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Root-knot nematodes: abundance in organic farming, differentiation among populations, microbes attached to juveniles in soil, and bacterial antagonists

Dissertationen aus dem Julius Kühn-Institut

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I dedicate this thesis to my late mother and my father, who will always be in my heart.

Mohamed Adam Braunschweig, January 2014

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SUMMARY

Two surveys were conducted to determine the frequency and abundance of plant-parasitic nematodes associated with different crops at an organic farm in Egypt, during autumn 2009 and 2011. Eleven genera of plant-parasitic nematodes were detected. Root-knot nematodes (*Meloidogyne* spp.) showed the highest abundance and frequency of all plant-parasitic nematodes during the two surveys. Commonly detected genera were *Tylenchorhynchus, Rotylenchulus, Helicotylenchus* and *Pratylenchus*. Further studies on *Meloidogyne* were carried out with regard to discrimination among populations, attachment of microbes to juveniles (J2) in soil, and biocontrol through bacterial strains which were known as antagonists of fungal pathogens.

Meloidogyne incognita populations and/or races that showed differential pattern of reproduction on a set of host plants, could be differentiated based on a newly developed PCR-DGGE system to electrophorecically separate variants of the pathogenicity gene *msp1*.

Three arable soils from different regions of Germany were shown to vary in the suppressiveness of their indigenous microbial communities against *Meloidogyne hapla*. Attachment of microbes to J2 in these three soils was investigated by cultivation-independent methods to identify those which specifically interacted with J2 in the most suppressive soil (Kleinwanzleben). The three soils differed in the microbes attached to J2. PCR-DGGE fingerprints of amplified ITS fragments or 16S rRNA genes showed many fungi and bacteria that were abundant on J2 but not in the surrounding soil, some of which seemed to be present in all three soils while most were soil type specific. Many bacteria associated with J2 from the most suppressive soil were closely related to infectious species like *Shigella* spp., while most abundant were *Malikia spinosa* and *Rothia amarae* as determined by 16S rRNA gene amplicon pyrosequencing.

Nematode-fungus disease complexes can cause dramatic synergistic yield losses. Bacterial strains known as antagonists of phytopathogenic fungi were evaluated with respect to their biocontrol potential towards *M. incognita*. Seed inoculation with most of the strains significantly reduced propagation of nematode on tomato roots. The best strains *Bacillus subtilis* Sb4-23, Mc2-Re2, and Mc5- Re2 were further studied for their mode-of-action. The strains were able to affect the nematodes

directly by metabolites present in culture supernatants and indirectly by induced systemic resistance of the plant. Experimental comparison of direct and plant-mediated effects suggested that the latter was the major control mechanism of these antagonists. Overall, these findings may improve the basis for integrated management strategies of root-knot nematodes in organic farming.

ZUSAMMENFASSUNG

In zwei umfassenden Untersuchungen im Herbst 2009 und 2011wurde die Verbreitung und die Abundanz pflanzenparasitärer Nematoden an den verschiedenen Feldfrüchten in einer Bio-Farm in Ägypten erfasst. Insgesamt wurden elf Genera gefunden, von denen Wurzelgallen-Nematoden (*Meloidogyne* spp.) in beiden Untersuchungen am häufigsten und mit der höchsten Anzahl in den Proben gefunden wurde. Weitere oft gefundene Genera waren *Tylenchorhynchus, Rotylenchulus, Helicotylenchus* und *Pratylenchus*. Davon ausgehend beschäftigten sich die weiteren Arbeiten mit *Meloidogyne,* und zwar mit der Differenzierung von Populationen, der Anheftung von Mikroorganismen an die Juvenile (J2) im Boden, und der biologischen Kontrolle durch Bakterienstämme, die als Antagonisten von pilzlichen Pathogenen bekannt sind.

Die untersuchten Populationen und/oder Rassen von *Meloidogyne incognita* zeigten phänotypische Unterschiede in ihren Vermehrungsmustern an einem Set von Wirtspflanzen. Damit einhergehend konnten auch genetische Unterschiede anhand einer neu entwickelten Methode zur Amplifikation des Pathogenitätsgens *msp1* und der elektrophoretischen Auftrennung der PCR-Produkte von Genvarianten im Denaturierungsgradienten (DGGE) nachgewiesen werden.

Für drei Ackerböden aus verschiedenen Regionen in Deutschland wurde unterschiedliche Suppressivität ihrer mikrobiellen Gemeinschaften gegen *Meloidogyne hapla* im Gewächshaus gezeigt. Mit Kultivierungs-unabhängigen Methoden wurde untersucht, welche Mikroorganismen an die J2 in den Böden anheften, um die zu identifizieren, die mit J2 im suppressivsten der drei Böden spezifisch interagierten (Kleinwanzleben). Die Mikroorganismen, die an den J2 nach Inkubation im Boden haften blieben, unterschieden sich zwischen den drei Böden. In PCR-DGGE Fingerprints von ITS-Fragmenten bzw. 16S rRNA Genen wurden viele Pilze und Bakterien detektiert, die an den J2 aber nicht im umgebenden Boden abundant waren. Während sich einige davon in allen drei Böden an den J2 anreicherten, waren andere spezifisch für einen Bodentyp. Mittels Pyrosequenzierung von 16S rRNA Gen-Amplikons konnten die mit J2 im suppressivsten Boden assoziierten abundantesten Bakterienarten beschrieben werden.

Viele davon waren verwandt mit infektiösen Arten wie *Shigella* spp., während *Malikia spinosa* und *Rothia amarae* am häufigsten detektiert wurden.

Krankheitskomplexe aus Nematode und Pilz können erhebliche synergistische Ertragsverluste verursachen. Bakterienstämme, die als Antagonisten von phytopathogenen Pilzen bekannt sind, wurden auf ihr Potential zur biologischen Kontrolle von *M. incognita* untersucht. Sameninokulation führte bei den meisten Stämmen zu einer signifikanten Reduktion der Vermehrung des Nematoden an Tomatenwurzeln. Für die drei besten Isolate, *Bacillus subtilis* Sb4-23, Mc2-Re2 und Mc5- Re2, wurden die zugrundeliegenden Mechanismen untersucht. Die Stämme konnten den Nematoden sowohl direkt durch Metabolite beeinträchtigen, die im Kulturüberstand zu finden sind, als auch indirekt über Induktion systemischer Resistenz der Pflanze. Im experimentellen Vergleich der direkten und Pflanzen-vermittelten Effekte zeigte sich, dass letzteres der dominierende Kontrollmechanismus dieser Antagonisten ist. Zusammen genommen könnten diese Befunde als Basis für eine verbesserte Strategie zum integrierten Management von Wurzelgallen-Nematoden im biologischen Landbau dienen.

Chapter I

General introduction and thesis outline

Root-knot nematodes (*Meloidogyne* spp.)

Distribution and economic importance

Root-knot nematodes (RKN) are plant parasites of the genus *Meloidogyne*, family *Heteroderidae*. Their common name refers to the characteristic galls or root-knots, associated with nematode infestation. This genus comprises more than 90 species, with some species having several races (Karssen, 2002). It also includes some of the most widespread and economically damaging nematodes worldwide, like *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, *M. chitwoodi* and *M. enterolobii*. Root-knot nematodes occur throughout the world with some species being primarily distributed in tropical and sub-tropical climates such as *M. incognita*, *M. javanica*, *M. arenaria* while others are well adapted to temperate or cool climates such as *M. hapla*, *M. chitwoodi* and *M. fallax*.

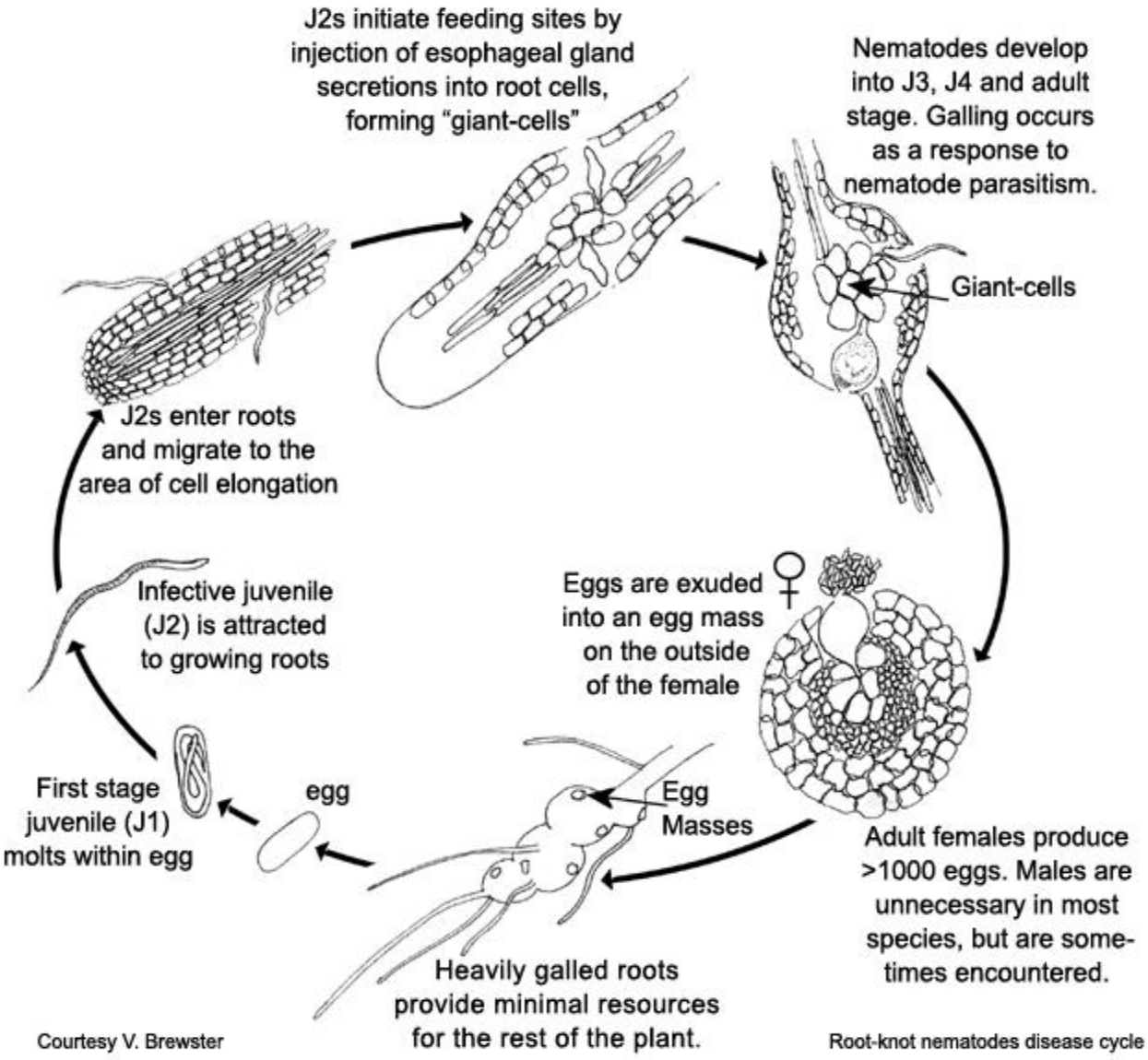
Overall, the most common species together parasite more than 5500 plant species, including annual and perennial crops (Trudgill and Blok, 2001). Their worldwide damage in terms of reduced yield and quality is estimated to be > $US 100 billion/year (Bird and Kaloshian, 2003). The damage symptoms caused by nematode infection may be apparent on parts of the plant both above and below the ground. Above-ground symptoms include varying degrees of stunting, yellowing of foliage, wilting, symptoms of nutrient deficiency and distortion of plant parts. Below-ground symptoms include galls, lesions, stunting, malformation, biforking and excessive formation of side roots (root beard). Severe infestation can cause plant death. Furthermore, nematode invasion provides entrance points for secondary pathogens such as soil-borne fungi or bacteria that can course synergistic yield losses.

Biology

Mature females of RKN remain with their head in the galled root tissue and deposit up to 1000 eggs into a gelatinous matrix (egg sac or egg mass) that is protruding to the posterior end of the female on the root surface (Fig. 1). The gelatinous matrix protects the eggs from dehydration and attack by microorganisms (Sharon and Spiegel, 1993).

Within eggs the embryo rapidly develops to a first-stage juvenile (J1) that molts within the egg to the infectious second-stage juvenile (J2). After hatching the J2 migrates through the soil in search of a suitable host which can be the parent plant or a nearby new host. Juveniles invade the root in the zone of elongation. With the help of their stylet they burrow into the root with no obvious damage. From the site of penetration juveniles migrate intercellularly towards the root tip where they turn around and move into the differentiating vascular cylinder (Wyss *et al.*, 1992; Abad *et al.*, 2003). Once they have reached the zone of protoxylem development they initiate a feeding by injecting pharyngeal gland secretions (the saliva) into root cells, which induces nuclear division without cytokinesis (Gheysen and Fenoll, 2002). This process gives rise to large, multinucleate cells, causing dramatic physiological changes in the parasitized cells, transforming them into giant cells, which seriously impair plant nutrient and water uptake (Trudgill and Blok, 2001).

Concomitant with giant cell formation, the surrounding plant cells enlarge and divide rapidly, resulting in the formation of a gall (Williamson and Hussey, 1996). At the site of penetration and/or gall formation secondary roots can form resulting in extensive formation of lateral roots. With further juvenile development, the metabolic capacity of the feeding site increases funnelling plant resources to the feeding nematode (Williamson and Gleason, 2003; Caillaud *et al.*, 2008). Within few days of feeding, the J2s grow thick like a sausage and undergo three more molts before transforming into adults**.** The fourth-stage juvenile (J4) already distinguishes between either male or female. The vermiform males emerge from the root and migrate into the soil. Females keep swelling to become saccate or pear-shaped. At this stage, the female is large enough to be seen with the naked eye when galled root tissue is teased apart. Although sexual propagation does occur in some RKN species, main propagation is by parthogenesis (Castagnone-Sereno, 2006). With egg deposition the cycle is completed. Under favourable condition, the life cycle may be completed within 30 days and several generations can develop in one season.


J2s initiate feeding sites by injection of esophageal gland secretions into root cells, forming "giant-cells"
Nematodes develop into J3, J4 and adult stage. Galling occurs as a response to nematode parasitism.
Giant-cells
J2s enter roots and migrate to the area of cell elongation
Infective juvenile (J2) is attracted to growing roots
Eggs are exuded into an egg mass on the outside of the female
First stage juvenile (J1) molts within egg
egg
Egg Masses
Adult females produce >1000 eggs. Males are unnecessary in most species, but are sometimes encountered.
Heavily galled roots provide minimal resources for the rest of the plant.
Courtesy V. Brewster
Root-knot nematodes disease cycle

Nematode problems in organic farming

Demand for organically grown food is increasing throughout the world, as a result of increasing concerns regarding food safety and environmental protection. This trend became a common perception amongst the general public and reflects the extent of consumers worrying about synthetic fertilizer, pesticides and their residuals routinely found in non-organic produce. The total market value of organic products reached over US-$55 billion in 2009 (Paull, 2011) and sales increased by over five billion US dollars per year (Willer *et al.*, 2009) Also, the land devoted to organic agriculture worldwide has increased in the years 2001-2011 from 15.8 million hectares to 37.2 million hectares at a compounding rate of 8.9% per annum (Paull, 2011).

Organic agriculture is a defined by the International Federation of Organic Agriculture Movements (IFOAM) as *"a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved" (IFOAM 2009).* Organic farming avoids or largely excludes synthetic inputs such as fertilizers, pesticides, hormones, feed additives etc to maintain the vitality of the soil as the basis for sustainable productivity, and relies as much as possible on natural processes and cycles for managing pests, diseases, weeds and crop nutrition. Like in conventional farming, continuing competition in global market requires that organic farmers supply high-quality, disease-free produce with an acceptable shelf-life. Disease management is therefore a critical consideration to the success of the organic farm. They depend on exclusive agronomic practices (e.g. crop rotation, green manure and compost) and natural pesticides (e.g. biocontrol agents or pesticide derived from organic sources) to manage soil productivity and soil-borne diseases which in terms of nematode control can be very challenging.

The management of PPN is difficult, especially in organic farming systems compared to foliar diseases and insect pests, because feasible control methods and

monitoring systems are not always available (Oka *et al.*, 2007). Nematodes mostly inhabit the soil and usually attack the underground parts of the plants, causing symptoms comparable to water or nutrition deficiency making it difficult to diagnose the disease (Stirling, 1991). Organic farmers struggle with nematode problems using cultural, physical and biological control methods, especially during the transition period from conventional to organic farming (van Bruggena and Termorshuizen, 2003). However, in other cases, problems with plant-parasitic nematodes started 5 to 10 years after conversion to organic system (Hallmann *et al.*, 2007). Reasons for nematode build-up under organic farming conditions are manifold, such as continuous growth of plants that provide food for PPN throughout the year; high incidence of legumes in the rotation for nitrogen fixation but being also an excellent host for RKN, and insufficient weed control creating a reservoir for RKN even when no host plant is grown. It is assumed that PPN should not be a problem in well-managed, long-term organic farms as stimulating soil life by organic means will enhance the antagonistic potential in the soil thus reducing PPN (Freckman, 1988; Griffiths *et al.*, 1994; Hallmann *et al.*, 2007). However, the question remains if this is really the case. Based on Hallmann *et al.* (2007) organic conditions can even stimulate nematode problems, at least under certain conditions. The overall data basis is too little to allow general conclusions. Information is especially missing for the tropical/subtropical regions. In this respect, the present study evaluates the current status of plant-parasitic nematodes on an organic farm in Egypt (chapter II).

Discrimination of *Meloidogyne* populations

Proper nematode management requires accurate information on the species, race or even virulence of a given nematode population causing the crop damage (Adam *et al.*, 2007; Robertson *et al.*, 2009). Most *Meloidogyne* species can be identified based on distinctive morphological characters and host ranges. However, some species are morphological very similar and even difficult to be distinguished by expert taxonomist. Furthermore, races or populations of a given species are morphologically very similar or even identical (Robertson *et al.*, 2009), requiring different tools to distinguish them

(Hussey, 1990). The North Carolina Differential Host Test is commonly used in identifying the races of the four major species, i.e. four races of *M. incognita*, two races of *M. javanica*, two races of *M. arenaria* and two races of *M. hapla* (Taylor and Sasser, 1978; Hartman and Sasser, 1985). Various populations/races of *Meloidogyne* spp. have been differentiated into virulent (aggressive) and avirulent (nonaggressive) based on their reproduction on different cultivars (Anwar and McKenry, 2007; Cortada *et al.*, 2008; Olowe, 2010). However, the identification of root-knot nematode populations/races based only on the differential host test can be uncertain in cases of mixed populations or occurrence of atypical populations (Fargette, 1987). Therefore this method should only be used in conjunction with morphological, biochemical or molecular assays (Hartman and Sasser, 1985). Identification of root-knot nematode species by isozyme analysis is an effective method, but it requires females which are usually not available in soil samples (Esbenshade and Triantaphyllou, 1990).

Recently, molecular techniques have been developed that overcome the limitations of classical diagnostic techniques and are more reliable and less time-consuming. Commonly used molecular methods for identifying root-knot nematode populations are based on the detection of DNA polymorphisms between species, such as restriction fragment length polymorphism (RFLP) of the amplified ITS region, random amplified polymorphic DNA (RAPD), satellite DNA probes, sequence characterized amplified regions (SCARs) primers, real-time PCR and high-resolution melting curve (HRMC) analysis (Adam *et al.*, 2007; Holterman *et al.*, 2012). The RAPD technique has been used to estimate the genetic relationship between individuals, populations and species of the major four *Meloidogyne* species, however, the detected intraspecific polymorphisms were rather low (Cenis, 1993; Chacon *et al.*, 1994; Guirao *et al.*, 1995). PCR-RFLP was a useful tool for differentiating six *Meloidogyne* species based on restriction site polymorphism (Fargette *et al.*, 1996; Stanton J *et al.*, 1997; Powers, 2004). The RAPD-marker specific to some species of RKN were selected to convert them into SCAR primers for identifying the species (Meng *et al.*, 2004; Williamson *et al.*, 1997; Zijlstra *et al.*, 2000). Microsatellites or Simple Sequence Repeats (SSRs) that are 1-6 base pair (bp) nucleotide motifs randomly dispersed throughout the genome could be served as

taxonomic markers to identify the species, such as the (GAAA) microsatellite region defined in *M. artiellia* by De Luca *et al* (2002). The real-time PCR assay was a rapid and precise method for the detection and quantification of *M. chitwoodi*, *M. fallax* (Zijlstra and van Hoof, 2006) and *M. incognita* (Toyota *et al.*, 2008). Moreover, HRMC analysis has been used successfully in distinguishing *M. chitwoodi*, *M. fallax* and *M. hapla* from each other and the group of the tropical species based on the second intergenic spacer (IGS2) region (Holterman *et al.*, 2012).

Denaturing gradient gel electrophoresis (DGGE) is a molecular tool that can be used to display differences in DNA sequences or mutations of various genes. The principle of this method is that sequence differences often cause a change in the melting behaviour of DNA fragments; therefore, DNA fragments of the same length but differing in sequence can be separated at different positions on the gel. DGGE was shown to be a powerful tool to reveal different sequence types of ITS-2 within and among geographical isolates of trichostrongyloid species (Gasser *et al.*, 1996) or to distinguish species of *Steinernema* from a mixed laboratory culture (Foucher and Wilson, 2002). Previous studies using DGGE focused on assessing soil or marine nematode communities (Bhadury *et al.*, 2006; Waite *et al.*, 2003; Foucher *et al.*, 2004; Cook *et al.*, 2005; Okada and Oba, 2008), but its potential to distinguish populations of plant-parasitic nematodes has not yet been explored. Here PCR-DGGE technique has been used to differentiate among populations/races of *M. incognita* (chapter III).

Suppressive soil

In biological control of PPN, the utility of nematode-suppressive soils is widely accepted (Stirling, 1991). Such soils have been described as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (Cook and Baker, 1983). In suppressive soils, it is expected that there are beneficial microorganisms that suppress plant pathogens. Nematode-suppressive soils are often first recognized when population densities of the nematodes decline or do not increase despite a susceptible host and

suitable environmental conditions. Ferris *et al.* (1976) observed that population densities of *M. incognita* were low in old peach orchards in California despite presence of a good host and favourable climatic conditions for nematode reproduction. Further in-depth studies identified the fungus *Dactylella oviparasitica* as the suppressive agent (Stirling and Mankau, 1978; Stirling *et al.*, 1979). Soils with specific suppressiveness to PPN are of interest to identify nematode-antagonistic microorganisms that could be developed to biocontrol agents. Moreover, understanding the ecological factors enabling these antagonists to persist, compete and function might help to optimize integrated management strategies of PPN (Bent *et al.*, 2008). *Meloidogyne incognita* egg masses and *H. glycines* cysts were analyzed to identify microbes specifically interacting with nematodes in suppressive soils based on culture-independent methods (Nour *et al.*, 2003; Yin *et al.*, 2003; Bent *et al.*, 2008). Although a broad range of bacteria and fungi can parasitize PPN, only few groups of microorganisms are associated with suppressiveness, such as egg-parasitic fungi, nematode-trapping fungi, endoparasitic fungi (Gray, 1985; Kerry, 1988; Carris *et al.*, 1989; Kim and Riggs, 1991; Stirling, 1991; Westphal and Becker, 2001) and obligate endoparasitic bacteria (Stirling, 1991; Weibelzahl-Fulton *et al.*, 1996). While nematode suppressiveness against cyst nematodes has been widely reported, only little is known about suppressiveness against root-knot nematodes (Bent *et al.*, 2008). To further explore this aspect in the present study, different soils were tested for their suppressiveness against *M. hapla* (chapter IV).

Microbe-nematode interaction

The soil around roots that forms the rhizosphere represents a preferable habitat for bacteria, fungi, viruses, protozoa and nematodes. About 30% of the carbon assimilated by the plant is released into the rhizosphere (Lynch and Whipps, 1990), leading to support microbial activity that may be 60 times greater for bacteria and 12 times greater for fungi compared with the bulk soil. Therefore bacteria-feeding nematodes and fungal-feeding nematodes are more abundant in this zone than in the bulk soil (Griffiths, 1989; Henderson and Katznelson, 1961). Furthermore, root exudates contain

compounds attractive to plant-parasitic nematodes. As a result, the rhizosphere of any crop plant provides diverse interactions between microorganisms and plant-parasitic nematodes (Mai and Abawi, 1987), ranging from mutualism to parasitism (Kerry, 2000; Rae *et al.*, 2008).

Plant-parasitic nematodes are obligate parasites that must pass the rhizosphere to reach their host. However, the time spent in the rhizosphere depends on their parasitic behaviour. The majority of PPN are ectoparasites that spend their entire time in the bulk soil/rhizsophere where they interact with soil/rhizosphere microorganisms. In contrast, endoparasites such as RKN spend only a short time in the bulk soil/rhizosphere before they enter the plant tissue, thus interaction with soil/rhizosphere microorganisms is limited. During their passage through the bulk soil/rhizosphere, plant-parasitic nematodes are most exposed to soil microbes including plant pathogens and beneficial as well as antagonistic microorganisms.

Some of those microorganisms might attach to the nematode cuticle. Well described is the specific attachment of *Pasteuria penetrans* to the cuticle of several plant-parasitic nematodes including *Meloidogyne* species (Davies *et al*., 2001). Various isolates of *Brevibacillus laterosporus* were able to attach to the cuticle surface of *Heterodera glycines* and *Bursaphelenchus xylophilus* (Tian *et al.*, 2007b). After attachment to the nematode surface, *B. laterosporus* strain G4 was able to penetrate the cuticle then digest the organs and eventually kill the nematodes (Huang *et al.*, 2005). Some endoparasitic nematophagous fungi are able to form adhesive spores that attach to the nematode cuticle, such as *Drechmeria coniospora*, *Catenaria anguillulae* and *Hirsutella rhossiliensis.* Such obligate parasites may affect nematodes movement reducing nematodes invasion of the roots (Kerry, 2000), or directly inhibiting or infecting nematode stages (Stirling, 1991). For example, both *P. penetrans* and *H. rhossiliensis* were found to limit nematode invasion of *M. incognita, M. hapla, H. glycines* and *H. avenae* (Siddiqui and Mahmood, 1999; Liu and Chen, 2005). *Aspergillus* sp., *Arthrobortis* sp. *and Cladosporium cladosporioides* have been shown to attach to the J2 of *Meloidogyne* spp and vermiform stages of *Rotylenchulus reniformis* (Amer-Zareen and Zaki, 2000; Castillo et al., 2010). Members of these fungal species have been

reported as biological control agents against PPN (Amer-Zareen *et al.*, 2001; Ayoub *et al.*, 2000; Kerry, 2000; Shamim A., 2012).

In general, attachment of microbes to the nematode cuticle may result in transport of those microbes to the roots where they can colonize the rhizosphere or endorhiza and might even induce plant defense mechanisms. Concerning to adhesion process, some microbes appeared specificity for the adhesion site on the cuticle of different nematode species. Conidia of *D. coniospora* adhere to the cuticle of many nematode species, which showed three patterns of binding sites i. specifically to the head and tail (*Pratylenchus penetrans*) ii. all over the body (*Ditylenchus dipsaci*) iii. very sparse no binding (*Aphelenchoides blastophthorus*) (Jansson *et al.*, 1988). Overall, there are complex tritrophic interactions in the rhizosphere, in which PPN and microorganisms act in antagonistic or synergistic associations affecting the plant host. In this thesis, three arable soils were assessed for their suppressiveness against *M. hapla* and whether there were specific soil microbes attached to the second-stage juveniles (chapter IV).

Interaction with soil-borne plant pathogens

Numerous interactions of PPN with the plant-pathogenic fungi and bacteria have been described (Back *et al.*, 2002). Plant-parasitic nematodes alone can invade the plant, but can also facilitate infection of secondary pathogens that alone cannot infect the plant. For examples, infection of roots by root-knot nematodes predisposes plants to infection by root-infecting fungi causing the development of root-rot and wilt disease (Armstrong *et al.*, 1976). Root-knot nematodes (*Meloidogyne* spp.) are probably the most recorded nematodes found in disease complexes with fungi, and the interaction with *Fusarium* on cotton represents the first report of the synergistic interaction between PPN and fungi (Atkinson, 1892). Since then, disease complexes caused by *Meloidogyne* and *Fusarium* have been described on several hosts such as alfalfa, beans, chickpea, tomatoes, peas, bananas and coffee (Bertrand *et al.*, 2000; Griffin and Thyr, 1986; France and Abawi, 1994; Siddiqui *et al.*, 1999; Jonathan and Rajendran G, 1998). Also, *Meloidogyne* has been reported to be involved in disease complexes with *Verticillium* and the root-rot pathogens *Pythium*, *Phytophthora* and *Rhizoctonia* (Back

et al., 2002). The interaction between plant-parasitic nematodes and plant pathogens is considered synergistic when the combined effects of both pathogens on the host plant cause more extensive damage than the sum of the damage caused by each pathogen individually (Wallace, 1983).

Bacterial pathogens are less in number compared to fungal pathogens and therefore fewer interactions between PPN and bacterial pathogens have been described. An Agrobacterium-RKN interaction has been reported on crops such as *A. tumefaciens-M. javanica* interaction on almond, *A. radiobacter -M. incognita* interaction on cotton and *A. vitis -M. hapla* interaction on grapevine (Orion and Zutra, 1971; Dhanvantari *et al.*, 1975 ; Sule and Lehoczky, 1993 ; Zutra and Orion, 1982; Rubio-Cabetas *et al.*, 2001). The interaction of *M. incognita* with *Ralstonia solanacearum* caused synergistic effects on wilt symptoms of several crops, especially tomato and eggplant (Reddy *et al.*, 1979; Napiere and Quinio, 1980; Swain *et al.*, 1987; Chindo *et al.*, 1991; Deberdt *et al.*, 1999). Simultaneous inoculation of *M. incognita* and *Ralstonia solanacearum* with *Fusarium* exhibited more early disease symptoms on *Coleus forskohlii* and *Withania somnifera* L. Dunal (Mallesh *et al.*, 2009). Use of a strategy that is able to simultaneously control several plant pathogens is highly desirable in this case. In the present work, bacterial isolates known as antagonists of fungal pathogens were investigated for their biocontrol potential against *M. incognita* (chapter V).

