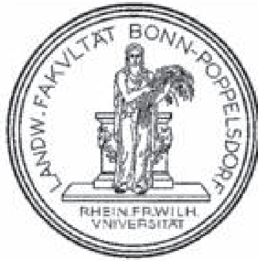

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Presence and Frequency of Occurence of Plant Parasitic Nematodes on Coffee (*Coffea Arabica* L, Rubiaceae) in Ethiopia and the Importance of Endophytic Microorganisms for Biocontrol

Institut für Nutzpflanzenwissenschaften und Ressourcenschutz
der
Rheinischen Friedrich-Wilhelms-Universität Bonn

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Abstract

Presence and frequency of occurrence of plant parasitic nematodes on coffee (C*offea arabica* l, Rubiaceae) in Ethiopia and the importance of endophytic microrganisms for biocontrol

A survey was conducted to determine the occurrence and distribution of plant-parasitic nematodes and associated endophytic fungi and bacterial antagonists on coffee in Ethiopia. Soil and root samples were collected from 15 coffee growing agroecologies during the wet season in August 2004 and dry season in April 2006. The dominant plant-parasitic nematode genera found were *Helicotylenchus, Scutellonema, Rotylenchus, Xiphinema, Heterodera, Tylenchorhynchus* and *Quinisulcius. Helicotylenchus* predominated throughout the area (65-74%), followed by *Xiphinema* (29-40%). Nematode densities were generally higher in the wet season than in the dry season, however, relative abundance of nematode taxa was in the same order. For the major plant-parasitic nematode genera, specimens were identified to the following species: *Helicotylenchus dihystera, H. multicinctus, H. californicus, H. gerti, Xiphinema insigne, X. basilgoodeyi, Scutellonema paralabiatum*, *Rotylenchus unisexus, Tylenchorhynchus agri* and *Quinisulcius capitatus.* Nematodes considered to be of economic importance and involved in coffee decline were not detected in this survey.

A total of 128 fungi growing endophytically were obtained from surface sterilized coffee roots. The most frequently detected genera were *Fusarium* and *Trichoderma*. Nematicidal effects of culture filtrates of these isolates on *M. incognita* juveniles were observed after 24 h of incubation. Juvenile inactivity ranged from 28% to 81% in which *Trichoderma* strain ET2 showed the highest level of inactivity up to 81%. Same isolates were tested for their pathogenicity towards *M. incognita* eggs. Isolates EF1, EF3, EF5, EF6, EF8, EF10, EF11, EU2 and EU4 showed the highest ability to infect eggs with values up to 89%. The percentage of egg pathogenicity was highest for *Fusarium spp.* Results of replicated greenhouse experiments demonstrated that isolates of *Fusarium* and *Trichoderma* reduced the number root-knot nematode egg masses up to 50%. Of all the tested isolates, the *F. oxysporum* isolate EF11 significantly reduced the number of egg masses in all sets of experiments. Reduction in nematode penetration ranging up to 30% was also recorded in the present study for isolates EF11 and EF10.

Endophytic bacteria were isolated and identified by Fatty Acid Methyl Ester-Gas Chromatography (FAME-GC). A total of 201 and 114 endophytic bacteria were isolated and identified during the wet and dry seasons, respectively. The most abundant genera found were *Pseudomonas, Bacillus, Agrobacterium, Stenotrophomonas and Enterobacter.* Higher population size densities ranging from 5.2×10^3 to 2.07×10^6 cfu g^{-1} fresh root were observed during the wet season. Culture filtrates of the bacterial isolates showed nematicidal effects ranged between 38% and 98%. The active strains were: *Agrobacterium radiobacter, Bacillus pumillus, B. brevis, B. megaterium, B. mycoides, B. licheniformis, Chryseobacterium balustinum*, *Cedecea davisae, Cytophaga johnsonae, Lactobacillus paracasei, Micrococcus luteus, M. halobius, Pseudomonas syringae* and *Stenotrophomonas maltophilia*. *Bacillus pumillus* and *B. mycoides* were the most effective strains in reducing the number of egg masses and galls up to 39 and 33%, respectively.

Zusammenfassung

Vorkommen und Häufigkeit von pflanzenparasitären Nematoden an Kaffee (C*offea arabica* I, Rubiaceae) in Äthiopien und die Bedeutung von endophytischen Mikroorganismen für die biologische Bekämpfung

In Äthiopien wurde an Kaffee eine Erhebung durchgeführt, um die Verbreitung pflanzenparasitärer Nematoden zu ermitteln und die mit Kaffee assoziierten endophytischen Pilze und Bakterien hinsichtlich ihrer antagonistischen Wirkung zu untersuchen. Hierzu wurden während der feuchten Jahreszeit im August 2004 und der trockene Jahreszeit im April 2006 Boden- und Wurzelproben aus 15 Kaffee-Agroökosystemen gesammelt. Bei den pflanzenparasitären Nematoden traten insbesondere Arten der Gattungen *Helicotylenchus*, *Scutellonema*, *Rotylenchus*, *Xiphinema*, *Heterodera*, *Tylenchorhynchus* und *Quinisulcius* auf. Die höchste Abundanz und größte Verbreitung zeigten die Gattungen *Helicotylenchus* (65-74%) und *Xiphinema* (29-40%). Die am häufigsten auftretenden Arten waren: *Helicotylenchus dihystera*, *H. multicinctus*, *H. californicus*, *H. gerti*, *Xiphinema insigne*, *X. basilgoodeyi*, *Scutellonema paralabiatum*, *Rotylenchus unisexus*, *Tylenchorhynchus agri* und *Quinisulcius capitatus*. Die Besatzdichte der Nematoden war in der feuchten Jahreszeit meist höher als in der trockenen Jahreszeit; das Artenspektrum blieb aber weitgehend unverändert. Wirtschaftliche Schäden durch pflanzenparasitäre Nematoden konnten in dieser Untersuchung nicht festgestellt werden.

Insgesamt wurden 128 endophytische Pilze von oberflächlichsterilisierten Kaffeewurzeln isoliert. Die meisten Isolate gehörten zu den Gattungen *Fusarium* und *Trichoderma*. Die Wirkung von Kulturfiltraten dieser Pilze auf Larven von *Meloidogyne incognita* wurde nach 24 h Inkubation ermittelt. Die Inaktivierungsraten lagen je nach Pilzisolat zwischen 28% und 81%. Die beste Wirkung zeigte das *Trichoderma* Isolat ET2. Weiterhin wurden die Pilzisolate hinsichtlich ihrer Fähigkeit zur Parasitierung von *M. incognita* Eiern untersucht. Die Pilzisolate EF1, EF3, EF5, EF6, EF8, EF10, EF11, EU2 und EU4 zeigten die beste Wirkung mit Parasitierungsraten bis zu 89%. Insbesondere Isolate von *Fusarium* spp. zeigten gute Parasitierungsraten. In Topfversuchen reduzierten einzelne Isolate von *Fusarium* und *Trichoderma* die Anzahl der Wurzelgallen und Eiermassen von *M. incognita* bis zu 50%. Die konstanteste Wirkung über alle Versuche zeigte das *F. oxysporum* Isolat EF11. Weiterhin verringerten die Isolate EF10 und EF11 die Eindringung von *M. incognita* in Tomatenwurzeln um bis zu 30%.

Endophytische Bakterien wurden aus oberflächensterilisierten Kaffeewurzeln isoliert und gaschromatographisch (FAME-GC) bestimmt. Insgesamt wurden 201 bzw. 114 endophytische Bakterienisolate aus Kaffeewurzeln während der feuchten bzw. trockenen Jahreszeit isoliert. Die am häufigsten vorkommende Bakteriengattungen waren: *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Stenotrophomonas* und *Enterobacter*. Die Populationsdichten waren während der feuchten Jahreszeit höher als in der trockenen Jahreszeit. Kulturfiltrate der endophytischen Bakterienisolate führten zu einer Inaktivierung von *M. incognita* zwischen 38% und 98%. Die beste Wirkung zeigten Isolate der Arten *Agrobacterium radiobacter*, *Bacillus pumilus*, *B. brevis*, *B. megaterium*, *B. mycoides*, *B. licheniformis*, *Chryseobacterium balustinum*, *Cedecea davisae*, *Cytophaga johnsonae*, *Lactobacillus paracasei*, *Micrococcus luteus*, *M. halobius*, *Pseudomonas syringae* und *Stenotrophomonas maltophilia*. In Topfversuchen führten Isolate von *Bacillus pumilus* und *B. mycoides* zur stärksten Verminderung der Anzahl Gallen und Eiermassen von bis zu 33% bzw. 39%.

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CHAPTER 1

1 Introduction

1.1 Coffee production and economic importance

Coffee is the most important agricultural commodity in the world, and is worth up to $14 billion annually. More than 80 countries cultivate coffee, which is exported as raw, roasted or soluble product to more than 165 countries worldwide. More than 50 developing countries, 25 of them in Africa, depend on coffee as an export, with 17 countries earning 25% of their foreign exchange from coffee. The United States Department of Agriculture forecasted coffee production in 2006/2007 at 123.6 million bags (60 kg or 132.276 pounds) which increased nearly 10 percent, or 11 billion bags over the previous year (USDA, 2006).

The plant genus *Coffea* comprises about ninety species but only a few are cultivated. Commercial coffee production relies on two species of coffee, *C. arabica* and *C. canephora (coffee robusta)*, with *C. arabica* being considered of superior quality and contributing to over 70% of the world's coffee production (Orozco-Castillo et al., 1994; Rani et al., 2000; Waller et al., 2007).

Coffea is a perennial woody shrub with a dimorphic growth characteristic, which consists of vertical and horizontal branches. *C. arabica* has its origin in the southwest highlands of Ethiopia (Harlan, 1969), but Thomas (1942) reported the existence of wild *C. arabica* on the Boma plateau in Sudan which have been introduced in the distance past from the Ethiopian highlands (Thomas, 1942). Berthaud and Charrier (1988) also reported the presence of *C. Arabica* populations on Mount Imantong in Sudan and Mount Marsabit in Kenya. However, it is generally believed that *C. arabica* has its highest genetic diversity in Ethiopia (Waller et al., 2007).

The root system of coffee consists of a short taproot and numerous lateral roots. Some of the lateral roots bend downward and reach depths of up to three meters. The remaining lateral roots form a dense mat of feeder roots which extend about two

meters deep and two meters laterally from the stem (Wrigley, 1988; Willson, 1999). Coffee leaves are sensitive to direct sunlight and both high and low temperatures. Shaded leaves are much more photosynthetically efficient than un-shaded leaves. In addition to this, full sunlight can raise the temperature of leaves as much as 20°C above the optimum and can damage them directly (Cannell, 1985; Wrigley, 1988; Willson, 1999; Waller et al., 2007).

While coffee is grown in various areas throughout the tropics, there is a relatively narrow range of environmental conditions under which it will flourish. With few exceptions, coffee is limited to tropical highland areas (Wrigley, 1988) which have climates similar to that of the Ethiopian highlands where coffee originated. The most important factors determining the suitability of an area for coffee cultivation are temperature, moisture and light intensity (Wrigley, 1988).

1.2 Major coffee pests and diseases

Limiting factors of coffee production includes species of fungi, bacteria, viruses, nematodes and insects. The major fungal diseases are coffee leaf rust (*Hemileia vastatrix*), coffee berry disease (*Colletotrichum kahawae*) and coffee wilt disease (*Fusarium xylarioides)* (Waller et al., 2007). The two important bacterial coffee diseases are halo blight of coffee incited by *Pseudomonas syringae* pv. *garcae* (Amaral et al., 1956) and coffee leaf scorch caused by the polyphagous bacterium *Xylella fastidiosa* (Wells et al., 1987). The former disease has been described in Brazil, Kenya, Uganda and China where it is becoming of some concern due to its high incidence and severity (Wen and Chen, 1995; Chen, 2002). Coffee leaf scorch has been recorded only in Brazil and Costa Rica where the bacterium *X. fastidiosa* attacks several other crops with high incidence, particularly in citrus and prunes.

Some records of virus diseases in coffee have sporadically been made in Brazil and Colombia but with no economic significance and of eventual misinterpreted diagnosis (Chen, 2002). However, the coffee ring spot virus CoRSV associated with a mite vector has been reported in several Brazilian states and recently found in Costa Rica. It causes conspicuous ring spot symptoms on leaves, berries, and less

frequently on twigs. A similar disease is known in the Philippines, but no information exists about its relationship to CoRSV (Chagas et al., 2003).

Stem and branch borers, berry feeders, bud, leaf, green shoot and flower feeding insects as well as, root and collar feeding insects are the most important insect pests recorded in coffee plantations of the world (Waller et al., 2007).

Furthermore, many genera and species of nematodes were found associated with coffee in many countries of the world. Root-knot nematodes, *Meloidogyne spp*., are among the most important parasites. They are widely distributed throughout the world in coffee plantations and are more frequently encountered than any other group of nematodes. The most important root-knot nematode species in terms of their damage and distribution are *M. exigua*, *M. incognita*, *M. coffeicola* and *M. paranaensis, w*hereas, *M. africana, M. decalineata, M. megadora, M. arabicida*, *M. javanica*, *M. arenaria* and *M. hapla* are less widespread (Campos and Villain, 2005).

1.3 Coffee production and its economic importance in Ethiopia

Since the establishment of agriculture 2000 years ago in Ethiopia, *C. arabica* has been grown in the natural forests of the south-western massive highlands of the Kaffa and Buno districts of the country. *C. arabica* has its center of origin in the highlands of southwest and southeast Ethiopia where wild coffee populations grow naturally in the undergrowth of the Afromontane rain forests at altitudes of 1370-1830 m (Waller et al., 2007).

The agriculture-based Ethiopian economy is highly dependent on *C. arabica* which provides employment in rural areas and is the means of livelihood for over 15 million people in Ethiopia. Ethiopia is currently the seventh largest coffee producer worldwide. Coffee is by far Ethiopia’s most important export crop which contributes 40% to the country’s foreign currency income (Tadesse et al., 2002; Denich and Gatzweiler, 2006).

The potential for coffee production in Ethiopia is very high, because of the country's suitable altitude, ample rainfall, optimum temperatures and fertile soil. The total area

covered by coffee is about 400,000 hectares, with a total production of 200,000 tones of coffee per annum.

In Ethiopia, coffee grows at various altitudes and the bulk of *C. arabica* is produced in the eastern, southern and western parts of the country, which have altitudes ranging from 1300 to 1800 m. Annual rainfall in the coffee growing regions of the country varies from 1500 to 2500 mm. Where precipitation is lower, as in the eastern part of the country, production is supplemented with irrigation. Rainfall distribution in the southern and eastern parts of the country is bimodal and in the western part monomodal. These distribution patterns enable the country to harvest coffee at different times of the year, ensuring a supply of fresh beans all year round.

All the coffee growing regions have fertile, friable, loamy soils, with a depth of at least 1.5 m. The topsoil is dominantly dark brown or brownish in color, with a pH ranging from 5 to 6.8. One outstanding characteristic of the soil is that fertility is maintained by organic recycling, i.e., through litter fall, pruning and root residue from the perennial, coffee and shade trees. In addition, the small-scale coffee farmers, who are the major producers, use organic fertilizers to supplement the natural fertility of the soil.

1.4 Coffee production systems in Ethiopia

In Ethiopia, coffee is grown under four main production systems. These include plantation coffee, forest, semi-forest and garden, which account for 5, 10, 35 and 50% of the total volume produced in the country, respectively (Tadesse et al., 2002; Mekuria, 2004; Anonymous, 2006).

Plantation coffee (5%) is grown on plantations owned by the state and some well managed smallholder coffee farms. In this production system, selected seedlings are used and proper spacing, mulching, weeding, shade regulation and pruning is practiced (Tadesse et al., 2002; Mekuria, 2004; Anonymous, 2006).

Forest coffee (10%) is found in south and south-western Ethiopia. These are considered the centers of origin of *C. arabica*. Forest coffee is self sown and grown

under the full coverage of natural forest trees. It has a wide genetic diversity (Tadesse et al., 2002; Mekuria, 2004; Anonymous, 2006).

Semi-forest coffee production systems (35%) are found in the south and south-western parts of the country. Farmers acquire forest land for coffee farms, and then thin and select the forest trees to ensure both adequate sunlight and proper shade for the coffee trees. They slash the weeds once a year to facilitate the coffee bean harvest (Tadesse et al., 2002; Mekuria, 2004; Anonymous, 2006).

Garden coffee (50%) is grown in the vicinity of farmer's residences, mainly in the southern and eastern parts of the country. The coffee is planted at low densities, ranging from 1,000 to 1,800 trees per hectare, is mostly fertilized with organic waste and is intercropped with different food crops (Anonymous, 2006; Mekuria, 2004).

1.5 Major coffee pests and diseases in Ethiopia

Limiting factors of coffee production in Ethiopia include diseases, such as coffee leaf rust, coffee berry disease (CBD) and coffee wilt disease (Derso et al., 2000).The antestia bug, *Antestiopsis intricata*, the blotch leafminer, *Leucoptera caffeine* and the coffee berry borer, *Hypothenemus hampei* are among the most prevalent insect pests of coffee in Ethiopia. However, they do not usually reach economic threshold levels because of the lack of environmental conditions conducive for their population growth (Million and Bayisa, 1985).

The only available information on coffee nematodes in Ethiopia is a general survey report that only covered two coffee growing areas. According to the report, seven genera of plant-parasitic nematodes were encountered (O'Bannon, 1975). Among these, the three genera *Meloidogyne*, *Pratylenchus* and *Rotylenchulus*, were identified as parasites of coffee.

1.6 Nematodes as plant parasites

Nematodes are round worms belonging to the phylum Nematoda and are present in almost every niche on the planet. They are aquatic animals found in marine and freshwater environments, in films of water in the soil, and as parasites within plant

and animal tissues. The first recorded history of phytoparasitic nematode was reported by Turbevill Needham in England in 1743 later called the wheat gall nematode, *Anguina tritici* (Mai, 1971). Next came the discovery of the root-knot nematode as a cause of galls on cucumber roots in 1855 by Berkeley. In 1859, Hermann Schacht described the sugar beet nematode that threatened the sugar industry of Germany. The sugar beet nematode was named *Heterodera schachtii* by Schmidt in 1871. These nematodes were easily associated with their hosts as they are all endoparasitic nematodes that cause distinct galling and symptoms on the plants (Mai, 1971).

Plant-parasitic nematodes are greatly affected by moisture, temperature and oxygen levels within the soil environment in which they persist. Distribution of parasitic nematodes is usually patchy, suggesting no specific pattern to their population distribution. They are vertically distributed within the top 30 cm of soil, but the highest numbers of nematodes are typically found in the top 15–20 cm (Norton, 1978). Nematode concentration is greatest near plant roots, as many are attracted to the root exudates. Nematodes move short distances in soil approximately 30 cm while long distance spread is primarily from equipment, irrigation, birds, and other animals or humans (Wallace, 1964).

Plant-parasitic nematodes have biological characteristics that distinguish them from other organisms that affect growth of plants. They spend at least part of their life cycle within the soil where they are dependent upon all events that happen in and on the field in which they live. Also any agronomic factor, such as organic matter content or cultivation technique will also affect nematode behavior (Yeates, 1984). Nematodes are highly dependent on the presence of host plants but also are influenced by biological, chemical and physical components of the soil environment.

Plant-parasitic nematodes are functionally divided into two major categories based on feeding habit as endoparasitic and ectoparasitic. Endoparasitic nematodes feed by either partially or completely entering root or shoot tissue whereas ectoparasitic nematodes feed on the surface. They inject their stylet into the epidermal cells of the root to feed from the outside. Economically, endoparasitic nematodes cause the greatest damage. Although direct mechanical injury from nematode feeding is

usually negligible, cell dysfunction as a result of the plant-nematode interaction leads to symptoms that include root-knots or galls, root lesions and deforming injured root tips and breakdown of the root tissue. In addition, indirect symptoms such as chlorosis, stunting and wilting often appear aboveground due to the root‘s inability to absorb and distribute water and nutrients needed for plant growth Furthermore, wounds created by the nematodes can act as entrance sites for secondary plant pathogens (Manzanilla-Lopez et al., 2003). In general, crop losses due to plant-parasitic nematodes are estimated annually to reach approximately 12% or approximately US$ 100 billion (Sasser and Freckman, 1987).

1.7 Nematode distribution in the soil

Most of the nematodes that feed on plants spend the majority of their lives in films of water between soil particles (Dropkin, 1980). Therefore, in addition to host availability, the physical and chemical characteristics of the soil environment play an important role in the survival and reproduction of plant-parasitic nematodes (Norton, 1979; 1989; Castro et al., 1990).

Population densities of plant-parasitic nematodes in the soil vary greatly in time and space. Patchy or aggregated spatial distribution of nematodes and changes of the typical polyspecific communities over time pose major sampling problems. The inherent tendency of nematode populations to increase or decrease, which is often enhanced by climate and other factors, is partly responsible for their patchy distribution. Some of the ecological factors that affect nematode population density and distribution in the soil are: soil texture, temperature, moisture, organic matter, pH, plant susceptibility and microorganisms (Norton, 1979; 1989).

Prevalence and virulence of many plant-parasitic nematodes of economic importance vary predictably in soils of different texture (Barker, 1998) because texture is related to soil porosity, water potential and other chemical and physical properties affected by particle size. Most plant-parasitic nematodes show higher population densities in sandy-loam soils than in clay soils. For example, the sting nematode (*Belonolaimus spp*), root-knot nematode (*Meloidogyne spp*.), needle nematode (*Longidorus spp*.), and stubby root nematode (*Trichodorus spp*) are found

in higher densities in sandy soils. Some root lesion nematodes such as *Pratylenchus zea* are common in sandy soils, but others of the same genus, such as *Pratylenchus hexincisus*, are abundant in the medium to heavy textured soils.

Soil moisture is a very important abiotic factor governing nematode populations, and directly or indirectly nematode survival in the soil. Movement, development and survival of nematodes in soil are regulated by the interaction between soil porosity and moisture. Depending on the pore size and moisture content, a nematode may reside in a pore that is filled with water or in a film of water adhering to the soil particle.

Temperature is one of the most important limiting factors in nematode reproduction as most nematodes have a temperature optimum within which they hatch and move through the soil (Norton, 1978). The soil temperature is usually related to the air temperature, although rapid changes in the soil temperature generally only occur in the top 15 cm. The temperature of a particular soil may determine the predominant species that will be found within it, as well as the population levels of each nematode, based on reproductive optima of each species.

Soil chemical properties such as pH, fertility and osmotic pressure are also potentially important for nematodes to thrive. Organic matter is also believed to affect nematode population indirectly as it has effects on soil properties (Norton, 1978). In addition to soil chemical properties, there are a number of management practices that can alter nematode populations in the soil. These include tillage, crop rotation, use of resistant varieties, and planting time.

Usually, the highest population density of plant-parasitic nematodes is detected when the plants are in the flowering stage. A low population is usually found in newly planted crops and in senescent plants when root growth has stopped. In crop cultivars with some degree of resistance, the nematode population density is generally low compared to susceptible cultivars. For banana, Davide and Marasigan (1992) showed that *R. similis* and *M. incognita* population densities were much higher in the susceptible cultivar Giant Cavendish than in resistant cultivars like Amas, Tanggung, Katsila and others.

1.8 Population dynamics of plant-parasitic nematodes

Population dynamics is the study of patterns of a population over space and time, and can include characteristics such as numbers of individuals, age distribution, sex ratio, and behavior of a population, especially in response to environmental factors (Norton, 1978; Ferris and Willson, 1987). Studies involving population dynamics can be conducted in attempt to predict nematode behavior in a particular field or in a larger region in the hopes of extrapolating that information to other regions of similar characteristics. These patterns can be studied and used to develop predictive models for a particular organism or for the interaction of a host and a parasite over time. These models can in turn be used to help understand complex relationships within a group of organisms or between different organisms (Duncan and McSorley, 1987). In order to gather data necessary to predict the dynamics of a nematode's population extensive survey's needed to be conducted in both time and space.

Plant-parasitic nematodes are responsible for over $100 billion dollars in economic losses worldwide to a variety of plants (Sasser and Freckman, 1987). These crops are grown in a variety of environments and are managed by completely different methods. Therefore, it would stand to reason that the nematode population fluctuation would vary depending on the crop, region and management practices. A survey of population diversity and fluctuation over time allows researchers to predict when nematode epidemics may occur and help suggest management practices to reduce population levels.

When performing a study of nematode population dynamics, it is important to understand the ultimate goal of the study. This may dictate the size of the area to be sampled, the sampling distribution, the type of extraction method and the data to be collected. Population dynamics will vary due to host plant availability, interaction with other genera or species of nematodes, and interaction with other parasites such as antagonistic fungi. Food source availability may be determined by the nature of the crop, or by the health of the host crop. Since presence of a food source will be one of the primary determining factors in increasing nematode populations (Ferris and Willson, 1987), an annual crop will undoubtedly lead to different dynamics than a perennial crop. Of equal importance is the geographical region that is being

surveyed. Regions with a year round growing season will be more likely to sustain higher nematode population levels than those with a shortened growing season, or where temperatures are too low for nematodes to have numerous generations.

Many studies conducted on nematode populations are done on areas that have not been previously surveyed, and their purpose is more to determine the levels and diversity of nematode populations within a single crop or a number of crops in a particular region. In other studies, nematodes are a known problem for a particular crop or region and research is done to determine population occurrence of a single nematode type for crop loss assessment or to determine control options to use in management.

1.9 Plant parasitic nematodes research in Ethiopia

Since 1975 only a few plant-parasitic nematodes from various crops have been reported from Ethiopia. Compared to other plant diseases, very little information is presently available on the occurrence of plant-parasitic nematodes in the country (O'Bannon, 1975; Godfrey et al., 1988; Abebe and Geraert, 1995; Swart et al., 2000; Wondirad and Mekete, 2002; Bogale et al., 2004).

The presence of *Meloidogyne* spp. on vegetables was the earliest plant-parasitic nematodes to be reported in the country. More detailed and comprehensive surveys of plant-parasitic nematodes were made by a FAO consultant thirty two years ago O'Bannon (1975). The survey covered the major agricultural areas in the Rift valley and the south-western part of the country. Out of 150 samples collected from 53 major crop species and potentially important plants, four plant-parasitic nematode genera were found to have the highest frequency of occurrence.

The root-knot nematodes, *Meloidogyne* spp. were the most predominant species found associated with about 40% of the crops sampled. Three species of this genus, namely, *Meloidogyne incognita*, *M. javanica* and *M. ethiopica* were identified in the country. Other genera, such as *Pratylenchus, Helicotylenchus* and *Rotylenchulus* were found on 34, 32 and 25% of the crops sampled, respectively. The other nematode genera found commonly from a range of crops were ring nematode

(*Criconemella* spp.), spiral nematode (*Helicotylenchus* spp.), lance nematode (*Scutellonema* spp.), needle nematode (*Longidorus* spp.) and dagger nematode (*Xiphinema* spp.). It was also found that, in cereal crops (Tef, barley, wheat, maize and sorghum) needle, dagger, lance and spiral nematodes occurred at high frequency. The citrus nematode (*Tylenchulus semipenetrans*) was identified in five different locations from oranges and grapes but was not widely distributed. Another important nematode species recorded was the burrowing nematode (*Radopholus similis*) on banana. Generally it was observed that, in most of the crops sampled in the highlands, nematode incidence was low, but in the lowlands, especially those under irrigation, they were high (O'Bannon, 1975). Low nematode numbers in the highlands is explained by: (1) high frequency of crop rotation with cereals, (2) long dry fallows, (3) short season crops with long dry spells and (4) high altitudes of above 2000 m which are less favorable for nematode activity. In contrast, lowlands are highly favorable for the following reasons: (1) monoculture, (2) continuous cropping, (3) high weed incidence, (4) long growing seasons, and (5) genetically homogenous germplasm (O'Bannon, 1975).

In 1995, Abebe and Geraert described one new and four known plant-parasitic nematodes from Ethiopia. These were: *Discocriconemella addisababa* sp. n, *Criconemella parava, Pratylenchus leptus, Scutellonema brevistyletum* and *Helicotylenchus abunaamai*.

Wondirad and Mekete (2002) conducted a survey in 1999-2000 to determine the frequency of occurrence of root-knot nematodes on vegetable crops in central and western Ethiopia. The result of this study confirmed the presence of three root-knot nematodes namely: *Meloidogyne incognita, M. javanica* and *M. ethiopica*. They were found associated with major vegetables in all growing regions of Ethiopia. Out of the 192 field samples taken, 62% were found to be infested. *M. incognita* was the predominant species with a total incidence of 55.3% and 57.7% in two sampling seasons. Results also showed similar trends in occurrence in both sampling years.

Another study was carried out in Ethiopia in 1998/1999, where Bogale et al. (2004) conducted a diagnostic survey to establish research priorities and generate baseline data on the occurrence, distribution and abundance of parasitic-nematodes

associated with enset (*Ensete ventricosum*) in Ethiopia. Results showed the presence of different plant-parasitic nematodes associated with enset in different enset growing agroecologies. The predominant nematode species found was *Pratylenchus goodeyi* with prominence value (PV) of 5640 per 100 g fresh root weight (FRW) followed by *Aphelenchoides ensete* (PV= 137 per 100 g FRW) and *Meloidogyne* spp (PV= 26 per 100 g FRW). *Helicotylenchus dihystera, H. multicinctus, Hoplolaimus* spp*., Pratylenchus coffeae, Radopholus similis, Scutellonema bradys* and *Tylenchus* spp*.* were also encountered, although less frequently and in relatively smaller densities. Moreover, the presence of *P. goodeyi* in enset roots and corms of wilting plants make the problem more complex, and indicate a possibility of disease interaction with wilt causing pathogen.

Van Den Berg et al. (2004) and Marais et al. (2005) described new records of Criconematidae and *Helicotylenchus* associated with different crops. The goal of the study was to describe detailed morphological characteristics of the respective genera. During a survey in the 2002 cropping season in the south, west and southwestern parts of the country, seven known Criconematidae, one new and seven known species of *Helicotylenchus* were described morphologically. Species of Criconematidae described were: *Criconema. crassianulatum, C mutabile, Criconemoides goodeyi, Discocriconemella limitanea, Ogma decalineatum, Pratylenchus leptos,* and *P. pandatus.* Species of the genus *Helicotylenchus* described were: *H. gerti sp. n, H. californicus, H. diagonicus, H. dihystera, H. egyptiensis, H. martini, H. multicinctus* and *H. willmottae*. Six of these seven species (*H. californicus, H. diagonicus, H. dihystera, H. egyptiensis, H. martini* and *H. willmottae*) were new records for Ethiopia.

1.10 Plant parasitic nematodes associated with coffee

Many genera and species of plant-parasitic nematodes have been found associated with coffee in many countries of the world including very damaging nematodes causing great losses to the coffee farmers and the local economy of developing countries (Campos et al., 1990). The most important and widely distributed nematodes in coffee plantations are *Meloidogyne* and *Pratylenchus* which cause great losses (Bertrand et al., 1995; Campos and Villain, 2005). Although, information

about their damage potential is lacking, species of the genus *Helicotylenchus, Hoplolaimus, Criconemella, Longidorus, Trichodorus, Paratrichodorus, Scutellonema, Xiphinema, Rotylenchus, Tylenchorhynchus* and *Ogma* have been reported associated with coffee (Campos and Villain, 2005).

In the neighboring Eastern African countries, plant-parasitic nematodes are one of the major coffee diseases. A unique group of root-knot nematode species known as the African coffee root-knot nematodes have also been found to occur in Africa causing severe root damage to coffee in Tanzania and Kenya. The species identified from the areas sampled are *Meloidogyne africana, M. decalineata, M. kikuyensis* and *M. megadora* (Swai, 1981; Bridge, 1984; Campos et al., 1990; Whitehead, 1996). In Tanzania, 16 genera of plant-parasitic species were recorded but only three *Meloidogyne* spp. were considered important. These are *M. decalineata, M. africana* and another unidentified species. A survey in Tanzania revealed a minimum of 20% loss attributable to nematode infection in coffee plantations in the northern coffee districts (Bridge, 1984).

