± 2^{b}	16.6 ± 2.9^{a}	18 ± 3.1^{a}	18 ± 1.9^{a}	19.8 ± 3.1^{a}
	(9.6–12.4)	(9–14)	(14.8–20)	(16.4–21)	(13–20)	(14–21)	(15–20)	(17–25)
Tail length	10.1 ± 0.8	9.8 ± 0.8	10.4 ± 1.5	10.8 ± 1.3	9.8 ± 1	9.8 ± 1.5	10.4 ± 1.9	9.6 ± 1.5
	(9–11)	(9–11)	(7.6–11.2)	(9–12)	(8–10.4)	(8–12)	(7.8–13)	(8.2–12)
(Spicule length	20.1 ± 5.7^{b}	27 ± 2.5^{a}	24.7 ± 1.4^{ab}	20.8 ± 1.4^{ab}	24.2 ± 3.3^{ab}	23.4 ± 3.2^{ab}	24.2 ± 3.7^{ab}	21.5 ± 2.6^{ab}
	(14.4–28)	(24–30)	(22.8–26.3)	(19.2–22.2)	(20.8–28.8)	(21–29)	(20–29.6)	(18.6–25)
Head width	10 ± 0.7	9.6 ± 0.5	9.6 ± 0.5	9.2 ± 0.8	9.4 ± 0.5	9.6 ± 0.5	10.3 ± 0.6	9.2 ± 0.4
	(9–11)	(9–10)	(9–10)	(8–10)	(9–10)	(9–10)	(9.6–11)	(9–9.8)
Head height	5 ± 0^{a}	3.8 ± 0.4^{b}	4.6 ± 0.5^{ab}	3.8 ± 0.8^{b}	4.2 ± 0.7^{ab}	4.4 ± 0.5^{ab}	4.6 ± 0.5^{ab}	3.8 ± 0.4^{b}
	(5–5)	(3–4)	(4–5)	(3–5)	(3.2–5)	(4–5)	(4–5)	(3–4)
Stylet knob width	3.6 ± 0.5	3.4 ± 0.5	4 ± 0	3.8 ± 0.4	3.6 ± 0.5	3.4 ± 0.5	4 ± 0	3.8 ± 0.4
	(3–4)	(3–4)	(4–4)	(3–4)	(3–4)	(3–4)	(4–4)	(3–4)
Stylet knob height	2.4 ± 0.5	2 ± 0	2.2 ± 0.3	2.2 ± 0.2	1.9 ± 0.2	2 ± 0	2.2 ± 0.5	1.9 ± 0.2
	(2–3)	(2–2)	(2–2.8)	(2–2.4)	(1.6–2)	(2–2)	(1.6–3)	(1.6–2)
a ratio	47.1 ± 10.5	45.1 ± 9	48. ± 4.9	42.5 ± 3.3	44.5 ± 5.3	50 ± 6.6	49.7 ± 5.6	42 ± 7.6
	(30.6–59)	(39.6–61.1)	(41.6–54.9)	(36.8–45.1)	(38.9–50.6)	(42.9–60.4)	(42.6–55).4	(34.5–51.4)
c ratio	119.2 ± 19.7	117.8 ± 11.8	124 ± 24	104.4 ± 16	119.5 ± 14.7	127.4 ± 14.2	136 ± 38.3	121.1 ± 17.9
	(97.8–150.7)	(105.2–133.4)	(95.7–162)	(86–128.1)	(99–137.5)	(105.8–145.3)	(98.8–201)	(96.9–137.1)
b1	13.9 ± 1.2	15.5 ± 1.9	15.6 ± 0.9	15 ± 1.8	15.4 ± 2	14.3 ± 1.6	15.2 ± 2.5	15.8 ± 0.8
	(12.5–15.4)	(14.3–18.8)	(14–16.3)	(12.3–17.2)	(12.5–18.2)	(11.8–16.4)	(10.8–16.8)	(14.5–16.6)
Stylet knob width/height	1.6 ± 0.5	1.7 ± 0.3	1.8 ± 0.2	1.8 ± 0.3	1.9 ± 0.3	1.7 ± 0.3	1.9 ± 0.4	2 ± 0.4
	(1–2)	(1.5–2)	(1.4–2)	(1.3–2)	(1.5–2.2)	(1.5–2)	(1.3–2.5)	(1.5–2.5)

[Z]Means in a row followed by the same letter are not significantly different at *P*=0.05, according to Tukey's HSD Multiple comparison test

3.3.1.2 Comparison between Ethiopian and other populations

For comparison of *M. hapla* from Ethiopia with populations from other countries all Ethiopian *M. hapla* isolates were considered to be of the same origin and therefore pooled to form the so called Ethiopian population of *M. hapla*. The Ethiopian population was then compared in terms of morphometric characters with populations described from Hawaii (Handoo *et al*., 2005), East Africa (Whitehead, 1968), those mentioned in Jepson (1987) and Chitwood (1949) (Table 6-8). In general, most morphometrics of the Ethiopian population of *M. hapla* fit closely within the range of the beforehand mentioned populations, but there were some exceptions.

Female body length (577µm vs. 781 µm) and width (410 µm vs. 492 µm) of the Ethiopian population was smaller than that of the population described by Jepson (1987). The stylet length was similar for the Ethiopian population (14.6 µm) and the population described by Jepson (14.6 µm) but greater than the East African population (11 µm). The inter phasmids distance of the Ethiopian population (20.2 µm) was similar to the population from Hawaiian (20.9 µm) but smaller than that of the original description (24.4 µm) by Chitwood 1949. In terms of length of vulva slit, the Ethiopian population (19 µm) could be distinguished from the Hawaiian population (23.9 µm).

The average J2 body length and tail length was greater for the Ethiopian population (369.7 µm; 47.9 µm) than for the Hawaiian population (323.1 µm; 42.6 µm) and East African population (337 µm; 43 µm). The population described in Jepson (1987) had the greatest mean tail length (59 µm) of all those populations. Moreover, both the East African and Hawaiian populations showed a lower *a* ratio (31 vs. 23.9) and a shorter length of hyaline tail terminus (14 µm vs. 10.9 µm) than the Ethiopian population, respectively.

Male morphometrics between the four populations only differed in the distance of the DGO to the stylet base which was similar for the Ethiopian and Jepson population, but greater than that of the East African population (4.3 µm vs 2.9 µm).

Table 6. Morphometrical comparison of *Meloidogyne hapla* **females** from Ethiopia, Hawaii, Jepson (1968) and East Africa. All measurements are in µm as mean ± s.d. (value range) except for the last column given as mean (value range).

Character	Ethiopia (This study)	Hawaii (Handoo *et al*.,2005)	Jepson 1987	East Africa (Whitehead, 1968) [y]
Body length	577.1 ± 86.1 (442–877.6) [a]	–	781 ± 76 (643–952) [b]	612 ± 118 (419–845) [a]
Body width	410 ± 55 (284–510.2) [ab]	342.3 ± 65.2 (225–495) [a]	492 ± 40 (426–559) [b]	430 ± 81 (311–561) [a]
Neck width	94.2 ± 22.2 (53.2–148)	75 ± 24.5 (50–120)	–	–
Stylet length	14.4 ± 1.4 (12.4–17.7) [b]	–	14.6 ± 0.53 (12–14) b	11 (10-13) [a]
Stylet knob height	2.6 ± 0.4 (2–3.2)	2.5 ± 0.1 (2.5–3)	–	–
Stylet knob width	3.1 ± 0.3 (2.5–4.1)	3 ± 0.5 (2.5–3.5)	–	2 (2-3)
DGO from stylet base	4.9 ± 0.6 (3.4–6.2)	5.2 ± 0.5 (5–6.5)	–	5 (4-6)
Excretory pore from anterior end	31.9 ± 4.8 (23–42)	36.4 ± 8 (25–50)	–	–
Inter-plasmid distance	20.2 ± 2.3 (14.5–26) [a]	20.9 ± 4.6 (12.5–30) [a]	–	24.4 (17-38) [b]*
Vulva slit length	19 ± 2 (13–24) [a]	23.9 ± 2.7 (20–30) [b]	–	19 (13-24.5) [a]*
Vulva slit to anus distance	16.7 ± 2 (13.6–23.6)	17.6 ± 2.7 (14–22.5)	–	14.6 (9.5-24.5) *
Distance from anus to tail terminus	16.7 ± 2 (12.8–22.8) [b]	15.4 ± 3.3 (12.5–25) [b]	–	9.3 (5.5-13) [a]*
a ration	1.4 ± 0.2 (1.1–1.9)	1.7 ± 0.2 (1.4–2.1)	–	–
Excretory pore/stylet length	2.2 ± 0.4 (1.5–3.2)	2.9 ± 0.7 (1.9–4)	–	–

*Measurements are from the original description of the species by Chitwood, 1949. [y] 'East Africa' does not include Ethiopia

Table 7. Morphometrical comparison of *Meloidogyne hapla* second-stage juveniles from Ethiopia, Hawaii, Jepson (1968) and East Africa. All measurements are in µm as mean ± s.d. (value range) except for the last column given as mean (value range).

Character	Ethiopia (this study)	Hawaii (Handoo *et al*., 2005)	Jepson, 1987	East Africa (Whitehead, 1968) [y]
Body length	369.7 ± 21.6 (315–417) [b]	323.1 ± 18.4 (284–355) [a]	–	337 ±11.4 (312-355) [a]
Body width	12 ± 1 (9.6–15)	10.8 ± 0.9 (10–12)	–	–
Head width	4.2 ± 0.4 (3.4–5)	4.8 ± 0.4 (4–5)	–	–
Head height	2.1 ± 0.3 (1.2–3)	2.5 ± 0.1 (2–2.5)	–	–
Center of median bulb from anterior end	51.6 ± 4.2 (40–57)	46.4 ± 2.3 (40–50)	–	–
Excretory pore to anterior end	73 ± 5 (60–84)	66.9 ± 7.6 (60–88)	–	–
Stylet length	13.4 ± 0.9 (10.4–15)	–	–	9.7 ± 0.9 (7.9-10.9)
DGO from stylet base	2.5 ± 0.3 (1.9–3.2)	2 .5 ± 0 (2.5–2.5)	–	–
Tail length	47.9 ± 3.5 (42–59.2) [b]	42.6 ± 4.2 (30–47.5) [a]	59 ± 5.3 (48.2– 69.8) [c]	43 ± 4 (33-48) [a]
Length of hyaline tail terminus	14 ± 2.1 (10–19.2) [b]	10.9 ± 2.1 (5–15) [a]	15.7 ± 1.7 (11.7–18.9) [b]	–
Body diameter at anus level	9.2 ± 1 (6–12)	–	–	–
Tail width at hyaline portion	3.7 ± 0.5 (3–5)	3.7 ± 0.5 (3–4.5)	–	–
Tail width 5µm from tail terminus	2.6 ± 0.5 (2–3.2)	2.4 ± 0.3 (2–3)	–	–
Ratios				–
a	31 ± 3 (24.3–38.9) b	30.2 ± 2.6 (24.1–35.5) [b]	–	23.9 ± 1.7 (20.1-26.6) [a]
c	7.7 ± 0.6 (6.4–9.1)	7.7 ± 0.6 (6.8–9.4	–	7.9 ± 0.2 (7.3-10.2)
c'	5.3 ± 0.7 (3.7–7.4)	5.4 ± 0.6 (4.5–6)	–	4.4 (3.7-4.7)
Head width/height	2.1 ± 0.4 (1.5–3.6)	1.9 ± 0.2 (1.6–2.5)	–	–
Caudal ratio A[a]	3.8 ± 0.7 (2.6–6.4)	3 ± 0.7 (1.7–5)	–	–
Caudal ratio B[b]	5.6 ± 1.42 (3.3-8.5)	4.7 ± 1.3 (2.4–7.5)	–	–

[y] 'East Africa' does not include Ethiopia

Table 8. Morphometrical comparison of *Meloidogyne hapla* males from Ethiopia, Hawaii, Jepson (1968) and East Africa. All measurements are in µm as mean ± s.d. (value range) except for the last column given as mean (value range).

Character	Ethiopia (This study)	Hawaii (Handoo *et al*., 2005)	Jepson (1987)	East Africa (Whitehead, 1968) [Y]
Body length	1200.1 ± 119.4 (990–1568)	–	–	1139 ± 166 (791-1432)
Body width	26.4 ± 3.2 (20–34.5)	–	–	–
Stylet length	20.2 ± 1.2 (17.6–22)	–	20 ± 0.7 (19.4–21.6)	20 ± 1.3 (17.3-22.7)
Center of median bulb from head	80.3 ± 11.3 (58–128)	–	–	–
DGO from stylet base	4.3 ± 0.5 (3–5.2) [b]	–	4.1 ± 1 (2.7–5.4) [b]	2.9 ± 0.2 (2.5-3.2) [a]
Excretory pore to head end	132.5 ± 17.1 (96–175)	–	–	–
Body width at anus level	16.5 ± 3.9 (9–25)	–	–	–
Tail length	10.1 ±1.3 (7.6–13)	–	–	–
Spicule length	23.2 ± 3.7 (14.4–30)	23.3 ± 2.5 (20–25)	–	25.7 ± 2.4 (21.6-28.1)
Head width	9.6 ± 0.7 (8–11)	–	–	–
Head height	4.3 ± 0.7 (3–5)	–	–	5.6 ± 0.8 (4.3-7.9)
Stylet knob width	3.7 ± 0.5 (3–4)	–	–	3.5 (2.5-5)
Stylet knob height	2.1 ± 0.3 (1.6–3)	2.7 ± 0.2 (2.5–3)	–	–
Ratios				
a	46.1 ± 6.9 (30.6–61.1)	–	–	41.7 ± 3.66 (33.3-47)
c	121.2 ± 20.9 (86–201)	–	–	118 ± 46.4 (73-283)
b1	15.1 ± 1.6 (10.8–18.8)	–	–	15.5 ± 1.6 (12.8-19.2)
Stylet knob width/height	1.8 ± 0.3 (1–2.5)	–	–	–

[y] 'East Africa' does not include Ethiopia

3.3.2 *Molecular analysis*

3.3.2.1 Sequence analysis

The amplification of the mtDNA (COII-16S) and 28S D2-D3 expansion segment of the 28S rDNA region produced single fragments of about 540 bp (Fig. 3) and 740 bp (Fig. 4), respectively. All tested 125 isolates gave the same fragment size for the D2-D3 region, similar the two *M. hapla* isolates form the Netherlands and the isolate from Germany. However, for the mtDNA, only 82 of the 125 isolates yielded a PCR product typical for *M. hapla* and even with varying PCR conditions, the remaining 43 isolates failed to yield any fragment for this region.

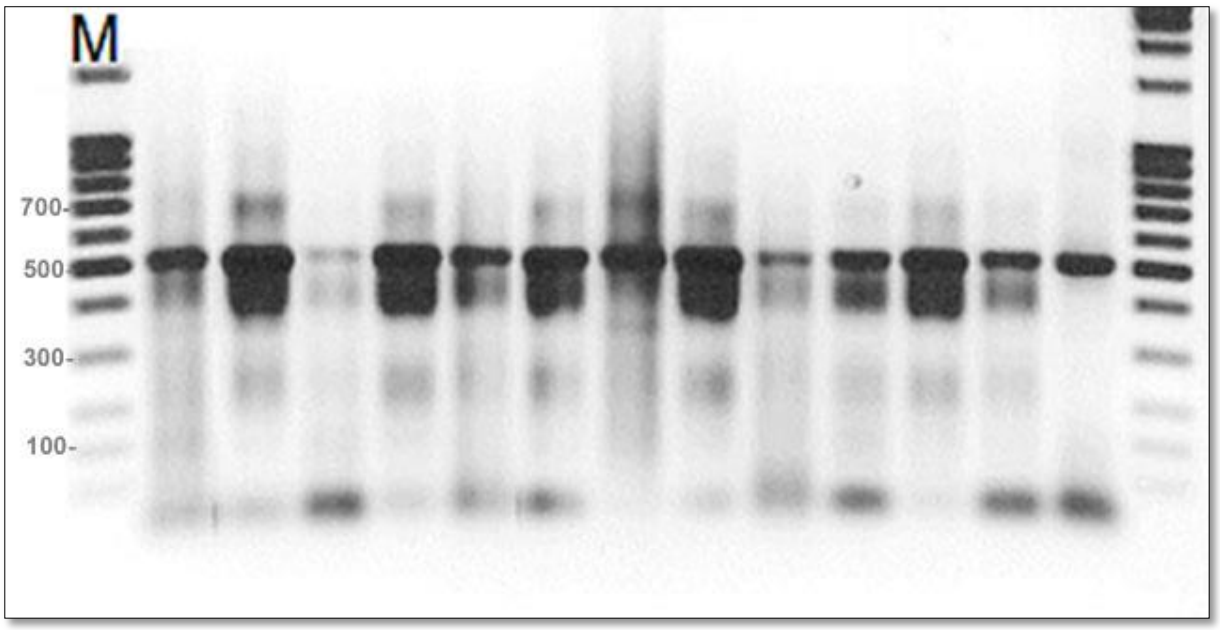

Figure 3. PCR products amplified using mtDNA COII-16S primers (C2F3 and 1108) from representative specimens of *Meloidogyne hapla* isolates from Ethiopia. M: 5000 bp ladder**.**

Twenty-one isolates were randomly selected and sequenced for both genes. A BLAST search at NCBI for both genes confirmed the species identity of the Ethiopian isolates (data not shown). For the mtDNA, each isolate revealed 99-100% sequence similarity with one or more previously published isolates of *M. hapla*. For the D2-D3 region, 93-99% similarity was obtained. Moreover, both sequence and phylogenetic analysis confirmed that the Ethiopian isolates represent *M. hapla*.

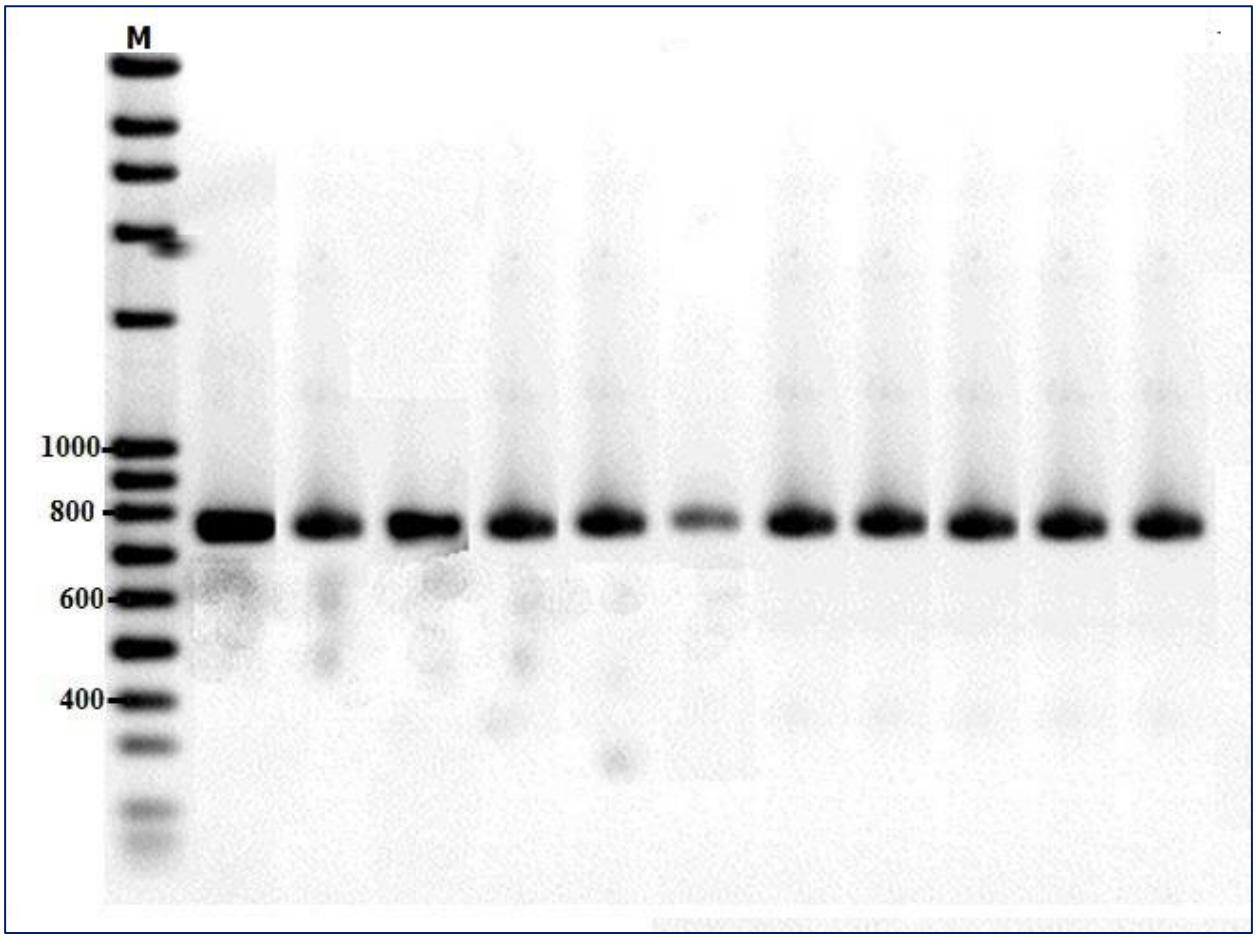

Figure 4. PCR products amplified using D2-D3 expansion segment of 28S RNA primers (D2A and D3B) from representative specimens of *Meloidogyne hapla* isolates from Ethiopia. M: 5000 bp ladder**.**

3.3.2.2 Nucleotide characteristic

The final mtDNA sequence alignment contained 511 positions with 162 parsimony informative sites. Within the Ethiopian isolates, there were four groups based on A+T richness: 83%, 87%; 88% and 89% (Table 9). The Ethiopian isolates shared an overall intra-sequence similarity value of 98.4%. The sequence similarity among all isolates of *M*. *hapla* sequences aligned in the sequence analysed was 91-100%. For comparison, mtDNA sequence similarity with other *Meloidogyne* species ranged between 23% for *M*. *partityla* and 29% for *M*. *javanica* (data not shown).

Similarly, the ClustalX alignments for the D2-D3 region consisted of 762 nucleotide positions with 255 parsimony-informative. When the %AT was compared among the Ethiopian isolates, three types of AT richness were observed (Table 9): 54%, 55%, and 56%. Out of those HRBB107 showed low %AT in both gene sequences.

3.3.2.3 Nucleotide position in D2-D3 sequence

The alignment for the 28S D2-D3 region was compared between three Ethiopian isolates (GUNB26, STRB6, FLMB24) and other isolates from Germany (JKI-de), the Netherlands (PSSC3093) and GenBank (GQ130139; China) (Fig. 5). Overall, the alignment consists of six sequences with 683 bp in length (after trimming to equal length). A total of 61 variable positions were found among sequences of those six isolates. The variability is described as 16 transitions (11 G ↔A; 5 C↔T); 43 transversions (8 A↔C, 9 G↔T, 12 A↔T and 14 G↔C) and two indels ↔T/C. The highest nucleotide variation (45 nucleotides) was between the Netherland isolate PSSC3093 and the Ethiopian isolate GUNB26. The other two Ethiopian isolates varied from PPSC3093 with 6 and 17 nucleotides for FLMB18 and STRB6, respectively. On the other hand, FLMB18 and STRB6 varied from GUNB26 with 39 and 28 nucleotides respectively. FLMB18 and STRB6 differ from each other by 11 nucleotides and from the German isolate JKI-de with 5 and 6 nucleotides, respectively.

Table. 9. Nucleotide composition (%) of rRNA and mtDNA of *Meloidogyne hapla* isolates.