Antagonists and their mechanisms for nematode suppression

All organisms that can parasite, inhibit, repel, or kill plant-parasitic nematodes are termed nematode antagonists. Fungi and bacteria are numerically the most abundant organisms in soil and some of them are able to specifically infect plant-parasitic nematodes, making them ideal candidates for biocontrol purpose (Dong and Zhang, 2006). Over the last decades, numerous bacteria and fungi have shown high potential as biocontrol agents of PPN (Siddiqui and Mahmood, 1999; Dong and Zhang, 2006; Sikora *et al.*, 2007; Tian *et al.*, 2007a). Although extensive work has been conducted to assess the biocontrol potential of bacterial antagonists, little information is available on their mechanisms in suppressing plant-parasitic nematodes when compared with that known about antagonistic fungi. A better understanding of those control mechanisms

will allow their optimization for a successful application in praxis (Sikora *et al*., 2007). Previous studies demonstrated that bacterial antagonists affect nematodes by a variety of mechanisms e.g. production of toxins or enzymes, parasitism, disruption of host recognition, repellence and induced systemic resistance (Hasky-Gunther and Sikora, 1995; Hasky-Gunther *et al.*, 1998; Siddiqui and Mahmood, 1999; Hallmann *et al.*, 2001; Reitz and Sikora, 2001; Sikora *et al.*, 2007; Tian *et al.*, 2007a).

Direct antagonism

Some bacteria produce metabolites or excretory enzymes that are harmful to plant-parasitic nematodes (Hallmann *et al.*, 1999). The adverse effect of these compounds include the inhibition of egg hatch, juvenile survival and nematode reproduction (Siddiqui and Mahmood, 1999). In *in vitro* assays, compounds produced by some bacteria isolated from the plant rhizosphere caused immobility of second-stage juveniles of *M. incognita* (Becker *et al.*, 1988). Culture filtrates of isolates of *Bacillus subtilis, Pseudomonas fluorescens* and *Burkholderia cepacia* inhibited egg hatch and J2 mobility of different root-knot nematode species (Meyer *et al.*, 2000; Li *et al.*, 2005; Elbanna *et al.*, 2011). *Bacillus cereus* produced an extracellular enzyme having collagenolytic and proteolytic properties that was able to damage the cuticle of *M. javanica* juveniles (Sela *et al.*, 1998). The antimicrobial metabolites 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin produced by *P. fluorescens* strain CHA0 inhibited egg hatch and juvenile survival of *M. javanica* (Siddiqui and Shahid Shaukat, 2003). However, all studies were conducted *in-vitro* leaving behind some uncertainty if such mechanisms also apply under field conditions.

Competition

Competition between plant-parasitic nematodes and antagonistic bacteria for space or nutrients is always present when they simultaneously occupy the same ecological niche within the root system such as *Meloidogyne* spp. and endophytic bacteria (Sikora *et al.*, 2007). Competition for nutrients was postulated by Oostendorp and Sikora (1990) as driving mechanisms for the interaction between the rhizobacterium *P. fluorescens* and the sugarbeet cyst nematode *Heterodera schachtii*. In contrast, Siddiqui and

Mahmood (1995) propagated niche exclusion as primary control mechanism of *Rhizobium* suppressing *Meloidogyne* spp. To be effective, competition requires high bacterial densities in close proximity to the nematode pathogen (Sikora *et al.*, 2007). Within this respect, Hallmann *et al.* (2001) observed high densities of the antagonistic bacterium *Rhizobium etli* G12 within root galls caused by *M. incognita*.

Induced systemic resistance

Some bacterial biocontrol strains can elicit a state of defensive capacity in plants against pathogens, termed induced systemic resistance (ISR). This process is based on plant defence mechanism activated by an inducing agent. ISR can protect the plant against a broad spectrum of pathogens (Wei *et al.*, 1996). With regard to nematode control, ISR was first reported by Hasky-Günther and Sikora (1995) and later shown for several other bacteria-nematode interactions. Using a spilt-root system, both *B. sphaericus* B43 and *R. etli* G12 caused ISR towards *M. incognita* on tomato resulting in reduced juvenile penetration on the non-treated responder side of the root system (Hauschild *et al.*, 2000; Schäfer *et al.*, 2006). ISR elicited by G12 resulted in a 36% reduction in the number of eggs per female while ISR elicited by B43 caused a 25% reduction in reproduction when compared to the untreated controls (Schäfer, 2007).

To better understand this mechanism, the bacterial determinants (inducer) of ISR were studied. In the split-root system, both living and heat-killed cells of *B. sphaericus* B43 and *R. etli* G12 caused ISR in potato against *G. pallida.* Quite interestingly, while culture filtrates of *B. sphaericus* B43 produced ISR, culture filtrates of *R. etli* G12 did not (Hasky-Gunther *et al.*, 1998). For *R. etli* G12 the heat-stable inducing agent was further studied indicating that the lipopolysaccharides functioned as main elicitor (Reitz *et al.*, 2000). On the plant side, ISR activates multiple defence mechanisms such as physical thickening of the cell walls by lignifications, deposition of newly formed callose and accumulation of phenolic compounds; increased activity of chitinase, peroxidase and other pathogenesis-related proteins; and synthesis of phytoalexin and other metabolites (Siddiqui and Mahmood, 1999; Tian *et al.*, 2007a; Anita and Samiyappan, 2012).

Few studies were conducted to have knowledge about physiological and biochemical changes associated with ISR in plants towards PPN. The importance of modified protein expression in bacteria-generated ISR in potato towards *G. pallida* has been reported by Hasky-Günther (1996), who studied protein expression in plants inoculated with B43 or G12. The results gave a novel protein band (38kDa) in plants inoculated with G12, which was different from any of the typical PR-proteins associated with ISR, but did not give any differences in protein patterns in the B43-inoculated plants when compared to those of untreated plants, indicating the lack of PR-protein involvement.

Anita *et al.* (2004) studied induction of defence enzymes by *P. fluorescens* isolate Pf, against challenge inoculation of *M. incognita* in tomato. The results showed that activities of peroxidise (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase, chitinase and catalase were significantly higher in bacterized tomato root tissues challenged with the nematode compared to plants inoculated with the nematode alone or untreated plants. Furthermore, isoform analysis revealed unique PO and PPO isoforms induced in *P. fluorescens*-treated plants.

Siddiqui and Shaukat (2004) studied the role of salicylic acid biosynthesis in the enhancement of defence mechanisms against *M. javanica*. They concluded that *Pseudomonas fluorescens* strain *CHA0* induce systemic resistance against a root-knot nematode *M. javanica* via a signal transduction pathway, which is independent of salicylic acid accumulation in roots. However, the signal transduction pathways for the systemic resistance towards PPN are still unknown.

Repellence and/or disruption of nematode-host recognition

Antagonistic bacteria can affect nematode attraction by producing substances that repel or inhibit nematodes movement toward the root (Oostendrop and Sikora, 1990). This can be done by either altering the root-exudates pattern to be less attractive to nematodes or depleting the oxygen in the root zone, making the root less attractive to nematodes (Sikora *et al.*, 2007). Within this respect, Padgham and Sikora (2007) studied the influence of *Bacillus megaterium* on *M. graminicola* attraction to rice plants

in a linked-pot chamber test. The results showed that *M. graminicola* preferentially penetrated the roots of plants not inoculated with *B. megaterium*. Nematode penetration into the root of plants treated with bacteria was 55% lower than in non-treated plants. In the present work, bacterial isolates with antagonistic activity against soil-borne fungal pathogens were investigated for their antagonistic potential against the root-knot nematode *M. incognita* and for their mode-of-action (chapter V).

THESIS OBJECTIVES

The overall objective of this thesis is to gain more knowledge about root-knot nematodes *Meloidogyne* spp.- an economically important plant pathogen and distributed worldwide. Focusing on some aspects that could provide new opportunities for enhancing crop protection strategies, especially in organic farming systems; including surveillance and monitoring, identification by molecular assay, interaction with soil microbes and biological control.

Therefore these studies were carried out for achieving the following specific objectives:

1. To study the occurrence of plant-parasitic nematodes associated with different organically grown crops in Egypt and to identify the predominant genera and species (chapter II).

2. To use PCR-DGGE molecular technique based on *msp1*gene to differentiate populations and/or races of the root-knot nematode *M. incognita* differing in their reproductive potential on different hosts (chapter III).

3. To detect bacteria and fungi attached to second-stage juveniles of the root-knot nematode *M. hapla* in suppressive soils using cultivation-independent techniques (chapter IV).

4. To evaluate the antagonistic potential of bacterial antagonists of fungal pathogens towards the root-knot nematode *M. incognita*, as well as, to investigate their modes-of-action (chapter V).

THESIS OUTLINE

Chapter I. General information about the distribution, economic importance, biology and life cycle the root-knot nematode *Meloidogyne* spp. is presented. Information on organic farming including prevalence and its problems with plant-parasitic nematodes and management challenges is given. Commonly used methods for identifying species, populations and races of *Meloidogyne* spp. including bioassay, morphological and biochemical assays are reported. Furthermore, suppressive soil, interaction between nematodes and soil microbes, microbial attachments to the nematode cuticle and mechanisms for nematode control including direct antagonism, competition, induced systemic resistance and repellence are given.

Chapter II. Information on the occurrence, frequency and population densities of plant-parasitic nematodes associated with different organic crops in SEKEM farm in Egypt during two surveys in 2009 and 2011 is presented. The most predominant genera during the surveys were identified to species level based on morphological characters. Comparison of nematode frequencies and population densities between both surveys is reported.

Chapter III. Within this chapter methods for differentiating *M. incognita* populations and/or races were studied. A bioassay was used to differentiate among populations and/or races based on variability in reproduction rate on different crops/cultivars. A PCR-DGGE protocol was developed for optimal separation of the pathogenecity gene *msp1*. The variation in the *msp1*fragments amplified from genomic DNA of populations/races was detected in DGGE analysis. Cloning and sequencing of different DGGE bands was performed to display sequence differences in variants of the *msp1*gene.

Chapter IV. The suppressive activity of arable soils against *M. hapla* was investigated. Microbial communities attached to second-stage juveniles of *M. hapla* were analysed using cultivation-independent techniques. PCR-DGGE of 16S-rRNA genes of bacteria and bacterial groups or fungal ITS from nematode and soil samples were proposed to investigate the total microbial communities. Cloning and sequencing was used to identify those bacteria or fungi based on nematode-specific bands in DGGE. Barcoded amplicon pyrosequencing was performed to determine bacterial 16S-rRNA gene sequences from nematode and soil samples.

Chapter V. Bacterial soil isolates were screened for their antagonistic effects on *M. incognita* juveniles in vitro (V-2). Assess bacterial isolates known as antagonists of fungal pathogens for their biocontrol potential against the root-knot nematode *M. incognita* in greenhouse (V-1). Seed treatment was used to identify the top strains for further studies on their mode-of-action. For the top strains the effect of bacterial culture supernatants towards nematode reproduction was evaluated. A linked twin-pot chamber was used to assess the effect of the antagonists on J2 attraction to tomato roots, while a split-root chamber was used to test the potential of those antagonists to induce systemic resistance. The effect of induced systemic resistance was compared with the effect caused by co-inoculation of bacterial antagonists and *M. incognita* in the same pot.

Chapter VI. Summarizes the main findings, gives general conclusion and future perspectives.

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Chapter II

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Occurrence of plant-parasitic nematodes in organic farming in Egypt

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Abstract

Two surveys were conducted at an organic farm, 60 km northeast of Cairo, Egypt, in 2009 and 2011 to study the occurrence, population density and distribution of plant-parasitic nematodes associated with different crops. A total of 216 soil samples were collected from vegetables, fruits and herbs during both surveys. Eleven genera of plant-parasitic nematodes were detected. The most abundant genus was *Meloidogyne*, which was detected in over 57% and 47% of all soil samples in 2009 and 2011, respectively. This genus also reached the highest mean population densities of all genera detected. *Tylenchorhynchus* was the next most abundant genus, occurring in over 29% of all soil samples in both surveys. Other nematode genera found were *Criconemella*, *Ditylenchus*, *Hoplolaimus*, *Paratylenchus*, *Pratylenchus*, *Tylenchulus*, and *Xiphinema*. High population densities with up to 2600 and 2300 nematodes per 100 g soil were recorded for the species *Rotylenchulus* and *Helicotylenchus*, respectively. In general, frequency and population densities of plant-parasitic nematodes were lower in the 2011 survey than in the 2009 survey which most likely was caused by variable agronomic and climatic conditions between those years. Overall, the data suggested that plant-parasitic nematodes pose a severe threat to organic farming under arid conditions, and that control measures should be further developed and implemented.

Keywords. *Meloidogyne incognita*, organic farming, plant-parasitic nematodes, *Pratylenchus*, root-knot nematode, survey, *Tylenchorhynchus.*

INTRODUCTION

Organic agriculture is increasing steadily in developed as well as developing countries. According to the latest statistics on organic farming worldwide, almost 37 million hectares (ha) in 160 countries are currently farmed organically, and the land devoted to organic agriculture worldwide has grown by 135% over the last decade (Willer and Kilcher, 2011). The total market value of organic products reached USD$55 billion in 2009 which is an increase of 16.3% compared with 2007 (Paull, 2011). In Africa, there are slightly more than one million hectares of certified organic agricultural land, representing 3% of the world's total organic agricultural land. The majority of certified organic produce is exported to the European Union. Certified organic products are currently known only in a few local markets, including Egypt, South Africa, Uganda, Kenya and Tanzania (Willer and Kilcher, 2011).

As for Egypt, the organic agricultural land has increased from 4,020 ha in 1998 to 9,342 ha in 2003, and 19,211 ha in 2008 representing an annual growth rate of 17% (Sadek and Shelaby, 2011). In 2011, Egypt already had a total of 56,000 ha grown organically (Paull, 2011). In the 1970's, the Egyptian organic agriculture was started by the SEKEM initiative and some growers in Fayoum and Kalubia governorates. Nowadays, most organic farms are concentrated in Fayoum governorate, and a few farms are located in reclaimed desert land in the Nile delta and in Upper Egypt (Radwan *et al*., 2011).

The SEKEM initiative (SEKEM is ancient Egyptian and means "life force") was founded in 1977 by the Egyptian pharmacologist and social entrepreneur Dr. Ibrahim Abouleish "to restore and maintain the vitality of the soil and food as well as the biodiversity of nature through sustainable, organic agriculture and to support social and cultural development in Egypt''. SEKEM was the first organization in the world to cultivate and harvest biodynamic cotton based on the application of composts made of various plant residues, fresh green material and manure (Merckens, 2000).

As organic farmers lack the use of synthetic fertilizers and pesticides, they often face problems with plant-parasitic nematodes, especially during the transition period from conventional to organic farming (Hallmann *et al*., 2007; van Bruggen and Termorshuizen, 2003). Nematode problems may not always be recognized as such by the farmers or they might be confused with water or nutrition deficiency. Monitoring systems and feasible control methods for plant-parasitic nematodes are less available compared to foliar pathogens and insect pests. Furthermore, scientific data about nematode problems in organic farming are still limited, and especially lacking in the case of Egypt. Therefore, the objectives of this study were to determine the frequency and abundance of plant-parasitic nematodes associated with organic crops in Egypt in two years, and to identify the predominant genera and species.

MATERIAL AND METHDOS

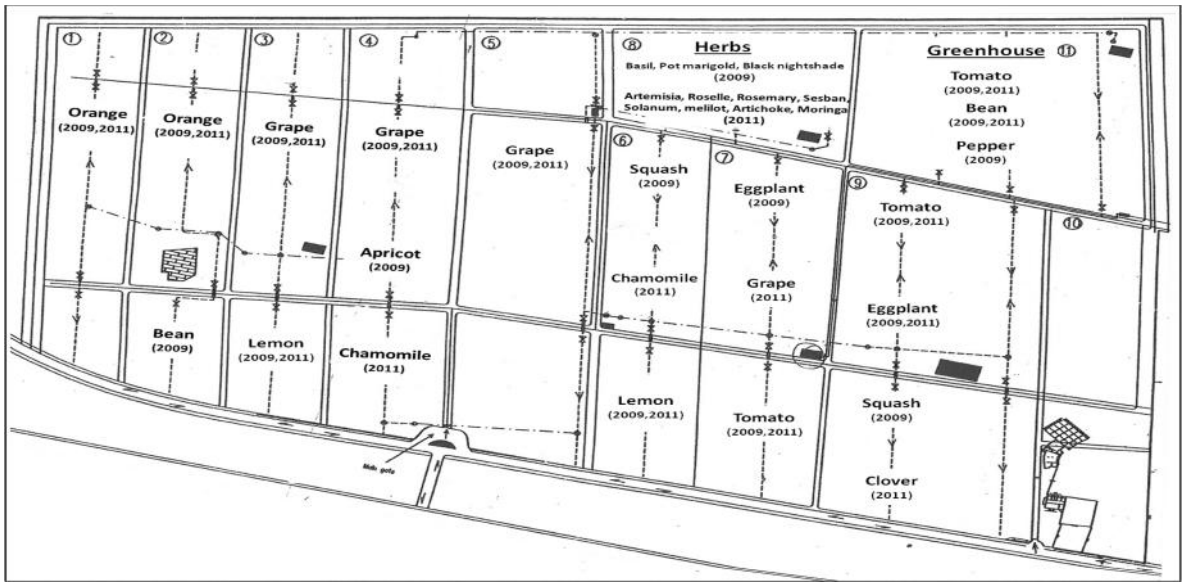
Sampling

The nematological surveys were conducted at the SEKEM organic farm located in El-Sharkia governorate 60 km northeast of Cairo. A total of 114 and 102 samples were taken during autumn 2009 and 2011, respectively. Samples were collected from field and greenhouse plots representing the different crops grown at the 120 acre farm (Fig. 1). As a rule, one sample was taken per acre and plot; however, additional samples were collected directly from poorly growing patches where the presence of plant-parasitic nematodes was suspected. Each sample consisted of approx. 1500 g composed of 20 cores taken from the top 20 cm of soil. All samples were taken following a zigzag pattern throughout the plot. Soil samples were kept in polyethylene bags and stored at 7°C until further processing.

For species identification of *Meloidogyne*, root samples were taken from bean (*Phaseolus vulgaris*), eggplant (*Solanum melongena*), tomato (*Solanum lycopersium*), pepper (*Capsicum annuum*), grape (*Vitis vinifera)* and various herbs such as artemisia (*Artemisia vulgaris*), artichoke (*Cynara cardunculus*), moringa (*Moringa oleifera*), roselle (*Hibiscus sabdariffa*), rosemary (*Rosmarinus officinalis*), sesban (*Sesbania sesban*), black nightshade (*Solanum nigrum*) and white melilot (*Melilotus distichum*). The roots were washed under tap water and females were isolated from the galled root tissue and identified based on the perennial patterns of ten adult females (Taylor and Sasser, 1978).

Nematode extraction

Soil samples were thoroughly mixed and 200 g aliquots were taken for nematode extraction by using Cobb's sieving and decanting technique followed by a modified Baermann technique (Hooper *et al*., 2005). Each soil sample was suspended in 3 litres of water, and after settling of coarse soil particles the supernatant was poured through two sieves of 200 µm and 25 µm aperture, respectively. This was repeated three times. The debris including the plant-parasitic nematodes retained on the two sieves was collected in 250-ml beakers. To clean the nematodes from remaining soil particles, the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Apricot
(2009)
⑤
Grape
(2009,2011)
⑧ Herbs
Basil, Pot marigold, Black nightshade
(2009)
Artemisia, Roselle, Rosemary, Sesban, Solanum, melilot, Artichoke, Moringa
(2011)
Greenhouse ⑪
Tomato
(2009,2011)
Bean
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Pepper
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Squash
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Chamomile
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Tomato
(2009,2011)
Squash
(2009)
Clover
(2011)

RESULTS

Genera and species of plant-parasitic nematodes at SEKEM farm

Eleven genera of plant-parasitic nematodes were found to be associated with different organically grown plants at SEKEM farm during the surveys in 2009 and 2011 (Tables 1 and 2). Nine of them were detected in both surveys including the bulb nematode *Ditylenchus*, the spiral nematode *Helicotylenchus*, the lance nematode *Hoplolaimus*, the root-knot nematode *Meloidogyne*, the lesion nematode *Pratylenchus*, the reniform nematode *Rotylenchulus*, the citrus nematode *Tylenchulus*, the stunt nematode *Tylenchorhynchus,* and the dagger nematode *Xiphinema*. The ring nematode *Criconemella* and the pin nematode *Paratylenchus* were only detected in the 2011 survey. Species identified were *Helicotylenchus dihystera*, *Meloidogyne incognita*, *Pratylenchus penetrans, Rotylenchulus reniformis* and *Tylenchulus semipenetrans.*

Frequency of genera in soil

The frequency of nematode genera in soil samples collected from different organically grown crops during the surveys in 2009 and 2011 are shown in Fig 2. Forty percent of the soil samples contained only a single genus in both surveys, while 43% and 58% of the soil samples contained two or more genera in 2009 and 2011, respectively. Plant-parasitic nematodes were not detected in 17% and 2% of the total samples in 2009 and 2011, respectively. *Meloidogyne* was the most frequent genus in both surveys, which occurred in 57% and 47% of all soil samples collected in 2009 and 2011, respectively. The next most frequent genus was the stunt nematode *Tylenchorhynchus* which occurred in 29% of all samples in both surveys. Other genera commonly detected were *Rotylenchulus, Helicotylenchus* and *Pratylenchus* which all occurred in between 20% and 29% of the samples. Genera observed with frequencies below 20% included *Ditylenchus, Hoplolaimus, Tylenchulus* and *Xiphinema* (Fig. 2).

Occurrence of plant-parasitic nematodes in different organically grown crops

Approximately 85% of the surveyed plants were infected with three or more genera while 15% of the surveyed plants were infected with only a single genus in both surveys (Fig. 1, Tables 1 and 2).

Table 1. Occurrence and population densities of plant-parasitic nematodes in different crops grown at the organic SEKEM farm in 2009.

Host plant[1)]	Genera	Occurrence (%)[2)]	Population density/100 g soil	
			Mean	Range (Minimum-Maximum)
Apricot (6) (*Prunus armeniaca*)	*Helicotylenchus*	100.0	118	40-150
	Meloidogyne	33.3	15	10-20
	Pratylenchus	16.6	150	150
	Rotylenchulus	50.5	154	53-263
	Tylenchorhynchus	33.3	260	260
	Xiphinema	16.6	60	60
Bean (6) (*Phaseolus vulgaris*)	*Hoplolaimus*	33.3	128	125-130
	Meloidogyne	100.0	410	10-990
	Rotylenchulus	16.6	20	20
	Tylenchorhynchus	16.6	60	60
Grape (24) (*Vitis vinifera*)	*Helicotylenchus*	75.0	650	40-2300
	Hoplolaimus	25.0	152	10-550
	Meloidogyne	62.5	429	10-2110
	Pratylenchus	58.3	126	10-280
	Rotylenchulus	100.0	429	10-2600
	Tylenchorhynchus	41.0	83	10-260
Lemon (8) (*Citrus limon*)	*Ditylenchus*	25.0	35	30-40
	Helicotylenchus	50.0	207	20-420
	Meloidogyne	50.0	182	20-420
	Pratylenchus	25.0	85	30-160
	Rotylenchulus	12.5	70	70
	Tylenchorhynchus	25.0	40	20-60
	Tylenchulus	37.5	63	30-110
Orange (12) (*Citrus sinensis*)	*Ditylenchus*	16.6	95	60-130
	Meloidogyne	75.0	38	10-130
	Tylenchulus	33.3	128	60-195
	Xiphinema	8.3	120	180
Squash (8) (*Cucurbita pepo*)	*Ditylenchus*	25.0	30	20-40
	Meloidogyne	62.5	108	65-175
	Rotylenchulus	12.5	70	70
	Pratylenchus	12.5	78	78
	Tylenchorhynchus	62.5	66	10-260
Tomato (8) (*Solanum lycopersicum*)	*Meloidogyne*	3.75	98	10-225
	Pratylenchus	12.5	10	10
	Tylenchorhynchus	25.0	30	10-50
Eggplant (4) (*Solanum melongena*)	*Ditylenchus*	50.0	45	20-70
	Pratylenchus	25.0	10	10
	Tylenchorhynchus	25.0	30	30
Pepper (35) (*Capsicum annuum*)	*Helicotylenchus*	2.8	10	10
	Meloidogyne	62.8	2891	10-17030
	Pratylenchus	5.7	150	150
	Tylenchorhynchus	5.7	77	77
Herbs (3)	*Meloidogyne*	66.6	1855	1110-2600

[1)] Number of collected samples is given in parenthesis.
[2)] Occurrence (%) = Number of positive samples / Number of total samples x 100

Table 1. Occurrence and population densities of plant-parasitic nematodes in different crops grown at the organic SEKEM farm in 2011.

Host plant[1)]	Genera	Occurrence(%)[2)]	Population density/100 g soil	
			Mean	Range (Minimum-Maximum)
Alfalfa (5) (*Medicago sativa*)	*Ditylenchus*	40.0	28	15-40
	Helicotylenchus	20.0	60	60
	Meloidogyne	100.0	62	10-105
	Pratylenchus	20.0	75	75
Bean (3) (*Phaseolus vulgaris*)	*Meloidogyne*	66.6	50	50
Chamomile (5) (*Chamomilla recutita*)	*Ditylenchus*	20.0	18	18
	Hoplolaimus	20.0	18	18
	Meloidogyne	20.0	20	20
	Pratylenchus	100.0	75	20-130
	Rotylenchulus	20.0	75	20
Eggplant (3) (*Solanum melongena*)	*Ditylenchus*	66.6	20	15-25
Grape (30) (*Vitis vinifera*)	*Helicotylenchus*	50.0	382	25-1870
	Hoplolaimus	6.6	44	42-45
	Meloidogyne	30.0	206	42-900
	Pratylenchus	20.0	70	25-100
	Rotylenchulus	76.0	396	25-2210
	Tylenchorhynchus	10.0	133	50-250
Lemon(9) (*Citrus limon*)	*Criconemella*	44.4	41	50-62
	Meloidogyne	44.4	56	18-138
	Paratylenchus	22.2	127	127
Orange (15) (*Citrus sinensis*)	*Ditylenchus*	20.0	55	12-90
	Meloidogyne	46.6	19	12-36
	Pratylenchus	20.0	46	12-90
	Tylenchorhynchus	53.0	198	12-450
	Tylenchulus	6.6	12	12
	Xiphinema	20.0	24	18-38
Tomato (23) (*Solanum lycopersicum*)	*Ditylenchus*	34.7	33	13-70
	Helicotylenchus	17.3	159	120
	Meloidogyne	69.5	488	130-1200
	Pratylenchus	13.0	55	25-100
	Tylenchorhynchus	62.1	54	13-140
Herbs (9)	*Ditylenchus*	11.1	20	20
	Helicotylenchus	11.1	25	25
	Meloidogyne	44.4	2091	75-5750
	Pratylenchus	22.2	197	115-280
	Tylenchorhynchus	44.4	263	20-420

[1)] Number of collected samples is given in parenthesis
[2)] Occurrence (%) = Number of positive samples / Number of total samples x 100

Overall, the most dominant genus for each crop was always identical in both surveys. *Meloidogyne* was found to be associated with over 88% of all crops in both surveys. Furthermore, it was the predominant genus associated with 70% and 55% of the crops sampled in 2009 and in 2011, respectively. *Meloidogyne* was the dominant genus on crops that were repeatedly planted in the same plots during both surveys, such as bean, herbs, lemon (*Citrus limon*), and greenhouse tomato but also on some crops that were planted during one or both surveys such as alfalfa (*Medicago sativa*), orange (*Citrus sinensis*), squash (*Cucurbita pepo*) and greenhouse pepper, with 46% to 100% occurrence. *Tylenchorhynchus* and *Pratylenchus* were found on the crops sampled. Few nematode genera occurred in all samples taken from one given crop (= 100% occurrence), such as *Helicotylenchus* being detected in all 6 samples taken from apricot (*Prunus armeniaca*) in 2009 or *Rotylenchulus* found in all 26 samples taken from grapes of the same year. Citrus was the preferable host for *Tylenchulus semipenetrans* which only occurred in these plots. *Criconemella* and *Paratylenchus* were only detected in samples taken from lemon and only in 2011. Furthermore, *Hoplolaimus* and *Xiphinema* were only associated with one or two hosts at low frequency in both surveys (Tables 1 and 2).

Population densities of plant-parasitic nematodes

According to mean population densities of nematode genera detected in soil samples collected from different crops grown in 2009 and 2011, *Meloidogyne* had the highest mean densities of all nematode taxa in both surveys (Fig. 3). *Rotylenchulus*, *Helicotylenchus, Hoplolaimus,* and *Tylenchorhynchus* achieved relatively high mean densities during one or both surveys. Other genera had rather low densities in both surveys including *Ditylenchus, Pratylenchus, Tylenchulus,* and *Xiphinema* (Fig. 3).

Concerning their mean and maximum densities on each crop separately, the highest mean and maximum densities of all plant-parasitic nematodes on any crop in both surveys was achieved by *Meloidogyne* with 2891 and 17,030 juveniles/100 g soil in pepper in the 2009 survey, respectively (Tables 1 and 2). Also this genus exhibited relatively high densities on herbs, grapes and tomatoes during both surveys (Tables 1

and 2). The highest population densities of *Rotylenchulus, Helicotylenchus* and *Hoplolaimus* were recorded on grapes in both surveys which reached up to 2600, 2300 and 550 nematodes per 100 g soil in 2009, respectively. The maximum densities for *Tylenchorhynchus* and *Pratylenchus* were 450 and 280 nematodes/100 g soil on herbs in 2009, respectively. Other genera that were found at low densities were *Criconemella, Ditylenchus, Paratylenchus, Tylenchulus* and *Xiphinema* (Tables 1 & 2).

Comparison of nematode frequencies and population densities between both surveys

In general, the frequency and mean population densities of most nematode genera in soil samples (n=114) collected from different organically grown crops in 2009 were lower than in soil samples (n=102) collected in 2011 (Fig. 2 and 3). The frequencies of *Meloidogyne, Rotylenchulus, Tylenchulus, Helicotylenchus* and *Hoplolaimus* in soil samples collected in the 2011 survey were significantly lower (less than 4-10%) in the 2009 survey. Also, in the 2011 survey, the first three genera were found in association with fewer crops than in the 2009 survey, and their occurrences on the same crops planted in both surveys were less in 2011, especially in case of *Meloidogyne* and *Rotylenchulus*. On the contrary, in the 2011 survey, *Ditylenchus* and *Xiphinema* were detected in more samples than in the 2009 survey, and *Ditylenchus* was associated with more crops and at higher occurrences than in 2009. *Pratylenchus* and *Tylenchorhynchus* exhibited almost consistent frequencies but inconsistent occurrence in different crops during both surveys. *Tylenchorhynchus* was associated with fewer crops in the 2011 survey than in the 2009 survey (Tables 1, 2 and Fig. 2).