In Brazil, according to the international *Meloidogyne* project, the average annual loss of coffee yield caused by *Meloidogyne* spp. is 24% (Sosa-Moss, 1985). Some species of coffee are resistant to *M. exigua* but *Coffee arabica*, the most economically important species of coffee is susceptible (Huang et al., 1983).

Although plant-parasitic nematodes are known to be the most important parasites in world coffee production, the level of knowledge in Ethiopia is very limited. The significance of coffee nematodes in Ethiopia has not been well researched. The only information on coffee nematodes is present in a report where a few nematode genera have been mentioned in two areas of the country. According to the report, seven genera of plant-parasitic nematodes were reported, among these, the genera *Meloidogyne*, *Pratylenchus* and *Rotylenchulus*, were the major parasites recovered from the coffee fields (O'Bannon, 1975).

1.11 Nematode control

Nematode management practices in traditional farming systems have been reviewed by different authors (Bridge, 1996; Sikora et al., 2005). The major objective of plant-parasitic nematode management is to prevent plant losses in quality and yield by keeping the nematode population below the threshold level (Whitehead, 1998). Common nematode control methods include cultural, physical, biological and chemical measures. Concerns regarding chemical measures in human health and environmental safety, have renewed interest in the use of alternative biological control strategies for use in integrated pest management.

Integrated pest management strategies (IPM) combines multiple methods such as plant hygiene, cultural practices, choice of planting material, physical methods, application of selective pesticides and biological control with antagonistic organisms (Sikora et al., 2005).

Biological control is defined as the use of one beneficial organism that either feeds on, or competes with the pest organism to reduce the population growth rate of, and the damage caused by the pest. Bacteria and fungi are the most common organisms in the soil and some of them have great potential as biological control agents against plant-parasitic nematodes. Biological control of plant-parasitic nematodes is mediated through different mechanisms such as antibiosis, parasitism, competition and predation (Stirling, 1991; Sikora, 1992).

Biological control of nematodes using rhizosphere micro-organisms was considered in several reviews to be a potential management tactic and effective alternative to nematicides (Sikora, 1992; Kerry, 2000). Biocontrol of plant-parasitic nematodes was reported for a great diversity of microorganisms including: plant growth promoting rhizobacteria (Becker et al., 1988; Spiegel et al., 1991; Racke and Sikora, 1992; Siddiqui and Shaukat, 2000; Siddiqui and Ehteshamul-Hauque, 2001), obligate fungal parasites and facultative fungal parasites (Nigh et al., 1980), competitors including endophytic fungi and endophytic bacteria (Hallmann and Sikora, 1994; Diedhiou et al., 2003; Dababat, 2007) as well as mycorrhizal fungi (Saleh and

Sikora, 1984; Pinochet et al., 2001; Elsen et al., 2003; Reimann and Sikora, 2003; Sikora and Reimann, 2004).

1.12 Endophytic organisms as biological control agents

Many promising microbial organisms demonstrate excellent performance in the laboratory and greenhouse against pests or diseases. However, this often translates in below-expected performance in the field because they have to compete with the native flora. This competition can be avoided by using endophytes living inside the plant where they are less exposed to environmental factors (Sikora, 1997; Dubois et al., 2006).

Endophytes are defined as ‘organisms that colonize internal plant tissue at some time in their life cycle without causing apparent harm to the host or any external disease symptoms (Carroll, 1988; Clay, 1990; Sikora, 1992; Schuster et al., 1995; Hallmann, 1999; Schulz and Boyle, 2006; Sikora et al., 2007).

Endophytes employ several modes of action against pests and diseases: parasitism, competition, antibiosis, indirect effects through plant growth stimulation and induced plant resistance (Schuster et al., 1995; Hallmann and Sikora, 1996; Pocasangre et al., 2000; Nitao et al., 2001; Dubois et al., 2006; Sikora et al., 2007).

Nothing has been done on nematode biological control in Ethiopia compared with achievements made in this field elsewhere in the world. The present study is therefore the first attempt to quantify the benefits of biological control of nematodes. It is including both isolation and evaluation of isolates under different experimental conditions.

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CHAPTER 2

2.a Geophytonematology of plant-parasitic nematodes associated with *Coffea arabica*, Rubiaceae in Ethiopia

1 Introduction

Coffee arabica is one of the most important cash crops originating from Ethiopia. It is the most widely distributed and cultivated species within the plant genus coffea in the country. The agricultural based economy of Ethiopia is highly dependent on coffee production and export, contributing 35 percent to the country's foreign currency earnings (Woods, 2003).

Plant-parasitic nematodes are considered a limiting factor in crop production because they cause serious root diseases resulting in decline of plant growth and yield. It is of paramount importance to have a comprehensive understanding of the occurrence of plant-parasitic nematodes to be able to predict potential nematode crop damage and yield losses. If nematode population have reached critical levels then control measures need to be initiated before damage occurs.

Survey data are also useful for the identification of nematode species associated with different crops and their distribution in a given locality. Before samples are taken, a detailed sampling plan including the collection pattern, as well as the number and size of samples must be developed based on available funds and resources. Based on the survey data, studies on the biology, ecology and control of plant-parasitic nematodes can be initiated and their role in crop loss and disease complexes with other pathogens like fungi, bacteria and viruses can be evaluated.

Geophytonematology can be considered as the study of nematode host distribution in agro-ecological zones. Although, plant-parasitic nematodes cause yield losses on many crops, their economic significance is generally not well understood or recognized by growers. Therefore, studies on the geophytonematology of plant-parasitic nematodes should be done to provide basic information on their importance (Noe and Sikora, 1990).

Many genera and species of plant-parasitic nematodes have been associated with coffee in many countries of the world including very damaging species (Campos et al., 2005). In East Africa, a unique group of root-knot nematode species known as the African coffee root-knot nematodes have been found to occur and cause severe damage in Tanzania and Kenya. The species identified from the areas sampled are *Meloidogyne africana, M. decalineata, M. kikuyensis,* and *M. megadora* (Swai, 1981; Bridge, 1984; Campos et al., 2005). In Tanzania, 16 genera of plant-parasitic species were recorded on coffee but only three *Meloidogyne* spp. were considered important. These are *M. decalineata, M. africana* and another unidentified species. A survey in Tanzania revealed a minimum of 20% yield loss attributable to nematode infection in coffee plantations in the northern coffee districts (Bridge, 1984).

Although plant-parasitic nematodes are known to be damaging to coffee production, the level of knowledge in Ethiopia is very limited. There is little to no available information on coffee nematodes other than a few unpublished reports where a few nematode genera have been mentioned (O'Bannon, 1975). The only available information of coffee nematodes in the country is a survey report that only covered two areas. According to the report, seven genera of plant-parasitic nematodes were encountered (O'Bannon, 1975). Among these, the three genera *Meloidogyne*, *Pratylenchus* and *Rotylenchulus*, were identified associated with coffee.

Therefore, more extensive information about the presence of plant-parasitic nematodes, their host associations, and frequency of occurrence in different coffee growing localities is essential. This study was therefore performed with the following objectives:

1. To determine the presence and frequency of occurrence of plant-parasitic nematodes in the coffee growing agroecologies of Ethiopia
2. To determine dynamics of nematode populations between dry and wet growing seasons.

2 Materials and methods

2.1 Survey methodology

According to the Ethiopian ministry of agriculture (MoA), current Agroecological Zonations of Ethiopia (AEZ) is based on the basic ecological elements of climate, physiography, soils, vegetation and farming systems. Accordingly, a total of 18 major AEZs are delineated and named by terms describing the broad moisture and elevation conditions of areas. Of these, coffee grows in 15 AEZs (MOA, 2000) (table 1).

Sampling was done during the wet season in August, 2004 and dry season in April, 2006 by dividing the coffee growing region in 20 km^2 grids, each representing a combination of agroecologies and plantation types. In total, 15 coffee agroecologies comprising four plantation types forest, semi-forest, garden and plantation) were studied. Sampling intensity for each agroecology was equivalent to its total size (table 1).

Soil samples were taken from 132 field sites (figure 1). Each sample of 1.0-1.5 kg consisted of four to ten cores taken from the top 25–30 cm of soil with a spade. Samples were taken predominantly from coffee trees with no or little ground cover vegetation. However, at sites with weeds and intercrops in close proximity to the coffee trees, the additional vegetation was recorded to later allow cross references of nematode data with non-target host plants. For each sampling site, the elevation, latitude and longitude were recorded using a Global Positioning System (GPS).

2.2 Nematode extraction

Extraction of nematodes was done at the Ambo Plant Protection Research Center, Ethiopia.

2.2.1 Nematode extraction from soil

From each field sample 200 g aliquots of soil were immersed in a bucket containing 2 liter water and hand stirred gently until all soil clumps disintegrated. The slurry was then passed through a 250 mm sieve and the passing suspension was collected in a

bucket. This step was repeated three times. The water in the bucket was used in the next sieving step with a 100 μm aperture sieve. Again this step was repeated three times and followed by further sieving steps with descending aperture size of 50 μm, 38 μm, and 25 μm. Retained material on each of the sieves was removed using 100 ml of water delivered as a fine stream. The final 400 – 600 ml sample from each sieve size was collected in beakers and the particulate contents allowed to settle overnight. The supernatant fluid was then siphoned from the beaker, leaving the nematodes and particulate debris at the bottom. To further clean the crude sample from the persisting debris nematodes were extracted by the Baermann technique for 24 h (Hopper, 1985a).

2.2.2 Nematode extraction from root

Roots were washed free of soil in tap water and examined for necrosis and root-knot infection. Nematodes were then extracted from 10 g of roots using a maceration/filtration technique by which the roots were washed, chopped with scissors and spread on a Baermann dish for 2 days (Hooper, 1985b).

2.3 Nematode counting, fixation and identification

After the extraction process, the samples were evaluated under microscope at 40X. The samples were placed in a round counting dish with vertical markings serving as counting lanes at 0.3 cm spacing. All plant-parasitic genera of nematodes were counted and recorded. Nematode population levels were determined and expressed as numbers per 100 g soil. The data obtained were subjected to community analysis to determine the frequency of occurrence, density and prominence value (Prominence value (PV) = Population density X $\sqrt{\text{frequency of occurrence}}$/10 of nematode genera) (De Waele et al., 1998). The PV value considers both population density and frequency occurrence which is useful to detect the order of importance for the nematode populations.

Specimens for further identification were processed and preserved as described by Hooper (1990a). Nematodes were fixed in a formal acetic acid fixative solution (F.A 4:1) of 10 ml formalin, 1 ml glacial acetic acid, 2 ml glycerol and distilled water up to 100 ml. As this solution irreversibly distorts the body contents of nematodes at room

temperature, the solution was heated to 60°C prior to the fixation of the nematodes. Samples were then mounted in glycerine for identification (Southey, 1985). Specimens were collected in a glass block and 4 ml of mounting solution of anhydrous glycerol, 2 ml; 96% ethanol, 1 ml; and distilled water, 90 ml were added. Specimens were covered loosely and left at room temperature for 3 weeks until the water and ethanol were evaporated. The genus composition of the plant-parasitic nematode community of each soil sample was determined under high magnification (400X) with a compound microscope mainly based on the characteristics of adult females (Mai and Mullin, 1996).

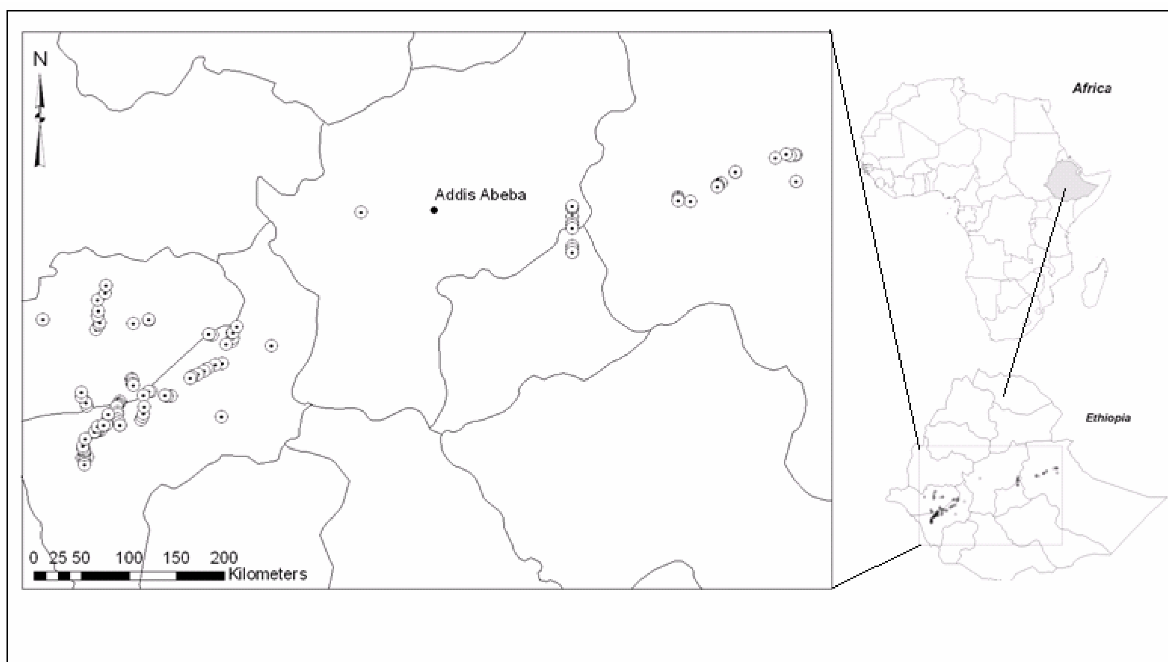

Figure 1: Distribution of sampling sites in major coffee growing regions of Ethiopia

Table 1: List of coffee growing agroecologies and sampling localities. Ministry of Agriculture (MoA) 2000, Natural Resources Management and Regulatory Department Addis Ababa, Ethiopia.

SH2: Tepid to cool sub-humid Mendi, Gimbi, TeferiKillo, Agaro, Aleta-wondo, Dedder, Bedessa, Bonga, Sodo, Boditi, Gelemso	**H2: Tepid to cool humid mid highlands** Bedele, Shebe, Gidole, Bore, Mechara, Masha, Wushush	**M2: Tepid to cool moist mid highlands** Yirgachefe, Woliso, Solemo, Kombolcha, Fisha Genet, Yirgalem
SH1: Hot to warm sub-humid Chanka (Wollega), Gimbichu (Hossaina), Bele, Metu, Waka, Dilla, Nekempte, ShewaGimira	**H1: Hot to warm humid lowlands** Tepi, Godere, Jinka (Bako), Jinka	**PH2: Tepid to cool per-humid highlands** MizanTeferi, ShewaGimira, Temenjayazhi
SM1: Hot to warm sub-moist Wenago, Dimtu, Kofi	**M1: Hot to warm moist** Asebe Teferi, Chelenko, Arjo, Hamusgebeya	**PH1: Hot to warm per-humid lowlands** Bebeka, Amaru
SM2: Tepid to cool sub-moist Harar, Kuyera, Babile, Mesela	**H3: Cold to very cold sub-humid** Ameya, Amaro Killo, Goche, Hageremariam	**SH3 :Sub-afroalpine to Afroalpine** Bonke Beza (Arbaminch)
A2: Tepid to cool arid mid highlands Dire Dawa	**A1: Hot to warm arid** Dire Dawa, Hurso	**M3: Cold to very cold moist** Wenchi

3 Results

The most dominant plant-parasitic nematode genera found were *Helicotylenchus, Scutellonema, Rotylenchus, Xiphinema, Heterodera* and *Tylenchorhynchus.* Genera observed with less frequency included *Ditylenchus, Trophurus, Criconemella, Hoplolaimus, Pratylenchus* and *Meloidogyne.* For the major plant-parasitic nematode genera, specimens were identified to the following species: *Helicotylenchus dihystera, H. multicinctus, H. californicus, H. gerti, Xiphinema insigne, X. basilgoodeyi, Scutellonema paralabiatum*, *Rotylenchus unisexus, Tylenchorhynchus agri* and *T. acti* (annex 1).

Eighty percent of the soil samples contained multiple genera of plant-parasitic nematodes while 6.3% of the soil samples contained only a single genus. Of the samples with multiple genera of plant-parasitic nematodes, 26.7% of the samples contained two genera, 20% contained three genera, 33.3% contained four and more genera and nematodes were not detected in 13.7% of the total samples.

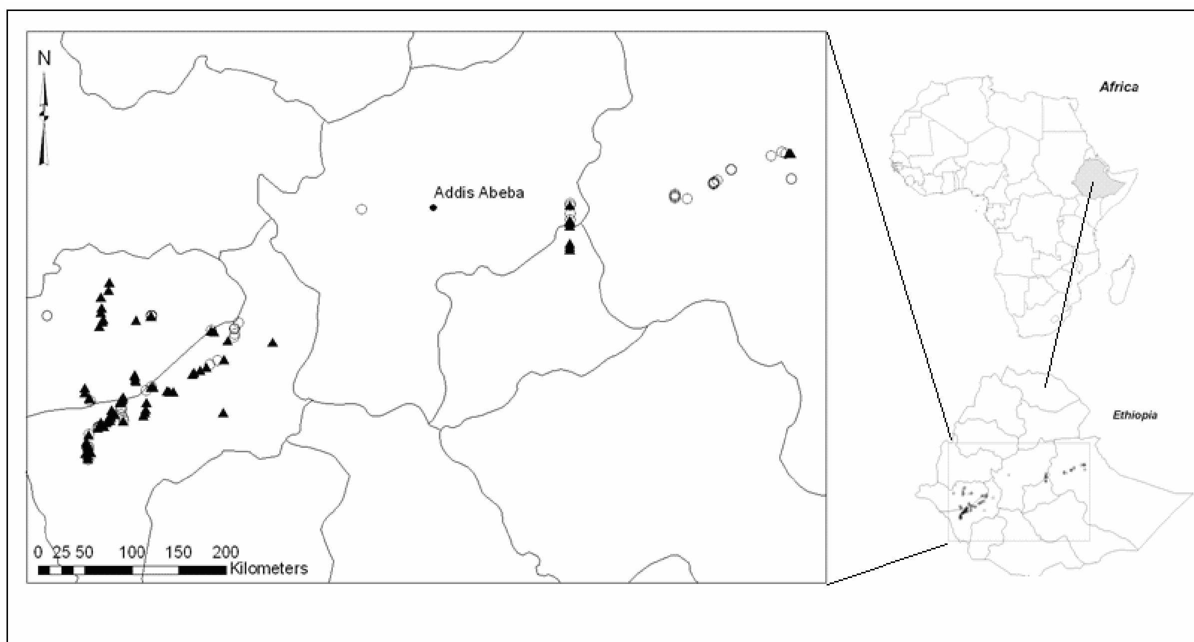

Figure 2: Distribution of *Helicotylenchus* species in different coffee agroecologies.

○ = negative sampling sites ▲ positive sampling sites

Helicotylenchus was found in 73.3% and 55% of the samples with maximum population densities of 1480 and 640 nematodes/100 g soil in the wet and dry

season samples, respectively. Prominence values were 639 for the wet season and 282 for the dry season (table 2, figures 2, 3). *Xiphinema* was recovered from the soil in 40% and 22% of the sampling times with maximum population density of 870 and 155 nematodes/100 g of soil and prominence values of 221 and 73 in wet and dry seasons, respectively (table 2, figures 3, 4).

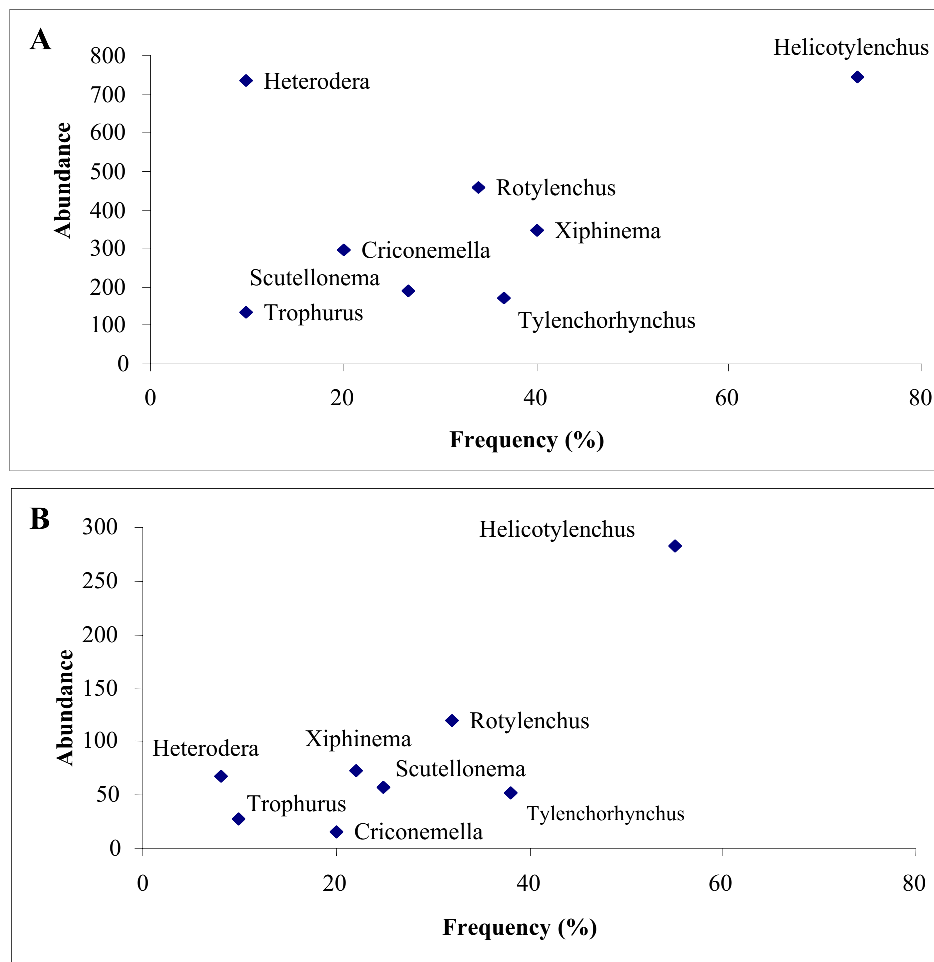

Figure 3: Frequency and abundance of the major plant-parasitic nematode species of coffee in the wet (A) and the dry (B) sampling season from Ethiopia

Heterodera is reported here for the first time in Ethiopia. Although species of *Heterodera* occurred in 10% and 8% of the soil samples in wet and dry season, respectively, this genus was specifically recovered from semi-forest coffee plantations of Bedele region reaching maximum population densities of 3310 juveniles/100 g soil and a prominence value of 233 and 67 in wet and dry seasons respectively (table 2, figures 3, 4).

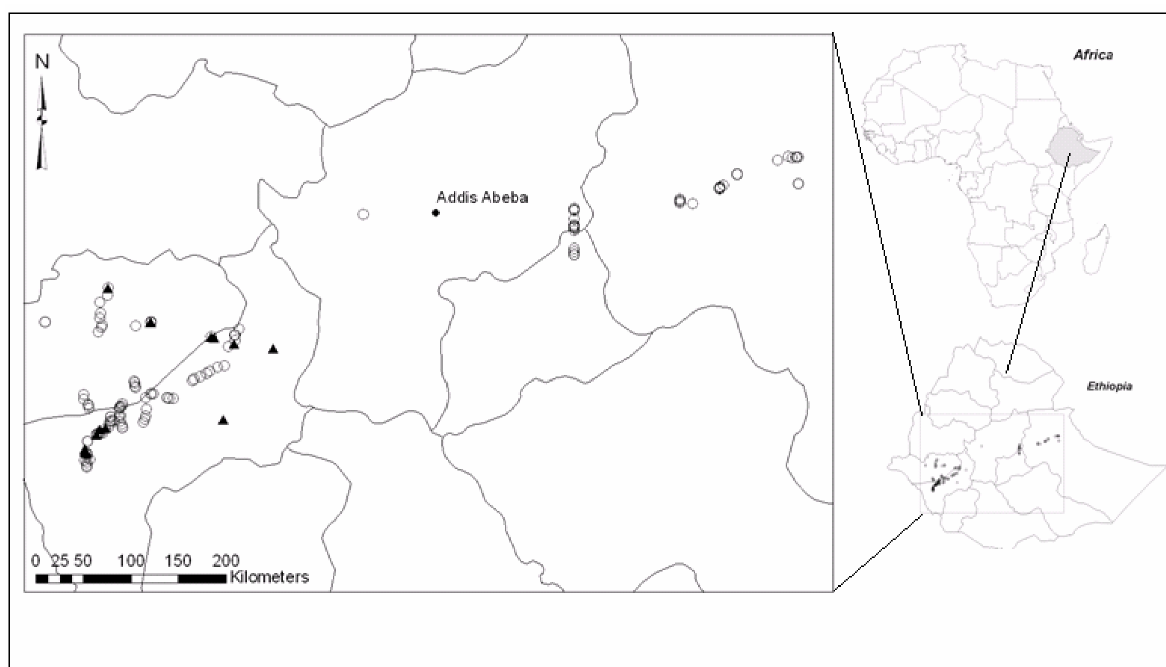

Figure 4: Distribution of *Heterodera* species in different coffee agroecologies. ○ = negative sampling sites ▲ positive sampling sites.

Table 2: Frequency of occurrence, population density and prominence value of major plant-parasitic nematode genera recorded from different plantation types of coffee growing agroecologies from Ethiopia.

Genera	Frequency of occurrence (%)		Population density/100 g soil				Prominence value	
			Mean		Maximum			
	Wet	Dry	Wet	Dry	Wet	Dry	Wet	dry
Helicotylenchus	73	55	373	190	1480	620	639	282
Xiphinema	40	22	174	78	870	155	221	73
Tylenchorhynchus	36	38	85.5	42	400	260	103	52
Rotylenchus	34	32	229	105	2175	670	268	119
Scutellonema	27	25	95	58	750	130	97	58
Criconemella	20	20	147	74	1410	160	132	15
Trophurus	10	10	66	44	250	70	43	28
Heterodera	10	8	369	119	3310	1195	233	67

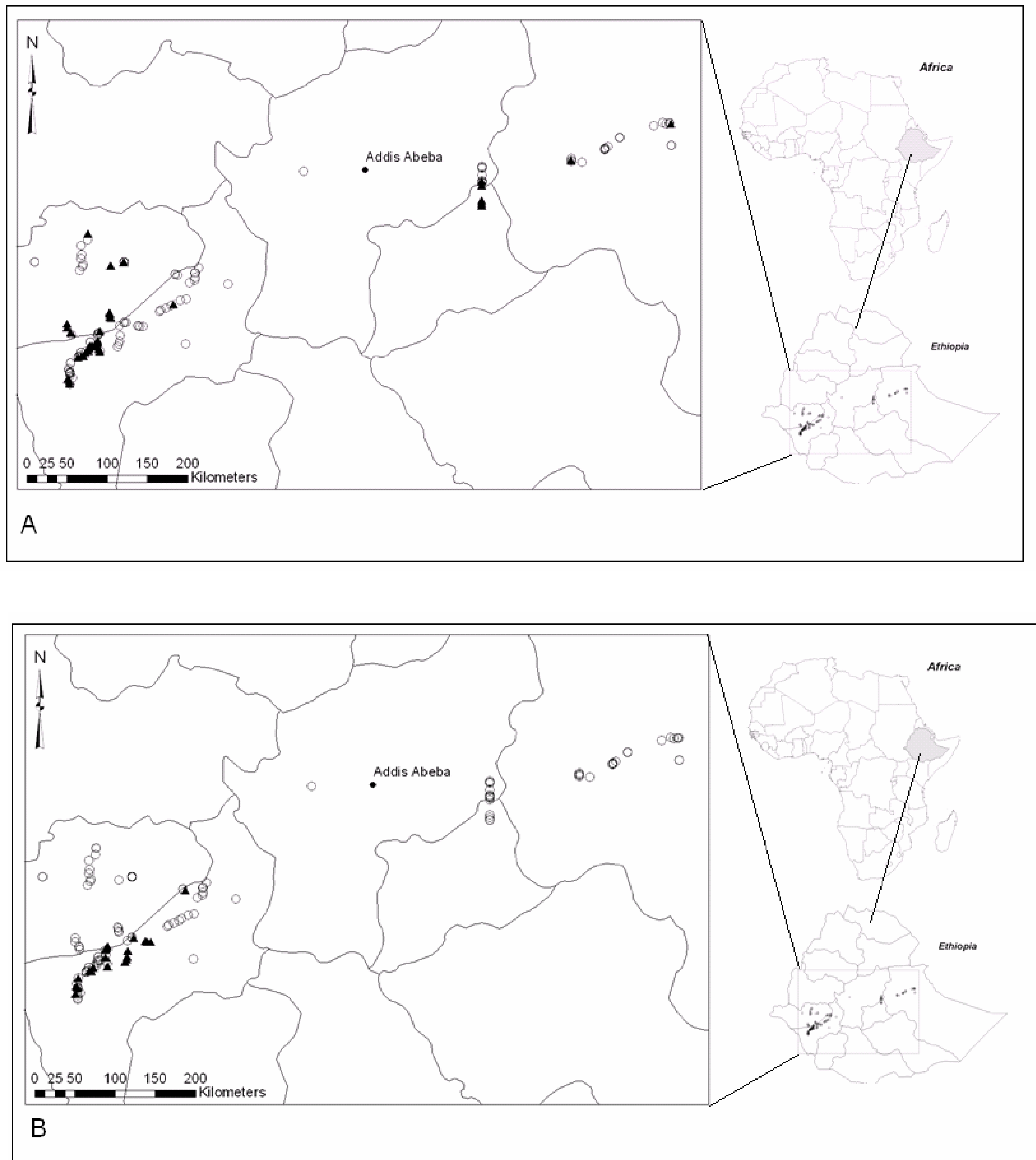

Figure 5: Distribution of the genus *Rotylenchus* (A) and *Scutellonema* (B) in different coffee agroecologies. ○ =negative sampling sites ▲ positive sampling sites.

Differences were observed in total nematode abundance between sampling seasons. The population densities of *Helicotylenchus, Xiphinema* and *Heterodera* were lower in the dry season than the wet season. The nematode density of *Scutellonema, Rotylenchus, Tylenchorhynchus*, *Ditylenchus, Trophurus,*

Criconemella and *Hoplolaimus* were similar in both sampling seasons. On the contrary, the population of *Meloidogyne* and *Pratylenchus* were not recovered during the dry season and only rarely recovered in the wet season (table 1 and figure 5).

In the survey, the root samples collected to detect for the presence of major endoparasitic nematodes contained no plant-parasitic nematodes.

