Isolation, characterization and interactions of soil microorganisms involved in the enhanced biodegradation of non-fumigant organophosphate nematicides

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I dedicate this work to my parents Elizabeth and Alfonso, to my sisters Vicky and Mercedes, to their families, and to my life's partner Véronique.

Isolation, characterization and interactions of soil microorganisms involved in the enhanced biodegradation of non-fumigant organophosphate nematicides

The most widely used pesticides utilized for the management of plant-parasitic nematodes belong to the organophosphorus group. Their efficacy may be reduced in areas where adapted microorganisms accumulate that are capable of rapidly degrading the active ingredients. The enhanced biodegradation process of non-fumigant nematicides is of particular concern in intensive agriculture. However, it remains unclear which microorganisms play the most important role in the rapid metabolization and how and why this process develops. Furthermore little is known as to whether the biodegradation process may be slowed down, stopped or reversed. Studies using soils with different nematicide history collected in four banana fields in the Atlantic region of Costa Rica demonstrated that the non-fumigant organophosphate nematicide terbufos had lower levels of efficacy and shorter effective activity against the burrowing nematode *Radopholus similis* when the soil had a prolonged terbufos application history. Lower levels of efficacy were related to the microorganisms capable of rapidly degrading the active ingredient. The analysis of soils collected in Germany with different nematicide application history demonstrated that fenamiphos, another organophosphate non-fumigant nematicide, was not rapidly biodegraded in soil with no previous pesticide exposure. This study also demonstrated that *Pseudomonas* spp. does not accumulate upon fenamiphos applications and may not be involved at all in fenamiphos degradation. The lack of surfactant production of the isolated *Pseudomonas* spp. could be a reason for their absence in the biodegradation process. Bacteria capable of degrading fenamiphos were isolated from another German soil with a large fenamiphos-history. These bacteria utilized fenamiphos as a sole carbon source. By comparison of the partial sequences of their 16S rRNA coding genes with those genes present in the GenBank sequence database, a fully resolved phylogenetic tree could be generated, showing that these fenamiphos degrading (Fd) isolates belonged to closely related *Microbacterium, Sinorhizobium, Brevundimonas, Ralstonia,* or *Cupriavidus* species. The Fd bacteria did not cross-degrade the novel organophosphate nematicide fosthiazate, thus suggesting that they are fenamiphos-specific. However, a combination of all microorganisms of the same soil from which the fenamiphos-degrading bacteria was isolated, was capable of degrading fosthiazate, thus demonstrating that there are other microorganisms capable of degrading nematicides even in the absence of an application history. This also revealed that the nematicide-history of one organophosphate nematicide does not intrinsically influence the degradation of another pesticide of this same chemical group.

The application of plant revitalizers enhanced soil microbial biomass over time which resulted in an enhanced biocontrol activity against the root-knot nematode *Meloidogyne incognita* and a delayed biodegradation process of fenamiphos.

In conclusion, this research demonstrated that many different soil bacteria can adapt when frequently exposed to a particular nematicide, thus offering them an alternative carbon source to grow. This effect can be slowed down by altering the microbial soil diversity through the application of natural plant enhancers that benefit nematicide non-degrading strains and simultaneously reduce nematode damage.

Isolierung, Charakterizierung und Wechselwirkungen von Bödenmikroorganismen verantworlich für den beschleunigten biologischen Abbau von nicht gas förmigen organophosphatishce Nematiziden

Die weitverbreitesten Pestizide gehören zur Wirkstoffgruppe der Organophosphate. Jedoch kann deren Wirkung durch das verstärkt Auftreten von Mikroorganismen, welche in der Lage sind diesen Wirkstoff zu degradieren, gemindert werden. Die verstärkte Degradierung von nicht gasförmigen Nematiziden betrifft vor allem Anbaugebiete mit intensiver Landwirtschaft. Bis heute ist ungeklärt welche Mikroorganismen bei dem Prozess der beschleunigten Metabolisierung von nicht gasförmigen organophosphatischen Nematiziden eine wichtige Rolle spielen oder wie und warum diese Prozess entsteht. Auch gibt es wenige Erkenntinsse darüber ob der Prozess der Bio-Degradierung verzögert, gestoppt oder umgekehrt werden kann. In diesen Untersuchungen wurden Böden von vier Bananenfeldern Costa Ricas, die zuvor mit verschiedenen Nematiziden behandelt wurden, genauer betrachtet. Es zeigte sich das die Behandlung mit dem Nicht-Begasungs Organophosphate Nematizid Terbufos einen Bekämpfungserfolg gegen den Nematoden *Radopholus Similis* zur Folge hatte sofern die Böden zuvor nicht so häufig mit dem Nematizid Terbufos behandelt wurden. Dieser Effekt konnte auf den hohen Anteil von Mikroorganismen in den Böden zurückgeführt werden, die den Wirkstoff im Boden schnell abbauten. Weiter Versuche mit verschiedenen Böden aus Deutschland zeigten, dass Böden die erstmals mit dem Nicht-Begasungs Organophosphate Nematizid Fenamiphos behandelt wurden, den Wirkstoff im Boden nicht ausreichend schnell biologisch abgebauen konnten. Verschiedene Bakterien der Gattung *Pseudomonas* konnten den Wirkstoff hier nicht metabolisieren. Ein Anstieg der *Pseudomonas* Population wurde nach einer Fenamiphos Behandlung nicht ermittelt. Der Mangel der Surfactant Produktion der bodenbürtigen Bakterien könnte ein Grund für den fehlenden biologischen Abbau sein. Folglich, könnten nur vereinzelte *Pseudomonas spp.* Stämme Nematizide abbauen. In weiteren Versuchen wurden aus deutschen Böden, die zuvor häufig mit Fenamiphos behandelt wurden, 17 Fenamiphos abbauende Bakterienstämme isoliert. Diese Bakterien bauten den Fenamiphos schnell ab. Weitere Versuche zeigten, dass ein Bakterienstamm den Wirkstoff als Kohlenstoffquelle für sein Wachstum nutzte. DNA Profile der Fenamiphos abbauenden Bakterienstämme wiesen 5 verschiedene RFLP Muster auf. Diese Bakterien wurden als *Microbacterium*, *Sinorhizobium*, *Brevundimonas*, *Ralstonia* oder *Cupriavidus* Spezies anhand ihrer partiellen 16S rRNA Gensequenzen identifiziert. Phylogenetische Analysen mit die Bakterien zeigten enge Verwandtschaft mit einander und haben gezeigt dass die Bakterien stammten von dem gleichen Vorfahren ab. Multiple Sequenz Analyse von den Fenamiphos abbauenden Bakterien ergaben identische Nucleotide Regionen mit Bakterien von ein Genebank. Die Fenamiphos abbauenden Bakterien bauten das neuartige Organophosphate Nematizid Fosthiazate nicht ab wodurch eine Fenamiphos Spezifizierung der Bakterien nachgewiesen werden konnte. Jedoch, in den Böden, in denen zuvor die Fenamiphos abbauenden Bakterien isoliert wurden, wurde der Wirkstoff Fosthiazate, aufgrund des hohen Mikroorganismen Anteil im Boden, abgebaut. Applikationen von Pflanzen revitalisierenden Mitteln erhöhte die mikrobielle Biomasse im Boden. Das frühe Eindringen des Wurzelgallen Nematoden *Meloidogyne incognita* wurde gehemmt. Der Abbau von Fenamiphos wurde verzögert. Zusammenfassend zeigte diese Arbeit, dass spezifische bodenbürtige Bakterien sich an bestimmte Nematizide anpassen und deren Wirkstoff als Kohlenstoffquelle für sich nutzen können. Dieser Effekt verlangsamte sich mit veränderter Populationsdichte der Mikroorganismen. Die Diversität durch Applikation von biologischen Pflanzenfördern hemmte den Nematodenbefall selbst wenn nicht Nematizid abbauende Stämme im Boden vorkommen.

1. GENERAL INTRODUCTION

1.1. Importance of plant-parasitic nematodes

Nematodes are microscopic, aquatic, elongated, tubular, spindle shaped worms that live in moist surfaces, films of water within soil and in moist tissues of different organisms and plants (Dropkin, 1989). Most plant-parasitic nematodes attack underground plant parts, especially roots (Whitehead, 1998). Other species are predominantly shoot parasites, attacking stems, leaves, flowers, seeds or combinations thereof. Nematodes may feed ecto-, semi-endo-, or endo-parasitically on host tissue using a narrow mouth spear or stylet. Some nematodes can transmit pathogenic viruses with their stylet (Evans et al., 1993). Most plant-parasitic nematodes are obligate parasites. The opening in the root tissue made with the stylet to penetrate or feed may be used later by pathogenic bacteria and fungi for secondary infections (Luc et al., 2005).