In the 2011 survey, mean population densities of *Ditylenchus, Meloidogyne, Helicotylenchus, Hoplolaimus, Tylenchulus,* and *Xiphinema* were significantly lower than in 2009 (Fig. 3). Also, maximum population densities recorded of these genera on different crops in 2011 were about 18-93% lower than those recorded in 2009, e. g. the maximum density of *Meloidogyne* on pepper in 2009 was 17,030 nematodes/100 g soil while it was 5750 nematodes/100 g soil on herbs in 2011. In contrast, mean population densities of *Rotylenchulus* and *Tylenchorhynchus* in all samples collected during the

2011 survey were significantly higher than in those collected during the 2009 survey. *Pratylenchus* had maximum and mean densities almost equal in both surveys (Fig. 3).

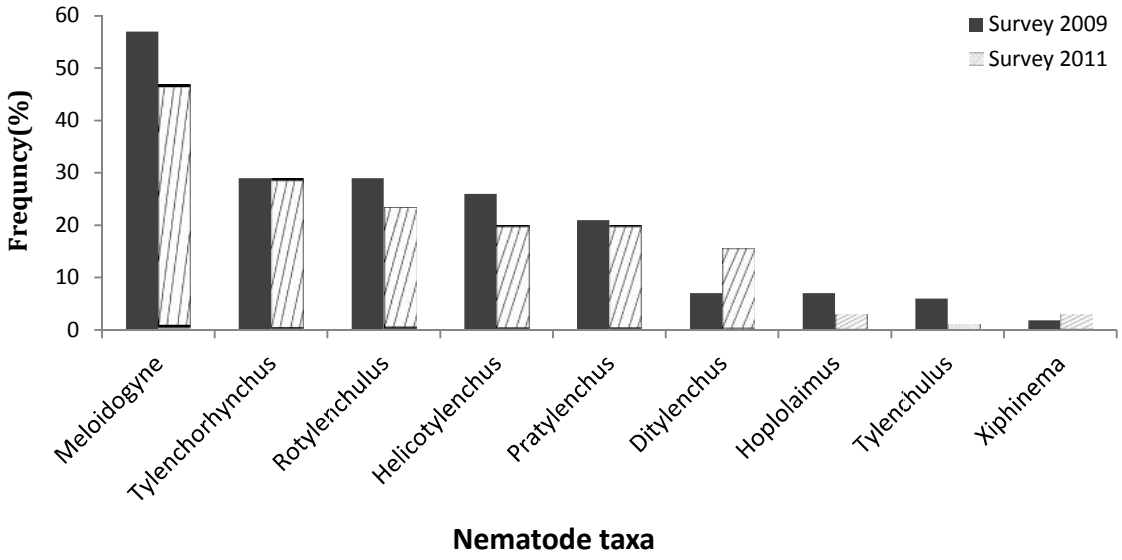

Fig. 2. Frequency of nematode genera in all soil samples collected from different crops during the surveys in 2009 and 2011.

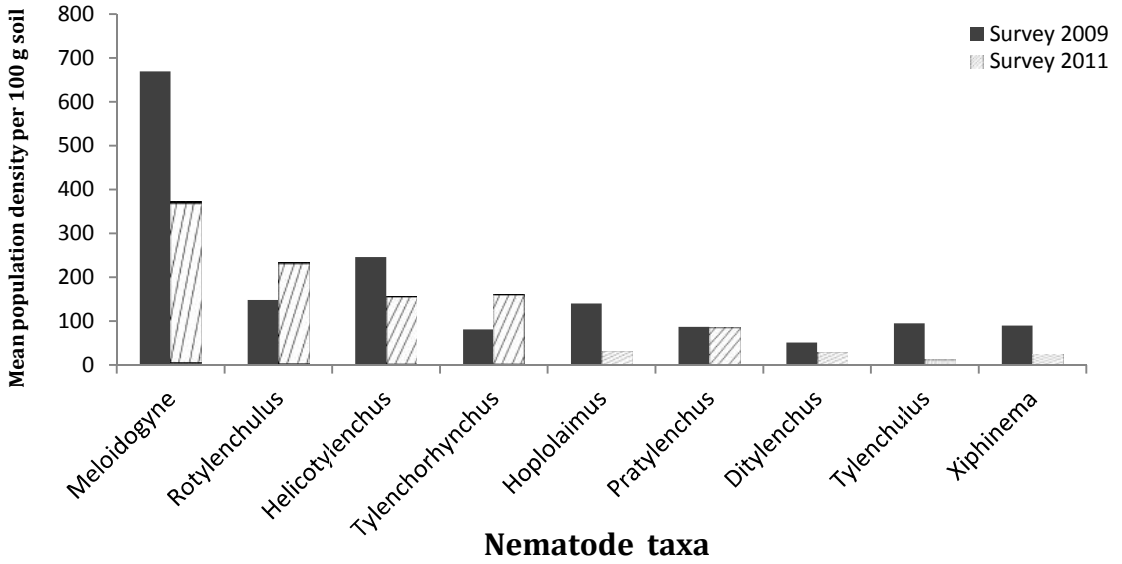

Fig. 3. Mean densities of frequent nematode genera detected at the SEKEM farm during the surveys in 2009 and 2011.

DISCUSSION

Organic agriculture has expanded in many countries worldwide as a consequence of the increasing demands for organic food in both domestic and export markets. Agricultural practices in organic farming are supposed to lead to a higher biodiversity of soil organisms and increase their activity, which may affect plant-parasitic nematodes (Freckman, 1988; Griffiths *et al*., 1994; Hallmann *et al*., 2007). However, our study on the frequency and abundance of plant-parasitic nematodes associated with different organic crops in SEKEM farm showed that current practices in organic farming are insufficient to prevent infection by plant-parasitic nematodes. All genera and species of plant-parasitic nematodes detected in surveys, have already been identified among 54 genera and 160 species under traditional farming system in Egypt (Tarjan, 1964; Oteifa and Tarjan, 1965; Ibrahim *et al*., 1976; Abou-Elnaga, 1979; Abou- Elnaga *et al.*, 1985; Ibrahim *et al*., 1986; Abou-Elnaga, 1989; Ibrahim, 1990; Ibrahim *et al*., 1994; Oteifa *et al*., 1997; Ibrahim *et al*., 2000; Ibrahim and El- Sharkawy, 2001; Mokbel *et al*., 2006; Ibrahim *et al*., 2010).

Plant-parasitic nematodes commonly detected in SEKEM organic farm were the genera *Meloidogyne, Tylenchorhynchus, Rotylenchulus, Helicotylenchus,* and *Pratylenchus,* which were the same genera mainly found in conventional farming in Egypt under similar environmental conditions, i. e. sandy soil and desert climate (Mokbel *et al.,* 2006; Ibrahim *et al*., 2000 and 2010). However, the same genera, albeit not the same species, have been reported from organically grown crops in Germany (Hallmann *et al.,* 2007). The most frequently found genus was *Meloidogyne,* which occurred in over 57% and 47% of soil samples in 2009 and 2011, respectively. This is comparable to the occurrence reported for non-organic farming in the new reclaimed lands in Egypt (62.5%) (Ibrahim *et al*., 2010), or for organic farming in Germany (51%) (Hallmann *et al.,* 2007). The next most abundant nematode was *Tylenchorhynchus* occurring in over 29% of soil samples in both surveys, which together with *Pratylenchus* was also the most dominant genus with an incidence of over 90% of the samples in organic farming in Germany (Hallmann *et al.*, 2007), and with 49.5% occurrence in conventional farming in Egypt (Ibrahim *et al*., 2010). In another study,

Tylenchorhynchus dubius was found significantly more frequently in organically managed soils, particularly in sandy soils than in conventionally managed soils (van Diepeningen *et al.*, 2006).

Meloidogyne incognita was associated with over 88% of all crops in both surveys, and particularly occurred at high densities on solanaceous crops. Pepper and tomato grown in greenhouses were preferable hosts for *M. incognita* which was able to reach mean densities up to 2891 juveniles/100 g soil in pepper. Van Bruggen and Termorshuizen (2003) observed that the root-knot nematode population increased in organic tomato production in greenhouses in the Netherlands over the past years, although other pests and diseases were kept at an acceptable level. In conventional vegetable production under similar environmental conditions as the SEKEM farm, *Meloidogyne* was reported to reach population densities of only 233 juveniles/100 g soil (Haroon and Osman, 2003; Bakr *et al*., 2011). *Pratylenchus*, which was associated with 67% - 70% of all crops at the SEKEM farm, was also commonly found on organically grown vegetables in Germany (Hallmann *et al*., 2007).

Despite the presence of lemon in both surveys, which is a good host for *Criconemella* and *Paratylenchus*, these genera were only detected in the second survey. This is likely as a result of the population densities which might have been below the detection level in the first survey, or both genera were associated with legumes that were intercropped with citrus before sampling in the second survey. *Tylenchulus* was only detected in citrus plots, which is considered a major plant-parasitic nematode on citrus in sandy soil especially in the new reclaimed lands in Egypt (Bakr *et al*., 2011). Also, *Ditylenchus* was found in association with citrus, cucurbits, legumes and Solanales, which was more common in the 2011 than in the 2009 survey, depending on the presence of its suitable hosts during each survey. It was also detected with these hosts in non-organic farming in Egypt (Mokbel *et al*., 2006).

Concerning their population densities in different crops, *Meloidogyne* on pepper had the highest mean and maximum densities of all plant-parasitic nematodes on any crop in

both surveys. The mean and maximum densities were higher than those that were achieved by the same genus on organically grown vegetables of 109 and 3312 nematodes/100 ml soil, respectively (Hallmann *et al*., 2007). Highest mean nematode densities detected in grape plots were achieved by *Helicotylenchus* and *Rotylenchulus*, with mean densities of 650 and 429 nematodes/100 g soil, respectively. Slightly lower densities were reported from conventional grape plots in Egypt, with 402 and 300 nematodes/100 g soil, respectively (Mokbel *et al*., 2006). *Tylenchorhynchus* and *Pratylenchus* with their preferable host (herbs) had relatively high mean densities of 263 and 197 nematodes/100 g soil, respectively, compared with 49 and 55 nematodes/100 ml soil, respectively, on organically grown vegetables in Germany (Hallmann *et al*., 2007).

Overall, in the 2011 survey the frequency and mean population densities of most nematode genera in soil samples were lower than in soil samples during the 2009 survey. For example, differences in mean densities of *Meloidogyne* were 44%. This might be caused by various factors such as, differences in the crops and their growth stages, (e.g. greenhouse tomato were in the senescence stage in the 2011 survey while they were in flowering and fruiting stages in the 2009 survey), or application of some agricultural practices that promote natural control such the application of composts or direct application of some biocontrol agents. It may also be due to some differences in the soil moisture and temperature between the two surveys.

In conclusion, plant-parasitic nematodes are a severe problem in the SEKEM farm and most probably also in other organic farms in Egypt. *Meloidogyne* showed the highest abundance and frequency of all plant-parasitic nematodes during the two surveys, which might result in economic damage to most crops. The results may help in identifying the most common nematode taxa occurring on each crop grown at the SEKEM farm as a prerequisite to develop effective nematode management strategies. Further research is needed to study dynamics and community structure of plant-parasitic nematodes during different seasons in several organic farms located in different regions of Egypt.

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Chapter III

The manuscript presented in this chapter has been submitted for publication in Journal of Nematology.

Differentiation of *Meloidogyne incognita* populations based on PCR-DGGE

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Abstract

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was used to differentiate among *M. incognita* populations and/or races based on *msp1* gene variation. Seven populations and races varied in their reproduction rate on different crops or cultivars. Principle component analysis (PCA) of the bioassay data separated them into two differential groups. A DGGE protocol was developed for optimal separation of *msp1* gene variants amplified from genomic DNA of populations/races. The UPGMA analysis of DGGE patterns separated the population/races into two major groups similar to those that were obtained from the phenotypic data, but it was more successful in separating each population/race in a separate cluster than PCA analysis that showed some of them overlapped. A correlation between the presence of a particular gene variant and the reproductive potential on particular hosts was not observed. The presented results indicated that PCR-DGGE could be a promising tool for answering unresolved questions regarding population genetics of plant-parasitic nematodes and genetic variation within the nematode species.

Keywords: genetics, host preference, *Meloidogyne incognita*, pathogenicity gene, PCR-DGGE, root-knot nematode, technique

INTRODUCTION

The root-knot nematode *Meloidogyne incognita* is one of the most economically damaging agricultural pests worldwide, with a wide host range of at least 1,700 plant species (Sasser et al., 1983). *This* sedentary endoparasite has evolved a highly *specialized* and *complex relationship with its host plants by inducing the root tissue* to form *specific* feeding sites, the so-called giant cells (Williamson and Hussey, 1996; Hussey and Grundler, 1998). Although *M. incognita* reproduces by obligate mitotic parthenogenesis, it exhibits high capacities of adaptation to environmental constraints, e.g. its ability to alter avirulent to virulent population able to reproduce on resistant cultivars (Castagnone-Sereno, 2006).

The use of resistant or non-host crops is an effective and environmentally friendly method to mange *M. incognita* on many crops and at the same time to reduce chemical nematicides (Williamson and Kumar, 2006). For *successful* nematode management using resistant plant cultivars or appropriate crop rotations, the differentiation among locally occurring nematode populations and/or races need to be known. Various populations of *M. incognita* have been differentiated into races based on their susceptibility to the differential hosts (Robertson et al., 2009; Devran and Sogut, 2011) or into virulent (aggressive) and avirulent (nonaggressive) populations based on their reproduction on different cultivars (Anwar and McKenry, 2007; Olowe, 2010). However, identification of *M. incognita* populations using solely differential hosts can be unreliable due to presence of atypical populations that in the past were identified as races of other species based on morphological and biochemical analysis (Fargette, 1987). Therefore, differentiation among *M. incognita* populations by method race biotest should be used in conjunction with morphological characterization or molecular methods to obtain a trustworthy diagnose.

In general, *Meloidogyne* species can be identified based on distinct morphological and biochemical characters, but populations or races of the same species cannot be directly defined by these techniques. High similarity among races of some species or poor taxonomic descriptions between populations resulted in intra-species merging of

morphological characters and difficulty in distinguishing between populations or races of *Meloidogyne* species based on morphological characters (Robertson et al., 2009).

Currently, molecular assays have become the preferred methods for routine identification of root-knot nematodes as they are faster and more accurate than morphological assay. A number of molecular methods to identify *Meloidogyne* species have been used, e.g. restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), satellite DNA probes, sequence characterized amplified regions (SCARs), real-time PCR and high-resolution melting curve (HRMC) analysis (Holterman et al., 2012). However, molecular assay that can determine race or virulence within the same species of *Meloidogyne* has not been obtained yet (Cortada et al., 2011). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is a molecular method that can detect differences in DNA sequences or mutations of various genes based on differential denaturing characteristics of the DNA. Despite its speed and potential to discern changes in a *single* nucleotide *base pair* of same length DNA fragment, PCR-DGGE in nematology has so far been limited to analysis of soil or marine nematode communities (Okada and Oba, 2008).

The *Mi-msp1* gene is highly expressed in preparasitic and parasitic J2 of *M. incognita* but not in adults (Ding et al., 2000). The *Mi-msp1* cDNA contained an open reading frame encoding 231 amino acids with the first 21 amino acids being a putative secretion signal. The secreted protein plays a key role in the initial infection of the host plant (Ding et al., 2000). It is considered a member of the SCP/TAPS family of secreted proteins that is found in several nematode species and is similar to the allergen antigen 5 of extracellular proteins from hymenopteran insect venom (King et al., 1990; Gao et al., 2001). The objective of this research was to differentiate *M. incognita* populations and/or races which showed variability in their reproduction rate on different hosts by PCR-DGGE based on *msp1*gene variation.

MATERIALS AND METHODS

Nematode sources: The seven nematode populations and races used in this study originated from three different countries (Table 1). Four populations (E1, E2, G1, G2) were identified as *M. incognita* by molecular diagnostic analysis using the intergenic spacer (IGS2) of the ribosomal DNA cistron, while three races were identified and supplied by Prof. Stephen Thomas, New Mexico State University in USA. All populations and races were isolated from single egg masses and propagated on tomato (*Solanum lycopersicum*) cv. Moneymaker under greenhouse conditions.

Table 1. *Meloidogyne incognita* populations or races used in this study and their origin.

Code	Geographic Origin	Original host
E1	Sekem organic farm, El-Sharkia, Egypt	Pepper
E2	Sekem organic farm, El-Sharkia, Egypt	Tomato
G1	Reichenau, Baden-Württemberg, Germany	Bur cucumber
G2	Reichenau, Baden-Württemberg, Germany	Cucumber
R1	Ken Barker, USA [a]	Tomato
R2	Ken Barker, USA [a]	Tobacco
R3	Mexico, USA [a]	Chile pepper

[a] Kindly provided by Prof. Stephen Thomas (New Mexico State University).

Greenhouse test: Different crops/cultivars were used to differentiate between populations and/or races, including pepper (*Capsicum annuum* cv. California wonder), cotton (*Gossypium hirsutum* cv. DP 61) and three cultivars of tomato (*Solanum lycopersicum*) cv. Moneymaker (susceptible), cv. Tomasa (tolerant), cv. Sparta (resistant). Two week-old seedlings were transplanted into 11-cm-diam. plastic pots containing about 400 g of pasteurized field-soil:sand mix (1:1, v:v). Two weeks later, each seedling was inoculated with 200 freshly hatched second-stage juveniles (J2) in 2 ml water by pipetting into four holes 3 cm deep around the plant base. The inoculum was prepared by extracting nematode eggs from tomato roots using 1.5% NaOCl as described by Hussey and Barker (1973). Suspension of eggs were placed on a modified

Baermann dish and incubated at 25 ± 2°C for 7-10 days to separate hatched J2 from eggs (Hooper et al., 2005). The hatched J2 were collected daily. Only freshly hatched J2 collected within 48 h were used for experiments. Eight replicates of each host and population or races combination were used. The pots were arranged in a randomized block design. The plants were watered as needed and fertilized weekly with 10 ml of commercial fertilizer (WUXAL® Super NPK fertilizer, 8-8-6 with micronutrients, 2.5 g liter^{-1}). Pots were kept in the greenhouse at 22 ± 2°C and 16-h photoperiod.

The experiment was terminated 50 d after inoculation when 30-50% of the eggs of one egg mass produced on Moneymeker showed folded juveniles inside the egg. Plants were removed from their pots, and root systems were carefully washed free of adhering soil by dipping the roots in a bucket of water with changing the water several times. Egg masses were stained by submersing the roots in 4% cochenille red (Brauns-Heitmann, Warburg, Germany) for 15 min to aid visualizing the egg masses. Immediately before examination excess stain was removed by gently washing the root in water. After counting the number of galls and egg masses on the entire root system of each plant, the root was transferred into a plastic bottle half filled with 2% chlorine solution. Roots were vigorously shaken for 3 min to free the eggs from the gelatinous matrices. The suspension was thoroughly washed with tap water through a 250 µm sieve sitting on a 20 µm sieve. The root debris on the top sieve was discarded. Eggs collected on the 20 µm sieve were transferred into a glass bottle and the number of embryonic eggs (black inside), juvenile eggs (folded juveniles recognizable within egg) and juveniles (hatched juveniles) were counted. By dividing total number of eggs per root with number of egg masses per root, the number of eggs per egg mass was obtained.

DNA extraction: Genomic DNA was extracted from individual J2 from the populations listed in Table 1 using ZR Tissue and Insect DNA MicroPrep^{TM} kit (ZYMO RESEARCH, USA). Ten individual newly hatched J2 for each of the populations or races were transferred by pipetting into ZR BashingBead^{TM} lysis tube and then lysed in a FastPrep instrument (MP Biomedicals, Heidelberg, Germany) for 40 s at high speed. The tubes were then centrifuged for 1 min at 10,000 *g*, the supernatant transferred to a

Zymo-spin™ IV Spin Filter and then processed according to the manufacturer's instructions.

PCR–DGGE to differentiate msp1 gene variants: The *msp1* gene fragments were amplified from DNA isolated from J2 for denaturing gradient gel electrophoresis (DGGE) using the primers msp410f (with GC-clamp) 5´GC-clamp-TTGATGATTGATGCCTGTAATGC and MImsp596r ATAACGACAATCAATCAAAT that were designed based on an alignment of published sequences of *Meloidogyne hapla* and *M. incognita*. PCR was conducted in a 25 µl volume of 1 µl of template DNA, 1x TrueStart buffer, 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 4% (vol/vol) acetamid, 0.2 µM of each primer, and 1.25 U TrueStart Taq polymerase (Fermentas, St Leon-Rot, Germany). PCR was carried out using the following thermal cycles: 95°C for 5 min, then 40 cycles at 94°C for 45 s, 46°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 5 min. Before DGGE analysis, the PCR products were examined by running 5 µl aliquots of the reaction mixtures in a 1% agarose gel. DGGE was performed with a gradient of 29% to 56% denaturants for analysis of *msp1* gene fragments (where 100% denaturant was defined as 7 M urea plus 40% formamide). Approximately 4-12 µl aliquots of PCR products prepared from DNA extracted from J2 of the populations and races were loaded side by side on a DGGE gel with four replicates each. The DGGE gel was run in a PhorU_2 apparatus (Ingeny, Goes, The Netherlands), in 1× Tris-acetate-EDTA buffer at 60°C with a constant voltage of 100 V for 16 h. The gel was silver stained as described by Heuer et al. (2001). GelCompar II 6.6 was used for pairwise comparisons of DGGE profiles of nematode populations using Pearson correlation for estimating similarity coefficients and the unweighted pair group method with arithmetic averages (UPGMA) for cluster analysis.

Cloning and sequencing: For sequencing of the different bands of *msp1*gene fragment that showed at different positions in the DGGE gel, PCR products obtained with the primers msp410f and MImsp596r were cloned using the vector pGEM-T and *Escherichia coli* JM109 high-efficiency competent cells (Promega, Madison, WI, USA). Based on PCR-DGGE, cloned amplicons corresponding in electrophoretic mobility to

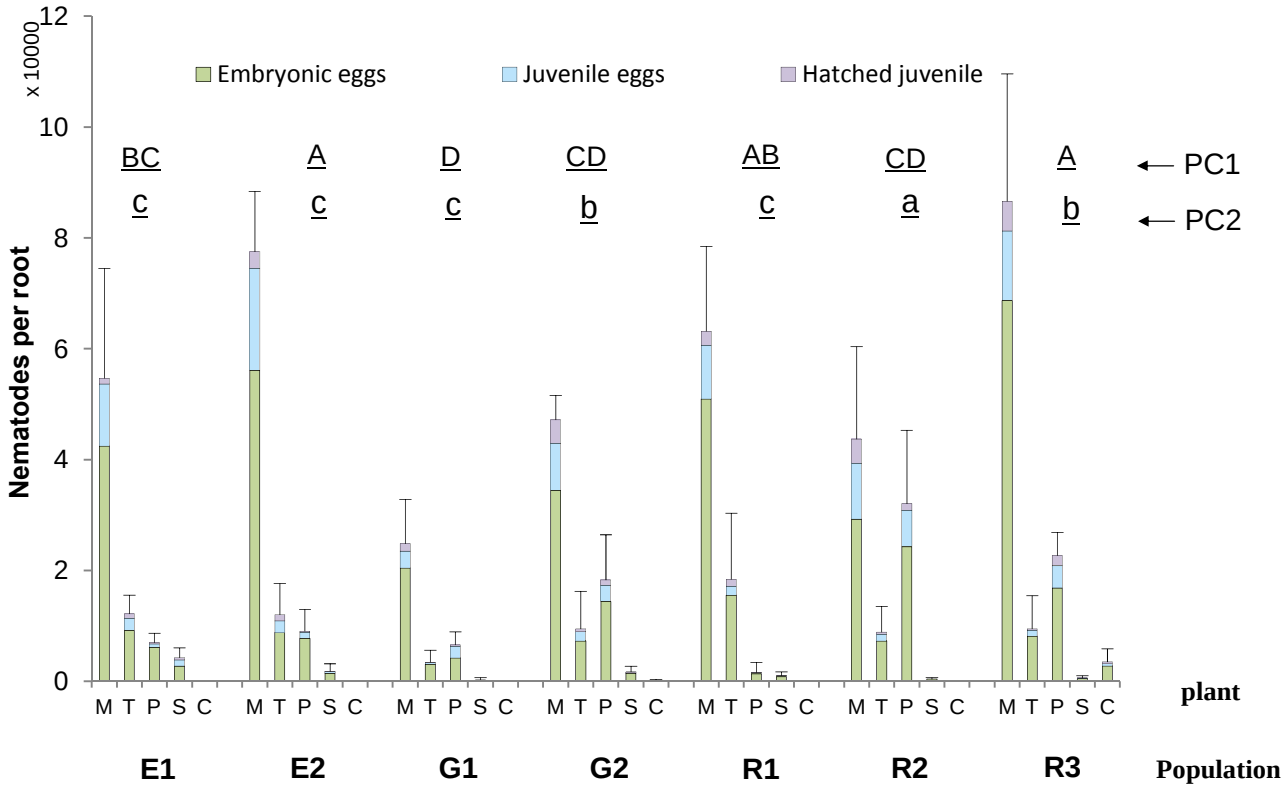
different bands were sequenced (Macrogen, Amsterdam, The Netherlands). The obtained sequences were compared with nucleotide sequences in the Genbank using the BLAST Software (http://blast.ncbi.nlm.nih.gov) to determine similarities. Sequences were aligned using Mega 5.1 program to show areas of variability and areas of conserved regions.

Statistical analysis: The numbers of egg masses, embryonic eggs, juvenile eggs, and hatched J2 from each of the five plants were compared between the seven nematode populations. To account for correlations in this multivariate dataset and to reduce dimensionality, principal component analysis (PCA) using SPSS Statistics 19 was performed. The first two principal components, which explained 89% of the variance, were used for univariate analyses of variance with Tukey adjustment to test for significant differences between the nematode populations.

RESULTS

Phenotypic differentiation among M. incognita populations: The patterns of embryonic eggs, juvenile eggs, and hatched J2 generated on the five host plants varied among populations and/or races (Fig. 1A). As expected, all populations and races reproduced well on the susceptible tomato cv. Moneymaker showing the highest number of eggs. This cultivar already allowed to differentiate among populations or/and races. For example, populations G1, G2, R2 and E1 produced fewer eggs and J2 on tomato cv. Moneymaker than R3 ($P \leq 0.05$). Eggs produced by population E2 on cv. Moneymaker developed faster to juvenile eggs than those from the other populations and races, with 23% of the total eggs developed to J1 ($P \leq 0.05$). In contrast, the resistant tomato cv. Sparta suppressed reproduction of all *M. incognita* populations and races, except for population E1 which was able to reproduce, achieving the greatest quantity and development of eggs. For the tolerant tomato cv. Tomasa, no significant differences in the quantity or development of eggs was observed among all populations or/and races, except that the total number of eggs produced by G1 was significantly lower than those produced by R1 ($P = 0.002$). Pepper cv. California wonder apparently differentiated between the three races but did not distinguish between German and Egyptian populations. On cotton cv. DP 61 only R3 and G2 were able to reproduce, with R3 resulting in a significantly higher number of eggs than G2 ($P \leq 0.05$). Principal component analysis on the selected nematode parameters for the different host plants and analysis of variance of the first and second principal component (PC1 and PC2) showed significant differences between all populations/races, expect that E1 and E2 were not different from R1 (Fig. 1A). The biplot of PC1 and PC2 showed good discrimination of the two populations E1 and G1 and the two races R2 and R3, but was overlapping for the populations E2 and G2 and race R1 (Fig. 1B). The PC1 that explained 73% of the total variance was mainly based on the number of embryonic eggs on tomato cv. Moneymaker, while PC2 explained an additional 16% of the total variance and was mainly based on the number of embryonic eggs on pepper. Based on PC1 the analyzed populations and races could be divided into two groups; one including R2, R3 and G2 and the other including E1, E2, G1 and R1 (Fig. 1B).

A.


x 10000
Embryonic eggs
Juvenile eggs
Hatched juvenile
BC
c
A
c
D
c
CD
b
AB
c
CD
a
A
b
PC1
PC2
Nematodes per root
M T P S C
plant
E1
E2
G1
G2
R1
R2
R3
Population

B.

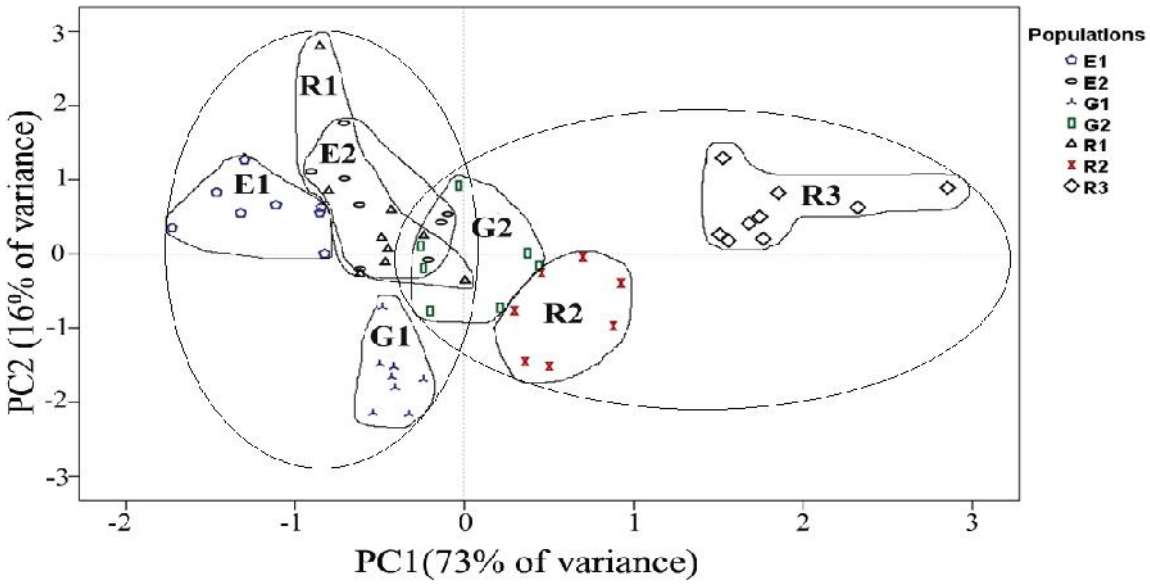

Populations
E1
E2
G1
G2
R1
R2
R3
PC2 (16% of variance)
PC1(73% of variance)

Fig.1. Phenotypic differentiation of *Meloidogyne incognita* populations by their reproduction on tomato cv. Moneymaker (M), tomato cv. Tomasa (T), tomato cv. Sparta (S), pepper cv. California wonder (P), and cotton cv. DP61 (C). **A**: Progeny and developmental stages of eggs were determined for each plant 50 days after inoculation of 200 J2 of Egyptian populations (E1, E2), German populations (G1, G2), or the races R1, R2, or R3. The reproduction pattern of the populations / races on the different host plants was compared by principal component analysis of the multivariate dataset and pairwise analysis of variance of principal components 1 (PC1) and 2 (PC2) using Tukey's adjustment (n = 10, $P < 0.05$). Different upper or lower case letters in a row indicate significant differences between populations with respect to PC1 or PC2, respectively. Error bars represent SD of total numbers of eggs. **B**: Biplot of PC1 and PC2.