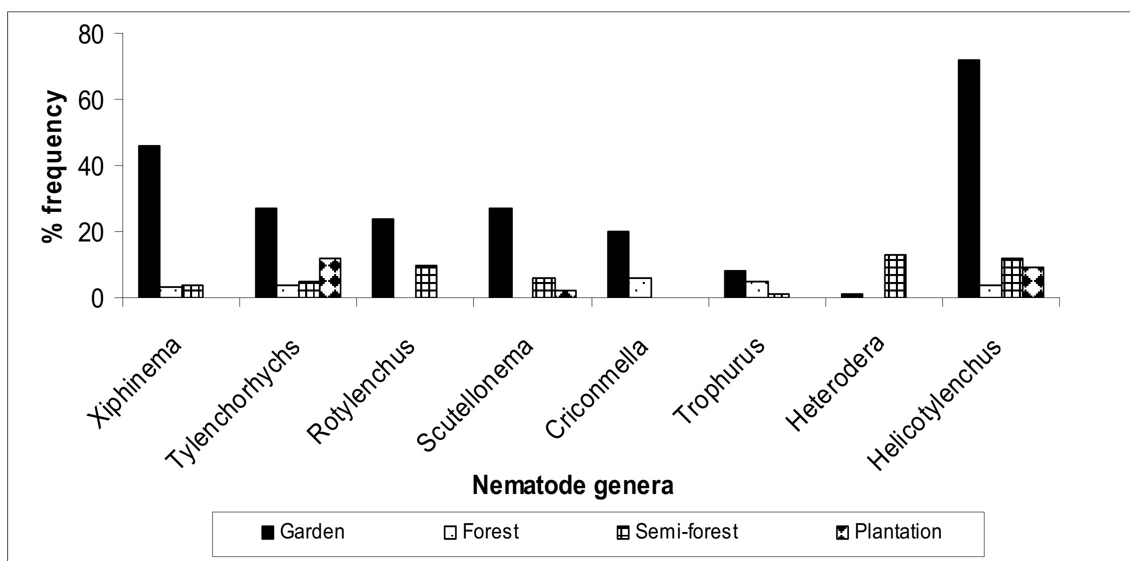

Figure 6: Frequency of the major plant-parasitic nematode genera in different coffee plantation types during wet season sampling.

4 Discussion

Plant-parasitic nematodes detected in the different coffee growing agroecologies of Ethiopia included species of *Helicotylenchus, Scutellonema, Rotylenchus, Xiphinema, Heterodera, Tylenchorhynchus, Ditylenchus, Trophurus, Criconemella, Hoplolaimus, Pratylenchus* and *Meloidogyne.* However, plant-parasitic nematodes were under detectable levels in 13.7% of the total areas of the eastern Ethiopia coffee growing regions. This could be because of the very high temperature in the region which resulted in extreme soil drying and death of all nematodes in the top 15 cm of the soil.

Nematode species belonging to the genus *Helicotylenchus* are ectoparasites or semi-endoparasites feeding on plant roots and have been sporadically associated with reduction in plant growth in many crops. However, no major host-parasite relationship of importance is proposed between this nematode and coffee in Ethiopia. Furthermore *Helicotylenchus* is mostly prevalent and a common parasite of grasses and weeds. The presence of ground cover near coffee plants makes it often difficult to draw accurate conclusions about their feeding relationships with coffee plants in Ethiopia. However, at least half of the sampling sites were free of weeds and in some of the weed-free sites high numbers of *Helicotylenchus* were recovered from the soil. Those nematodes might be parasitic on coffee. High population density of this genus can lead to root damage and the establishment of entry point for other soil borne pathogens that can increase root damage. *Helicotylenchus* was found to be one of the common nematode genera in coffee plantations of Brazil, Hawaii and India (Souza et al., 1999; Giribabu and Saha, 2002; Campos and Villain, 2005; Hue et al., 2005). Significant damage and yield loss by species of *Helicotylenchus* were reported in other crops (O'banon and Inserra, 1989; Gowen and Queneherve, 1990; Yeates and Wouts, 1992; Speijer et al., 1993; Bridge et al., 1995).

High numbers of *Xiphinema* were prevalent in weed-free sampling sites and suggest parasitism on coffee. The survey data showed the presence of specimens from the *Xiphinema americanum* group with restricted distribution in garden coffee plantations. These nematodes are ectoparasites and can cause root tip galling and root stunting in woody plants. They are of considerable importance as vectors of

viruses. However, a *Xiphinema* virus interaction on coffee has not yet been established. However, these species tend to be associated with numerous hosts worldwide especially in woody plants (Sturhan et al., 1997; Shurtleff and Averre III, 2000; Kumari et al., 2005). Further studies are necessary to study their economic importance in coffee.

Species of *Heterodera* were recovered specifically from semi-forest coffee plantations and were all juveniles. Even though never reported from coffee, this genus has worldwide economic importance and detailed research on their distribution, identity and their damage potential in coffee and also to other important crops of Ethiopia should be studied.

Observations of different species from the genus *Scutellonema, Rotylenchus* and *Tylenchorhynchus*, which generally feed ectoparasitically, revealed low frequency and numbers which do not suggest economically important host-parasite relationships with coffee and required further study. These species, however, are reported as minor pests on other crops.

The impact of *Ditylenchus, Trophurus, Criconemella* and *Hoplolaimus* on coffee is not known. They only were detected in few numbers and sporadically. It is likely that these groups might be associated with neighboring plant species.

The root-knot nematode *Meloidogyne,* and the root lesion nematodes, *Pratylenchus,* were detected in low numbers and were found only in three and two sampling sites, respectively and only in the wet season. Both genera were only recovered as juveniles. It is probable that a host-parasite interrelationship exists between *Meloidogyne* and *Pratylenchus* and coffee. However, based on the low numbers observed at each site and the low frequency of positive sites, these two genera do not seem to be important in Ethiopia. Root-knot nematodes known to infect coffee, such as *M. africana, M. decalineata, M. kikuyensis, M. megadora* and *M.* exigua were not detected in this survey. This might be as a result of co-evolution of the host and the parasite and sources of resistance may exist in the coffee biodiversity of Ethiopia. As these groups of nematodes are serious pests in coffee growing countries of the world, fields in Ethiopia need to be monitored more closely to

determine the presence of these nematodes in other areas and especially in nurseries. It is also of paramount importance to regulate these genera in the quarantine list as the possible introduction in to the country. In addition, tests of Ethiopian coffee germplasm for resistance to root-knot nematodes would be of worldwide importance.

The population of *Helicotylenchus, Xiphinema* and *Heterodera* was lower during the dry season sampling compared with the wet season sampling which might indicate their association with wet season plant cover. This could be directly affected by changes in the abiotic environment or indirectly by the effect of abiotic changes on the quantity and quality of the food source, and by interfering with the biotic interactions with other organisms. However, population densities were often the highest among the nematodes identified in both sampling seasons.

The nematode community of *Scutellonema, Rotylenchus, Tylenchorhynchus Ditylenchus, Trophurus, Criconemella* and *Hoplolaimus* were relatively abundant and almost similar in both sampling seasons. On the contrary, the population of *Meloidogyne* and *Pratylenchus* were not recovered during the dry season sampling compared with the rare recovery in wet season.

Few differences were detected in distribution of plant-parasitic nematodes. The notable exception was the presence of *Heterodera* that was specifically recovered from semi-forest coffee plantations (figure 4).

Most genera were generally recovered from across the region sampled. Plant-parasitic nematodes were however not detected in the four Eastern Ethiopia coffee growing regions of Sororo, Deder, Chelenko and Harar. This could be because of the arid environmental conditions of the area where the soil is characterized by very dry and compact and therefore not suitable for the nematodes to survive.

Consistent differences in total plant-parasitic nematode abundance were observed among plantation types with greater abundance in garden coffee than other plantation types. Differences were also observed in total nematode abundance between sampling season. The results revealed some clear seasonal trends in the

population dynamics of plant-parasitic nematode groups, which could reasonably be interpreted as changes affected by seasonal environmental factors i.e. soil temperature, soil moisture content as well as ground cover. The seasonal variation in population density could be ascribed to abiotic factors especially for nematode species with high nutrient and energy needs such as Xiphinema, *Helicotylenchus* and *Tylenchorhynchus* (Norton and Niblack, 1991).

Nematodes were not recovered from roots in this survey. This could be explained in that, all root samples were collected from surface lateral roots of coffee which are 5-10 cm below the soil surface. As this root zone is very near to the soil surface, it is probable that endoparasitic nematodes preferred the lateral roots in deeper soil surface.

2.b Description of plant parasitic nematode species of Tylenchida (Nematoda) associated with coffee (*Coffea arabica*, L) from Ethiopia

1 Introduction

During the survey in 2004 and 2006, nematodes of the order Tylenchida were found in soil samples taken from the rhizosphere of coffee. The present work characterizes the most important species found during that survey: Species of the genus *Helicotylenchus, Scutellonema, Xiphinema, Tylenchorhynchus* and *Rotylenchus* are described and discussed with the objective to:

1. Characterize the most common and potentially important plant-parasitic nematodes associated with coffee by morphological and morphometric characters
2. Illustrate and describe key features of the nematodes as well as their distribution in coffee growing regions.

2 Materials and methods

2.1 Nematode extraction and fixation

Samples were taken from 132 different coffee plantations and examined in the laboratory. Nematode extraction and fixation was done as explained in chapter 2.1

2.2 Permanent slide mounts

The nematode specimens were dehydrated through rapid glycerine method and mounted in pure glycerine on glass slides supported by the glass rod of slightly higher width than the nematodes (Hooper, 1985a). A paraffin wax ring was made at the center of the glass slide and a small drop of anhydrous glycerol was placed in the center of the paraffin ring. The nematodes were then transferred on to the slide and arranged in the center of the drop so that they were touching the slide surface and not floating. A cover slip was then gently heated to 60-65°C on a hot plate. With the melting of the paraffin wax ring the cover slip settled down and confined the glycerol to the center of the mount.

2.3 Nematode identification

Species of plant-parasitic nematodes were identified using a compound microscope (400X) mainly based on the characteristics of adult females using different keys for the respective species (Sher, 1963; Tarjan, 1973; Loof and Luc, 1990; Castillo et al., 1993; Firoza and Maqbool, 1994; Lamberti et al., 2004).

3 Results and discussion

The following numbers of species were identified: 4 *Helicotylenchus*, 1 *Scutellonema*, 1 *Rotylenchus*, 1 *Tylenchorhynchus,* 1 *Quinisulcius* and 4 *Xiphinema.* Detailed descriptions complemented with morphometrics, illustrations, key futures and some additional data are given for each species. Species of *Scutellonema paralabiatum, Tylenchorhynchus agri* and *Quinisulcius capitatus* are new records for Ethiopia.

1 *Helicotylenchus* Steiner, 1945

The genus *Helicotylenchus* as reviewed by Frioza and Maqbool (1994), is classified to species level by a combination of 16 diagnostic characters: habitus, body length, morphometric values a, c, c´, V%, dorsal gland opening, stylet length, shape of stylet knobs, head shape and annulation, tail shape and annulation, phasmid distance from anus expressed in terms of number of cuticular annules, presence and absence of males and female posterior genitial branch. Four species were identified from different agroecologies of coffee, namely: *H. dihystera, H. multicinctus, H. californicus* and *H. gerti.*

1.1 *Helicotylenchus multicinctus* (Cobb, 1893), Golden, 1956

Female body C shaped; hemispherical lip region, slightly set offset with 3-5 annules and sclerotized framework. Reproductive system is didelphic with opposed branches; spermatheca rounded and slightly off set; intestine is not overlapping rectum. Tapering tail with a hemispherical annulated terminus; no mucro or any ventral projection; pore like phasmid, 1-6 annules anterior to anus (figure 1). The morphological and morphometric characters are given in table 1.

These specimens correspond well with various descriptions by Siddiqi (1973) and Sher (1966). Damage to crops by this species alone and in association with other species is reported in Costa Rica, Panama and India causing considerable yield reduction in banana and as minor pest to coffee (Siddiqi, 1973). This species was collected from five sampling locations at Abella- Morrocho, Limu, Aposto, Jimma and Hagere Selam.

1.2 *Helicotylenchus dihystera* (Cobb, 1983)

Females assume a spiral body shape; lip region is hemispherical with 4-5 annules. Cephalic region well developed extending 3-4 annules posteriorly; stylet well developed of 23-29 μm long; stylet knobs rounded posteriorly and hooked anteriorly. Dorsal oesophageal gland opening (DGO) 10-17 μm posterior to the base of the stylet. Reproductive system is didelphic with opposed branches; intestine is not overlapping to rectum. Tail dorsally convex-conoid with broadly pointed hyaline terminus; phasmids located 5-8 annules anterior to anus (figure 1). Morphological and morphometric descriptions are given in table 2

The morphological and morphometric characters are in line with the descriptions by Siddiqi (1972), Van den Berg and Heyns (1975), Van den Berg and Kirby (1979) and Firoza and Maqbool (1994). Specimens of this species were collected from coffee in all field sites of four different sampling locations at Shebe, Agaro, Mechara and Metu. *Helicotylenchus dihystera* is especially known as parasite of several tropical crops such as rice, sugarcane, maize and Banana (Coyne and Plowright, 2002). Detailed studies on host ranges and damage to crops were recorded by Sher (1966), Siddiqi (1972) and O'Bannon and Inserra (1989). It has not been considered as important pest of coffee to date.

1.3 *Helicotylenchus californicus* (Sher, 1966)

The body is curved ventrally and lip region rounded but not set off. It has 4–5 distinct body annules. The stylet is well developed and 25.4-29.1 μm long. Epiptygma is not seen. Spermatheca is distinct and filled with sperm. Intestine is not overlapping to the rectum. Tail region is with ventral projection (figure 1). Phasmid located at 4-10

annules anterior to anus. Detailed morphological and morphometric descriptions are measurements are given in table 3

These specimens correspond with descriptions from Van den Berg and Heyns, 1975**.** Specimens of this species were collected from coffee grown in the region Areka, Jimma-Seka, Bonga, and Agaro.

1.4 *Helicotylenchus gerti (*Marais et al., 2005)

Body spiral; lip region hemispherical, not set off with 3-4 annules; well developed stylet (23-25 μm). Epiptygma folded into vagina (n = 4); reproductive system didelphic with opposed branches. Intestine not overlapping to rectum. Non-annulated, digitate ventral tail projection; phasmid located in middle of lateral field (figure 1). Detailed morphological and morphometric characters are given in table 3.

These specimens correspond well with the description by Marais *et al*. (2005), which is the only report for Ethiopia. There is no evidence in the literature that *H. gerti* caused damage to coffee or any other crops. Specimens of this species were collected from four sampling locations at Chelenko, Chena, Aletawondo and Aposto.

Table 1: Comparison of morphometric characteristics of *Helicotylenchus multicinctus*. All measurements are in μm unless stated.

Characters	Specimens from Ethiopia	After Siddiqi, 1973	After Sher, 1966
n (females)	13		3
L (mm)	0.7 ± 0.1 (0.6 – 0.8)	0.54 (0.46 – 0.68)	0.47 – 0.53
a	31.2 ± 3.2 (21.4 – 34.4)	24.47 (23.8 – 28.5)	24 – 30
b	-	4.8 (4.5 – 6.0)	4.7 – 5.4
b´	-	-	3.4 – 4.1
c	47.6 ± 6.5 (33.4 – 57.2)	53.8 (48 – 63)	35 – 46
c´	1.3 ± 0.2 (0.9 – 1.6)	-	0.8 – 1.0
V (%)	-	69 (64.6 – 71.8)	65 – 69
Stylet length	23.1 – 28.1	20 – 24	22 – 24
O	0.6± 0.1 (0.6 – 0.8)	-	-

Table 2: Comparison of morphometric characteristics of *Helicotylenchus dihystera*. All measurements are in μm unless stated.

Characters	Specimens from Ethiopia	After Firoza and Maqbool, 1994	After Siddiqi, 1972	After Van den Berg and Heyns, 1975	After Van den Berg and Kirby, 1979
n (females)	42	-	12	476	50
Habitus	Spiral	Spiral	Spiral	Spiral	Spiral
L (mm)	0.7 ± 0.1 (0.6 – 0.8)	0.59 – 0.79	0.67 (0.61 – 0.68)	0.6 (0.5 – 0.9)	643 (555 – 768)
a	26.9 ± 2.3 (20.8 – 32.3)	27 – 35	29.5 (26 – 34)	27.9 (19.4 – 36.0)	23.2 (18.6 – 30.2)
b	-	-	5.7 (5.1 – 6.4)	5.6 (4.6 – 6.8)	4.5 (3.6 – 5.1)
b´	-	-	4.6 (4.3 – 5.2)	4.6 (4.0 – 5.6)	5.0
c	50.3 ± 6.8 (37.7 – 72.2)	35 – 49	48 (40 – 65)	43.7 (30.0 – 62.0)	42.6 (31.2 – 51.4)
c´	1.0 ± 0.1 (0.8 – 1.4)	0.8 – 1.2	1.0 – 1.3	1.1 (0.7 – 1.4)	1.0 (0.8 – 1.4)
V	60.3 – 65.8	60 – 65	60 – 66	-	-
Stylet length	26.7 ± 1.8 (22.5 – 29.1)	24 – 29	26 (24.5 – 27.5)	24.7 (20.9 – 27.6)	25.0 (23.2 – 27.6)
O	50.1 ± 5.7 (38.6 – 60.1)	-	47 (42 – 49)	42.8 (19.0 – 62)	49.7 (41.5 – 57.1)
Head annuli	4 – 5	4 – 5	4 or 5	-	-
Tail annuli	-	6 – 12	8 – 12	-	-
Phasmid position	5 – 8	5 – 11	6 - 12	-	-

Table 3: Comparison of morphometric characteristics of *Helicotylenchus californicus* and *Helicotylenchus gerti*. All measurements are in μm unless stated.

Characters	Specimens from Ethiopia	After Van den Berg and Heyns, 1975	Specimens from Ethiopia	After Marais et al., 2005
n (females)	12	25	7	15
Habitus	Spiral	Spiral	Spiral	Spiral
L (mm)	0.7 ± 1.8 (0.6 – 0.8)	0.7(0.6 – 0.8)	637.4 ± 9.9 (622.3 - 649.3)	603 ± 52.9 (527 – 694)
Number of lip annuli	4- 5	3 - 5	3 - 4	3 - 4
a	27.7 ± 2.1(24.0 – 32.3)	28.8(23.4 – 35.0)	29.7± 1.2 (28.2-31.4)	26.8 ± 1.4(24.9 – 28.7)
c	49.5 ± 2.5(46.3 – 53.8)	39 (30.8 – 53.0)	47.7 ± 6.7 (40.1-57.2)	37.2 ± 3.5 (32.7 – 44.3)
c´	1.1 ± 0.1 (0.8 – 1.3)	1.1 (0.7 – 1.5)	1.2 ± 0.1 (1.1- 1.3)	1.4 ± 0.2 (1.1 – 1.8)
V	61 ± 2.2 (58 - 63)	63 (60 – 64)		65 ± 1.0 (63 - 66)
Stylet length	27.1 ± 1.3(25.4-29.1)	24.1 (22.8 27.2)	22.0 ± 1.6 (20.4-24.7)	23 ± 0.7 (22 - 24)
DGO	14.5 ± 1.9 (11.5 – 16.8)	-	12.2 ± 1.4 (10.4 – 13.8)	10 ± 1.4 (8 - 12)
O	53.7 ± 4.5 (43.4 – 59.9)	40.9 (29.7 – 49.3)	-	-

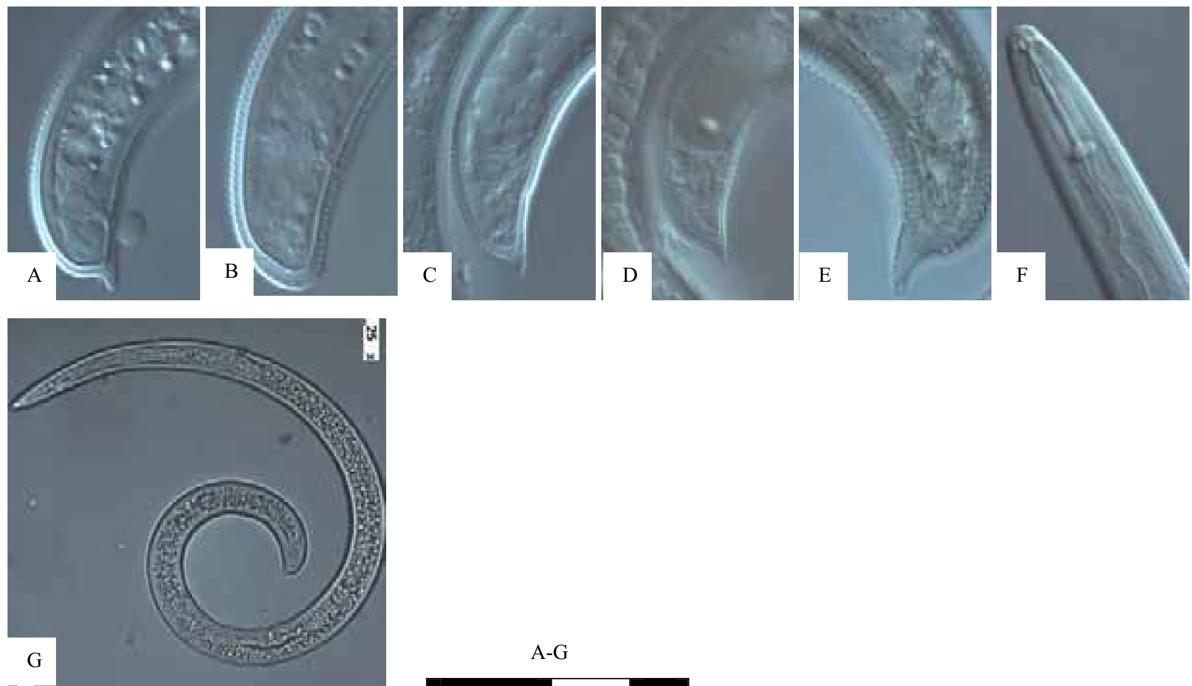

Figure 1: Photomicrographs of *Helicotylenchus* species associated with coffee from Ethiopia. A: Rounded tail with terminal projection of *H. dihystera.* B: Hemispherical annulated tail terminus of *H. multicinctus*. C & D: Irregular tail projections of *H. californicus.* E: Tail with non annulated ventral projection of *H. gerti*. F: Dorsal gland opening distance from stylet end. G: Spiral habitus of *Helicotylenchus* spp. Scale bars: A-F: 40X; G: 25X.

2. *Scutellonema* Andrassy, 1958

Identification of the genus *Scutellonema* was done following the key developed by Sher, 1963 and original descriptions of species since then. *Scutellonema paralabiatum* is the only species found associated with coffee soils in Ethiopia.

2.1 *Scutellonema paralabiatum* (Siddiqi and Sharma, 1994)

The body is ventrally curved and the maximum body width is 0.83 mm. Cephalic region is not offset, slightly tapering to a flat disc with 4-6 annules and the frame work is strongly sclerotized, with outer margins extending into body. The stylet is 20.7–26.6 µm long with rounded knobs. The DGO is 4.2–6.9 µm behind stylet knobs. The spermatheca is not developed. The intestine is not extending over rectum. The tail is

rounded to hemispherical and the lateral field is not areolated in scutellum. Only in few specimens, the epiptygma is projected outward from the vulva, other wise in most of the specimens the epiptygma is one and is not projected (figure 2). Morphological and morphometric measurements are given in table 4.

Scutellonema paralabiatum is reported for the first time in Ethiopia and is the only species of the genus found associated with the rhizosphere of coffee soils in Ethiopia. This species was originally described from pigeon pea in Kenya and also found on sweet potato in Uganda (Siddiqi and Sharma, 1994). Specimens of this species were found in Ethiopia at Agaro, Bonga, Wuswush, Mizan, Areka-Hossaina, Areka, Boditi-Shone, Aleta-Wendo and Abela-Morrocho on coffee.

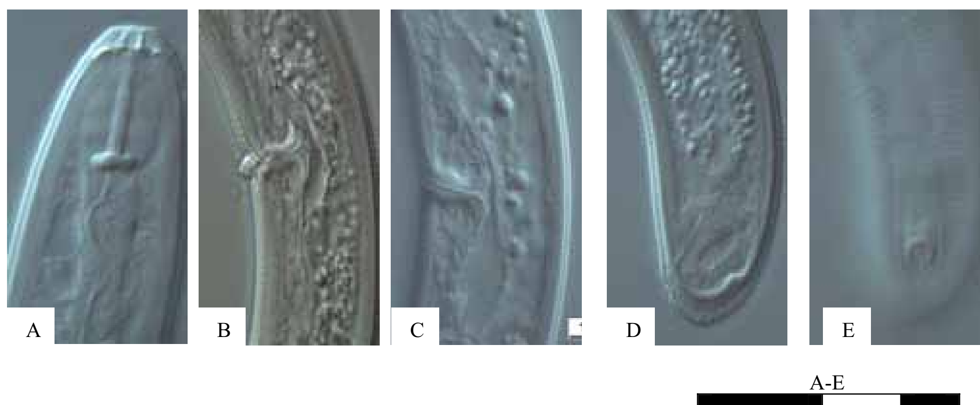

Figure 2: Photomicrographs of *Scutellonema paralabiatum* from Ethiopia. A: Head shape and DGO. B: Outward projected epiptygma. C: Epiptygma not projected. D: Rounded to hemispherical tail shape. E: Lateral field not aerolated in scutellum. Scale bars: A-E, 40X

Table 4: Comparison of morphometric characteristics of *Scutellonema paralabiatum*. All measurements are in μm unless stated.

Characters	*Scutellonema paralabiatum* from Ethiopian coffee	After Siddiqi and Sharma, 1994
n (Females)	29	8
L (mm)	0.75 ± 0.03 (0.68 – 0.83)	0.87 ± 0.58 (0.65 – 0.87)
a	28.45 ± 1.19 (26.36 – 31.39)	26.5 ± 3.2 (22– 31)
b´	5.81 ± 3.26 (4.29 – 20.98)	5.6 ± 0.38 (5.2 – 6.28)
c	86.85 ± 12.79 (62.83 – 103.84)	-
c´	0.49 ± 0.06 (0.39 – 0.65 0)	61.8 ± 9.7 (49 – 77)
Stylet length	24.4 ± 1.29 (20.7 – 26.6)	26.7 ± 0.9 (25 – 28)
O	10.38 ± 7.43 (4.7 – 26.57)	-
Scutella	3.68 ± 0.36 (2.7 – 4.8)	3.5 (2.5 – 3.8)
Tail length	8.66 ± 1.37 (6.1 – 11.5)	10 (8 – 13)

3. *Rotylenchus* Filipjev, 1936

The identification of species of the genus *Rotylenchus* was done following the tabular key developed by Castillo and Vovlas (2005). The most important differential and diagnostic characters used are: lip annulation, lip region shape, lateral field areolation, body longitudinal striations, tail shape, separation of lip region, stylet length and vulval position. Only one species was found.

3.1 *Rotylenchus unisexus* (Sher, 1965)

Specimens of this species have 4-5 lip annules and the lateral field is aerolated only at the oesophageous region. Lip region is hemispherical, continuous with the body contour. Labial frame work is well developed. Vulva is located at 50–70% of body length. Tail is 15.4–23.1 μm long with hemispherical shape (figure 3). Morphological and morphometric measurements are given in table 5.

Rotylenchus unisexus was originally described from Rhodes grass in Kenya (Sher, 1965). My specimens fit the original and other descriptions of the species very well.

They were collected from 10 sampling locations at Mechara, Mesela, Jimma-Seka, Tmenja-Yaje, Mizan, Tepi, Metu, Hossaina, Areka, Aletawendo.

Table 5: Comparison of morphometric characteristics of *Rotylenchus unisexus* females. All measurements are in μm unless stated.

Character	Specimens from Ethiopia	After Sher, 1965	After Van den Berg and Heyns, 1974
n (females)	12	20	309
L (mm)	0.85 ± 0.23 (0.84 – 1.02)	0.7 – 1.0	0.5 – 0.9
a	31.23 ± 2.04 (28.44 – 34.15)	24 – 31	19 – 33.5
b	7.81 ± 1.40 (5.99 – 10.18)	6.3 – 8.4	5.3 – 9.0
b´	8.25 ± 1.33 (6.46 – 9.92)	5.1 – 6.8	4.4 – 7.0
c	47.36 ± 6.85 (38.66 – 61.16)	35 – 52	30.3 – 105.6
DGO	4 – 6.8	-	-
Stylet length	26.79 ± 1.27 (24.6 – 29.1)	25 – 29	19.5 – 28.7
O	0.19 ± 0.03 (0.16 – 0.25)	16 – 24	10.1 – 33.0
Head annules	4 – 5	-	-
Tail length	15.4 – 23.1	-	-

Figure 3: Photomicrographs of *Rotylenchus unisexus* associated with coffee from Ethiopia. A: Scelortized head and DGO distance from stylet end. B: Lateral lines joined at tail tip. C: Rounded tail. Scale bars A-C 40X

4. *Tylenchorhynchus* Cobb, 1913

The genus *Tylenchorhynchus* was established by Nathan A. Cobb in 1913 when he described *T. cylindricus* found in soil from reclaimed coastal swamp lands in southern California. The original genus has now been subdivided in several others (Tarjan, 1973). One of the most important characters used in distinguishing genera previously considered *Tylenchorhynchus* is the number of lines in the lateral field, ranging from three to six. The genus considered now *Tylenchorhynchus* comprise of the species having four lines in the lateral field. Tarjan (1973) gave a valuable key and a table of diagnostic data of species.

*4.1 Tylenchorhynchus agri (*Ferris, 1963)

The lateral field is with 4 lines. Stylet is 14.2–21.4 μm long. Execratory pore opens at a level of anterior end of basal oesophageal bulb. Hemizonid is 1-2 annules anterior to excretory pore and has clavate tail shape (figure 4). Morphological and morphometric measurements are given in table 6.

Tylenchorhynchus agri was originally described from corn fields at the University of Illinois, USA, in 1963 (Ferris, 1963). Specimens from Ethiopia fit the original descriptions of the species well. This is the first report of this species from Ethiopia. It was found at Bedessa, Mechara, Hosaina, Shebe, Aposto, Bonga - Wushwush and Teppi.

5 *Quinisulcius* Siddiqi, 1971

The genus *Quinisulcius* was established by Siddiqi, 1971. One of the most important character used in distinguishing this genus is the presence of five lateral lines (Siddiqi, 2000). In our survey we described one species called *Quinisulcius capitatus.*

5.1 *Quinisulcius capitatus* (Allen, 1995)

Lateral field with five lines, ends near the tail terminus; stylet slender with well developed knobs (15-18 μm long); Dorsal oesophageal gland opens 3 μm behind the stylet base; oval shaped median oesophageal bulb; tail conoid bluntly pointed with a

characteristic enlarged tip with annules (figure 4). Morphological and morphometric measurements are given in table 6.

Quinisulcius capitatus was first described as *Tylenchorhynchus acti* by Hopper 1959 and later synonymized by Allen, 1995 (Siddiqi, 2000). It was originally described from roots of okra from Alabama, USA, 1959 (Hopper, 1959). Specimens from Ethiopian coffee fit the original descriptions of the species well. They were found in three sampling locations at Mechara, Wendo Genet and Dire Dawa. This is the first report of this species from Ethiopia.

Table 6: Comparison of morphometric characteristics of *Tylenchorhynchus agri* and *Quinisulcius capitatus* females from Ethiopia with type populations. All measurements are in μm unless stated.