M. hapla isolates*	D2-D3 28S					mtDNA				
	T	C	A	G	AT	T	C	A	G	AT
ETDB44	30	19	26	25	56	50	2	39	9	89
ETDB47	30	20	26	24	56	50	2	40	8	89
ETDB54	30	18	26	26	56	49	3	39	9	88
FLMB18	29	18	26	26	56	50	2	40	9	89
FLMB24	29	19	26	26	55	50	2	40	8	89
FYRB60	30	20	26	25	56	50	2	39	8	89
FYRB66	29	19	26	25	55	50	2	40	8	89
M.hapla (JN005873)	30	19	26	25	56	-	-	-	-	-
GUNB26	29	21	25	25	55	47	5	40	8	87
HRBB100	30	19	26	25	56	50	2	39	9	89
HRBB103	30	19	26	25	56	50	2	39	9	89
M.hapla (DQ328685)	30	18	26	26	56	-	-	-	-	-
HRBB115	30	18	26	26	56	49	2	39	9	88
HRBB82	30	19	26	26	55	50	2	39	9	89
FYRB62	50	3	39	8	89	-	-	-	-	-
HRBB107	28	23	26	23	54	45	8	38	8	83
HRBB98	30	18	26	26	56	49	3	39	9	88
JKI-de	30	19	26	25	56	50	2	39	9	89
GUNB42	30	20	25	25	55	50	2	39	8	89
HRBB95	29	19	26	25	55	49	2	39	9	89
M.hapla (GQ130139)	29	18	26	26	56	-	-	-	-	-
STRB4	29	20	27	25	56	50	2	39	9	89
PPSC3093-nl	30	19	26	26	56	-	-	-	-	-
STRB6	30	20	26	25	55	50	2	39	9	89
M.hapla (AY539839)	-	-	-	-	-	49	3	39	9	88
HRBB112	29	19	26	26	56	50	2	39	9	89
M.hapla (DQ145641)	29	18	26	27	55	-	-	-	-	-
M.hapla(AY757889)	-	-	-	-	-	49	2	40	9	89
PPS11-nl	27	18	26	29	53	-	-	-	-	-
FYRB67	30	19	26	26	56	49	3	39	9	88

*Isolates from GenBank are indicated by the corresponding accession nr. in parenthesis; PPSC3093-nl and PPS11-nl = newly obtained sequences of isolates from the Netherland; JKI-de = newly obtained sequence of isolate from Germany.

Table 10. Sequence similarity (%) of D2-D3 expansion segments of 28S rDNA of *Meloidogyne hapla* isolates from Ethiopia, the Netherlands (nl) , Germany (de) , China (cn) and Moldova (md). *PPSC3093* and JKI-de are newly obtained sequences. Additional sequences from GenBank database are shown with theirs corresponding accession number.

		M. hapla populations																									
		01	**02**	03	04	**05**	06	07	08	09	10	11	12	13	14	**15**	**16**	17	18	19	20	21	22	23	24	**25**	**26**	**27**
01.	HRBB115	**-**																									
02.	**DQ328685 (md)**	100	-																								
03.	ETDB54	100	100	-																							
04.	HRBB98	100	100	100	-																						
05.	**JN005873 (cn)**	100	100	100	100	-																					
06.	STRB4	96	96	96	96	96	-																				
07.	*ETDB47*	94	94	94	94	94	93	-																			
08.	*GUNB26*	92	92	92	92	92	92	90	-																		
09.	*HRBB107*	84	84	84	84	84	84	83	86	-																	
10.	GUNB42	92	92	92	92	92	91	89	93	84	-																
11.	HRBB95	98	99	99	99	99	96	93	92	83	92	-															
12.	*FYRB60*	99	99	99	99	99	97	94	93	85	93	97	-														
13.	*FYRB66*	99	99	99	99	99	96	94	92	85	92	98	100	-													
14.	*STRB6*	98	98	98	98	98	97	94	93	84	92	97	99	99	-												
15.	JKI-de **(de)**	99	99	99	99	99	95	93	92	83	91	98	98	98	97	-											
16.	***PPSC3093* (nl)**	100	100	100	100	100	96	94	92	84	92	99	99	99	98	99	-										
17.	FLMB24	99	99	99	99	99	97	95	92	84	92	98	98	98	98	98	99	-									
18.	*HRBB100*	100	100	100	100	100	96	94	92	84	92	99	99	99	98	99	100	99	-								
19.	HRBB82	100	100	100	100	100	96	94	93	85	92	99	99	99	98	99	100	99	100	-							
20.	HRBB103	98	99	99	99	99	97	94	93	84	92	98	98	98	97	98	99	99	99	99	-						
21.	FYRB67	99	100	100	100	100	97	94	92	84	92	99	98	99	98	99	100	100	100	100	99	-					
22.	*ETDB44*	97	98	98	98	98	95	93	91	84	91	97	96	97	97	97	98	98	98	98	97	98	-				
23.	HRBB112	98	99	99	99	99	96	93	93	85	92	98	98	98	98	98	99	99	99	99	98	99	97	-			
24.	*FLMB18*	99	99	99	99	99	96	94	93	85	92	98	98	99	98	98	99	99	99	100	99	100	98	99	-		
25.	**GQ130139(cn)**	100	100	100	100	100	96	94	92	84	92	99	99	99	98	99	100	99	100	100	99	100	98	99	99	**-**	
26.	**DQ145641 (nl)**	100	100	100	100	100	96	94	93	85	92	99	99	99	98	99	100	100	100	100	99	100	98	99	100	100	**-**
27.	***PPS11* (nl)**	80	80	80	80	80	76	73	74	63	70	78	78	79	77	78	80	80	80	80	79	79	77	78	79	80	80	**-**

```
PPSC3093  :  TGAGGAGGACACGGATAGAGTCGGCGTATCTTGCAAGTATTCAATTACTTTATTATTGTGTTGTTATCTCTGAGCT :  76
GQ13013   : .........A.......................................................................... :  76
FLMB24    : .........A.......................................................................... :  76
JKI-de    : .........A.......................................................................... :  76
STRB6     : .........A.......................................................................... :  76
GUNB26    : .........A.....................................................G.................... :  76

PPSC3093  : CCAGATTGGGACAGAGGAAAGCAGCATGATTTAATGTGATGCATTTACTTGTCTGGTGTGTGGGGGTATCTTAAGA :  152
GQ13013   : ..............................................................................-.... :  151
FLMB24    : ..............................................................................-.... :  151
JKI-de    : ..............................................................................-.... :  151
STRB6     : ..............................................................................-.... :  151
GUNB26    : ...............................TT............G....C...................-G... :  151

PPSC3093   : TGGATTTGCAACCAATGTTTTGAGGCCAGCTTGCTGGTACCCAAACATTGTTAACATTTTTTATCTTGGATATTCG : 228
GQ13013   : ........................................................................... :  227
FLMB24    : ..........................................G.............................. :  227
JKI-de    : ........................................................................... :  227
STRB6     : ........................................................................... :  227
GUNB26    : .........TG..................................G.....T.....................T :  227

PPSC3093  : AGTACGGCTTACGTGCATTTTTTGTATTGATCTAAGTGCAAGTTACGGTCGCATGCGACACGTGCTTTTCATTTAG :  304
GQ13013   : ........................................................................... :  303
FLMB24    : ..................................A.....................................A.... :  303
JKI-de    : ........................................................................... :  303
STRB6     : ..................................A..........C.........C................... :  303
GUNB26    : .A..........C...........G...C.A.......G.......C.....C...C..C..............C :  303

PPSC3093  : TTCGGTGCAGTTAATGCTCTCGTACTCTCCCCCCATGTAAAAGCCGGTCATCTATTTGACCCGTCTTGAAACACGG :  380
GQ13013   : ........................................................................... :  379
FLMB24    : ..................................A........................................ :  379
JKI-de    : ........................................................................... :  379
STRB6     : ..........................................................................C.C. :  378
GUNB26    : ...C.....................C.........A...........G...T.....C.A.....C......C.C. :  379

PPSC3093  : ACCAAGGAGTTTATCGTGTGCGCAAGTTTTTGGGTGTTAAAAACCTAGAAGCGAAATGAAAGTAAATGATTCTTTA :  456
GQ13013   : ........................................................................... :  455
FLMB24    : ........................................................................... :  455
JKI-de    : ..............................................A............................ :  455
STRB6     : ..............................................C.................A...... :  455
GUNB26    : .......G....T..C................................C............................ :  455

PPSC3093  : GAGTCTGATGTGCAATCTTTTTTCAGAAAAGTGCATCATGGCCCC-ATTCTAACTGTTTACAGTAGGGTGGCGGAA :  531
GQ13013   : ..............................................-............................... :  530
FLMB24    : ..............................................-............................... :  530
JKI-de    : ........................A...........C.......-............................... :  530
STRB6     : .......................TCA...........C.......-............................... :  530
GUNB26    : T.........................A...................C...................A.......... :  531

PPSC3093  : GAGCGTACGCGGTGAGACCCGAAAGATGGTGAACTATTCCTGAGCAGGACGAAGCCAGAGGAAACTCTGGTGGAAG :  607
GQ13013   : ........................................................................... :  606
FLMB24    : ........................................................................... :  606
JKI-de    : .........................T................................................... :  606
STRB6     : ..................................................A.....C.................. :  606
GUNB26    : ........................................................................... :  607


PPSC3093  : TCCGAAGCGGTTCTGACGTGCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTA :  683
GQ13013   : ..............................................................T.......... :  682
FLMB24    : ........................................................................... :  682
JKI-de    : ......C.T..................T.............T................................. :  682
STRB6     : .................C.....C..................................................... :  682
GUNB26    : ..............................................ATA..............................:  683
```

Figure 5. Alignment of D2-D3 28S expansion segment gene of six selected *Meloidogyne hapla* isolates. Isolates *GUNB26*, *STRB6* and FLMB24 are from Ethiopia. Whereas isolate *PPSC3093* and *JKI-de* are newly obtained sequences from the Netherlands and Germany, respectively. GQ130139 is from GenBank originally from China. Sequences were trimmed to equal length manually in GeneDoc software. Identical nucleotide positions are indicated with '.' Indels are indicated with '-'.

Sequence similarity of the D2-D3 region was compared between Ethiopian isolates, three other isolates from GenBank (DQ328685, JN005873, and GQ130139), two isolates from the Netherlands (PSSC3093, PSS11) and one isolate from Germany (JKI-de). Results indicated that Ethiopian isolates vary in sequence homology between each other and isolates originating from other countries (Table 10). Isolate PPS11 from the Netherlands revealed a very high sequence divergence (37%). Excluding isolate PPS11, sequence similarity ranged from 83-100%. Isolate HRBB107 shared low similarity (83%) with isolate ETDB47, HRBB95 and JKI-de. The Isolate JKI-de from Germany shared a maximum of 99% similarity with five Ethiopian isolates. Isolates originated from the Netherlands (PPSC3093), Moldova (DQ328685) and China (JN005873, GQ130139) shared 100% similarity with at least 6 Ethiopian isolates.

3.3.2.4 COII-16S PCR-RFLP

Digested PCR product of the COII-16S separated on agarose gel showed that both endonucleases (*DraI* and *AluI*) were able to digest the PCR product from selected Ethiopian *M. hapla* isolates. *DraI* produced three fragments of 250, 200 and 50 bp (Fig. 6) while *AluI* produced one fragments of 400 bp (Fig. 7). On the contrary, *HinfI* did not produce any restriction fragment (not shown).

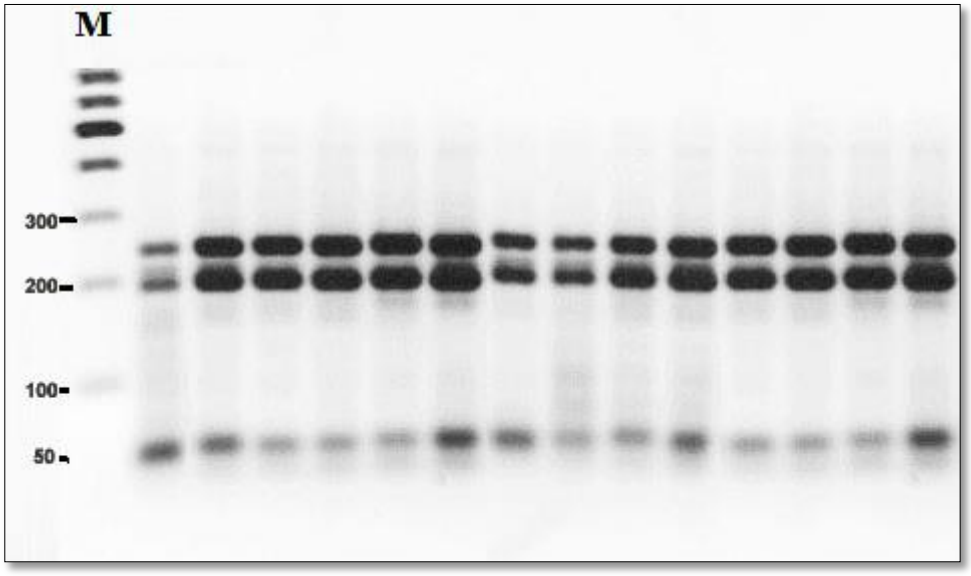

Figure 6. *DraI* restriction fragment pattern of the PCR-amplified COII-16S region of the mtDNA of *Meloidogyne hapla* representative isolates on 2% agarose gel. The size marker is 1kb DNA ladder.

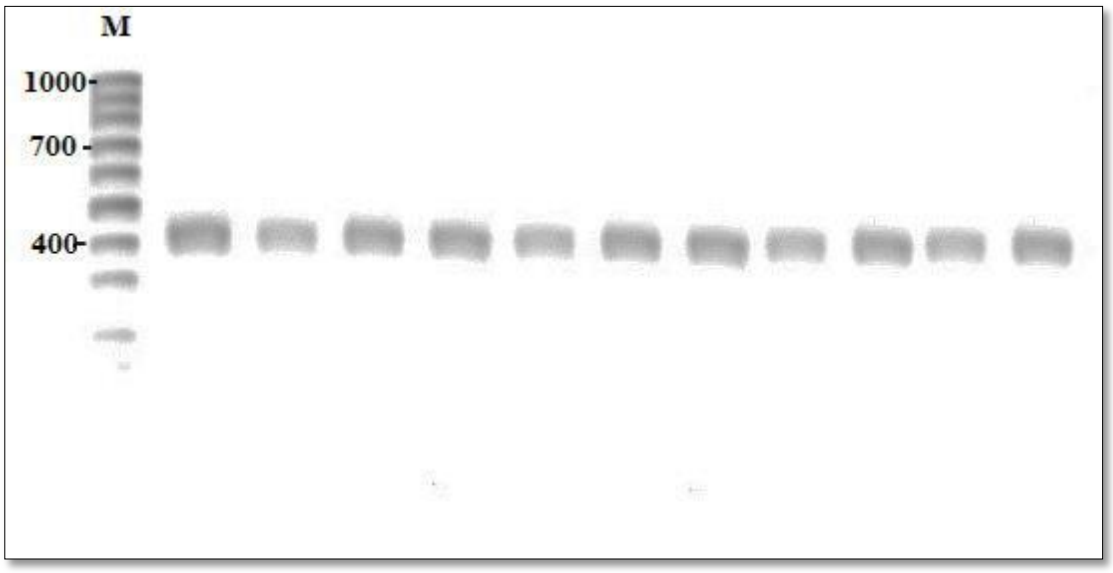

Figure 7. *AluI* restriction fragment pattern of the PCR-amplified COII-16S region of the mtDNA of *Meloidogyne hapla* isolates on 2% agarose gel. The size marker is 5kb DNA ladder.

3.3.3 *Phylogenetic analysis*

The Maximum Parsimony (MP) a total of 44 sequences for mtDNA and Maximum Likelihood (ML) analysis from 38 sequences for D2-D3 were able to infer the phylogenetic relationship among the root-knot nematodes (Fig. 8 and 9). Based on default parameters, the best sequence evolution model for mtDNA was found to be T92+G representing the model Tamura 3-parameter (Nie and Kumar, 2000). The base frequency varied in that both A and T had 0.44 while C and G on the other hand had same 0.06. The estimated gamma distribution shape parameter was 0.67 and the phylogenetic tree had a –ln likelihood of -2433.68. With a similar default search, the best sequence evolution model for the D2-D3 region obtained was K2+G representing the model Kimura 2-parameter (Nie and Kumar, 2000). Each base had equal frequency of 0.25 and the estimated gamma distribution shape parameter was 1.32. The phylogenetic tree obtained had a –ln likelihood of 5737.45.

Identification of *M. hapla* isolates based on sequences of the mtDNA (COII-16S) and rDNA region of the D2-D3 was in agreement with both morphological and morphometrical data. Phylogenetic trees of both genes demonstrated that *M. hapla* isolates from Ethiopia formed highly supported clades with those of published *M. hapla* isolates of different geographical origin (Fig. 9).

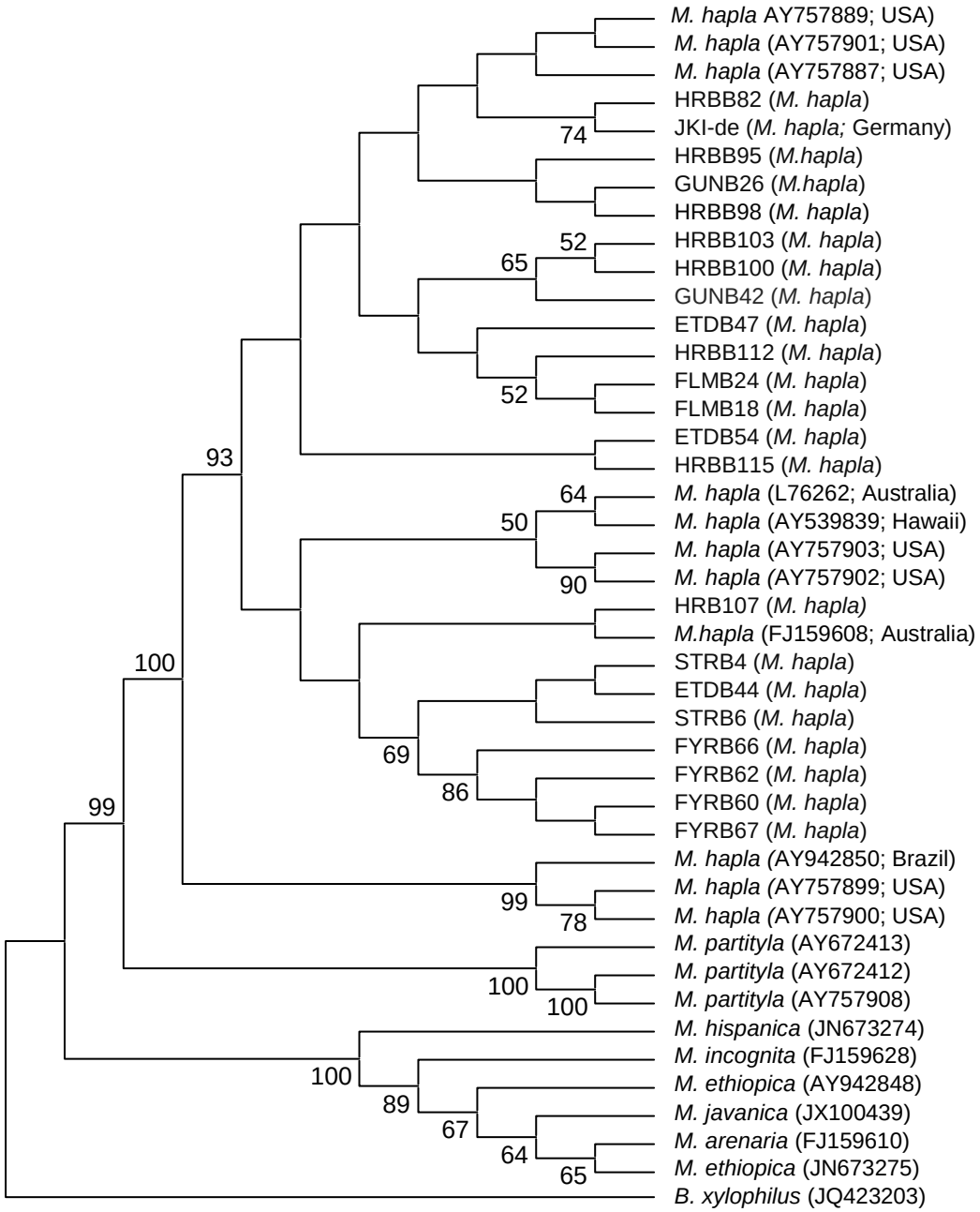

Figure 8. Maximum Parsimony analysis of mtDNA (COII-16S) of *Meloidogyne hapla* isolates from Ethiopia (red coloured) and other related sequences. Sequences from GenBank are given with their corresponding accession number in parenthesis. The newly obtained sequence for the isolate from Germany is in bold. Whereas sequences for Ethiopian isolates are given by farm codes (STR, HRB, FYR, FLM, GUN, and ETD) followed by 'B' + isolate number. All ambiguous positions were removed for each sequence pair. The analysis was performed using 1000 bootstrap replicates. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm by the random addition of sequences (10 replicates). Corresponding bootstrap values above 50% are shown on each node. *Bursaphlenchus xylophilus* has been used as an outgroup taxon.

Phylogenetic analysis of mtDNA of the Ethiopian *M. hapla* isolates with *M. hapla* and other *Meloidogyne* species obtained from GenBank using Maximum parsimony formed three highly supported clades (Fig. 8): Clade I includes *M*. *hispanica*, *M*. *javanica*, *M*. *arenaria*, and *M*. *ethiopica*; Clade II consists of only *M*. *partityla*; and Clade III contains *M. hapla* which was separated from the other *Meloidogyne* species with a bootstrap value of 99%. In turn, two *M. hapla* isolates from USA (AY757899, AY757900) and Brazil (AY942850) formed an independent subclade with 100% bootstrap while other isolates from USA (Fig. 8) were placed into two highly supported subclades (93% bs). One subclade consists of *M. hapla* isolates from USA (AY539839, AY757903, and AY757902), Australia (L76262, FJ159608) and half of the Ethiopian isolates originally collected from higher altitude regions of Ethiopia except isolate *HRBB107*. The second subclade includes three isolates from USA (AY757889, AY757901, AY757887), one isolate from German (JKI-de) and the remaining isolates from Ethiopia. In summary, the Ethiopian *M. hapla* isolates were placed on the tree sandwiched between isolates originating from USA.

Phylogenetic trees obtained with both ML and MP revealed consistent topology and thus the ML tree is here presented (Fig. 9). In ML phylogenetic analysis, *Meloidogyne* species formed two highly and moderately distinct clusters supported by bootstrap value 98% and 61%, respectively. The first highly supported cluster comprises the species *M*. *marylandi* and *M*. *graminis*, the second cluster two highly supported clades of 95% and 78% bootstrap value. The first clade encompassed the tropical *Meloidogyne* species (*M. incognita, M. javanica, M. arenaria, M. ethiopica, M. enterolobii, M. thailandica* and *M. hispanica*). The second clade with the exception of the *M. hapla* isolate from the Netherlands (*PSS11*), formed three subclades of *M. hapla* populations including: a) one isolate from the Netherlands (DQ145641); b) isolates from China (JN005873, GQ130139), Moldova (DQ328685), The Netherlands (PPSC3093), Germany (JKI-de) and four Ethiopian isolates (HRBB100, HRBB115, HRBB98, ETDB54) and c) all remaining Ethiopian isolates.

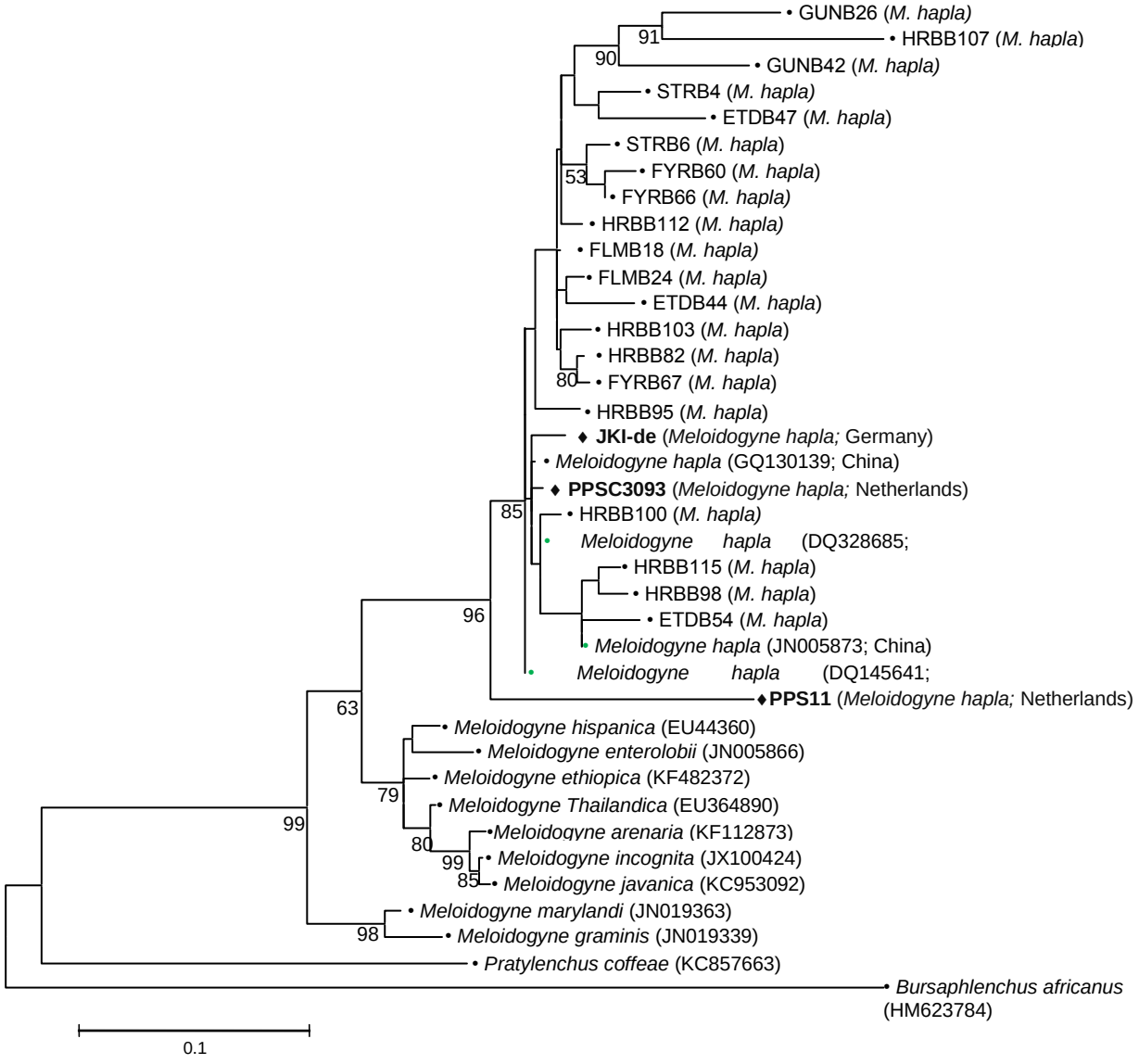

Figure 9. Maximum Likelihood analysis of the D2-D3 expansion segment of 28S ribosomal DNA of *Meloidogyne hapla* isolates from Ethiopia and other related sequences. Sequences from GenBank are given with their corresponding accession number. Newly obtained sequences for isolates from Netherlands and Germany are in shown in bold. Whereas sequences for Ethiopian isolates are given by farm codes (STR, HRB, FYR, FLM, GUN and ETD) followed by 'B' + isolate number. The analysis was performed using 1000 bootstrap replicates. Corresponding bootstrap support more than 50% are given for each appropriate clade. *Bursaphlenchus xylophilus* and *Pratylenchus coffeae* have been used as an outgroup taxon.