Differentiation of M. incognita populations based on msp1 gene variation: The *msp1* gene was used to differentiate between *M. incognita* populations and/or races by separation of amplified sequence variants in DGGE analysis. DGGE profiles of all populations and races showed a good separation of *msp1* fragments by five major bands detected at different positions in the denaturing gradient (Fig. 2A). Among them, the variants R1-A and R2-B were abundant in all replicate DNA samples from all populations and races. R1-A was the dominant band in all samples. Other bands seemed to be specific for some populations or races. For example, gene variants G2-A and G2-B were abundant in the population G2, R2, and R3 (but only in two of the replicates from ten J2), while much less abundant in the populations E1, E2, G1, and R1. Band E1-B was weak and only appeared for population E1 and R2 (Fig 2A).

UPGMA analysis of DGGE patterns of the different populations and races revealed a clear separation in two main clusters (Fig 2B). One large cluster was formed by G2, R2, R3 (two replicates) and E1 (one replicate) and the other one by G1, E2, R1, E1 (except for one replicate) and R3 (two replicates). With the exception of R3, replicates of each population or race (at least three replicates) were clearly separated forming together a separate cluster with < 95% similarity. Sequencing of cloned amplicons, which corresponded to different bands, displayed 97-99% similarity with sequences of *msp1* genes of *M. incognita* in the Genbank (AF013289, ASM18041v1). Sequence variations close to the reverse primer among the gene variants corresponding to the five DGGE bands could explain the different melting behavior in the denaturing gradient (Fig 2C).

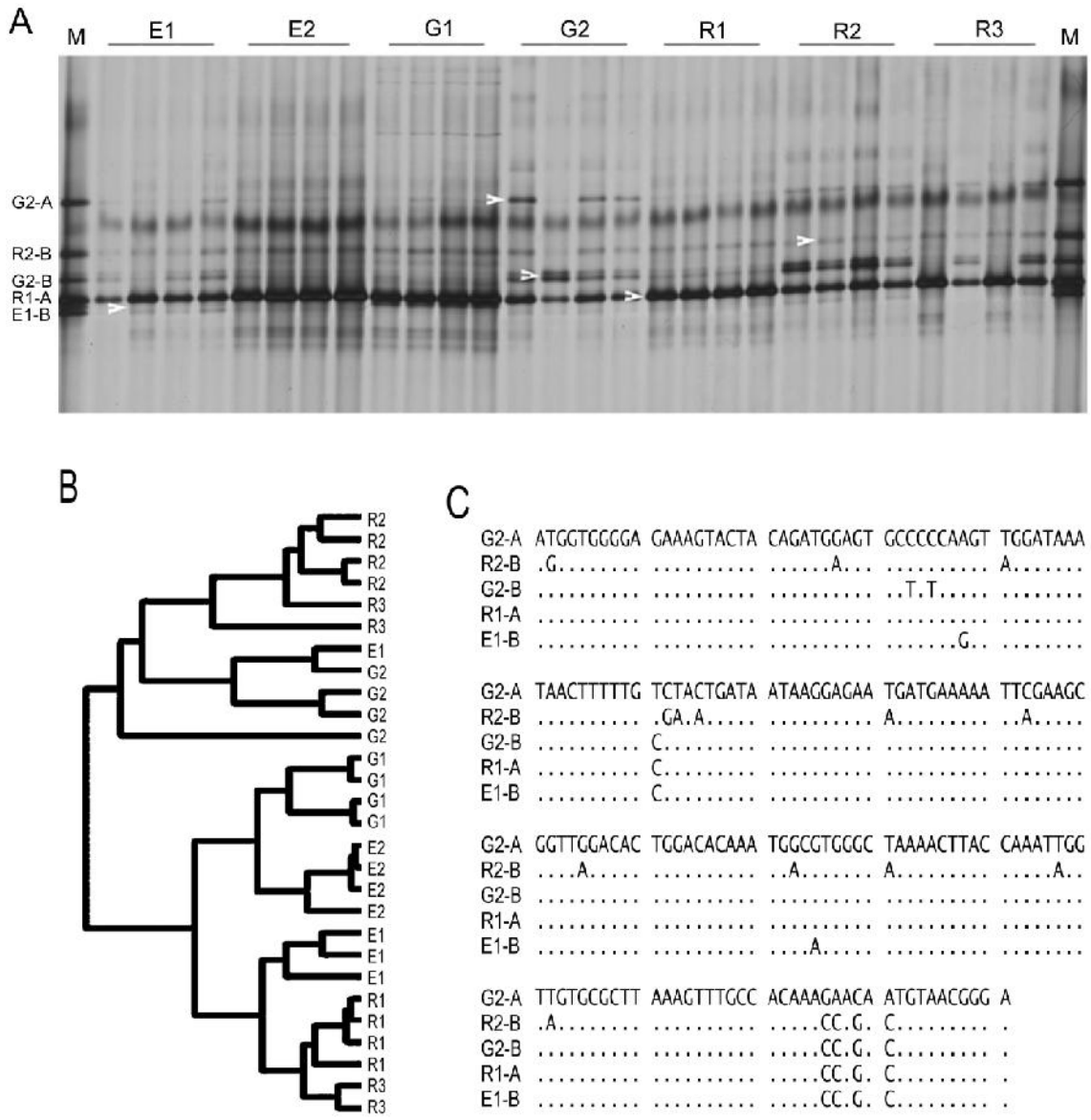


Fig. 2. Genetic differentiation of *Meloidogyne incognita* populations based on variants of their pathogenicity gene *msp1*. **A**: Denaturing gradient gel electrophoresis (DGGE) of the *msp1* genes from two Egyptian populations (E1, E2), two German populations (G1, G2), and the races R1, R2, and R3. Each of four replicates per population were derived from DNA of ten J2. Cloned and sequenced amplicons representing the different gene variants were combined in a marker (M) as indicated on the left side. **B**: UPGMA cluster analysis of the DGGE fingerprints. **C**: Alignment of DNA sequences of the *msp1* gene variants representing major bands in DGGE. Dots indicate the same base as in the sequence of the G2-A variant. Primer sequences were not included.

DISCUSSION

Recently, molecular techniques became alternative ways to overcome some gaps of the traditional diagnostic techniques. In this study, a PCR-DGGE technique was developed to differentiate populations and/or races of *M. incognita*, which have exhibited variability in their reproduction on different crops/cultivars. PCA scatter plot analysis was able to separate the seven populations/races into two differential groups. Parameters that were related to egg production and embryonic development were good indicators for characterizing populations/races, and might be better discriminating than gall numbers as described by Anwar and McKenry (2007) and Verdejo-Lucas et al. (2012). However, PCA did not allow complete separation of all populations and/or races, as populations the Egyptian populations and Race 1 could not be distinguished.

None of the selected host plants on its own was able to differentiate among all populations and/or races. Reproduction on tomato cv. Moneymaker discriminated well among the populations, while mostly pepper enabled to distinguish among the races. Tomato cv. Sparta differentiated population E1 from others, while cotton cv. DP 61 differentiated Race 3 from other population or races. These results indicate that the differentiation using plant hosts affords many crops / cultivars and nematode parameters to achieve a discrimination among *M. incognita* populations. Despite its problems with speed and accuracy, bioassays are still the most common method used to differentiate *M. incognita* populations (Anwar and McKenry, 2007; Olowe, 2010; Thies, 2011).

To the best of our knowledge, this is the first report on the use of PCR-DGGE to distinguish among populations of one species of plant-parasitic nematodes. Despite its high rapidity and efficiency as a diagnostic tool, DGGE in nematology has so far only been applied to compare soil or marine nematode communities based on the 18S rRNA gene (Cook et al., 2005; Okada and Oba, 2008). Here, we developed a DGGE protocol for optimal separation of *msp1* gene variants. We were able to show that *M. incognita* populations / races differ in their *msp*1 variants and therefore, this method was able to differentiate populations or races. Hence, DGGE banding patterns could be used to

visualize similarity or dissimilarity among populations / races based on these different bands.

Interestingly, UPGMA analysis of DGGE patterns separated the populations or races into two major groups similar to those that were obtained from PCA analysis of the bioassay data. Although the variability in the *msp1* gene was sufficient for separation, no relation between the gene variants and the reproductive potential of the population on different hosts was observed. This agrees with Gerič Stare et al. (2012) showing that sequence variability of the *expB2* gene was not sufficient to distinguish pathotypes of *Globodera rostochiensis*.

In general, the PCR-DGGE method should be taken into account as a molecular tool that could address many unresolved questions of genetic variation and population genetics of plant parasitic nematodes. This approach is expected to be a useful tool to resolve allele frequencies to differentiate populations using effector genes of plant-parasitic nematodes, or to study population-level epidemiology and population-specific infectivity.

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Chapter IV

The manuscript presented in this chapter has been accepted for publication in Applied and Environmental Microbiology

Specific microbial attachment to root-knot nematodes in suppressive soil

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ABSTRACT

Understanding the interactions of plant-parasitic nematodes with antagonistic soil microbes could provide opportunities for novel crop protection strategies. Three arable soils were investigated for their suppressiveness against the root-knot nematode *Meloidogyne hapla*. For all three soils, *M. hapla* developed significantly fewer galls, egg masses, and eggs on tomato plants in non-sterilized than in sterilized infested soil. Egg numbers were reduced by up to 93%. This suggested suppression by soil microbial communities. The soils significantly differed in the composition of microbial communities and in suppressiveness to *M. hapla*. To identify microorganisms interacting with *M. hapla* in soil, second-stage juveniles (J2) baited in the test soil were cultivation-independently analyzed for attached microbes. PCR-denaturing gradient gel electrophoresis of fungal ITS or 16S rRNA genes of bacteria and bacterial groups from nematode and soil samples were analyzed, and DNA sequences from J2-associated bands were determined. The fingerprints showed many species that were abundant on J2 but not in the surrounding soil, especially in fungal profiles. *Fungi* associated with J2 from all three soils were related to the genera *Davidiella* and *Rhizophydium*, while *Eurotium*, *Ganoderma*, and *Cylindrocarpon* were specific for the most suppressive soil. Among the 20 highly abundant operational taxonomic units of bacteria specific for J2 in suppressive soil six were closely related to infectious species like *Shigella* spp., while most abundant were *Malikia spinosa* and *Rothia amarae*, as determined by 16S rRNA amplicon pyrosequencing. In conclusion, a diverse microflora specifically adhered to J2 of *M. hapla* in soil and presumably affected female fecundity.

Keywords: *Meloidogyne hapla*; biocontrol; soil suppressiveness; cuticle; plant-parasitic nematodes; bacterial antagonists; fungal antagonists.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are among the most damaging pathogens of many crops worldwide, and are important pests in Europe (1). Chemical nematicides are costly and restricted due to their adverse impact on the environment and human health, whereas cultural control or host plant resistance are often not practical or not available (2). Alternative management strategies could include biological control methods. Microbial pathogens or antagonists of root-knot nematodes have high potential for nematode suppression. Many fungal or bacterial isolates have been found that antagonize root-knot nematodes either directly by toxins, enzymatically, parasitically, or indirectly by inducing host plant resistance (3). Indigenous microbial communities of arable soils were occasionally reported to suppress root-knot nematodes (4-7).

Soils that suppress to *Meloidogyne* spp. are of interest for identifying antagonistic microorganisms and the mechanisms that regulate nematode population densities. Understanding the ecological factors that enable these antagonists to persist, compete and function may improve the basis for integrated management strategies. Cultivation-independent approaches were used in several studies to analyze the diversity of bacteria or fungi associated with the plant-parasitic nematode genera *Bursaphelenchus* (8), *Heterodera* (9-11), or *Rotylenchulus* (12). Papert *et al.* (13) showed by PCR-DGGE of 16S rRNA genes that the bacterial colonization of egg masses of *Meloidogyne fallax* differed from the rhizoplane community. A rRNA sequence most similar to that of the egg-parasitizing fungus *Pochonia chlamydosporia* was frequently detected in egg masses of *Meloidogyne incognita* that derived from a suppressive soil (4).

Root-knot nematodes spend the majority of their life protected inside the root. After hatch second-stage juveniles (J2) of root-knot nematodes migrate through soil to penetrate host roots. During this searching, they are most exposed to soil microbes. Root-knot nematodes do not ingest microorganisms, and their cuticle is the main barrier against microbes. The collagen matrix of the cuticle is covered by a continuously shed and renewed surface coat mainly composed of highly glycosylated proteins, which

likely is involved in evasion of host immune defense and microbial attack (14). Attachment of microbes to the J2 cuticle while dwelling through soil may result in transport of microbes to roots, endophytic colonization, co-infection of roots, or defense response of the plant triggered by microbe-associated molecular pattern. Attached microbes may also directly inhibit or infect J2, or later colonize eggs of nematodes (15). Despite its potential ecological importance, the microbiome associated with J2 of root-knot nematodes has not yet been analyzed by cultivation-independent methods.

In this study three arable soils were investigated for their suppressiveness against the root-knot nematode *Meloidogyne hapla*. The bacteria and fungi attached to J2 incubated in these soils were analyzed based on their 16S rRNA genes or internal transcribed spacer (ITS), respectively, and compared to the microbial communities of the bulk soil. The objectives were (i) to test whether a specific subset of soil microbes attaches to J2 of *M. hapla*, (ii) to test whether attached species differ between soils of varying suppressive potential, and (iii) to identify bacteria and fungi that putatively interact with J2 of *M. hapla*.

MATERIALS AND METHODS

Soils. Soils were obtained from three different locations in Germany and included a Luvic-Phaeozem with medium clayey silt and 17.2% clay (loess loam, pH 7.3, organic carbon content C_{org} 1.8%) from a field of the plant breeder KWS Saat AG in Klein Wanzleben (Kw), a Gleyic-Fluvisol with heavy sandy loam and 27.5% clay (alluvial loam, pH 6.7, C_{org} 1.8%) from a lettuce field in Golzow (Go), and an Arenic-Luvisol with less silty sand and 5.5% clay (diluvial sand, pH 6.1, C_{org} 0.9%) from a field in Großbeeren (Gb). These soils were selected because of a low abundance of *M. hapla* despite the presence of suitable environmental conditions and susceptible plants. The soils were previously characterized in detail (16), and data on microbial communities were available. Soil samples were collected from eight plots within each field. Each sample consisted of approximately 3 kg composed of 12 soil cores taken from the top 30 cm. All samples were kept in polyethylene bags and stored at 4°C until further processing.

Greenhouse assay for soil suppressiveness. The suppressiveness against *M. hapla* of the microbial communities in the three soils was determined by comparing the reproduction of inoculated J2 on tomato plants in natural and sterilized soil. Native soil without inoculated J2 served as control for putative indigenous root-knot nematodes. Thus, each of the eight replicate soil samples of each soil was divided into three portions for the three treatments. The portion for the J2-inoculation into sterilized soil was autoclaved at 134°C for 10 min to kill indigenous microbes, followed by a 20 min dry cycle,. Each portion of the soil samples was separately mixed with steamed loamy sand at a ratio of 1:1 to improve physical soil properties for greenhouse culture, and placed in 1.2 kg portions in 15-cm diameter pots. Two week-old seedlings of *Solanum lycopersicum* 'Moneymaker' were transplanted into the pots. One week after transplanting, 1,600 freshly hatched J2 of *M. hapla* were inoculated into each pot, except the control for putative indigenous root-knot nematodes. The J2 were inoculated by transferring 1 ml of a suspension with 200 J2 ml^{-1} into each of eight holes at the periphery of the pot (7 cm from stem base, 2 cm deep), so that the J2 could interact with soil microbes before penetrating tomato roots. The pots were arranged in a randomized

block design, so that in total 72 pots (8 replicate blocks x 3 soils x 3 treatments) were maintained in the greenhouse at 20 ± 2°C at ambient light. Plants were watered and fertilized as needed. Two months after inoculation, root systems were washed free of adhering soil and weighted. Egg masses attached to the roots were stained with 0.4% cochenille red solution (Brauns-Heitmann, Warburg, Germany) for 15 min. Galls and egg masses were counted. Roots were vigorously shaken for 3 min in 2% chlorine to free the eggs from the gelatinous matrices. The suspension was poured through a 250 µm aperture sieve to remove roots. Eggs were collected on a 20-µm sieve and counted.

Soil baiting with J2 and DNA extraction. To analyze the microorganisms attaching to J2 when they move through soil, J2 were inoculated in each soil and extracted after exposure to the microbial communities in the three soils. Four replicate tubes per soil type with 2000 inoculated J2 in 50 g soil were kept at 20 ± 2°C in the dark for 7 days. The soil moisture was adjusted to 15%. J2 were extracted from the soil by centrifugal flotation with $MgSO_4$ solution (17), collected on 25 µm aperture sieves, and transferred with sterile water into Petri dishes. Under the stereomicroscope 100 J2 from each replicate, which were morphologically identified as root-knot nematodes, were captured by needle. DNA from J2 with adhering microorganisms was extracted using the FastPrep FP120 bead beating system (MP Biomedicals, Santa Ana, CA) for 30 s at high speed, the FastDNA Spin Kit for soil (MP Biomedicals), and the GENECLEAN Spin Kit (MP Biomedicals) for further purification. In parallel, total soil DNA was extracted from 0.5 g bulk soil of each tube by the same method for comparison of the microbial communities from nematode samples to those of the surrounding soil.

PCR-DGGE of fungal ITS and bacterial 16S rRNA gene fragments. PCR amplifications of fungal ITS and of 16S rRNA genes of bacteria or bacterial groups from total DNA of soil and J2 samples, and separation of the PCR products in DGGE was done as previously described (18). Shortly, bacterial 16S rRNA gene fragments were amplified either directly from total DNA using the primer pair F984GC / R1378, or via PCR with primers that were designed to target the bacterial groups *Alphaproteobacteria, Betaproteobacteria, Pseudomonas, Actinobacteriales, Enterobacteriaceae*, or *Bacillus* (all primer sequences are shown in Table S1). The

fungal ITS fragments were amplified using a nested PCR approach with primer pairs ITS1F / ITS4 and ITS1FGC / ITS2. DGGE was done using the PhorU$_2$ system (Ingeny, Goes, The Netherlands) as previously described (18).

Analysis of ribosomal sequences of microbes attached to J2. For the DGGE fingerprints of bacterial groups and fungal ITS fragments that showed nematode-specific bands, PCR products were cloned and sequenced to identify the corresponding microbial species by sequence comparison to GenBank entries. For *Alphaproteobacteria* and *Pseudomonas*, PCR products obtained with primers F984GC / R1378, for *Bacillus*, products produced with primers BacF / R1378, and for fungal profiles, products of the primers ITS1FGC / ITS2 were used (Table S1). PCR products were cloned using the vector pGEM-T and *Escherichia coli* JM109 high-efficiency competent cells (Promega, Madison, WI). Based on the PCR-DGGE analysis, cloned amplicons corresponding in electrophoretic mobility to nematode-specific bands were sequenced (Macrogen, Amsterdam, The Netherlands).

Barcoded amplicon pyrosequencing was used to analyze 16S rRNA genes of total J2-associated bacteria. PCR with universal bacterial primers F27 / R1494 was done as previously described (19). The products were purified with the Minelute PCR Purification Kit (Qiagen, Hilden, Germany) and used as target to amplify the V3–V4 region of 16S rRNA genes with fusion primers containing the Roche-454 A and B Titanium sequencing adapters, an eight-base barcode sequence in adaptor A, and specific sequences V3F / V4R targeting the ribosomal region. Library preparation and sequencing were done on a 454 Genome Sequencer FLX platform according to standard 454 protocols (Roche – 454 Life Sciences, Branford, CT) by Biocant (Cantanhede, Portugal). Pyrosequencing data were evaluated according to Ding *et al*. (20). Briefly, sequences matching the barcode and primer were selected for blastn searches in the database SILVA 115 SSU Ref (21) and a subset of that containing the strains with species name. Chimera were truncated, barcodes and primers removed, and sequences shorter than 200 bp discarded. Multiple alignments and operational taxonomic unit assignment (OTU, > 97% similarity) were done using the software package Mothur v1.14.0 (22). OTU were regarded as specific for J2 that comprised more than 1% of all

sequences of J2 samples, and that were not detected in soil or had at least 100 times higher relative abundance on J2 compared to soil.

Statistical analysis. For the greenhouse experiment, numbers of galls, egg masses, eggs per gram of root, and eggs per egg mass after propagation of inoculated J2 were compared between pots with native and sterilized soil for each soil type. Data were log-transformed and a linear model with soil, treatment, and soil*treatment as fixed effects, and block as random effect was applied (Table S2). For pairwise comparisons between soil types the Tukey-Kramer adjustment was applied.

Sequence accession numbers. Sequences for DGGE bands were deposited in GenBank with accession no. KF225704-KF225718 and KF257370-KF257399. Pyrosequencing data were deposited at the NCBI Sequence Read Archive under the study accession number SRP029944.

RESULTS

Microbes of the three soils reduced progeny of *M. hapla* to different extent. To assess the suppressive effect of the microbial soil communities on *M. hapla*, the nematode propagation on tomato was compared between sterilized and native soils. Significantly fewer galls, egg masses, eggs, and a reduced rate of fecundity (eggs per egg mass) were found on roots from native soils than in sterilized soils eight weeks after J2 inoculation ($P<0.001$, ANOVA with soil origin and sterilization as fixed effects, see Table S2). Also soil origin had a significant effect on nematode counts and fecundity ($P<0.015$), except for egg masses ($P=0.055$). In non-sterilized soil Kw the lowest numbers of galls, egg masses, eggs and eggs per egg mass were found compared to soils Go and Gb (Table 1). The number of eggs was reduced by 93% in native soil Kw compared to the sterilized control and was significantly lower than for the other soils, suggesting that the microbial community of soil Kw had a more suppressive effect. The reduction in galls and egg masses for soil Kw was less pronounced than egg reduction (58% and 68%, respectively). The least suppressive soil Go had significantly more galls, egg masses, and eggs in the non-sterilized treatment than soil Kw (Table 1), with significantly lower reductions compared to the sterilized control (30%, 38%, and 63%, respectively).

In contrast to the native soils, in sterilized soils the numbers of galls and egg masses were highly similar between soils. Egg numbers and fecundity in sterilized soils were fewest for Go and highest for Gb, while sterilized soil Kw did not show the lowest counts among the soils as seen for the soils with indigenous microbial communities (Table 1). This suggested a minor role of the physico-chemical soil differences compared to biotic factors. In control pots without J2 inoculation, indigenous root-knot nematodes developed only 5 galls on one tomato plant in soil Kw, which was too low to confound nematode counts of the inoculated non-sterilized pots (data not shown).

TABLE 1. Effect of soil biota on fertility of *M. hapla* on tomato planted in three infested soils

Parameter	Soil treatment	$\log_{10}$ (number g^{-1} root fresh weight) ± SD [a]		
		Soil Kw	Soil Go	Soil Gb
Galls	Sterilized	1.53 ± 0.18 [A]	1.57 ± 0.21 [A]	1.54 ± 0.11 [A]
	Non-sterilized	1.09 ± 0.33 [a]	1.45 ± 0.06 [b]	1.17 ± 0.19 [a]
Egg masses	Sterilized	1.47 ± 0.17 [A]	1.49 ± 0.20 [A]	1.45 ± 0.11 [A]
	Non-sterilized	0.86 ± 0.44 [a]	1.28 ± 0.13 [b]	0.91 ± 0.39 [ab]
Eggs	Sterilized	4.48 ± 0.08 [AB]	4.45 ± 0.14 [A]	4.58 ± 0.12 [B]
	Non-sterilized	3.31 ± 0.19 [a]	3.95 ± 0.27 [b]	3.86 ± 0.21 [b]
Fecundity (Eggs / egg mass)	Sterilized	3.01 ± 0.13 [AB]	2.96 ± 0.07 [A]	3.13 ± 0.10 [B]
	Non-sterilized	2.45 ± 0.35 [a]	2.67 ± 0.24 [ab]	2.95 ± 0.41 [b]

[a] Values are means of eight replicate root systems. Different letters within a row indicate a significant difference between means either for sterilized or native soils (P<0.05, Tukey-Kramer adjustment).

Fungal attachment to *M. hapla* in soil. The fungi sticking to J2, which were extracted from the three soils and washed, were analyzed by PCR-DGGE of fungal ITS fragments. ITS profiles of DNA from J2 showed 20 (for soil Kw) to 40 (for soil Gb) clearly visible bands while profiles of fungal soil communities were much more complex (Fig. 1). Several fungal ITS-types were abundant in all replicate DNA samples from J2 of one or more soils but not in the surrounding soil suggesting specific attachment to the J2 in soil (Fig. 1; bands 2, 3, 4, 6, 9, 11, 13, 15).

Some of the fungal ITS types associated with J2 were also abundant in soil but the relative band intensity within the profile was higher for the J2 samples than for soil which indicated an enrichment on J2 (Fig. 1; bands 1, 5, 7, 8, 10, 12, 14). The most reproducible patterns were detected on J2 from replicates of the most suppressive soil Kw evidencing the most specific fungal attachment compared to those from the other two soils. The DNA sequences of ITS types were determined to identify fungal species that potentially interacted with the J2 in soil. The sequences corresponded to fungal ITS of eight genera of Ascomycota, five genera of Basidiomycota, *Rhizopodium* (Chytridiomycota), and *Mortierella* (Fungi incertae sedis) (Table 2).

Bands 9 and 15, of which the DNA was most closely related to the genera *Davidiella* and *Rhizophydium*, respectively, were associated with J2 from all three soils, even though they were mostly below detection limit in the soil fungal communities. Some bands were common on both nematodes and soil samples in the three soils, such as bands 1, 12 and 14 that were corresponding to *Malassezia restricta*, *Mortierella* sp. and *Ascomycete* sp., respectively (Table 2). Eight of the ITS-types associated with J2 were soil type specific, four of which were only detected on J2 (Table 2; bands 3, 4, 6, 13), while the other four were obtained from both J2 and soil samples (Table 2; bands 5, 7, 8, 10). The sequences of these bands exhibited 98-100% similarity with known sequences of fungal species in GenBank (Table 2).

Furthermore, two of the attached ITS-types seemed to be specific for J2 samples in two of the three soils (Table 2; bands 2, 11). The ITS-type of band 2 was found in J2 samples from the two most suppressive soils, Kw and Gb, and corresponded to *Aspergillus penicillioides* (99.7% identities). In contrast to J2 from soils Go and Gb, those extracted from the most suppressive soil Kw were specifically associated with ITS-types closely related to *Eurotium* sp., *Ganoderma applanatum*, and *Cylindrocarpon olidum* (Table 2; bands 6, 7, 13).

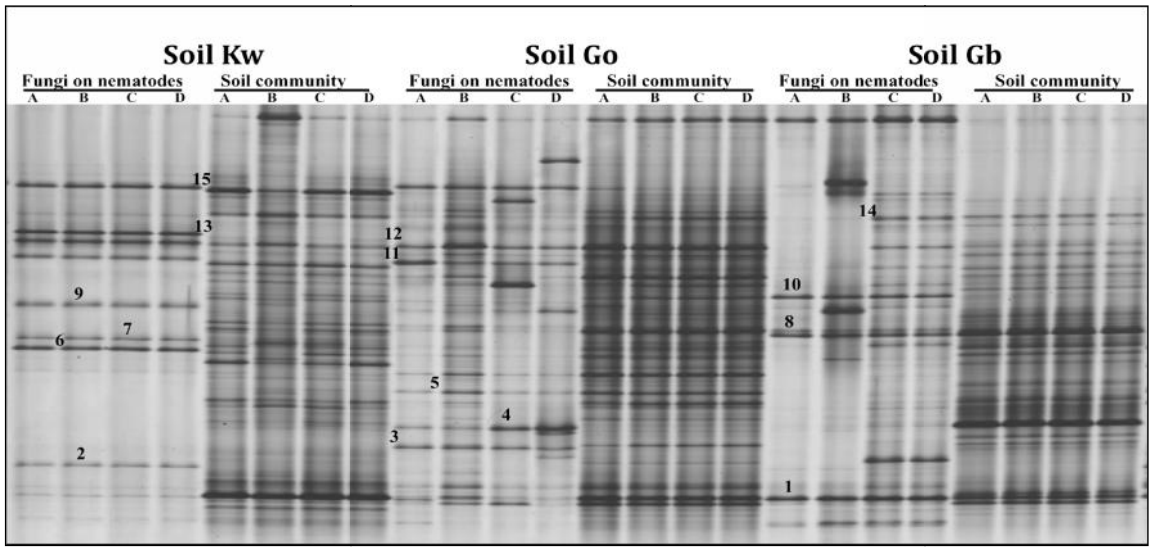

FIG. 1. DGGE profiles of fungal ITS fragments amplified from DNA of *M. hapla* J2 from three arable soils, and from total soil DNA. Fungal ITS-types are marked that were enriched in nematode samples and characterized by sequencing (Table 2).