Plant parasitic nematodes are an important limiting factor to crop production in temperate, tropical and sub-tropical agriculture (Evans et al., 1993; Luc et al., 2005). The damage done to a plant depends on the nematode species and the number of nematodes feeding on it (Whitehead, 1998). Most crops including cereals, vegetables, fruit trees and fibre plants are susceptible to several nematode species. Yield losses and crop quality reduction caused by nematodes have negative economic consequences on farmers, consumers and society (Webster, 1972).

The aim of nematode control is to restrict significant yield losses and quality in vulnerable crop plants and, in the longer term, to keep plant-parasitic nematode populations under the threshold level (Whitehead, 1998). Despite the use of crop rotation, soil amendments, resistant/tolerant varieties, catch crops and biocontrol agents, the control of plant parasitic nematodes still relies heavily on the use of chemical nematicides worldwide.

1.2. Use of nematicides in agriculture

The role of chemicals in nematode control has been well reviewed by Whitehead (1998), Hague and Gowen (1987) and Johnson (1985). Sikora and Marczok (2005) provided a list of the most common chemicals available on the market used for nematode control. Chemicals

which paralyse or kill nematodes are referred to as nematicides (Whitehead, 1998). They are classified as fumigant or non-fumigant types. Fumigant nematicides have large vapour pressures and diffuse rapidly through the network of soil pores in the gas phase. Most of these chemicals are either halogenated aliphatic hydrocarbons or methyl isothiocyanate precursor compounds with toxic effect on almost all living organisms including bacteria, fungi and plants. The fumigant nematicides are highly effective in nematode control. To prevent plant damage they must be applied long before planting or transplanting. However, the use of fumigant nematicides, such as methyl bromide, has decreased in modern agriculture due to an environmental concerns which resulted in a restriction of use (Santos et al., 2006; Webster et al., 2001).

Non-fumigant nematicides are granular or liquid compounds which are water soluble and have either contact or nematistatic and systemic activity against nematodes (Sikora and Fernandez, 2005). Some of these nematicides are also used as insecticides. The non-fumigant nematicides are applied to soil at concentrations that paralyze nematodes and do not kill them. This type of nematicides can be divided into two groups, the organophosphates and the carbamates, according to their molecule structure. In most cases, the mechanism of action of both groups is associated with suppression of nematode mobility during the period when adequate concentrations are present in the soil. These non-volatile types of nematicides are preferred nowadays in modern agriculture since they are more specific than the fumigants, have less environmental risk and are generally not phytotoxic (Cabrera et al., 2009a). They can be applied to the soil even when the crop has been established or as seed treatment (Cabrera et al., 2009b). The lack of new non-fumigant nematicide molecules has lead to the repetitive application of the same compounds for nematode management. It takes about 12 years to develop a new non-fumigant nematicide for the market. The repeated application of the same nematicide is practiced specially in monoculture systems, for example in banana production, where nematicide treatments are performed according to an application calendar that can vary from 2 to 3 times per year. These repeated applications may influence the emergence of specific soil microorganisms that can degrade the active substances at accelerated rates (Smelt et al., 1987; Ou et al., 1994).

1.3. Enhanced biodegradation of non-fumigant nematicides

The accelerated microbial degradation of pesticides was first described in 1951 with the herbicide 2,4-D (Audus, 1951). Since then over 375 publications have appeared which describe the biodegradation of more than 50 different soil applied crop protection products (Anderson et al., 1998) such as insecticides (Felsot et al., 1981; Read, 1983; Racke and Coats, 1988; Morel-Chevillet et al., 1996) fungicides (Walker, 1987; Thom et al., 1997) and herbicides (Kirkland and Fryer, 1972; Torstensson et al., 1975; Wilson, 1984; Gray and Joo, 1985; Skipper et al., 1986).

Enhanced biodegradation is the rapid microbial degradation of a pesticide, in the present case nematicides, by a specialized fraction of the soil microflora (Karpouzas and Giannakou, 2002). Soil microorganisms have adapted in such a way that they can rapidly metabolize specific nematicides. The degradation products (metabolites) of nematicides have been implicated in an enrichment emergence of soil microflora capable of accelerated degradation of parent compounds (Ou and Rao, 1986; Jones and Estes, 1995). The nematicide biodegradation in monoculture systems was reported in the early 1990's from soil cultivated with tomato after repetitive nematicide applications using the same active substance (Stirling et al., 1992). A similar effect was observed later in soil cultivated with turfgrass (Ou et al., 1994) and potato (Karpouzas et al., 1999). Mclean and Lawrence (2003) found a lack of nematicide efficacy in a soil cultivated with cotton which was treated often with the same chemical.

At the present time, the most widely used pesticides belong to the organophosphorus group (Singh and Walker, 2006). The enhanced biodegradation of several insecticides of this chemical group, such as diazinon, chlorpyrifos, parathion and dimethoate has been reported (Sethunathan and Pathak, 1972; Singh and Walker, 2006; Drufovka et al., 2008; Li et al., 2008). The organophosphate herbicide glyphosphate and the acaricide coumaphos have also been found to rapidly biodegrade (Singh and Walker, 2006). However, studies on organophosphate nematicides are rarer. Even though there are reports of biodegradation on this nematicide group it remains unclear which microorganisms play the most important role in the rapid metabolization and how and why this process develops. To date, there is little information whether the enhanced biodegradation process can be slowed down or reversed by manipulating the natural soil microbial populations.

1.4. Cross biodegradation

Cross biodegradation is referred to when microorganisms have been frequently exposed to one nematicide and the microbes are able to simultaneously degrade other nematicides. The cross-enhancement of selected pesticides in soil can be dependent on the structural similarity of the compounds (Singh et al., 2005). For example, the bacterial population of a cadusafos-adapted soil was able to rapidly degrade the chemically related nematicide ethoprophos (Karpouzas et al., 2004b). The cross adaptation of microorganisms decreases exponentially the efficacy of nematicides but it also can be considered an important process in the rapid degradation of pesticides from the soil environment (Ankumah et al., 2008). However, not all microorganisms that can degrade one nematicide are able to metabolize another one (Karpouzas and Walker, 2000; Karpouzas et al., 2004a). To avoid the onset of biodegradation, and therefore cross-degradation, sufficient chemical rotation, i.e. the use of active ingredients from different chemical groups, in combination with crop rotation and the use of resistant cultivars is recommended (Karpouzas and Giannakou, 2002). However, the cross-degradation process is still poorly understood (Suett and Jukes, 1988) and whether adapted microorganisms to old molecules can also degrade the new nematicides at accelerated rates is currently unknown.

1.5. Natural plant enhancers

Application of residue amendments derived from crops or animals is known to improve crop yield. The use of amendments has become an important agronomic tool and an efficient way of improving plant nutrition and therefore they are considered to be plant growth enhancers (Mulawarman et al., 2001; Mulawarman, 2002). A beneficial effect of adding extra nutrients to soil is to increase crop yield and performance (Muller and Gooch, 1982; Mullins and Mitchell, 1995). Most of these residue derived compounds are organic amendments. The use of organic amendments which can provide extra nutrients to soil, increase crop yield and control plant-parasitic nematodes has been previously reviewed (Mankau, 1962/1968; Muller and Gooch, 1982). More recently, it has been reported that in some cases the benefits of adding organic material or residues to the soil is mainly due to a decrease in the levels of soil pathogens (Mullawarman et al., 2001). This is caused by an alteration in soil structure and ecology, and/or by the action of residue derived chemicals on the soil fauna and flora. Organic amendments deliver nutrients which specifically support the growth of antagonistic

fungi on other organisms (Muller and Gooch, 1982; Rodriguez-Kabana, 1986). However, it has still not been reported whether the addition of organic amendments or plant enhancers can also support or suppress the growth of microorganisms responsible for the enhanced degradation of nematicides. It is known that once the enhanced biodegradation of nematicides is present in a soil it can stay active for prolonged periods after the last application (Smelt et al., 1996). If the microorganisms involved in the degradation process could be reduced through the application of organic amendments or plant enhancers and nematicide-efficacy re-establish is still unknown.

1.6. Scope of the study

The overall goal of the present study was to investigate the enhanced biodegradation process in soils with different histories of non-fumigant nematicide applications. The objectives of the research in this thesis were:

1. Demonstrate the enhanced biodegradation process occurring in soil of a banana monoculture system.

2. Study the microorganisms involved in the rapid nematicide metabolization.

3. Isolate, indentify and characterize nematicide-degrading microorganisms.

4. Determine the occurrence of cross-degradation from old to new nematicide molecules.

5. Determine whether natural plant enhancers can slow down or reverse the rapid nematicide breakdown process caused by microorganisms.