3.4 DISCUSSION

In this study, on the bases of comprehensive molecular and morphological analysis, *M. hapla* from Ethiopia was characterized and compared to populations from other countries. In Ethiopia, several plant-parasitic nematode genera were reported associated with rose plants cultivated in different regions (Meressa *et al*., 2012). Of these genera, *Meloidogyne* was frequent as well as abundant. Based on preliminary observation on morphology and morphometry of some characters, we realized the presence of this species uncommon to tropical environment particularly in Ethiopia. Thus we propagated a pure culture and studied its morphology, morphometry and molecular characteristics in more detail.

3.4.1 *Morphology and morphometrical analysis*

The identification of *M. hapla* to species level might depend on a specific population that bears varying forms of morphological characters (Eisenback, 1982). Based on sampling locality, our populations were roughly grouped into those from lowland (≈1600 m) or highland (≥ 2300 m) regions. In this regard, isolates obtained from Ziway locality represented the low land regions. There were differences in morphometrics of some characters in females, males and J2's between the Ethiopian isolates. However, the variation observed lacks consistent correlation with sampling localities to infer the type of environment from which they were sampled, but is sufficient to draw some general conclusion.

Morphometrical measurements were found to distinguish the Ethiopian population from others. The Ethiopian female population was different from the Hawaiian in possessing a wider body diameter (410 µm vs 342.3 µm) and narrower vulva slit (19 µm vs 23.9 µm). One population from the Netherlands was previously reported to have an average vulva slit length of 18.9 µm which is almost similar to the Ethiopian population (Handoo *et al*., 2005). Second-stage juveniles were comparably larger in body size (369.7 µm vs 323.1 3µm) and tail length (48 µm vs 42.6 µm).

The Ethiopian population differed from a population described from East Africa (Tanzania) in some morphometrics of female and J2 (Whitehead 1968). The Ethiopian females had a relatively shorter (577 µm vs 612 µm) and narrower (410 µm vs 430 µm) body compared to the East African population. There was also a considerable difference in female stylet length (11 µm vs 14.4 µm). On the other hand the body of the Ethiopian J2 was longer than for the East African J2 (369.7 µm vs 337 µm). The tail length of the Ethiopian population (48 µm) falls between the East African population (43 µm) and the population described by Jepson (1987) (59 µm). The smaller *a* ratio (23.6) of the East African J2 compared with the Ethiopian J2 (31) indicates the fact that the Ethiopian population possess a thinner body.

On the bases of J2 body length, the possible geographical origin of the Hawaiian population was suggested to be associated with the origin of *Coffea arabica* in Ethiopia (Handoo *et al*., 2005). Although, a warmer environment might correlate with smaller J2 body size (Handoo *et al*., 2005), the Ethiopian J2 populations were apparently larger in size than both the East African and Hawaiian populations. Hence, the Ethiopian population may unlikely share similar origin with these two populations.

3.4.2 *COII-16S RFLP-PCR*

The approximate size of the PCR product of the mitochondria DNA was in agreement with previous studies (Powers and Harris, 1993). A PCR restriction profile of mitochondria DNA was conducted as this is a suitable method for species identification within root-knot nematodes (Orui, 1998; Powers *et al*., 1986). The PCR digestion with *AluI* and *DraI* resulted in a restriction pattern typical for *M. hapla,* which was in agreement with previous work by Orui (1998). As revealed by WebCutter analysis, the exact size of the middle fragment produced by *DraI* was either 199 or 198 bp. Isolates ETDB44, FYRB60, FYRB62, FYRB66 and FYRB67 had 198 bp which was the same as AY757887. The rest of the isolates had 199 bp similar to isolate WYA2-25A E (accession nr. AY757889) retrieved from GenBank. Moreover, unlike the Hawaiian isolate (AY539839), none of our isolates produced 200 bp for this middle fragment. The restriction pattern for *AluI* and the absence of the *HinfI* restriction site was consistent with Orui (1998).

DNA based molecular diagnostics avoids ambiguous diagnosis in root-knot nematodes (Hyman, 1990). Amplification of mtDNA (COII-16S) produced a fragment size of approximately 540 bp which was in agreement with the one report by Orui (1998) and Powers and Harris (1993). Using the same primer pairs used here, the three tropical root-knot nematode species consistently produced a typical mtDNA PCR fragment size of 1.7 kb for *M. incognita* and *M. javanica* and 1.1 kb for *M. arenaria* (Powers and Harries, 1993). Hence, our PCR product size gave the first impression in discriminating *M. hapla* at least form those known tropical root-knot nematode species. The presence of unusual PCR size polymorphism even within isolates of same species as reported for *M. incognita* (Blok *et al*., 2002) showed that mere use of PCR fragment size is not convincing for the correct identification of this species.

The PCR fragment size (740 bp) for D2-D3 expansion segment of 28S rDNA was not species specific because other *M. incognita* and *M*. *chitwoodi* included as a control also yielded the same fragment size (data not shown). Nevertheless, sequence analysis of the PCR products of both genes confirmed the identity of our specimens as *M. hapla*.

3.4.3 *Phylogenetic analysis*

In our study, the MP analysis of the mtDNA gene of *M. hapla* isolates formed a highly supported clade together with other isolates retrieved from GenBank. Nevertheless, considerable nucleotide variation has been observed within the Ethiopian isolates. For example, the overall sequence variability within isolates was 11.5%. On the other hand the sequence variability among those *M*. *hapla* isolates retrieved from the GenBank was 12%. Therefore, the sequence disparity observed between the Ethiopian isolates can be considered realistic. The rapid evolution nature of mtDNA (Power *et al*., 1986) combined with the reproduction behaviour of *M*. *hapla* (Hugall *et al*., 1994) could explain the sequence variation observed between isolates.

Sequence variation in the D2-D3 was observed within isolates of *M*. *hapla* (excluding isolate PPS11) that ranged from completely identical (0%) to 17%. When isolate PPS11 was considered, the disparity to isolates HRBB107 and PPS11 was 36.5%. Subbotin *et al*. (2011) reported higher sequence variability of 35 nucleotides within clones of the same PCR for *Helicotylenchus martini*. Sequence variability in the rDNA within individuals of the same species has been previously reported (De Ley *et al*., 2002). On the other hand Blok *et al*. (2002) found identical D2-D3 sequence for three isolates of *M*. *hispanica* collected from different geographical origins. However, given our Ethiopian isolates were collected from different geographical regions; the level of heterogeneity observed between isolates could be considered within the acceptable range. Both ML and MP analysis of the two gene regions placed all the *M. hapla* isolates within a strongly supported clade separated from other *Meloidogyne* species.

3.4.4 *Biogeography of Meloidogyne hapla*

Accurate information on the species and distribution of particular plant-parasitic nematodes is significant to develop preventive or control strategies. The occurrence of *M*. *incognita*, *M*. *javanica*, *M*. *arenaria* and *M*. *ethiopica* in Ethiopia is already known for several decades (O'Bannon, 1975), whereas *M. hapla* was only recently reported to occur in Ethiopia (Meressa *et al*., 2014a).

The most common tropical *Meloidogyne* species *M. incognita*, *M. arenaria* and *M. javanica* occur in areas with an average temperature up to 36^{o}C (Taylor *et al*., 1982). *M. hapla* is generally distributed in cooler parts of the world where it survives mean annual low temperature of -15^{o}C and in some highland areas of tropical and subtropical countries where temperature is below 27^{o}C (Taylor *et al*., 1982). However, its occurrence in the rift valley of Ethiopia (Meressa *et al*., 2014a), South Africa (Onkendi and Moleleki, 2013) and the

highlands of Tanzania (Whitehead, 1968) might indicate a possible wider climate range of this species. The temperature of our sampling localities generally variedthroughout the year but can reach a maximum of about 35^{o}C that would have challenged the survival of *M. hapla.* In this case, the fact that all rose greenhouses were conditioned with optimum irrigation schedule might have supported *M. hapla* to flourish under Ethiopian climatic conditions.

A greenhouse test carried out to evaluate the host suitability of statice, carnation, gypsophila and freesia showed that they all support the reproduction and development of *M. hapla* (Meressa *et al*. 2014b). However, *M. hapla* was not detected on those cut-flower species in Ethiopian greenhouses (Meressa *et al*., 2012), only from roses. The fact, that the occurrence of *M. hapla* was linked to roses might be attributed to the import of infested roxes from Europe. In fact, the D2-D3 of 28S rDNA of isolate PPSC3093 from the Netherlands shared a sequence similarity of 99-100% with 81% of the Ethiopian isolates.

In conclusion, we presented here *M*. *hapla* as a new record for Ethiopia making it the 4th country in Africa where this species is found. Its occurrence associated with rose cut-flowers is alarming and will be a challenge to the growers in the country to develop sustainable management strategies.

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Chapter Four

4. Host suitability of cut-flowers to *Meloidogyne* spp. and population dynamics of *M. hapla* on the rootstock *Rosa corymbifera* ˈLaxaˈ*

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ABSTRACT

The host suitability of cut-flowers to *Meloidogyne* spp. was tested under greenhouse conditions. The reaction of seven cut-flower species viz. *Dianthus plumarius*, *Dianthus caryophyllus*, *Gypsophila paniculata*, *Limonium sinuatum* (Fortress Dunkelblau), *Limonium sinuatum* (Petite Bouquet Mix), *Rosa corymbifera* ˈLaxaˈ and *Freesia laxa* against the root-knot nematodes *Meloidogyne hapla* and *M. incognita* was evaluated. There were significant (*P*< 0.001) differences in plant species as host for either *M. hapla* or *M. incognita*. *Freesia laxa* appeared to be a poor host for *M. hapla* and *M. incognita* with a reproductive factor of 0.5 and 1.1, respectively. *Gypsophila paniculata* and *R. corymbifera* were not suitable hosts for *M. incognita* resulting in a reproductive factor below one. On the other hand *M. hapla* reproduced significantly (P< 0.05) higher on *R. corymbifera* ˈLaxaˈ than on the other plant species assessed. In all plant species, nematode infected plants were less vigorous than their uninfected controls. In the second test, the pathogenicity and population dynamics of *M. hapla* on the rootstock *R. corymbifera* ˈLaxaˈ was evaluated. Within 24 hours after inoculation, about 2% of the juveniles had penetrated the root system. A week later, nematode penetration reached 14%. First eggs appeared 43 days after root infection. At final termination of the experiment 78 days after inoculation the reproduction factor of *M. hapla* was 58.9. In infected plants number of leaves per plant was lower than in the respective controls. In conclusion, the tested flower plants were hosts for *M. hapla* and *M. incognita*; however, the host status varied between plant and nematode species. *Rosa corymbifera* ˈLaxaˈ turned out to be a very good host for *M. hapla* allowing high nematode reproduction.

4.1 INTRODUCTION

Within the past decade, Ethiopia developed to one of the main cut-flower producing countries in East Africa. About 80% of all cut flowers produced are roses, mainly for export to Europe. Other cut flower species include carnation, statice, gypsophila and freesia. While fungal diseases and insects are already considered as a major pest problem on cut flowers in Ethiopia (DLV-plant, 2011), plant-parasitic nematodes have been ignored for a long time. Only recently, thirteen genera of plant-parasitic nematodes were reported to be associated with cut flowers produced in Ethiopia (Meressa *et al*., 2012).

Among plant-parasitic nematode, root-knot nematodes *(Meloidogyne* spp.) are generally considered the economically most important group worldwide (Sasser, 1977). Three of the most common tropical species, i.e. *M. incognita*, *M. arenaria* and *M. javanica*, are known to occur in Ethiopia (O'Bannon, 1975), although they have not yet been detected on cut flowers. Recently, *Meloidogyne hapla* was found to be the most frequent and abundant species in rose greenhouses in Ethiopia (Meressa *et al*., 2014). For temperate regions, the damage potential of *M*. *hapla* on cut flowers is well documented throughout the world (Pizetta *et al*., 2010; Voisin *et al*., 1996; Towson and Lear, 1982; Santo and Lear, 1976; Coolen and Hendrickx, 1972). However, little is known about the damage potential of *M. hapla* on cut flowers other than roses as well as of the tropical root-knot nematode species on cut flowers grown in Ethiopia. A good understanding of the host suitability of cut-flower species grown in Ethiopia to *M. hapla* and *M. incognita* is essential for future management strategies.

Therefore, the objective of the present study was to i) evaluate the host status of seven cut-flower species/cultivars to *M. hapla* and *M. incognita* and to ii) describe the pathogenicity and development of *M*. *hapla* on the most commonly grown rootstock *R*. *corymbifera* 'Laxa' over time.

4.2 MATERIAL AND METHODS

4.2.1 *Plant material and growth condition*

In experiment 1 the following cut-flower species were evaluated: carnation (*Dianthus plumarius* and *Dianthus caryophyllus*), gypsophila (*Gypsophila paniculata*), statice (*Limonium sinuatum* cv. Fortress Dunkelblau and *L. sinuatum* cv. Petite Bouquet Mix), rose (*Rosa corymbifera* 'Laxa') and freesia (*Freesia laxa*). *Rose corymbifera* 'Laxa' seeds were provided by Klei (Heidgraben, Germany), while bulbs of freesia and seeds of the other species were obtained from Volmary GmbH (Münster, Germany).

Seeds were germinated in plastic trays filled with growth substrate (Floragard®, Oldenburg, Germany). Two weeks after germination, individual seedlings were transplanted into 75 ml multi-well plates filled with steam-sterilized field soil. One month old seedlings were finally transplanted into 1 l capacity plastic pots filled with steam-sterilized field soil and silver sand (2:1, v:v), respectively. Bulbs of freesia were directly planted into the plastic pots. Each cut-flower species/cultivar was inoculated with either *M. hapla*, *M. incognita* or left uninoculated (control). Each treatment was replicated 10 times and pots were randomly arranged in the greenhouse at about 20±3^{o}C. The photocycle was adjusted to 16 h light and 8 h dark period using 600 W, 58500 lumen lamps (Norka-Lighting®, Hamburg, Germany). Plants were watered daily as needed and once weekly fertilized with 0.3% WUXAL® Super liquid foliar fertilizer (Agrarversand Oberland, Schongau, Germany).

In the second experiment the pathogenicity and population dynamics of *M*. *hapla* on *R. corymbifera* ˈLaxaˈ was assessed. Non-inoculated plants served as control. Planting procedure and growth conditions were the same as described above. Sampling dates were 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71 and 78 days after nematode inoculation (DAI). Each treatment was replicated 5 times.

4.2.2 *Nematode inoculation*

A stock population of both *M. hapla* and *M. incognita* originating from Germany was maintained on tomato (*Solanum lycopersicum* cv. Moneymaker) in the greenhouse. For collecting juvenile inoculum, galled tomato roots were placed in a misting chamber as described by Hooper *et al.* (2005). Freshly hatched second-stage juveniles (J2) were collected every other day in a 1 l glass beaker and stored at 6°C. Only juveniles less than two weeks old were used in the experiments. For inoculation the nematode density was adjusted to 200 J2s per ml by adding tap water. Four vertical holes about 6 cm deep and 1 cm wide were bored in the soil around the plant stem using a rounded conical-end plastic stick. Then, 5 ml of the nematode suspension per plant was injected into the four holes using a Multipette® plus dispenser (Eppendorf®, Hamburg, Germany). Hence, each pot received a total of 1000 J2s. Control plants received a similar volume of tap water without nematodes. In Experiment 1 pots were inoculated either with *M. hapla*, *M. incognita* or remained untreated (control), in Experiment 2 pots were inoculated with *M. hapla* or remained untreated (control).

4.2.3 *Assessments*

Experiment 1 was terminated nine weeks after nematode inoculation. Shoots were cut off at soil level and fresh weight was recorded. Roots were then washed free from the adhering soil, carefully blotted on tissue paper and weighed. Eggs were dislodged from the root system using a 0.52% sodium hypochlorite (NaOCl) solution following the method described by Hussey and Barker (1973). J2 numbers in the soil were recovered from 100 ml soil aliquot using the modified Baermann technique (Hooper *et al*., 2005). Both eggs and J2 were counted at 63× magnification using a compound microscope. The final population was determined as the sum of eggs extracted from the roots and J2s extracted from the soil.

Within the second experiment the first sampling was taken one day after inoculation. The remaining eleven samples were taken every seven days until 78 DAI. At each sampling date, shoot height, leaf number, shoot fresh weight, root fresh weight and number of galls was recorded. For the first six sampling dates until 36 DAI, nematodes within the root system were directly counted under a stereomicroscope after staining with 2% acid fuchsin (Byrd *et al*., 1983). For the final six sampling dates from 43 DAI to 78 DAI, number of eggs and J2s extracted from the roots using a 2% hypochlorite solution and number of J2s from soil extracted by Baermann were evaluated. In addition, symptoms such as wilting and leaf chlorosis were visually assessed.

4.2.4 *Statistical analysis*

In the first experiment, shoot fresh weight and root fresh weight, final nematode population (*Pf*) and nematode reproductive factor (RF = Pf/Pi)) were subjected to analysis of variance followed by mean separation ($P \leq 0.05$) with Duncan's multiple-range test using STATISTICA 7 software (StatSoft, 2004). Each plant species or cultivar was ranked for 'host status' on the basis of the nematodes RF according to Ferris *et al.* (1993): poor host ($0 < RF < 1$), maintenance host ($RF \approx 1$), good host ($1 < RF < 10$) and excellent host ($RF \geq 10$).

In the second experiment, all plant growth parameters, final nematode population densities (Pf), number of galls per root system and reproduction factors were subjected to analysis of variance taking number of days after inoculation as a factor. Means were compared as described above. Linear models were estimated to the relationship between gall numbers per root system and weeks after nematode inoculation as well as for nematodes in the root and days after nematode inoculation.

4.3 RESULTS

4.3.1 *Host plant suitability*

Cut-flower species and cultivars significantly differed ($P < 0.001$) in their host suitability to either *M. hapla* or *M. incognita* (Table 1). For *M. hapla*, *R. corymbifera* 'Laxa' was found to be the most susceptible host with RF = 12.7, followed by *L. sinuatum* cv. Fortress Dunkelblau with RF = 9.2. On the contrary, *F. laxa* was rated nonhost or poor host with RF = 0.5. The other cut flower species and cultivars were good hosts for *M. hapla* with RF's varying between 2.0 (*G. paniculata*) and 7.1 (*L. sinuatum* cv. Petite Bouquet Mix).

Regarding *M. incognita*, the most susceptible cut-flower species was *D. plumarius* with RF = 7.2 (Table 1), followed by *D. caryophyllus* (RF = 6.7) and *L. sinuatum* (RF = 6.2). In contrary, *R. corymbifera* 'Laxa' (RF = 0.6) and *G. paniculata* (RF = 0.8) were non hosts to poor hosts for *M. incognita. Freesia laxa* (RF = 1.1) turned out to be maintenance host.

Infestation with *M. hapla* significantly ($P< 0.05$) reduced shoot and root fresh weight of *R. corymbifera*, *D. plumarius* and *D. caryophyllus*, and root fresh weight of *F. laxa* in comparison with their respective controls (Table 1). In all other cases, except for shoot fresh weight of *L. sinuatum* cv. Petite Bouquet Mix, shoot and root fresh weight in nematode infested plants was less than in non-infested plants, although not significantly. For *M. incognita* the nematode effect on plant growth was less pronounced (Table 1). A significant reduction was only observed for shoot and root fresh weight of *D. plumarius* and shoot fresh weight of *F. laxa*. Although shoot and root fresh weight of all other flower crops (except for shoot fresh weight of *L. sinuatum* cv. Petite Bouquet Mix) was less in nematode-infested plants compared to non-infested plants, albeit differences were not significant.

Table 1. Host suitability of selected cut-flower species and cultivars to *Meloidogyne hapla* and *M. incognita* under greenhouse conditions.

Plant species	Inoculation	Shoot fresh weight (g)[a]	Root fresh weight (g)[a]	Nematodes per g root fresh weight	Reproductive factor (RF)[b]	Host rating [c]
Dianthus plumarius	Control	5.8 ± 0.31[b]	8.9 ± 1.11[b]	-	-	-
	M. incognita	4.1 ± 0.28[a]	5.2 ± 0.81[a]	143.3 ± 19[cd]	7.2 ± 0.6[c]	G
	M. hapla	4.2 ± 0.25[a]	5.6 ± 0.82[a]	99.3 ± 21.2[bc]	2.5 ± 0.8[ab]	G
Dianthus	Control	4.6 ± 0.14[b]	9.5 ± 0.90[b]	-	-	-
caryophyllus	*M. incognita*	3.9 ± 0.09[ab]	7.6 ± 0.96[b]	5.3 ± 1.2[a]	6.7 ± 0.5[c]	G
	M. hapla	3.7 ± 0.18[a]	4.3 ± 0.91[a]	69.3 ± 17 [b]	3.9 ± 0.9[b]	G
Gypsophila	Control	2.9 ± 0.22[a]	9.0 ± 0.80[a]	-	-	-
paniculata	*M. incognita*	2.6 ± 0.16[a]	7.2 ± 0.36[a]	8.0 ± 2.0[a]	0.8 ± 0.4[a]	P
	M. hapla	2.6 ± 0.09[a]	7.4 ± 0.96[a]	7.3 ± 0.9[a]	2.0 ± 0.5[ab]	G
Limonium sinuatum	Control	4.0 ± 0.27[a]	7.0 ± 0.38[b]	-	-	-
(Fortress Dunkel	*M. incognita*	3.6 ± 0.19[a]	4.8 ± 0.59[a]	133.0 ± 17.2[c]	4.9 ± 0.9[b]	G
Blau)	*M. hapla*	3.4 ± 0.21[a]	5.3 ± 0.67[ab]	182.7 ± 7.5[d]	9.2 ± 2.5[d]	G
Limonium sinuatum	Control	4.0 ± 0.28[a]	8.1 ± 1.38[a]	-	-	-
(Petite bouquet Mix)	*M. incognita*	4.1 ± 0.21[a]	6.9 ± 0.97[a]	75.3 ± 9.4[b]	6.2 ± 1.4[c]	G
	M. hapla	4.2 ± 0.20[a]	7.6 ± 0.98[a]	102.3 ± 9.9[c]	7.1 ± 0.9[cd]	G
Rosa corymbifera	Control	11.0 ± 1.85[b]	11.1 ± 1.66[b]	-	-	-
'Laxa'	*M. incognita*	9.0 ± 1.14[b]	9.2 ± 0.98[ab]	14.3 ± 2.0[a]	0.6 ± 0.1[a]	P
	M. hapla	6.0 ± 0.57[a]	6.7 ± 0.43[a]	357.0 ± 20.1[e]	12.7± 1.8[e]	E
Freesia laxa	Control	8.5 ± 1.1[b]	5.3 ± 0.6[b]	-	-	-
	M. incognita	6.4 ± 1.8[a]	3.3 ± 0.7[ab]	59.7 ± 10.7[b]	1.1 ± 0.6[a]	M
	M. hapla	8.1 ± 1.5[b]	2.7 ± 0.6[a]	46.0 ± 15.0[ab]	0.5 ± 0.2[a]	P

Means in columns followed by the same letter do not differ ($P \leq 0.05$) according to Duncan's multiple-range test (n = 10).
[a]Means were compared with the uninfected control and infected plants of the same species or cultivars.
[b]RF = Reproduction factor, i.e. final nematode density/initial nematode density
[c]Host status category: poor to nonhost (P) (0 < R < 1); maintenance (M) (≈1); good (G) (1 < R < 10); and excellent (E) (R ≥ 10).