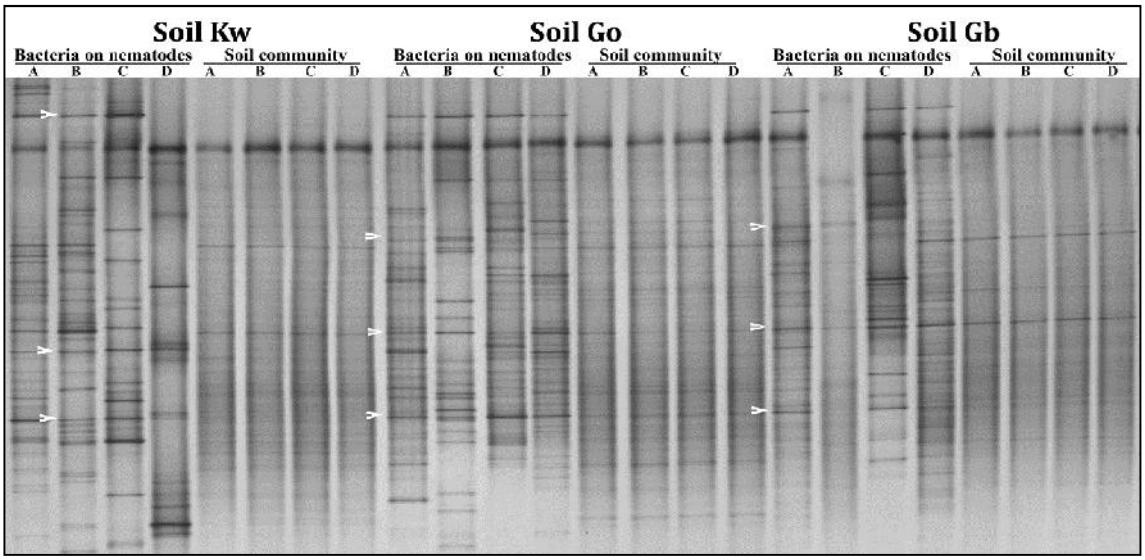
Soil Kw
Bacteria on nematodes
Soil community
Soil Go
Bacteria on nematodes
Soil community
Soil Gb
Bacteria on nematodes
Soil community
A B C D A B C D
A B C D A B C D
A B C D A B C D

TABLE 2. Identification and frequency of the dominant nematode-specific DGGE bands

DGGE band no.	Closest GenBank match	% Iden-tities	Number of samples where band was found					
			Nematodes			Soil		
			Kw	Go	Gb	Kw	Go	Gb
Fungi DGGE								
1	*Malassezia restricta* EU400587	98.7	4	4	4	4	4	4
2	*Aspergillus penicillioides* GU017496	99.6	4	0	2	0	0	0
3	*Cryptococcus pseudolongus* AB105353	100	0	4	0	0	0	0
4	*Chaetomium globosum* JX501299	98.2	0	4	0	0	0	0
5	*Arthopyreniaceae* FJ439584	100	0	4	0	0	4	0
6	*Eurotium* sp. AM901702	100	4	0	0	0	0	0
7	*Ganoderma applanatum* JX501311	99.6	4	0	0	2	0	0
8	*Cladosporinum cladosporioides* AJ300335	100	0	0	4	0	0	4
9	*Davidiella* sp. JX164064	99.6	4	4	4	0	0	0
10	*Cryptococcus* sp. JX164076	100.0	0	0	4	0	4	4
11	*Trichosporonales* EF060720	98.3	4	4	0	0	0	0
12	*Mortierella* sp. JF439489	99.6	4	4	2	4	4	4
13	*Cylindrocarpon olidum* GU198183	99.0	4	0	0	0	0	0
14	*Ascomycete* AM410609	99.2	4	4	4	4	4	4
15	*Rhizophydium* sp. DQ485617	98.7	4	4	2	0	0	0
Bacillus DGGE								
1	*Bradyrhizobium pachyrhizi* NR_043037	97.9	0	3	0	0	0	0
2	*Sphingomonas insulae* NR_044187	99.4	1	1	3	0	0	0
3	*Staphylococcus epidermidis* NR_036904	100	4	4	4	0	0	0
4	*Staphylococcus epidermidis* NR_036904	99.6	4	4	4	0	0	0
5	*Micrococcus endophyticus* NR_044365	98.6	3	3	4	0	0	0
6	*Bacillus megaterium* NR_043401	99.7	4	4	4	0	0	0
7	*Micrococcus luteus* NR_037113	99.2	4	4	4	4	4	4
8	*Propionibacterium acnes* NR_040847	100	4	4	4	4	4	4
9	*Methylobacterium rhodesianum* NR_041028	97.2	2	1	3	0	0	0
10	*Streptococcus thermophilus* NR_074827	100	0	0	3	0	0	0
Alphaproteobacteria DGGE								
1	*Solirubrobacter soli* NR_041365	99.8	2	3	1	3	3	0
2	*Janthinobacterium lividum* NR_026365	99.8	1	0	3	0	0	0
3	*Rhizobium phaseoli* NR_044112	99.8	4	4	4	0	0	0
4	*Pedomicrobium australicum* NR_026337	96.0	1	3	3	4	4	4
5	*Ochrobactrum anthropi* NR_074243	99.5	4	3	2	2	4	0
6	*Maricaulis maris* NR_041967	91.0	3	2	3	4	4	4
7	*Nitrospira moscoviensis* NR_029287	96.3	2	3	0	0	0	0
8	*Anderseniella baltica* NR_042626	92.8	2	2	2	0	0	0
9	*Devosia chinhatensis* NR_044214|	96.6	0	3	2	4	4	4
10	*Kaistia soli* NR_044302	96.0	0	2	3	0	0	0
11	*Magnetospirillum gryphiswaldense* NR_027605	96.3	1	3	1	0	0	0

12	*Bosea eneae* NR_028798	95.5	4	4	4	0	0	0
13	*Rhodobacter blasticus* NR_043735	96.3	4	4	4	4	4	4
Pseudomonas DGGE								
1	*Pseudomonas asplenii* NR_040802	99.5	0	3	0	0	0	0
2	*Pseudomonas tuomuerensis* NR_043990	99.1	2	3	2	4	4	4
3	*Pseudomonas koreensis* NR_025228	100	3	0	0	0	0	0
4	*Pseudomonas jessenii* NR_024918	99.3	1	3	3	0	4	3
5	*Pseudomonas jessenii* NR_043314	99.1	1	1	3	0	0	4
6	*Pseudomonas koreensis* NR_074834	99.8	3	0	1	0	0	0
7	*Pseudomonas taetrolens* NR_036909	98.9	4	4	4	4	4	4

Nematode-specific bands representing attachment to J2 in the three soils were mainly detected in DGGE fingerprints generated with primers, which were designed to preferentially target 16S rRNA genes of *Alphaproteobacteria, Bacillus,* and *Pseudomonas*. Bacterial 16S rRNA genes amplified based on the selective specificity of primer BacF were most clearly enriched in J2 samples (Table 2). Among them, four intense bands were detected in most J2 samples from all soils (Table 2; Fig S1 A, bands 3-6), of which the sequences belonged to the genera *Staphylococcus*, *Micrococcus*, and *Bacillus* (Table 2).

The majority of cloned 16S rRNA genes amplified based on the specificity of primer F203α belonged to the *Alphaproteobacteria* (Table 2). Despite the high variability of these bacteria from nematode samples, a few bands were dominant on most J2 from the three soils (Table 2; Fig. S1 B), which were related to *Rhizobium phaseoli* (99.8% identities) or *Bosea* sp., respectively. Bacteria from J2 samples that were much more abundant for the most suppressive soil Kw were not apparent, but more intense bands were related to sequences of the actinobacterial species *Solirubrobacter soli,* and the alphaproteobacterial species *Ochrobactrum anthropi* and *Anderseniella* sp. (Table 2).

In *Pseudomonas*-specific DGGE fingerprints, bands related to *P. koreensis* were most clearly associated with J2 from soil Kw (Table 2, bands 3, 6; Fig. S1 D). Other pseudomonads that were relatively more abundant in J2 samples than in the soil samples were similar to *P. asplenii*, *P. tuomuerensis*, *P. jessenii*, or *P. taetrolens*. DGGE

fingerprints from 16S rRNA genes of *Actinobacteriales, Betaproteobacteria*, and *Enterobacteriaceae* showed high variability among replicate J2 samples so that bacteria specifically attached to the nematodes were hardly distinguishable from randomly attached bacteria (Fig. S1 C, E, F).

Bacteria on J2 based on 16S rRNA gene amplicon pyrosequencing. Bacterial 16S rRNA gene sequences from nematode and soil samples were determined by barcoded amplicon pyrosequencing. In total 22,347 sequences from 12 nematode samples were obtained and analyzed together with sequences from all three bulk soils. The sequences were grouped, based on 97% identity, into 12,425 OTU, of which 87% were unique to soil samples, 9% had a higher relative abundance on J2 than in soil, and 6% were unique to J2 samples. Thus the diversity of bacterial OTU associated with the J2 in soil was strongly reduced compared to soil. The overlap of abundant OTU between J2 and soil samples was low. The 24 OTU that were most abundant in nematode samples (>1%) but not detected in soil, or that were at least 100 times higher in relative abundance on J2 than in soil, are shown in Table 3. They mainly belonged to the *Alpha-, Beta-*, and *Gammaproteobacteria, Firmicutes*, and *Actinobacteria*.

Nineteen of the OTU had more than 99% sequence identity with strains of well studied species, nine of which are associated with infectious diseases (*Streptococcus salivarius, Peptoniphilus gorbachii, Mycoplasma wenyonii*, *Brucella* sp., *Paracoccus yeei*, *Neisseria mucosa*, *Shigella flexneri*, *Acinetobacter schindleri*, *Acinetobacter johnsonii*). In the most suppressive soil Kw, J2 were especially associated with 18 OTU, of which the most abundant OTU were related to the species *Rothia amarae, Malikia spinosa, Shigella* spp., *Janthinobacterium lividum, Geobacillus stearothermophilus*, and *Pseudomonas kilonensis*. Three of the OTU, which were mainly detected on J2 from soil Kw but also on J2 from soil Gb, were closely related to yet uncultured bacteria of the *Gemmatimonadetes, Deltaproteobacteria*, or *Rhodospirillaceae*, respectively.

TABLE 3. OTU of bacteria that were highly enriched on soil-derived J2 of *M. hapla* compared to the bacterial community in soil, based on 16S rRNA gene amplicon pyrosequencing

Most similar cultured species or environmental sequence of the OTU specific for J2 (Acc. No., identities) [a]	Number of sequences		
	J2 from Kw	J2 from Gb	J2 from Go
Micrococcus yunnanensis (KC469953, 100%)	9	21	612
Rothia amarae (T) (AY043359, 100%)	835	0	0
Geobacillus stearothermophilus (T) (AB021196, 99.2%)	394	74	0
Streptococcus salivarius (T) (AY188354, 100%)	0	651	0
Anaerococcus octavius (T) (Y07841, 99.2%)	91	4	177
Peptoniphilus gorbachii (T) (DQ911241, 100%)	118	0	28
Clostridium disporicum (T) (Y18176, 99.6%)	202	3	0
Mycoplasma wenyonii (CP003703, 99.7%)	110	1	3
Uncultured *Gemmatimonas* in rhizosphere (EU159980, 98.9%)	101	1	0
Uncultured delta proteobacterium (HE613616, 100%)	96	3	0
Ochrobactrum sp. / *Brucella* sp. (AJ242584 / AY594216, 99.8%)	147	17	0
Hirschia maritima (T) (FM202386, 96.0%)	128	0	0
Haematobacter missouriensis (T) (DQ342315, 100%)	222	0	0
Paracoccus yeei (T) (AY014173, 100%)	161	0	0
Uncultured *Rhodospirillaceae* (GQ263062, 100%)	261	5	0
Malikia spinosa (AB077038, 98.5%)	962	0	48
Janthinobacterium lividum (T) (Y08846, 99.8%)	480	13	0
Neisseria mucosa (HG005351, 99.8%)	104	0	0
Vogesella indigofera (AB021385, 99.2%)	0	421	0
Shigella flexneri / *S. fergusonii* (T) (X96963 / AF530475, 100%)	518	0	109
Acinetobacter schindleri (T) (AJ278311, 99.6%)	0	76	305
Acinetobacter johnsonii (X81663, 100%)	0	229	67
Enhydrobacter aerosaccus (T) (AJ550856, 100%)	172	3	67
Pseudomonas kilonensis (T) (AJ292426, 99.8%)	281	9	0
Total sequences	7647	8664	6164

DISCUSSION

This study has revealed by cultivation-independent techniques that diverse microbial communities attached to J2 of *M. hapla* when they were moving through soil. Several fungal and bacterial types were abundant on J2 but not in the surrounding soil, while other types detectable in soil were highly enriched on J2 relative to other soil microbes. This suggested a specific attachment of these microbes to the cuticle surface of J2. Evidence is gathering that species-specific characteristics of cuticle and surface coat determine microbial attachment to J2, and that the highly glycosylated mucins of the surface coat play a role in specificity (14). Bacterial adhesion changes with genetically determined modification of the complex carbohydrates of the surface coat (23, 24). The Gram-positive obligate parasites of root-knot nematodes, *Pasteuria* spp., are highly host specific in endospore attachment to the cuticle. So far only a few examples for non-parasitic attachment of bacteria or fungi to the cuticle of plant-parasitic nematodes have been described (25, 26), and images of the J2 surface by scanning electron microscopy indicated a rather low abundance of microorganisms with the exception of highly specialized parasites (27). Also we found evidence for a rather low number of microbes on the cuticle, evidenced by high variation between microbial DGGE fingerprints from J2, and low amounts of direct PCR products from DNA of J2 samples. The importance of the surface coat of the nematode cuticle in the recognition by nematode parasites has been recognized but studies have focused on highly specialized nematode parasites (28), and more recently on potential human pathogens (29).

In our study, soil suppressiveness to *M. hapla* was most likely caused by indigenous soil microbes as it was not observed in sterilized controls. In addition, differences in suppressiveness between the three soils investigated corresponded to differences in microbial soil communities and J2 attached microbes, while progenies of *M. hapla* in the sterilized soils were rather similar or did not correlate with the differences in the soils with indigenous microbial communities. However, some fungi and bacteria were found attached to J2 from all three soils, which therefore have not severely contributed to the differences in suppressiveness between the soils. It cannot be ruled out that some of these common microbes were already associated with the

inoculated J2. In previous studies, sensitivity to pasteurization or biocide treatment also provided evidence of the biological nature of soil suppressiveness to plant-parasitic nematodes (4, 30).

For all three soils, the reduction in numbers of egg masses and eggs was more pronounced than the effect on galling. This observation suggested a mode of action directed against nematode reproduction rather than against J2 vitality or the initial infection by juveniles. We surmised that reduction of reproduction was mediated by microbial attachment to juveniles in soil while searching for host plant roots. This attachment may have resulted in the transport of microbes into the root to the location of egg development. Although no indication of the presence of known parasites became evident this mode of action points to the involvement of antagonists that get attached to J2 in soil and then reduce the fecundity in females of the target nematode, as reported for *Pasteuria penetrans,* or egg-parasitic fungi (31, 32). Accordingly, a similar baiting assay as we used had been successful in searching for egg-parasites of root-knot nematodes (33). Transport of cuticle-attached microbes, which are not egg-parasites, to the host plant of the nematode has been shown for the phytopathogenic fungus *Dilophospora alopecuri* adhering to the J2 cuticle of *Anguina funesta* (34). Other attached microbes may establish as endophytes. Specific endophytes were observed to significantly reduce progeny of root-knot nematodes probably by indirect mechanisms based on endophyte-plant interactions rather than directly by nematicidal activity (35).

In our study by cultivation independent methods, we identified bacteria and fungi associated with J2 in soils with different suppressiveness against *M. hapla*. Two fungi were found on J2 from all tested soils that have been reported as attachments to nematode surface. A fungus of the genus *Rhizophydium* was previously reported as attachment to *Criconemoides* sp. (36), and fungi related to *Malassezia restricta* have been found in association with the soil nematodes *Malenchus* sp. and *Tylolaimophorus typicus* (37). In our study, a fungus related to *Cylindrocarpon olidum* was only abundant on J2 from the most suppressive soil Kw. Isolates of this genus were shown to reduce the number of galls of *M. javanica* on tomato roots (38), or to inhibit egg hatch of *Meloidogyne* spp. by metabolites (39). *Cladosporinum cladosporioides*, that was only

associated with J2 from the Gb soil, was previously found to be associated with *Meloidogyne* sp. females (40), and with *Rotylenchulus reniformis* vermiform stages and eggs (12).

Genera or species of the bacterial attachments to J2 from the three soils were also found in association with different plant-parasitic nematodes in previous studies (8, 9, 41, 42). J2 from the most suppressive soil Kw were often associated with OTU similar to species that were reported to be involved in infectious diseases (*Mycoplasma wenyonii*, *Peptoniphilus gorbachii*, *Brucella* sp., *Paracoccus yeei*, *Neisseria mucosa*, *Shigella flexneri*). These OTU may have in common with their pathogenic relatives that they efficiently attach to tissue surfaces as part of their life style, and thereby become enriched on the cuticle of J2. Other J2-enriched OTU were related to soil bacteria as *Rothia amarae*, *Malikia spinosa*, *Janthinobacterium lividum*, *Geobacillus stearothermophilus*, or *Pseudomonas kilonensis*. These bacteria might antagonize *M. hapla* after cuticle attachment but have not yet been found associated with root-knot nematodes. This can be explained by the bias of cultivation approaches which were used in most previous investigations. In a study on the bacterial community associated with cysts of *Heterodera glycines*, less than 5% of the bacteria could be cultured, and there was limited resemblance of the dominant species detected by DGGE analysis and the plating method (9).

In conclusion, a diverse microflora specifically adhered to J2 of *M. hapla* in soil, which might lead to colonization of eggs and play a role in nematode suppression. Several bacteria and fungi from soil enriched on the baiting J2 extracted from soil reportedly possess some nematicidal properties against plant parasitic nematodes. These should be evaluated for their potential as biocontrol agents. The sequence tags of these microbes could be useful to develop targeted cultivation methods for these species, for cultivation-independent study of the *in situ* interaction with *M. hapla*, and to survey their population increase in response to soil treatments. Management of arable soils to increase the abundance of antagonistic bacteria and fungi could become a substantial part in nematode control.

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TABLE S1. Primers used in this study

Specificity	Primer	Sequence (5'–3')	T_a °C	Refe-rence
Bacteria	F984GC	GC-clamp[a]-AACGCGAAGAACCTTAC	53	
	R1378	CGGTGTGTACAAGGCCCGGGAACG		(1)
Bacteria	F27	AGAGTTTGATCMTGGCTCAG	56	(2)
	R1494	CTACGGYTACCTTGTTACGAC		(3)
Bacteria	R1492	TACGGYTACCTTGTTACGACT	56	(2)
Bacteria	V3F	ACTCCTACGGGAGGCAG	44	(4)
	V4R	TACNVRRGTHTCTAATYC		
Alpha-proteobacteria	F203α	CCGCATACGCCCTACGGGGGAAAGATTTAT	56	(5)
Beta-proteobacteria	F948β	CGCACAAGCGGTGGATGA	64	(6)
Actino-bacteriales	$F243_{HGC}$	GGATGAGCCCGCGGCCTA	63	(1)
Entero-bacteriaceae	F234	GATGWRCCCRKATGGGA	57	(7)
	R1423	AKCTAMCTRCTTCTTTTGCAA		
Pseudomonas	F311Ps	CTGGTCTGAGAGGATGATCAGT	63	(8)
	R1459Ps	AATCACTCCGTGGTAACCGT		
Bacillus and related taxa	BacF	GGGAAACCGGGGCTAATACCGGAT	65	(9)
Fungi	ITS1FGC	GC-clamp-CTTGGTCATTTAGAGGAAGTAA	55	(10)
	ITS4	TCCTCCGCTTATTGATATGC		
	ITS2	GCTGCGTTCTTCATCGATGC		

[a] 5' GC-clamp CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG

TABLE S2. Statistical analysis of the effect of soil biota on fertility of *M. hapla* feeding on tomato planted in infested soils

Dependent variable	Significance of fixed effects [a]		
	soil	sterilized	soil*sterilized
logGalls	*P*=0.015	*P*<0.001	*P*=0.070
logEggmasses	*P*=0.055	*P*<0.001	*P*=0.096
logEggs	*P*<0.001	*P*<0.001	*P*<0.001
logEggs	*P*<0.001 with sterilized=0 *P*=0.044 with sterilized=1	*P*<0.002 with soil=Kw, Go, or Gb	not applicable
fecundity	*P*=0.003	*P*<0.001	*P*=0.097

[a] Data and statistical analysis by SAS package 9.3:

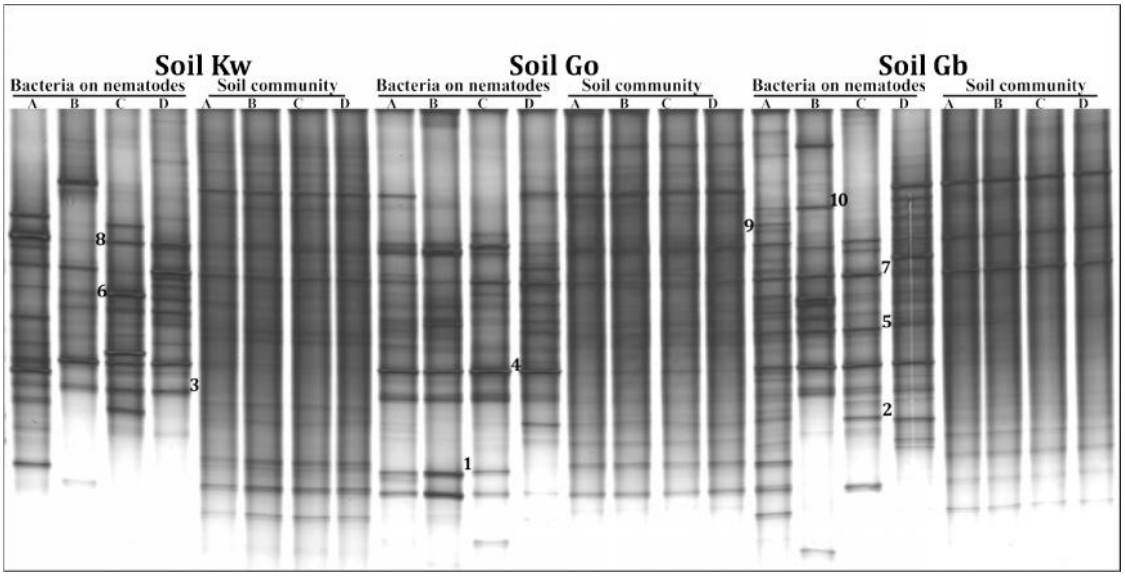
```
data M_hapla;
input block soil$ sterilized galls eggmasses eggs; /* per gram root freshweight*/
datalines;
1 Kw   0      26.1   19.9   3569.7
2 Kw   0       6.2    4.0   1968.7
3 Kw   0      14.3   10.1   1204.5
4 Kw   0      18.7   14.1   2145.9
5 Kw   0       3.8    1.0   1085.6
6 Kw   0      23.3    8.6   3506.5
7 Kw   0       5.7    3.7   1824.8
8 Kw   0      23.9   20.5   2424.2
1 Kw   1      45.6   38.6   32362.2
2 Kw   1      37.1   32.8   32861.9
3 Kw   1      37.8   32.4   25784.0
4 Kw   1      37.8   30.1   29014.1
5 Kw   1      31.2   25.9   26210.2
6 Kw   1      49.9   48.1   44296.4
7 Kw   1      32.5   31.8   30723.2
8 Kw   1      13.4   12.5   26215.0
1 Go   0      23.6   14.2   4829.7
2 Go   0      34.6   28.7   20323.7
3 Go   0      24.5   11.0   7300.6
4 Go   0      27.9   18.5   7541.0
5 Go   0      27.8   17.9   15363.6
6 Go   0      31.5   21.5   17770.4
7 Go   0      25.2   20.7   3952.9
8 Go   0      34.2   24.3   6818.2
1 Go   1      28.3   25.8   25055.9
2 Go   1      43.6   35.1   32651.0
3 Go   1      40.9   31.1   27985.5
4 Go   1      13.8   11.9   13071.9
5 Go   1      39.0   33.5   33246.5
6 Go   1      34.3   27.4   28133.0
7 Go   1      63.9   50.0   37230.2
8 Go   1      65.0   50.6   35150.5
1 Gb   0       9.1    8.1   2673.9
```

```
2 Gb   0      17.0   17.9   9242.2
3 Gb   0       7.8    2.0   6736.8
4 Gb   0      15.7    8.1   7037.0
5 Gb   0      12.5    2.3   10242.8
6 Gb   0      19.4   12.7   7870.0
7 Gb   0      17.5   12.6   5909.5
8 Gb   0      29.9   24.0   14403.0
1 Gb   1      21.3   17.2   29278.6
2 Gb   1      40.4   35.1   43593.3
3 Gb   1      48.6   35.1   38557.2
4 Gb   1      33.0   22.1   23832.9
5 Gb   1      35.2   31.9   45328.3
6 Gb   1      42.2   30.8   31615.6
7 Gb   1      31.6   27.8   50319.1
8 Gb   1      34.6   30.3   53750.7
;
data M_hapla; set M_hapla; /* log transformation */
fecundity = log(eggs / eggmasses);
logGalls= log(galls); logEggmasses= log(eggmasses); logEggs=log(eggs);

/* General tests */
proc mixed data=M_hapla; /* Effect of soil and soil sterilization on gall no.
*/
class block soil sterilized;
model logGalls = soil sterilized soil*sterilized / ddfm=kr; random block;
proc mixed data=M_hapla; /* Effect of soil and soil sterilization on eggmass
no. */
class block soil sterilized;
model logEggmasses = soil sterilized soil*sterilized / ddfm=kr; random block;
proc mixed data=M_hapla; /* Effect of soil and soil sterilization on eggs no.
*/
class block soil sterilized;
model logEggs = soil sterilized soil*sterilized / ddfm=kr; random block;
proc mixed data=M_hapla; /* Effect of soil and sterilization on eggs per
eggmass */
class block soil sterilized;
model fecundity = soil sterilized soil*sterilized / ddfm=kr; random block;

/* Tukey-Kramer tests for sterilized and native soils separately: */
proc sort data=M_hapla; by sterilized soil block;
proc mixed data=M_hapla; by sterilized; /* difference between soils in gall
no. */
class block soil;
model logGalls = soil / ddfm=kr; random block;
lsmeans soil / ADJUST=TUKEY;
proc mixed data=M_hapla; by sterilized; /* egg masses compared between soils
*/
class block soil;
model logEggmasses= soil / ddfm=kr; random block;
lsmeans soil / ADJUST=TUKEY;
proc mixed data=M_hapla; by sterilized;/* no. of eggs compared between soils
*/
class block soil;
model logEggs = soil / ddfm=kr; random block;
lsmeans soil / ADJUST=TUKEY;
proc mixed data=M_hapla; by sterilized; /* fecundity compared between soils */
class block soil;
model fecundity = soil / ddfm=kr; random block;
lsmeans soil / ADJUST=TUKEY;
proc sort data=M_hapla; by soil sterilized block;
proc mixed data=M_hapla; by soil;  /* For verification of general effects
after */
class block sterilized;                    /* a significant interaction
soil*sterilized */
```

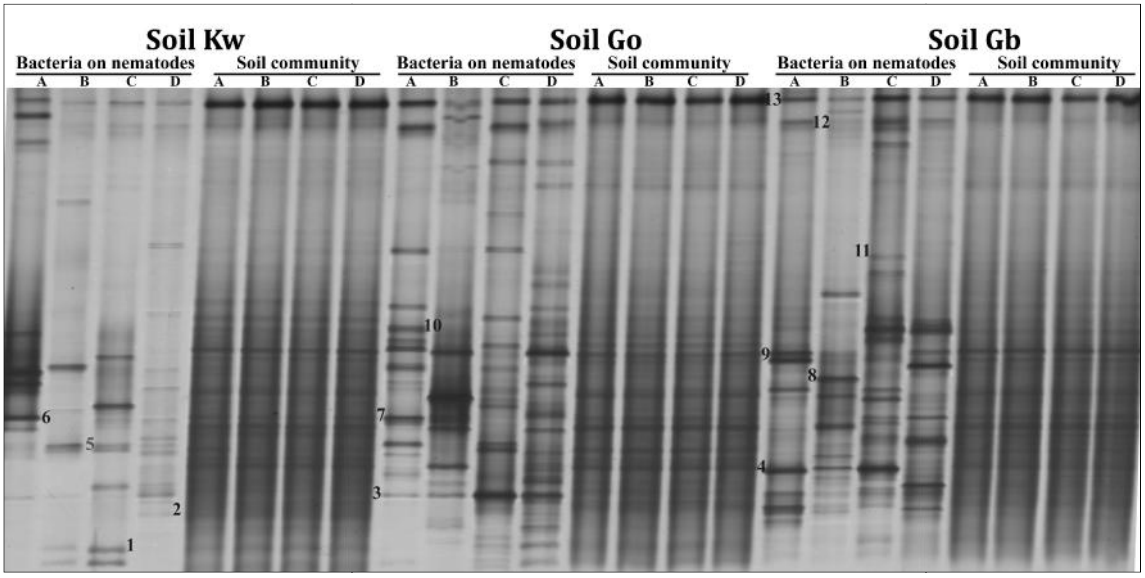
```
model logEggs = sterilized / ddfm=kr; random block;run;
```

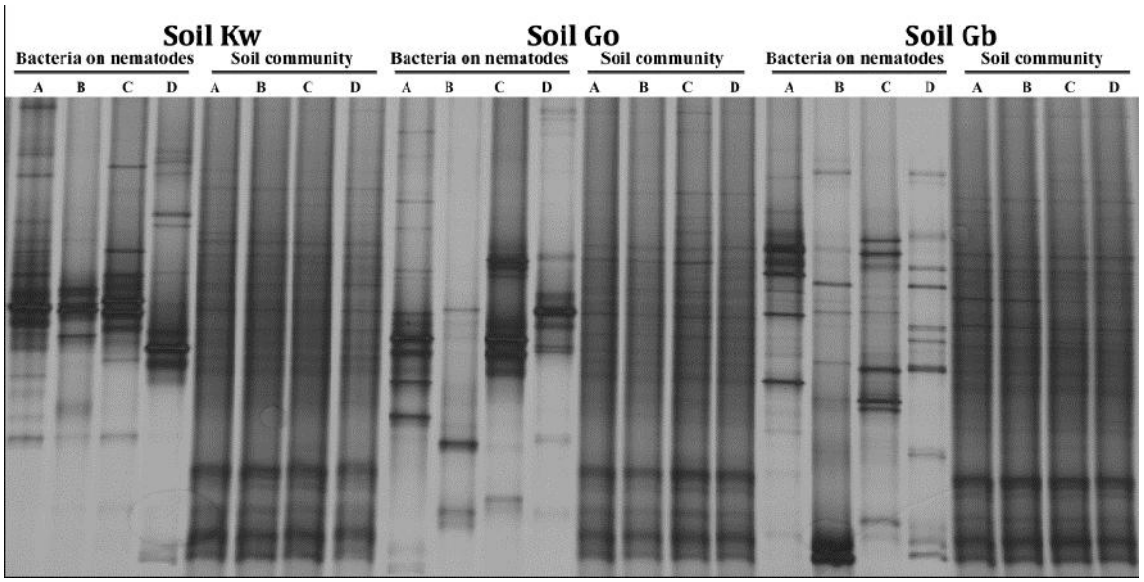
Fig. S1. PCR-DGGE profiles of 16S rRNA genes of bacterial subgroups amplified in nested PCR from DNA of *M. hapla* juveniles from three arable soils (Kw, Go, Gb), and from total soil DNA. Ribotypes are marked that were enriched in nematode samples and characterized by sequencing (Table 2).