Character	Specimens of *Tylenchorhynchus agri* from Ethiopian	After Hopper, 1959	Specimens of *Quinisulcius capitatus* from Ethiopia	After Ferris, 1963
n (females)	10	5	7	10
L (mm)	0.74 (0.63 – 0.79)	0.65 – 0.71	0.63(0.56 – 0.74)	0.7 (0.66 – 0.77)
a	34.0 (30.9 – 38.6)	30.2 – 36.2	30.5 (28.5 – 33.4)	30 (28 – 33)
b	-	4.6 – 4.9	-	5.1 (4.7 – 5.5)
c	16.4 (15.3 – 17.6)	14.5 – 16.1	14.7 (12.9 – 16.2)	18 (15 – 21)
v %	57.5 (54.7 – 63.6)	55.2 – 56.9	55.4 (52.9 – 56.9)	56 (55 – 58)
Stylet length	16.8 (15.4 – 18.2)	16.5 – 17.3	18.1 (14.2 – 21.4)	21 (20 – 23)

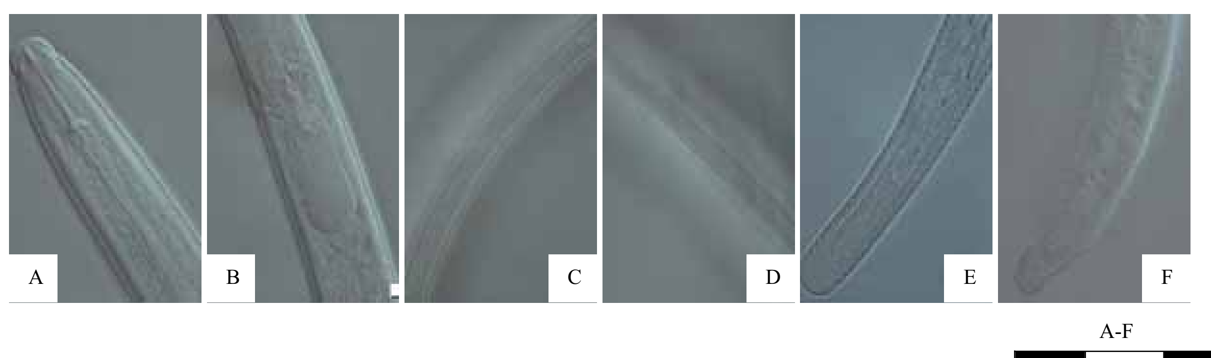

Figure 4: Photomicrographs of *Tylenchorhynchus* and *Quinisulcius* species associated with coffee from Ethiopia. A: Slender stylet with well developed knobs inclined posteriorly and DGO opening. B: Oval shaped median bulb. C: Lateral fields with 4 lines of *Tylenchorhynchus. agri.* D: Lateral fields with 5 lines of *Quinisulcius capitatus.* E: Clavate tail shape of *T. agri*. F: Conoid tail of *Q. capitatus* Scale bars A-D 40 X.

6. *Xiphinema* Cobb, 1913

The genus *Xiphinema* Cobb (1913) as revised by Loof and Luc (1990) has a total of 213 nominal species including 172 valid species, 25 synonymised species, 13 species inquirendae and 3 transferred to other genera. Species belonging to this genus can cause damage to many economically important crops by direct feeding on their roots and some by transmitting plant viruses.

The specimens were identified using the polytomous keys by Loof and Luc (1990) and Lamberti et al. (2004). The morphology and morphometric descriptions were compared with previous descriptions of the same species from different parts of the world (Coomans, 1964; Loof and Maas, 1972; Luc and Southey, 1980).

Several populations of *Xiphinema* belonging probably to four different species were found. Two of them are *Xiphinema insigne* and *X. basilgoodeyi* whose morphological and morphometric characters are discussed and compared with report in the literature (Loof and Maas, 1972; Luc and Southey, 1980; Loof and Luc, 1990). Both species are reported for the first time from Ethiopia.

In the survey two species of the *X. americanum*-group were found. *X. americanum* group is considered to be a complex of more than 51 species, many of them difficult to identify (Lamberti et al., 2000). Species of this group are characterized by a small body, vulva position between 50 and 56%, short tail, and bacteria like inclusions in the genital tract.

6.1 *Xiphinema insigne (*Loof, 1949)

The body habitus is an open C shape with an average length of 2.51 mm. It has a rounded shape head and lip region is separated by a weak depression from the head. The odontostyle is robust, odontophore is flange and guide ring is typical of the genus. The cuticle is smooth, with two layers. Vulva is situated at about 34% of the body length. The genital tracts are amphidelphic and reflexed and has a well developed anterior genital tract but small in size. The Z-organ is absent. Tail is long, conoid, ventrally arcuate and the tail tip is sharply curved with two lateral pores. Juveniles have the same body habitus and tails as an adult female (figure 5). Males not found

The Ethiopian *X. insigne* population is in line with populations described from different parts of the world (Loof and Maas, 1972; Luc and Southey, 1980; Loof and Luc, 1990). This species has a worldwide distribution and has been originally reported from the soil around the roots of soursop, coconut and grasses from Kurengala, Ceylon. *X. insigne* is a highly variable species with different forms. These forms are regarded primarily as an expression of geographical variance. Tarjan and Luc (1963) reported great variation in tail length of *X. insigne*. The Ethiopian *X. insigne* population has a longer tail length compared with other descriptions. The only population comparable in tail length with the Ethiopian population is that from Malawi (table 8). This is the first report of this species from Ethiopia. It was found in 13 sampling locations at Mechara, Agaro, Jimma-Seka, Bonga Wusush, Chena, Temenja Yaje, Mizan, Tepi, Gore, Metu, Areka and Aleta Wondo.

6.2 *Xiphinema basilgoodeyi* (Coomans, 1965)

The cuticle is with two layers. The lip region is continuous with the body. The oesophago-intestine junction consists of a small conically rounded structure. Female gonads are didelphic and reflexed. Tail is ventrally curved with small peg of 9.3 µm (8.5–10) long (figure 5). Juveniles have similar body habitus and tails as in adult female. Males not found.

All morphometric measurements coincide with the original descriptions with the exception of the high b ratio. Even though there are some reports of the presence of *X. basilgoodeyi* in coffee (James, 1983), morphometric description of the species except for the original description by Coomans (1964) were not found. This species has been originally reported from the soil around the roots of coffee from Congo. This species was only found in one sample site at Gore Masha in a semi-forest coffee plantation type. This is the first report of this species from Ethiopia.

6.3 *Xiphinema americanum* 'a'

The body is ventrally curved at the posterior than in the anterior part. The lip region is continuous and cylindrical. The odontostyle, odontophore and guide are typical for the *X. americanum* group. Female reproductive system is amphedelphic and both branches equally developed. Ovaries are filled with bacteria. Tail has a conoid terminus and bluntly round (figure 5). Specimens of this species were collected in one sampling location at Bedele and are the first report from Ethiopia.

The morphometric measurements were*:* Female (n =5): L = 2-2.2 mm; odontostyl = 97.6-100 µm; vulva position = 52.2-56; tail length = 21.7-23. Juveniles (n =2): L = 364 µm; total spear length = 24 µm.

6.4 *Xiphinema americanum* 'b''

This species can be distinguished from the other species of the *X. americanum*-group by its short odontostyle and conoid tail shape. Specimens of this species were collected in two sampling locations at Aposto and Wendogenet and are the first report from Ethiopia.

The morphometric measurements were*:* female (n =3): L = 1.6-1.8 mm; odontostyl = 76-89 μm; vulva position = 49.8-54; tail length = 20-21. Juveniles and males were not found. For both *X. americanum*-group populations, the species identity requires confirmation and detailed descriptions. In this survey only a small number of specimens were recovered which makes the species identification difficult.

Table 7: Comparison of morphometric characteristics of *Xiphinema basilgoodeyi* from Ethiopia with the type population. All measurements are in μm unless stated.

Characters	After Coomans, 1964	Specimens from Ethiopia (n = 9)
L (mm)	2.93 (2.53 –3.3)	3.01 (2.68 – 3.47)
a	53.0 (46 – 59)	48.15 (40.69 – 53.52)
b	6.9 (6.0 – 7.7)	13.81 (8.03 – 16.57)
V	47.5 (43.5 – 50)	46.7 (42.1 – 52.6)
Stylet	202 (185 – 220)	207.96 (216 – 191.2)
Odontostyl	128.5 (116 – 138)	128.33 (125 – 132)
Basal portion of the stylet	73.7 (66 – 82)	79.22 (76 – 83)
Thickness of body cuticle	3 (2.5 – 3.5)	2.9 (2.3 – 3.7)

Table 8: Comparison of morphometric characteristics of *Xiphinema insigne* females from Ethiopia with the type population and other descriptions from different regions. All measurements are in μm unless stated.

Populations	n	L (mm)	a	Tail length	c	c´	V	odontostyl	odontophore	Total stylet
Type population										
. Lecto type	4	2.24	55	107	20	4.8	30	102	59	161
. Loos paralecto		2.02	–	101	20	–	29.0	98	62	160
. Florida paralecto		2.20	50	84	26	3.8	29.6	95	59	154
. Riverside paralecto		2.32	66	109	21	5.1	32.4	94	62	156
Ethiopia (Coffee)	15	2.48 (2.34 – 2.70)	55.79 (50.53 – 62.50)	148.49 (n=7 (132.6 – 165.6)	16.98 (16.26 – 18.39)	7.03 (6.23 – 7.52)	34.37 (32.45 – 38.31)	111.18 (n=7) (108.5 – 114.0	60.3 (n=7) (56.3 – 64.7)	171.48 (n=7) (166.7 – 176.3)
Sri Lanka	2	1.98 (1.99)	42 (44)	123 (132)	16 (25)	5.6 (6.0)	32 (30.7)	103 (104)	54 (57)	157 (161)
Japan	6	2.74 (2.58 – 2.94)	68.4 (56.1 – 74.7)	127 (126 - 128)	22 (21 - 23)	5.0 (4.9 – 5.1)	35.4 (33.8 – 36.9)	103 (101 – 104)	68 (65 – 71)	171 – (167 – 175)
U.S.A. Raleigh. N.C	6	2.51 (2.34 – 2.83)	56.9 (49.2 – 63.6)	120.8 (100.7 – 138.6)	20.9 (18.9 – 24.4)	5.1 (4.3 – 5.6)	34.3 (33.3 – 36.4)	105 (100 – 110)	62.4 (58.4 – 67.1)	167.5(160 – 177.1)
U.S.A. Frenso. Ca	11	2.86 ± 0.14 (2.65 – 3.08)	67.8 ± 3.9 (59.6 – 72.7)	128.5 ± 5.4 (118.6 – 137.9)	22.3 ± 1.5 (19.2 – 24.0)	5.1 ± 0.3 (4.7 – 5.6)	35.9 ± 1.7 (33.5 – 39.7)	104.1 ± 2.9 (97.1 – 107.9)	63.5 ± 4.1 (52.9 – 66.4)	167.6 ± 5.3 (151.1 – 174.3)
Philippines	20	2.68 ± 0.15 (2.44 – 3.09)	59.4 ± 4.5 (50.4 – 67.9)	123 (n=4) (113 - 140)	18.4 (n=4) (16.4 – 20.0)	5.7 ± 0.5 (4.9 – 6.5)	34.6 ± 1.2 (32.3 – 37.1)	93 ± 2.5 (89 – 96)	62 ± 2.6 (56 – 66)	155 ± 2.3 (152 – 160)
Mauritius (sugar-cane)	7	2.52 (2.30 – 2.65)	60.2 (55.7 – 64.6)	123 (108 – 131)	21 (18– 24)	5.3 (4.5 – 6.4)	33.6 (31.7 – 34.8)	94 (92 – 97)	59 (55 – 62)	154 (152 – 157)
Malawi	9	2.68 (2.42 – 2.89)	68 (62 – 74)	148 (125 – 170)	18 (16 – 19)	6.4 (n=8) (6.0 – 7.2)	38 (36 – 41)	98 (n=8)(95 – 102)	65 (62 – 67)	163(n=8) (157 – 169)

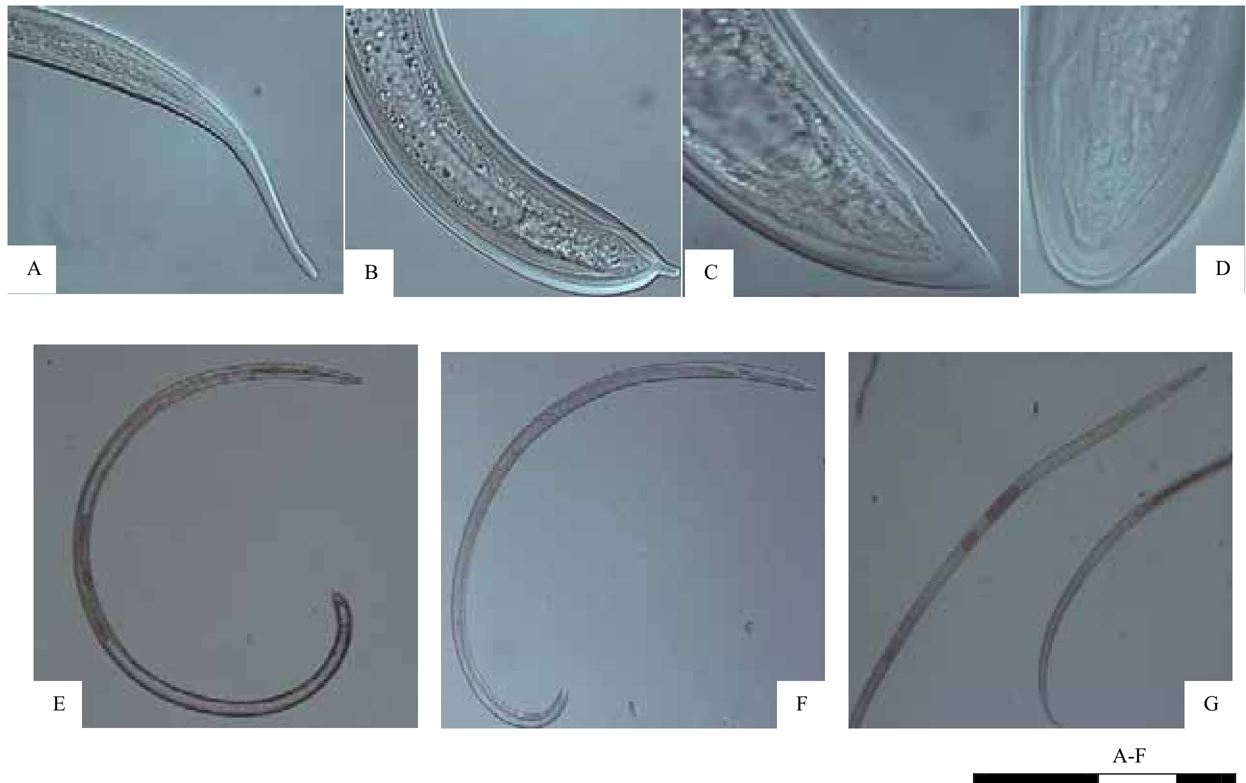

Figure 5: Photomicrographs of X*iphinema* spp. associated with coffee from Ethiopia. A: Ventrally arcuate long conoid tail of *X. insigne.* B: Ventrally curved tail of *X. basilgoodeyi* with small peg. C: Dorsally convex tail and bluntly rounded tail of *X. americanum* 'a'. D: *X. americanum* 'b'. E - G: Body habitus of *Xiphinema* species. Scale bars A-C 25X and D-F 10X.

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CHAPTER 3
Studies on the natural communities of endophytic fungi in coffee (*Coffea arabica* L) from Ethiopia and their antagonistic potential towards *Meloidogyne incognita*

1 Introduction

Fungal endophytes are natural inhabitants of the plant system. They can be separated in three major groups: 1) specialized fungal endophytes of grasses, belonging to the family Claviciptaceae within the phylum Ascomycota, 2) arbuscular mycorrhizal fungi (AMF) belonging the order of Glomales within the phylum Ascomycota and 3) fungal endophytes comprised of mostly hyphomyceteous fungi that have a saprophytic stage in the rhizosphere (Morgan et al., 1990; Glenn, 1996; Niere, 2001; Reimann, 2005; Dubois et al., 2006).

Endophytic fungi have been reported from many plants world-wide such as rice (Fisher and Petrini, 1992), maize (Fisher et al., 1992), tomato (Hallmann and Sikora, 1994), grasses and woody plants (Clay, 1990; Redlin and Carris, 1996), banana (Brown et al., 1998; Pereira et al., 1999; Pocasangre et al., 1999; Dubois et al., 2006), wheat (Marshall, 1999), pharmaceutical plants (Yaojian et al., 2001), cacao (Rubini et al., 2005) and coffee (Santamaría and Bayman, 2005; Vega, 2006).

Isolation of endophytic fungi requires complete elimination of surface colonizers. Therefore, the surface disinfestation procedure becomes a critical step in working with saprophytic endophytic fungi. Surface disinfestation is commonly done with aqueous solutions of hypochlorite, ethanol, hydrogen peroxide, mercuric chloride or formaldehyde. The choice of disinfectant concentration and incubation time are dictated by the thickness of the plant tissue, the relative permeability of its surface, and the texture of its surface (Quadt-Hallmann et al., 1997; Hallmann et al., 2006).

Fungal endophytes are known to produce bioactive products that may play important

ecological and biological roles in the host plant. In mutualistic associations, endophyte infected plants are protected from attack by specific insects, nematodes and fungi, while in return the endophyte gains shelter and nutrition from the host plant. Endophytic fungi may provide additional benefit to the host plant such as growth promotion via secretion of phytohormones or mobilization of nutrients (Schulz, 2006; Sikora et al., 2007).

Fungal endophytes also employ several modes of action against pests and diseases: parasitism, competition, antibiosis, indirect effects through plant growth promotion and induced plant resistance (Cayrol et al., 1989; Schuster et al., 1995; Hallmann and Sikora, 1996; Anke and Sterner, 1997; Pocasangre et al., 2000; Nitao et al., 2001; Sharon et al., 2001; Sikora et al., 2003; Niere et al., 2004; Dubois et al., 2006).

The use of fungal culture filtrates to demonstrate toxicity against plant-parasitic nematodes can help to rapidly identify strains with biocontrol potential (Nitao et al., 1999). The main method by which nematodes are inhibited *in vitro* is through antibiosis. Antibiosis is the antagonism resulting from the production of secondary metabolites by one microorganism that is toxic to another microorganism (Alabouvette and Lemanceau, 1999). Various endophytic fungi have been shown to produce filtrates toxic to plant-parasitic nematodes *in vitro* (Ciancio, 1995; Hallmann and Sikora, 1996; Pocasangre, 2000; Meyer et al., 2004; Nitao et al., 2001; Niere, 2001; Niere et al., 2004; Dababat, 2007).

Results from previous studies suggested a wide range of endophytic fungi in tropical regions. For example, the biodiversity and occurrence of endophytic fungi for the biological control of nematodes, insects and fungal pathogens have been investigated in major food crops such as maize, tomato and banana (Hallmann and Sikora, 1994; Kimenju, 1998; Griesbach, 1999; Niere et al., 1999).

The genus *Fusarium* and *Trichoderma* seems to be the most frequent and effective towards plant-parasitic nematodes (zum Felde et al., 2005; Sikora et al., 2007). *Fusarium* is a cosmopolitan fungus. The species *F. oxysporum* is well studied among

the communities of soil-borne fungi and has some pathogenic and many non-pathogenic strains. The pathogenic strains can cause root-rots and when they invade the vascular system are responsible for wilt on many plant species of economic importance. The non-pathogenic forms of which some occur as endophytes play an important role in soil microbial ecology, especially in soil suppressiveness (Pocasangre, 2000; Alabouvette et al., 2001; Niere, 2001; zum Felde et al., 2005; Sikora et al., 2007).

Genetic diversity exists between and within pathogenic and non-pathogenic populations of *F. oxysporum*, and various methods have been used to detect such variation. The amplification of variable ribosomal DNA (rDNA) regions allows for discrimination of the genus and species and intraspecific level (Edel et al., 1995). Of particular importance is the intergenic spacer (IGS) region which shows considerable divergence within closely related species (Appel and Gordon, 1994; 1995). The use of PCR amplified rDNA regions as a substrate for restriction fragment length polymorphism (RFLP's) is one of the widely used methods use to evaluate genetic diversity in *F. oxysporum* populations. Several researchers have also reported genetic diversity of endophytic *F. oxysporum* from various plants based on analysis of IGS region.

Studies on endophytic fungal communities of coffee are recent, but practical aspects have been already evaluated in some coffee growing regions of South America. A survey for fungal endophytes in various coffee tissues was conducted in Colombia, Hawaii, Mexico, and Puerto Rico. More than 700 endophytic fungal isolates have been identified and the most common genera were *Colletotrichum, Fusarium, Penicillium,* and *Xylaria*. Various genera containing fungal entomopathogens were also isolated, including *Acremonium*, *Beauveria*, *Cladosporium, Clonostachys* and *Paecilomyces* (Santamaría and Bayman, 2005; Vega, 2006).

In Ethiopia, the importance of mutualistic endophytic fungi as part of microbial biodiversity and their use for biological control has not been researched. The association between fungal endophytes and coffee plants has not been described. The use of these fungi in IPM programs is of great potential. Therefore, the objective of this

study was to determine the endophytic fungal populations of coffee roots and evaluate their potential in a biocontrol program of nematodes. In our research we selected the root-knot nematode *M. incognita* as a model organism as it is one of the most widely distributed and economically important plant-parasitic nematode in tropical agriculture and an important parasite of coffee world wide. The major objectives of the research were:

1. Isolation and identification of fungal endophytes from major coffee growing regions of Ethiopia
2. *In vitro* screening of fungal endophytes for nematode inactivating potential
3. *In vivo* studies on colonization and plant growth promotion with promising fungal isolates under greenhouse conditions and
4. Morphological and molecular characterization of *Fusarium* isolates

2 Materials and methods

2.1 Sample collection

Coffee roots were collected from 60 representative sampling sites in the different coffee growing agroecologies of Ethiopia during the wet season (August 2004) and dry season (April 2006), respectively. Selection was done based on coffee plantation type and their total area. Four samples were taken from state farms, 12 samples from forest plantations and 14 from semi forest plantation type and 30 from garden plantation according to the total area coverage of each plantation type. At every site 5–6 random sampling points around each coffee tree were selected. At each sampling point between 2–3 g of coffee roots were collected. Root samples were kept in a refrigerator ($4^{\circ}C$) until isolation of endophytic fungi was done at the Institute of Plant Diseases, University of Bonn, Germany.

2.2 General media and reagents used

The following media and reagents were used:

PDA

24 g Potato Dextrose Broth (PDB) (Difco)

18 g Agar

1 l Sterilized distilled water

150 ppm streptomycin and 150 ppm of penicillin

1/10 strength PDA

2.4 g PDB

30 g Agar

1 l sterilized distilled water

150 ppm streptomycin and 150 ppm of penicillin

Nutrient broth

100 ml distilled water and 0.8 g nutrient broth

2.3 Isolation of endophytic fungi

To determine the optimum NaOCl concentration for disinfestation, 1%, 1.5%, 2% and 2.5% concentrations were tested on three coffee root samples. Roots were incubated in the NaOCl solution for three minutes and then washed three times in sterile water. To confirm successful surface disinfection, the roots were pressed onto 10% PDA. In addition aliquots of the final rinse were plated onto the same medium. Plates were examined for fungal growth after incubation at 25°C for 10 days. Only if no growth occurred in both forms of surface disinfection was the concentration considered successful.

For isolation of endophytic fungi, between 10-15 coffee root pieces were cut into pieces (0.5–1 cm long), and surface disinfested in 2.5% (w/v) NaOCl for 3 min and rinsed 3 times in sterile water. Root pieces were then placed on 10% PDA. To avoid bacterial contamination, the PDA was supplemented with 150 ppm Streptomycin and 150 ppm Penicillin. The roots were then incubated at 25°C in the dark and checked for fungal growth for 15 days. Mycelia growing out of the roots were transferred to new PDA plates and pure fungal cultures were maintained for further use.

2.4 Culturing and storage of endophytic fungi

Single colonies were cultured on PDA in Petri-dishes at 25°C in the dark. The fungi were stored in test tube slants with PDA at 4°C for short term use (till 8 months). For permanent storage, the fungi were stored at - 80°C in Cryobank storage vials. (CRYOBANK™,MASTE group Ltd., Merseyside, UK).

2.5 Nematode inoculum

In all experiments the root-knot nematode *M. incognita* was used as a model nematode because of its economic importance in coffee and in tropical agriculture in general. *M. incognita* was multiplied on tomato plants cv. ‘Furore’ and nematode inoculum was extracted from 7-8 weeks old tomato plants. The plants were uprooted and nematode eggs were extracted using the technique described by Hussey and Baker (1973). Galled tomato roots were washed with tap water to remove debris. The roots were then cut into

1-2 cm pieces and macerated in a blender for 20 s. The macerated roots were filled in a 1 L bottle containing 600 ml of a 1.5% NaOCl solution and manually shaken for three minutes to free the eggs from the gelatinous egg masses. The egg suspension was then rinsed over a sieve combination of 200, 100, 45 and 25 µm mesh size to remove debris and excess NaOCl. The eggs on the 25 µm sieve were collected in a beaker and agitated for 10-12 days at room temperature in a dark place using an aquarium pump to facilitate hatching. Freshly hatched second stage juveniles were collected by a modified Baermann dish technique. The juveniles suspension was adjusted to 1000 J2/ ml of water and used as inoculum for the experiments.

2.6 Effect of fungal culture filtrates towards *Meloidogyne incognita*

A total of 30 fungal isolates were tested in 4 sets of *in vitro* experiments to determine the effect of fungal culture filtrates towards *M. incognita* juveniles. Erlenmeyer flasks containing 100 ml PDB were each inoculated with 5 to 8 mycelia plugs of the different isolates and incubated at 25°C for 7-10 days in darkness on a rotary shaker at 120 rpm. Fungal hyphae were separated from the media by centrifugation at 4000 rpm for 20 min. The supernatant was then filtered successively through 5 µm and 0.2 µm pore size cellulose triacetate filters (Millex-SV, Millipore cooperation, Bedford, USA) and the filtrate was collected in sterilized flasks. The effect of culture filtrates on the inactivity of infective juveniles (J2's) was tested in 12-well tissue culture plates. In each well, three concentrations, 25, 50 and 90% of the original stock filtrates were prepared by diluting it with a solution containing 200 *M. incognita* juveniles and 200ppm Streptomycin sulphate solution to prevent growth of microbes. Juveniles kept in sterile filtered PDB or sterile distilled water served as controls. Each treatment was replicated 6 times and wells were kept in an incubator at 24°C. After 24 h of incubation, the number of inactive/ immobile juveniles was counted. Differences in juvenile inactivity/ immobility between test and control solutions were analyzed with one way ANOVA followed by Dunnett's test to compare each treatment against the media and water controls (SPSS 14.0 for windows, 2005).

2.7 *In vitro* egg pathogenicity of endophytic fungi towards *Meloidogyne incognita and Meloidogyne exigua*

Egg masses of *M. incognita* were hand picked from galled tomato roots, disinfected in 0.5% NaOCl solution for 30s, rinsed three times in sterile distilled water, and then added to PDA plates with sporulating cultures of the different endophytic isolates. After 24 h, 20 egg masses were transferred again to 10% PDA (4 egg masses per Petri dish) and incubated at 25°C for 2 weeks. Egg masses unexposed to the endophytes were served as a control. The number of egg masses exhibiting dense fungal colony of the respective isolate was counted and the percentage of egg mass infection was then calculated. Egg pathogenicity were determined following staining with Rose Bengal (0.5 g Rose Bengal /100 ml of 5% ethanol) and examining 100 arbitrary selected eggs with a compound microscope (10–100 x) (Viaene and Abanoi, 2000).

Populations of *M. exigua* were obtained from Laboratório de Controle Biológico de Fitonematóides, Universidade Federal de Vicosa, Brazil. *M. exigua* were multiplied on coffee seedlings and pepper plants to get sufficient inoculum for experimentation. Egg mass collection and egg pathogenicity test was done as described above for *M. incognita*

2.8 Egg pathogenicity in soil

A four percent alginate solution was prepared by mixing 4 g sodium alginate per 100 ml distilled water and blended for 5-10s. The solution was heated to approximately 80°C in a microwave oven and kept for 3–4 h at room temperature till a clear solution free of bubbles was obtained. The solution was mixed with an equal amount of *M. incognita* egg suspension to bring the final alginate concentration of 2% (Rodriguez-Kabana et al., 1994).

Fiberglass screens coated with polyvinyl chloride with a 1.5 mm^2 mesh were used as a carrier to deliver the eggs into the soil. The screens were cut into 2.5 x 5 cm pieces and immersed in 95% ethanol for 24 h to remove oil and stiffen them. The clean screen was then immersed in the alginate egg suspension with forceps, lifted off, and excess liquid

was allowed to run off. The screen was then pulled through two glass pipette rods with distance holder of approximately 1 mm to produce a film of uniform thickness and immediately dipped into 0.25 M $CaCl_2$ to facilitate polymerization of the alginate.

Thickened alginate films containing *M. incognita* eggs were buried vertically in a 4.5 cm sized pots filled with a sterilized sand inoculated with a spore suspension of $1x10^5$ g^{-1} soil. Twelve days after fungal inoculation, films were picked from the soil and washed gently with tap water to remove soil. The percentage of eggs parasitized by the fungus was determined from 100 randomly selected eggs under a compound microscope (20-100 x).

2.9 *In vivo* effect of endophytic isolates against M*eloidogyne incognita* on tomato

Nine endophytic fungal isolates were selected based on their nematicidal and pathogenic ability in the previous *in vitro* experiments. Three replicated greenhouse experiments were conducted to select the endophytes effective under semi-field conditions.

All endophytic isolates were cultured on PDA containing 150 ppm streptomycin and 150 ppm of chloramphenicol in Petri dishes in an incubator at 25°C for 2 weeks. The mycelia and conidia formed were carefully scraped from the media and suspended in tap water. Spores were separated from mycelia by sieving through cheese cloth. The spore suspension was then adjusted to the desired concentration by counting spore density using a Thoma Haemocytometer.

Tomato seeds of cultivar Hellfrucht Frühstamm were sown in 70-plug commercial seedling trays. Seedlings were propagated for 3 weeks in the greenhouse, before being transplanted into 500 ml pots of a sterile sand/soil mixture (2:1, v/v). One week after transplanting, seedlings were inoculated with a fungal spore concentration of 5×10^6 g^{-1} of soil of the respective isolates. The inoculum was delivered in 3 holes 2 cm deep made by a`plastic rod around the stem base. Control plants received an equal amount of tap water. Five days after fungal inoculation, pots were inoculated with 600 J2's of *M.*

incognita in 3 ml of water as described for the fungi. Plants were grown in the greenhouse at 22 ± 2 ºC with 18 h of suplemental artificial light per day, irrigated as needed, and fertilized weekly with Polycrisol (N: P: K =14:10:14) at 2 g l^{-1}.

The experiment consisted of a non-inoculated control, a control inoculated with only *M. incognita* (J2's) and the treatments with nine different fungal endophytes with *M. incognita*. Each treatment was replicated six times. The experiment was terminated 8 weeks after nematode inoculation and fresh shoot and root weight as well as number of egg masses were recorded. For the latter, roots were removed from the soil, washed free of soil particles and stained in 0.015% Phloxine B for 20 min to facilitate egg masses counting (Shurtleff and Averre III, 2000).

2.10 Determination of fungal colonization

The level of endophytic colonization of the nine selected endophytic fungi was examined under greenhouse conditions. Tomato plants were raised for 4 weeks as described in section 2.9. Each treatment consisted of six replicates. Re-isolation of the inoculated fungi was accomplished by root surface sterilization with 1.5% NaOCl for three minutes, followed by three rinses in sterilized water. A sterility check was carried out by pressing each root section onto PDA. Root sections of approximately 0.5 cm length were then placed onto PDA containing 150 ppm streptomycin and 150 ppm chloramphenicol. Successful re-isolation was confirmed using different phenotypical characteristics of the respective endophytic fungi. From each root of the different treatments, 9 segments were selected randomly and 3 segments each were plated on 3 plates of PDA. Petri dishes were incubated for 10 days in an incubator at 25°C and percentage endophytic colonization was determined for each isolate as well as the controls.

2.11 Effect of *Fusarium* and *Trichoderma* isolates on nematode penetration

The effect of 2 *Fusarium* and 1 *Trichoderma* isolates on the penetration of *M. incognita* into tomato roots was investigated under greenhouse conditions. The isolates were selected out of 9 isolates in a preliminary screening (see section 2.9). The experiment

consisted of a non-inoculated control, a *M. incognita* inoculated control and the three treatments with the isolates with *M. incognita*. Each treatment was replicated six times. The tests were designed and treated as described in section 2.9.