The degree of *M*. *hapla* reproductive potential on each plant species/cultivars resulted in a sequence of host suitability *R. corymbifera* 'Laxa' > *L. sinuatum* (FDB) > *L. sinuatum* (PBM) > *D. caryophyllus*> *D. plumarius*> *G. paniculata*> *F. laxa.* The host suitability for *M. incognita* on the other hand showed different sequence as *D. plumarius* > *D. caryophyllus* >*L. sinuatum* (PBM) >*L. sinuatum* (FDB) >*F. laxa* >*G. paniculata* >*R. corymbifera* 'Laxa'.

4.3.2 *Population dynamics and pathogenicity of* Meloidogyne hapla *in* Rosa corymbifera *'Laxa'*

Within the first 36 days following inoculation, number of *M. hapla* in the root system of *R. corymbifera* 'Laxa' significantly increased (P<0.001) over time from 13.8 the day after inoculation to about 216.8 at 36 DAI (Table 2). At 36 DAI, root galls were apparent

throughout the root system (Fig. 1). At each sampling date, gall counts per root system exceeded 100 (Fig. 2). The female had reached its typical sac-like shape (Fig. 1A-E) within 36 DAI. First eggs were extracted from roots at 43 DAI. Since then, number of extracted eggs increased over time until final determination at 78 DAI when a reproductive factor of 58.9 was reached (Table 2). The nematode density extracted from roots significantly (P<0.001) increased over time (Fig. 3). This increase in nematode density over time was found to have a moderately positive relationship with root fresh weight (r^2 = 0.342; p = 0.001). J2 density in the soil also significantly (P = 0.003) increased over time (Fig. 4). However, no statistically significant relationship was found between nematode eggs extracted from the root and juvenile population density in the soil.

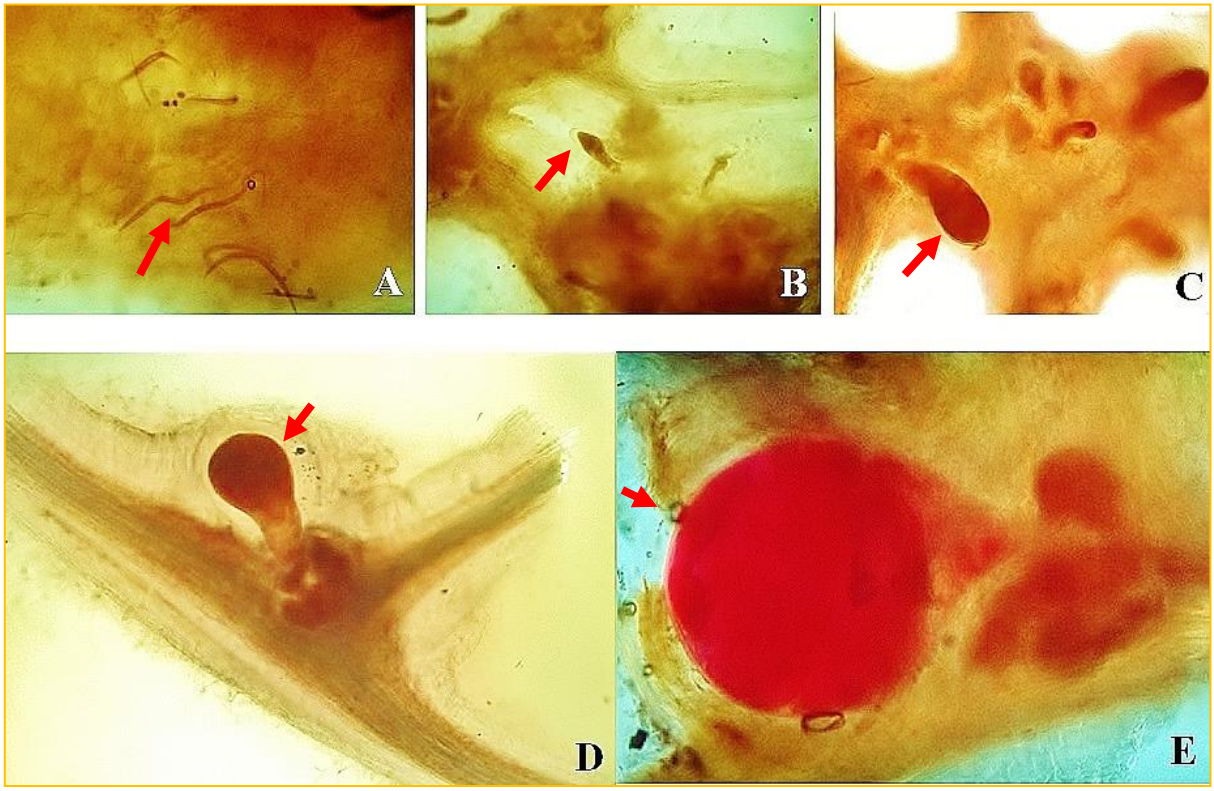

Figure 1. *Meloidogyne hapla* stained with acid fuchsin in the root tissue of *Rosa corymbivera* 'Laxa' at different developmental stages. A. eight days; B. fifteen days; C. twenty-two days; D**.** twenty-nine days and E**.** 36 after nematode inoculation. Arrows indicate the nematode stained in root tissue.

Infected plants showed reduced growth compared to uninfected control, especially towards the end of the experiment (Table 2). For instance, the number of leaves per plant was significantly (P < 0.05) less over time. Moreover, leaves of infected plants had a typical shrivelled margin, yellow blade and petiole and shortly dropped-off. No significant difference in shoot fresh weight was observed between 50 and 71 days after nematode inoculation. However, a significant reduction (P < 0.001) in shoot fresh weight was recorded in infected

plants compared with uninfected plants at the final sampling date (Table 2). Uninfected plants showed a consistent increase in shoot height over time until the final date of harvest. On the other hand, infected plants revealed a significant (P = 0.03) increase in plant height only until 50 days, after which no significant increase was found. At the end of the experiment, the difference in plant height between infected and uninfected control plant is shown (Fig. 5A and B). No significant difference in root fresh weight between infected and uninfected plants was found until 57 DAI. However, at 64 DAI, roots were heavier (P < 0.001) than those of uninfected plants. Visually, roots of infected plants were shorter and bushier (Fig. 5 C and D). In addition, infected roots were greyish and had a very loosen root bark.

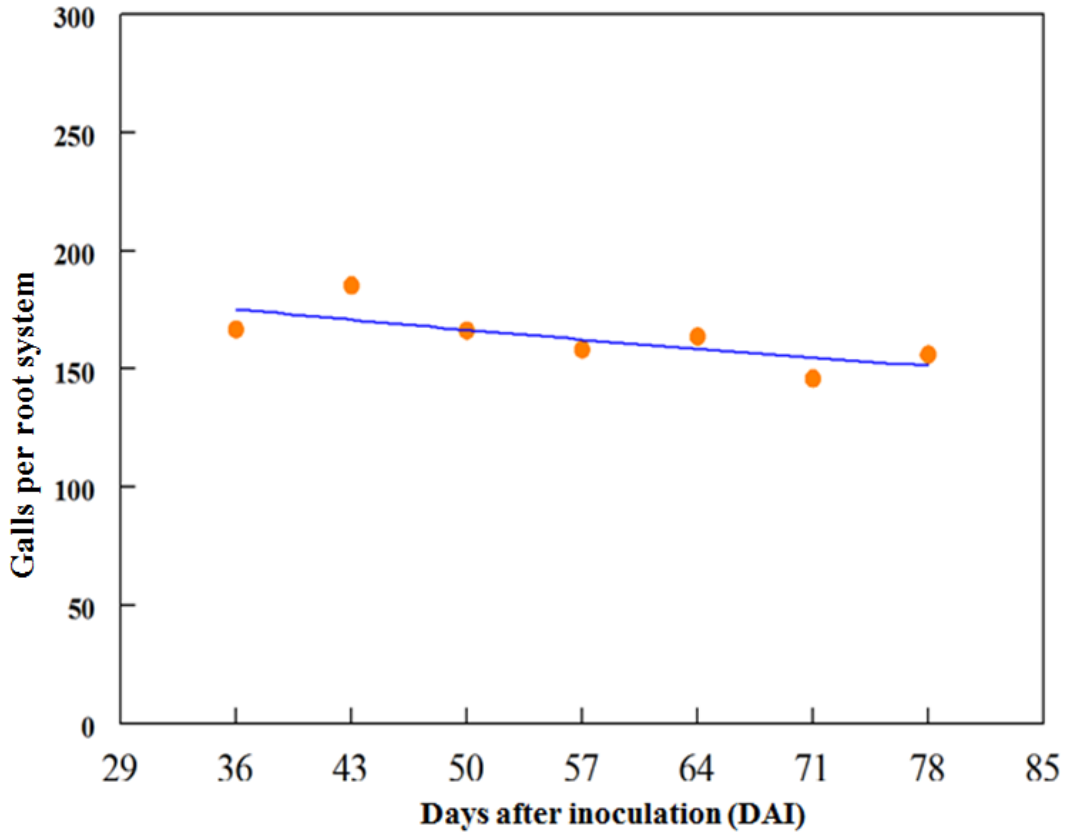

Figure 2. Linear relationship between number of root galls and time after inoculation of *Meloidogyne hapla* on *Rosa corymbifera* 'Laxa' (n = 5).

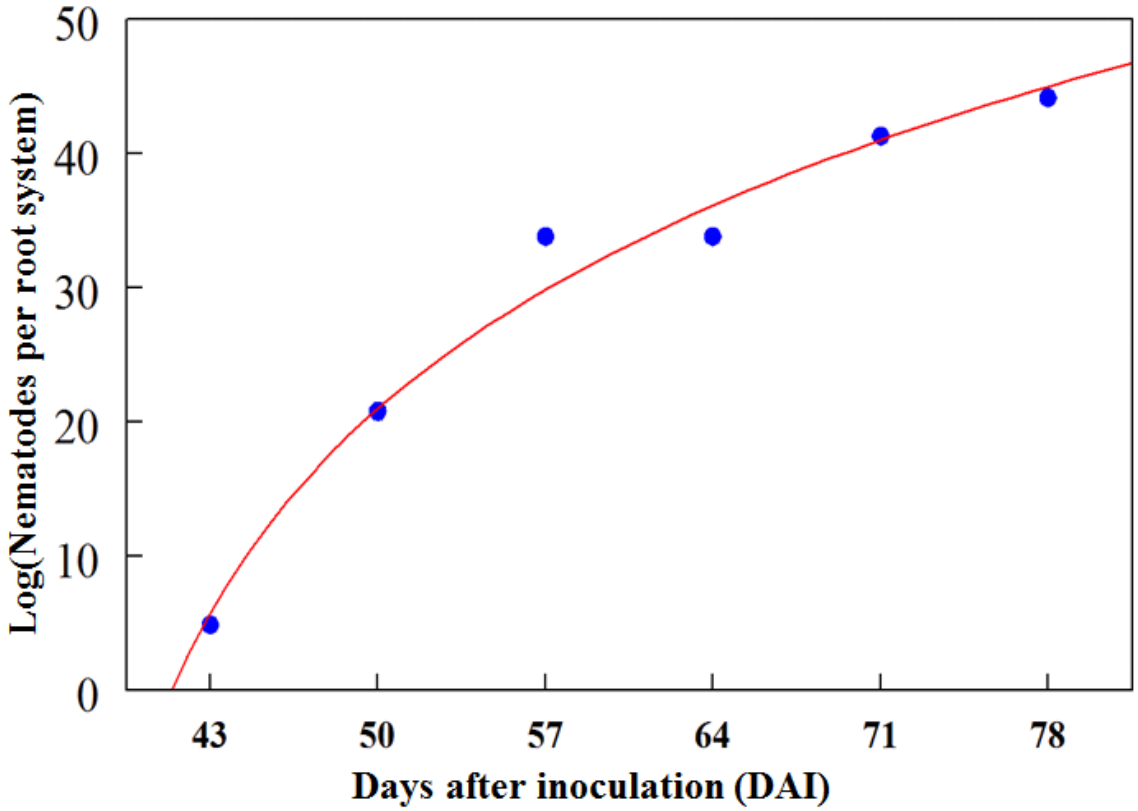

Figure 3. The relationship between *Meloidogyne hapla* eggs and J2 per root system and days after inoculation (n = 5).

Table 2. Plant growth performances and nematode numbers over time on *Rosa corymbifera* ˈLaxaˈ infested by *Meloidogyne hapla.*

DAI	Treatment	Leaf Nr.*	Shoot fresh weight (g)	Root fresh weight (g)	Shoot height (cm)	Total number of nematode per root system	Final nematode population (soil and root)	RF (Pf/Pi)
01	Control	4.6 ± 0.40^{l}	0.2 ± 0.02^{k}	0.2 ± 0.02^{k}	5.10 ± 0.29^{j}	-	-	-
	infected	5.8 ± 0.37^{k}	0.4 ± 0.03^{k}	0.2 ± 0.03^{k}	5.3 ± 0.26^{j}	13.8 ± 2.9^{d}	-	-
08	Control	7.0 ± 0.32^{j}	0.6 ± 0.04^{j}	0.3 ± 0.03^{k}	7.1 ±0.34^{i}	-	-	-
	infected	8.2 ± 0.58^{i}	0.6 ± 0.09^{j}	0.3 ± 0.05^{k}	7.2 ± 0.45^{i}	139.2 ± 31.9^{b}	-	-
15	Control	11.2 ± 0.58^{h}	1.4 ±0.12^{i}	0.8 ± 0.08^{j}	13.2 ± 0.67^{h}	-	-	-
	infected	8.4 ± 0.24^{i}	1.2 ± 0.07^{i}	1.1 ± 0.10^{j}	12.8 ± 0.45^{h}	144.2 ± 16.3^{b}	-	-
22	Control	11.8 ± 0.80^{gh}	2.1 ± 0.08^{h}	1.2 ± 0.09^{j}	17.8 ± 0.44^{g}	-	-	-
	infected	11.8 ± 1.16^{gh}	1.9 ± 0.10^{h}	1.6 ± 0.17^{i}	16.5 ± 0.91^{g}	124.8 ± 17.1^{b}	-	-
29	Control	13.2 ± 1.56^{defg}	2.9 ± 0.15^{g}	2.7 ± 0.18^{h}	20.7 ± 0.83^{f}	-	-	-
	infected	12.0 ± 0.77^{gh}	2.3 ± 0.20^{h}	2.4 ± 0.15^{h}	20.1 ± 0.92^{f}	232.6 ± 45.7^{a}	-	-
36	Control	13.8 ± 1.16^{df}	3.5 ± 0.23^{f}	3.9 ± 0.21^{g}	22.2 ±0.32^{e}	-	-	-
	infected	12.2 ± 0.37^{gh}	2.9 ± 0.15^{g}	3.5 ± 0.40^{g}	24.3 ± 0.29^{d}	216.8 ± 23.2^{a}	-	-
43	Control	14.0 ± 0.55^{d}	3.6 ± 0.21^{f}	6.5 ± 0.47^{e}	25.3 ± 0.68^{cd}	-	-	-
	infected	12.4 ± 0.24^{gh}	3.1 ± 0.08^{g}	5.22 ± 0.43^{f}	25.7 ± 0.55^{c}	-	6094 ± 1111.3^{d}	6.1 ± 1.1^{d}
50	Control	15.2 ± 1.98^{bcd}	3.7 ± 0.24^{f}	7.5 ± 0.81^{de}	26.6 ± 0.95^{c}	-	-	
	infected	13.8 ± 0.80^{d}	4.0 ± 0.25^{de}	6.98 ± 0.46^{de}	28.3 ± 1.1^{bc}	-	22610 ± 5789.8^{cd}	22.6 ± 5.8cd
57	Control	15.6 ± 1.03^{c}	4.6 ± 0.17^{b}	7.4 ± 0.49^{d}	28.2 ± 0.51^{b}	-	-	-
	infected	14.2 ± 0.73^{d}	4.8 ± 0.15^{b}	7.62 ± 0.54^{d}	30.5 ± 0.80^{a}	-	35546 ± 4972.5^{bc}	35.5 ± 4.9bc
64	Control	16.0 ± 0.55^{c}	5.1 ± 0.32^{ab}	8.8 ± 0.22^{c}	29.5 ± 0.72^{a}	-	-	-
	infected	13.2 ± 0.49^{ef}	4.3 ± 0.18^{cd}	8.48 ± 1.05^{cd}	30.2 ± 0.93^{a}	-	35896 ± 11765.2^{bc}	35.9 ± 11.7^{bc}
71	Control	17.4 ± 0.87^{ab}	4.5 ± 0.41^{bc}	9.4 ± 0.46^{bc}	29.8 ± 0.46^{a}	-	-	-
	infected	13.0 ± 0.32^{f}	5.0 ± 0.26^{ab}	10.02 ± 0.50^{ab}	30.1 ± 1.08^{a}	-	45725 ± 7066.5^{ab}	45.7 ± 7.1^{ab}
78	Control	18.4 ± 0.49^{a}	5.6 ± 0.40a	9.9 ± 0.42^{b}	31.1 ± 0.88^{a}	-	-	-
	infected	12.2 ± 0.37^{g}	4.2 ± 0.19^{d}	10.8 ± 0.24^{a}	30.8 ± 1.73^{a}	-	58930 ± 5460.8^{a}	58.9 ± 5.5^{a}

* All measurements are means ± standard deviation. Means in columns followed by the same letter do not differ ($P \leq 0.05$) according to Duncan's multiple-range test (n = 5).

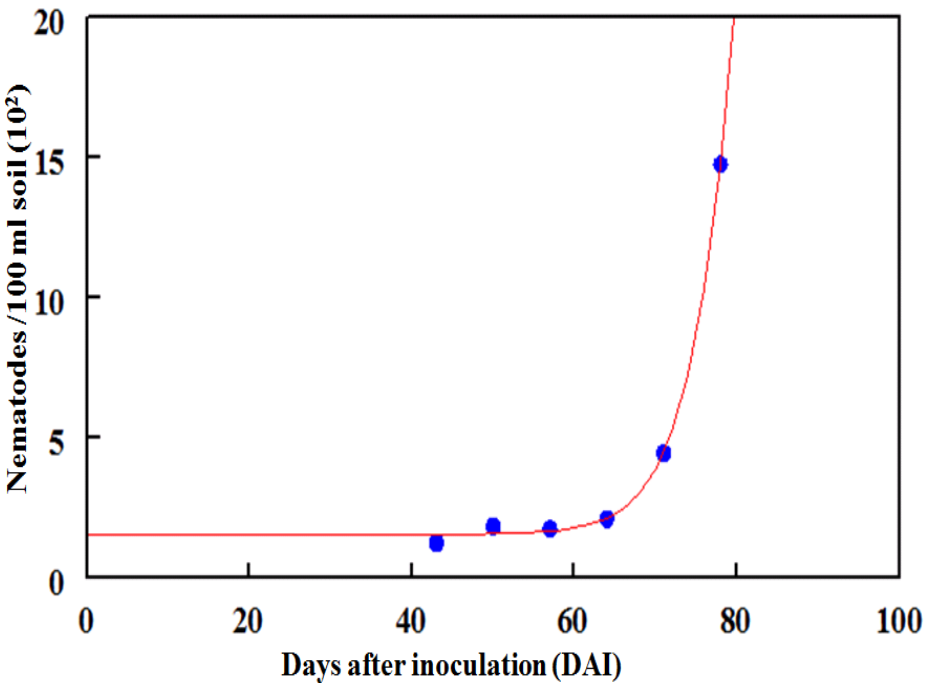

Figure 4. Population dynamics of *Meloidogyne hapla* in the soil 43-78 days after plants were inoculated (n = 5). J2s were recovered from 100 ml by modified Baermann tray method.

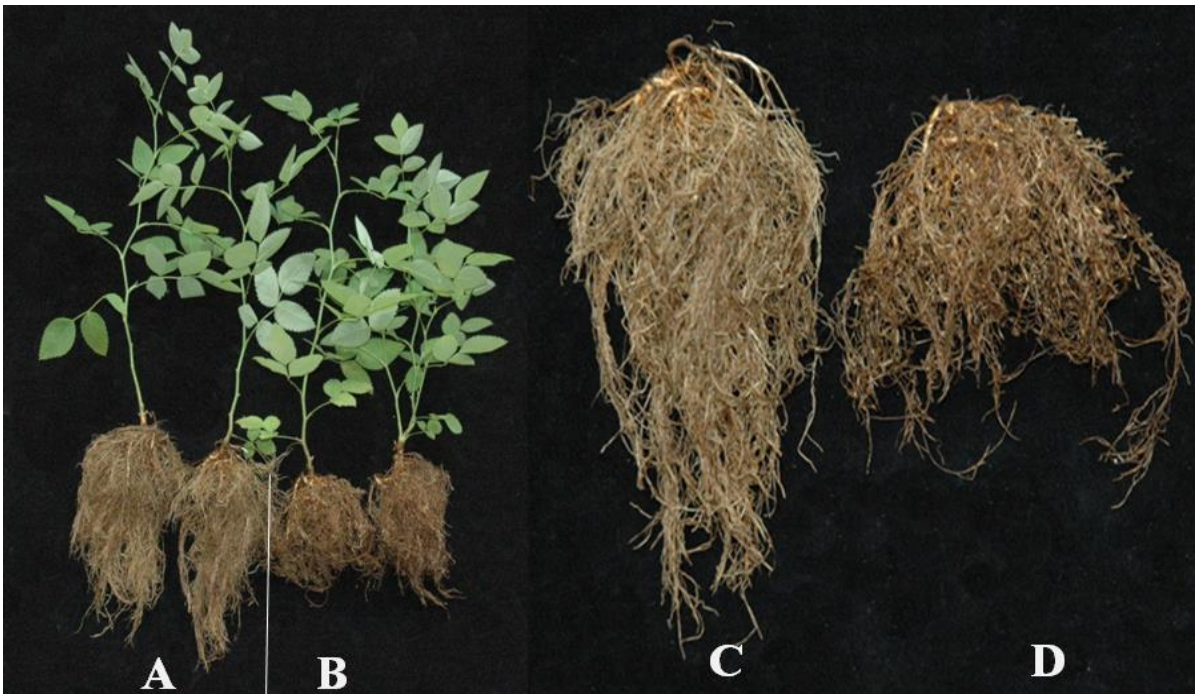

Figure 5. Effect of *Meloidogyne hapla* infection on growth of *Rosa corymbifera* 'Laxa' 78 days after nematode inoculation. Uninfected control (A and C) and infected plants (B and D).

4.4 DISCUSSION

Over the last decade, Ethiopia developed to a major producer of cut flowers for the international market. Meressa *et al.* (2012) detected plant-parasitic nematodes associated with roses grown in greenhouses in Ethiopia. Infested greenhouse sites were often associated with poor plant stand. The question rose, if common root-knot nematodes species such as *M. hapla* and *M. incognita* could also multiply on cut flower species grown in Ethiopia. Both scenarios would mean a major threat to cut flower production in Ethiopia.

All cut flower species and cultivars tested in this study turned out to be good or excellent hosts for *M. hapla* except for *F. laxa*, and good hosts for *M. incognita*, except for *R. corymbifera* 'Laxa' and *G. paniculata*. However, there was a substantial difference in host status of the tested cut-flower species and cultivars for both root-knot nematode species. Unfortunately, none of the tested species and cultivars was resistant for both nematode species at the same time. In general, rotation between host and nonhost crops is an important component of IPM of plant-parasitic nematodes (Neo *et al*., 1991) and knowing the host status of any given crop might enable the farmer to use this crop for nematode management. However, this tool might be of limited value if cut-flowers are produced in monoculture.

In our study *R. corymbifera* 'Laxa' was a host for *M. hapla*. This is confirmed by Coolen and Hendrickx (1972) who tested 13 rose rootstocks in the field and all rootstocks turned out to be hosts for *M. hapla*, except for *Rosa canina* cv Success and *R. canina* cv Heinsohn's Rekord, being poor hosts. Similarly, Pizetta *et al.* (2010) found all nine rootstocks of *Rosa multiflora* and *R. indica* being hosts for *M. hapla*, although at variable degree. However, it should not be ignored, that the host plant response can be influenced by the origin of the *M. hapla* isolate. This was shown by Wang *et al*. (2004) for rose rootstocks of *R. multiflora* and *R. indica* using 4 geographic isolates of *M. hapla*. In their studies host suitability of *R. multiflora* clone K1 ranged from intermediate to resistant depending on the *M. hapla* isolate used. Similarly, *R. indica* was a good to excellent host for a specific isolate of *M. hapla* from Canada but resistant to three other isolates, thus expressing an isolate-specific resistance. This clearly shows the importance of testing host plant suitability under local conditions.