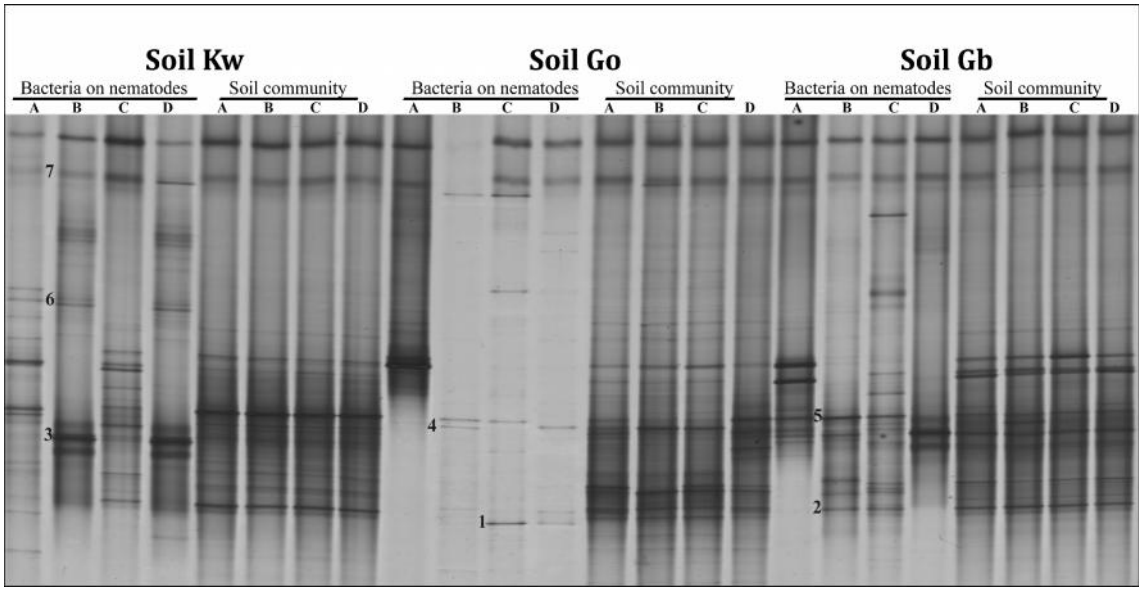
A) PCR-DGGE for "Bacillus" (based on specificity of primer BacF):



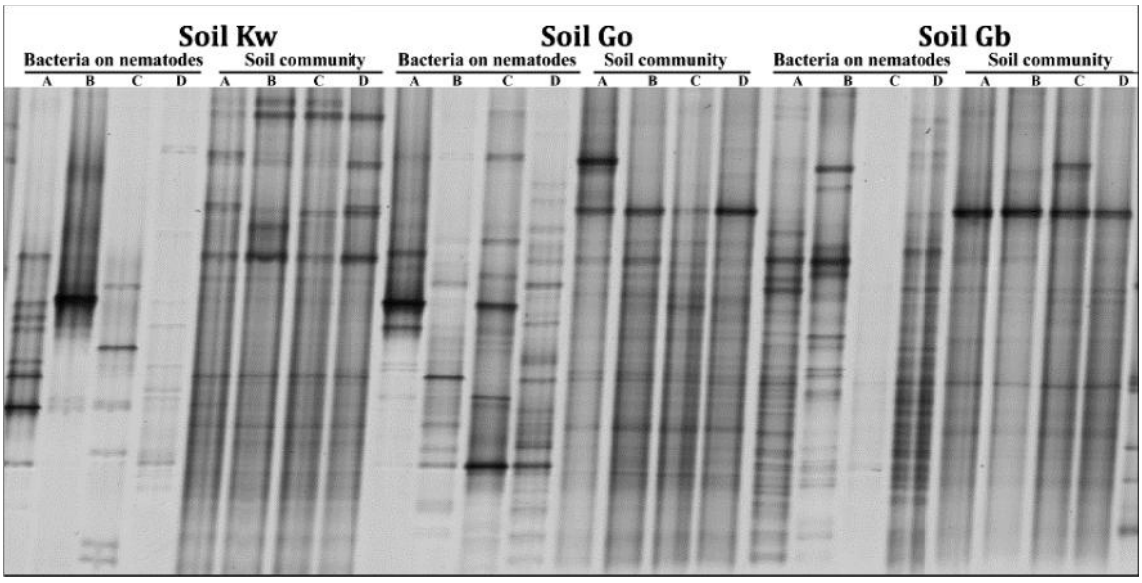
B) PCR-DGGE for *Alphaproteobacteria* (based on specificity of primer F203α):



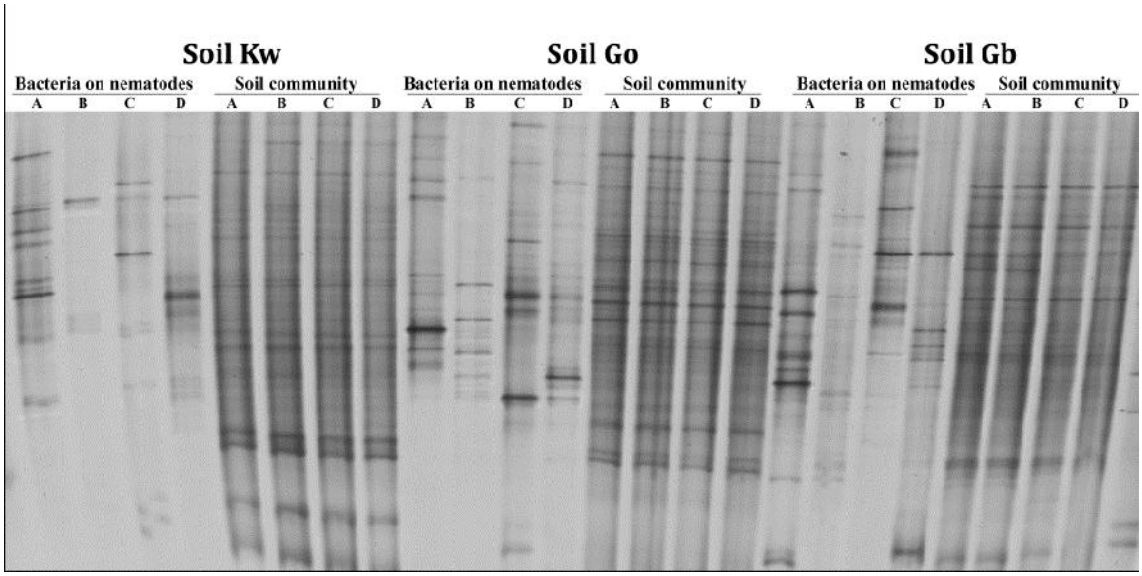

Soil Kw
Soil Go
Soil Gb
Bacteria on nematodes
Soil community
A B C D


Soil Kw
Soil Go
Soil Gb
Bacteria on nematodes
Soil community
A B C D
7
6
3
4
1
5
2

E) PCR-DGGE for *Enterobacteriaceae* (based on specificity of primers F234 / R1423):



F) PCR-DGGE for *Actinobacteriales* (based on specificity of primer F243HGC):



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Chapter V

The manuscript V.1 presented in this chapter has been published in in PLoS One.

The manuscript V.2: the thesis author preformed *the screening for in-vitro activity against nematodes experiment*.

Bacterial antagonists of fungal pathogens also control root-knot nematodes by induced systemic resistance of tomato plants

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Key words: *Meloidogyne incognita*, *Bacillus subtilis*, antagonism, induced systemic resistance, plant-parasitic nematode, repellence

Short title: Bacterial antagonists of *Meloidogyne incognita*

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Abstract

The potential of bacterial antagonists of fungal pathogens to control the root-knot nematode *Meloidogyne incognita* was investigated under greenhouse conditions. Treatment of tomato seeds with several strains significantly reduced the numbers of galls and egg masses compared with the untreated control. Best performed *Bacillus subtilis* isolates Sb4-23, Mc5-Re2, and Mc2-Re2, which were further studied for their mode of action with regard to direct effects by bacterial metabolites or repellents, and plant mediated effects. Drenching of soil with culture supernatants significantly reduced the number of egg masses produced by *M. incognita* on tomato by up to 62% compared to the control without culture supernatant. Repellence of juveniles by the antagonists was shown in a linked twin-pot set-up, where a majority of juveniles penetrated roots on the side without inoculated antagonists. All tested biocontrol strains induced systemic resistance against *M. incognita* in tomato, as revealed in a split-root system where the bacteria and the nematodes were inoculated at spatially separated roots of the same plant. This reduced the production of egg masses by up to 51%, while inoculation of bacteria and nematodes in the same pot had only a minor additive effect on suppression of *M. incognita* compared to induced systemic resistance alone. Therefore, the plant mediated effect was the major reason for antagonism rather than direct mechanisms. In conclusion, the bacteria known for their antagonistic potential against fungal pathogens also suppressed *M. incognita*. Such “multi-purpose” bacteria might provide new options for control strategies, especially with respect to nematode-fungus disease complexes that cause synergistic yield losses.

Key words: *Meloidogyne incognita, Bacillus subtilis*, antagonism, induced systemic resistance, plant-parasitic nematode, repellence

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are among the most damaging sedentary endoparasitic nematodes worldwide. The various species within this genus have an overall host range covering approximately 5500 plant species [1]. The species *Meloidogyne incognita* which is the most important under economic aspects can infect 1,700 plant species [2]. Root-knot nematodes also interact with fungal pathogens. A nematode-fungus interaction was first recorded by Atkinson in 1892, who observed that infection by root-knot nematodes always increased the severity of *Fusarium* wilt [3]. Such interactions often result in a disease complex causing synergistic yield losses [4] as described for root-knot nematodes and soil-borne fungal pathogens like *Thielaviopsis basicola*, *Rhizoctonia solani, Verticillium dahliae* and *Fusarium oxysporum* [5]. Controlling just one of the pathogens might not fully solve the problem. Combinations of nematicidal and fungicidal treatments are possible but not always desired due to their negative impact on the environment and human health. An alternative could be the use of microorganisms with dual antagonism against both the nematode and the fungal pathogen**.**

Bacteria represent an important group of biocontrol agents and several commercial products are nowadays available to control plant-parasitic nematodes [6] or fungal pathogens [7]. Only few studies previously investigated concomitant effects of bacterial antagonists against fungal and nematode pathogens. Bacterial isolates of the genera *Pseudomonas* and *Streptomyces* were described to control both *V. dahliae* and *M. incognita* [8]. A strain of *Pseudomonas aeruginosa* was found to be antagonistic towards *Meloidogyne javanica* and the fungal pathogens *Macrophomina phaseolina*, *R. solani*, *Fusarium solani*, and *F. oxysporum* [9]. Considering the broad spectrum of microbial antagonists reported over the past decades, different and more efficient microbial antagonists might be around waiting for discovery. The present work focused on bacterial strains. The mechanisms of bacteria to antagonize plant-parasitic nematodes include parasitism, pathogenesis, competition, repellence and induced systemic resistance [10-13]. Understanding their mode of action will help improving their effectiveness [10].

In the present work, bacterial isolates of the species *Bacillus subtilis, Pseudomonas trivialis, Pseudomonas jessenii*, and *Serratia plymuthica* were selected to study their antagonistic potential against the root-knot nematode *M. incognita* on tomato under greenhouse conditions. All strains have previously shown antagonistic potential towards soil-borne fungal pathogens [14-17]. From the first experiment, the top three bacterial strains plus *Rhizobium etli* G12 as positive control were selected for further studies on their mode of action. The objectives of this study were i) to evaluate the biocontrol potential of fungal antagonists towards *M. incognita*, and ii) to investigate their mode of action.

MATERIALS AND METHODS

Bacterial isolates. In total, nine bacterial isolates were tested in various experiments (Table 1). Four bacterial isolates (Sb3-24, Sb4-23, Mc5-Re2, Mc2-Re2) have previously shown *in-vitro* activity against fungal pathogens and *M. incognita* juveniles [17]. Three bacterial isolates (3Re2-7, C48, Ru47) are known antagonists of fungal pathogens [14-16]. Finally, the nematode antagonistic bacterium *R. etli* G12 served as positive control and *Escherichia coli* JM109 as negative control, respectively.

Table 1. Bacterial isolates used in this study.

Strain	Bacterial species	Isolation source	Pathogen suppressed	Reference	Source [a]
Sb3-24	*Bacillus subtilis*	Soil	*Verticillium dahliae, Rhizoctonia solani, Fusarium culmorum, Meloidogyne incognita*	[17]	GB
Sb4-23	*Bacillus subtilis*				GB
Mc5-Re2	*Bacillus subtilis*	Endorhiza of chamomile			GB
Mc2-Re2	*Bacillus subtilis*				GB
3Re2-7	*Pseudomonas trivialis*	Endorhiza of potato plants	*Rhizoctonia solani*	[14]	GB
C48	*Serratia plymuthica*	Rhizosphere of oilseed rape	*Verticillium dahliae*	[16]	GB
Ru47	*Pseudomonas jessenii*	Suppressive soil	*Rhizoctonia solani*	[15]	KS
G12	*Rhizobium etli*	Rhizosphere of potato plants	*Meloidogyne incognita*	[37]	RS
JM109	*Escherichia coli*		Non-antagonistic		P

[a] GB: G. Berg, University of Technology, Graz, Austria; KS: K. Smalla, Julius Kühn-Institut, Braunschweig, Germany: RS: R. Sikora, Bonn University, Germany; P: Promega, Mannheim, Germany.

Nematodes. The root-knot nematode *M. incognita* used in all experiments was propagated on tomato (*Solanum lycopersicum*) cv. Moneymaker under greenhouse conditions. For gaining nematode inoculum, eggs were extracted from heavily galled tomato roots. Roots were cut into 1-2 cm pieces, transferred to a 500 ml plastic bottle half filled with a 1.5% chlorine solution and vigorously shaken for 3 min to free the eggs from the gelatinous matrix [18]. The suspension was then thoroughly washed with tap water through a 250 µm aperture sieve, and eggs retained on the 20 µm sieve. To separate hatched second-stage juveniles (J2) from eggs the egg suspension was placed

on a modified Baermann dish and incubated at 25 ± 2°C for 7-10 days [19]. Hatched J2 were collected daily and stored at 6°C until further use in the experiments.

Plants and growing conditions. Tomato cv. Moneymaker was used in all experiments. Tomato seeds were grown in plastic pots containing a mixture of field-soil and sand (1:1, v:v). The plants were watered as needed and fertilized weekly with 10 ml of commercial fertilizer (WUXAL® Super NPK fertilizer, 8-8-6 with micronutrients, 2.5 g liter^{-1}). Pots were kept in the greenhouse at 25 ± 2°C and 16-h photoperiod.

Experimental evaluation. Nematode penetration was determined seven days after inoculation by staining the roots with a 1% acid fuchsine solution. Stained roots were kept in the refrigerator overnight to intensify the staining process. Excess acid fuchsine was removed by washing the roots in tap water. Roots were cut into 1 cm pieces and macerated twice for 15 s with a commercial blender (Waring, Torrington, CT, USA) and the number of juveniles in the root suspension was counted at 20 x magnification under a stereomicroscope.

Nematode reproduction was determined 50 days after nematode inoculation by counting the number of galls, egg masses and eggs produced by *M. incognita* on the tomato roots. Roots were gently washed to remove adhering soil. Fresh weights of shoots and roots were taken. Egg masses attached to the roots were stained with a 0.4% cochenille red (Brauns-Heitmann, Warburg, Germany) solution for 15 min. After excessive stain was removed by washing the roots in tap water the number of galls and egg masses was counted. Thereafter, roots were cut in 1-2 cm pieces and transferred into a glass bottle half filled with a 2% chlorine solution. Roots were heavily shaken for 3 minutes and the suspension was then thoroughly washed with tap water through a 250 µm sieve to remove root debris. Eggs collected on a 20 µm sieve were transferred into a glass beaker and counted.

Experiment 1: Potential of seed-inoculated strains to control *M. incognita*. Seven bacterial strains were investigated for their antagonistic activity against *M. incognita* in pot experiments. Tomato seeds were mixed in a bacterial lawn grown overnight on tryptic soy agar (Merck, Darmstadt, Germany) at 28°C for 24 h until the seed surface was completely covered by bacteria. The treated seeds were left a few minutes under a laminar flow hood for drying, and then each seed was transferred in 11-cm diameter plastic pots containing 400 g of soil watered to field capacity. Pots containing seeds that were treated with cells of strain G12 served as positive control, and pots with *E. coli* treated or untreated seeds served as negative controls. Each treatment was replicated 12 times. Pots were arranged in randomized block design in the greenhouse and kept under the experimental conditions described above. Three weeks later, each pot was inoculated with 1,000 freshly hatched J2 in four holes of 2 cm depth at 3 cm distance from the stem base. The numbers of generated galls and egg masses per plant were counted 50 days after J2 inoculation.

Experiment 2: Effect of bacterial culture supernatants towards *M. incognita*. As an outcome of experiment 1 the top three bacterial isolates were selected for studying their mode of action: Sb4-23, Mc2-Re2, and Mc5-Re2. Bacterial isolates G12 and *E. coli* served as positive and negative control, respectively. Bacterial cultures were grown from 200 µl pre-culture in 100 ml tryptic soy broth (TSB, Merck, Darmstadt, Germany) for 24 h at 28°C with shaking, and centrifuged at 7500 g for 20 min. Three-week-old tomato seedlings were grown in 7x7x8 cm pots, each containing 300 g soil. The top soil layer (2 cm) was removed. The soil surface was drenched with 20 ml of the respective bacterial culture supernatant or sterile TSB and covered with the previously removed soil. Three days later, a suspension with 1,000 J2 was inoculated into four holes at 2 cm distance from the stem of each plant. Each treatment was replicated ten times and arranged in a randomized block design in the greenhouse. All plants were kept under the experimental conditions described. Fifty days after nematode inoculation the fresh weight and length of shoot and root, and the numbers of leaves, galls, egg masses, and eggs were determined for each pot.

Experiment 3: Effect of antagonistic strains on repellence of J2. This experiment was conducted using the linked twin-pot chamber as described in a previous study [20]. The two plastic pots of 7x7x8 cm were filled with 300 g soil and connected by a plastic tube of 1 cm diameter and 4 cm length filled with soil (Fig. 1A). Tomato seeds were coated with bacterial cells as described. The treated seeds were grown in the right pot while untreated seeds were grown in the left pot. In the control both pots received untreated seeds. The bacterial culture of these bacterial isolates was prepared following the procedure described above, and then centrifuged at 7,500 g for 20 min. The supernatant was discarded and the resulting pellet was washed then resuspended in sterile tap water. The bacterial density was adjusted to 0.8 at 560 nm, corresponding to 3.2×10^7 cfu ml^{-1} (Sb4-23), 2.4×10^7cfu ml^{-1} (Mc2-Re2), 1.8×10^7cfu ml^{-1} (Mc5-Re2), 1.2×10^7cfu ml^{-1} (*E. coli*) and 4×10^7cfu ml^{-1} (G12). Three weeks later, the right pots were inoculated with 10 ml of a bacterial suspension (OD_{560} = 0.8). The bacterial suspension was added into four holes of a depth of 2 cm around the stem base. After three days, 2,000 J2 in 1 ml water were inoculated through a small hole in the centre of the tube. The hole was sealed with plastic to maintain moisture. Each treatment was replicated ten times. The linked twin-pot chambers were arranged in a randomized block design in the greenhouse and kept under the experimental conditions described. Seven days after nematode inoculation the numbers of J2 penetrated into the roots on both sides of the linked twin-pot chambers were determined.

Experiment 4: Induced systemic resistance towards *M. incognita*. Tomato plants were grown in a split-root system as described in a previous study [21]. Three 7x7x8 cm plastic pots were used with one pot placed on top of two pots (Fig. 2A). One tomato seed was placed in the centre of the upper pot half filled with soil. Roots grew through holes in the bottom equally into the two lower pots which were completely filled with soil. After three weeks, one of the two bottom pots termed inducer side was inoculated with 20 ml of a bacterial suspension in tap water (OD_{560} = 2, corresponding to 8×10^9 cfu ml^{-1} for strain Sb4-23, 5×10^9 cfu ml^{-1} for Mc2-Re2, 4×10^9 cfu ml^{-1} for Mc5-Re2, 1×10^9 cfu ml^{-1} for *E. coli*, or 1.2×10^{10} cfu ml^{-1} for G12). Plants treated at the inducer side with an equivalent amount of tap water served as control. Three days

later, each bottom pot opposite to the inducer side, termed responder side, was inoculated with 1,000 J2. Each treatment was replicated ten times, and arranged in a randomized block design. Fifty days after nematode inoculation galls and egg masses were counted on the roots of the inducer and the responder side.

Experiment 5: Comparison of the effects by direct and plant-mediated antagonism. In this experiment it was evaluated whether the indirect effect of the bacteria via the plant could fully explain the inhibition of *M. incognita*, or whether co-inoculation in the same pot could enhance the effect through direct antagonism. Three-week-old tomato seedlings grown in the spilt-root systems as described above were divided into three groups: i) plants treated with bacteria on the inducer side and J2 on the responder side, ii) plants kept untreated on the inducer side and treated with bacteria and J2 on the responder side, and iii) plants kept untreated on the inducer side and inoculated with J2 on the responder side (control). Bacteria were applied by drenching 20 ml of a bacterial suspension (OD_{560} = 2) into holes made at the inducer side. Three days later, 1,000 J2 in 2 ml water were inoculated into holes made at the respective pot side. Each treatment was replicated ten times and arranged in a randomized block design in the greenhouse. A duplicated setup of the experiment was sacrificed after seven days to evaluate J2 penetration into roots as described above. After 50 days the numbers of galls, egg masses, and eggs per plant were determined.

Statistical analysis. Analysis of variance was done using the procedure GENMOD of the statistical software SAS 9.3 (SAS Institute Inc., Cary, NC, USA) to fit generalized linear models. For count data (numbers of galls, egg masses, eggs, J2 in roots) the procedure was used to perform a Poisson regression analysis with a log link function and specification of a scale parameter (Pearson) to fit overdispersed distributions. Class variables were treatment (strain or uninoculated control) and block (accounting for the randomized block design of experiments). For multiple comparisons of strain effects the *p*-value was adjusted by the method of Tukey. Repellence (experiment 3) was statistically tested using the procedure GENMOD as explained to compare the numbers of J2 in roots at the uninoculated side of the linked twin-pot system between treatments. The effect of the different strains on growth of plants

infected by *M. incognita* was tested by MANOVA using the SAS procedure GLM, with the dependent variables root weight, root length, shoot weight, shoot length, and number of leaves. For multiple comparisons of the effect of antagonistic strains to the *E. coli* control the *p*-value was adjusted by the method of Dunnett.

RESULTS

Potential of seed-inoculated strains to control *M. incognita*. In total nine bacteria were tested for their antagonistic potential towards *M. incognita* by seed inoculation (experiment 1). The number of galls and egg masses developed by *M. incognita* was highest in the non-inoculated control and the treatment with the non-antagonistic strain *E. coli* JM109 (Table 2). Significantly less galls and egg masses than in these controls were found in the treatments with the biocontrol strains, except for Sb3-24 and 3Rc2-7. The highest control potential was achieved by strain Sb4-23, which did not significantly differ from the well studied positive control G12. It caused 86% reduction in the number of galls and 96% reduction in number of egg masses compared with the untreated control. Good biocontrol was also achieved by the two other *Bacillus subtilis* isolates Mc2-Re2 and Mc5-Re2 with over 60% reduction in number of galls and over 70% reduction in number of egg masses. Based on these results, the isolates Sb4-23, Mc2-Re2, and Mc5-Re2 were selected for studying their mode of action in nematode suppression.

Table 2. Effect of bacterial seed treatment on number of galls and egg masses of *M. incognita* after propagation on tomato plants.

Bacterial inoculant	Galls per plant (± stdev)	Treatment effect on no. of galls [a]	Egg masses per plant (± stdev)	Treatment effect on no. of egg masses [a]
Culture medium	331 ± 35	A	269 ± 38	A
E. coli JM109	316 ± 39	B A	193 ± 48	B
Sb3-24	267 ± 87	B A C	164 ± 64	B
3Rc2-7	240 ± 58	B D C	135 ± 37	C B
C48	195 ± 48	D C	104 ± 31	C D
Ru47	185 ± 62	E D	76 ± 32	D
Mc2-Re2	122 ± 73	E F	70 ± 47	E D
Mc5-Re2	80 ± 27	G F	35 ± 17	E F
G12 (+ control)	48 ± 25	G	12 ± 10	G F
Sb4-23	45 ± 24	G	11 ± 14	G

[a] Tukey-Kramer grouping for least squares means ($\alpha = 0.05$): Means followed by the same letter are not significantly different (n = 12).

Effect of bacterial culture supernatants on *M. incognita*. The isolates Sb4-23, Mc5-Re2, and Mc2-Re2 selected from experiment 1 were tested for negative effects of their metabolites on *M. incognita* (experiment 2). Application of cell-free culture supernatants of all three tested strains and the positive control G12 significantly reduced the number of galls, egg masses, and eggs on tomato roots compared to the treatments with *E. coli* culture supernatant or sterile culture medium (Table 3). The lowest average number of galls was observed in the Sb4-23 treatment, which did not significantly differ from Mc2-Re2 and the positive control G12 but from Mc5-Re2. Among the bacterial antagonists, no differences were observed in numbers of egg masses and eggs per root. The number of eggs per egg mass was significantly lower for the treatments with Sb4-23 and G12 metabolites than for the negative controls.

Table 3. Effect of bacterial culture supernatants on reproduction of *M. incognita* on tomato plants.

Applied culture supernatant	Average no. per plant ± stdev. [a]			
	Galls	Egg masses	Eggs (x 1,000)	Eggs / egg mass
Culture medium	172 ± 14 A	129 ± 16 A	41 ± 6 A	322 ± 67 A
E. coli JM109	136 ± 16 B	98 ± 15 A	32 ± 7 A	330 ± 67 A
Mc5-Re2	98 ± 20 C	67 ± 22 B	19 ± 6 B	282 ± 31 AB
G12	83 ± 20 CD	60 ± 18 B	14 ± 7 B	224 ± 41 C
Mc2-Re2	80 ± 13 CD	49 ± 11 B	13 ± 3 B	275 ± 49 ABC
Sb4-23	75 ± 17 D	54 ± 21 B	13 ± 5 B	253 ± 23 C

[a] Tukey-Kramer grouping for least squares means: Means followed by the same letter within each column are not significantly different ($\alpha = 0.05$, $n = 10$).

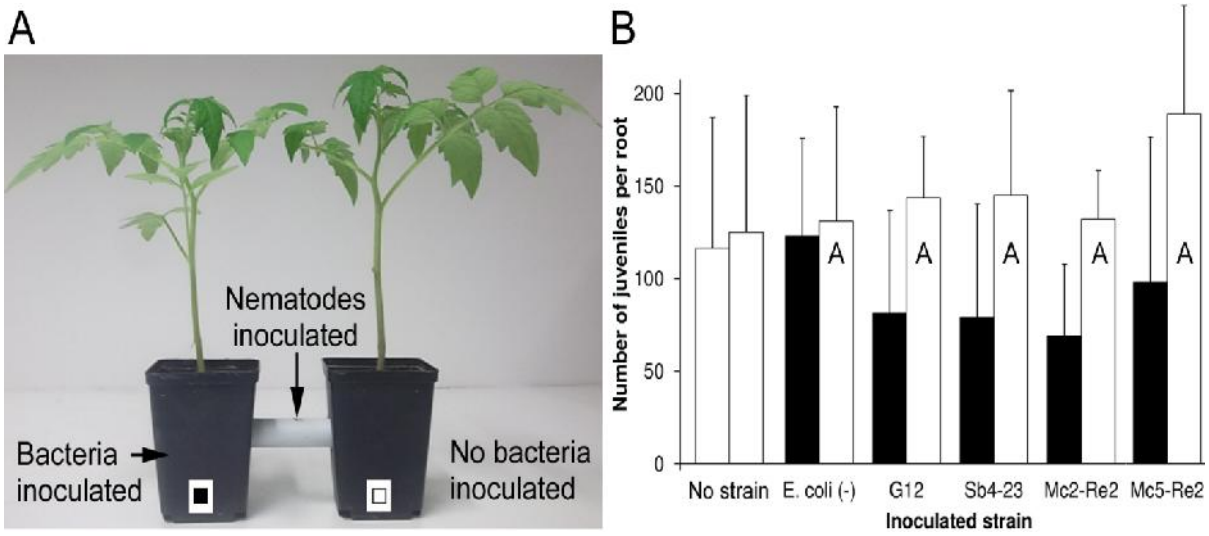
The culture supernatants of the strains significantly differed in their effect on plant growth during nematode exposure, as revealed by MANOVA of the length and weight of root and shoot, and the number of leaves 50 days after nematode inoculation (P = 0.005, Table 4). Among the three strains tested, only metabolites of Mc2-Re2 significantly enhanced plant growth compared to the *E. coli* control, as evidenced by increased root length (P = 0.006, Dunnett test) and number of leaves (P = 0.03). G12 had a positive effect on root length.