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2. GENERAL MATERIALS AND METHODS

2.1. Location of field research in Costa Rica

To investigate the enhanced biodegradation in a mono culture system, 4 commercial banana plantations were selected in the Atlantic region of Costa Rica (Figure 1). This region contains 95% of all commercial banana plantations of the country. The Reventazon River (RR) was a natural soil dividing factor. Soils of banana fields located west of the river (F1, F3 and F4) were of volcanic origin and the east one (F2) of river sediments (Serrano et al., 2006). The average mean temperature and the average relative humidity in this area between 2000 and 2005 was 25.5 °C and 83.6% as reported by the meteorological station at Siquirres, Costa Rica, respectively.

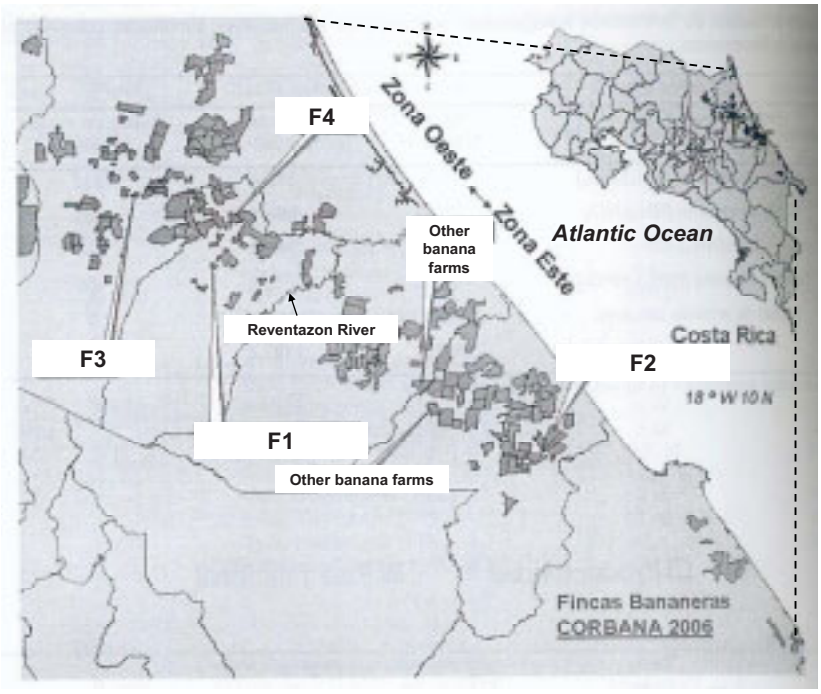

Figure 1. Enlargement of the Atlantic region of Costa Rica showing fields with banana cultivation in the area (dark gray blocks). Collection of soil and roots was performed in the four commercial banana plantations indicated as F1, F2, F3 and F4. Map of Costa Rica (above right). This map was adapted from Serrano et al. (2006).

2.2. German soils used for enhanced biodegradation screening

To investigate the enhanced biodegradation phenomena at laboratory and greenhouse level samples from four soils were used in this investigation. All soil samples were collected from the top 20 cm of soil in fields of experimental stations in Nordrhein Westfalen, Germany.

Soil 1 (S1): was obtained from the field 1 of the experimental farm Laacher Hof of Bayer CropScience AG. This soil was composed of 24.5% clay, 62.6% silt and 12.9% sand and had a pH of 4.06. This soil was treated 42 times with fenamiphos in the last 16 years.

Soil 2 (S2): was obtained from the same field as S1 but has received 25 fenamiphos treatments in 12 years. This soil was composed of 23.1% clay, 60.8% silt and 16.1% sand, and had a pH of 4.51.

Soil 3 (S3): was obtained from the field Am Hohenseh 4a, Burscheid, of the experimental farm Höfchen of Bayer CropScience AG. This soil was composed of 16.2% clay, 78.4% silt and 5.4% sand, and had a pH of 6.17. This soil has received 25 fenamiphos treatments in 12 years.

Turf grass was grown in these three soils before arrival to our laboratory. The soil samples collected in these soils were stored at 20 ± 2 °C in the dark. The last fenamiphos treatment was performed about 1 month before this investigation started.

Soil 4 (S4): was soil obtained from the research station of the University of Bonn at Klein Altendorf, Rheinbach. This soil was composed of 14.6% clay, 77.6% silt and 7.8% sand, and had a pH of 6.75. The field was previously cultivated with wheat during summer season and has never received fenamiphos or other nematicide treatment before arrival to the laboratory.

During the research period all soils were kept in slightly capped plastic containers at 8 °C in the dark and have received autoclaved water to their holding capacity to maintain moisture.

2.3. Culture media, antibiotics, fungicides and reagents

Acetonitrile: HPLC grade (Sigma-Aldrich).

Agar: AppliChem GmbH.

Ampicillin: A stock solution was prepared dissolving Ampicillin (AppliChem) in autoclaved (121 ºC for 20 min) distilled water to a desired concentration following filter-sterilization (0.4 µm). The stock solution was kept at -20 °C.

Blue media: 20 g Mannitol ($C_6H_{14}O_6$), 0.7 g Potassium dihydrogen (KH_2PO_4), 0.9 g Sodiumphosphatedibasic heptahydrate (NA_2HPO_4), 2.0 g Sodiumnitrate ($NaNO_3$), 0.4 g

Magnesiumsulfate heptahydrate ($MgSO_4$ $7H_2O$), 0.1 g Calcium chloride anhydrous ($CaCl_2$ $2H_2O$), 2 ml Trace Elements solution, 0.2 g CTAB (Cetyl trimetrilamonium bromide, $C_{l9}H_{42}NBr$), 0.005 g Methylene blue and 15 g bacteriological agar dissolved in 1 L autoclaved distilled water.

Buffer phosphate solution (BPS): 1.39 g HK_2 O_4P (136.09 g mol^{-1}, Merk) and 1.08 g H_2KO_4P (136.09 g mol^{-1}, Merk) dissolved in 800 ml autoclaved distilled water.

Chloramphenicol: A stock solution was prepared dissolving Chloramphenicol (AppliChem) in 80% ethanol (v:v) to a desired concentration following filter-sterilization. The stock solution was kept at -20 ºC.

Cycloheximide: A stock solution was prepared dissolving Cycloheximide (Sigma) in 80% ethanol (v:v) to a desired concentration following filter-sterilization. The stock solution was kept at -20 ºC.

Ethanol: HPLC grade (Merck).

Glycerol: (Merck).

GC-TSB: 24 g purified and fine Trypticase Soy Broth (Becton Dickson) made for gas chromatography and 16 g Bacto Agar in 800 ml autoclaved distilled water.

Hexan: HPLC grade >95% purity (Merck).

KBM: 26.3 g King B Agar (Fluka, Biochemika) and 8 ml glycerol in 790 ml autoclaved distilled water.

KMB$^+$: 26.3 g King B Agar (Fluka, Biochemika) and 8 ml de glycerol in 790 ml autoclaved distilled water amended with filter-sterilized stock solutions of 13 µg Chloramphenicol ml^{-1}, 40 µg Ampicillin ml^{-1} and 100 µg Cycloheximide ml^{-1} final concentrations.

Methanol: HPLC grade (Sigma-Aldrich).

PDA$^+$: 19.2 g potato dextrose broth (Oxid LTD) and 14.4 g agar in 800 ml autoclaved distilled water amended with 150 ppm of Streptomycin sulphate and 150 ppm of Chloranphenicol.

Reagent 1: 45 g sodium hydroxide (certified ACS) pellets dissolved in 150 ml of methanol (reagent grade) and 150 ml of deionised distilled water.

Reagent 2: 325 ml 6.0 N hydrochloric acid supplemented with 275 ml methanol (reagent grade).

Reagent 3: 200 ml Methyl-tert Butyl Ether in 200 ml hexane (HPLC grade).

Reagent 4: 10.8 g sodium hydroxide pellets dissolved in 900 ml deionised distilled water.

Soil extract agar medium (SEAM): This medium was used since it contains low levels of carbon (close to 0). Every 500 g of autoclaved S4 were mixed with 900 ml of autoclaved

distilled water in a Duncan bottle and shaken vigorously by hand for 1 minute. After shaking, the solution was kept at room temperature for 72 hours. After this period, the solution was transferred into cylindrical tubes and centrifuged for 10 min at 20 ºC, 10 brake at 2000 G. The liquid was then filtered through folded filter paper (ø 385 mm, Whatman, Schleicher and Schuell) and collected in glass flasks. 20 g of Difco Bacto Agar were added per liter of soil extract, stirred and the pH was adjusted to 6.0. This mixture was autoclaved and poured in sterile conditions into sterile 5 cm diameter Petri dishes. Each Petri dish was filled with 5 ml of medium.