Quite interestingly, *R*. *corymbifera* 'Laxa' was a poor to nonhost for *M*. *incognita*. If this can be confirmed under field conditions, *R*. *corymbifera* 'Laxa' could be a useful tool to control *M. incognita* at infested sites.

In our study, both species of carnation and both cultivars of *Limonium* were good hosts for *M. hapla* and *M. incognita.* However, host suitability varied between plant genotype and nematode species, e.g. both carnation species allowed a higher reproduction of *M. incognita*

than of *M. hapla*. For carnation, *M. incognita* is considered the main cause for yield reduction estimated to reach 20% worldwide (Sasser *et al.*, 1987). Differences in host status among carnation cultivars for *M. incognita* have been reported by Cho *et al*. (1996). The 33 screened cultivars fall into three groups ranging from highly and moderate resistant to susceptible.

Freesia laxa was a poor host for *M. hapla* and a maintenance host for *M. incognita*. For both nematode species, only few small galls were observed on freesia roots. However, numerous small dark necroses were associated with the bulbs. Unfortunately, no information on *M. hapla* and *M. incognita* affecting freesia was found in the literature. However, for another tropical root-knot nematode species, *M. javanica*, Tyler (1941) reported that infected roots showed light galling and plants suffered a lot even at low densities of *M*. *javanica*. If the poor host status of freesia can be used in the field to reduce high densities of *M. hapla* or *M. incognita* still needs to be proofed. In commercial rose production, there is usually a short fallow period between two consecutive crops in which old plants are uprooted and left to dry on the soil surface. This fallow period might be used to grow resistant short season freesia before new rose seedlings are transplanted.

As shown here *G. paniculata* was a poor host for *M. incognita* and only allowed little multiplication (RF = 2.0) of *M. hapla*. In contrast, McSorley (1994), Goff (1936) and Wilcken and Ferraz (1998) found *G*. *paniculata* being susceptible to *M*. *incognita*. This discrepancy might be attributed to differences in the used nematode isolate or cultivar as discussed above.

Root invasion, development and duration of the life cycle of *M*. *hapla* depend on both host plant species and environmental conditions (Kinloch and Allen, 1971). Within this respect, the development and population dynamics of *M. hapla* was studied more in detail on *R*. *corymbifera* ˈLaxaˈ. First juveniles penetrated the root tips within 24 hours after inoculation. Under the given temperature of 20 ± 3^{o}C, *M*. *hapla* completed its life cycle between 36 and 43 DAI. Unfortunately, the exact date could not be determined as sampling was done on a weekly basis. At final termination of the experiment 78 DAI a RF = 58.9 was achieved indicating the enormous reproduction potential of *M. hapla* on *R*. *corymbifera* ˈLaxaˈ. Nematode infection was associated with a lower number of leaves per plant compared with uninfected controls. However, results on shoot and root fresh weights showed no clear tendency between infected and uninfected plants. Although visual inspection indicated a reduced root system in *M. hapla* infested plants, root fresh weight was for most sampling dates not significantly reduced. Most likely reduced root length was compensated by the weight of the root galls (Santo and O'Bannon, 1982). Moreover, gall numbers per root

virtually decreased over time which most likely can be contributed to the fusion of neighbouring galls in older roots thereby impaired the counting.

Growing roses over several years in combination with the high reproduction rate of *M. hapla* under greenhouse conditions will facilitate *M. hapla* causing severe losses (Epstein and Bravdo, 1993; Santo and Lear, 1976). Although little is known about the overall economic damage Johnson *et al.* (1969) reported a reduction of 19,000 harvestable flower stems per ha and year. In infested soil, roots are damaged and water and nutrient uptake is disturbed resulting in wilting, leaf discoloration and senescence (Bird, 1974). Our observation of leave chlorosis on nematode infested plants was most likely attributed to nutrient deficiency. As reported by Xu *et al*. (2010) root-knot nematode infection can cause reduction of leaf nitrogen, an important component of leaf chlorophyll.

In conclusion, the tested cut-flower species were in general good hosts for *M. hapla* or *M. incognita*. Especially the rose rootstock *R. corymbifera* ˈLaxaˈ turned out to be an excellent host for *M. hapla* and thus should be excluded from being used as a rootstock in *M. hapla* infested greenhouses. Overall, *M. hapla* and *M. incognita* present a severe threat to cut-flower production.

4.5 REFERENCE

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Chapter Five

5. Impact of *Meloidogyne hapla* initial population densities on damage threshold to three rose rootstock species

ABSTRACT

The relationship between initial population densities (*Pi*) of *Meloidogyne hapla* on growth of three rose rootstocks (*Rosa corymbifera* 'Laxa', *R*. *multiflora* and *R*. *canina* ' Inermis') and nematode population development was studied. Each plant species was inoculated with ranges of nematode densities of 0, 0.062, 0.125, 0.25, 0.50, 1, 2, 4, 8, 16, 32, 64 and 128 second-stage juveniles g^{-1} soil and were allowed to grow for 80 days. Seinhorst yield model ($y= Y_{max}*(m+ (1-m)*0.95\text{^}(Pi-T/Pi))$) was fitted to total fresh weight and root fresh weight data of all the three rose rootstocks. The tolerance limits (*T*) for total fresh weight was 0.04, 0.09 and 0.01 J2 per gram soil and a minimum yield (*m*) 0.65, 0.471 and 0.427 for *R*. *corymbifera* 'Laxa', R. *multiflora* and *R. canina*, respectively. Similarly, estimated tolerance limits for root fresh weight of *R. corymbifera* 'Laxa' was 0.09 J2 per gram soil and minimum yield was 0.58. In comparison, *R. multiflora and R. canina* showed a lower tolerance limit (*T*) of 0.011 J2 g^{-1} soil and a minimum yield of 0.71 and 0.47, respectively. The reproductive factor (Pf/Pi) was higher at low initial nematode population densities for all rootstocks and then decreased to below maintenance level with increasing initial population density. Root gall severity consistently increased with initial nematode population density. Further, number of root-galling against final nematode population per gram root fresh weight showed a strong positive relationship. The relation between Pi and Pf was fitted to the Seinhorst population model (*Pf*= (*M* * *Pi*)/ *Pi* + *M*/*a*). *Rosa multiflora* supported best the population of *M*. *hapla* to a maximum population density of (*M*) 27.53 J2 g^{-1} soil with an estimated average multiplication rate (*a*) of 24.39. The nematode For *R. corymbifera* 'Laxa' and *R. canina* the multiplication rate was 4.34 and 3.62 and the maximum population densities 6.08 and 4.78 J2 per g dry soil, respectively. Hence, it was demonstrated that all three rootstocks are sensitive to even low initial nematode densities and are excellent host for *M. hapla*.

5.1 INTRODUCTION

The northern root-knot nematode, *Meloidogyne hapla* Chitwoodi, is a major problem of cut rose production under greenhouse conditions (Pizetta *et al*., 2010; Voisin *et al*., 1996; Towson and Lear, 1982; Coolen and Hendrickx, 1972; Santo and Lear, 1975). Severe infestation of rose greenhouses with *M. hapla* was recently reported also for Ethiopia (Meressa *et al*., 2014; Meressa *et al*., 2012). Severe deformation of the root system, intense leaf chlorosis and reduction in stem size are among the damages caused by this nematode. Any reduction in stem size will directly reduce the amount of marketable flower stems.

In rose greenhouses, soil fumigation with nematicides (Hugue and Goven, 1987) and chemical or hot-water bare-root dips (Towson and Lear, 1982; Dale, 1973) have been employed to control *M*. *hapla*. However, nematicide as an option for nematode control has declined over time for both economical and environmental reasons (Wachira *et al*., 2009). Further, application of nematicides on roses can reduce the number of marketable flower stems by 25-58% due to reduction in photosynthesis and transpiration process (Epstein and Bravdo, 1973). Thus, alternative control measures such as resistance cultivars are urgently needed (Wang *et al*., 2004; Trudgill, 1991). For rose rootstocks, there are few reports available indicating partial resistance of *Rosa canina* and *R*. *multiflora* cultivars to *M*. *hapla* (Coolen and Hendrickx, 1972). However, those resistance evaluations were carried out mainly based on one nematode density, in which case the resistance might appear different at various ranges of nematode initial densities, as crop damage becomes evident when nematode densities at planting are above the tolerance limit (Viaene and Abawi, 1996). Hence, the initial soil nematode density at planting above which plant growth is impaired needs to be evaluated to predict nematode damage and apply control measures (Ferris, 1978; Barker and Olthof, 1976).

Most cut flower growers in developing countries give no or very little consideration to human and environmental health and often apply nematicides prophylactic even in cases were there is no need. This could be avoided by providing threshold levels for nematode damage and executing a proper nematode sampling before planting (Vovlas *et al*., 2008; Noling and Ferris, 1986).

Todate, the relationship between nematode population densities at planting and plant growth and yield has been described for several plant-nematode combinations using the Seinhorst model (Schomaker and Been, 2013, Norshie *et al*., 2011; Vovlas *et al*., 2008; Vrain, 1996; Viaene and Abawi, 1996). In this case, damage threshold density or tolerance limit of crop species or cultivars is determined (Barker and Olthof, 1976), which allows minimizing the cost of nematode control measures.

However, no such information is yet available for the relationships between *M. hapla* and rose rootstocks. Therefore, the objective of the present study was to investigate the effect of different initial population densities of *M. hapla* on the growth of the three rose rootstocks *Rosa corymbifera* 'Laxa', *R*. *multiflora* and *R*. *canina* cv. Inermis and estimate the host efficiency of the rootstocks to the nematode.

5.2 MATERIAL AND METHODS

5.2.1 *Plant material*

Three species of rose rootstocks, *Rosa corymbifera* ' Laxa', *R. multiflora* and *R. canina* cv. Inermis were evaluated for their damage caused by *M. hapla*. One month old rooted plant material raised from seeds was obtained from Klei (Heidgraben, Germany). Each plant was individually transplanted into a 2 kg capacity plastic pot filled with steam-sterilized field soil and silver sand (4:1, w/w), respectively. Throughout the experimental period, temperature was set at 23^{o}C during the day and 20^{o}C at night. Photocycle was adjusted to 16 h light and 8 h dark using 600 W, 58500 lumen lamps (Norka-Lighting®, Hamburg, Germany). Plants were watered daily with 15 ml tap water and fertilized weekly with 0.3% WUXAL® Super liquid foliar fertilizer (Agrarverstand Oberland, Schongau, Germany).

5.2.2 *Nematode inoculum*

A stock population of *M. hapla* originating from Germany was reproduced on tomato (*Solanum lycopersicum*) cv. Moneymaker in pots containing steam sterilized field soil, maintained in a greenhouse adjusted to 21 ± 2^{o}C. For collecting nematode inoclum, heavily-galled tomato roots were placed in a misting chamber (Hooper *et al*., 2005). Hatched juveniles were collected every two days and stored at 6^{o}C until used for the experiment. Every two days the water in the flask was replaced with fresh tap water to facilitate aeration and thereby maintain juvenile fitness. At the day of nematode inoculation, **t**he J2 density was adjusted to give initial population densities of 0, 0.062, 0.125, 0.25, 1, 2, 4, 8, 16, 32, 64, and 128 J2 g^{-1} dry soil.

5.2.3 *Nematode inoculation*

Four vertical holes about 6 cm deep and 1 cm wide were bored into the soil around the plant stem using a rounded conical-end plastic stick. Then, 20 ml of the nematode suspension per

plant was directly injected into the four holes with a calibrated Multipette® plus (Eppendorf®, Hamburg, Germany) dispenser. Control plants received a similar volume of tap water. The pots were arranged in a randomized block design on raised benches in a greenhouse. Each treatment was replicated six times.

5.2.4 *Measurements*

Nine weeks after nematode inoculation, the top plant parts was cut off and shoot fresh and dry weights were recorded. The dry weight was measured after 24 h of oven drying at 110^{o}C. Root fresh weight was measured after roots were washed free from adhering soil and carefully blotted on tissue paper.

Root galls per root system were counted with the aid of a magnification lens (Zevatron®, Arolsen, Germany). Eggs and juveniles were then dislodged from egg masses using a 1.6% sodium hypochlorite solution following the method described by Hussey and Barker (1973). In addition, juveniles were extracted from 100 g soil for 48 h using the modified Baermann technique (Hooper *et al*., 2005). Both eggs and J2 were counted at 63× magnification using a compound microscope.

The final nematode population (*Pf*) was then determined by adding the number ofeggs and juveniles extracted from the whole root system and the number of juveniles extracted from soil

5.2.5 *Statistical analysis and model fitting*

Regression analysis for estimating yield and population dynamical parameters were carried out using a script written in *R*-version 3.0.1 and edited in Tinn-*R* version 2.4.1.7. The damage functions on the plant fresh biomass from the Pi ranges of *M. hapla* were described using the Seinhorst exponential model (Schomaker and Been, 2013; Seinhorst, 1979). In this model, a nonlinear regression function was used to estimate its coefficients and fit the data to $Y = Y_{max}*(m+ (1-m)*0.95^{(Pi-T/Pi)})$ When $P \geq T$, and $y = Y_{max}$ when $P \leq T$ (Schomaker and Been, 2013;); where y is the relative average value of plant weight; Y_{max} is the yield at densities lower than T; m is the relative minimum value of y at a very large Pi; Pi is the initial nematode population density; T is the tolerance limit (the initial nematode density below which plant growth is not affected); 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 indicate the goodness-of-fit of data to the model. The relationship between Pi and Pf densities were

described by fitting the data to the Seinhorst population dynamics model $Pf = (M * Pi)/(Pi + \frac{M}{a})$, where, *M* is an estimate of the maximum population density at high *P*i values and *a* maximum multiplication rate of the nematode at lower *P*i values (Seinhorst, 1967 and 1970) as described by Schomaker and Been (2013).

The linear model was estimated to the relationship between gall numbers and final population densities per gram of root fresh weight. The nematode reproduction factor (RF) was expressed as *Pf/Pi* for all density ranges. Since dry weight, RF and shoot-to-root ratio were not adequately described by the Seinhorst model, data were subjected to ANOVA. Means were separated by Duncan's test at *P*<0.05 level.

5.3 RESULTS

5.3.1 *The relation between initial nematode densities and plant weight*

All initial population densities of *M. hapla* caused a significant (*P* < 0.05) reduction in shoot fresh weight of all three rose rootstock species (Fig. 1A and B). The relationship between total shoot fresh weight of the plants at harvest and initial population density was adequately described by the Seinhorst model with R^2= 0.80, 0.87 and 0.82 for *R. corymbifera*, *R. multiflora* and *R. canina*, respectively. Moreover, all rootstocks had similar response that the total fresh weight of inoculated plants was significantly less than their corresponding control plants.

Increasing initial nematode densities were significantly (*P* = 0.012) correlated with a decrease in root fresh weight of all three rootstock species. At the highest nematode density (128 J2 g^{-1} soil), the reduction in root fresh weight compared to the control was 50%, 31% and 65% for *R. corymbifera*, *R. multiflora* and *R. canina*, respectively.

The shoot dry weight of the uninoculated control plants of *R. corymbifera* and *R. canina* was significantly greater than for plants inoculated with *M. hapla* regardless the initial densities (Table 1). Therefore, a significant negative correlation between shoot dry weight and initial nematode densities was observed (*R. corymbifera*: R^2 = 0.618, *P* = 0.003; *R. canina:* R^2 = 0.551, *P* = 0.006). In contrast, for *R. multiflora*, no such statistically significant negative correlation was observed.

The shoot-root ratio of all three rootstock species was poorly correlated with the initial nematode density and thus not statistically different from the corresponding uninoculated control plants (Table 1). Damage threshold

The tolerance limit (*T*) to *M. hapla* varied between the rootstock species (Table 2; Fig. 1A). According to the best fit of Seinhorst model for total plant fresh weight the tolerance limit was estimated at 0.04 J2 g^{-1} soil for *R. corymbifera* ˈLaxaˈ, 0.09 J2 g^{-1} soil for *R. multiflora* and 0.01 J2 g^{-1} soil for *R. canina* ˈInermisˈ. The minimum relative yield (*m*) was predicted for *R. canina* ˈInermisˈ (0.427) and *R. corymbifera* ˈLaxaˈ (0.471) at a significantly ($P < 0.05$) lower initial nematode density than for *R. multiflora* (0.65) (Table 2; Fig. 1 A).

Similarly, fitting the data of the root fresh weight to the Seinhorst model (Table 2; Fig. 1 B) the tolerance limit for *R. corymbifera* ˈLaxaˈ was estimated at 0.09 J2 g^{-1} soil and a minimum relative yield of 0.58. In comparison, *R. multiflora* and *R. canina* ˈInermisˈ showed a lower tolerance limit of 0.011 J2 g^{-1} soil and a minimum yield of 0.71 and 0.47, respectively.

Damage symptoms such as wilting of apical shoot tip, stunting of tops, leaf yellowing and leaf fall were first observed on R. multiflora 35 days after nematode inoculation irrespective of the initial population density. On *R. corymbifera* ˈLaxaˈ and *R. canina* ˈInermisˈ leaf yellowing and leaf fall became apparent after 45 days of inoculation, and apical shoot wilting was rarely seen.

Table 2. Effect of initial nematode density (Pi) on reproduction of *Meloidogyne hapla* and on shoot-root ratio and shoot dry weight of three rose rootstock species.

Nematode initial density/g soil (Pi)	Shoot-root ration			Shoot dry weight			Reproductive factor Pf / Pi		
	R. corymbifera	*R. multiflora*	*R. canina*	*R. corymbifera*	*R. multiflora*	*R. canina*	*R. corymbifera*	*R. multiflora*	*R. canina*
0	0.58	0.68	0.72	7.00 a	4.40	5.05 a	-	-	-
0.062	0.32	0.46	0.49	3.68 b	3.38	2.32 b	6.5 a	27.16 b	9.28 a
0.125	0.33	0.49	0.54	3.67 b	3.33	1.80 bcd	5.1 b	60.50 a	4.81 b
0.25	0.32	0.43	0.63	3.47 b	3.17	1.85 bcd	3.5 c	8.64 bc	4.04 bc
0.5	0.38	0.49	0.59	3.00 ab	3.08	2.07 bc	1.8 d	13.71 bc	1.62 cd
1	0.28	0.44	0.79	3.50 b	3.12	1.68 bcde	1.7 d	8.80 bc	0.91 d
2	0.34	0.53	0.76	3.17 ab	3.03	1.63 bcde	1.2 de	7.98 bc	0.91 d
4	0.31	0.53	0.79	2.92 ab	2.83	1.60 cde	0.70 de	5.91 bc	0.72 d
8	0.51	0.48	0.67	2.55 ab	2.57	1.47 cde	0.48 e	2.56 bc	0.39 d
16	0.49	0.60	0.66	3.13 ab	3.08	1.47 cde	0.27 e	1.63 c	0.20 d
32	0.48	0.46	0.71	2.50 ab	2.82	1.22 cd	0.16 e	1.13 c	0.16 d
64	0.51	0.65	0.72	2.67 ab	3.15	1.38 cde	0.08 e	0.41 c	0.24 d
128	0.31	0.68	0.74	1.98 c	2.45	0.97 e	0.06 e	0.26 c	0.14 d
P	*0.216*	*0.713*	*0.877*	*<0.001*	NS	*<0.001*	<0.001	<0.001	<0.001

[a]Numbers within columns followed by the same letter are not significantly different according to Duncan's multiple-range test at p≤ 0.05 level

NS = not significant, *P*= significance at p≤ 0.05

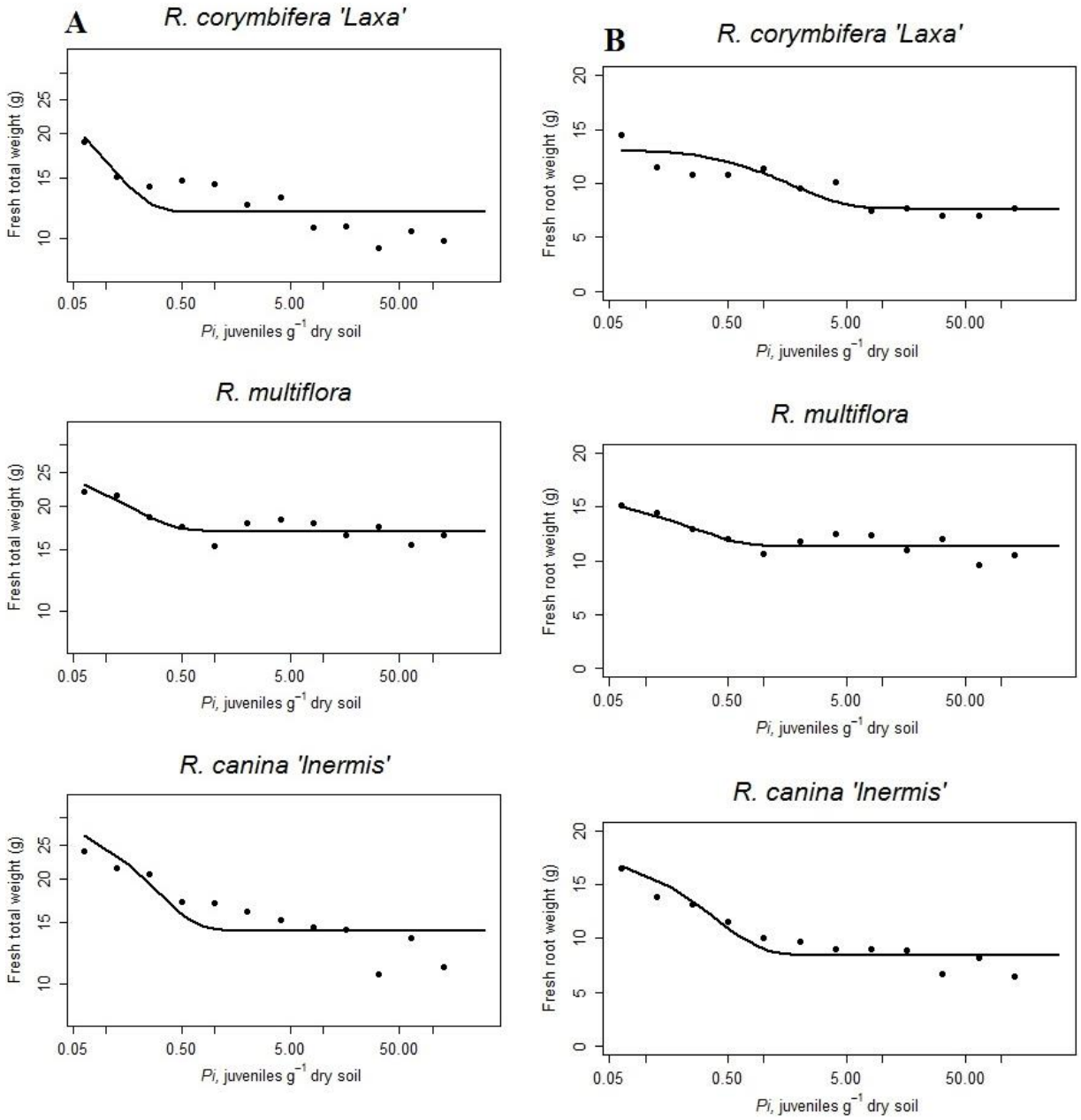

Figure 6. Relationships between initial population densities (*P*i) of *Meloidogyne hapla* and fresh total weight (A) and fresh root weight (B) of three rose rootstock species. Plants were grown in greenhouse and harvested after 80 days. Each point represents a mean of 6 plants and the line is the predicted function obtained by fitting the data to Seinhorst model Y= Ymax* (m+(1-m)*0.95^(Pi-T/Pi)) where Y_{max} is the maximum yield at *Pi* < *T* or Pi = 0 and *y* yield in terms any weight, *m* the minimum relative yield, and *T* the tolerance limit.

Table 3. Parameter estimates of the damage threshold (*T*), minimum relative yield (*m*) of the Seinhorst equation for the relationship between plant weight (*y*) and initial population densities of *Meloidogyne hapla* (*Pi*) on three rose rootstock species. Their corresponding graphs are shown in Figure 1 A and B.