The experiment was terminated 15 days after nematode inoculation when nematode penetration was measured. The roots were then submerged in a staining solution containing Fuchsine acid and then heated in a microwave for 2 min to stain the nematodes inside the roots. The roots were then washed with tap water to free them of excessive stain and afterwards macerated with an Ultra Turrax® T25 for 1 minute. The macerated roots were then diluted in 100 ml tap water and mixed thoroughly and 10 ml aliquots were examined for nematodes in a counting dish using a binocular microscope

2.12 Morphological characterization of *Fusarium* isolates

For good macroconidia production, Carnation leaves technique is used in which young banana leaves were harvested from actively growing parts and were dried in an oven at 55°C for overnight and cut into pieces (Fisher et al., 1982). Several leaf pieces were put on SNA medium and single spores of 20 isolates were plated and incubated at 25°C under near-ultraviolet light. The plates were examined after 7 days or until sporulation occurred. Only conidiophores were examined.

2.13 Molecular characterization of *Fusarium* isolates

2.13.1 DNA extraction:

Fusarium cultures were started by transferring fungal spores from the original cultures stored at -80°C into 50 ml of PDB. The cultures were grown at 25°C for 7 days on a rotary shaker at 121 rpm in the dark. Mycelia from the cultures were harvested by filtration through Miracloth (Calbiochem®, EMD Bioscience, Inc. La Jolla, CA) and dried in freeze-drier overnight. Approximately 5 g of freeze-dried mycelia was placed in a test tube and crashed to powder in liquid nitrogen. Genomic DNA was isolated using a high pure PCR template preparation kit according to the manufacturer's recommendation (ROCHE, Cat. 11 796 828 001).

2.13.2 PCR amplification of IGS and ITS regions

IGS – PCR: A fragment of the IGS was amplified by polymerase chain reaction (PCR) with oligonucleotide primers PNFO (5' CCCGCCTGGCTGCGTCCGACTC-3') and PN22 (5' CAAGCATATGACTACTGGC -3'). Amplifications were performed in volumes of 50 µl containing: 2 µl DNA template and 48 µl of PCR mix (28.75 µl of distilled H2O, 10 of Go taq 3µl of 5xRB MgCl2 (25 µM), 4 µl of DNTP 2.5 mM, 0.25 of Go taq (Promega) DNA and 0.1 of each primer PNFO and PN 22).

PCR reactions were performed in an Whatman Biometra, Eppendorf, PCR machine set at 45 cycles of denaturation at 95°C for 90 s, followed by primer annealing at 52°C for 60 s and extension at 72°C for 90 s. DNA of a known *F. oxysporum* (FO 162) isolate was included as a positive control. The PCR products were run on 2% agarose gel for 90 min and stained for 30 min in ethidium bromide and visualized under UV light. A 1kb DNA marker (Promega) was used to determine the size of the PCR products.

IGS – RFLP: 20 µl of PCR products were digested for 3 h at 37°C with 5 µl of IGS restriction enzyme mix (2.5 µl RE buffer 10X, 0.25 µl BSA, 0.5 µl respective restriction enzymes and 1.75 µl distilled H_2O). Restriction enzymes used were: *HinfI, HindIII, XhoI* and *HpaI* (Promega, USA). The restriction fragment length polymorphisms (RFLP) were separated by electrophoresis in 1% TAE buffer using 2% agarose gels. Electrophoresis was carried out at 80 v for 90 min and stained in Ethidium Bromide solution in the dark (200 ml distilled water plus 20 µl ethidium Bromide).

ITS – PCR: Nine isolates which were similar to the positive control of FO 162 in the IGS – RFLP test were further tested for their similarity using ITS – RFLP. The primer's ITS 1 (5'TCCGGTGAACCTGCGG- 3') and ITS 4 (5'- CCTTGGTCCGTGTTTCAAGACGGG 3') were used to amplify a fragment of the rDNA. Primers were synthesized by Promega, USA. PCR amplification were performed in a total volume of 50 µl by mixing 2 µl of the template DNA with 13.75 µl distilled H_2O, 5 µl Go taq 5 x RB, 1.5 µl $MgCl_2$ (25 mu), 2 µl NTP 2.5 mM, 0.25 µl each ITS 1 and ITS and 0.125 µl of Go taq. PCR reactions were performed in an Eppendrof Whatman Biometra PCR machine with an

initial denaturation of 3 min at 95°C followed by 33 cycles of primer annealing at 55°C for 60 s and extension at 72°C for 60 s. DNA of FO 162 isolates was included as a positive control. The PCR products were run on 2% agarose gel for 90 min and stained for 30 min in ethidium bromide and visualized under UV light. A 1kb DNA marker was used to determine the size of the PCR products.

ITS – RFLP: 20 µl of PCR products were digested for 3 h at 37°C with 5 µl of ITS restriction enzyme mix (2.5 µl RE buffer 10X, 0.25 µl BSA, 0.5 µl of respective restriction enzymes and 1.75 µl distilled H_2O). Restriction enzymes used were: *HaeIII, Hind III, HinfI, HhaI, Alu, XhoI and NotI* (Promega, USA). The restriction fragments RFLP were separated by electrophoresis in 1% TAE buffer using 2% agarose gels. Electrophoresis was carried out at 80 v for 90 min and the gel was stained in Ethidium Bromide solution in the dark.

3 Results

3.1 Isolation of endophytic fungi from coffee roots

A total of 128 fungi growing endophytically were obtained from surface disinfested coffee roots during the wet and dry seasons of sampling. Fungal isolates growing out of one root piece were considered to be identical. Therefore, only one fungal species per root piece was counted. The most frequently detected genera were *Fusarium* and *Trichoderma*. Their relative occurrence was higher during the dry season than wet season. In terms of plantation type, higher numbers of isolates were detected from garden plantations followed by forest plantations (annex 3).

During the wet season sampling a total of 78 isolates were obtained and 16 were selected for further testing. The selection was done based on the fungal capacity to produce conidia under standard growth conditions. Isolates that didn't produce conidia were discarded as they are not suitable for mass production and application. Tentative identification of the 16 isolates showed that 7 were *Fusarium*, 4 were *Trichoderma* and 5 could not be identified. In the dry season sampling 50 endophytic fungi were isolated

and 14 were selected for further testing. Preliminary identification revealed that out of the 14 isolates, 6 belonged to the genus *Fusarium*, 5 to the genus *Trichoderma* and 3 could not be identified.

Table 1: List of selected endophytic fungal isolates from coffee plantations of Ethiopia.

Isolate Id*	Sampling season	Fungus sp	Isolate Id*	Sampling season	Fungus sp
EF1	Wet	*Fusarium oxysporum*	EU3	Dry	Unidentified
EF2	Wet	*Fusarium oxysporum*	EU4	Wet	Unidentified
EF3	Dry	*Fusarium oxysporum*	EU5	Wet	Unidentified
EF4	Wet	*Fusarium oxysporum*	EU6	Dry	Unidentified
EF5	Wet	*Fusarium oxysporum*	EU7	Wet	Unidentified
EF6	Wet	*Fusarium oxysporum*	EU8	Wet	Unidentified
EF7	Dry	*Fusarium oxysporum*	ET1	Wet	*Trichoderma sp*
EF8	Dry	*Fusarium oxysporum*	ET2	Wet	*Trichoderma sp*
EF9	Dry	*Fusarium oxysporum*	ET3	Wet	*Trichoderma sp*
EF10	Dry	*Fusarium oxysporum*	ET4	Wet	*Trichoderma sp*
EF11	Dry	*Fusarium oxysporum*	ET5	Dry	*Trichoderma sp*
EF12	Wet	*Fusarium oxysporum*	ET6	Dry	*Trichoderma sp*
EF13	Wet	*Fusarium oxysporum*	ET7	Wet	*Trichoderma sp*
EU1	Dry	Unidentified	ET8	Dry	*Trichoderma sp*
EU2	Dry	Unidentified	ET9	Dry	*Trichoderma sp*

* EF= Ethiopian *Fusarium*, ET= Ethiopian *Trichoderma*, EU= isolates which were not identified.

3.2 Effect of fungal culture filtrates towards *Meloidogyne incognita*

Nematicidal effects of culture filtrates on *M. incognita* juveniles were observed after 24 h of incubation. Inactive/ immobile nematodes appeared straight while active/mobile ones assumed their normal sigmoid shape and exhibited slight movement. The fungal isolates differed significantly in their ability to inactivate juveniles compared with the media control (Dunnett's test $P < 0.05$). In general, juvenile inactivity increased with increasing filtrate concentration. The percentage of inactivity differed significantly between the different fungal isolates ($P < 0.05$). The percentage of juvenile inactivity

ranged from 28% to 81% (table 2).

Trichoderma strain ET2 showed the highest level of toxicity followed by *Fusarium* strains EF10 and EF12 at metabolite concentration of 90% resulting in 81%, 75% and 70% juvenile inactivity respectively (table 2). When the culture filtrates were diluted to 25% of the original concentration, 6 isolates (EU1, EU3, EU4, ET2, EF10 and EF6) produced significant levels of juvenile inactivity that ranged from 12.2% to 31.2% compared with the media control after 24 h of exposure. Most fungal isolates were weakly effective in the 25% concentration when compared with the media control. In 50% concentration, 12 isolates (EF2, EF3, EF7, EU2, ET5, ET6, ET7, EF10, EF6, EF9, EF12 and EU7) showed significant effects on nematode inactivity ranging from 21–54% when compared with the media control after 24 h of exposure. Culture filtrates of 16 from the tested 30 strains caused high inactivity of *M. incognita* juveniles at a 90% concentration. Two isolates (EF10 and EF6), however, showed significant effects on juvenile inactivity compared with the media control at all levels of concentration (table 2).

Table 2: Effect of different endophytic fungi on inactivity (mean ± S.E) of *Meloidogyne incognita* juveniles in culture filtrates of 25%, 50% and 90% concentration after 24 h of exposure.

Isolate Id	Set 1			Isolate Id	Set 2		
	25%	50%	90%		25%	50%	90%
EF1	21.6 ± 1.8	28.6 ± 1.7	45.2 4.2*	EU3	12.2 4.9*	16 ± 6.5	21.6 ± 8.8
EU1	31.2 ± 2.7*	30.7 ± 2.5	44.3 ± 8.1*	EU4	13.5 ± 5.5*	28 ± 11.4	20.2 ± 8.2
EF2	17.8 ± 1.2	54 ± 2.1*	62.3 ± 5.7*	EU5	6 ± 2.5	11.5 ± 4.7	23 ± 9.4
EF3	17.3 ± 2.1	47.8 ± 9.5*	65.3 ± 6.6*	ET8	10 ± 4.1	20.8 ± 8.5	62.8 ± 3.9*
EU2	14.1 1.7	28.5 ± 11	23.5 ± 2.3	EU6	11 ± 3.8	11.5 ± 6.8	25.5 ± 4.3
EF4	7 ± 2.9	19 ± 7.8	24.2 ± 9.9	EF7	10.8 ± 6.8	41.8 ± 17.1*	47.3 ± 9.3*
ET1	14.2 ± 1.7	28.5 ± 8.9	16.5 ± 6.7	EU2	11.8 ± 8.1	38.5 ± 15.7*	51.8 ± 2.2*
EF9	18.8 ± 2.7	21.5 ± 2.3	25.5 ± 8.9	ET2	20 ± 8.2*	29.5 ± 12.1	81.2 ± 2.7*
Media	20 ± 3.8	29.5 ± 8.1	31.2 ± 2.7	Media	9.2 ± 2.5	26.8 ± 4.1	29.5 ± 6.7
Water	9.5 ± 3.8	17.5 ± 7.1	20.3 ± 8.3	Water	4.5 ± 1.8	17.8 ± 7.3	22.2 ± 9.1

Isolate Id	Set 3			Isolate Id	Set 4		
	25 %	50%	90%		25%	50%	90%
EF5	10.8 ± 0.9	15 ± 1.6	23.8 ± 1.5	EF9	14 ± 1.2	26.8 ± 2.1*	54.5 ± 3.3*
ET3	10.3 ± 1.1	20.2 ± 2.4	25.8 ± 1.9	EF10	15.5± 1.4*	24.2 ±2.4*	75.5± 3.4*
ET4	10.3 ± 2.9	20.2 ± 2.3	45.3 ± 2.9*	EF6	15.5± 1.1*	24.2 ±1.5*	55.5 ± 2.2*
ET5	9.8 ± 2.2	35.8 ± 3.8*	65.5 ± 2.1*	EF12	13.2 ±1.1	21.3±2.4*	69.8 ± 2.4*
EF11	8.6 ± 0.6	12.8 ± 1.1	22.2 ± 1.3	EU7	10.3±0.9	21 ± 1.5*	27.8 ± 1.2*
ET6	19.5 ± 2.2	28.5 ± 4.5*	24.8 ± 1.5	EU8	11.5 ± 1.6	18.2 ± 1.6	23.3 ± 1.7
ET7	17.3 ± 3.3	28.7 ± 4.9*	38.2 ± 3.1*	EF13	9.8 ± 0.6	16.5 ± 1.1	19 ± 1.6
Media	14.2 ± 1.1	14.2 ± 0.4	18.2 ± 1.4	Media	8.8 ± 1.1	13.2 ± 1.2	17.8 ± 1.4
Water	9.8 ± 0.5	16.± 1.6	16 ± 1.9	Water	4.2 ± 0.4	12 ± 1.1	15.3 ± 1.1

Means followed by * are significantly different from media control in the same set based on Dunnett's test $P < 0.05$.

3.3 *In vitro* egg pathogenicity of endophytic fungi towards *M. incognita* and *Meloidogyne exigua*

A total of 30 fungal isolates were tested for their pathogenicity towards *M. incognita* eggs. Of those, 9 infected >30% of *M. incognita* eggs. Isolates EF1, EF3, EF5, EF6, EF8, EF10, EF11, EU2 and EU4 showed the highest ability to infect eggs with values up to 89%. The percentage of egg pathogenicity was the highest for *Fusarium* isolates, although variability occurred between isolates. *Trichoderma* sp. (ET2), which showed the highest inactivity in the *in vitro* culture filtrate test, showed less infection compared with the other strains tested. Low infection was observed in the controls (0–7.4%), which indicates that the egg masses used were sufficiently sterilized and/ or they did not contain high levels of egg pathogenic fungi (figure 1).

Further *in vitro* tests were conducted with 9 isolates which were selected from the preliminary screening. The pathogenicity of these isolates towards *M. incognita* and *M. exigua* eggs is shown in tables 2 and 3. All of the isolates infected the eggs at a considerably higher rate than the controls. Egg pathogenicity ranged from 52 to 89%. Strains of EF5, EF10, EF11, EF1 and EF3 which belong to the genus *Fusarium* had relatively higher levels of infection reaching up to 89% as compared with the other strains mainly *Trichoderma* (tables 2, 3 and figure 1).

Similar trends in egg pathogenicity were observed towards *M.* exigua with the same fungal isolates. Up to 86% of infection was recorded with isolate EF10. Isolates belonging to the genus *Fusarium* showed significant egg infection toward *M. exigua* eggs ranging from 45 to 86% (table 4).

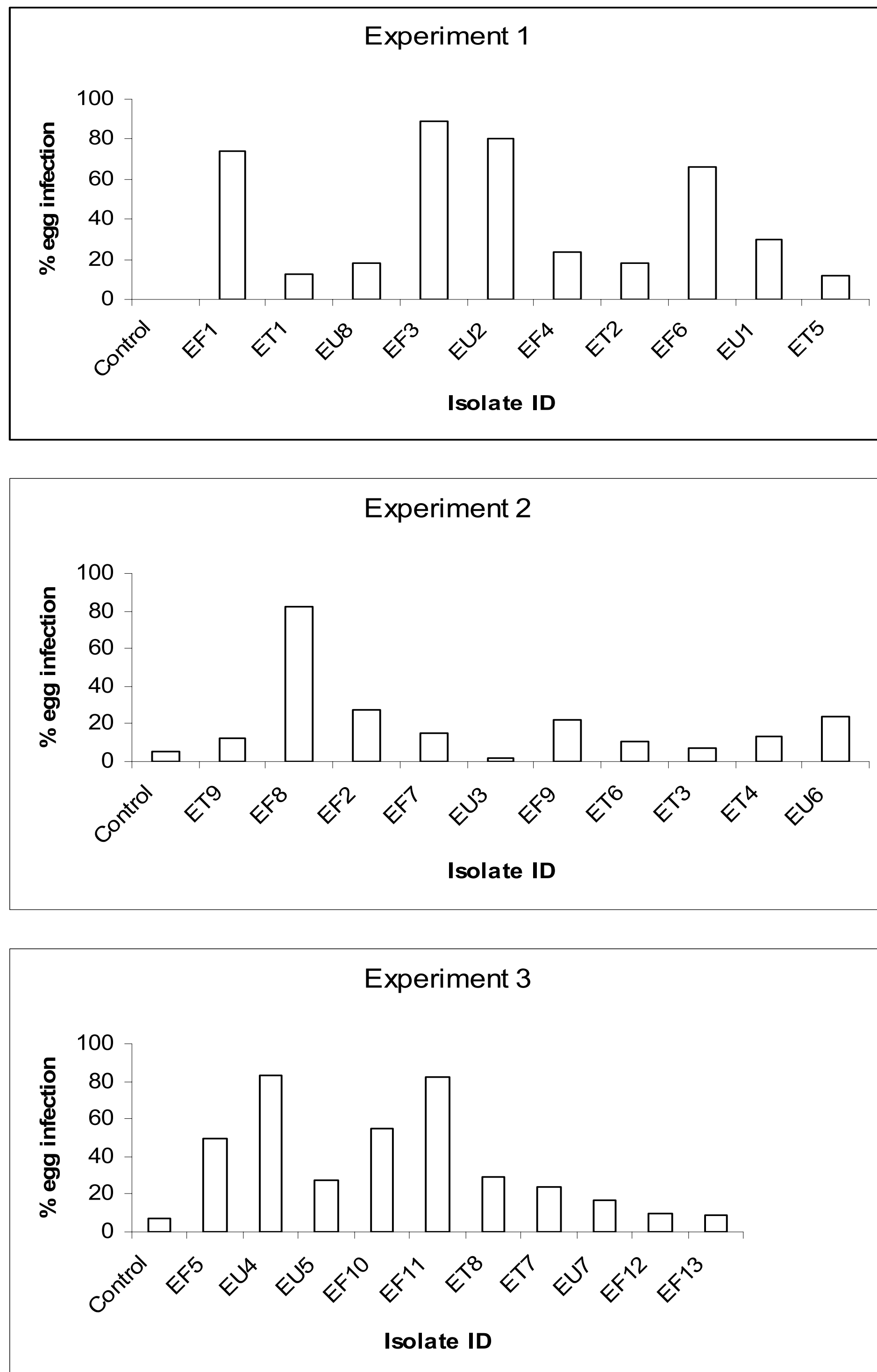

Figure 1: *In vitro* pathogenicity of *Meloidogyne incognita* eggs by different endophytic fungi 2 weeks after incubation. Figures showed results from separate experiments.

Table 3: *In vitro* infection of egg masses and eggs of *Meloidogyne incognita* by different endophytic fungi 12 days after incubation.

	Experiment I		Experiment II	
Isolate ID	Average no. of egg masses colonized (%)	% eggs infected	Average no. of egg masses colonized (%)	% eggs infected
EF5	100	89.3	86.7	82
EU4	80	56.3	93.3	68
EF10	83.3	84	88.33	60. 3
EF8	80	68.4	93.3	69. 3
EF11	96.7	86.3	93.3	82
EF1	76.7	86	81.7	85
EF3	90	86	86.7	89
EU2	73.3	66.7	100	77.7
EF6	52	43.7	43.3	56
Control	1.7	0	10	1.6

3.4 Egg pathogenicity in soil

Fungal strains varied in level of infection toward *M. incognita* eggs in soil. High infections were recorded for strains EF10 (38%), EF11 (35%) and EF5 (29%). The lowest infection was recorded for EF8 (17%) and the control in the first experiment. Experiment 2 showed similar differences between fungal isolates as in experiment 1, but at a lower level. Egg pathogenicity was highest for strain EF10 in both experiments (figure 2).

Table 4: *In vitro* colonization and pathogenicity of egg masses and eggs of *Meloidogyne exigua* by different endophytic fungi after 12 days of incubation.

	Experiment I		Experiment II	
Isolate ID	Average no. of egg masses colonized (%)	% eggs infected	Average no. of egg masses colonized (%)	% eggs infected
EF5	50	78.3	58.3	78
EU4	75	86	66.6	62.3
EF10	75	45.7	41.6	86
EF8	58.3	52.3	50	52.3
EF11	66.7	88.3	66.6	56
EF1	83.3	66.7	66.6	77.3
EF3	50	68.3	41.6	84.3
EU2	58.3	52.3	66.6	68.3
EF6	66.7	54.	75	66
Control	25	33.3	41.6	42

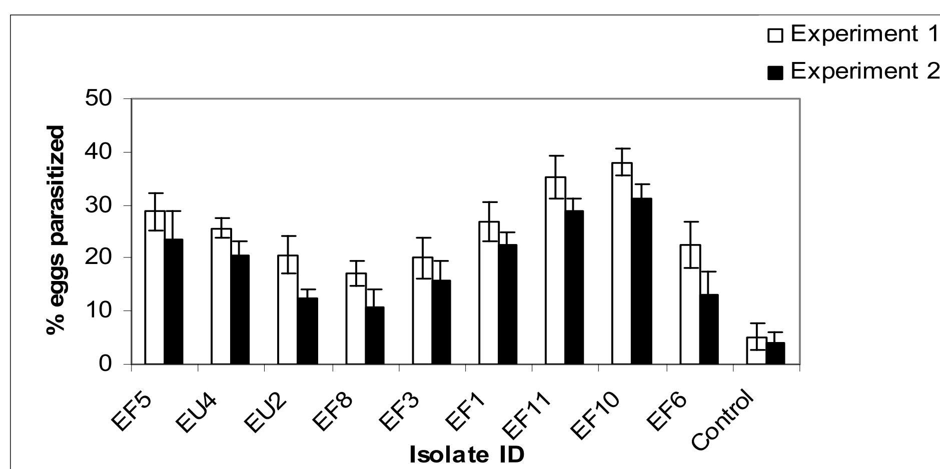

Figure 2: *In vivo* infection of *Meloidogyne incognita* eggs in soil 12 days after endophytic fungal inoculation.

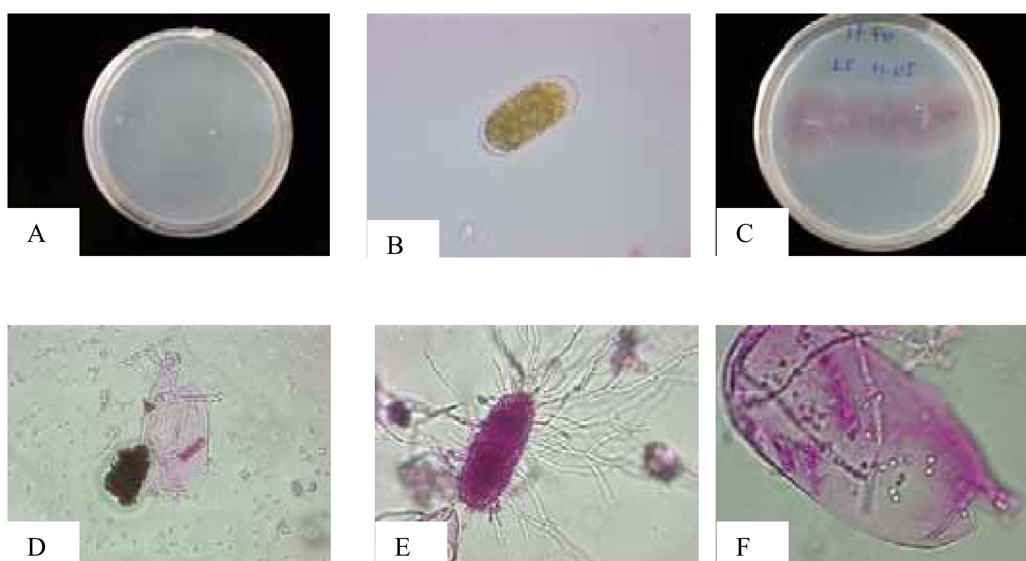

Figure 3: *In vitro* pathogenicity of *Fusarium* isolates on *Meloidogyne incognita* eggs 10 days after fungal inoculation. A & B control, C, D, E & F infected eggs masses and eggs.

3.5 *In vivo* effect of the selected endophytic isolates against *Meloidogyne incognita*

In experiment 1, five of the 9 isolates reduced (P < 0.05) the number of egg masses formed compared with the fungal free nematode control. The effective isolates were 5 strains of *Fusarium* EF5, EF9, EF1, EF10 and EF1. The rate of reduction however varied slightly among the different isolates. Isolate EF5 caused a significant reduction in number of egg masses by 36% whereas, EF9, EF1, EF10 and EF1 reduced egg mass formation by 31%, 28%, 27% and 25%, respectively (figure 4).

In experiment 2, two of the 9 isolates tested reduced the number of egg masses compared with the fungal free nematode control (P < 0.05). Isolate ET11 and EF1 showed a significant reduction in the number of egg masses of 40% and 50% compared to the control, respectively (figure 4).

In experiment 3, three of the 9 isolates tested significantly reduced the number of egg masses compared with the nematode control (P < 0.05): isolate EF10, EF9 and EF1.

The rate of reduction ranged from 17% to 22% and was lower when compared with the previous experiments. In all three experiments, isolate EF1 demonstrated significant biocontrol activity with the number of eggs killed ranging from 22% to 40% (figure 4).

Shoot and root fresh weight measurements differed significantly between fungal isolates ($P < 0.05$). However, within each experiment, no significant differences in growth were observed between fungal treatment and the fungal free nematode control treatment. Although no significant differences in root fresh weights were detected a general trend toward higher root fresh weight was observed for plants treated with the different fungal isolates compared to the fungal free nematode control plants (figure 5).

Within each experiment, nematode galling was not significantly different between the endophyte treatments and the control. The only exception was in experiment 3 ($P < 0.05$) where plants treated with fungal isolate EF9 and EF10 caused a significantly lower level of nematode galling compared to the control (data not shown).

3.6 Endophytic fungal colonization

Percent root colonization of the different fungal isolates ranged from 41% to 92%. Percent colonization of plant roots treated with the endophytic *Fusarium* isolates was statistically different from the control plants ($p< 0.05$). A low percentage of colonization was observed for *Trichoderma* isolates (figure 6). Fungal endophytes were not recovered from the control treatments indicating an absence of secondary contamination. However, an unidentified fungal species was detected in the control plants which were different from the inoculated strains.

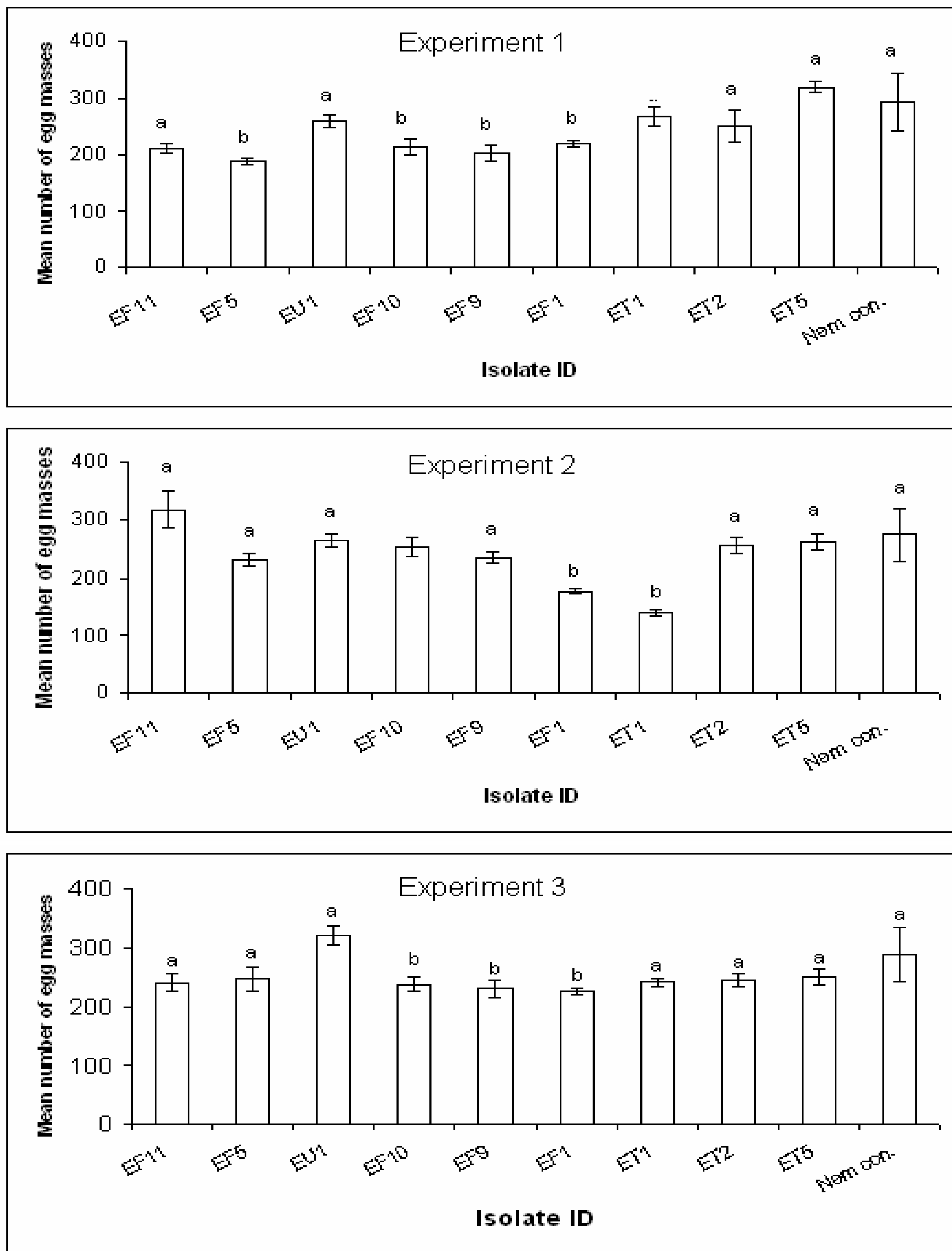

Figure 4: Influence of *Fusarium* and *Trichoderma* endophytic isolates on number of *Meloidogyne incognita* egg mass on tomato 8 weeks after fungal inoculation. Means with the same letter are not significantly different based on Dunnett's test (P< 0.05; n= 6).

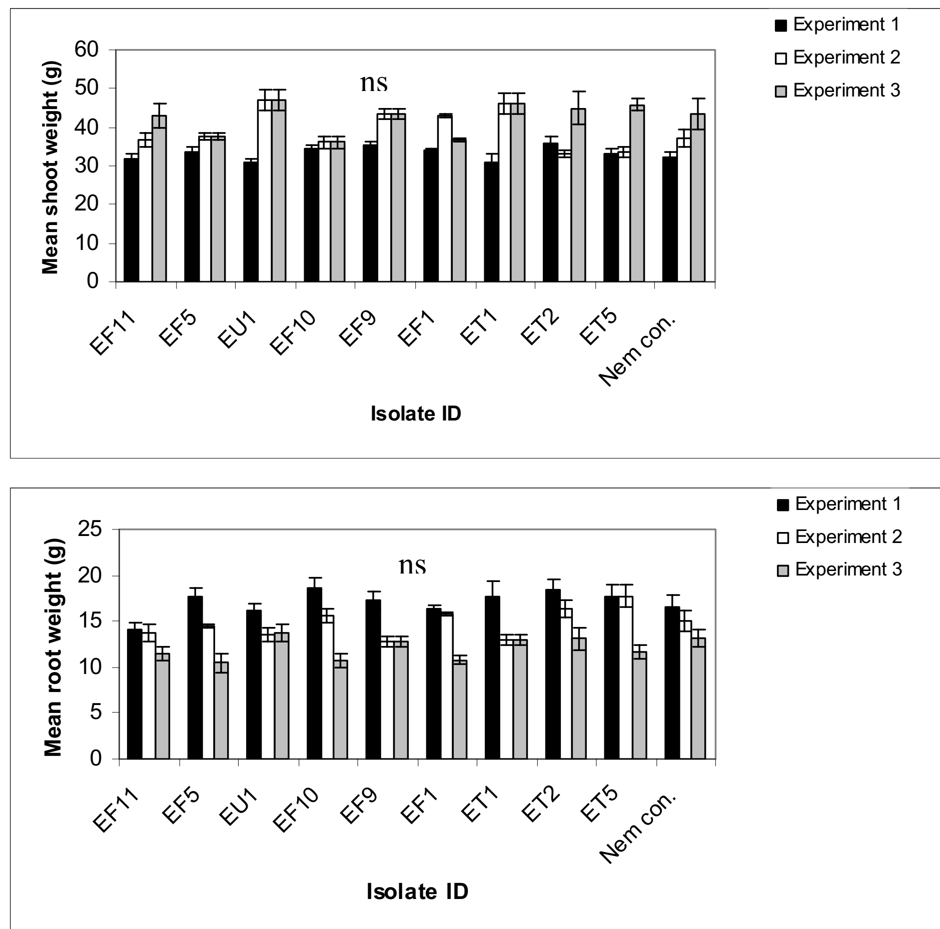

Figure 5: Influence of *Fusarium* and *Trichoderma* endophytic isolates on tomato shoot and root fresh weight. Means were not significantly different based on Dunnett's test ($P < 0.05$; $n = 6$; ns= non significant).