Soil extract liquid medium (SELM): The soil extract liquid medium was prepared as described in the SEAM except for the addition of agar. pH = 6.

Streptomycinsulphate: A stock solution was prepared by dissolving Streptomycinsulphate (AppliChem) in autoclaved distilled water to a desired concentration following filter-sterilization. The stock solution was kept at -20 °C.

Trifluoroacetic acid: 99% spectrophotometric grade (Sigma-Aldrich).

TSA 100%: 9.6 g agar (Agar Bacteriology grade, AppliChem) and 24 g Tryptone Soya broth (Oxoid) in 800 ml autoclaved distilled water.

TSA$^+$: 9.6 g agar (Agar Bacteriology grade, AppliChem) and 24 g Tryptone Soya broth (Oxoid) in 800 ml autoclaved distilled water amended with a filter-sterilized stock solution of Cycloheximide to a final concentration of 100 µg ml^{-1}.

Trace elements: 2 g iron sulphate heptaydrate ($FeSO_4$ $7H_2O$), 1.5 g Magnese sulphate monohydrate (MNS_4) and 0.6 g Amonium molybdate tetrahydrate [$(NH_4)_6$ Mo_7O_{24} H_2O] in 1 L of distilled water.

2.4. Isolation of bacteria from soil

Bacteria were isolated by transferring 10 g of soil to an autoclaved 300 ml Erlenmeyer flask containing 100 ml of autoclaved distilled water. Flasks were shaken at 120 rpm for 16 h at 28 ± 2 °C in an incubator in the dark. After incubation and under laminar flow, 1 ml of the solution was transferred into 9 ml autoclaved BPS in a glass test tube. Serial dilutions to 1 X 10^{-3} in BPS were prepared. From the final dilution, 50 µL were transferred to TSA 100% in 10 cm diameter Petri dishes. About 10 autoclaved glass beads were placed in every Petri dish which was closed and shaken by hand for 10 s. After shaking, the beads were removed and the Petri dishes were sealed with Parafilm and incubated in the dark at 28 ± 2 ºC for 48 h. Single colonies were picked with a sterile 4 mm loop and transferred onto fresh TSA$^+$.

2.5. Origin and culture of plant-parasitic nematodes

2.5.1. *Radopholus similis*

Radopholus similis was isolated from banana roots in a commercial farm in Costa Rica. A pure *R. similis* culture was maintained and multiplied in sterile carrot discs, as described by Speijer and De Waele (1997). Carrots were cleaned with tap water, dried with paper tissue and sprayed with 70% ethanol. The ethanol was burned off in the laminar flow. Both carrot ends were removed and the carrot was peeled with a sterile scalpel. The carrots were cut into discs and placed in sterile 3 cm Ø Petri dishes. *R. similis* was treated with Streptomycin sulphate (2000 ppm) on a 20 µm aperture sieve. 2 hours after treatment, the nematodes were collected from the sieve and inoculated at the outer edge of the carrot discs. Petri dishes were sealed with Parafilm and kept in an incubator at 25 ºC in dark conditions for 6 weeks. After this period of time, Petri dishes were taken out of incubation and opened under laminar flow. Nematodes on the surface of the carrot disc were washed and transferred with tap water into a beaker. The remaining carrot disc was macerated twice for 10 s at low speed in a commercial kitchen blender with tap water. The macerated suspension was poured onto a 100 µm aperture sieve to separate nematodes from carrot tissues. Nematodes that passed through the sieve were collected in a beaker and mobile nematodes were counted and used in the experiments as required.

2.5.2. *Meloidogyne incognita*

Meloidogyne incognita race 3 was obtained from a natural infested soil in Florida, USA, and maintained on the tomato cv. Furor permanently cultivated in a green house at 27 ± 5 °C. Tomato seedlings, 3-4 weeks old, were planted in autoclaved field soil mixed with sand (2:1, v:v) inoculated with high numbers of *M. incognita* juveniles and eggs. Plants were fertilized with 0.2% Polycrescol (14:10:14, N:P:K) once a week and watered as needed. Nematode eggs were extracted from 8 weeks old tomato roots using 1.5% NaOCl following the method described by Hussey and Barker (1973). Plants were removed from soil and uprooted. Roots were gently washed with tap water, cut in 1-2 cm pieces and macerated 2 times for 10 s each time in a Warring blender (Bender and Hohbein) with tap water. Every 500 ml of the macerated solution was mixed with 258 ml of 4% NaOCl (AppliChem) and manually shaken for 3 min. This suspension was poured over four nested sieves; 250 µm on the top, followed

by 100 µm, 45 µm and 25 µm aperture sieve. Eggs remaining in the 25 µm sieve were rinsed with tap water to separate nematodes from NaOCl and were collected on a beaker for experimental use.

2.6. Nematicides

DiTera® DF: Biological nematicide with 90% of non-viable *Myrothecium verrucaria* strain AARC-0255 its metabolites and production substrates (Valent BioSciences U.S.A.).

Fenamiphos: Ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate analytical grade (Sigma-Aldrich) was mixed with methanol to a desired concentration and filter sterilized (0.4 µm).

Fosthiazate 150 EC: (*RS*)-*S*-*sec*-butyl-*O*-ethyl 2-oxo-1,3-thiazolidin-3-ylphosphonothioate liquid formulation 15% active ingredient (Syngenta Crop Protection, Switzerland).

Nemacur 5 GR: Ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate. Commercial fenamiphos granular formulation 5% active ingredient (Bayer Crop Science, Germany) was mixed with methanol to a desired concentration and filter sterilized (0.4 µm).

Nemathorin 10 WG: granular formulation 10% fosthiazate ((*RS*)-*S*-*sec*-butyl-*O*-ethyl 2-oxo-1,3-thiazolidin-3-ylphosphonothioate) active ingredient (Syngenta Crop Protection, Switzerland) was mixed with methanol to a desired concentration and filter sterilized (0.4 µm).

Terbufos 10 GR: *S*-*tert*-butylthiomethyl *O*, *O*-diethylphosphorodithioate granular formulation 10% active ingredient (Industrias Bioquim Centroamericana S.A.).

2.7. Identification of soil bacteria

2.7.1. Gas chromatography technique (GC-FAME)

Well separated bacterial single colonies were identified by bacterial fatty acids extraction as described by Sasser (1990). Single bacterial colonies were transferred from TSA 100% to GC-TSB with a sterile plastic loop in 4 quadrants. The bacteria were scratched onto the GC-TSB in 4 quadrants. The first and second quadrants were performed with the same loop and a new sterile plastic loop was used to perform a third and fourth quadrant. Plates were incubated for 24 h in the dark at 28 °C. After incubation, to harvest bacterial cells, a 4 mm sterile loop was used to transfer the cells from the third quadrant to the bottom of culture polypropylene tubes (13 X 100 mm) which were previously cleaned with hexane for 10 min

in a tube rotator. To release the fatty acids the cells were lysed by adding 1.0 ml of Reagent 1 to each tube containing cells. The tubes were closely with Teflon lined caps, vortexed briefly and heated in a water bath for 5 min at 100 °C. After heating, the tubes were vigorously vortexed for 10 s and returned to the water bath at 100 °C for 25 min and then cooled slowly to room temperature.

To generate fatty acid methyl esters 2 ml of Reagent 2 were added to every sample. The tubes were capped, briefly vortexed and heated for 10 min at 80 °C in a water bath. Subsequently the tubes were cooled rapidly in cold tap water. To transfer the fatty acids from the aqueous phase to an organic stage, each cooled sample received 1.25 ml of Reagent 3. After capping the tubes were gently mixed on a tube rotator for 10 min. The tubes were uncapped and the aqueous (lower) phase was discarded. The organic phase was washed by adding 3 ml of Reagent 4 to every sample. The tubes were recapped and mixed for 5 minutes. About 2/3 of the organic phase was transferred into a GC glass vial and stored at -20 °C until the analysis by gas chromatography was performed.