Parameters*	*Rosa corymbifera* 'Laxa'		*R. multiflora*		*R. canina* 'Inermis'	
	TSFW	RFW	TSFW	RFW	TSFW	RFW
Y_{max}	27.94	13.1	26.09	15.89	30.08	17.77
m	0.427	0.58	0.65	0.71	0.471	0.47
T	0.04	0.09	0.07	0.01	0.011	0.02
SeY_{max}	8.793	1.19	1.93	1.03	4.655	1.53
Se_m	0.124	0.07	0.048	0.05	0.068	0.04
Se_T	0.004	0.12	0.003	0.01	0.009	0.01
r^2	0.80	0.75	0.87	0.77	0.82	0.89

* Y_{max} is the yield at densities lower than T; *m* = minimum yield: *T* = tolerance limit Se = standard error; df = degree of freedom; N = number of observations; r^2 = coefficient of determination; SFTW = total shoot fresh weght; RFW= root fresh weight

5.3.2 *Root galling severity*

Root infestation of *M. hapla* resulted in distinct gall formation on roots of the three rose rootstocks. In general, the galling severity increased with increasing nematode population densities (Fig. 2). There was a statistically significant difference in gall counts per root system among rootstocks (P < 0.001), where *R. multiflora* showed the highest (627) and *R. canina* 'Inermis' the lowest (323.5) number of root galls. According to rootstock species, the highest number of root galls was found at the initial *M. hapla* densities of 64, 32, and 128 J2 g^{-1} soil for *R. corymbifera*, *R. multiflora* and *R. canina* roots, respectively.

Number of galls and final population density of *M. hapla* revealed a significant strong positive relationship (*R. corymbifera* 'Laxa': R^2= 0.916, *P*<0.001; *R. multiflora*: R^2= 0.941, *P*<0.00, *R. canina* 'Laxa': R^2= 0.972, *P*<0.00) (Fig. 3). For all rootstock species, the lowest number of galls and the lowest final population density per gram root fresh weight was found at the lowest inoculum density. In general, *R. canina* 'Inermis' roots formed fewer number of root galls per gram root fresh weight than the other two species (Fig. 3).

5.3.3 *Nematode population dynamics*

The population dynamics of *M. hapla* differed depending on the rootstock species (Table 3, Fig. 4). *Rosa multiflora* supported the highest population of *M*. *hapla* to a maximum population density of (*M*) 27.53 J2 g^{-1} soil representing an average multiplication rate (*a*) of 24.39. In comparison, the nematode multiplication rates on *R. corymbifera* 'Laxa' and *R. canina* 'Inermis' were 6.08 J2 g^{-1} soil and 4.78 J2 g^{-1} soil, demonstrating a multiplication rates of 4.34 and 3.62, respectively.

In general, fitting the data for each rootstock species to the equation ($Pf = (M \times Pi) / (Pi + M/a)$) revealed that, the final population density (*Pf*) at time of harvest increased with increasing initial population density (*Pi*) in that their 95% confidence interval on curve lies above the equilibrium line (*Pf* = *Pi*), until the root size and food supply became limiting (Fig 5).

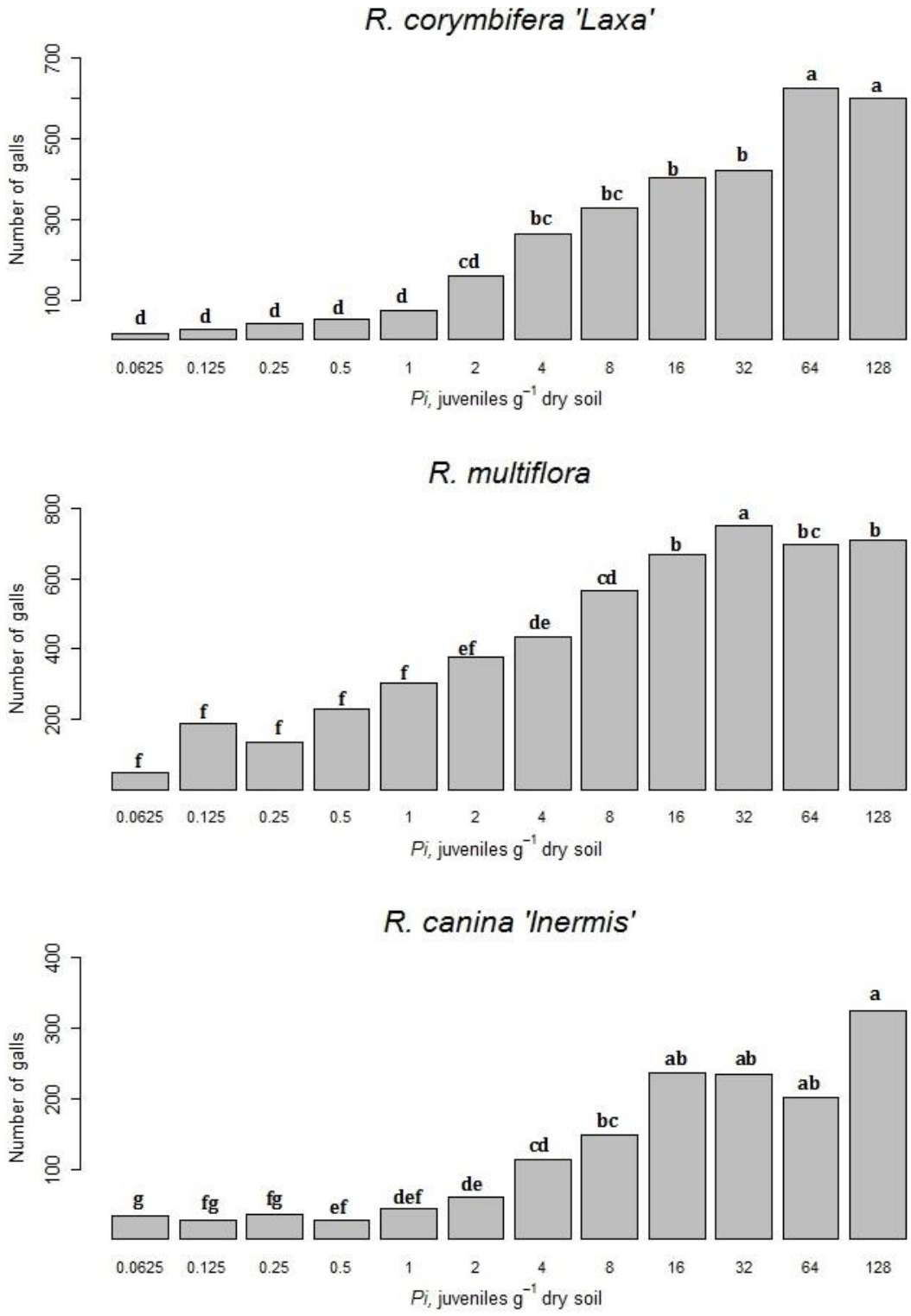

Figure 7. Relationship between initial population densities (*P*i) of *Meloidogyne hapla* and number of galls on three rose rootstock species. Plants were grown in greenhouse and harvested after 80 days (n = 6).

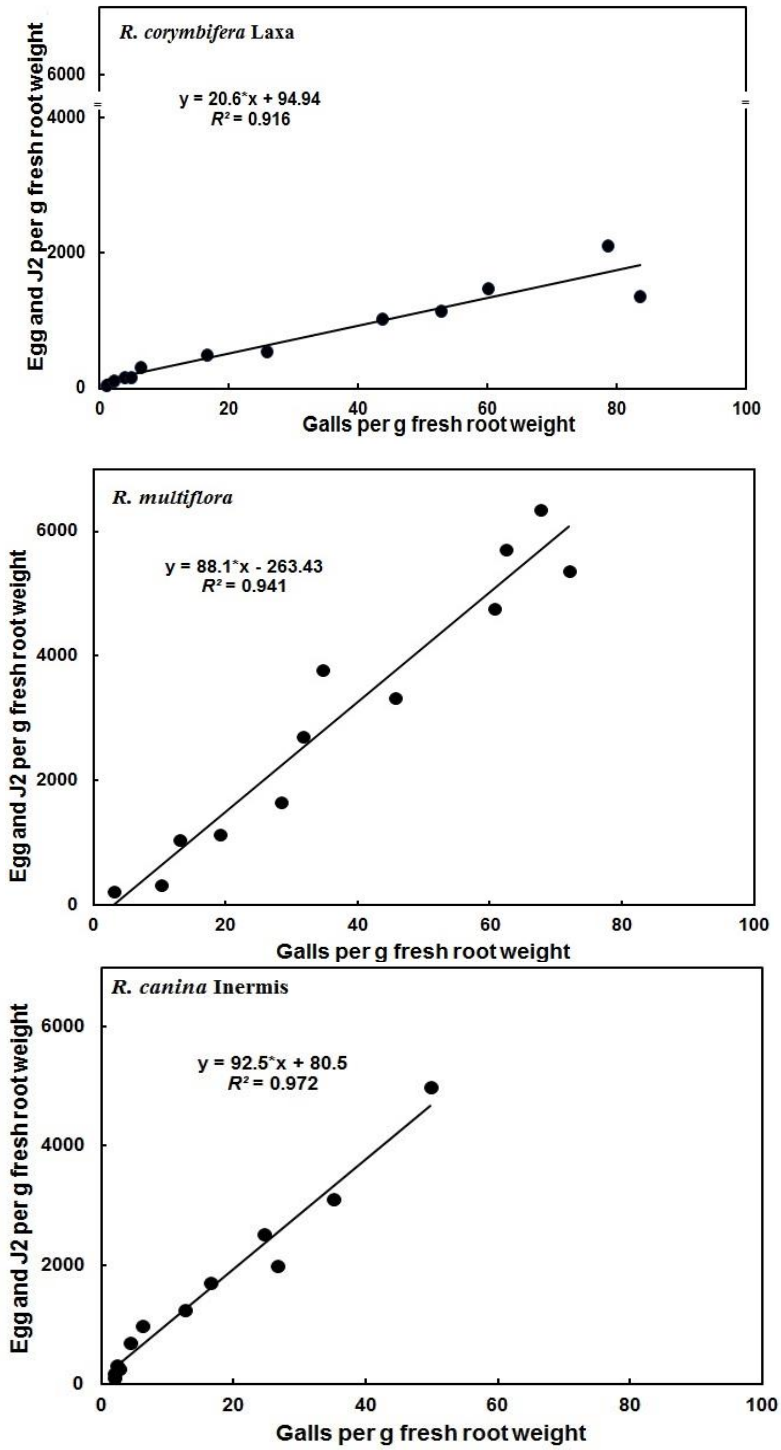

Figure 8. Linear relationship between final population (*Pf*) densities of *Meloidogyne hapla* and number of root galls both expressed per unit gram root fresh weight of *Rosa corymbifera* ˈLaxaˈ, *R. multiflora* and *R. canina* ˈInermisˈ. Each point is the mean of six replications**.**

It seemed that nematode reproduction factor (*Pf*/*Pi*) on *R. multiflora* increased until an initial population density of 32 J2 g^{-1} soil was reached and then dropped with further increasing initial nematode densities. In contrast, for *R. corymbifera* ˈLaxaˈ and *R. canina* ˈInermisˈ, the

reproduction factor increased only until an initial inoculum density of 2 J2 g^{-1} soil was reached.

Table 4. Parameter estimations of the population dynamic model for *Meloidogyne hapla* on three rose rootstock species.

Rootstocks	N	*a*	*M*	Se_a	Se_M	df	R^2
R. corymbifera 'Laxa'	12	4.34	4.78	0.881	0.71	10	0.88
R. multiflora	12	24.39	27.53	6.755	5.67	10	0.80
R. canina 'Inermis'	12	3.62	6.08	1.641	2.3	10	0.61

N = number of observations: *a* = maximum rate of multiplication, *M* = maximum population density; df = degree of freedom; R^2 = coefficient of determination; Se_a = standard error *a*; Se_m = standard error for *M*.

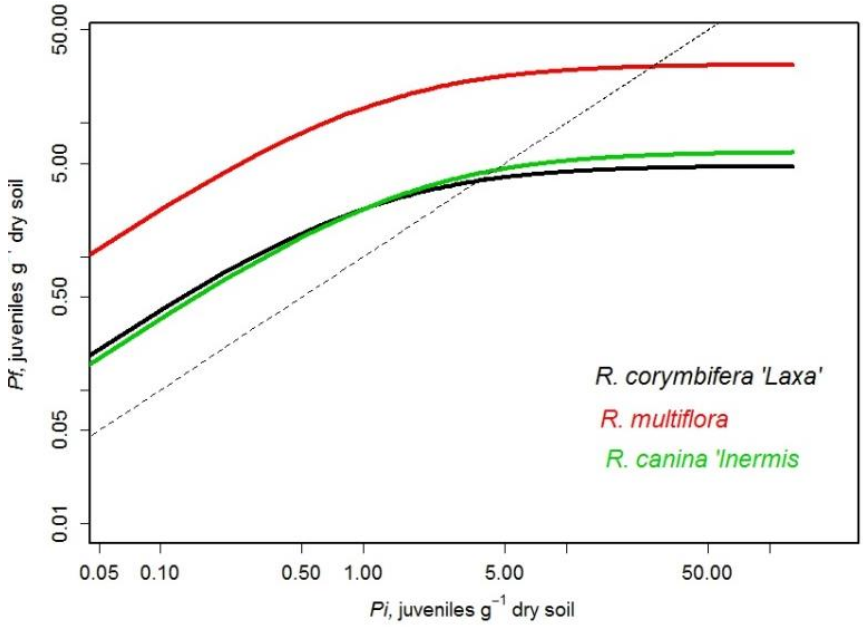

Figure 9. The relation between Pi and Pf of *Meloidogyne hapla* on rose rootstock species. Curve was fitted to the equation $Pf = (M \times Pi) / (Pi + M/a)$. Solid lines: 50% quintile of each rootstock in different colours; dotted line: equilibrium line ($Pf = Pi$).

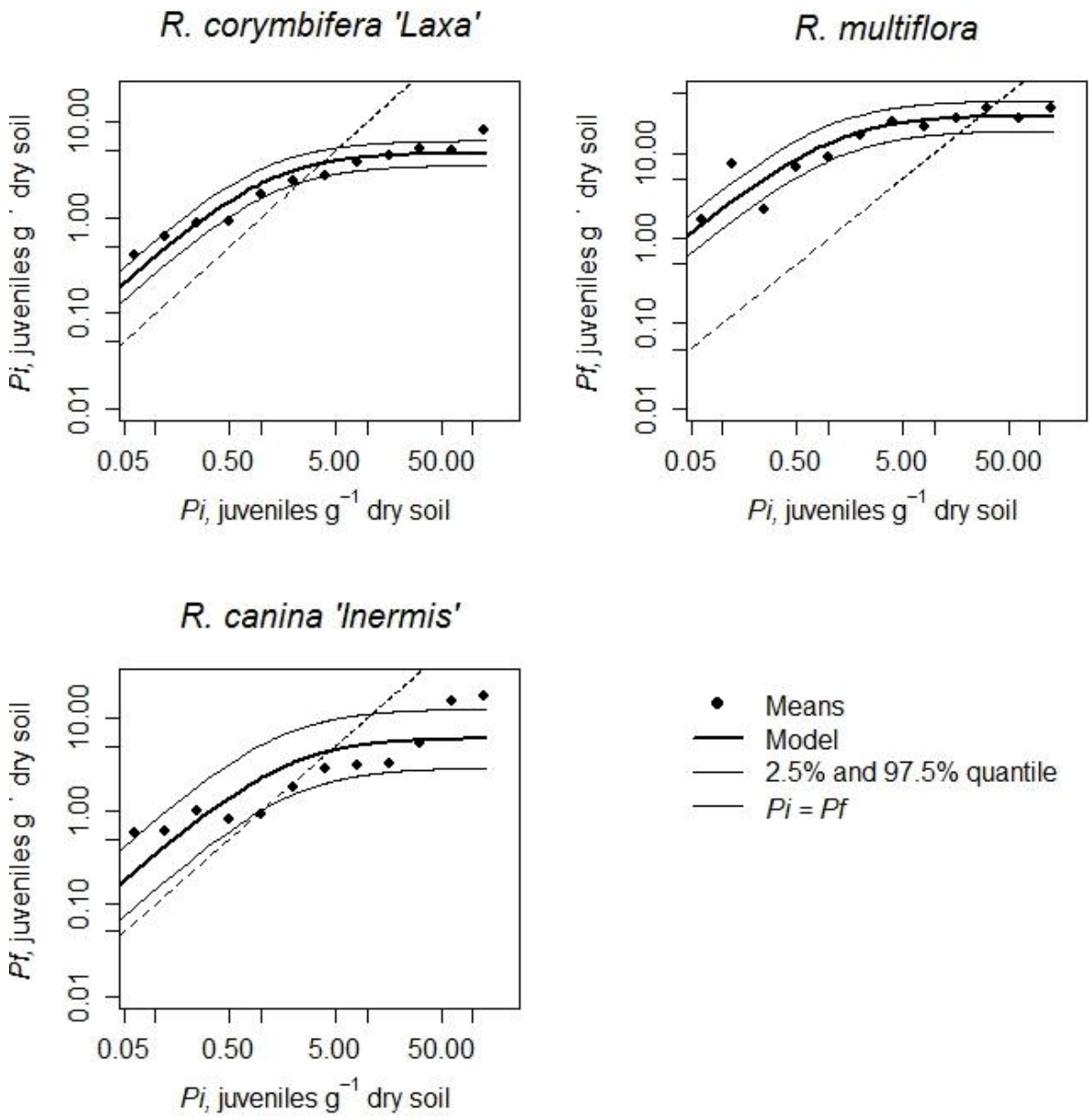

Figure 10. Relationship between initial (*Pi*) and final (*Pf*) populations of *Meloidogyne hapla* on rose rootstocks. Data were fitted to the population dynamic model $Pf = (M \times Pi)/\ Pi + M/a$, where, *Pf* the final population density, *M* maximum population density and *a* maximum multiplication rate of the nematode.

5.4 DISCUSSION

5.4.1 *Plant weight*

The results demonstrated that all three rose rootstock species tested were susceptible to *M. hapla* and allowed high nematode reproduction. The good host status of rose rootstock species for *M. hapla* was confirmed by the work from Pizetta *et al*. (2010); Voisin *et al*. (1996) and Santo and Lear (1975).

Total shoot fresh weight and root fresh weight was negatively correlated with increasing initial nematode densities. All rootstock species suffered from *M. hapla* damage even at the lowest initial densities and plant weight declined upto nematode densities about 1 J2 g^{-1} soil after which it became stable. Damage of rose rootstocks due to *M. hapla* infestation was also reported by Coolen and Hendrickx (1972).

Rosa species are perennial woody shrubs and in commercial farms seedlings are transplanted into the field or greenhouse usually when they are 12 to 16 weeks old.In the present study, seedlings were inoculated at four weeks after germination which is earlier than standard practice. Griffin and Hunt (1972) found that plant tolerance to nematodes is influenced by the age of the plant at the time of nematode inoculation and that older plants have a higher chance to escape from excessive damage, asseedlings have already developed a strong root system able to better compensate nematode damage. Therefore, our results might have overexpressed disease severity at the seedling stage, but nonetheless provides a good picture of the damage potential that might build up over time considering the long cropping time or roses.

The estimates of the minimum relative yield and the tolerance limits were quite low in comparison with other Meloidogyne spp and crops (Kim and Ferris, 2002; Di Vito *et al*., 1986; Di Vito *et al*., 1983). The low values of tolerance limit and minimum relative yield indicate the high pathogenicity of *M. hapla* on those rose rootstock species. Growers should be aware of the enormous damage potential of *M. hapla* and pre-sampling prior to new plantings of rose cultivars known to be susceptible to *M. hapla* is highly recommended. Although tolerance limits developed in greenhouse tests might not fully represent field conditions (MacGuidwin *et al*., 1986), they nevertheless will provide value orientation about the threshold level of *M. hapla*.

For the tested rose rootstock species our results indicated a threshold level for *M. hapla* of 0.07 J2 g^{-1} soil. Meressa *et al*. (2012) reported a *Meloidogyne* population density of 2.88 J2 g^{-1} soil from rose greenhouses in Ethiopia, where *Meloidogyne hapla* was the most abundant species associated with roses (Meressa *et al*., 2014). Under those conditions, planting of the

current rootstock species would result in high damage as confirmed by visual inspection of those fields.

5.4.2 *Galling severity*

All three rootstock species tested showed high numbers of galls, whereat number of galls of *R. corymbifera* 'Laxa' and *R. multiflora* was almost twice as high as for *R. canina* 'Inermis'. For *R. canina* and *R. multiflora* those results are contrary to the observations of Voisin *et al*. (1996) who only found light root galling following infection with *M. hapla*. Differences in tested culitvar, *M. hapla* population and nematode density might explain those discrepancies in plant reaction.

Acoording to De Meijer (1993) and Fourie *et al*. (2010), root galling seemed to be less important to evaluate plant resistance. However, the linear relationship observed for number of root galls and *Pf* per g root fresh weight suggests that number of galls could be suitably used as indicator to predict plant resistance or susceptibility. Flower stem length and diameter are the most important quality parameters for cut rose export (Ahmad *et al*. 2011). Indeed, plant growth regulators such as gibberellins that induce growth of plant stem are partly of root-origin (Kefeli, 1978). Therefore, change on root phsiology due to root root galling might suppress the activity of this hormone and impare the development of the stem; thereby reduce the quality of marketable yield. Although Fourie *et al*. (2010) and De Meijer (1993) overlooked root galling as a parameter of plant resistance.

5.4.3 *Population dynamics*

The maximum population density (*M*) and maximum multiplication rate (*a*) of *M. hapla* were estimated by the population dynamic model of Seinhorst (1967). The *M*/*a* ratio for *R. corymbifera* 'Laxa' and *R. multiflora* were 1.1 J2 g-1 soil, while for *R. canina* 'Inermis' it was 1.7 J2 g-1 soil. Those values indicated that *M. hapla* had a small maximum population density and a high multiplication rate. However, the fact that those parameters are influenced by environmental conditions (Schomaker and Been, 2013) and no susceptible standard for rose rootstocks was available, further studies using different genotypes need to show if our results can be generalized.

The nematode reproductive factor (RF) was high at low initial nematode densities and declined with increasing initial nematode densities. Overall, the RF ranged from 0.1 - 6.5 for *R. corymbifera* 'Laxa', 0.3 - 60.5 for *R. multiflora* and 0.2 - 9.3 for *R. canina* 'Inermis'. In fact,

at nematode densities above 2, 16 and 0.5 J2 g-1 soil for R. corymbifera, R. multiflora and R. canina, respectively, the RF fell below 1 indicating that the final *M. hapla* density was below the initial density. This is a well known phenomenon caused by resource limitations of plants damaged at high population densities (Ferris, 1985). On the other hand, the high RF at low nematode density suggests a high susceptibility of the tested rootstock species for *M. hapla* which is in line with previous results by Pizetta *et al*. (2010). In the present experiment, roots were relatively confined in a small pot (2 l) compared to 7.5 l (Santo and Lear, 1975) that might have hidered full root growth that might provide sufficient new roots that supports better nematode multiplication.

The decline in RF with increase in *Pi* is consistent to previous findings that reproduction rates of *M. incognita* on sweet pepper (Di Vito *et al*., 1985; Lindsey *et al*., 1982), soybean (Fourie *et al*., 2010), tobacco (Di Vito *et al*., 1983), tomato (Di Vito *et al*., 1991), *M. hapla* on alfalfa (Noling and Ferris, 1986) and *M. javanica* on potato (Vovlas *et al*., 2005) have declined with increased inoculum densities. Several crops have been evaluated for host status of *Meloidogyne* species based on reproductive factor, where a value >1 is taken to consider a crop as host for a nematode (Meressa *et al*., 2014; Maleita *et al*., 2012; Fourie *et al*., 2006; Ferris *et al*., 1993; Lamondia, 1996). However, reproductive factor is exclusively density dependent that ignores the effect of high initial population densities (Schomaker und Been, 2006). In our results, the values of reproductive potential at the highest *Pi* were 0.06, 0.26 and 0.14 for *R. corymbifera*, *R. multiflora* and *R. canina*, respectively, which appears literary as if the rootstocks were poor hosts. Therefore, use of different initial nematode densities is very useful to evaluate plant resistance for parasitic nematodes because the the maximum multiplication rate (*a*) and maximum population density (*M*) values could be estimated.

According to Nicola and Di Vito and (2009), yield and growth evaluations over a range of initial nematode densities under greenhouse conditions have less use for prediction under field conditions. However, the fact that roses are typically grown under greenhouse conditions (Meressa *et al*., 2012; DLV plant, 2011; Mercurio, 2007) may indicate that the present evaluationof the rootstocks should be useful to estimate damages in the cut rose production systems. Yet, it should also be considered that the host-nematode relationship varies with environment, nematode race and host cultivar (Barker and Olthof, 1976).