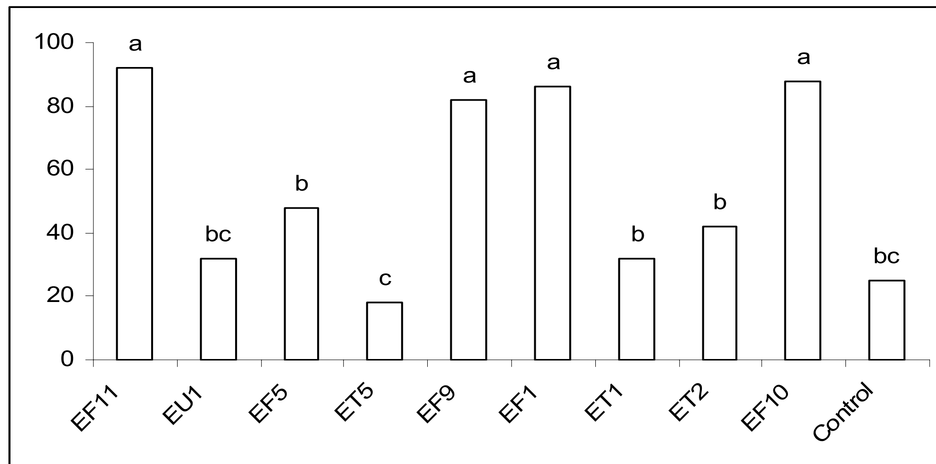

Figure 6: *Fusarium* and *Trichoderma* endophytic colonization of tomato roots 20 days after inoculation. Means with the same letter are not significantly different based on Dunnett's test (P< 0.05; n= 6).

3.7 Effect of *Fusarium* and *Trichoderma* isolates on nematode penetration

The most effective isolates of *Fusarium* (EF11 and EF10) and of *Trichoderma (*ET9) from the *in vitro* experiments (see section 3.6) were further tested for their effect on the penetration of *M. incognita* into tomato roots. Pre-inoculation of tomato with isolates EF11 and EF10 caused a significant reduction in nematode penetration, compared with *Trichoderma* sp (ET9*)* and the fungal free nematode control. The reduction in the number of J_2 that penetrated the roots ranged between 28-30% over the control (figure 7). No significant differences were observed in either shoot or root fresh weight based on Dunnett's test (P< 0.05; n= 6; data not shown).

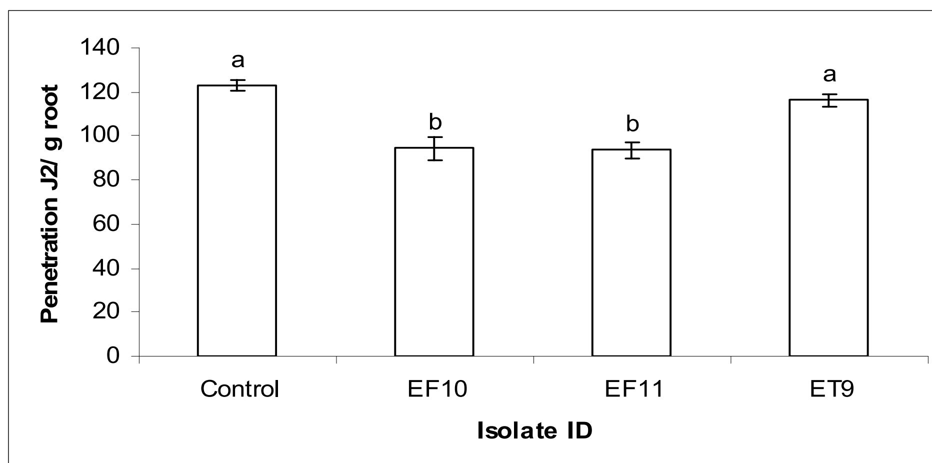

Figure 7: The influence of pre-inoculation of tomato with endophytic fungal isolates of (*Fusarium* EF11 and EF10 and *Trichoderma* ET9) on the penetration of *Meloidogyne incognita* juveniles 15 days after nematode inoculation. Means followed by the same letter are not significantly different from the control based on Dunnett's test. ($P < 0.05$; $n = 6$).

3.8 Cultural and morphological identification of *Fusarium* isolates

The isolates of *Fusarium* grew rapidly and covered the PDA plate within 8-12 days. The fungi produced white aerial mycelium and had a purple colour that varied from dark purple to light purple or pink. *Fusarium* isolates produced abundant oval to kidney shaped microconidia. Macroconidia were slightly sickle shaped, with a foot-shaped basal cell (figure 8).

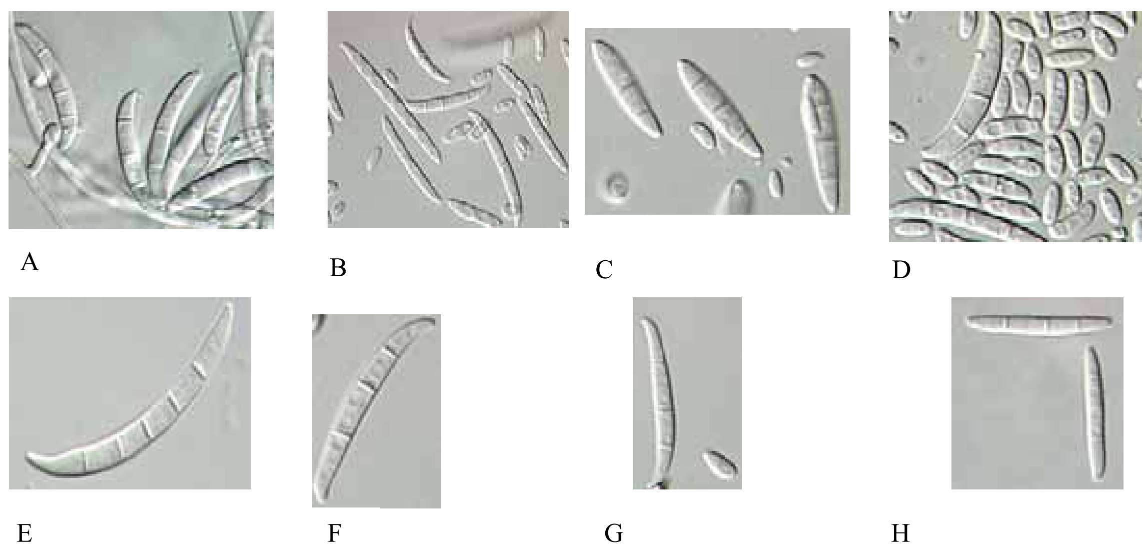

Figure 8: Morphological similarities of sickle shaped macroconidia from different endophytic *Fusarium oxysporum* isolates from coffee roots of different regions of Ethiopia. A= EF1; B= EF2; C= EF4; D= EF5, E= EF11; F= EF12; G= EF8; H= EF9.

3.9 Molecular identification of *Fusarium* isolates

Oligonucleotide primers PNFO and PN22 showed the amplification of a single fragment of about 1500 bp for each of the 15 endophytic isolates from coffee as well as for FO162 (figure 9). One to five distinct restriction patterns were resolved depending on the restriction enzymes. Enzyme *Hinf*I produced five; *XhoI* three and *HindI* one different restriction patterns (figure 11). Among the 3 restriction enzymes tested, *XhoI* and *HinfI* detected two polymorphisms among the isolates. No polymorphism was shown with *HindIII*.

Primers ITS 1 and ITS 4 showed amplification of a single DNA fragment of about 550 bp for each of the 10 isolates tested (figure 10). All PCR products were digested with each restriction enzyme in to 1 to 4 fragments depending on the restriction enzymes and isolates. Enzyme *HaeIII* and *HinfI* produced 4; *AluI* two; *NotI*, *XhoI*, *HindIII* and *HhaI* one each restriction patterns (figure 13). Most isolates selected from IGS – RFLP showed no

polymorphism among themselves and the positive control *Fusarium oxysporum* (FO162).

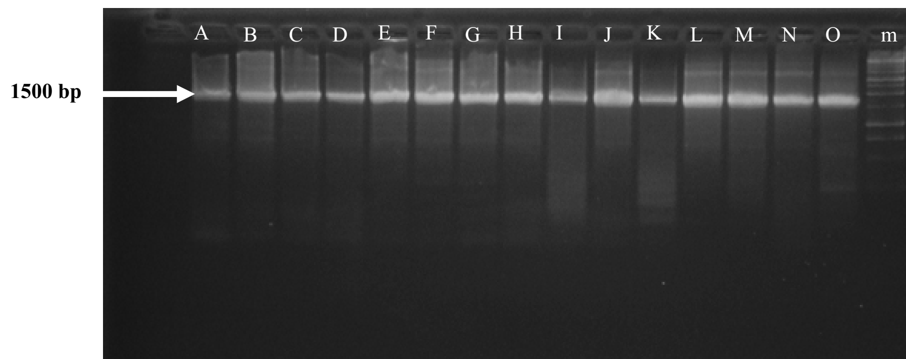

Figure 9: PCR amplification products of the intergenic spacer (IGS) region of the ribosomal DNA of endophytic *Fusarium* isolates from coffee roots. PCR products visualized on a 2% agarose gel stained by ethidium bromide. Lanes A-P: amplified from *Fusarium* isolates; Lane m: molecular-weight marker (1kb DNA ladder, Promega).

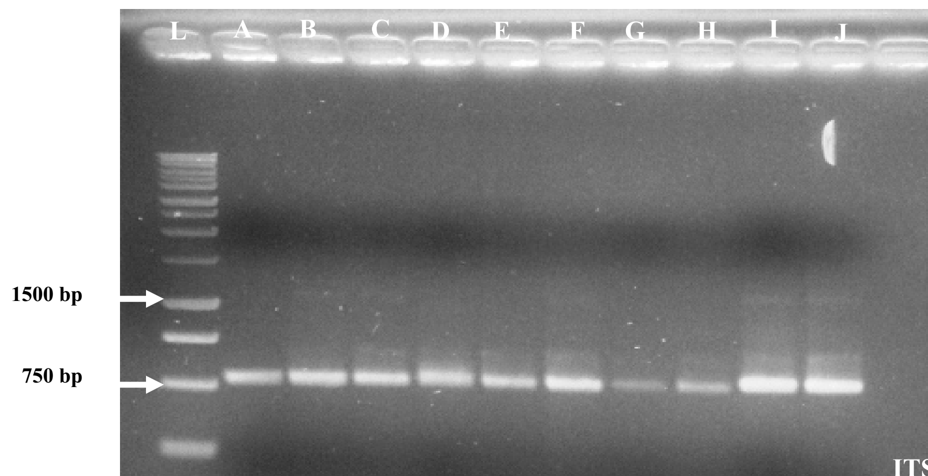

Figure 10: PCR amplification products of the internal transcribed spacer (ITS) region of the ribosomal DNA of endophytic *Fusarium* isolates from coffee roots. PCR products visualized on a 2% agarose gel stained by ethidium bromide. Lanes A-J: amplified from *Fusarium* isolates; Lane L: molecular-weight marker (1kb DNA ladder, Promega).

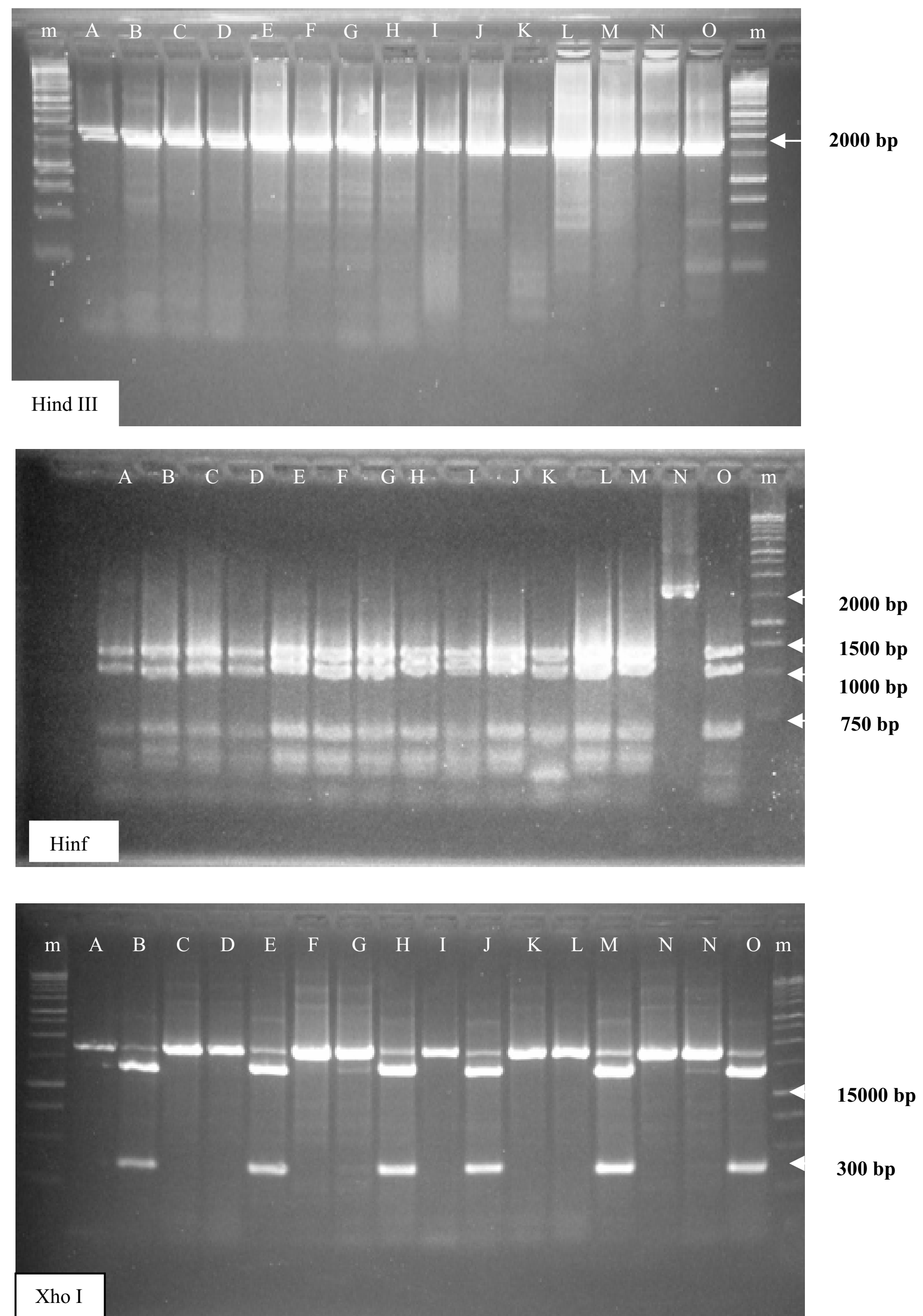

Figure 11: Restriction fragment of amplified intergenic spacer (IGS) products of various *Fusarium* isolates digested with *XhoI, HindIII* and *HinfI.* Lanes A-N: endophytic *Fusarium* isolates from Ethiopia; lane O is FO162 control and lane m is a 1 kb molecular size marker (Promega).

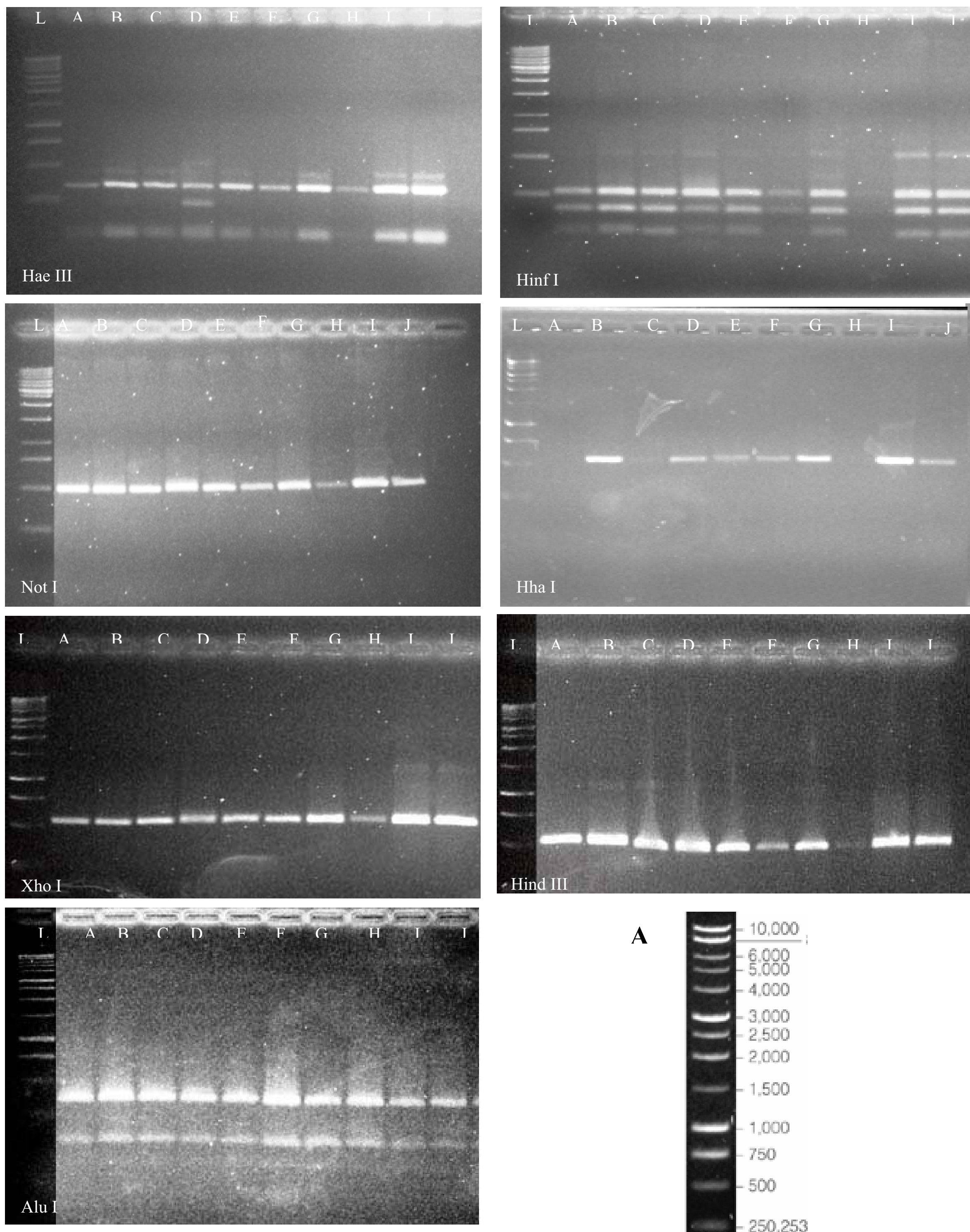

Figure 12: Restriction fragment of amplified internal transcribed spacer (ITS) products of various *Fusarium* isolates from coffee roots digested with *HaeI, HinfI, NotI, HhaI, XhoI, HindIII* and *AluI*. Lane L is a molecular size marker and lane J is FO162 control. **A**: is a 1 kb marker (Promega) used in all experiments.

4 Discussion

4.1 Isolation of endophytic fungi from coffee roots

The fungal endophytes used in this study were obtained by surface disinfestation of coffee roots with 2.5% (w/v) NaOCl for 3 min. This concentration was used to ensure total surface disinfestation and to protect internal tissues and endophytes. Disinfestations were confirmed by pressing roots on PDA, which showed no surface fungal growth. Different surface sterilization methods can be used depending on plant species, plant age, plant part and tissue size (Petrini, 1991; Fisher et al., 1992; Schuster, 1995; Hallmann et al., 1996; Hallmann et al., 2006). Hallmann et al. (2006) reviewed different procedures and sterilizing agents to isolate endophytic fungi from different plant roots. For example, surface sterilization of roots from various species of *Ericacaceae* and *Lycopersicon esculentum* was achieved by treatment in 0.3%-2% sodium hypochlorite for 1-3 min. The reagent and incubation time used in these experiments was similar and effective for complete disinfestation of coffee roots confirming that the isolates were truly endophytic.

The isolation of 128 endophytic fungi showed the presence of a diverse natural endophytic population in coffee. Endophytic microorganism have been found in all plant families, which representing many species in different climatic regions of the world (Spurr and Welty 1975; Luginbuhl and Muller 1980; Petrini and Carroll 1981; McInroy and Kloepper 1991; Petrini, 1991; Fisher et al., 1992). However, the amount of published information available on the occurrence of endophytic fungi from coffee is small. Santamarya and Bayman (2005) reported 340 endophytic fungal species from coffee leaves. The most common were: *Pestalotia, Botryosphaeria, Xylaria, Colletotrichum, Guignardia, Aspergillus, Cladosporium, Coprinus, Fusarium, Penicillium, Mucor, Rhizopus,* and *Trichoderma.* In another study, Peterson et al. (2005) reported hundreds of endophytic fungal species from coffee roots, leaves, stems and various berry parts. Of all *Penicillium* occurred most frequently.

A number of surveys were conducted to determine the presence and frequency of endophytic fungi in other plants in different parts of the world. Amin (1994) isolated 86 different fungal endophytes from banana roots from Indonesia. Similar investigations were made in Kenya on tomato by Hallmann and Sikora (1994a) who isolated 142 different endophytic fungi. Niere (2001) identified a total of 285 endophytic fungi associated with banana roots from Thailand.

The principal groups of endophytic fungi obtained in this study were non-pathogenic *Fusarium* and *Trichoderma*. The occurrence of *Fusarium* and *Trichoderm*a varied between sampling seasons from 25% to 44% of all fungi isolated. These results correspond well with findings by Fisher and Petrini (1992); Fisher et al. (1992); Amin (1994); Hallmann and Sikora, (1994a), who have isolated endophytic fungi from maize, banana and tomato. Hallmann and Sikora (1994a) reported frequency of occurrence of endophytic *Fusarium* up to 53% from tomato. Niere (2001) reported *Acremonium* and *Fusarium* were the most commonly isolated from banana at a frequency of 20% and 30% of all isolates, respectively.

The number of isolates obtained varied between the wet and dry season sampling. The higher number of isolates, generally obtained during wet season sampling, may be explained by the effect of optimum moisture and temperature conditions and the presence of vegetation cover. The latter adding higher nutrients and organic matter levels into the root zone. Sieber and Gruning, (2006) stated that the biodiversity of root endophyte communities varies in relation to environmental factors, type of vegetation, spatiotemporal patterns of the root microcosm and interaction among microorganisms.

The present study showed that coffee plants were found to host a diverse spectrum of endophytic fungi. *Fusarium* and *Trichoderma* were the predominant endophyte present in high numbers during the dry season sampling which indicates the ability of the two genera to survive dry soil conditions. This characteristic may be important in coffee since it is a perennial crop needing protection year round.

4.2 *In vitro* culture filtrates activity towards *M. incognita*

Results from the *in vitro* culture filtrate screening revealed that 13% of the isolates have significant biological control activity at all tested concentrations. The percentage of juvenile inactivity ranged from 28% to 81% after 24 h of exposure. These isolates can be considered as promising candidates for biocontrol but activity had to be verified under *in vivo* conditions since the endophytes may act in a different manner within the plant tissue. However, the goal of this initial study was to develop a procedure for rapid screening of fungal isolates and this was only possible under such *in vitro* conditions.

The production of secondary metabolites and toxins by soil-borne and fungal endophytes is well researched and is believed to be one of the most common mechanisms for plant protection against different groups of plant-parasitic nematodes (Amin, 1994; Hallmann and Sikora, 1996; Meyer et al., 2004; Athman et al., 2006; Dababat, 2007). The activity of culture filtrates of non pathogenic *Fusarium oxysporum* has shown different toxic effects towards different group of nematodes. For example, Hallmann and Sikora (1996) reported 60%–100% inactivation of different trophic groups of nematodes after 24 h exposure. In similar studies, Dababat (2007) also showed that culture filtrates of *F. oxysporum* 162, a strain originally isolated and tested by Hallmann and Sikora (1994), caused up to 82% mortality of *M. incognita* after 24 h exposure. Culture filtrates of endophytic fungi isolated from banana from Uganda and Indonesia caused 44 and 90% inactivation of the banana nematode *Radopholus similis*, respectively (Amin, 1994; Schuster et al., 1995).

In the present study, culture filtrates of *Trichoderma* isolates showed higher levels of inactivation compared with the *Fusarium* isolates. Meyer et al. (2000) reported that culture filtrates of *Trichoderma* significantly increased the percentage of immobile juveniles of *M. incognita* by 1.75 to 5 times compared with the control. However, these results are in contrast to observations made by Dababat (2007) who reported a non significant difference between *Fusarium* and *Trichoderma*.

4.3 *In vitro* screening of endophytic isolates for egg pathogenicity towards *M. incognita*

Differences in egg pathogenicity of the fungal isolates tested were observed whereby 30% of the isolates caused > 30% pathogenicity. Maximum pathogenicity was observed with *Fusarium* compared with *Trichoderma*. Verdejo-Lucas et al., 2002 reported up to 5% egg parasitism of *Meloidogyne* species by different fungal isolates including *Fusarium* spp. Similarly, parasitism of eggs of *H. schachtii* by *F. oxysporum* was reported from California (Nigh et al., 1980a). In contrast to our finding Santos et al. (1992) reported that up to 31% and 53% parasitism were recorded for *Fusarium* and *Trichoderma* species, respectively. Wang et al. (2005) screened 117 isolates of *Pochonia chlamydosporia* and reported that 12 of the *in vitro* tested isolates parasitized > 15% of the eggs of *Rotylenchulus reniformis*.

In view of these observations, complementary studies were undertaken to determine the egg pathogenicity of the same isolates in oil in Alginate films. Although egg pathogenicity of the same fungal isolates tested in the Petri dish assays were higher than the Alginate test, significant levels of control up to 38% were recorded in the soil compared with the control.

In conclusion, the results from the different *in vitro* tests showed that there exists a wide spectrum of endophytic fungi in coffee and that they have a potential as biocontrol agents for the control of root-knot nematode. Results obtained from the screening process must be seen as preliminary in the sense that experiments were carried out under laboratory conditions. The next step is therefore evaluation of promising isolates under *in vivo* greenhouse conditions.

4.4 *In vivo* antagonism of selected endophytic isolates against the root-knot nematode

The *Trichoderma* and *Fusarium* isolates used in these experiments demonstrated their potential as biocontrol agents under the *in vitro* conditions described above. Studies were therefore designed to study the biocontrol activity of the most promising isolates

under *in vivo* conditions that simulated field conditions. Results of three replicated greenhouse experiments demonstrated that isolates of *Fusarium* and *Trichoderma* reduced the number of *M. incognita* egg masses up to 50%. The 9 isolates investigated, however, were not equally effective. *Fusarium oxysporum* EF1 produce a significant reduction in the number of egg masses in all three experiments and was the most effective isolate. In similar experiments, Hallmann and Sikora (1994) demonstrated a reduction of > 50% in the number of egg masses/g of root after application of different non-pathogenic endophytic *Fusarium oxysporum* isolates. Dababat (2007) reported a greater impact of the mutualistic endophyte *F. oxysporum* (FO162) in reduction of number of *M. incognita* egg masses when compared to *Trichoderma* isolates.

Comparable results to those obtained with *M. incognita* in my studies on tomato were obtained with the burrowing nematode *Radopholus similis* on banana following biological enhancement of tissue culture plants with mutualistic endophytic *F. oxysporum* isolates (Niere et al., 1998; Pocasangre, 2000; Pocasangre et al., 2000; Niere 2001; Vu, 2005). In these studies, significant reductions of up to 90% in nematode infection were recorded in greenhouse experiments.

The isolates tested in my experiments did not have any effect on plant growth. These results confirm observations by Hallmann and Sikora (1994), Meyer et al. (2001), Sankaranarayanan et al. (2002) and Dababat (2007) who have reported non-significant results on plant growth parameters after treating plants with biocontrol agents. Contrary to these findings, Windham et al. (1986), Kleifeld and Chet (1992) and Spiegel and Chet (1998) reported growth stimulation of *Trichoderma* on different plants. Whether or not growth stimulation occurs, is often related to the *in vitro* screening criteria used to make the first selection. If screening targets nematode control, growth promotion is not always to be expected.

4.5 Determination of fungal colonization

In my experiment different strains of *Fusarium* sp successfully recolonised tomato plants up to 92% within 4 weeks of inoculation. The high percentage of endophytic root

colonization is similar with results obtained by Hallmann and Sikora, 1994; Pocasangre et al., 2000; Niere et al., 2001; zum Felde, 2002; Vu, 2005; Dababat, 2007. Dababat (2007) found that inoculation of commercial tomato varieties with FO162 resulted in root colonization up to 100% three weeks after fungal inoculation. Similar colonisation trials were conducted on banana by Niere (2001), Pocasangre (2000) and Vu (2005) who reported that different endophytic *Fusarium* isolates could colonized 38-49%, 45-70% and 40-45% of the root tissue sampled on different banana cultivars, respectively, after biological enhancement of tissue culture plantlets. A low level of fungal colonization was observed in the roots of non-inoculated control plants, due to contaminant fungi other than *Fusarium* spp. and was much lower than in the fungal inoculated plants.

4.6 Effect of *Fusarium* and *Trichoderma* isolates on nematode penetration

The reduction in nematode penetration obtained in the present study with the *F. oxysporum* isolates EF1 and EF10 ranged up to 30%. Similar effects were reported by Sikora (1997) who discussed that enhancement of seedlings with biological control agents reduces early nematode penetration of roots in the pathozone and therefore increases protection directly at the site of nematode infection. *F. oxysporum* has been shown to decrease the penetration rate of the sedentary endoparasitic nematode *M. incognita* on tomato (Hallmann and Sikora, 1994). Dababat (2007) reported that tomato plants enhanced with *F. oxysporum* reduced penetration by up to 56% over the controls. In a related experiment, Vu et al. (2006) found up to 41% reduction in penetration of enhanced banana transplants with different *F. oxysporum* towards *R. similis*. This reduction in penetration may be related to changes in root exudates, production of fungal metabolites with nematostatic action or to a delay in penetration due to repellent activity as postulated by different authors.

Endophytic *F. oxysporum* isolates obtained from coffee roots were grouped in to 2 IGS-RFLP groups. This showed the little genetic difference among isolates. Athman et al., 2006 reported 9 IGS-RFLP genotypes of *F. oxysporum* from banana roots. A further investigation on the genetic variation of endophytic fungi from coffee using different molecular techniques is required for better understanding.