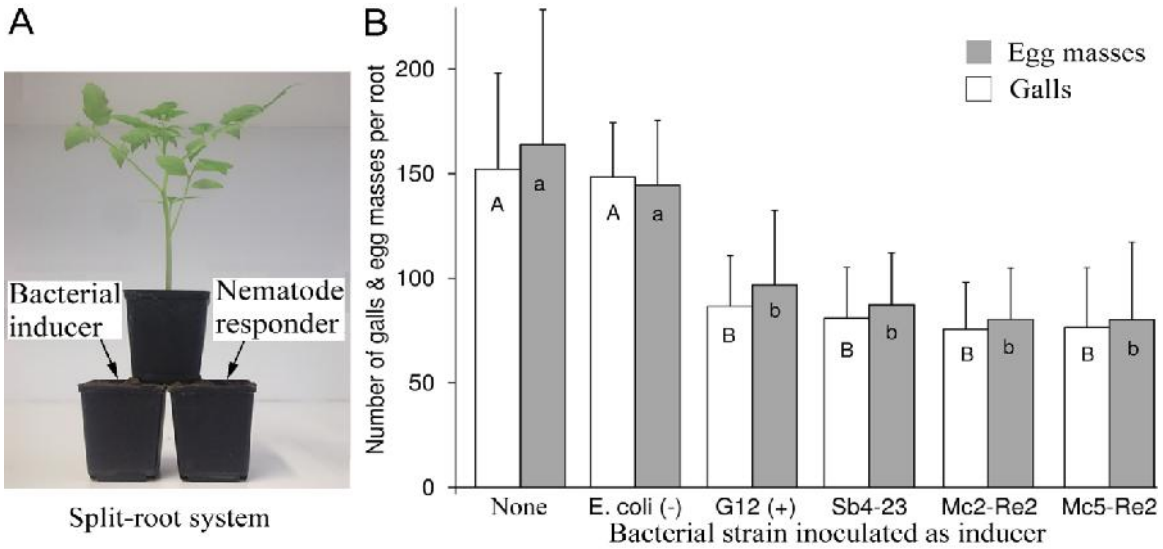
Table 4. Effect of bacterial culture supernatants on plant growth of tomato infected with *M. incognita.*

Applied culture supernatant	Root		Shoot		No. of leaves
	Length (cm)	Weight (g)	Length (cm)	Weight (g)	
Culture medium	12.1 ± 1.7	2.9 ± 0.5	33.8 ± 3.8	11.4 ± 1.5	8.5 ± 1.3
E. coli JM109	12.9 ± 1.3	3.2 ± 0.7	35.5 ± 1.8	11.9 ± 0.8	8.8 ± 0.8
Mc5-Re2	14.1 ± 1.9	3.2 ± 0.5	39.1 ± 3.8	12.6 ± 0.8	9.4 ± 0.7
Sb4-23	14.1 ± 1.4	3.3 ± 0.4	37.2 ± 4.4	11.6 ± 0.9	9.6 ± 0.8
G12	14.8 ± 1.4 *	3.5 ± 0.6	34.0 ± 2.0	12.2 ± 0.8	9.5 ± 0.7
Mc2-Re2	15.0 ± 1.1 *	3.1 ± 0.6	38.0 ± 3.6	12.0 ± 1.1	9.7 ± 0.7 *

* Significantly different ($P \leq 0.05$, Dunnett adjustment, n = 10) to both control treatments (JM109 culture supernatant and sterile culture medium).

Effect of antagonistic strains on repellence of J2. A linked twin-pot set-up was used to evaluate the effect of bacterial antagonists on attraction of *M. incognita* J2 to tomato roots (experiment 3, Fig. 1A). One week after inoculating the nematodes at the centre of a tube connecting two pots planted with tomato, the numbers of J2 that moved to one or the other side and penetrated the roots were counted (Fig. 1B). As a trend, slightly more J2 were found in the roots at the uninoculated side of linked twin-pot systems that were treated with biocontrol strains compared to the treatment with *E. coli* or the control. However, the difference was not statistically significant (P = 0.10). None of the treatments with biocontrol strains significantly differed from that with *E.*

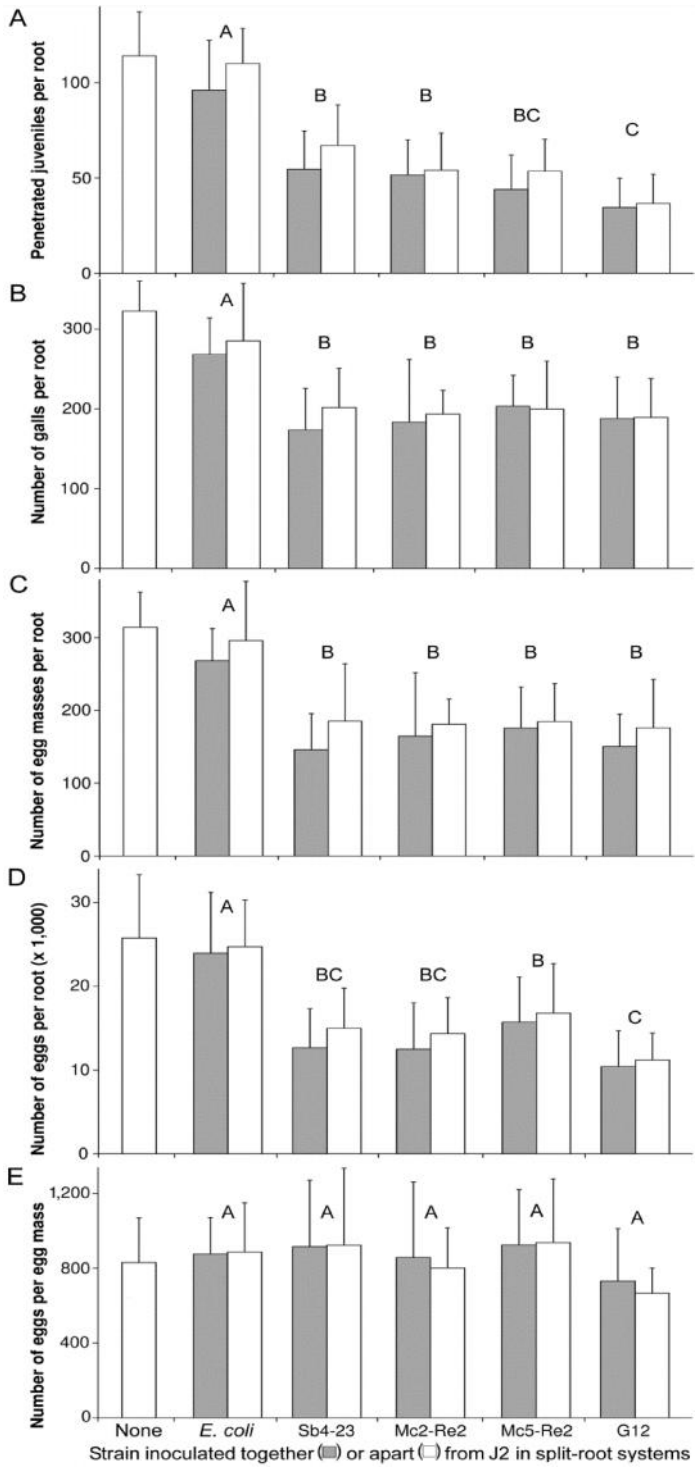

A
Nematodes inoculated
Bacteria inoculated
No bacteria inoculated
B
Number of juveniles per root
200
150
100
50
0
A
A
A
A
A
No strain
E. coli (-)
G12
Sb4-23
Mc2-Re2
Mc5-Re2
Inoculated strain



Split-root system

Plant-mediated rather than direct effect of biocontrol strains on *M. incognita*. In split-root systems the plant-mediated effect of the bacteria on *M. incognita* was compared to the combined plant-mediated and direct effect when bacteria and J2 are co-inoculated in the same pot (experiment 5). One week after nematode inoculation, all antagonistic bacteria significantly reduced J2 penetration compared to the negative control *E. coli*, which did not differ from the control without inoculated bacteria (Fig. 3A). The lowest numbers of penetrated juveniles were observed for G12 and Mc2-Re2, corresponding to of 67% and 52% reduction compared to the control, respectively. Three-factorial analysis of variance revealed a significant difference between stains in their effect on root penetration of J2 ($P < 0.0001$), and a significant decrease of J2 by co-inoculation with bacteria ($P = 0.01$). However, J2 in roots were only slightly decreased by co-inoculation of J2 and biocontrol strains, so that most of the biocontrol effect on J2 can be explained by induced systemic resistance alone.

Fifty days after nematode inoculation, in all treatments with bacterial antagonists significantly less galls, egg masses, and eggs were found compared to the treatment with *E. coli*, or the untreated control (Fig. 3B-D). Three-factorial analysis of variance revealed a significant difference between stains in their effect on nematode reproduction ($P < 0.0001$). Co-inoculation did not have a detectable effect on numbers of galls or eggs ($P = 0.3$ or 0.2, respectively), and only a slight effect on egg masses ($P = 0.049$). Thus, most of the biocontrol effect on reproduction can be explained merely by induced systemic resistance. The three tested biocontrol strains did not significantly differ in their potential to suppress *M. incognita*. The positive control strain G12 could slightly better reduce the number of eggs compared to strain Mc5-Re2 in this experiment. No significant effect of the bacteria on the number of eggs per egg mass was detected (Fig. 3E).


A
Penetrated juveniles per root
100
50
0
A
B
B
BC
C
B
Number of galls per root
300
200
100
0
A
B
B
B
B
C
Number of egg masses per root
300
200
100
0
A
B
B
B
B
D
Number of eggs per root (x 1,000)
30
20
10
0
A
BC
BC
B
C
E
Number of eggs per egg mass
1,200
800
400
0
A
A
A
A
A
None
E. coli
Sb4-23
Mc2-Re2
Mc5-Re2
G12
Strain inoculated together (▩) or apart (□) from J2 in split-root systems

DISCUSSION

Within this study seven antagonistic bacteria with known antagonism towards fungal pathogens were selected and tested for their potential to control *M. incognita* on tomato. Five of the bacterial antagonists significantly reduced *M. incognita* infestation on tomato after seed treatment. It was shown that individual bacterial antagonists have a much broader control spectrum than originally thought by concomitantly controlling fungal pathogens and plant-parasitic nematodes. The results are in accordance with previous work where potato-associated strains of *Pseudomonas* and *Streptomyces* inhibited both the soil-borne fungal wilt pathogen *V. dahliae* and the root-knot nematode *M. incognita* [8]. Similarly, Tariq et al. [9] were able to show that a strain of *P. aeruginosa* inhibited both the root-rotting fungi *M. phaseolina, R. solani*, *F. solani*, and *F. oxysporum* as well as the root-knot nematode *M. javanica* infecting chili roots.

In the present study nematode antagonism was shown for strains belonging to the species *B. subtilis*, *P. jessenii*, and *S. plymuthica*. All antagonistic bacteria were able to significantly reduce galls and egg masses on tomato compared with the untreated control. While other strains of *B. subtilis* and *S. plymuthica* have been reported as nematode antagonists before [22-25], strains with biocontrol potential belonging to the species *P. jessenii* were first reported in this study. The positive control *R. etli* G12 confirmed its good biocontrol potential [26]. Within experiment 1, bacterial isolates were applied as a seed treatment. The good results achieved by this method raises optimism that seed treatment could be an efficient and economical way for bacterial delivery in practise as already reported for other bacterial antagonists [27, 28].

Besides seed treatment also a soil drench with culture supernatants of the antagonistic bacteria resulted in a significant reduction in galls, egg masses, and eggs produced by *M. incognita*. Nematode suppression by bacterial culture supernatants has previously been reported when testing for antibiosis under *in vitro* conditions [29, 30]. Unfortunately, still very little is known about the active compounds of culture supernatants causing nematode antagonism. Siddiqui et al. [31] found that for *P. aeruginosa* the ethyl acetate extract caused 64% inactivity of *M. javanica* juveniles

within 24 h and assumed that the active compound was of proteinaceous or glycoproteinaceous nature. The active compound was described as heat sensitive, sensitive to extreme pH values, polar in nature and with a molecular weight smaller than 8,000 Da [32].

Padgham and Sikora reported that *Bacillus megaterium* caused repellence of *Meloidogyne graminicola* from rice roots [12]. Production of repellent substances or modification of the plant's exudates by the antagonistic bacteria were suggested as mechanisms for this effect [10]. In our study, a trend for repellence of *M. incognita* by the tested biocontrol strains was observed, although it was not statistically significant due to high variation between replicates. A complete different mechanism involved in bacteria-mediated nematode control is induced systemic resistance of the plant. In relation to nematode control, induced systemic resistance was first reported by Hasky-Günther and Sikora [33]. In our study using a split-root system, all four antagonistic bacteria tested induced systemic resistance towards *M. incognita* in tomato. Galls and egg masses were reduced between 40% and 51%, respectively, which was in the range of control rates reported for similar studies [34-36]. For the positive control strain *R. etli* G12 used in the present study it was shown that viable as well as dead bacterial cells were able to trigger the systemic resistance response in potato against the potato cyst nematode *Globodera pallida*. Furthermore, it turned out to be the oligosaccharides of the core-region of the bacterial lipopolysaccharides to be the main trigger of the resistance response [36].

Our experimental setup allowed for the first time to compare between the plant-mediated antagonistic effect of the strains and direct effects of the bacteria on the nematode caused by nematicidal, nematostatic or repellant bacterial compounds or parasitism on juveniles or eggs. In comparison with induced systemic resistance the application of the antagonistic bacteria together with the nematodes on the responder side of the split-root system only slightly enhanced the biocontrol effect. Thus induced systemic resistance was identified as the major control mechanism of the antagonists in this study (experiment 5). For all tested strains bacterial cells and cell-free culture

supernatants caused similar reductions in galls, egg masses, and eggs. Together with the just mentioned result of experiment 5, this suggested that systemic resistance in tomato was induced by compounds from the bacteria that can also be found in the culture supernatants.

In conclusion, all bacterial antagonists with known antifungal capacity tested in this study also showed antagonistic activity against the root-knot nematode *M. incognita*. The control potential of the three *B. subtilis* strains Sb4-23, Mc2-Re2, and Mc5-Re2 was within the range of the positive control *R. etli* G12. For all tested strains seed treatment with bacterial cells as well as bacterial culture supernatants caused similar reductions in number of galls, number of egg masses and total number of eggs per plant. The results achieved with *B. subtilis* were especially stimulating since it produces spores that are a lot easier to formulate and store than Gram-negative bacteria such as *R. etli* G12 or the tested *Pseudomonas* strains. Overall best nematode control in this study was achieved by *B. subtilis* Sb4-23 making this isolate a promising candidate for dual biocontrol of *M. incognita* and seed-borne fungal pathogens under field conditions.

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RESEARCH LETTER

Bacillus and *Streptomyces* were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt

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Abstract

Plant protection via disease-suppressive bacteria in desert farming requires specific biological control agents (BCAs) adapted to the unique arid conditions. We performed an ecological study of below-ground communities in desert farm soil and untreated desert soil, and based on these findings, selected antagonists were hierarchically evaluated. In contrast to the highly specific 16S rRNA fingerprints of bacterial communities in soil and cultivated medicinal plants, internal transcribed spacer profiles of fungal communities were less discriminative and mainly characterised by potential pathogens. Therefore, we focused on in vitro bacterial antagonists against pathogenic fungi. Based on the antifungal potential and genomic diversity, 45 unique strains were selected and characterised in detail. *Bacillus*/*Paenibacillus* were most frequently identified from agricultural soil, but antagonists from the surrounding desert soil mainly belonged to Streptomyces. All strains produced antibiotics against the nematode *Meloidogyne incognita*, and one-third showed additional activity against the bacterial pathogen *Ralstonia solanacearum*. Altogether, 13 broad-spectrum antagonists with antibacterial, antifungal and nematicidal activity were found. They belong to seven different bacterial species of the genera *Bacillus* and *Streptomyces.* These Gram-positive, spore-forming bacteria are promising drought-resistant BCAs and a potential source for antibiotics. Their rhizosphere competence was shown by fluorescence in situ hybridisation combined with laser scanning microscopy.

Introduction

While desertification is recognised as a major threat to biodiversity, the conversion of desert soil into arable, green landscapes is a global vision (Clery, 2011; Marasco *et al*., 2012). Desert farming, which generally relies on irrigation, is one way to potentially realise this goal. In Australia, Israel, California and Africa, desert farming areas are expanding. For example, desert farming in Egypt will have grown by 40% by 2017 (Reuters, 2007). However, emerging problems with soilborne pathogens, which can substantially limit crop yield, are often reported after several years of agricultural land use (Krikun *et al*., 1982). These soilborne pathogens include various taxonomic groups, for example, fungi (*Fusarium culmorum*, *Rhizoctonia solani*, *Verticillium dahliae*), bacteria (*Ralstonia solanacearum*) and nematodes (*Meloidogyne incognita*) (Klosterman *et al*., 2009; Messiha *et al*., 2009; Neher, 2010). Because of its depleting effect on the ozone layer, the extensively used broad-spectrum soil fumigant methyl bromide was banned by the Montreal Protocol in 1987 and phased out in most countries by 2005. Now, there is an urgent demand for ecologically compatible and efficient strategies to suppress soilborne pathogens in both conventional and organic desert agriculture (Bashan & de-Bashan, 2010).

Biological control based on naturally occurring antagonists offers sustainable solutions for plant protection (Weller, 2007; Berg, 2009; Lugtenberg & Kamilova, 2009; Raaijmakers *et al*., 2009). However, beneficial plant–microorganism interactions are highly specific, and only a few broad-spectrum antagonists have been reported (Zachow *et al*., 2008; Hartmann *et al*., 2009). Gram-negative bacteria, especially those from genus Pseudomonas, were identified as the dominant members of the indigenous antagonistic communities under humid conditions (Berg *et al*., 2005; Haas & Defago, 2005; Costa *et al*., 2006; Zachow *et al*., 2008) and as a major group of disease- suppressive bacteria through pyrosequencing (Mendes *et al*., 2011). Although there are problems with the for- mulation and shelf life of *Pseudomonas*, strains have still been developed as commercial BCAs (Weller, 2007; Berg, 2009). Gram-positive bacteria have also been widely used as BCAs and plant growth-promoting rhizobacteria (PGPRs), even though their ability to colonise the rhizo- sphere has been controversial (Hong *et al*., 2009; Fan *et al*., 2011). Their ability to form durable, heat-resistant endospores allows for easy formulation (Emmert & Han- delsman, 1999; Adesemoye *et al*., 2009), but their use as BCAs in desert agroecosystems is not been established so far.

Desert soils are characterised by arid conditions, which include a combination of extreme temperatures and desiccation, high soil salinity, low nutrient levels, high UV radiation levels and physical instability caused by strong winds (Cary *et al*., 2010). In one of the most prominent examples of organic desert farming in Sekem (Egypt), we found a strong correlation between long-term organic agriculture and bacterial community composition in soils. Bacterial communities in agricultural soil showed a higher diversity and a better ecosystem function for plant health compared to the surrounding natural desert soil (Köberl *et al*., 2011). A comprehensive analysis explained these structural differences: the proportion of Firmicutes represented by antagonistic *Bacillus* and *Paenibacillus* in field soil was significantly higher (37%) than in the desert soil (11%). In contrast, *Actinobacteria* occurred in farmland in lower concentrations (5%) than in the desert (21%), and antagonistic isolates of *Streptomyces* were only isolated from native desert soil (Köberl *et al*., 2011). A high presence of *Actinobacteria* in soil of the North American Sonoran Desert was also found by 454-pyrotag analyses (Andrew *et al*., 2012) as well as in soil of the hyperarid Atacama Desert in north-west Chile (Neilson *et al*., 2012). From the latter, several so far unknown Streptomyces spp. were recently described (Santhanam *et al*., 2012a,b, 2013). In addition, a study examining soil bacterial communities in the Negev Desert in the south of Israel even revealed a higher abundance of *Actinobacteria* in barren soils compared to soils under shrub canopies (Bachar *et al*., 2012). However, the indigenous desert microbiome should contain BCAs that are adapted to the specific biotic and abiotic conditions of desert habitats as well as strains that produce novel bioactive compounds, because the genus Streptomyces is known as a unique source of novel antibiotics (Goodfellow & Fiedler, 2010; Niraula *et al*., 2010; Nachtigall *et al*., 2011). The potential for both has been until now poorly understood and used.

The objective of this study was to analyse microbial communities from agricultural desert habitats (e.g. from the rhizospheres and endorhiza) in comparison with the surrounding desert soil for their biocontrol potential and to specifically select and characterise broad-spectrum antagonists against soilborne pathogens regarding this potential.

Materials and methods

Experimental design and sampling

Microbial diversity in organic desert farming was studied at Sekem farms (www.sekem.com) in Egypt (30°22′88″N, 31°39′41″E) in comparison with surrounding desertsoil (30°35′01″N, 32°25′49″E; 35°59′0″N, 41°2′0″E). The sampling strategy is described in detail in Köberl et al. (2011). Briefly, at each site, four composite samples of soil in a horizon of 0–30 cm depth were collected. Furthermore, roots with adhering soil were obtained from three different species of medicinal plants (Matricaria chamomilla L., Calendula officinalis L. and Solanum disti- chum Schumach. and Thonn.) planted on a Sekem farm. From each plant species, four independent composite samples consisting of 5–10 plants were taken. Samplings were performed in October 2009 and in April 2010. Phys- icochemical data of the soil are provided in Luske & van der Kamp (2009).

Microbial fingerprints from single-stranded conformational polymorphism analysis of the ITS and 16S rRNA region (PCR-SSCP)

Total community DNA was isolated from bulk soil, rhizosphere and endorhiza of the medicinal plants according to Köberl et al. (2011). Fingerprinting of microbial communities by SSCP was performed as described by Schwieger & Tebbe (1998). Amplification of the fungal internal transcribed spacer (ITS) fragment was performed by a nested PCR approach with primer pairs ITS1/ITS4 and ITS1/ITS2^{P} (White et al., 1990). Nested PCR was performed as described by Zachow *et al*. (2008). SSCP analysis of bacterial 16S rRNA gene sequences is specified in Köberl *et al*. (2011). Sequences of excised and re-amplified

bands were submitted to EMBL Nucleotide Sequence Database under accession numbers FR854281-FR854290, FR871639-FR871646 and HE655458-HE655480.

SSCP profiles of the microbial communities generated with universal fungal and bacterial primers were further applied for multivariate analysis. According to the distance of the bands, the SSCP gels were theoretically divided into operational taxonomic units (OTUs). The presence or absence of individual amplified product DNA bands in each group was scored. OTUs served as response variables for principal component analysis (PCA) using Canoco 4.5 for Windows (Lep s & Smilauer, 2003). Matrices based on Pearson correlation were subjected to significance tests of pairwise similarities by applying permutation analyses ($P < 0.05$) using the permtest package of R statistics version 2.13.1 (The R Foundation for Statistical Computing, Vienna, Austria) with 10^5 random permutations of sample elements (Kropf et al., 2004; R Development Core Team, 2011).

Screening for in vitro activity against soilborne bacteria and nematodes

Forty-five promising strains with antagonistic activity against pathogenic fungi (Köberl et al., 2011) were tested for antibacterial activity against *Ralstonia solanacearum* 1609 and B3B. The activity of all isolates against both *R. solanacearum* strains was identical; therefore, the data in Table 2 are presented in singular form. For the screening, yeast peptone glucose (YPG) medium was used, and Tetrazolium Violet (Sigma-Aldrich, Saint Louis, USA) was added to the medium prior to pouring as a redox indicator of bacterial growth (Adesina et al., 2007; Tsukatani et al., 2008).

For testing the activity of the selected antagonists towards the phytopathogenic nematode *Meloidogyne incognita* (Kofoid and White) Chitwood, culture supernatants from the bacteria were prepared. For this, the bacterial isolates were grown at 28 °C for 24 h on R2A agar (Merck, Darmstadt, Germany). A preculture was grown over night from a single colony in 5 mL of tryptic soy broth (TSB) (Merck) with 50 mg L^{-1} rifampicin at 28 °C with shaking at 150 r.p.m. 200 μL of the preculture were added to 100 mL sterile TSB and incubated for 24 h at 28 °C with shaking. The bacteria were then removed from the culture by centrifugation at 7500 g for 20 min, followed by sterile filtration of the supernatants through membranes with 0.22 μm pore size. The sterile culture supernatants were kept at 4 °C until application. To study the effect of extracellular bacterial products on the mortality of *M. incognita* juveniles (J2), 500 μL of a juvenile suspension containing approximately 100 freshly hatched J2 was mixed with 1 mL of each bacterial filtrate in a Petri dish with 500 μL of an antibiotic solution containing 300 mg L^{-1} streptomycin and 300 mg L^{-1} penicillin to suppress microbial growth. Each treatment was replicated 4 times. Controls consisted of TSB, water and a culture supernatant of the nonantagonistic strain Escherichia coli JM109, respectively. All dishes were kept at 25 ± 2°C in the dark. Numbers of motile and nonmotile nematodes were counted after 6, 12, 24 and 48 h using a binocular microscope. To distinguish between nonmotile and dead J2, the nematodes were transferred to water at the end of the exposure time. Juveniles that did not recover and become motile again were considered dead. The rate of mortality was determined using linear regression of the percentages of dead J2 after 0, 6, 12 and 24 h.

Fluorescence in situ hybridisation (FISH) and confocal laser scanning microscopy (CLSM)

Samples were fixed in 4% paraformaldehyde and stained by in-tube FISH according to the protocol of Cardinale et al. (2008). An equimolar mixture of Cy3-labelled EUB338, EUB338II and EUB338III probes (Amann et al., 1990; Daims et al., 1999) was used for the detection of all bacteria and a Cy5-labelled HGC236 probe (Erhart et al., 1997) for the detection of *Actinobacteria*. As a negative control, nonsense FISH probes labelled with both fluorochromes (NONEUB; Wallner et al., 1993) were applied. Confocal images were obtained using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems GmbH, Mannheim, Germany).

Results

Molecular fingerprinting of microbial belowground communities

All investigated SSCP fingerprints of the ITS and 16S rRNA gene fragments from both the rhizosphere and endorhiza of the medicinal plants and bulk soil showed a high diversity. According to the statistical cluster analysis, there is a clear plant-specific effect on both communities in the rhizosphere (Fig. 1, Table 1). Furthermore, microenvironment-specific SSCP patterns of the microbial communities were detected, and statistically significant differences between the rhizosphere and the endorhiza of the medicinal plants were calculated (Fig. 1, Table 1). Additionally, plant-associated microenvironments were compared with the surrounding soil. The composition of the bacterial and fungal communities in soil differed significantly from the plant-associated communities (P values: fungal communities 0.0241; bacterial communities 0.0266) and between agricultural and desert soil (P values: fungal communities 0.0291; bacterial communities 0.0289).

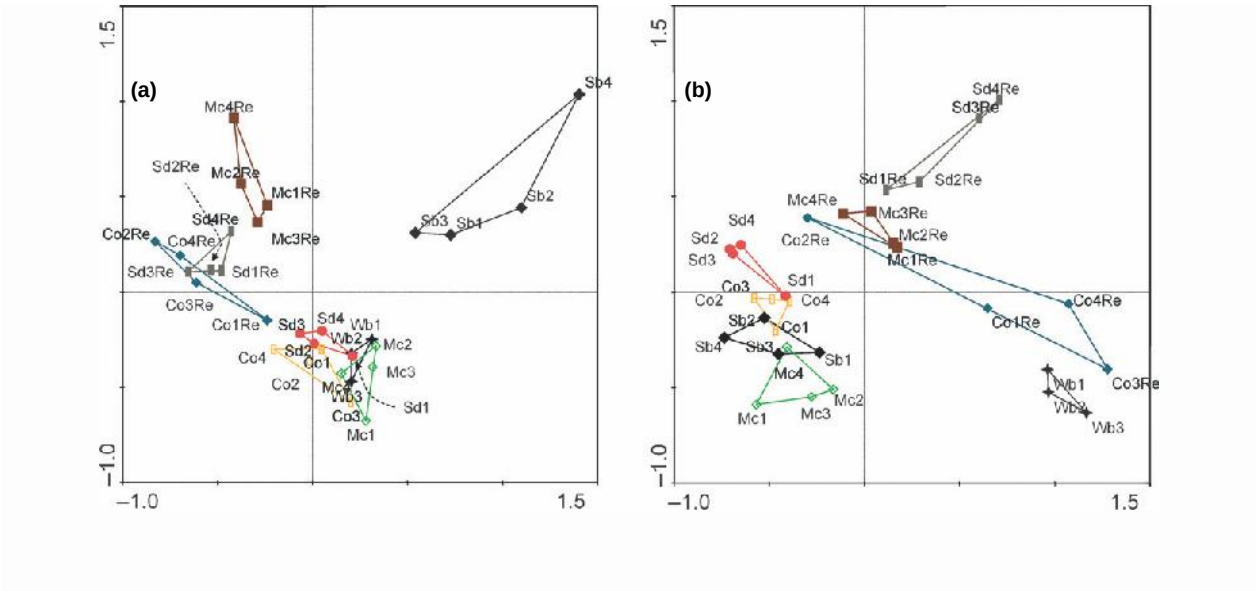

Fig. 1. PCA of OTUs identified by SSCP fingerprinting for fungal (a) and bacterial (b) communities. Samples were encoded using a combination of letters and numbers indicating (1) soil type or plant species (Wb = desert soil, Sb = Sekem soil, Mc = *Matricaria chamomilla*, Co = *Calendula officinalis*, Sd = *Solanum distichum*), (2) replicate (1–4) and (3) microenvironment (Re = endorhiza, rhizosphere and soil have no further designation).

Table 1. Statistical analysis of microbial fingerprints obtained by PCR-SSCP.

Microenvironment	Fungal communities	Bacterial communities
	P values for pairwise comparisons between medical plants*	
Rhizosphere†		
Mc-Co	0.0276	0.0281
Co-Sd	0.0284	0.0286
Mc-Sd	0.0296	0.0286
Endorhiza†		
Mc-Co	0.0297	0.0556
Co-Sd	0.0719	0.0283
Mc-Sd	0.0282	0.0293
Medicinal plant	P values for comparisons between rhizosphere and endorhiza*	
Matricaria chamomilla	0.0290	0.0287
Calendula officinalis	0.0288	0.0287
Solanum distichum	0.0287	0.0281

*Analysed by permutation test ($P < 0.05$) using R statistics.
†Mc, *Matricaria chamomilla*; Co, *Calendula officinalis*; Sd, *Solanum distichum*.