To date, the use of resistance rootstocks for perennial crops is of importance to minimize nematode damages and avoid the use of expensive toxic nematicides (Wang *et al*., 2004). Nevertheless, except for some indications of partial resistance to specific isolates of *M. hapla* in *R. multiflora, R. indica* and *R. canina* rootstock cultivars (Wang *et al*., 2004), resistance rootstocks are not yet commercially available. However, determination of the nematode-crop

relationship provides a guide to employ for crop management strategies. The threshold levels provided here should have practical value to determine the damage level that may be caused by *M. hapla* in roses. Indeed, additional information on the relationship between *Pi* and marketable yield (flower stems) under different environmental conditions is necessary to further comprehend the threshold level.

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Chapter Six

6. Nematicidal potential of plant extracts against the root-knot nematode *Meloidogyne hapla*

ABSTRACT

Nematicidal potential of both aqueous and ethanol extracts from corm of *Rumex abyssinicus* Jacq, roots of *Plumbago dawei* Rolfe and inflorescence of *Maesa lanceolata* Forssk was evaluated against *Meloidogyne hapla*. For evaluating*in vitro* mortality of second-stage juveniles (J2), each extract was tested at five concentrations: 100%, 50%, 25%, 12.5% and 6.25% and five exposure times (10 min, 30 min, 1 h, 2 h, and 20h). In addition, ethanol extracts of each plant species were tested in the greenhouse at three different concentrations of 3, 1.5 and 0.75 ml per tomato plant (*Solanum lycopersicum* cv. Moneymaker) inoculated with 1000 J2. The *in vitro* test revealed that extracts of all three plant species significantly killed *M. hapla* compared to the control. At the highest concentrations and longest exposure periods, ethanol extracts of all three plant species achieved 100% mortalitiy of the nematode. In general, the 100% ethanolic extract of *P. dawei* and both ethanolic and aqueous extracts of *M. lanceolata* were as effective as to kill 50% of the test nematodes within the first 10 min of exposure. In the greenhouse experiment shoot fresh weight of tomatoes treated with the highest extract concentration of *P. dawei* and *M. lanceolat*a was higher (P<0.001) than for untreated control plants. In contrast, the root and shoot fresh weight of plants treated with *R. abyssinicus* did not differ from untreated control plants. Final nematode population densities and number of egg masses were significantly (P< 0.001) reduced following application of all extracts of the three plants. In conclusion, application of those plant extracts as botanical nematicides against *M. hapla* is highly promising as these plants are abundantly available for growers in Ethiopia.

6.1 INTRODUCTION

Plant-parasitic nematodes are among the widely overwhelming pests in the world agriculture causing significant yield losses. An overall annual yield loss was previously estimated to range from 12.3 to 20% depending on the type of crop which can certainly be more than 100 billion dollar (Bird and Bird, 2001; Sasser and Freckman, 1987). Usually, only few of the nematode genera are responsible for most of the crop damage, of which the root-knot nematodes are of particular importance (Sasser and Freckman, 1987; Sasser, 1989).

A survey carried out in 2011 and 2012 in Ethiopia showed that root-knot nematodes are the prominent genus associated with rose flowers causing severe leaf chlorosis, though the damage level has not been determined (Meressa *et al*., 2012).

Use of nematicides for nematode control is increasingly banned due to environmental concern and thus several effective nematicides have been withdrawn from the market (Fernandez *et al*., 2011; Nyczepir *et al*., 2010). As a result, searching for alternative control strategies became more and more important. In annual crops, rotation with non-host crops and use of cover crops grown between the main crops are effective measures to reduce populations of *Meloidogyne* (Belair and Parent, 1996; Sasser, 1989). However, in perennial crops such as roses, rotation as a control measure becomes difficult. Consequently, managing root-knot nematodes using plant derived products with nematicidal potential provide an environmentally friendly and affordable alternative (Chitwood, 2002). Recent investigations have shown the potential of plant products as biocontrol agents (Boursier *et al*., 2011; Ayazpour *et al*., 2010). The fact that many plant extracts constitute of active compounds such as isothiocyanates, glucosinolates, cyanogenic glycosides, alkaloids, terpenoids, sesquiterpenoids, diterpenoids, steroids, triterpenoids, and phenolics, makes them a promising sources for natural pesticides (Oka *et al*., 2006; Chitwood, 2002).

Plant extracts are different from synthetic nematicides as they are generally less toxic, degrade easily, reasonably available and cheap to subsistent farmers (Kahn *et al*., 2008; Chitwood, 2002). Plant species such as castor bean, rapeseed, tobacco, clove, sweet flag, marigold, eucalyptus, *Euphorbia hirta*, neem, *Cassia alata*, *Chromolaena odorata*, *Gliricidia maculate* (Katooli *et al*., 2011; Ononuju and Nzenwa, 2011; Ugwuoke *et al.*, 2011; Nimbalkar and Rajurkar, 2009; Pakeerathan *et al*., 2009; Wiratno *et al*., 2009; Khan *et al*., 2008) are among those reported exhibiting a significant nematicidal potential against *Meloidogyne* species.

Overall, plant extracts are a promising source for nematode control (Chitwood, 2002) particularly bio-extracts from plants that are already used for related purposes. Within these respect three plants *viz. Rumex abyssinicus* Jacq, *Plumbago dawei* Rolfe and *Maesa*

lanceolata Forssk native to Ethiopia were selected. Here, we evaluated the nematicidal potency of their aqueous and ethanolic extract on *in vitro* mortality of second-stage juveniles of *M. hapla* and for control of *M. hapla* on tomato under greenhouse conditions.

6.2 MATERIAL AND METHODS

6.2.1 *Plant material*

The three plant species *Rumex abyssinicus* Jacq, *Plumbago dawei* Rolfe and *Maesa lanceolata* Forssk were collected from Hurumu district in Illubabor Zone, Ethiopia (Lat: 8.35°; Lon: 35.67°) during summer 2011. The rhizome of *R. abyssinicus* and root of *P. dawei* were manually uprooted and washed under running tap water to remove adhering soil. The inflorescence of *M. lanceolata* was collected from 3 m high shrubs. Plant species identification was confirmed by National Herbarium, Science Faculty, Addis Ababa University, Ethiopia.

6.2.2 *Extracts preparation*

Plant parts of the respective species were carefully allowed to dry under shade in Ethiopia. They were then packed in paper bags and transported to Julius Kühn-Institut, Münster, Germany. The plant parts were then cut into small pieces and powdered in a commercial Waring® blender to a fine powder. Subsequently, 50 g powder of each plant material was added to 250 ml 80% ethanol or 250 ml distilled water and shaken for 4 hrs at 500 rpm using an orbital shaker (IKA®-Werke GmbH, Staufen, Germany). The paste obtained was squeezed through a 250 µm nylon cloth followed by vacuum filtration through an MN 615 Nr.1 filter paper (Macherey-Nagel, Düren, Germany). The solution was further separated from the plant debris by centrifugation at 3000 rpm for 10 min. The ethanol extracts obtained in beakers were placed in a water bath at 50^{o}C until all ethanol has evaporated and yielded in a gooey substance which was then diluted with distilled water to a volume of 50 ml.

The aqueous extract was similarly treated in the water bath till the volume was reduced to 50 ml.The 50 ml final volume was considered to correspond to the initial amount of plant powder (50 g) and taken as stock extract (= 100%). Extracts were stored at 6^{o}C room for a couple of days until used for the assay. The final filtrates obtained are described hereafter as ethanol extract (ETE) and aqueous extract (WTE) of the three plant species.

The extract yield was determined by taking triplicated aliquots of 1 ml of each stock solutions and oven drying at 50^{o}C for 24 h.

6.2.3 *Test nematode*

Meloidogyne hapla was multiplied on tomato (*Solanum lycopersicon*) cv. Moneymaker in the greenhouse. Freshly hatched second-stage juveniles (J2) were extracted from infested tomato roots by the spray-mist technique described in Hooper *et al*. (2005). Twenty-four hours before use in the *in vitro* experiment, the juvenile suspension was passed over a Baermann funnel to obtain only vital J2s, after which the J2 density was adjusted to 2000 J2 ml^{-1}. For the pot experiment, the density was adjusted to 200 J2 ml^{-1}.

6.2.4 *In vitro nematode mortality bioassays*

As a preliminary study J2's were incubated for 24 h at the highest concentration (100%) of both the ethanol and aqueous extracts of each plant species. All juvenile in all treatments were completely inactivated. Hence, to test the efficacy of each plant extract on J2 activities the extracts of each plant species were serially diluted following a logarithmic scale of 2^x where x equals to 0, 1, 2, 3 and 4. The final test concentrations were therefore designated as 100%, 50%, 25%, 12.5% and 6.25% of the stock extract. The exposure time was set for 10 min, 30 min, 1 h, 2 h and 20 h. In this way, the experiment was designed as 5×5 (extract concentration and incubation periods) factorial giving 25 treatments in triplicate.

One millilitre of each test concentration was pipetted into a 24-well plate and mixed with a drop of Tween 80 (Applichem GmbH, Darmstadt, Germany). A drop of Tween in 1 ml distilled water and only distilled water served as control. Subsequently, 100 µl nematode suspensions containing about 200 J2s were added to the test substances irrespective controls and incubated for the exposure time described above. After exposure time was completed, the nematodes were immediately pipetted onto 1 cm diameter 20 µm nylon sieves, thoroughly rinsed with distilled water and evaluated under a compound microscope at 63× magnification.

At each rating point, status of the first 100 J2's was checked for activity. Nematodes were considered dead when no sign of movement in the first three seconds was observed. Dead nematode were found to have a straight-like shape whereas inactive but alive nematodes had anteriorly twisted manner, thust assisted the discrimination between the dead or alive.

6.2.5 *Greenhouse experiment*

The experiment consisted of ethanol extracts from all three plant species, each applied at three concentrations, i.e. 0.75, 1.5 and 3 ml of the stock solution added to water to give a final volume of 6 ml. Tomato plants inoculated with *M. hapla* but without plant extracts and

plants without any treatment served as controls. The experiment was carried out in 5 replicates.

One month old tomato (*Solanum* l*ycopersicon*) cv. Moneymaker seedlings were transplanted into 1 l plastic pots filled with sterilized field soil and silver sand (1:1, v:v). After three days, three vertical holes about 6 cm deep and 1 cm wide were made in the soil around the tomato stems with a rounded conical-end plastic stick. Then, 5 ml of the nematode suspension (1000 J2 per plant) was directly released into each hole with a calibrated Multipette® plus (Eppendorf AG, Hamburg, Germany) dispenser. Two hours later, 6 ml of each concentration of the ethanol extracts was separately injected.

Pots were randomized on a raised greenhouse bench with plant extract type as main factor. Pots were kept in a greenhouse at a temperature varying between 20 and 23^{o}C. Supplemental light was provided to extend day length to 16 h per day. Throughout the experimental period, plants were watered daily with about 15 ml of tap water and fertilized weekly with 0.3% WUXAL® Super liquid foliar fertilizer (Agrarverstand Oberland, Schongau, Germany).

The experiment was evaluated eight weeks after nematode inoculation. The shoot part of the tomato plants was removed and fresh weight was determined. The root system was carefully washed free of soil, plotted dry on paper tissue and fresh root weight was recorded. Egg masses were stained by immersing in 0.4% (w/v) solution of red food color cochenill a red E 124 (Brauns-Heitmann, Warburg, Germany) for 15 min (Thies *et al.,* 2002) and counted. Eggs and juveniles were then dislodged from the roots following the NaOCl technique described by Hussey and Barker (1973) and counted at 63× magnification using a compound microscope.

6.2.6 *Statistical analysis*

For the *in vitro* bioassay, mortality of juveniles exposed to plant extracts (M*p*) was corrected to mortality in water (M*w;* which was obetained to be 2.7%) using Abbott's formula: *Corrected mortality* = $(\%\mathrm{M}p - \%\mathrm{M}w)/(100 - \%\mathrm{M}w) * 100$ (Abbott, 1925). The corrected mortality was plotted against plant extract concentrations and log fitted using Slide Write Plus 7.01 (Advanced Graphics Software Inc.). The corrected mortality was transformed by probit against $\log_{10}$ of the concentration to determine the LC_{50} value using SPSS version 17 (SPSS, Inc., Chicago IL).

For the greenhouse experiment, both nematode and plant data were subjected to univariate analysis of variance using SPSS version 17, with plant species as main factor and plant

extract concentration a sub-factor. Duncan's multiple-range Test was used to compare means at the 0.05 level.

6.3 RESULTS

6.3.1 *Extract yield*

Plant extract yield obtained was 15 mg ml^{-1} for *Plumbago dawei*, 12.5 mg ml^{-1} for *Rumex abyssinicus* and 10 mg ml^{-1} for *M. lanceolata*, whichcorrelated to 0.3, 0.25 and 0.2 mg g^{-1} of the initial powder used. These yields were considered to be 100% (= of the stock extract).

6.3.2 *Effect of plant species and extraction solvent on J2 mortality*

The results demonstrated that the nematicidal potency significantly varied depending on extraction solvent and plant species. The ethanol extract of *M*. *lanceolata* and *P. dawei* showed a higher (*P*<0.001) J2 mortality than the ethanol extract of *R*. *abyssinicus* (Fig. 1). While the ethanol extract of *M. lanceolata* was more potent than its aqueous extract, no such effect was seen for the extracts achieved from *R. abyssinicus* and *Plumbago dawei*. The aqueous extracts of all plant species did not differ from each other in their J2 mortality.

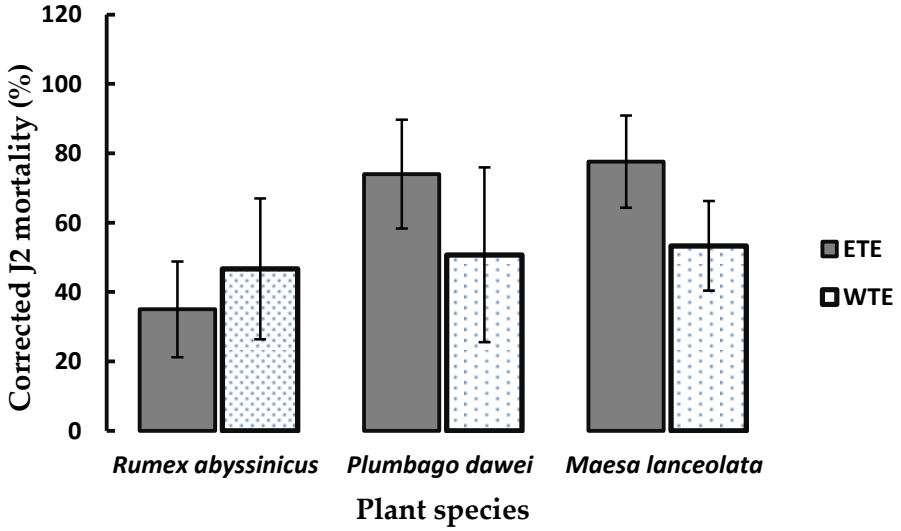

Figure 11. Effect of extraction solvent and plant species on mortality of second-stage juvenile of *Meloidogyne hapla* (Mean ± SD). SD= standard divaion; ETE= ethanolic extract; WTE= aquoes extract

6.3.3 *In vitro nematode mortality assay*

Nematode mortality significantly varied with extract concentration level and duration of exposure (Fig. 2-4). For all extracts types, higher concentrations of extract resulted in a significant increase (P<0.001) of J2 mortality. Similar, an increase in exposure time also resulted in an increase (P<0.001) of J2 mortality. Nematode mortality at the highest extract concentration and maximum exposure time ranged from 91.3% for the aqueous extract of *M. lanceolata* (Fig. 4B) to 100% for all three ethanol extracts and the aqueous extracts of *R. abyssinicus* and *P. dawei* (Fig. 2-4).

The ethanolic extract of *M. lanceolata* killed all nematodes following 30 min exposure at 100% concentration, 1 h at 50% concentration or 20 h at 25% concentration (Fig. 4A). Likewise, for *P. dawei* all nematodes were killed when exposed for 2 h at 100% and 50% concentration or for 20 h at 25% concentration (Fig. 3A).

In general, juvenile mortality was lowest at the lowest extract concentrations of each species over the minimum exposure period. For example, exposure for 10 min at the lowest extract concentration of the ethanolic extract of *R. abyssinicus* and *P. dawei* resulted in in 12.7 % and 42% mortality, respectively (Fig 2A and 3B). Hence, for all plant species and extract types, nematode mortality increased as the exposure duration was increased. The ethanolic extract of *M. lanceolata* however, resulted in a significantly higher mortality (90%) even when juveniles were exposed for 10 min at 50% concentration (Fig. 4B).

In this regard, ethanolic and aqueous extract of *R. abyssinicus* and aqueous extract of *P. dawei* require were able to kill 50% of the J2 in 2 h time at 100%, 25% and 12.5% concentration, respectively. Whereas ethanolic and aqueous extract of *M. lanceolata* and ethanolic extract of *P. dawei* were more potent that caused 50% mortality in 10 min at a concentration of 50%, 25%, 100%, respectively.

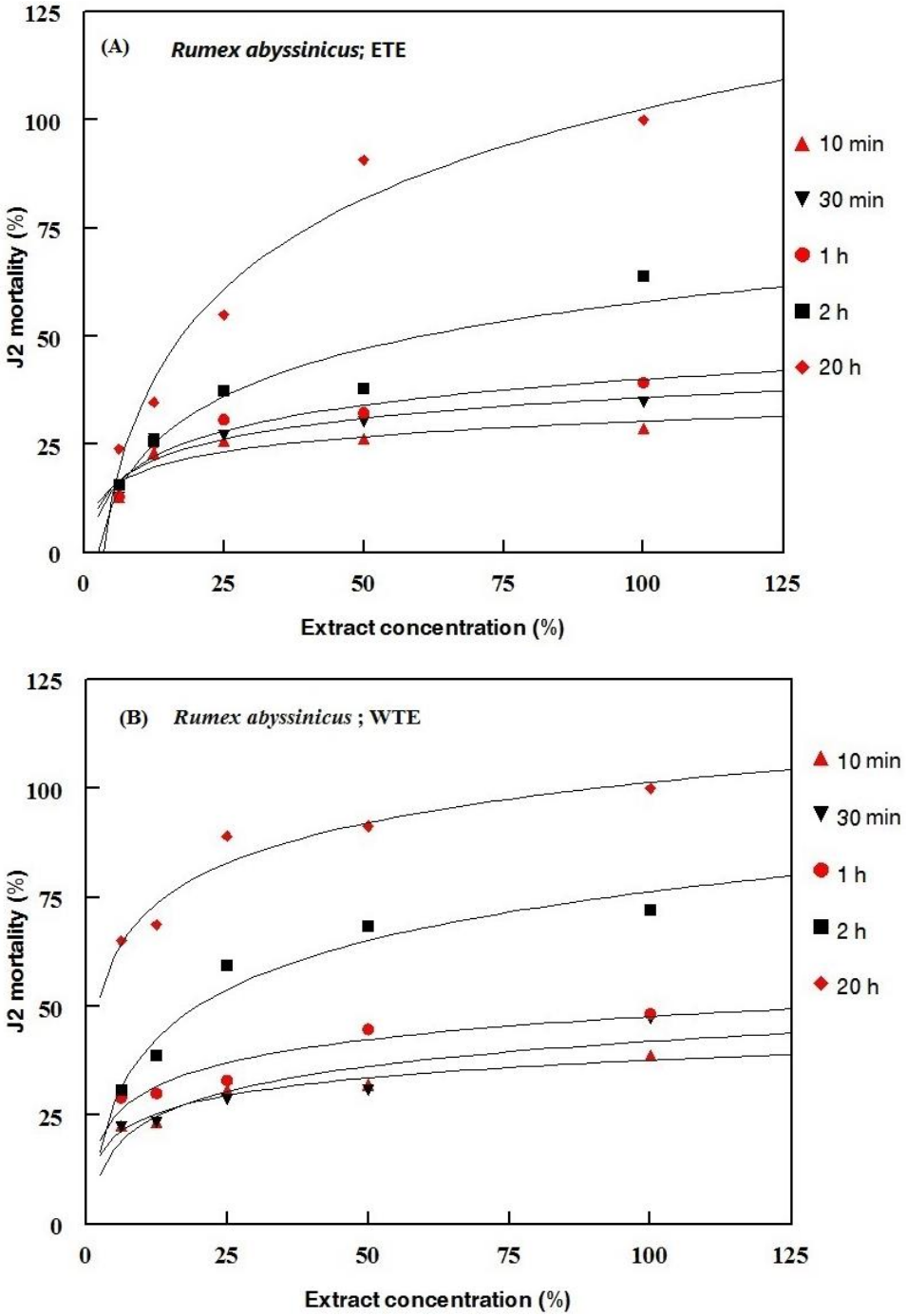

Figure 12. Dose-response relationship between concentrations of ethanolic (A) and aqueous (B) extract of *Rumex abyssinicus* and second-stage juveniles (J2) of *Meloidogyne hapla* after exposure for 10 min, 30 min, 1 h, 2 h and 20 h (n = 3). J2 mortality was corrected for mortality in water (control) using Abbot's formula.

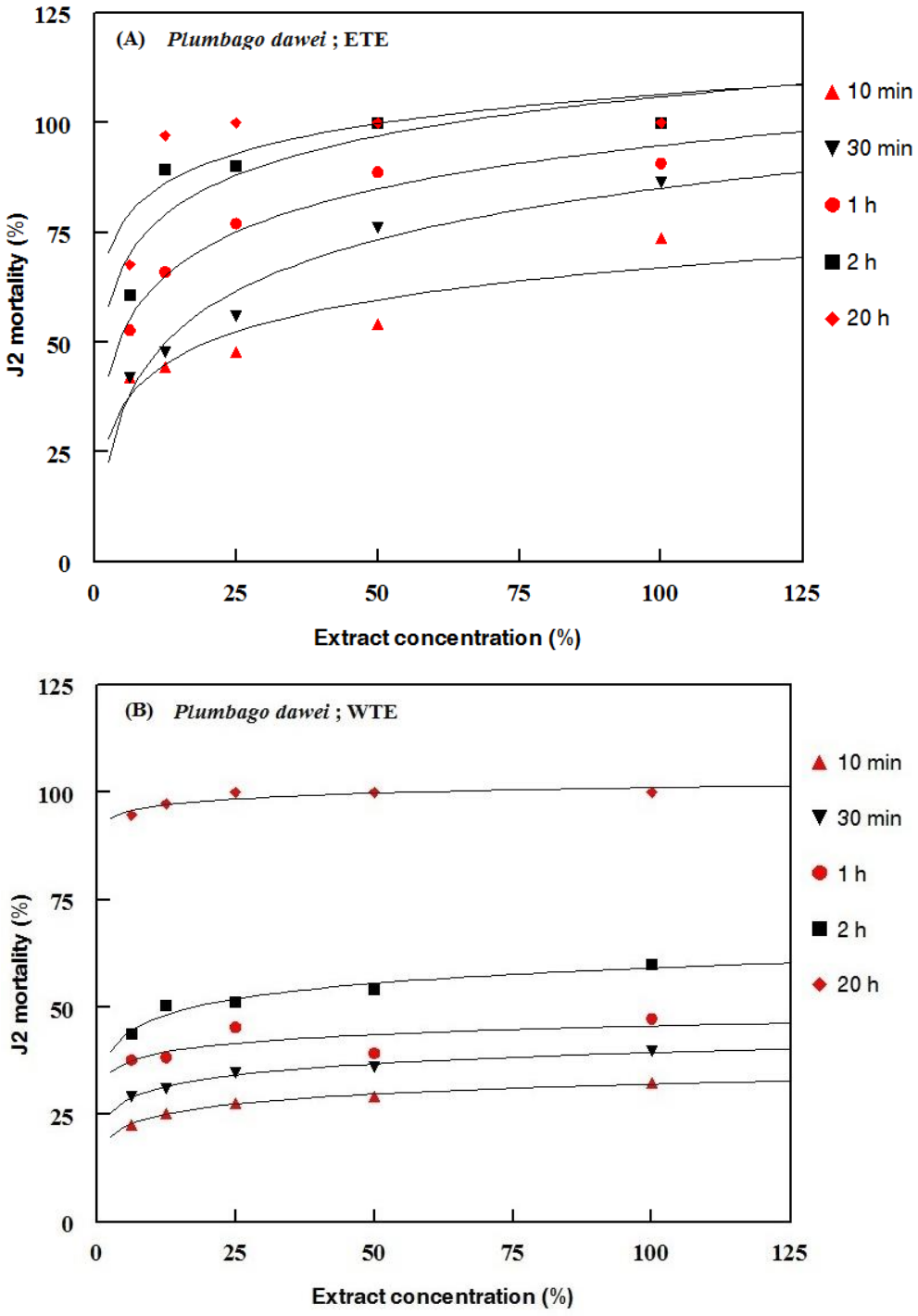

Figure 13. Dose-response relationship between concentrations of the ethanolic (A) and the aqueous (B) extract of *Plumbago dawei* and second-stage juveniles (J2) of *Meloidogyne hapla* after exposure for 10 min, 30 min, 1 h, 2 h and 20 h (n = 3). J2 mortality was corrected for mortality in water (control) using Abbot's formula.