5 Conclusions

In this study, endophytic fungi were isolated from different coffee plantations in Ethiopia. The isolates tested could have a paramount importance for further use in coffee growing regions of the world. It is therefore concluded that:

1. A wide range of endophytic fungi are associated with coffee.
2. Endophytic fungi can be isolated based on surface-sterilization followed by tissue maceration. A concentration of sodium hypochlorite of 2.5% is optimum for coffee roots.
3. The major fungal genera found were *Fusarium* and *Trichoderma*
4. Endophytic fungal isolates obtained from coffee exhibit different level of culture filtrate activity, egg parasitism and reduced nematode penetration.
5. *Fusarium* spp. are found effective and consistent in biocontrol activity
6. *Fusarium* spp are able to colonize tomato roots which is an important phenomena for further use of endophytic fungi for biocontrol.
7. The use of these beneficial endophytic fungi in integrated pest control programs is an important strategy to control root-knot nematodes.
8. Little genetic variations were observed between the different *Fusarium oxysporum* isolates.

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CHAPTER 4

Studies on the natural communities of endophytic bacteria in coffee (*Coffea arabica* L.) from Ethiopia and their antagonistic potential towards *Meloidogyne incognita*

1 Introduction

Endophytic bacteria are defined as those bacteria that can be isolated from surface disinfested tissue or extracted from within the plant, and that do not visibly harm the plant (Hallmann et al., 1997). They have been found in numerous plant species (Chanway, 1998), with most being members of common soil bacterial genera such as *Pseudomonas*, *Bacillus*, and *Azospirillum* (Chanway, 1996). Although the interaction between endophytic bacteria and their host plants is not fully understood, many strains can promote plant growth (Hallmann et al., 1997; Chanway, 1998) and increase plant resistance towards pathogens and parasites (Hallmann et al., 1997; Hasky-Günter et al., 1998).

Endophytic bacteria are omnipresent in plant tissues and have been isolated from flowers, fruits, leaves, stems, roots, seeds and root nodules (McInory and Kloepper, 1995; Sturz et al., 1997; Hallmann et al., 1997, 1999; Hallmann, 2001). Endophytic bacteria reside inter-cellularly or intra-cellularly within host tissues and therefore are able to form more intimate relationships with the host plant than most other plant associated bacteria. By residing within plant tissues, endophytic bacteria also are sheltered from environmental stresses and microbial competition. Although some endophytic strains may inhibit plant growth (Sturz et al., 2000), it has been shown that a higher proportion of bacterial endophytes are plant growth promoters than is the case for bacteria on the rhizoplane or in the rhizosphere (Hallmann et al., 1997).

Significant variation in the population dynamics of indigenous and introduced endophytes in plants has been reported. These variations are attributed to plant source, plant age, tissue type, time of sampling and environmental conditions. Generally,

bacterial populations are higher in roots than in the stems and leaves (Hallmann, 2001; Lamb et al., 1996 as quoted by Denise et al., 2002)

Endophytes are promising candidates for use in agriculture for biocontrol and plant protection. They colonize the same niche as plant pathogens and may therefore be better suited than rhizosphere bacteria to either out compete or directly antagonize pathogens. Because of the intimate contact between endophytes and plant cells, induced systemic resistance may be an important mechanism of biocontrol (Benhamou et al., 1996; Hasky-Günter et al., 1998; Nejad and Johnson, 2000).

Analysis of fatty acids has been routinely used in identification of anaerobic bacteria. The fatty acids between 9 and 20 carbons in length have also been used to characterize genera and species of bacteria, especially non fermentative Gram negative organisms. With the advent of fused silica capillary columns, it has become practical to use gas chromatography of whole cell fatty acid methyl esters to identify a wide range of organisms (Sasser, 1990). More than 300 fatty acids and related compounds have been found in bacteria analyzed in the MIDI Research and Development Laboratory. The information contained in these compounds can be estimated by considering not only the presence or absence of each acid, but also by using the data in quantitative fashion (Sasser, 1990).

Many studies have been conducted to determine the diversity of bacterial communities in different habitats (Hallmann et al., 1997; Mahaffee and Kloepper, 1997; Krechel et al., 2002). Bacterial diversity generally refers to the genetic diversity, i.e. the amount and distribution of genetic information within the bacterial communities. Diversity is a function of two components: 1) The total number of species present (species richness) and 2) The distribution of individuals among those species (evenness). Diversity indices characterize the species composition of the community at a given site and time. One of the most popular formulas for species diversity is the Shannon index (H’). For a given number of species, H’ is maximum, when the organisms are equally distributed among

the relative proportion of species and H’ is lower when there is a stronger dominance of one or a few species.

Root-knot nematodes (*Meloidogyne* spp.) occur worldwide and are regarded as one of the most important pests causing severe losses to economically important crops. These important crop pests occur in intensive cropping system and until recently have been controlled mainly by nematicides. Since many of the most commonly used nematicides are expensive or are being withdrawn from the market due to their harmful effect on humans, their persistence in the soil or their contamination of ground water, investigators are concentrating their efforts on integrating biological control agents in plant disease management strategies (Jatala, 1986). Therefore, application of microorganisms to the soil or plant as a biological control agent offers an alternative method to control plant-parasitic nematodes (Stirling, 1991).

Research has demonstrated that bacterial endophytes can improve plant growth and reduce disease symptoms caused by several plant pathogens such as *Fusarium oxysporum* on cotton (Chen et al., 1995) or *Verticillium albo atrum* and *Rhizoctonia solani* on potato and cotton (Kloepper et al., 1992; Pleban et al., 1995). Hallmann et al. (1995) reported that endophytic bacteria may contribute to control of plant-parasitic nematodes. They evaluated 7 strains of endophytic bacteria isolated from cucumber and cotton roots against the root-knot nematode, *M. incognita* and they found a significant reduction of 50% in the number of galls on cucumber. In another study, Munif et al. (2000) screened endophytic bacteria isolated from tomato roots towards *M. incognita* on tomato under greenhouse conditions. They showed antagonistic properties of 21 out of 181 endophytic bacteria towards *M. incognita*.

Much of the work with endophytic bacteria has been done with agricultural and horticultural plant species. However, little is known about endophytic bacteria in coffee plants, which is a commercially important crop in the world. As Ethiopia is the center of origin and diversity for coffee, there is a high probability that endophytic bacteria play an important role in natural pest and disease control. With this hypothesis, endophytic

bacteria were isolated from coffee and tested against the root-knot nematode *Meloidogyne incognita* with the objectives of:

1. Collection, isolation and identification of endophytic bacteria associated with coffee
2. Screening endophytic bacteria for their biological control activity potential towards *M. incognita* under *in vitro* conditions
3. Screening endophytic bacteria for their antagonistic potential towards *M. incognita* under *in vivo* conditions

2 Materials and methods

2.1 Culture media and reagents

The following culture media and reagents were used to grow bacterial pure cultures and for extraction of bacterial fatty acids:

Tryptic Soy Agar (TSA): 9.6 g agar, 24 g Trypton Soy Broth (TSB) and 800 ml of deionized water

Tryptic Soy Agar 5% (TSA): 9.6 g agar, 1.5 g TSB and 800 ml deionized water

Phosphate buffer solution (PB): 1.74 g K_2HPO_4 and 1.36 g KH_2PO_4 per 1000 ml

GC-TSA: Purified and fine agar and TSB made for gas chromatography. 9.6 g agar, 24 g Trypton Soy Broth (TSB) and 800 ml of deionized water.

Reagent 1:

Sodium hydroxide (certified ACS)	45 g
Methanol (reagent grade)	150 ml
Deionized distilled water	150 ml

Reagent 2:

6.0 N Hydrochloric acid	325 ml
Methanol (reagent grade)	275 ml

Reagent 3:

Hexane (HPLC grade)	200 ml
Methyltert buthyl ether (MTBE)	200 ml

Reagent 4:

Sodium hydroxide	10.8 g
Deionized distilled water	900 ml

2.2 Sample collection and isolation of endophytic bacteria

Sampling methodologies and procedures are the same as described in chapter 3 section 2.1. Sixty root samples were collected from the different coffee growing agroecologies of Ethiopia, during the wet season in August, 2004 and again during the dry season in April, 2006.

Endophytic bacteria were isolated from 5 g of coffee root segments using a surface sterilization and titration plating technique as described by Hallmann et al. (1997). The technique involved shaking 5 g of coffee roots in 2.5% (w/v) NaOCl for 3 min, followed by three of 3 min rinses in phosphate buffered saline solution (PBS). To check for surface contamination (sterility check), samples were imprinted on 5% TSA and incubated at 28°C for 24 h. The edges of each root sample were cut off with a sterile scalpel and discarded to avoid possible contaminations. Tissues were then triturated by hand using a sterile mortar and pestle in 10 ml of PBS. Triturated tissues were serially diluted in sterile PBS and 0.1 ml of the 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated on 5% TSA using a spiral platter (Eddy Jet, Version 1.2, IUL Instruments, Königswinter, Germany). Following incubation for 1-3 days at 28°C, representative bacterial colonies were selected from the dilution plates based on colony size, shape, morphology, and colour. Single colonies were selected and purified by re-streaking onto fresh plates of TSA. The bacteria were then incubated at 28°C and bacterial purity was checked after 48 h. A loop of bacteria cells was then transferred into 1.2 ml vials containing sterile Tryptic Soy Broth (TSB) plus 20% glycerol. The samples were stored at -80°C until processed for identification.

2.3 Identification and diversity of endophytic bacteria

Purified strains were identified by gas chromatographic analysis of bacterial fatty acids methyl esters (FAME-GC) using the MIDI system (Microbial ID, Inc., New Jerssey, USA). FAME-GC peaks were named by Microbial Identification System (MIS) software of Hewlett Packard Co. and Microbial ID, Inc., Newark, Delware, USA. MIS calibrated the gas chromatograph with a commercial FAME calibration mix prior to running samples and after every ten samples.

The bacteria from stock cultures were streaked with a loop on to pure GC-TSA media and incubated at 28°C for 24 h. Bacterial fatty acids were extracted by the following steps (Sasser, 1990):

Harvesting: Culture tubes were cleaned using hexane solution for 15 min in a rotary shaker. A 4 mm loop was used to harvest bacterial cells from the third quadrant of the streaked plate (second or first quadrant if slow growing). The cells were then placed in a clean 13 x 100 culture tube.

Saponification: One ml of reagent 1 was added to each tube containing cells. The tubes were sealed with teflon lined caps, vortexed briefly and heated at 100°C for 5 min, at which time the tubes were vigorously vortexed for 10 seconds and returned to the water bath for 25 minute heating.

Methylation**:** The cooled tubes were uncapped and 2 ml of reagent 2 was added. The tubes were capped again and briefly vortexed. After vortexing, the tubes were heated for 10 min at 80°C.

Extraction: Addition of 1.25 ml of reagent 3 to the cooled tubes was followed by recapping and gentle tumbling on a clinical rotator for about 10 min. The tubes were then uncapped and the lower phase was pipetted out and discarded.

Base Wash: Three ml of reagent 4 was added to the organic phase remaining in the tubes and the tubes were recapped, and agitated for 5 min. Following uncapping, about 2/3 of the organic phase was pipetted into a GC vial which was capped and was then ready for analysis.

Extracted samples were stored at -20°C until analyzed in the gas chromatograph. After GC analysis bacterial strains with a similarity index (SI) > 0.2 were considered positively identified, whereas strains with SI < 0.2 were considered tentatively identified.

The diversity of the endophytic bacteria detected was determined by the Shannon diversity index at species level using the computer program Biodiversity Professional version 2.0, 1997.

2.4 *In vitro* screening of endophytic bacteria culture filtrates against *Meloidogyne incognita*

Forty identified endophytic bacterial strains which were stored at -80°C were pre-cultured on 100% TSA for 24 h at 28°C. A loop full of bacteria was then transferred into a liquid culture of tryptic soy broth medium (TSB) and grown in 200 ml flasks on a rotary shaker at 121 rpm for 2 days at 25°C. Culture filtrates were obtained after centrifugation of the bacterial cells at 4000 rpm for 20 min. Pellets were discarded and the supernatant was collected in a sterilized flask. The supernatant was passed successively through 5 µm and 0.2 µm pore size cellulose triacetate filters (Nalgene®, Rochester, New York) and the sterile filtrate was collected in sterilized flasks.

M. incognita juveniles (J2) were used as inoculum in all experiments. Nematode inoculum was extracted from tomato roots as described in chapter 3 section 2.5. Three ml of the culture filtrate was transferred to each of the 12-wells in the tissue culture plates to which 1 ml sterile water containing 200–300 surface sterilized J2 was added. Juveniles kept in filtered TSB or sterile distilled water served as controls. To avoid any contamination, every treatment received 2 ml of a 6000 ppm streptomycin solution, so that the final concentration was 2000 ppm. Each treatment was replicated 6 times and the wells were incubated at 24°C. After 1, 2 and 3 days of incubation, the number of dead J2 were counted and percentage mortality was calculated. Differences in J2 mortality between the treatment and control solutions were tested with one way analysis of variance (ANOVA) followed by Dunnett's test to compare each treatment against the control using a statistical software SPSS for windows Ver. 14:0.

2.5 Preliminary screening of endophytic bacteria towards the root-knot nematode *Meloidogyne incognita* on tomato

Coffee is a perennial plant and coffee beans need 4-5 months to germinate. Therefore, a preliminary screening was done using tomato as an indicator plant. To determine the nematicidal activity of 28 bacterial strains, 5 sets of experiments each containing bacterial strains and 2 controls (Ringer´s solution and Nematicide) were conducted.

Seedlings were produced of the cv. Hellfrücht Fruhstamm which were sown in a tray containing a sterile sand/ soil mixture (2:1 v/v). Bacterial cells were grown on TSA at 28°C. A loop of bacteria was then transferred into TSB and placed on a rotary shaker for two days at 25°C. The liquid culture was centrifuged at 4000 rpm for 20 min. The bacterial pellets were re-suspended in ¼ strength Ringer's solution and adjusted photometrically to OD_{560nm}= 2.0 equal to approximately 10^8 cfu/ml.

Two week old tomato seedlings were dipped in the bacterial suspensions for 20 min and transplanted individually into 100 ml transparent containers (14 x 2 x 4 cm) filled with the sterile sand/ soil mixture (2:1 v/v). After five days, 5 ml of the same bacterial suspension was added in to each plant as a soil drench. Control plants received 5 ml of Ringer's solution or 2 ml of the nematicide Nemathorin (3 ppm). Five days later, 300 J2 of *M. incognita* were inoculated into the root zone of each plant in three 2 cm deep holes made with a plastic rod. Five weeks after nematode inoculation, the plants were harvested and root and shoot weight as well as root-knot galling and the number of galls were recorded.

2.6 Antagonistic activity of endophytic bacteria against the root-knot nematode *Meloidogyne incognita*

Five endophytic bacterial isolates were selected on their performance in the preliminary screening experiments (see section 2.5) and tested under greenhouse conditions. Tomato seeds cv. *Hellfrucht Frühstamm* were sown in a 70 plug seedling tray with each plug measuring 4 x 4.5 x 2.5 cm and filled with a sterile sand soil mixture. Three weeks old tomato seedlings were then treated two times by dipping and drenching with each of the following isolates: *Bacillus megaterium, B. pumillus, B. mycoides, Agrobacterium radiobacter* and *Pseudomonas coronafaciens.* The bacterial suspensions were prepared after centrifugation at 8000 rpm for 20 min, the supernatant was discarded and the pellet was re-suspended in a sterile ¼ strength Ringer-solution. Bacterial densities were adjusted with a spectral photometer to an optical density of OD_{560nm}= 2.0 representing approximately 10^8 cfu/ ml.

Seedlings were dipped in 10^8 cfu/ ml of the respective bacterial suspension before transplanting and then transplanted into 500 ml pots containing 400 g of the sterilized sand: soil mixture (2:1). Five days after transplanting a second inoculation of 10^8 cfu/ ml was made as a soil drench. The bacteria in the Ringer-solution were inoculate in a 5 ml suspension (10^8 cfu/ ml) per plant in three holes made around the plants to a depth of 2 cm. Control plants were treated with 5 ml of Ringer-solution.

Each plant was then inoculated with 800 *M. incognita* J2 per plant by pipetting the nematode in water solution into three holes in the root zone made with a plastic rod five days after treating the plants with the second bacterial suspension. Each treatment was replicated 8 times and plants were arranged in a completely randomized design in the greenhouse at 25 ± 3°C with 16 hours of supplemental artificial light per day. Plants were watered every day and fertilized weekly with Poly Crescol (N: P: K = 14: 10: 14) at 2 gl^1 of water.

Eight weeks after nematode inoculation, the plants were harvested and fresh shoot weight, fresh root weight, number of galls, gall index, and number of egg masses were recorded. Gall index was measured as described by Zeck (1971) on a scale from 0-10 where 0= no galls and 10= completely galled. Number of egg masses was counted under a dissecting microscope after staining in an aqueous solution of Phloxine B at 0.15 g l^{-1} (Shurtleff and Averre III, 2000).

Data were analyzed according to the standard analysis of variance procedures using SPSS (Version 14.0 for windows, 2005). Treatment means were separated using Dunnett's test to compare each treatment against the control.

3 Results

3.1 Collection, isolation and identification of endophytic bacteria from coffee

The abundance of endophytic bacteria communities associated with the roots of coffee was assessed during the wet season of 2004 and dry seasons April 2006. A wide range of bacteria species were recovered from the different coffee growing agroecologies. Results of viability tests showed that root prints on TSA incubated at 28°C for 24 h after surface disinfection with 2.5% (w/v) of NaOCl for 3 min showed no colony growth. Therefore, samples were free of surface colonizers and the isolated bacteria were considered true endophytes.

Endophytic bacteria were identified by Fatty Acid Methyl Ester-Gas Chromatography (FAME-GC). For the wet season sampling, taxonomic identities of 201 bacterial isolates belonging to 43 genera were identified by the MIDI system. Of these 201 isolates, 115 (57%) were identified with a similarity index (SI) of > 0.2 and 30 (14.9%) could not be identified by the MIDI system (No match and unidentified*)*. A further 56 isolates (27.9%) were identified with SI of < 0.2 which indicates a tentative identification (annex 2).

In the dry season sampling 114 bacterial isolates belonging to 16 genera were identified. Of these 114 isolates 77 (67.6%) were identified with SI of > 0.2 and 12 (10.5%) could not be identified by the MIDI system (No match and unidentified*)*. A further 25 isolates (21.9%) were identified with SI of < 0.2 which indicates a tentative identification (annex 2).

Of the total isolates recovered during the wet season sampling, 56% were Gram-negative and 30% were Gram-positive, with the Gram-negative bacteria comprising 29 of the 43 genera identified. During the dry season sampling, 72% were Gram-negative and 24 were Gram-positive, with the Gram-negative bacteria comprising 9 of the 16 genera identified (annex 2).

The Gram-negative genus *Pseudomonas* was the most frequently encountered genera with 57% and 39% occurrence in wet and dry season samplings, respectively (figure 1). In terms of species *P. putida* was the most frequently occurring with 13.4 and 21.9% in wet and dry seasons, respectively. Other *Pseudomonas* species recovered more frequently were *P. fluorescens* (up to 21%) and *P. chlororaphis* (up to 15%) *(*annex 2).

Gram positive bacteria of the genus *Bacillus* were also distributed throughout most sampling sites with 11% and 4% occurrence in the wet and dry seasons, respectively (figure 1). Species within this genus include: *Bacillus brevis, B. licheniformis, B. megaterium, B. mycoides, B. pumillus* and *B. sphaericus* (annex 2).

Genera of bacteria previously known to be associated with nematode biocontrol or plant growth promotion (*Pseudomonas*, *Bacillus* and *Agrobacterium*) were found in this survey in both wet and dry seasons. The most abundant genera found were *Pseudomonas, Bacillus, Agrobacterium, Stenotrophomonas and Enterobacter* which were homogeneously distributed throughout all coffee plantation types. The genera *Micrococcus* and *Acinetobacter* were recovered from all plantation types except from state farm plantations (figure 2).

The highest levels of species diversity was recorded from the semi-forest plantation type (H'= 2.55 and 1.98 in wet and dry season sampling, respectively) followed by the garden plantation (H'= 2.50 and 2.17 in wet and dry season sampling, respectively) and then the forest plantation type (H= 2.34 and 2.00 in wet and dry season sampling, respectively) (figure 2). This indicated that, genera distribution didn't follow a defined distribution pattern between plantation types, and that close similarities between the three plantation types were evident. The endophytic community of the state farm plantation type was less diverse (H'= 1.84 and 1.56 in wet and dry season sampling, respectively) compared with the other plantation types (figure 2).

Bacterial densities clearly varied with the season sampled. Higher population densities were observed during the wet season ranging from 5.2×10^3 to 2.07×10^6 cfu g^{-1} fresh

root compared with the dry season bacterial densities that ranged from 2.7×10^3 to 1.23×10^4.

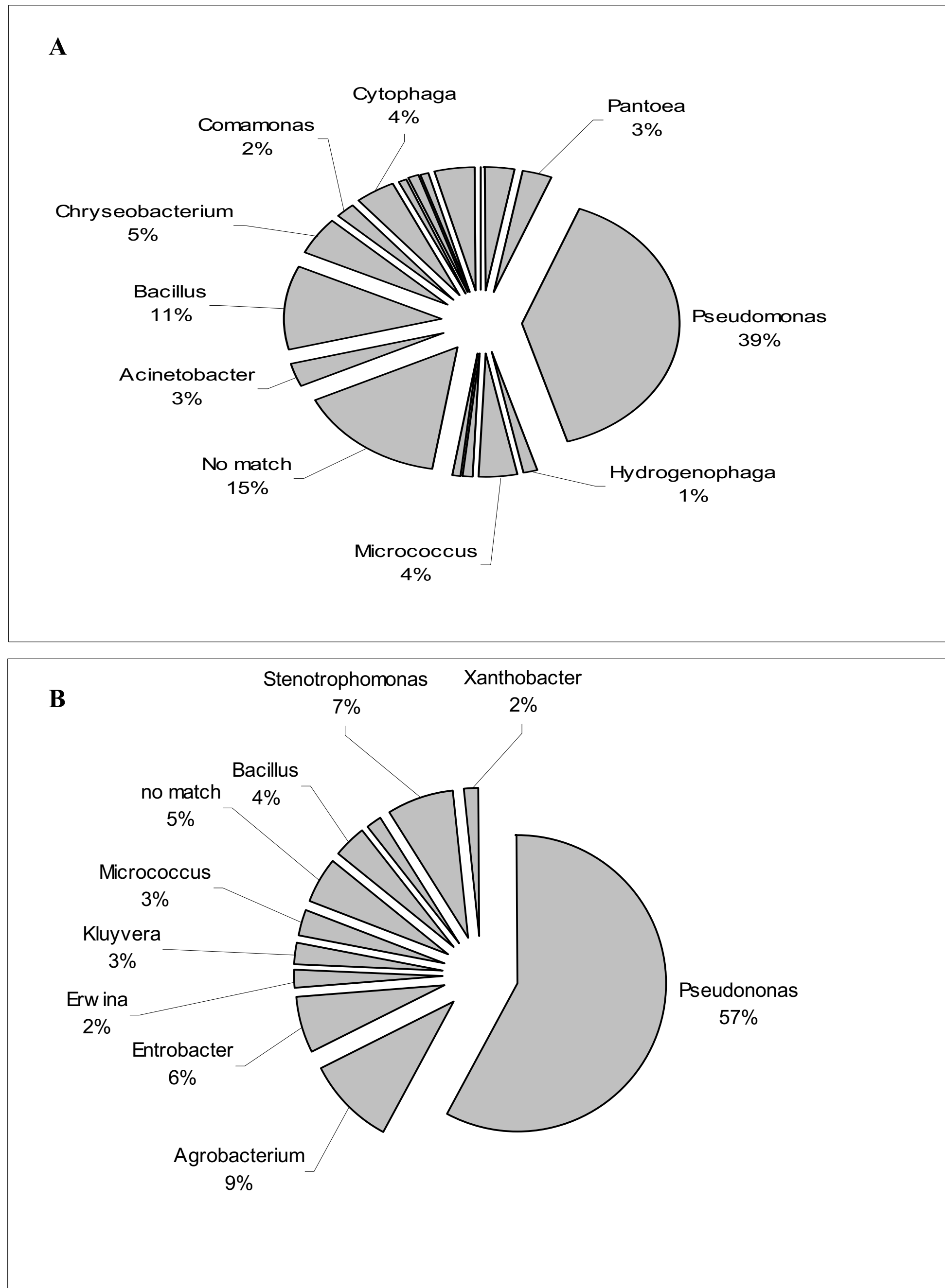

Figure 1: Relative population density of endophytic bacteria from coffee roots during the wet (A) and dry (B) season from Ethiopia

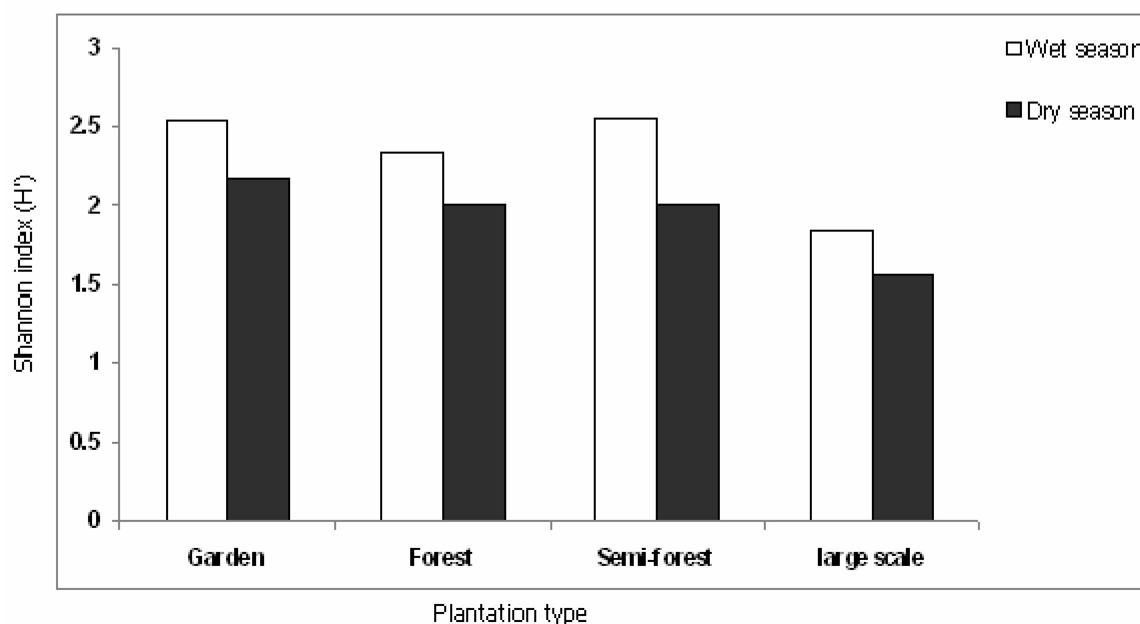

Figure 2: Shannon diversity (H') index of endophytic bacterial communities associated with coffee roots

3.2 *In vitro* screening of endophytic bacterial culture filtrates against *Meloidogyne incognita*

Culture filtrates of the bacterial isolates showed nematicidal effects toward the J2 of the root-knot nematode *M. incognita*. Of the 40 endophytic bacterial strains tested, 14 showed significant J2 mortality compared to the media control (table 1). Mortality ranged between 38% and 98%. The active strains were: *Agrobacterium radiobacter, Bacillus pumillus, B. brevis, B. megaterium, B. mycoides, B. licheniformis, Chryseobacterium balustinum*, *Cedecea davisae, Cytophaga johnsonae, Lactobacillus paracasei, Micrococcus luteus, M. halobius, Pseudomonas syringae* and *Stenotrophomonas maltophilia*.

The degree of J2 mortality increased with increased time of exposure. The highest mortality in all cases was observed 3 days after exposure. The highest level of mortality was recorded for *B. megaterium, A. radiobacter* and *C. davisae* with 98.3%, 97.3% and 94.6%, respectively (table 1).

Table 1: Effect of culture filterates from endophytic bacteria on mortality of *Meloidogyne incognita* juveniles 1, 2 and 3 days after incubation at 24°C.

Set 1	Day 1	Day 2	Day 3	Set 2	Day 1	Day 2	Day 3	Set 3	Day 1	Day 2	Day 3
Water	1.7	9.3	11.3	Water	6.7	12.4	20.3	Water	5	6.3	12
Media	2	5	28.6	Media	13.3	33	38.3	Media	31.3	36.7	38.3
Chryseobacterium indologenes	8.6	15	27.6	Stenotrophomonas maltophilia	70*	70*	86*	Agrobacterium radiobacter	73*	87.6*	97.3*
Bacillus pumillus	27*	76.3*	87.6*	Bacillus brevis	19.6	57	76.3	Pseudomonas syringae	53.6*	59*	79.5*
Paenibacillus pabuli	6.3	14.6	43	B43	34	67*	87.3*	Chryseobacterium balustinum	20.3	22.6	28.7
Cytophaga johnsonae	8	8	14.3	Acinetobacter lwoffii	16	20.3	29.6	Pantoea agglomerans	9.6	10.3	18.6
Cellulomonas gelida	9.3	24.3	34.6	Chryseobacterium balustinum	19	27.3	28.6	Pseudomonas putida	14.3	20	30.6
Comamonas acidovorans	6	14.6	43	Pseudomonas chlororaphis	32	40.3	31.6	Hydrogenophaga pseudoflava	33.6	37.6	35.4
Chryseobacterium balustinum	84.3*	92*	93*	Stenotrophomonas maltophilia	73.6*	93.6*	100*	Micrococcus luteus	64.6*	83*	87*
Set 4	Day 1	Day 2	Day 3	Set 5	Day 1	Day 2	Day 3	Set 6	Day 1	Day 2	Day 3
Water	4.6	3.6	3.6	Water	8.3	11	20.6	Water	10	18.6	21.6
Media	13.3	19.6	28.6	Media	22.3	28.3	38	Media	17	21.6	27.3
Bacillus licheniformis	34	41*	41.6*	Pseudomonas coronafaciens	21.6	19.6	22.6	Acinetobacter baumannii	16	25	43
Chryseobacterium balustinum	22	25.6	31	Bacillus mycoides	70*	78.3*	87.3*	Acinetobacter johnsonii	22.6*	24.3	34
Paenibacillus pabuli	24.6	23.3	25.6	Brevibacterium helvolum	22.6	24.3	34	Micrococcus halobius	31.3*	36.6*	38.3*
Cellulomonas fimi	18.3	25	27	Micrococcus kristinae	25.6	33.3	31.3	Bacillus megaterium	81*	97.3*	98.3*
Cedecea davisae	75.6*	78.3*	94.6*	Aeromonas salmonicida	22	32.3	21.6	Burkholderia cepacia	12.7	14.6	24.3
Pseudomonas corrugata	20.3	27	31.6	Arthrobacter ilicis	19.3	28	21.6	Lactobacillus paracasei	18	65.6*	77.3*
Cytophaga johnsonae	55.3*	76.3*	87.3*	Micrococcus varians	21	32.6	34	Kluyvera ascorbata	19	29.6	32

(*) The mean difference is significant at 0.05% based on observed means from the water and media control.