The purified samples were identified by gas chromatographic analysis of bacterial fatty acids methyl esters using the Gaschromatograph Hewlett Packard Co. (HP) 5890E Serie II Plus, with electronic pressure programming. The chromatographer used a FED Split (Splitless-Einlassblock) detector and a 25 m + 0,2 mm + 0,33 mm Kap Soile Ultra 2 column. The identification program used was the Sherlock MIDI Identification system, Version 3.9, TSBA (Microbial Identification System, Newark, Delaware, USA). The program conditions for sample identification were; Injector A at 250 °C, Det Temp A at 300 °C, Aux E Press: 38,0 Psi and Inj A Press: 9,0 Psi. A calibration mix sample (commercial FAME calibration mix:Reagent 3, 1:1, v:v) was used prior running samples and after every ten samples. GC-FAME peaks were annotated by the microbial identification system software.

2.7.2. Molecular characterization and identification technique (16S rRNA)

To characterize and identify soil bacteria Restriction Fragment Length Polymorphism (RFLP) patterns of the 16S rRNA gene and partial 16S rRNA sequence analysis was performed. For the characterization and identification the following procedure was developed based on Chen and James (2002) adapted to the conditions of the Security 1 (S1) laboratory of the INRES-Phytomedizin University of Bonn, Germany.

2.7.2.1. Bacterial culture and preparation of compounds for the PCR Master Mix

Single colonies were grown for 48 h on TSA^+ under dark conditions at 28 °C. Two different primers were used to amplify the 16S rRNA genes by Polymerase Chain Reaction (PCR). The forward primer was 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer was the 9rev (5'-AAGGAGGTGATCCAGCC-3') both obtained from Sigma.

The primers were originally dissolved in milliQ water to a concentration of 100 μM and were diluted to a final concentration of 10 μM with milliQ water. The PCR buffer was prepared mixing 5 X Green GoTaq Flexi Buffer (Promega) with Magnesium Chloride ($MgCl_2$ at 25 miliMolar, Promega) in a 10:3, v:v ratio. Deoxynucleotide triphosphate (dNTP) at 2.5 mM was prepared by adding 10 μL 100 mM dCTP (Promega), 10 μL 100 mM dATP (Promega), 10 μL 100 mM dGTP (Promega) and 10 μL 100 mM dTTP (Promega) to 1160 μL autoclaved milliQ water on an autoclaved Eppendorf tube. Taq Polymerase (Promega) was the enzyme used for PCR and was kept on ice prior adding it to the PCR Master Mix.

2.7.2.2. PCR Master Mix preparation

The PCR Master Mix was prepared in an autoclaved Eppendorf tube kept on ice, by mixing 10 μL 5 X PCR buffer, 4 μL dNTP (2.5 mM), 1 μL 27F (10 μM), 1 μL 9rev (10 μM), 0.25 μL Taq polymerase and 33.75 μL autoclaved MilliQ water for every bacterial sample.

From the TSA^+ plates containing the growing bacteria a small colony was picked with a sterile pipette tip and transferred to the bottom of an autoclaved plastic microfuge PCR vial. To each PCR vial 50 μL of the PCR Master Mix was added. All microfuge vials were closed properly with autoclaved plastic caps. Centrifugation for 2 s was performed in a microcentrifuge (Labnet International) to bring all PCR Master Mix to the bottom of the plastic vials where bacterial cells were located.

2.7.2.3. PCR procedure

The microfuge vials were placed on a PCR thermal cycler (T Gradient, Biometric). Amplification was performed by an initial denaturation at 95 °C for 4 minutes, followed by 35 cycles of 95 °C for 1 minute, 50 °C for 1 minute and 72 °C for 1 minute, and with a final extension cycle of 72 °C for 5 minutes. After the PCR procedure, samples were kept at 4 °C.

2.7.2.4. Gel electrophoresis analysis

After PCR the amplification was verified by agarose gel electrophoreses. The gel was prepared with 1 X Tris-Acetate EDTA-Buffer (TAE, AppliChem). To every 100 ml 1 X TAE, 1 g Agarose (Sigma) was added in an Erlenmeyer flask and heated for 5 min in a microwave (MW800, Continent) at 650 Watts. After cooling at aprox. 50 °C every 100 ml of this mixture received 1 µL of 10 mg ml^{-1} Ethidium Bromide (AppliChem). This solution was poured into an electrophoresis tray and left in rest for 30 min until the gel had solidified. The gel was subsequently transferred to the gel electrophoresis chamber filled with 1 X TAE solution. From every PCR sample obtained, 5 µL were taken and transferred into the wells of the agarose gel. In the first slot of the agarose gel the 1 kb DNA Ladder (Promega) was loaded. After transferring all samples to the gel, the electrophoresis analysis was performed for 60 min at 120 V and 400 mA. To visualize and analyze the DNA bands, the gel was placed on ultraviolet transilluminator and a picture with a digital camera (S9500, Finepix, Fujifilm) was taken. The remaining 45 µL of PCR product of each sample was kept on refrigeration at 4 °C until further use.

2.7.2.5. Restriction enzyme analysis

In order to digest the bacterial DNA bands to detect different bacterial species it was necessary to conduct a restriction enzyme analysis. The enzyme *Cfo*1 (Promega) was used to prepare the Restriction Enzyme Master Mix (REMM) since it has been shown to be informative for 16S RFLP analysis (Karpouzas et al., 2000). In a sterile Eppendorf tube the REMM was prepared by adding 0.2 µL acetylated bovine serum albumin (BSA, Promega), 2 µL Buffer B (Promega) and 1 µL *Cfo*1 enzyme to 1.8 µL autoclaved distilled milliQ water for each bacterial sample.

From the 45 µL PCR product left per sample 15 µL were taken and transferred to a new autoclaved microfuge PCR plastic vial and mixed with 5 µL of REMM. The plastic vials were closed, centrifuged for 2 s and incubated for 3 h at 37 °C. After incubation, the samples were stored at 4 °C. To analyze the restriction enzyme analysis the procedure described in 2.7.2.4. for gel electrophoreses analysis was performed with two modifications. Firstly, the time for electrophoresis was extended to a total of 120 min, and secondly, the DNA marker

used was the 50 bp ladder (Promega). The remaining PCR product was kept in refrigeration and used later for purification.

2.7.2.6. DNA purification

The purification of the amplified DNA fragment was performed using the GFX PCR DNA and Gel Band Purification Kit (Illustra, General Electric, Health Biosciences) following the procedure described in the user manual. Briefly, in a sterile Eppendorf tube 150 µL Capture Buffer type 2 were added to every 30 µL PCR product sample. The mixture was directly pipette onto the filter units of the plastic vials of the purification kit and centrifuged at 14000 rpm for 30 s at 28 °C on a 5402-Eppendorf Centrifuge. The supernatant in the filter was recovered and used further. 25 ml Buffer type 1 were mixed with 100 ml ethanol (analytical grade) and 500 µL of this mixture was added to each sample on the filter and centrifuged as previously described. The supernatant in the filter was used further. The filter containing the DNA was transferred to a new Eppendorf tube. 50 µL Elutio-Buffer 4 was added to each sample and incubated for 60 s at room temperature. Subsequently samples were centrifuged for 60 s (14000 X G at 28 °C) and the DNA was eluted.

From every purified sample 2 µl were taken and mixed with 1 µl loading dye (Sigma) to quantify the amount of purified DNA by gel electrophoresis. Agarose gel electrophoreses analysis was performed as previously described in section 2.7.2.4. To calculate the amount of DNA present in the samples the molecular marker Lambda DNA (Promega) digested with *Eco* RI, *Hin*d III, and *Bam*H I was used. To prepare this marker every 100 µL of Lambda DNA were mixed with 20 µL Buffer E (Promega), 3.3 µL *Eco* RI (Promega), 3.3 µL *Hin*d III (Promega), 3.3 µL *Bam*H I (Promega) and 70 µL distilled milliQ water and incubated for 3 h at 37 °C. To the digested Lambda DNA loading dye was added. To prepare the loading dye 4 mg Bromophenol blue were mixed with 1 ml milliQ water in an Eppendorf tube. 650 µL of this mixture were transferred to 350 µL milliQ water in another Eppendorf tube and from this dilution 250 µL were transferred to another clean Eppendorf tube. The same procedure was performed with Xylene cyanol and the final 250 µL were mixed with the 250 µL obtained previously with Bromophenol blue. The 500 µL dye solution was mixed with 300 µL of glycerol and 200 µL milliQ water to obtain a final volume of 1000 µL loading dye. Every 4 µL of Lambda DNA (250 ng/µL) was mixed with 1 µL of the loading dye to obtain a final concentration of 200 ng of DNA per µL.

The band intensity obtained with the bacterial samples was related to individual bands of the marker.

2.7.2.7. DNA Sequencing and bacterial identification

Samples containing the bacterial DNA were sent to GATC Biotech AG (Konstanz, Germany) and sequenced. Sequences were edited with the BioEdit Sequence Alignment Editor (Hall, 1999) and compared to the sequences of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) to identify the bacterial isolate using blast analysis.