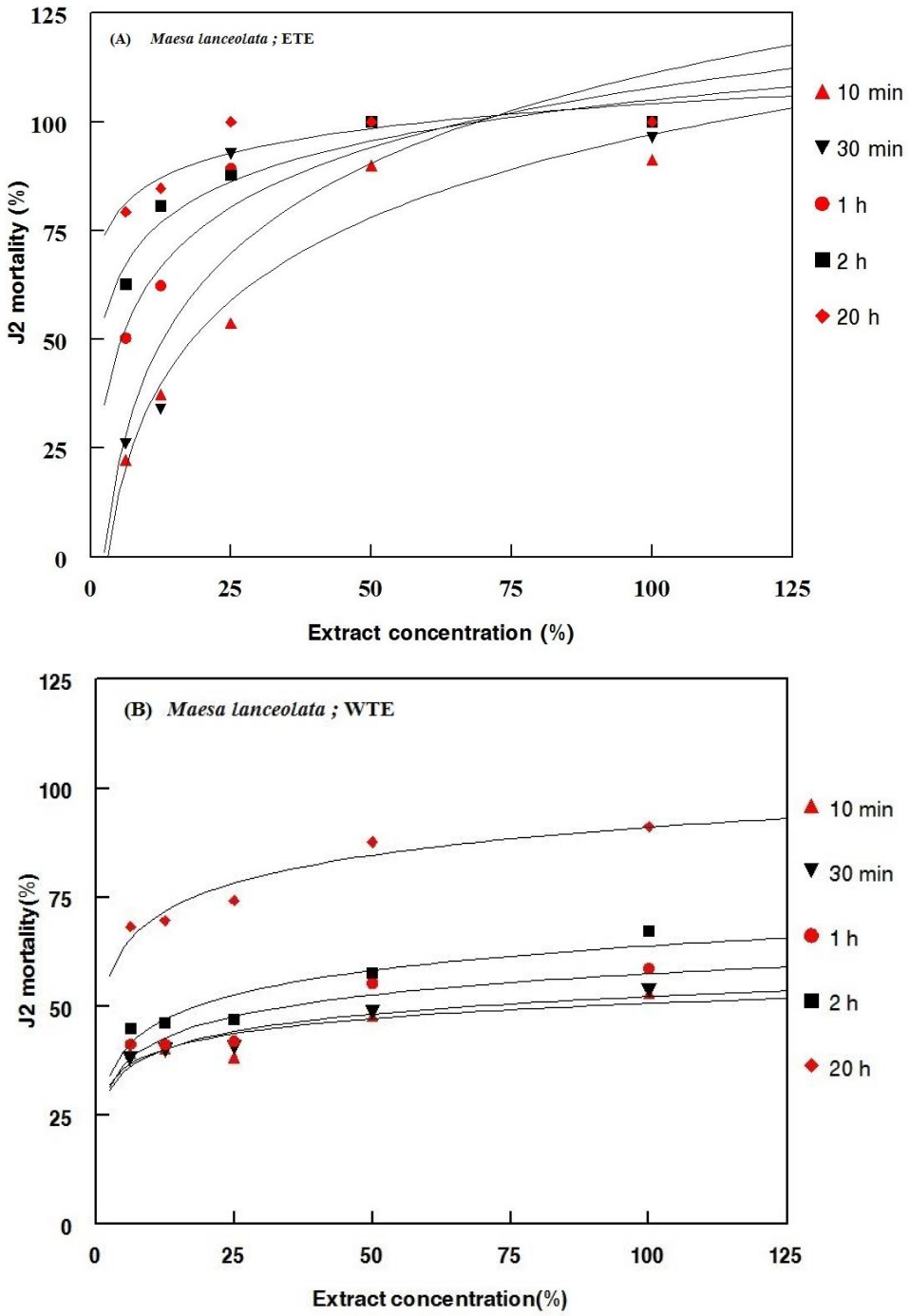

Figure 14. Dose-response relationship between concentrations of ethanolic (A) and aqueous (B) extract *Maesa lanceolata* and second-stage juveniles (J2) of *Meloidogyne hapla* after exposure for 10 min, 30 min, 1 h, 2 h and 20 h (n = 3). J2 mortality was corrected for mortality in water (control) using Abbot's formula.

6.3.4 *Lethal concentration*

The results revealed that the ethanol extracts of *P. dawei* and *M. lanceolata* were more potent at a lower concentration regardless of the duration of exposure (Fig. 5). However, the efficacy of all aqueous extracts and ethanol extract of *R. abyssinicus* was time dependent. For instance, similar dilution levels aqueous extract were required to kill 50% of the juveniles when exposed for 2 h in the three plant species. At the highest exposure time, except for the

ethanol extract of *R. abyssinicus*, time of exposure had little influence on lethal concentration level.

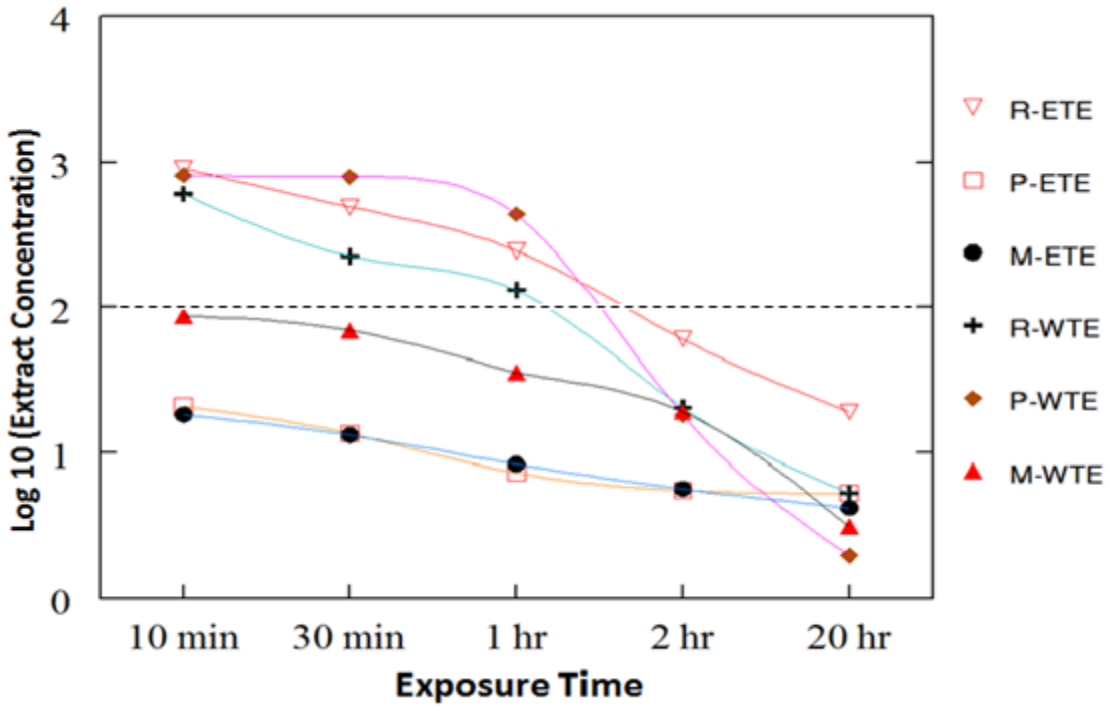

Figure 15. Extract concentration that is required to kill half of the *Meloidogne hapla* juveniles in a given period of time. The broken line indicates the concentration of the stock extract (100%) of *Rumex abyssinicus* (R), *Plumbago dawei* (P) and *Maesa lanceolata* (M). ETE and WTE are ethanolic and aqueous extracts, respectively.

6.3.5 *Appearance of dead nematodes*

The effect of the aqueous extract on the morphological appearance of *M. hapla* juveniles was evaluated under a compound microscope and photographed. Juveniles killed by plant extracts showed a distinct structural disintegraty which was different to dead nematodes from the control. *Rumex abyssinicus* caused continuous bubbles mainly in the lower part of the digestion system (Fig. 6C). Likewise, leakage of body content and separation of the cuticle from the endoderm was apparent on nematodes killed by extracts of *P. dawei* (Fig. 6B). Nematodes killed by *M. lanceolata* showed typically broken small and larger bubbles along the upper digestive system and slight shrinkage of the intestine (Fig. 6C).

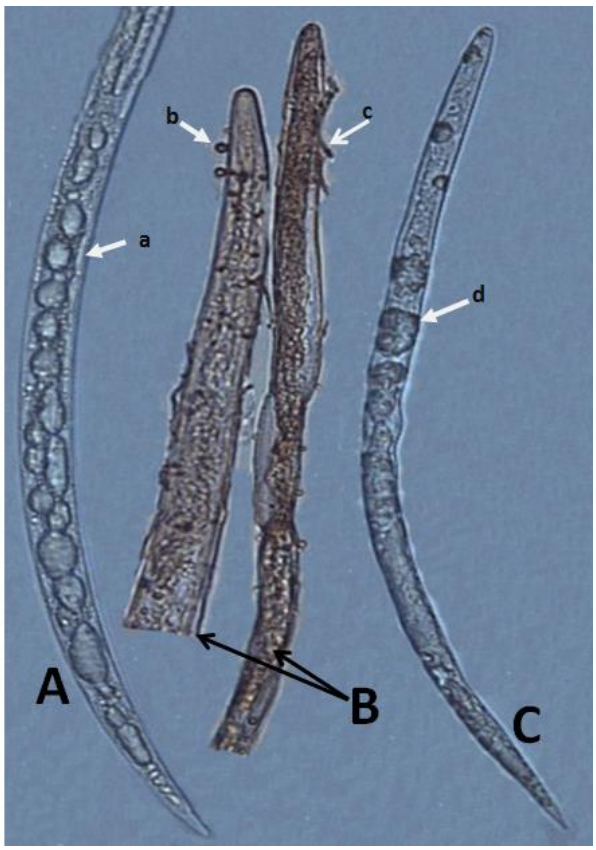

Figure 16. Characteristic appearance of the dead nematodes after 20 h exposure in aqueous extracts of A) *Rumex abyssinicus* B) *Plumbago dawei* and C) *Maesa lanceolata.* Distinct features that appeared on the dead nematodes are indicated with white arrows.

6.3.6 *Effect of ethanolic extracts on growth of tomato and nematode population*

The effects of extract treatments on the growth performance of tomato plant and population of *M. hapla* in roots are shown on Table 1. All treatments significantly (*P*<0.001) reduced the number of egg masses per root system compared to the control. At the highest extract concentration (100%), number of galls per g root fresh weight did not differ between the three plant species. achieved similar activity to reduce the.

All plant extracts significantly (*P*< 0.001) reduced the multiplication rate of *M. hapla* on tomato in comparison to the control. Moreover, the effect of the plant extracts was concentration-dependent. For example, application of 100% ethanolic extract of *M. lanceolata* resulted in the lowest nematode reproduction (RF=5.1) compared to its 50% and 25% levels. For *R. abyssinicus* both 100% and 50% concentrations showed similar significant effect on the nematode reproduction (RF=6.0) different from its 25% (RF=20.4). The 100% extract concentration of *P. dawei* caused a decline in nematode reproduction to 16.4 but was not different from the 50% and 25% concentrations of *M. lanceolata* and *R. abyssinicus*, respectively.

At the highest concentration (100%) of *R. abyssinicus,* root fresh weight of tomato was rather less than for palnts inoculated with nematodes only. Moreover, plants have exhibited intense shoot wilting may be attributed to direct effect of the extract on tomato roots. In comparison to the control plants, application of all concentration levels of *M*. *lanceolata* or the highest concentration of *P. dawei,* significantly increased the shoot fresh weight of tomato.

Table 1. Influence of ethanolic plant extracts on growth of tomato and egg mass formation and population of *Meloidogyne hapla* under greenhouse conditions.

Plant species	Concentration	Egg mass*	Egg mass/g root	Root fresh weight (g)	Shoot fresh weight (g)	Pf/Pi
Rumex	100x	143.6 ± 47.4^{cd}	26.3 ± 10.9^{bc}	5.7 ± 1.2^{c}	12.6 ± 5.7^{bc}	5.7 ± 0.6^{d}
abyssinicus	50x	139.4 ± 34.7^{cd}	17.4 ± 3.5^{b}	7.9 ± 0.6^{b}	10.4 ± 3.8^{d}	6.0 ± 0.5^{d}
	25x	295.8 ± 44.9^{b}	31.8 ± 8.5^{bc}	9.6 ± 1.6^{a}	12.9 ± 3.6^{bc}	20.4 ± 1.4^{c}
Plumbago	100x	112.8 ± 37.4^{d}	14.9 ± 7.8^{b}	8.3 ± 1.9^{b}	15.1 ± 1.6^{b}	16.1 ± 2.7^{c}
dawei	50x	155.8 ± 56.3^{c}	16.9 ± 6.5^{b}	9.3 ± 1.0^{b}	11.6 ± 3.5^{c}	39.8 ± 4.2^{b}
	25x	253.4 ± 52.1^{b}	41.5 ± 9.1^{c}	6.2 ± 1.0^{b}	12.8 ± 4.7^{bc}	54.9 ± 7.2^{b}
Maesa	100x	142.4 ± 50.0^{cd}	16.4 ± 7.3^{b}	9.1 ± 2.2^{a}	16.6 ± 1.3^{ab}	5.1 ± 1.1^{d}
lanceolata	50x	257.4 ± 34.3^{b}	25.9 ± 5.3^{bc}	10.2 ± 0.9^{a}	15.2 ± 3.5^{b}	22.5 ± 2.2^{c}
	25x	312.4 ± 37.3^{b}	26.6 ± 4.3^{bc}	11.8 ± 0.9^{a}	18.3 ± 3.7^{a}	37.0 ± 2.9^{b}
Control (+)	0x	471.4 ± 69.9^{a}	57.0 ± 26.6^{a}	9.3 ± 2.7^{ab}	13.1 ± 1.5^{c}	115.4 ± 9.2^{a}
Control (-)	0x	0 ± 0.0^{e}	0 ±0.0^{d}	8.5 ± 0.7^{ab}	19.4 ± 4.0^{a}	0 ± 0.0^{e}

*Means with the same letter with in column are not significantly different according to Tukey's studentized Range Test at p=0.05. Control (+): Plants inoculated with *Meloidogyne hapla* only. Control (-): plants uninoculated with nematodes and untreated with extracts. Means are values of 5 replicates. *Pf/Pi* was calculated based on *Pi* = 1000 J2.

6.4 DISCUSSION

The potential of plant extracts to control root-knot nematodes is a well known phenemenom (D'Addabbo *et al*., 2010; Oka *et al*., 2006; Agbenin *et al*., 2005). In the present study ethanolic and aqueous extracts of rhizomes of *R.abyssinicus*, roots of *P. dawei* and inflorescence of *M. lanceolata* were used to evaluate their nematicidal activity *in vitro*. These plant species are available in most part of Ethiopia. Their respective plant parts are traditionally used by the local community for different medicinal purposes including treating wound and expelling gastrointestinal parasites of their cuttels (Mekonnen *et al*., 2010; Belachew, 1995).

Results indicated that ethanolic and aqueous extracts of all three plant species showed toxic effects towards *M. hapla* juveniles under *in vitro* conditions. Overall control efficacy varied between extraction solvent and plant species. Best control was achieved by *M. lanceolata*. Wiratno *et al*. (2009), previously rated plant extracts as highly toxic when mortality was above 80%. In this regards, ethanolic extract of *M*. *lanceolata* is considerd highly toxic even at the lowest concentration used when nematodes were exposed for more than 2 h. The aqueous extract of *M*. *lanceolata* could also be rated as highly toxic when the exposure time extended 20 h, in which case above 91% mortality was observed. Comparing these to previously reported results for brassica and datura extract where 60-70% J2 mortality was obtained after 48 h exposure indicates a high nematicidal effect of our plant extracts to *M. hapla* (Ayazpour *et al*., 2010).

The toxicity nature of these three plants against plant-parasitic nematodes has not been previously reported. Nevertheless, many plant extracts constitute a broad spectrum of metabolites nematicidal potential such as glycosides, alkaloids, terpenoids, phenols, and tannin (Widmer and Abawi, 2000; Chen *et al*., 1997; Bauske *et al*., 1994; Viglierchio and Wu, 1989; Osman and Viglierchio, 1988; Fassuliotis and Skucas, 1969).

The nematicidal activity of *M. lanceolata* might be ascribed to one or more of its biological active constituents such as triterpenoid saponins (Sindambiwe *et al*., 1996), alkylated benzoquinones (Muhammad *et al*., 2003), benzophenons (Schmutterer, 1990), tannins and phenols (Chifundera *et al*.,1993). The findings of D'Addabbo *et al*. (2009) that saponin extracts from *Medicago sativa* were toxic to *M*. *incognita* and *Xyphinema index* causing 90-100% mortality within 16 h of exposure suggests that the saponin from *M*. *lanceolata* might have a similar toxic effect on *M*. *hapla*. Bagalwa and Chifundera (2007) reported that 1 mg/l leaf extract of *M. lanceolata* significantly controlled aquatic adult insects and moustiquitoes. Furthermore, the aqueous extract of leaves and fruit of *M. lanceolata* is used in Rwandan traditional medicine against infectious hepatitis, bacillary dysentery, and neuropathies (Sindambiwe *et al*., 1996) confirming its biological effectiveness.

Plumbago dawei produces several secondary metabolites including tannins, flavonoids and cardiac glycosides (Omwenga and Paul, 2012) as well as plumbagin and β-Sitosterol (Maniafu *et al*., 2009). The latex of this plant is known for its high toxicity to human skin causing injury similar to boiling water (local people personal comm.). This raises the question if this compound might also be responsible for the characteristic appearance of dead *M. hapla* J2 in which the cuticle was disintegrated and body content oozed. Another compound, Plumbagin, has been reported to have antifungal and antibacterial activity through inducing cell cycle arrest (Mohana and Purushothaman, 1980). Maniafu *et al*. (2009) indicated that the crude extract of *P. dawei* possess a very high larvicidal activity against mosquito larvae. Flavonoids and tannins have been known to form complexes with bacterial cell walls and denature the cell membrane, respectively (Akiyama et al., 2001). Similar mechanisms might be responsible for the high mortality of *M. hapla*. The fact that tannin-rich plants are effective against parasitic nematodes in ruminants (Hoste et al., 2006) as well as against the plant-parasitic nematode *Heterodera glycine* (Chen et al., 1997) indicates that they might also be active against *M. hapla*.

The third tested plant species, *R. abyssinicus*, contains chemical derivatives such as chrysophanol-8-P-D-glucoside and emodin-8-f3-D-glucoside (Yohannes *et al*., 1984). Unlike the other two plants where the ethanolic extract showed a higher potency of nematode control than the aqueous extract, for *R*. *abyssinicus* it was the opposite, i.e. the aqueous extract showed a higher nematicidal effect than the ethanolic extract. This different behaviour might be attributed to the chemical nature of the toxic compounds, if they are more hydrophilic or hydrophobic. For example, emodin as a major constituent of *R*. *abyssinicus* is less soluble in ethanol than in water (Yangcheng et al., 2009). Besides its nematicidal activity, the aqueous extract of *R*. *abyssinicus* also showed an anthelmintic activity against earth worms (Raju and Ali, 2010).

For the pot experiment, all concentrations of the ethanolic extracts of the three plant species tested resulted in reduced *M. hapla* reproduction compared with the control. The effect of plant extracts on the nematode could be expected either through inhibiting root penetration or reproduction in the root. Terpenoids have been reported to inhibit reproduction of *Meloidogyne incognita* and *M. javanica* (Bauske et al., 1994; Osman and Viglierchio, 1988). The reproduction rate of *M. hapla* on a tomato treated with the highest dose of *M*. *lanceolata* was also quite low (*Pf/Pi* =5.1) that may account for the inhibition effect of terpenoids content which inhibit root penetration and later retardation in different activities of the nematode including feeding and reproduction (Bunt,1975).

The application of plant derived products into the soil for management of plant-parasitic nematodes is in general environmentally compatible and mainly cost effective for the local

farmers compared to conventional nematicides (Pakeerathan et al., 2009; Chitwood, 2002). In the pot experiment, the amount of ethanolic extract applied per plant was conceivably little (0.75-3 kg/m^3) compared to the amount suggested by Khan *et al*. (2008). However, for practical application it needs to be considered that *R. abyssinicus* can express some phytotoxicity, while it should be applied in time before the next planting. For *P. dawei* the farmer needs to take precaution to avoid any contact of the plant extract with the skin. Those limitations might be avoided if not the extract is used for nematode control but the plant part itself, either as fresh biomass or dried and ground to a powder. Hence, our results are promising particularly for the local farmers in the region where other control strategies are not still well known (Chitwood 2002).

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SUMMARY

Plant-parasitic nematodes feed on living plant tissues thereby cause serious damage to their host plants. The level of damage varies depending on the type of nematode species and the plant cultivar or species. Cut-flowers just like any other crops harbour several plant-parasitic nematodes species that may cause significant economic losses. In Ethiopia, floriculture is becoming the most successful and economically important sector of agriculture. Therefore, the recently growing damage caused by plant-parasitic nematodes is of major concern for the floriculture industry. The goal of this study was to establish baseline information on plant-parasitic nematodes associated with the various cut-flower species, determine their level of interaction and evaluate alternative methods for minimizing their damage.

A nematological survey was carried out covering five cut-flower species, i.e. rose, carnation, freesia, statice and gypsophila, to investigate the plant-parasitic nematodes associated with those plant species. A total of thirteen major plant-parasitic nematode genera were detected, namely *Helicotylenchus, Meloidogyne, Longidorus, Paratylenchus, Pratylenchus, Criconemella, Rotylenchus, Rotylenchulus, Merlinius, Tylenchorhynchus, Paratrichodorus, Hemicycliophora,* and *Ditylenchus*. Rose harboured all of the detected nematode genera while freesia harboured only *Helicotylenchus*. Furthermore, *Meloidogyne* was only detected from roses. Nematode diversity and abundance was season dependent in that only nine out of the thirteen genera detected during the wet season were also detected detected during the dry season sampling. Moreover, *Helicotylenchus* and *Meloidogyne* were distributed irrespective of altitudinal changes.

The root-knot nematode *Meloidogyne hapla*, a serious pest of roses in the north hemisphere, was detected for the first time in Ethiopia. Subsequently, it was both morphologically and molecularly characterized and compared to other *M. hapla* populations originating from different countries. With few exceptions, the morphology and morphometrics of the Ethiopian *M. hapla* population closely fitted with the description of populations described from Hawaii, East Africa (Tanzania) and by Jepson (1987). The identity of *M. hapla* as well as its close similarity with other *M. hapla* populations was confirmed by molecular characterization and phylogeny reconstruction based on the D2-D3 expansion segment of the 28S-rRNA and mtDNA.

Because *Meloidogyne* was the most abundant genus found in the survey, the host suitability of the five cut-flowers mentioned above was evaluated for its host status regarding *M. hapla* and *M. incognita*. The results indicated that rose was an excellent host for *M. hapla* but a poor host for *M. incognita*, whereas, freesia was a relatively poor host to both species.

Nevertheless, none of the cut-flowers species was resistant to either of these *Meloidogyne* species. While the survey showed that *M. hapla* was so far only found on roses any precaution should be taken to avoid spread of this species to the other four cut-flower species.

Indeed, the relation between different initial nematode population densities of *M. hapla* and the most economically important cut-flower, the rose, was essentially important to be determined. Evaluation of three rose rootstocks (*Rosa corymbifera* Laxa, *R. multiflora* and *R. canina* Inermis) indicated that, all the three rootstocks are susceptible to *M. hapla* with quite low tolerance limit (*T*). Hence, use of these rootstocks in *M. hapla* infested soils is by no means recommended. Currently several other varieties of rootstocks are used in Ethiopia of which little is know about their resistance towards *M. hapla*. Screening of those rootstock varieties for *M. hapla* resistance or tolerance is of paramount importance to develop sustainable management strategies.

A promising option as alternative root-knot nematode control might be botanicals. Within this respect, the nematicidal potential of three plant species, namely *Rumex abyssinicus*, *Plumbago dawei* and *Maesa lanceolata*, native to Ethiopia was evaluated. Interestingly, ethanolic extracts of all three plant species significantly killed J2 of *M. hapla.* In *M. lanceolata*, even the aqueous extract was as potent as its corresponding ethanolic extract. Out of those three plant species, *M. lanceolata* is the most promising as it grows in large biomass and abundantly throughout the country.

In conclusion, the Ethiopian floriculture sector faces several nematode problems with *M. hapla* being identified as a major pest on roses. Hence, further investigations aiming at nematode control are urgently needed to protect the Ethiopian floriculture industry from further harm.