The fingerprints of the fungal community represented a high diversity in all microenvironments and were similar for the first and second samplings (Fig. 2). In general, potential plant pathogens were frequently found within the fungal communities. In fingerprints from both samplings, *Alternaria* (closest database match Alternaria tenuissima, 100% similarity to JN620417) and *Fusarium* (closest database matches *Fusarium chlamydosporum*, 100% similarity to HQ671187 and *Fusarium solani*, 99% similarity to FJ865435) were most commonly found. *Alternaria* was also found in desert soil from Sinai (first sampling) as well as from Saqqara (second sampling). In addition, *Cladosporium* (teleomorph *Davidiella*) was identified in fingerprints from both samplings. In rhizosphere and soil samples from the first sampling, *Epicoccum* (closest database match *Epicoccum nigrum*, 100% similarity to JN578611) was assigned to a dominant band. In soil from the Sinai desert, the black fungus *Aureobasidium* (closest database match *Aureobasidium proteae*, 99% similarity to JN712490) was additionally identified. Similarly, *Verticillium dahliae* (closest database match *V. dahliae var. longisporum*, 100% similarity to AB585937) was identified as a dominant band found in almost all plant samples from the second sampling time, which apart from Fusarium spp. was one of the main soilborne phytopathogens on the Sekem farms. In samples from the second sampling, the obligate root-infecting pathogen *Olpidium* (closest database match *Olpidium brassicae*, 99% similarity to AB625456), belonging to the fungal phylum *Chytridiomycota*, and *Sarocladium* (closest database match Sarocladium strictum, 100% similarity to JN942832; previously recognised in *Acremonium*) were found. Although several other ITS fragments were not identified, due to this high content of potential phytopathogens in the fungal communities, the selection of antagonists was focused on the bacterial communities.

Detailed characterisation of selected antagonistic strains

A screening of 1212 bacterial isolates resulted in 162 antifungal antagonists against the main fungal soilborne

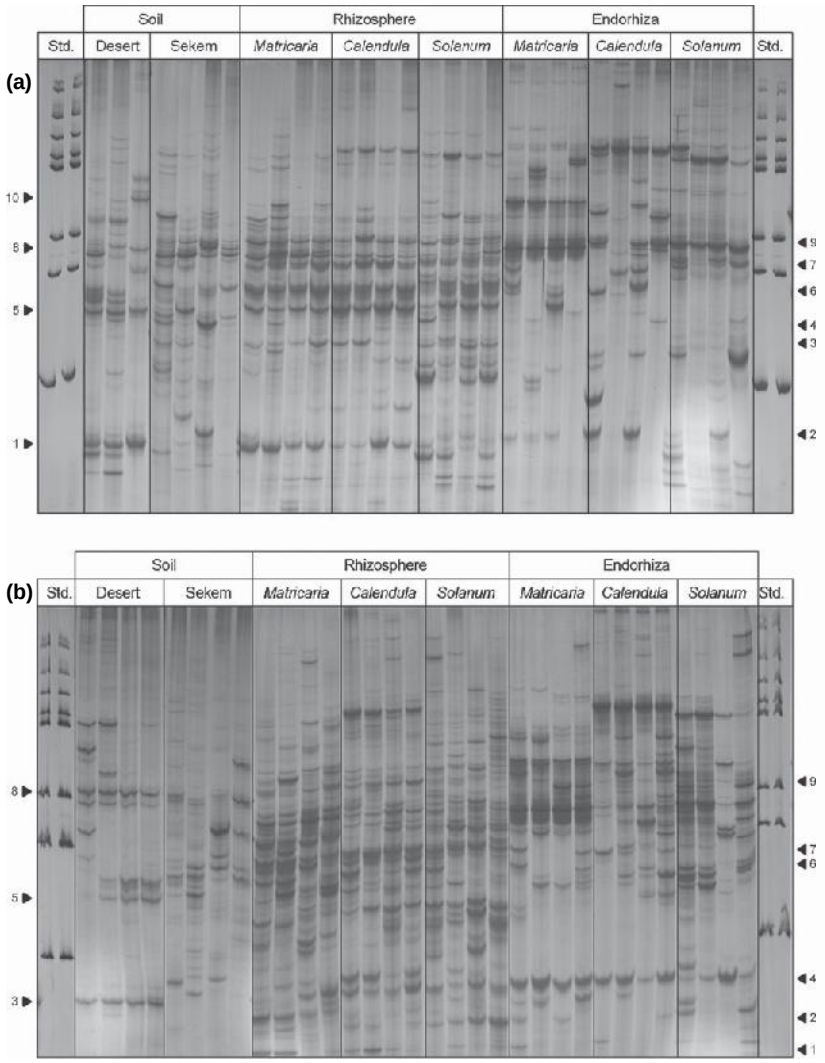

Fig. 2. ITS PCR-SSCP profiles of the fungal communities in soil, rhizosphere and endorhiza of the medicinal plants from first (a) and second (b) sampling time. Std.: 1 kb DNA ladder. (a) From fingerprints of the first sampling (October 2009), the following bands were identified as: 1. *Epicoccum nigrum*, 100% similarity to JN578611; 2. *Pichia jadinii*, 99% similarity to FJ865435; 3. *Gibellulopsis nigrescens*, 100% similarity to JN187998; 4. Emericella nidulans, 99% similarity to JN676111; 5. *Alternaria tenuissima*, 100% similarity to JN620417; 6. *Davidiella tassiana*, 99% similarity to JN986782; 7. *Fusarium chlamydosporum*, 100% similarity to HQ671187; 8. *Exserohilum rostratum*, 99% similarity to JN179081; 9. *Fusarium solani*, 99% similarity to FJ865435; 10. *Aureobasidium proteae*, 99% similarity to JN712490. (b) From the second sampling (April 2010), the following bands were identified: 1. *Cryptococcus carnescens*, 99% similarity to GU237051; 2. *Olpidium brassicae*, 99% similarity to AB625456; 3. *Preussia minimoides*, 96% similarity to AY510422; 4. *Verticillium dahliae var. longisporum*, 100% similarity to AB585937; 5. *Alternaria tenuissima*, 100% similarity to JN620417; 6. *Fusarium chlamydosporum*, 99% similarity to EU556725; 7. *Cladosporium cladosporioides*, 100% similarity to JN986781; 8. *Ulocladium oudemansii*, 100% similarity to FJ266488; 9. *Sarocladium strictum*, 100% similarity to JN942832.

pathogens (*V. dahliae*, *R. solani* and *F. culmorum*) (Köberl *et al*., 2011). These fungi were identified in Sekem soil by cultivation and, with the exception of *R. solani*, in the molecular fingerprinting analyses. Altogether, 45 genotypically unique antifungal strains were selected to assess their antibacterial activity against *R. solanacearum* (Table 2). Of these isolates, 33.3% were able to inhibit the growth of the soilborne bacterial pathogen in vitro, including most isolates of *Streptomyces* (3 of 4 isolates) and some strains of the *Bacillus subtilis* group (12 of 30 isolates).

Plant-parasitic nematodes often positively interact with soilborne fungal pathogens. Therefore, the selected bacterial isolates were additionally evaluated *in vitro* for their effects against juveniles of the root-knot nematode *M. incognita*. All bacteria accumulated inhibitory substances in the culture medium to some degree, while the medium itself and water had no effect. The percentage of dead J2 continuously increased during the incubation period of 48 h reaching over 70% for 11 strains with a maximum of 89% for strain Mc5Re-2, while only 28% of J2 were dead in the *E. coli* control (Table 2). On average, the increase in mortality was highest within the first 12 h of exposure and declined thereafter. The ten most efficient strains caused between 47% and 63% mortality in the first 24 h, with the highest rates observed for strains Sb4-23, Mc5Re-2, Mc1Re-3 and Sb3-24 (Fig. 3). The seven most efficient antagonists were all isolates of *Bacillus subtilis* obtained from either agricultural soil or from the endorhiza of *M. chamomilla*.

In situ visualisation of *Actinobacteria* in the rhizosphere

FISH-CLSM analysis confirmed generally high bacterial abundances and occurrence of *Actinobacteria* in below-ground habitats under arid conditions. Using an

Table 2. List of selected bacterial antagonists isolated from different microenvironments with their antagonistic properties.

			Antagonistic activity towards‡					
							Meloidogyne incognita¶	
ARDRA group*	Isolate number	Closest database match† (accession number), similarity (%)	*Verticillium dahliae*§	*Rhizoctonia solani*§	*Fusarium culmorum*§	*Ralstonia solanacearum*	Dead J2 after 48 h (%)‖	Mortality rate (% J2 per day)**
A	Wb2n-1	*Bacillus vallismortis* (NR_024696), 99%	+	++	+	+	73 ± 6	49 ± 4
A	Sb1-6	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	–	+	+	54 ± 4	32 ± 2
A	Sb3-5	*Bacillus subtilis* subsp. *subtilis* (NR_027552, 99%	+	++	+	+	46 ± 3	25 ± 3
A	Sb3-13	*Bacillus atrophaeus* (NR_024689), 99%	+	++	+	+	33 ± 3	17 ± 1
A	Sb3-21	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	+	–	68 ± 7	52 ± 4
A	Sb3-24	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	++	+	–	78 ± 7	57 ± 4
A	Sb4-14	*Bacillus vallismortis* (NR_024696), 99%	+	+	+	–	45 ± 5	23 ± 1
A	Sb4-23	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	+	+	84 ± 5	63 ± 3
A	Mc3-4	*Bacillus mojavensis* (NR_024693), 98%	+	++	++	+	67 ± 8	30 ± 2
A	Mc5-18	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	++	++	++	–	29 ± 2	14 ± 2
A	Mc5-19	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	–	+	35 ± 4	17 ± 2
A	Co1-6	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	++	++	++	+	70 ±7	37 ± 3
A	Co2-14	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	+	++	–	72 ± 12	40 ± 5
A	Co7-19	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	++	+	+	–	48 ± 5	26 ± 1
A	Sd1-14	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	++	–	56 ± 5	35 ± 3
A	Sd3-12	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 100%	+	+	++	–	29 ± 2	17 ± 1
A	Sd3-21	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	+	–	57 ± 4	35 ± 5
A	Sd7-15	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	+	++	+	–	43 ± 4	26 ± 2
A	Mc1Re-3	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	++	++	–	80 ± 4	56 ± 7
A	Mc2Re-2	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 99%	+	++	+	+	83 ± 4	54 ± 4
A	Mc2Re-9	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	++	–	61 ± 3	38 ± 2
A	Mc2Re-18	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	++	–	82 ± 2	50 ± 6
A	Mc2Re-21	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	–	+	++	–	66 ± 5	46 ± 3
A	Mc3Re-13	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 98%	+	+	+	+	61 ± 3	43 ± 3
A	Mc5Re-2	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	+	+	+	–	89 ± 3	59 ± 3

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Table 2. Continued

ARDRA group*	Isolate number	Closest database match† (accession number), similarity (%)	Antagonistic activity towards‡				*Meloidogyne incognita*¶	
			Verticillium dahliae§	*Rhizoctonia solani*§	*Fusarium culmorum*§	*Ralstonia solanacearum*	Dead J2 after 48 h (%)‖	Mortality rate (% J2 per day)**
A	Mc5Re-15	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	+	+	+	+	33 ± 2	22 ± 1
A	Sd2Re-10	*Bacillus mojavensis* (NR_024693), 100%	++	++	++	–	52 ± 7	24 ± 2
A	Sd8Re-6	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	+	+	+	+	22 ± 2	13 ± 2
A	Sd8Re-7	*Bacillus subtilis* subsp. *subtilis* (NR_027552), 99%	++	++	++	–	24 ± 2	12 ± 1
A	Sd8Re-23	*Bacillus subtilis* subsp. *spizizenii* (NR_024931), 100%	++	+	+	–	26 ± 2	14 ± 1
C	Wb1-13	*Bacillus endophyticus* (NR_025122), 99%	–	+	+	–	21 ± 2	14 ± 2
C	Mc4-18	*Bacillus endophyticus* (NR_025122), 99%	–	+	+	–	56 ± 5	21 ± 2
D	Wb2-3	*Paenibacillus polymyxa* (NR_037006), 99%	–	+	+	–	49 ± 4	34 ± 4
D	Sb3-1	*Paenibacillus kribbensis* (NR_025169), 99%	+++	++	+	–	44 ± 6	23 ± 1
D	Mc2-9	*Paenibacillus brasilensis* (NR_025106), 99%	++	++	+	–	64 ± 6	24 ± 1
D	Mc5-5	*Paenibacillus brasilensis* (NR_025106), 99%	++	–	++	–	58 ± 5	26 ± 1
D	Mc6-4	*Brevibacillus limnophilus* (NR_024822), 99%	+++	–	++	–	77 ± 4	39 ± 2
D	Mc2Re-16	*Paenibacillus brasilensis* (NR_025106), 98%	++	+	–	–	57 ± 9	31 ± 4
D	Mc5Re-14	*Paenibacillus polymyxa* (NR_037006), 99%	++	+	++	–	52 ± 3	38 ± 1
D	Sd5Re-24	*Paenibacillus brasilensis* (NR_025106), 99%	++	+	++	–	20 ± 2	11 ± 2
E	Wb1n-4	*Streptomyces scabiei* (NR_025865), 98%	+	++	+	+	70 ± 2	47 ± 4
E	Wb2n-2	*Streptomyces peucetius* (NR_024763), 98%	++	++	+	+	66 ± 3	40 ± 1
E	Wb2n-11	*Streptomyces subrutilus* (NR_026203), 99%	+++	+++	+	+	76 ± 7	48 ± 6
E	Wb2n-23	*Streptomyces peucetius* (NR_024763), 98%	++	+++	+	–	26 ± 3	15 ± 1
F	Mc1-3	*Lysobacter enzymogenes* (NR_036925), 99%	+	++	++	–	63 ± 6	23 ± 2

*The letters represent the different amplified rRNA gene restriction analysis patterns (A-F); group B (Bacillus cereus group) was completely excluded (Köberl *et al.*, 2011).

†According to 16S rRNA gene sequencing.

‡Dual culture assay: +…0–5 mm, ++…5–10 mm, +++…> 10 mm radius of zone of inhibition, - …no suppression.

§Results of a previous study performed by Köberl et al. (2011).

¶Control with Escherichia coli showed 28% dead J2 after 48 h, and a mortality rate of 21%, at controls with media and water both values were 0%.

‖Standard deviation.

**Determined by linear regression of the percentages of dead J2 after 0, 6, 12 and 24 h, ± error of slope.

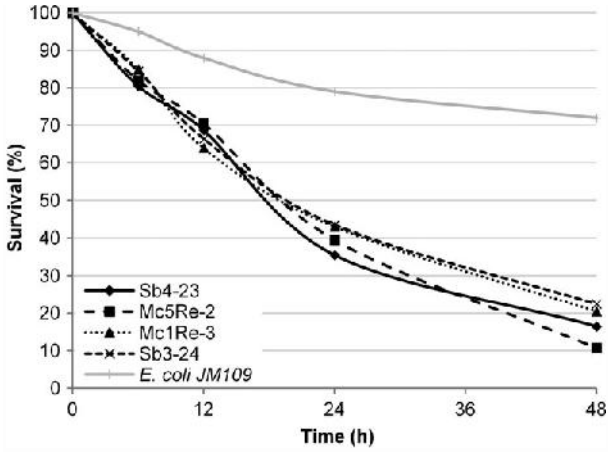

Fig. 3. In vitro effects of extracellular bacterial products on the mortality of Meloidogyne incognita juveniles. Depicted are the impacts of the four most efficient isolates in comparison with the control with Escherichia coli JM109.

Actinobacteria-specific probe, some of these bacterial colonies could be identified in the rhizosphere of Matricaria chamomilla as well when grown under organic desert farming conditions (Fig. 4).

Discussion

One of the major challenges of the 21st century will be to develop an environmentally sound and sustainable crop production. Desert agriculture opens up new possibilities to address diverse problems: to produce enough food for poor regions, to produce renewable crops for industrial applications, and to capture and restore CO_2 in soil. The accumulation of soilborne pathogens is another important ecological problem, which can cause dramatic yield losses. To solve this problem, we analysed associated microbial communities, which were found specific for each plant species and microhabitat. ITS profiles of fungal communities were less discriminative than bacterial fingerprints and characterised mainly by potential pathogens. Therefore, we selected bacterial antagonists against these and the well-known pathogens.

The dominance of Gram-positive bacteria in the group of antagonists in plant-associated and soil communities under arid conditions is in contrast to other studies performed under humid, temperate climate conditions. Here, mainly members of the genus *Pseudomonas* were found as antagonists (Berg et al., 2006; Costa et al., 2006; Weller, 2007), as it is well-studied for its beneficial plant–microorganism interaction (Haas & Defago, 2005; Lugtenberg & Kamilova, 2009). To verify our result, *Pseudomonas*-selective medium was used to monitor *Pseudomonas* isolates (King *et al*., 1954), but only a few colonies were detected (data not shown). This differing ecology between arid and humid environments can be explained by the extreme abiotic conditions, such as the combination of extreme temperatures and desiccation, high soil salinity, low nutrient levels and high UV radiation levels in deserts. Recently, in a farm located in the northwestern desert region of Egypt, Marasco *et al.* (2012) reported a predominant role of *Bacillus* within the plant growth-promoting microbiome associated with the drought-sensitive pepper plant, which supported this conclusion. In addition, in the rhizosphere of Antarctic vascular plants, another extreme environment, *Firmicutes* were also identified as the most abundant phylum using a deep-sequencing approach (Teixeira *et al*., 2010). However, in the microbiome of the sugar beet rhizosphere, *Firmicutes* represent 20% of the bacterial phyla with *Proteobacteria* as the dominant member (39%) (Mendes *et al*., 2011). *Bacillus*, *Paenibacillus* and *Streptomyces* are spore-forming bacteria, and spore production aids in survival under suboptimal conditions

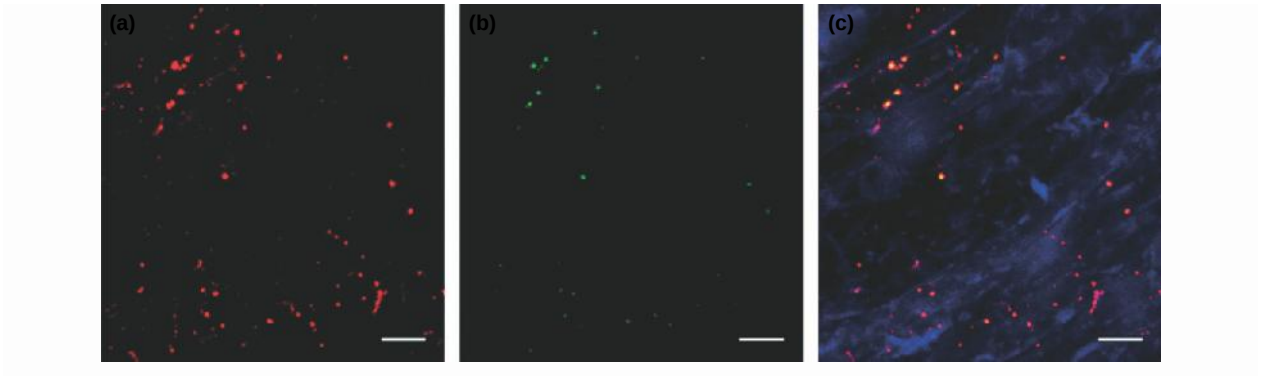

Fig. 4. *In situ* visualisation of *Actinobacteria* in the rhizosphere of *Matricaria chamomilla*. Fluorescent *in situ* hybridisation (FISH) showed a high colonisation of chamomile roots with bacteria in general (a), of which some colonies could be identified as *Actinobacteria* (b). The overlay (c) of the fluorochrome signals (a and b) with the autofluorescence of the root (blue) shows examples for *Actinobacteria* (yellow) amidst other eubacteria (red). Scale bar = 5 μm.

(Nicholson, 2002). However, it is still unclear whether these Gram-positive bacteria were alive and active in soil. Once considered their habitat, the soil may simply just serve as a reservoir (Hong *et al*., 2009). While rhizosphere colonisation was recently shown by the BCA *Bacillus amyloliquefaciens* FZB42 (Fan *et al*., 2011), we also found *Actinobacteria* colonisation as well.

Bacillus/Paenibacillus and *Streptomyces* species are well-known for their biocontrol potential (Schisler *et al*., 2004; Berg, 2009). Several strains of *Bacillus subtilis* are already in use as biological pesticides (Fan *et al*., 2011), and the antagonistic potential of *Paenibacillus* polymyxa towards a wide range of mycotoxin-producing fungi such as *F. culmorum* is well documented (Tupinamb a *et al*., 2008). Furthermore, a broad disease-suppressive activity has been detected for strains of Lysobacter (Postma *et al*., 2011), the only Gram-negative genus selected. Despite this fact, we know that the biocontrol effect and mode of action are strongly strain-specific (Berg *et al*., 2006; Berg, 2009). In our study, we detected plant species and microhabi- tat-specific bacterial antagonists, but also strain specificity was confirmed. Altogether, 13 broad-spectrum antagonists with antibacterial, antifungal and nematicidal activity were found which belong to seven different bacterial species of the genera *Bacillus* (*B. atrophaeus, B. mojavensis, B. subtilis subsp. div., B. vallismortis*) and Streptomyces (S. peucetius, S. scabiei, S. subrutilus). On their basis, biocontrol products specifically for arid conditions can be developed.

In this study, we linked ecological data with the selection strategy for antagonists. Within the fungal community, mainly potential phytopathogens were identified. Therefore, we focused on the selection of bacterial antagonists. In the cultivation-independent and dependent approach, strains of *Bacillus/Paenibacillus* were found as the key players in bacterial communities in arid agricultural systems. Conversely, members of the genus Streptomyces were important in the natural desert ecosystem. This was also confirmed by a comparative deep-sequencing approach of desert and field soil (Köberl *et al*., 2011). Gram-positive, spore-forming bacteria of the genera *Bacillus*, *Paenibacillus* and *Streptomyces* were selected using our hierarchical procedure; all of them belong to risk group 1 (no risk for humans and the environment) and are promising drought-resistant and heat-resistant biocontrol candidates. Furthermore, they showed a remarkable antibiotic activity.

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Chapter VI

Main findings, General conclusion and Future perspectives

MAIN FINDINGS

- Plant-parasitic nematodes occur widely on the SEKEM organic farm; eleven genera were detected during the surveys 2009 and 2011, with nine genera being detected in both surveys.

- *Meloidogyne* was the most frequently encountered genus in both surveys. Other genera commonly detected were *Tylenchorhynchus, Rotylenchulus, Helicotylenchus* and *Pratylenchus*.

- *Meloidogyne incognita* populations and/or races varied in their reproduction rate on different hosts and based on PCR-DGGE of the *msp1* gene.

- Three arable soils from different regions in Germany significantly differed in the composition of microbial communities and suppressiveness towards *M. hapla*.

- DGGE fingerprints of those three German soils showed many ribotypes that were abundant on *M. hapla* second-stage juveniles (J2) but not in the surrounding soil, some of which seemed to be present in all three soils while most were soil type specific.

- Determination by 16S rRNA amplicon pyrosequencing indicated that *M. hapla* J2's from the most suppressive German soil Kw were associated with OTU's that were closely related to *Shigella* spp., while most abundant were *Malikia spinosa* and *Rothia amarae*.

- Following application as seed treatment, all tested bacterial antagonists of fungal pathogens also showed antagonistic potential against *M. incognita*; the highest nematode control was achieved by *B. subtilis* strains Sb4-23, Mc2-Re2, Mc5- Re2 and *R. etli* G12.

- The top four bacterial antagonists controlled *M. incognita* by a combination of mechanisms including metabolites causing nematode inhibition and repellency, or inducing systemic resistance.

GENERAL CONCLUSION

Plant-parasitic nematodes (PPN) are one of the most important groups of pests on many crops worldwide. Control of PPN is difficult, especially in organic farming systems, because feasible control methods and monitoring systems are not always available compared to foliar diseases and insect pests. Most nematodes are soil-borne pathogens that rarely cause symptoms on the foliage, or cause symptoms similar to water or nutrition deficiency making it difficult to diagnose the disease. Therefore, surveillance and monitoring for PPN is an important requisite for developing effective nematode management strategies (chapter II). Regular monitoring of PPN will also allow to detect increasing infestation levels long before economic damage appears but also to identify suppressive soils (chapter IV).

In this study, the occurrence, distribution and abundance of PPN associated with different organic crops at SEKEM farm in Egypt were determined (chapter II). Our results showed that *Ditylenchus*, *Helicotylenchus*, *Hoplolaimus*, *Meloidogyne*, *Pratylenchus*, *Rotylenchulus*, *Tylenchulus*, *Tylenchorhynchus*, and *Xiphinema* occurred widely at the SEKEM farm and threatened most agricultural crops. The commonly detected genera *Meloidogyne*, *Tylenchorhynchus*, *Rotylenchulus*, *Helicotylenchus* and *Pratylenchus* in both surveys have a broad host spectrum making their management difficult. Genera that were only detected depending on the presence of their suitable hosts during each survey suggested that the use of resistant or non-host crops is useful to limit their prevalence. Especially the population densities of *Meloidogyne*, *Rotylenchulus* and *Helicotylenchus* reached levels that were damaging to most crops. Other genera were detected at relatively low densities, but might increase under susceptible crops to damaging levels within a relatively short period of time. Overall, the monitoring identified the most common nematode taxa occurring on each

crop grown at the SEKEM farm. Especially the wide distribution of root-knot nematodes (RKN) across all sectors of the farm covering different crops poses a significant threat to organic farming in Egypt. Therefore, this nematode taxe received main focus in this thesis (chapter III, IV, and V).

Accurate identification of RKN is crucial to select the appropriate management strategy. Within this study *Meloidogyne incognita* was identified as the primarily occurring RKN species (chapter II). As populations of this species can vary a lot regarding their virulence on resistant cultivars, further differentiation is required, which is not possible using morphological analysis and may be uncertain using differential hosts. Within this respect, one of the thesis objectives was to accurately and rapidly discriminate *M. incognita* populations/races using molecular methods. PCR-DGGE was applied to differentiate *M. incognita* populations/races originated from different countries (chapter III). These populations and races of *M. incognita* differed in their reproduction rate on specific crops and/or cultivars. PCR-DGGE of the *msp-1*gene amplified from those populations and races facilitated the discrimination among them based on variants of this gene. Interestingly, the UPGMA analysis of the DGGE patterns separated the population/races into two major groups. Compared to principle component analysis, DGGE was more successful in separating each population/race in a separate cluster. This indicates that DGGE is a useful tool to differentiate *M. incognita* populations/races. Furthermore, it is a promising tool for studying population genetics between and within PPN species.

Studying the interaction of root-knot nematodes with soil microbes may result in discovering natural antagonists as potential candidates for biocontrol purpose. This might

especially be the case when using suppressive soils. The discovery of low densities of *M. hapla* in threearable soils from Germany despite the presence of a susceptible host and suitable environmental conditions lead to in-depth studies on the biological origin of those low nematode numbers (chapter IV). Suppressive soils are known to contain numerous beneficial microorganisms that reduce plant-parasitic nematodes. The three German soils were used for baiting *M. hapla* second-stage juveniles (J2) to determine whether specific microbes attached to the J2 cuticle. PCR-DGGE and 454-pyrosequencing of 16S rRNA genes techniques were useful tools to reveal diverse microbial communities attached to J2 and to directly identify the specific attachment of bacteria and fungi without the need to culture. The sequences of these microbes could be useful to develop cultivation methods for these species, or for cultivation-independent analysis of the interaction with *M. hapla*. Results showed that species of fungi and bacteria attached to the J2 cuticle that were not detected from the surrounding soil, indicating a specific attachment to the nematode cuticle. Furthermore, it was shown that differences in suppressiveness to *M. hapla* among the three German soils corresponded to differences in microbial soil communities and microbes attaching to J2 cuticle. In particular, fungi and bacteria from J2's of the most suppressive soil Kw were more abundant and diverse than those from the other two soils. Some of those "enriched" microorganisms have been previously reported as antagonist of root-knot nematodes. Thus managing arable soil towards increased abundance of antagonistic bacteria and fungi could become a substantial part in nematode control.

Root-knot nematodes are probably the most recorded nematodes found in disease complexes with fungal plant pathogens. Therefore, the use of microorganisms with dual antagonism against RKN and fungal pathogens are highly desirable. This study has shown that bacterial isolates with antagonistic activity against soil-borne fungal pathogens also possessed antagonistic potential against the root-knot nematode *M. incognita* (chapter V). By applying bacterial cells on seeds, all antagonists caused a significant reduction in numbers of galls and egg masses on tomato compared with the untreated control. Based on seed treatment results, the top four strains *B. subtilis* Sb4-23, Mc2-Re2, Mc5-Re2 and *R. etli* G12 were selected for studying their mode-of action. Understanding their mechanisms in suppressing the nematode will allow optimization of the biocontrol potential for a successful application in praxis. Our results demonstrated that these four isolates affected nematodes by a variety of mechanisms including direct effect by culture supernatants, repellency and induced systemic resistance. The latter was identified as the major control mechanism of the antagonists based on two reasons: i) In split-root experiment to compare direct antagonism and induced systemic resistance, the co-inoculation of bacterial antagonists with *M. incognita* in the same pot did not enhance suppression of the nematode compared to spatially separated inoculation; ii) In the repellence test the effect of the antagonists was within the range of that achieved by induced resistance, and repellance therefore did not add to this effect.

RECOMMENDATIONS FOR FURTHER WORK.

The research that has been undertaken for this thesis has provided the following insights for future work:

- To study dynamics and community structure of plant-parasitic nematodes throughout the seasons in organic farms located in different regions of Egypt.

- PCR-DGGE techniques should be developed for other effector genes of *Meloidogyne* spp., which could resolve allele frequencies to differentiate populations and study population-specific epidemiology and infectivity.

- Investigation should be strengthened to confirm if the German soils possess specific suppressiveness against *M. hapla*, and if those microorganisms attached to the nematode cuticle are involved in this suppressiveness.

- The bacteria and fungi found to be attached to the cuticle of J2's should be evaluated for their biocontrol potential of *M. incognita*.

- The dual control potential of the three *B. subtilis* strains Sb4-23, Mc2-Re2, Mc5-Re2 against *M. incognita* and fungal plant pathogens should be further exploited under field conditions.

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Publications

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Languages

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