2.8. High pressure liquid chromatography (HPLC analysis)

To separate, identify and quantify nematicides High Pressure Liquid Chromatography analysis (HPLC analysis) was performed. The HPLC analysis was performed on a HEWLETT PACKARD (HP) system using a LiChrospher®100 C18 reversed phase column (250 by 4.0 mm, 5 µm), preceded by a LiChrospher® C18 reversed phase guard column (4.0 by 4.0 mm, 5 µm). The HPLC was controlled by ChemStation for LC 3D systems and consisted of a HP 1050 pump unit, HP 1050 diode array detector, HP 1046A fluorescence array detector, and 1050 (717S) autosampler. Before samples were injected, the column had been equilibrated with 90% (v/v) water and 0.1% (v/v) trifluoroacetic acid (TFA) (solvent A) and 10% acetonitril (solvent B). After injection the samples were eluted at a flow rate of 1.0 ml/min using an isocratic flow of solvent A for 2 min, a linear gradient to 90% solvent B for 28 minutes, followed by a isocratic flow for 5 minutes with 90% solvent B. Before the next sample was injected the column was re-equilibrated by a 1 minute linear gradient to 10 % solvent B, followed by a 4 minute isocratic flow of 4 minutes. The retention time for fenamiphos and fosthiazate was 23 and 19 minutes, respectively. The molecule and spectral analysis of fenamiphos and fosthiazate is shown in Figure 2. The linear relationship between the peak area and the nematicide amount is shown in Figure 3.

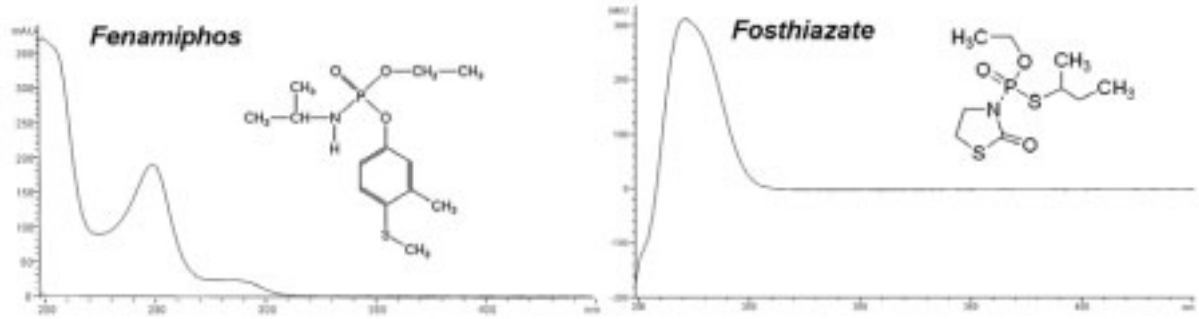

Figure 2. Molecule and spectral analysis of fenamiphos and fosthiazate as detected by HPLC analysis at 250 nm wave length in a retention time of 23 and 19 minutes, respectively.

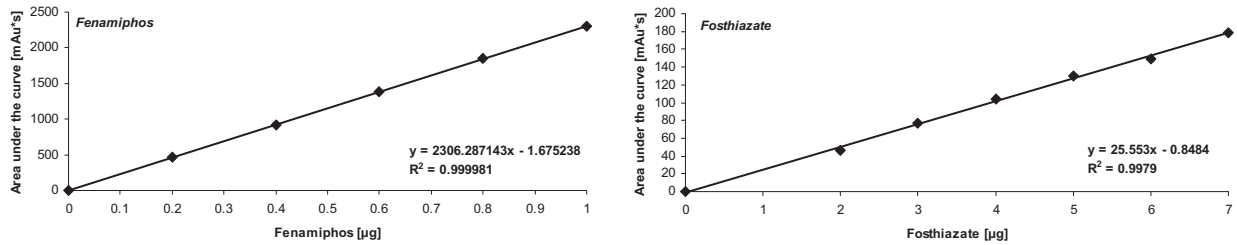

Figure 3. Standard curve of fenamiphos and fosthiazate detected by HPLC analysis at 250 nm wave length.

2.8.1. Quantification of nematicides in soil extract liquid medium

To quantify the nematicides in soil extract liquid media, a 400 µL sample of each treatment was mixed with 1600 µL methanol in an Eppendorf tube under laminar flow. This mixture was filter-sterilized (0.4 µm) and transfer to a HPLC glass vial. The glass vials were capped tightly. From each sample, 20 µL was injected onto the column for HPLC analysis. The nematicide area under the peak was determined. Using the standard curve the amount of nematicide in the sample was determined.

2.8.2. Extraction and quantification of nematicides in soil extract agar medium

Under laminar flow, 5 cm diameter Petri dishes containing soil extract agar amended with nematicides were opened. A triangular shaped sample was taken with a sterile scalpel from an area near the bacterial colony, transferred to an Eppendorf tube and weighted. For every 0.1 g of agar 100 µL of methanol was added and incubated over night. Subsequently, the tubes containing the agar-methanol mixture were centrifuged for 5 min at 14000 X G at 24 °C. After centrifugation, 200 µL of the solution were transferred to HPLC plastic vials and caped tightly. From each sample, 20 µL was injected onto the column and the remaining nematicide was detected by retention time and spectral pattern. The amount of nematicide was calculated using the standard curve obtained with the pure compound.

2.9. References

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3. FIELD EVIDENCE OF TERBUFOS ENHANCED BIODEGRADATION IN BANANA CULTIVATION

3.1. Introduction

The burrowing nematode, *Radopholus similis* (Cobb 1893) Thorne 1949, is the most common and damaging nematode in banana cultivation worldwide (Araya, 1995; Araya et al., 1995; Araya and Moens, 2005; Gowen et al., 2005). This nematode is responsible for restricted root growth and diminished bunch weight, leading to overall decreases in yield (Frison et al., 1998; Moens and Araya, 2002; Araya, 2003; Araya and Moens, 2005). Consequently, *R. similis* root infestations are correlated to a decline in banana yield (Sarah et al., 1996; Gowen et al., 2005; Cabrera et al., 2006). Because of the lack of effective alternative management tools in this perennial crop, the control of *R. similis* in commercial plantations is usually based on up to three applications of non-fumigant nematicides on a yearly basis (Araya, 2003). One of the most commonly used nematicides currently being applied is the organophosphate terbufos. However, after terbufos application, root decline associated with high nematode densities were observed, suggesting that the efficacy of the nematicide is declining, which is most likely caused by an elevated nematicide degradation process in the soil.

Accelerated degradation of the nematicides fenamiphos, carbofuran and ethoprophos has been shown to occur in soils with a history of repeated exposure to these specific types of nematicides (Ou, 1991; Ou et al., 1994; Smelt et al., 1996; Karpouzas et al., 1999; Karpouzas and Walker, 2000). In soils treated with aldicarb, oxamyl and fenamiphos a specific microflora established which was capable of rapidly degrading the active nematicidal components (Ou and Rao, 1986; Jones and Estes, 1995). For example, the fungi *Bjerkandera adusta*, *Pleurotus ostreatus* and *Phanerochaete chrysosporium* have been shown to degrade 50 to 96% of terbufos after four days of exposure (Jauregui et al., 2003). The performance of terbufos in differently treated soils was reported in maize cultivation in relation to the corm root worm (Little et al., 1992). However, little is known about the biodegradation of terbufos in soils cultivated with banana where other agroecological conditions and use of pesticides are encountered.

In the commercial cultivation of *Musa*, the use of tissue-culture bananas instead of transferring the follower production suckers is an attractive alternative when fields have to be replanted (Quénéhervé, 1993). The *in-vitro* propagated bananas are propagated under sterile laboratory conditions and, consequently, these healthy plants, generally promise higher yields, when compared to suckers, which acquired almost always nematodes and other pests and diseases during their development in the field. The growth of the delicate *in-vitro* propagated plants could be affected by the repeated application of non-fumigant nematicides. Previous reports in other crops have shown a phytotoxic side effect caused by terbufos treatments (Sinclair et al., 1992; Kennedy, 2002). Therefore, it is of importance to the banana production industry to elucidate whether or not terbufos applications negatively affect the quality and functioning of the banana root system.