3.3 Preliminary *in vivo* screening of endophytic bacteria towards *Meloidogyne incognita*

Thirty six percent of the 28 strains screened reduced the number of galls up to 54% (table 2). The results of the greenhouse test were slightly contrary to those achieved in the filtrate *in vitro* tests (section 3.2). Isolates which gave significant differences in control in the *in vitro* test gave non-significant levels of controlling the *in vivo* screen. Based on these tests, a total of 10 strains belonging to the species, *Agrobacterium radiobacter, Bacillus licheniformis, B. pumillus, B. mycoides, B. megaterium, Pseudomonas syringae, Pantoea agglomerans, Paenibacillus pabuli, Cellulomonas fimi* and *Pseudomonas coronafaciens* were selected as potential biocontrol agents for advanced studies. *B. licheniformis* exhibited the highest reduction in the number of galls followed by *A. radiobacter, P. syringae* and *B. megaterium.*

In all the experimental sets conducted, no growth stimulation in either fresh shoot or root weight was observed with the exception of *Pseudomonas coronafaciens* which caused an increase in shoot fresh weight of 51% compared with control (table 2).

Therefore, isolates of *A. radiobacter, B. licheniformis, B. pumillus, B mycoides, B megaterium* and *Pseudomonas syringae* gave significant biocontrol results in both *in vitro* and *in vivo* tests.

Table 2: Effect of different endophytic bacterial isolates on shoot fresh weight (g) and number of galls of *Meloidogyne incognita* on tomato 8 weeks after nematode inoculation. Each set is the result of a separate experiment.

Isolate (Set 1)	Shoot fresh weight	No of galls/ g root	Isolate (Set 2)	Shoot fresh weight	No of galls/ g root
Ringer's solution	8.1	33.3±0.40	Ringer's solution	6.8	45.1±3.22
Nematicide	8.3	0±2.76*	Nematicide	8.3	2.4±1.21*
Chryseobacterium indologenes	3.4	31.1±1.96	*Bacillus licheniformis*	7.5	23.2±1.91*
Bacillus pumillus	7.2	19.1±1.45*	*Pseudomonas syringae*	7.1	25.8±1.62*
Paenibacillus pabuli	7.7	32.9±1.81	*Stenotrophomonas maltophilia*	7.2	36.5±1.67
Cytophaga johnsonae	7.6	28.5±3.25	*Pantoea agglomerans*	7.8	29.4±3.00*
Bacillus megaterium	7.6	18.9±1.90*	*Pseudomonas putida*	7.6	38.1±4.53
Comamonas acidovorans	8.1	30.1±2.43	*Hydrogenophaga pseudoflava*	8.7	39.4±3.16
Chryseobacterium balustinum	7.9	23.8±2.4	*Micrococcus luteus*	7.9	41.3±2.38
Isolate (Set 3)	**Shoot fresh weight**	**No of galls/ g root**	**Isolate (Set 4)**	**Shoot fresh weight**	**No of galls/ g root**
Ringer's solution	6.8	43.4±2.91	Ringer's solution	4.7	79.7±7.62
Nematicide	6.9	2.5±2.50*	Nematicide	6.8	28.5±4.31*
Agrobacterium radiobacter	7.4	23.3±1.00*	*Pseudomonas coronafaciens*	7.1*	57.9±7.78*
Bacillus brevis	7.1	37.7±3.13	*Bacillus mycoides*	6.2	53.1±4.81*
Paenibacillus pabuli	6.4	27.4±2.17*	*Brevibacterium helvolum*	6.4	65.3±5.03
Cellulomonas fimi	5.6	25.6±4.51*	*Micrococcus kristinae*	6.3	60.8±5.61
Cedecea davisae	5.5	42.4±3.64	*Aeromonas salmonicida*	6.6	66.2±5.59
Pseudomonas corrugata	6.2	52.9±5.36	*Arthrobacter ilicis*	6.7	66.6±3.26
Cytophaga johnsonae	7.1	44.1±3.1	*Micrococcus varians*	5.9	64.4±3.24

* indicate significant differences compared with the control of the same set (*P<0.05, n=8*).

3.4 Antagonistic activity of endophytic bacteria against *Meloidogyne incognita in vivo*

The five most active endophytic bacterial isolates applied as a plug dip and soil drench reduced the number of egg masses to varying degrees compared to that of the nematode control (figure 3). The results obtained in the first experiment show that strains of *B. pumilis, B. mycoides* and *P. coronafaciens* caused a significant reduction in the number of egg masses of 30%, 35% and 37%, respectively (figure 3). In the second experiment, *B. megaterium*, *B. pumillus*, and *B. mycoides* significantly reduced the number of egg masses by 31%, 30% and 39%, respectively.

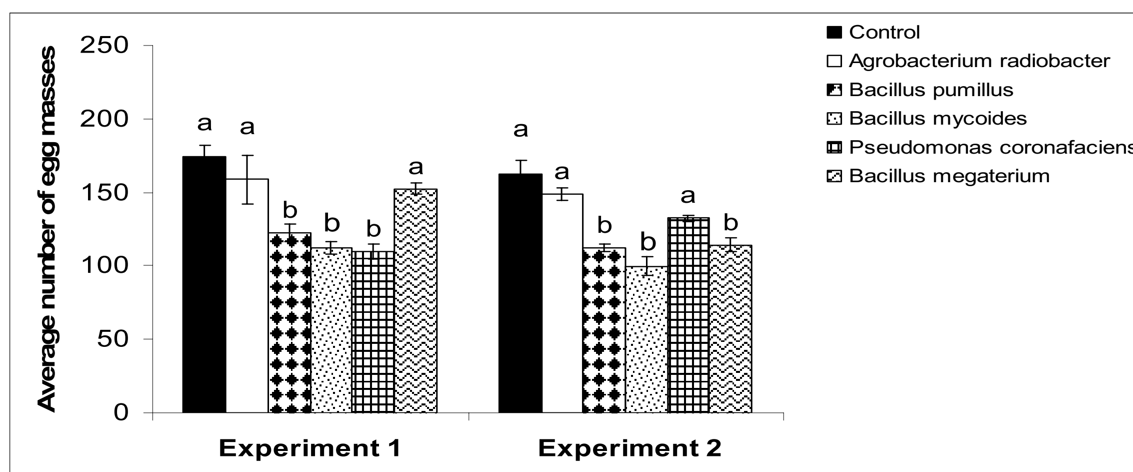

Figure 3: Effect of the endophytic bacteria *Agrobacterium radiobacter, Bacillus pumillus, B. mycoides, Pseudomonas coronafaciens* and *B. megaterium* on the number of egg masses formed by *Meloidogyne incognita* on tomato in replicated experiments. Means in the same experiment with the same letters are not significantly different based on Dunnett's test ($P < 0.05$).

Figure 4 shows the data obtained in replicated experiments studying the influence of the five endophytic bacteria on the number of galls caused by *M. incognita*. The number of galls was reduced between 28% and 34% in experiment 1 and 2 respectively. In experiment 1, *B. mycoides, B. pumillus* and *P. coronafaciens* caused a significant reduction in the number of galls by 28%, 26% and 22%, respectively, when compared to the nematode control. Whereas, a non-significant reduction of number of galls was recorded for *A. radiobacter* and *B. megaterium.*

In experiment 2, the number of galls formed on roots following treatment with *B. mycoides* and *B. pumillus* was 33% and 23% lower, respectively, than on roots treated with *M. incognita* alone. In contrast, plants treated with *A. radiobacter, P. coronafaciens* and *B. megaterium* showed no effect on number of galls compared with the control. In both experiments, *Bacillus mycoides* and *B. pumillus* were the most effective strains compared with the others.

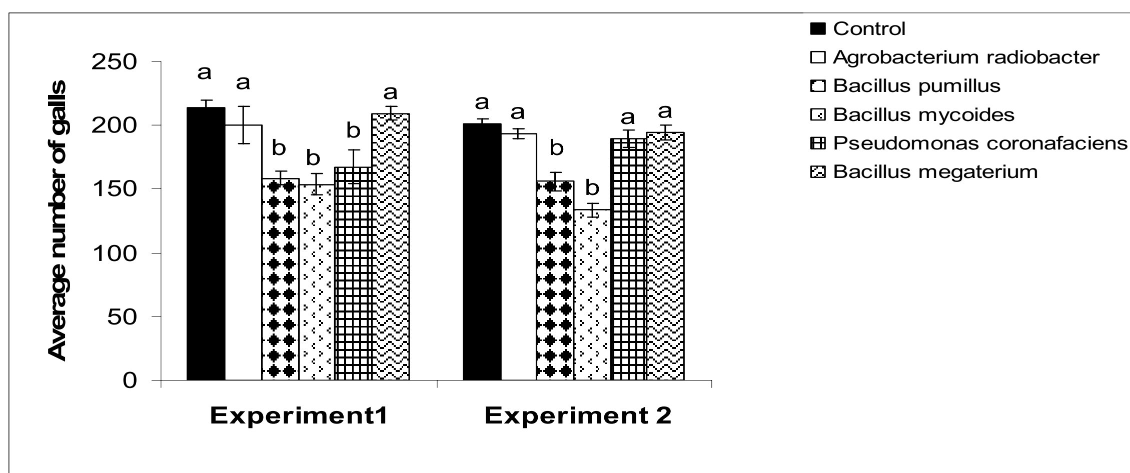

Figure 4: Effect of the endophytic bacteria *Agrobacterium radiobacter, Bacillus pumillus, B. mycoides, P. coronafaciens* and *B. megaterium* on the number of galls formed by *Meloidogyne incognita* on tomato in replicated experiments. Means in the same experiment with the same letters are not significantly different based on Dunnett's test ($P < 0.05$).

Although the values for fresh plant weight measurements were not significant, the data revealed a trend to higher shoot fresh weight for tomato plants treated with *Bacillus mycoides* and *P. coronafaciens* in both experiments and with *B. megaterium* in experiment 2. None of the treatments had an effect on root fresh weight of tomato plants (figure 5).

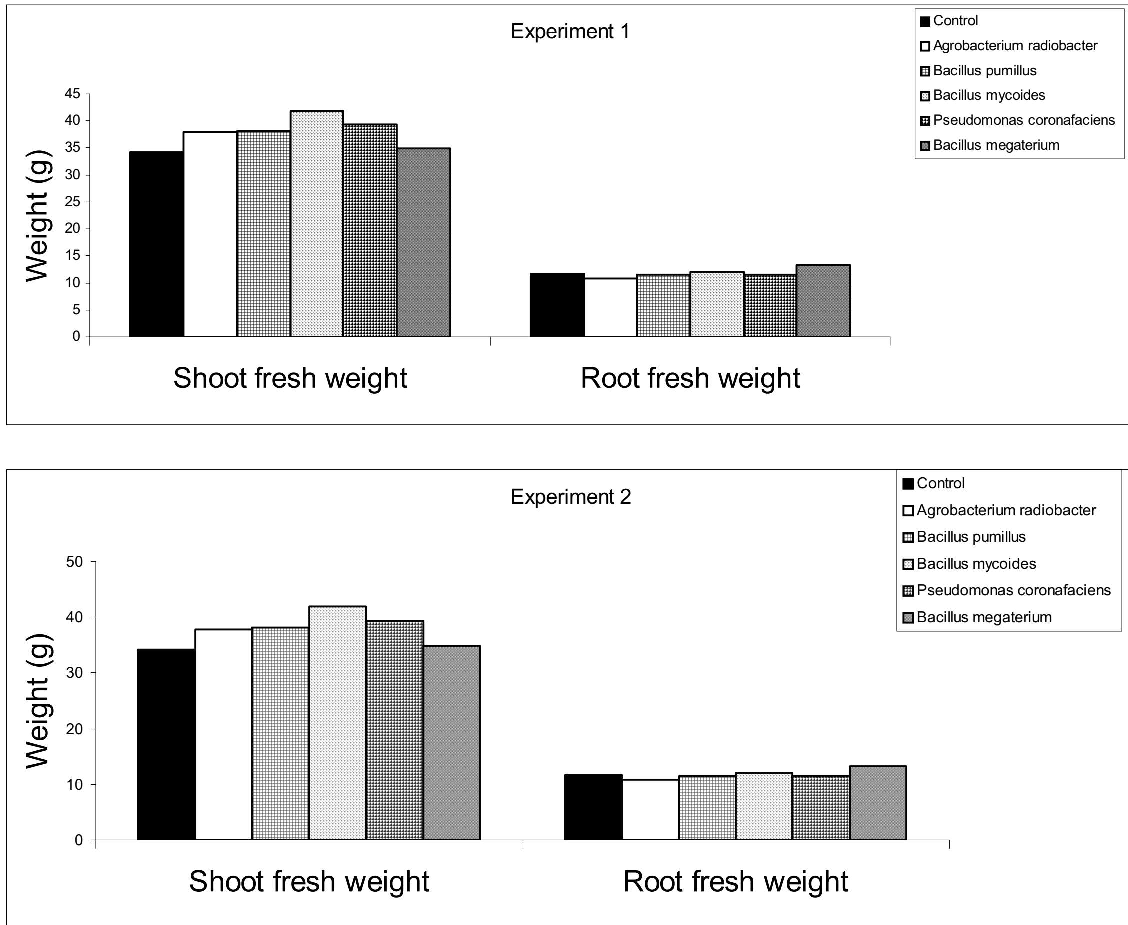

Figure 5: Effect of endophytic bacterial isolates on shoot and root fresh weights of tomato plants eight weeks after inoculation. Treatment means are not significantly different from the control according to Dunnett's test ($P < 0.05$).

4 Discussion

4.1 Isolation and identification of endophytic bacteria

The endophytic bacterial community associated with coffee roots were determined to provide data on their occurrence, distribution and antagonistic potential towards the root-knot nematode *M. incognita*. The recovery of different endophytic bacterial isolates from coffee roots after surface disinfestation with 2.5% (w/v) NaOCl demonstrated that these isolates are endophytic bacteria according to the definition of Hallmann et al., (1997).

It has been suggested that the majority of endophytes originate from the rhizosphere soil and can colonize both the rhizosphere and endorhiza simultaneously. Therefore, many of the modes of action described in the past for rhizobacteria-nematode interaction probably apply directly to endophytic bacteria. The finding in my experiment reflects this hypothesis in that almost all endophytic bacteria detected in coffee were also reported as rhizobacteria in other studies (McInroy and Kloepper, 1995; Sturz et al., 2000; Sikora et al., 2007).

Identification of the endophytic bacteria by fatty acid analysis showed that coffee plants supported a large population of diverse genera and species of endophytic bacteria. Endophytic bacteria have been isolated from many plants such as from sugar beets (Jacobs et al., 1985), pear (Whitesides and Spotts, 1991), oak (Brooks et al., 1994), maize (Fischer et al., 1992; McInroy and Kloepper, 1995) and coffee (Vega et al., 2005).

In my study, 43 and 16 genera were isolated and identified by the MIDI system in the wet and dry sampling seasons, respectively. The spectrum of endophytic bacteria isolated was similar to that found in other studies on different plants from different regions of the world. Vega et al. (2005) reported 87 culturable endophytic bacterial isolates belonging to 19 genera from coffee plants collected from Colombia, Hawaii and Mexico. McInroy and Kloepper, 1995 reported 46 species in 31 genera and 45 species in 32 genera from roots and stems of sweet corn and cotton, respectively. Comparable results were also reported from clover roots, 31 species in 14 genera (Sturz et al.,

1997), in cotton roots, 43 bacterial species of 28 genera (Hallmann et al., 1997) in potato tubers, 24 species of 13 genera (Sturz et al., 1998) and in tomato, 88 species of 51 genera (Munif, 2001). A more comprehensive review on spectrum of endophytic bacteria from roots of various cultivated plants is given by Berg and Hallmann (2006).

The current study demonstrated that *Pseudomonas* was the predominant endophyte with 39% and 57% of the total endophytic bacterial population in the wet and dry season sampling, respectively. The percentage occurrence of the genus *Bacillus* with 11% and 4% in the wet and dry seasons, respectively, was also relatively high compared with the other bacteria. The occurrence of *Pseudomonas* spp. and *Bacillus* spp. is consistent with result of endophytic bacteria isolated from other plants such as cotton and sweet corn (McInroy and Kloepper, 1995), pea (Elvira–Recuenco and van Vuurde, 2000), potato (Garbeva et al., 2001), citrus (Araujo et al., 2001), maize (Seghers et al., 2004), soybean (Kuklinsky-Sobral et al., 2005) and rice (Padgham and Sikora, 2007). The results of my findings on endophytic bacteria in coffee showed that they are very diverse and *Pseudomonas* spp. and *Bacillus* spp. are the most abundant genera.

The population densities of endophytic bacteria isolated from coffee roots ranged from 5.2×10^3 to 2.07×10^6 cfu g^{-1} fresh root during the wet season and from 2.7×10^3 to 1.23×10^4 cfu g^{-1} during the dry season sampling. In other findings, my results showed that the bacterial density is within the range of most endophytic bacterial populations of around 10^3–10^6 cells per gram fresh weight (McInroy and Kloepper, 1995; Hallman et al., 1997). Hallmann et al. (1997) reported that population densities of indigenous endophytic bacteria in roots averaged about 10^5 g^{-1} fresh root weight. The population densities of bacterial endophytes obtained from the roots of different plants ranged from 10^4 to 10^6 cfu g^{-1} for cotton and sweet corn (McInroy and Klopper, 1994), 4.0×10^2 to 1.3×10^4 cfu g^{-1} for cotton (Misaghi and Donndelinger, 1990; Hallmann et al., 1997), 10^6 cfu g^{-1} for potato (Krechel et al., 2002), 10^4 to 10^6 for leguminous tree (Wang et al., 2006).

The diversity of endophytic bacteria differed depending on coffee plantation type. The Shannon diversity index (H') for the different coffee plantation types varied with the highest values being obtained for semi-forest, forest and garden plantations and the lowest for the large scale plantations. The differences in the diversity of endophytic bacteria may reflect the differences in agricultural management practices. Generally, it is only in the large scale coffee plantation type that intensive cultivation practices are undertaken such us application of chemical fertilizers, herbicides and mechanical power. My results show that the diversity of endophytic bacteria is influenced by the intensive agricultural management practices undertaken in large scale farms. Various reports demonstrated that environmental pollution and intensive soil cultivation along with use of mechanical power and agrochemicals affects soil microbial diversity (Giller et al. 1997; Steenwerth et al., 2002; Garbeva et al., 2004). Previous findings also have shown that variations in the composition of endophytic bacteria can be caused by changes in abiotic factors such as temperature, rainfall, cropping practice or soil amendments (Hallmann et al., 1999; Siciliano et al., 2001; Berg and Hallmann, 2006).

4.2 *In vitro* screening of endophytic bacteria culture filtrates against *Meloidogyne incognita*

The culture filtrates of 14 strains showed significant levels of mortality towards *M. incognita* juveniles ranging from 38% to 98%. These strains were: *Agrobacterium radiobacter, Bacillus pumillus, B. brevis, B. megaterium, B. mycoides, B. Licheniformis,* Chryseobacterium balustinum, *Cedecea davisae, Cytophaga johnsonae, Lactobacillus paracasei, Micrococcus luteus, M. halobius, Pseudomonas syringae* and *Stenotrophomonas maltophilia*. The *in vitro* screening method enabled efficient screening of many isolates with a minimum requirement on labor and time. It was apparent from the results that the various isolates tested had different nematicidal activity towards *M. incognita* juveniles.

Similar tests by Becker et al. (1988), who screened more than 5000 rhizobacteria revealed the presence of detectable compounds that affected the vitality of *M. incognita*

juveniles *in vitro*. Ali et al. (2002) tested culture filtrates of 20 *Pseudomonas* spp. towards *M. javanica* juveniles *in vitro*. Seventy percent of the strains were reported to produce > 25% juvenile mortality of *M. javanica* and 30% of these caused > 50% juvenile mortality. Tian et al. (1995) reported that out of the 833 rhizosphere bacteria isolated from vegetable crops, 10 of them caused above 90% inactivation *in vitro* after 24 h exposure. Some strains were also reported causing high mortality of *Xiphinema thornei, Trichodorus pakistanensis, Scutellonema clathricaudatum, Criconemoides profusus* and *M. hapla* after 6 h of exposure. Spiegel et al. (1991) reported that culture filtrates of *Pseudomonas chitinolytica* caused high levels of juvenile mortality of *M. javanica*.

The number of effective strains (35% of the total strains) in my studies with respect to their nematicidal activity is far greater than that detected in other studies. For rhizosphere bacteria, it has been reported that 1% of the total number of bacteria screened showed antagonistic activity towards *M. incognita* (Becker et al., 1988), 7.2% towards *Heterodera schachtii* (Oostendorp and Sikora, 1989), 9% towards *Globodera pallida* (Racke and Sikora, 1992), 12% against *M. incognita* (Zavaleta-Meija and Van Gundy, 1982), and 6.1% towards *M. javanica* (Siddiqui et al., 2001). Sikora (1992) also reported that 7%-10% of the rhizosphere bacteria isolated from potato, sugar beet or tomato root systems have antagonistic activity against cyst and root-knot nematodes.

4.3 Preliminary *in vivo* screening of endophytic bacteria towards *Meloidogyne incognita*

Endophytic bacteria isolated from coffee roots were screened in the greenhouse for their antagonistic effect towards *M. incognita* on tomato. This assay for biological control activity allows selection of multiple mechanism of antagonism other than pure antibiosis *in vitro*. Ten of the 28 strains (36%) screened showed antagonistic activity towards to *M. incognita*. The percentage of endophytic bacteria in my studies with antagonistic effects was higher compared with similar studies. Hallmann et al. (1995) reported that 10% of the evaluated isolates of endophytic bacteria isolated from cucumber and cotton roots were active against the root-knot nematode, *M. incognita.* They found a significant

reduction of 50% in the number of galls on cucumber. Munif et al. (2000) screened several endophytic bacteria isolated from tomato roots under greenhouse conditions and reported that 12% of the tested 181 endophytic bacteria had antagonistic properties towards *M. incognita*.

In my study, strains of *Agrobacterium radiobacter, Bacillus licheniformis, B. pumillus, B. mycoides, B. megaterium, Pseudomonas syringae P. coronafaciens, Pantoea agglomerans, Paenibacillus pabuli* and *Cellulomonas fimi* significantly reduced the number of galls caused by *M. incognita*. The results are comparable with other findings which showed that one or more of the above mentioned bacterial species are antagonistic toward different nematode species (Becker et al., 1988; Oostendorp and Sikora, 1989; Kloepper et al., 1992; Hallmann et al., 1998; Hasky-Gunther et al., 1998; Neipp and Becker, 1999; Siddiqui and Ehteshamul-Haque, 2000; Ali et al., 2002).

4.4 Antagonistic activity of endophytic bacteria against *Meloidogyne incognita in vivo*

Bacillus pumillus and *B. mycoides* were the most effective strains in reducing both nematode infection parameters i.e. number of galls and number of egg masses. The number of galls produced represent the number of nematodes that successfully invaded the root and developed to adults, while the number of egg masses represents the presence of egg-laying females which indicates rate of development. The significantly lower number of egg masses (up to 39%) and galls (up to 33%) in plants inoculated with *B. pumillus* and *B. mycoides* confirms reports on the ability of endophytic bacteria to inhibit plant-parasitic nematodes in the root (Zavaleta-Meija and Van Gundy, 1982; Sikora, 1988; Oostendorp and Sikora, 1989, 1990; Spiegel et al., 1991; Kloepper et al., 1992; Santhi and Sivakumar, 1995; Keuken, 1996, Hallmann et al., 1997).

Among the rhizobacteria tested, *Pseudomonas* spp. and *Bacillus* spp. appear to have the greatest potential as biocontrol agents as they are dominant in the rhizosphere (Mahaffee and Kloepper, 1997).

Certain *Bacillus* spp. can protect plants against plant-parasitic nematodes and they have been proposed for use as biocontrol agents. For instance, *Bacillus cereus* reduced the number of galls and egg masses up to 50% caused by *M. incognita, M. javanica* and *M. arenaria* (Mahdy et al., 2000). Padgham and Sikora (2007) reported a reduction of greater than 40% in nematode penetration and gall formation after treatment with *B. megaterium* against *Meloidogyne graminicola* on rice.

Species of *Bacillus* are also good candidates for biocontrol because they produce endospores that are tolerant to heat and desiccation (Weller, 1988; Kloepper et al., 2004), and can therefore be stored as commercial products. The mechanisms responsible for the reduction in gall and egg mass number by *B. pumilus* and *B. mycoides* might be similar to those reported for other endophytic and rhizosphere bacteria. This includes reduced penetration, direct antibiosis through toxic metabolites or induced systemic resistance (Sikora et al., 2007).

Kloepper and Ryu (2006) reviewed the effect of different endophytic bacterial strains that have showed induced systemic resistance. Among those listed, *Bacillus pumilus* is reported as one of the most important endophytes to elicit induced systemic resistance towards various plant diseases. Bargabus et al. (2002; 2004) found two strains of *B. pumilus* and one of *B. mycoides* that reduced the severity of Cercospora leaf spot of sugar beet, caused by *Cercospora beticola*.

My results clearly demonstrate that coffee hosts several endophytes belonging to different taxonomic groups that exhibit properties important for biocontrol research. The proportion of strains showing biocontrol activity towards *M. incognita* was comparably high. However, only direct antibiosis and antagonistic effects were tested. These strains may exhibit other indirect mechanisms, such as induction of systemic resistance. Therefore, further studies have to be conducted for a better understanding of their mechanisms of action and subsequent successful use in biocontrol.

5 Conclusions

Studies on the indigenous populations of endophytic bacteria associated with coffee and their antagonistic activity towards the most economically important nematode species, *Meloidogyne incognita* was studied. The conclusions from this study are:

1. This is the first report of the isolation, identification and characterization of endophytic bacteria from coffee in its center of origin.
2. Coffee plants were found to host a diverse spectrum of endophytic bacteria. The most abundant genera were to *Pseudomonas, Bacillus, Agrobacterium, Stenotrophomonas* and *Entrobacter.*
3. Out of the 40 endophytic bacteria tested, 14 isolates caused *in vitro* mortality of *M. incognita* juveniles between 38% to 98% compared to the controls.
4. Consistent *in vivo* reductions in the number of egg masses and the number of galls were obtained with isolate of *Bacillus pumillus* and *B. mycoides.*
5. The positive results of the *in vitro* and *in vivo* tests demonstrated that the biological control potential and future practical significance of these endophytes in controlling root-knot nematodes in coffee and other crops.

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Annex 1: Occurrence of plant-parasitic nematodes in coffee fields of Ethiopia (+ and – shows presence and absence of the respective nematode genera)

Localities	*No of samples (132)*	*Helicotylenchus*	*Scutellonema*	*Rotylenchus*	*Xiphinema*	*Tylenchorhynchus*	*Hoplolaimus and Pratylenchus (++)*	*Ditylenchus (+) and Meloidogyne(++)*	*Trophurus*	*Criconemella*	*Heterodera (+) and Cephalenchus(++)*
Sororo	2	-	-	-	-	-	-	-	-	-	-
Asebe teferi	3	+	-	-	-	-	-	-	-	-	-
Bedesa	5	+	-	-	-	+	-	++	+	-	-
Mechara	5	+	-	+	+	+	-	-	-	+	-
Mesela	5	-	-	+	-	+	++	-	-	-	+
Deder	5	-	-	-	-	-	-	-	-	-	-
Chelenko	3	-	-	-	-	-	-	-	-	-	-
Harar	2	-	-	-	-	-	-	-	-	-	-
Belewa	3	+	-	-	-	-	-	-	+	-	-
Dire Dawa	3	-	-	-	-	+	-	-	-	-	-
Limu	6	+	-	-	-	-	-	-	-	-	-
Agaro	4	+	+	-	+	+	-	-	-	-	+
Jimma Seka	4	+	-	+	+	-	-	-	-	-	-
Shebe	4	+	-	-	-	+	-	-	-	+	-
Bonga wusush	5	+	+	-	+	+	-	-	-	+	-
Chena	3	+	-	-	+	-	-	-	-	-	-
Temenja yaji	5	+	-	+	+	-	-	-	-	-	-
Mizan	3	+	+	+	+	-	-	-	-	+	-

Bebeka	3	-	-	-	-	-	-	-	-	-	-
Tepi	10	+	-	+	+	+	+	-	-	+	++
Gore	5	+	-	-	+	-	-	-	-	-	-
Metu	4	+	-	+	+	-	-	-	-	+	-
Hosaina	5	+	-	+	-	+	-	-	+	-	-
Areka Hossaina	5	+	+	-	-	-	-	+	-	-	-
Areka	5	-	+	+	+	-	-	-	-	-	-
BoditiShone	5	+	+	-	-	-	-	-	-	-	-
AletaWondo	5	+	+	+	+	-	-	-	-	-	-
Aposto	5	+	-	-	-	+	-	-	-	-	-
Abela Morocho	5	+	+	-	-	-	-	-	-	-	+
Wondogenet – Tola	5	+	-	-	-	+	-	-	-	-	-

Annex 2: Percent occurrence of endophytic bacteria isolated from different coffee growing agroecologies in Ethiopia in the wet and dry seasons

Bacterial species	Wet season		Dry season		Bacterial species	Wet season		Dry season	
	No of isolate	% occurrence	No of isolate	% occurrence		No of isolate	% occurrence	No of isolate	% occurrence
Acinetobacter baumannii	1	0.50	0	0	*Kluyvera cryocrescens*	0	0	3	2.63
Acinetobacter calcoaceticus	4	1.99	0	0	*Kluyevera ascorbata*	1	0.50	0	0
Acinetobacter johnsonii	1	0.50	0	0	*Lactobacillus paracasei*	1	0.50	0	0
Acinetobacter lwoffii	1	0.50	0	0	*Micrococcus halobius*	2	1.00	0	0
Aeromonas salmonicida	2	0.50	0	0	*Micrococcus lylae*	0	0	3	2.63
Agrobacterium radiobacter	1	0.50	11	9.65	*Micrococcus kristinae*	1	0.50	0	0
Arthrobacter ilicis	3	1.49	0	0	*Micrococcus luteus*	4	1.99	0	0
Bacillus brevis	2	1.00	0	0	*Micrococcus varians*	1	0.50	0	0
Bacillus licheniformis	2	1.00	0	0	*Pantoea agglomerans*	6	2.99	0	0
Bacillus megaterium	7	3.48	4	3.51	*Pantoea agglomerans*	0	0	3	2.63

Bacillus mycoides	3	1.49	0	0	*Paenibacillus pabuli*	2	0.50	0	0
Bacillus pumillus	7	3.48	0	0	*Paracoccus denitrificans*	1	0.50	0	0
Bacillus sphaericus	1	0.50	0	0	*Pseudomonas chlororaphis*	20	9.95	17	14.95
Brevibacterium helvolum	1	0.50	0	0	*Pseudomonas coronafaciens*	1	0.50	0	0
Shigella dysenteriae	0	0	1	0.88	*Pseudomonas aeruginosa*	0	0	2	1.75
Burkholderia cepacia	1	0.50	0	0	*Pseudomonas corrugate*	6	2.99	0	0
Flavobacterium resinovorum	1	0.50	0	0	*Pseudomonas fluorescens*	16	7.96	6	5.26
Cedecea davisae	1	0.50	0	0	*Pseudomonas putida*	27	13.43	25	21.93
Cellulomonas fimi	1	0.50	0	0	*Pseudomonas syringae*	8	3.98	16	14.04
Cellulomonas gelida	1	0.50	0	0	*Salmonella typhimurium**	1	0.50	0	0
Chryseobacterium balustinum	9	4.48	0	0	*Salmonella typhi*	0	0	1	0.88
Chryseobacterium indologenes	1	0.50	0	0	Stenotrophomonas maltophilia	5	4.5	8	7.02
Comamonas	5	2.49	0	0	Variovorax	1	0.50	0	0

acidovorans					paradoxus*				
Cytophaga johnsonae	9	4.48	0	0	*Xanthomonas campestris**	3	1.49	0	0
Entrobacter tylorae	0	0	3	2.63	*Xanthobacter agilis*	0	0	1	1.75
Erwina carotovora	0	0	2	1.75	*No match & unidentified*	30	14.93	6	5.26
Enterobacter intermedius	0	3	2.63	2.63	Total number of isolate	201	100.00	114	100
Hydrogenophaga pseudoflava	1	0.50	0	0					

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