Knowledge about the level of both nematicide efficacy and biodegradation of a pesticide or pesticide group is important for the grower in order to develop management strategies that can effectively reduce crop losses caused by plant-parasitic nematodes. The current lack of knowledge with respect to these ongoing processes are leading to an increase in numbers of nematicide applications, thus increasing the production costs and the chemical pressure on human health and the environment. Therefore, the aims of this study were to:

1. Determine the presence of terbufos biodegradation in soils infested with *R. similis* from banana plantations in Costa Rica.
2. Establish the effect of the nematicide application history on the current level of nematicide biodegradation.
3. Investigate whether terbufos has phytotoxicity side-effects on banana root development.
4. Elucidate the plant parasitic nematode diversity in banana roots.

3.2. Materials and Methods

3.2.1. Soil and root collection

Soil samples were collected from four commercial banana plantations (*Musa* AAA) of the province of Limon along the Atlantic coast of Costa Rica. The soil samples of these plantations will be named F1, F2, F3 and F4 hereafter. The samples were taken from

November 2005 to January 2006. The plantations were located west (F1, F3 and F4) or east (F2) Reventazon River (RR) as described in section 2.1. The soil pH of the six farms ranged between 5.51 and 5.68 and the organic matter content between 1.81 and 4.44 percent. The nematicide application history from 2000 to 2005 in every farm is shown in Table 1.

Table 1. Nematicide history from 2000 to 2005 of the four commercial banana plantations evaluated.

	Soil identification			
	F1	F2	F3	F4
		Nematicide name		
Year applied				
2000	No application	Oxamyl[x]	n.a.	Carbofuran[x] Terbufos[z] Ethoprophos[z]
2001	No application	Oxamyl[x]	n.a.	Carbofuran[x] Terbufos[z] Ethoprophos[z]
2002	No application	Oxamyl[x]	n.a.	Carbofuran[x] Terbufos[z] Ethoprophos[z]
2003	No application	Oxamyl[x]	Oxamyl[x] Terbufos[z]	Carbofuran[x] Terbufos[z] Fenamiphos[z]
2004	No application	Oxamyl[x]	Carbofuran[x] Ethoprophos[z] Terbufos[z]	Carbofuran[x] Terbufos[z] Cadusafos[z]
2005	No application	Oxamyl[x]	Oxamyl[x] Fenamiphos[z]	Oxamyl[x] Terbufos[z] Cadusafos[z]

[x]carbamate nematicide. [Z]organophosphate nematicide. n.a. = nematicide application was performed but name of compound was not available. The nematicide history of every field was provided by CORBANA, Costa Rica.

For each plantation 12 composite soil samples were taken by random sampling for use in the greenhouse test. Each composite sample was composed of 5 subsamples, taken from the area around different individual plants. For every subsample an excavation of 15 cm deep, 10 cm wide and 30 cm long and located 10 cm in front of an individual follower sucker was performed with a spade. This area falls within to the 40 cm band in front of the follower sucker where nematicides are regularly applied. Each composite sample therefore contained about 22,500 cm^3 of soil. The composite soil samples were transported in plastic bags to the nematology laboratory and greenhouse of the Tropical Agricultural Research and Higher Education Centre in Turrialba, Costa Rica.

In every field 12 composite root samples were taken to extract the plant-parasitic nematodes. Roots were taken from follower production suckers performing a similar excavation as the one used for soil sampling. The follower production suckers for extracting root samples were different from those used for soil sampling. The follower suckers to be sampled were selected according to their mother plant stage by using the method described by Speijer and De Waele (1997).

3.2.2. Determination of terbufos biodegradability and side-effect

To determine the presence of enhanced biodegradability of terbufos and its effect on root growth a modification of the green house bioassay described by Pattison et al. (2000) was used. All 12 composite soil samples from the same plantation received one of the following three treatments; i) non-autoclaved and no nematicide treated control, ii) non-autoclaved and nematicide treated, or iii) autoclaved and nematicide treated. Soil autoclaving was performed for 60 minutes twice at 121 °C over two consecutive days. One-hundred µg of Terbufos 10 GR per gram of soil was applied once to the respective treatments at the beginning of the experiment (day 0). The nematicide granules were applied to a plastic bag containing the soil and mixed thoroughly. From each treatment six sub-samples were taken at 0, 15, 30, 45 and 60 days after nematicide application. Each sub-sample was composed of 60 g soil and was transferred to a 10 cm diameter plastic pot. One *in-vitro* propagated banana (*Musa* AAA) cv. Grande Naine, 15 to 20 cm long from the first leaf to the crown, was planted in each pot at the time the soil-subsamples were taken. After planting, treatments were arranged in a randomized block design at the greenhouse. After nematicide application, all soil treatments were stored in the dark at room temperature.

Nematodes were inoculated at the same moment when the six soil-subsamples per treatment were taken. Per pot, a suspension containing 500 mixed stages of *R. similis* was inoculated into three holes 3 cm deep previously made with a 1 ml pipette tip around the banana stem. The plants were carefully watered daily as required with tap water. Seven days after nematode inoculation, the banana plants were harvested. The roots were gently rinsed free of soil with tap water and root fresh weigh was measured. Nematode extraction from the roots was performed as described by Araya (2002) and quantified visually, using a microscope.

3.2.3. Determination of nematode diversity in banana roots

Every composite sample was gently washed and dead roots were discarded. Plant parasitic nematodes were extracted from 25 g of functional roots, which were macerated for 10 s at high speed followed by 5 s at low speed in a commercial blender containing 300 ml tap water. The macerated solution was filtered using nested sieves with apertures of 250 μm, 106 μm and 0.25 μm, respectively. Nematodes recovered on the 0.25 μm sieve were transferred to a glass beaker and the volume adjusted to 200 ml using tap water. Of each sample, 4 ml was taken and all nematodes encountered in the first 2 ml were counted and identified. For identification the key described by Hunt et al. (2005) was used.

3.2.4 Statistical analysis

To conform to assumptions of normally distributed data, nematode numbers were transformed using a log(x+1) transformation before statistical analysis was performed. One-way analysis of variance (ANOVA) was performed for each set of data separated. One set of data corresponded to one evaluation date of the five dates possible. Nematode numbers and root fresh weight were analyzed in the same way. Fisher's Least Significant Difference (LSD) was performed where significant differences ($P < 0.05$) were detected. The statistical software SPSS 12.0 was used to perform the analysis.

3.3. Results

3.3.1 Biodegradability of terbufos

The number of recovered nematodes from roots in the non-treated control soil differed significantly ($p < 0.05$) from roots which were treated with nematicides from the plantation F1 (Figure 1A). In this soil a high level of nematode control was obtained from 15 days after nematicide treatment up to 60 days. In the bioassays where soil from F2 and F3 were used, nematode numbers in banana roots were significantly lower in both nematicide treatments when compared to the non-treated control during all the evaluations period (Figures 1B and 1C, respectively). The number of nematodes recovered from the banana roots in the non-treated control soil from F4 were not significantly different from the non-autoclaved terbufos treated soil 0, 15, 30 and 60 days after nematicide treatment (Figure 1D). Forty five days after nematicide application there was no significant difference between the non-autoclaved

terbufos treatment and autoclaved terbufos treatment. Nematode numbers in roots of the autoclaved nematicide treated soil remained low during the entire evaluation period.

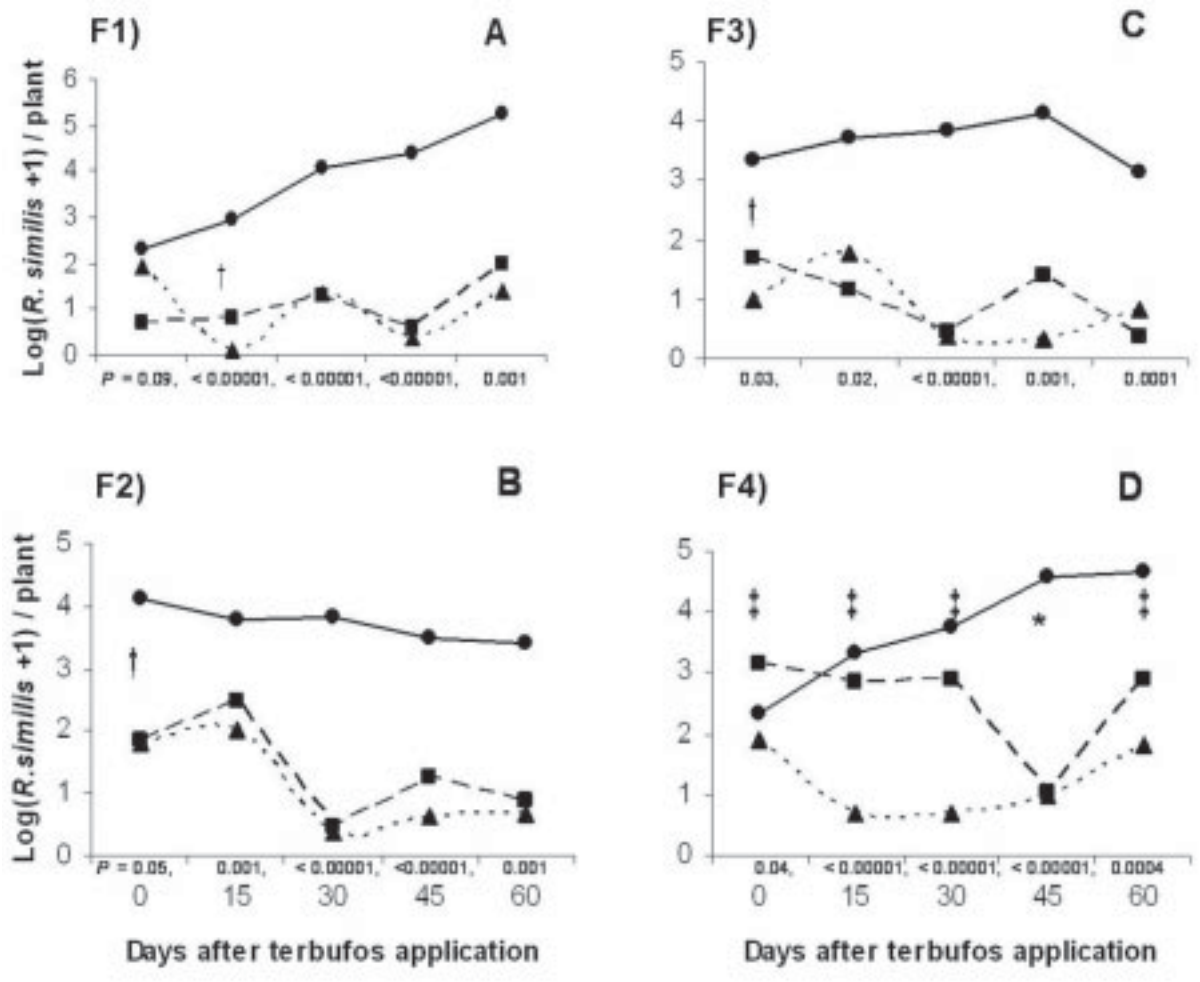

Figure 1A-D. Mean log (number of *R. similis* +1) recovered from banana roots, 7 days after inoculating 500 nematodes and 0, 15, 30, 45 or 60 days after terbufos application in differently treated soils. Non-treated control soil (—•—), non-autoclaved and terbufos-treated soil (--■--), and autoclaved and terbufos-treated soil (--▲--). A) F1 = soil from a field with no nematicide application history B) F2 = soil from a field where oxamyl has been applied once a year. C) F3 = soil from a field with alternate application of carbamate and organophosphate nematicides two or three times per year. D) F4 = soil from a field where nematicides have been applied three times per year, first a carbamate followed by two organophosphate treatments and in which terbufos was always the second nematicide applied. † = Beginning date of significant differences between non-treated soil control and the other two soil treatments up to the end of evaluation period. ‡ = Non-significant differences between non-treated soil control and non-autoclaved nematicide treated soil, but significant difference between autoclaved nematicide treated soil and the other two treatments. * indicates a statistical significant difference between the non-treated control soil and the other two nematicide treatments. N= 6.

3.3.2. Terbufos side-effect

The terbufos application did not significantly affect root fresh weight of the *in-vitro* propagated bananas, cv. Garnde Naine, with three exceptions (Table 2). The banana root weight in the tests using F1 and F4 were significantly higher in autoclaved and nematicide treated soil than in the non-treated control, but not significantly different from the nematicide treated soil 45 days after terbufos application. Similar results occurred in tests using F2, evaluated thirty days after terbufos application. In all these cases a positive effect was found, resulting in an increase in root weight when compared to roots extracted from the non-treated soils. Banana roots in the tests with F3 were not significantly different at any time of evaluation.

Table 2. Comparison of root fresh weight (g) of *in-vitro* propagated *Musa* (AAA) cv. Grande Naine after 7 days planted in different terbufos treated and untreated soils with different nematicide history.

	Soil identification																			
	F1					F2					F3					F4				
	Days after terbufos application																			
	0	15	30	45	60	0	15	30	45	60	0	15	30	45	60	0	15	30	45	60
	Root fresh weight [g]																			
Soil treatment																				
Not-treated soil	1.60	2.08	3.43	1.85b	2.52	3.50	4.05	4.20b	4.63	2.67	4.10	4.20	4.40	4.47	1.63	1.52	1.58	3.25	1.70b	2.52
Nematicide treated soil	1.85	1.88	2.95	2.08ab	2.88	2.82	4.05	5.05ab	4.80	2.02	4.02	3.48	3.80	4.70	1.68	1.43	1.68	2.95	2.48a	2.30
Autoclaved and nematicide treated soil	1.67	1.85	2.95	2.38a	2.77	3.10	4.58	5.93a	4.68	2.43	3.93	3.77	5.22	4.60	2.07	1.95	1.75	4.25	2.32a	2.68
Fisher's LSD	n.s.	n.s.	n.s.	0.43	n.s.	n.s.	n.s.	0.90	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.44	n.s.
P	0.67	0.40	0.65	0.05	0.32	0.15	0.50	0.01	0.87	0.24	0.97	0.45	0.32	0.98	0.33	0.97	0.80	0.25	0.004	0.84

Numbers in same column with different letter are significantly different. n.s. = not significantly different. A) F1 = soil from a field with no nematicide application history. F2 = soil from a field where oxamyl has been applied once a year. F3 = soil from a field with alternate application of carbamate and organophosphate nematicides two or three times per year. F4 = soil from a field where nematicides have been applied three times per year, first a carbamate followed by two organophosphate treatments and in which terbufos was always the second nematicide applied. N = 6.

3.3.3. Nematode diversity in banana roots

Radopholus similis was the most abundant plant-parasitic nematode in banana roots, representing over 70% of the total population in all four fields evaluated (Figure 2). *Helicotylenchus* spp. was present in all fields been the 18, 30, 2 and 5% in the farms F1, F2, F3 and F4, respectively. *Pratylenchus* spp. was present only in F2 conforming 1% of the

total nematode population. *Meloidogyne incognita* was found only in F4 with a total population of 8%.

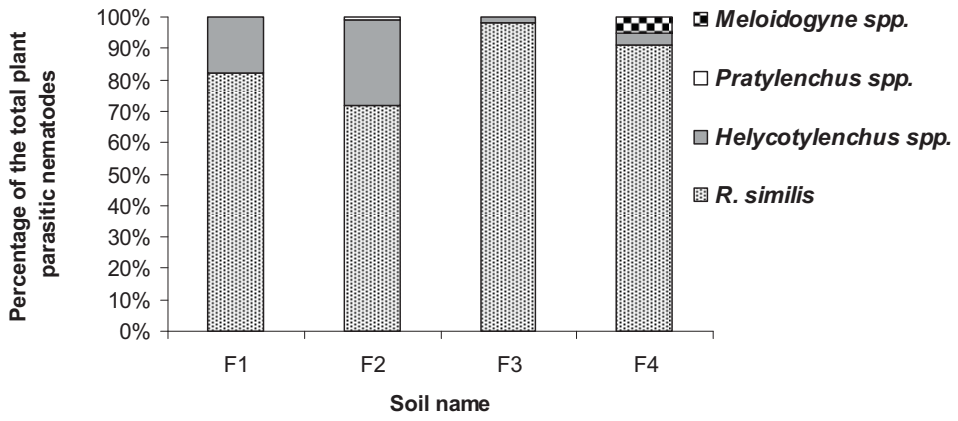

Figure 2. Plant-parasitic nematode diversity in roots of follower production suckers *Musa* AAA taken in four different commercial plantations of Costa Rica. F1 = soil from a field with no nematicide application history. F2 = soil from a field where oxamyl has been applied once a year. F3 = soil from a field with alternate application of carbamate and organophosphate nematicides two or three times per year. F4 = soil from a field where nematicides have been applied three times per year, first a carbamate followed by two organophosphate treatments and in which terbufos was always the second nematicide applied. N = 12.

3.4. Discussion

The bioassay indicated that terbufos was rapidly degraded in F4, the soil sample from the plantations where this nematicide was applied on a yearly basis. Since terbufos applied to the non-autoclaved treatment lost effectiveness at all evaluation times, except when the soil had been autoclaved in advance, microbial activity must have been responsible for this degradation process. Similar results in a comparable bioassay were obtained from soil frequently treated with Furadan®, Mocap®, Nemacur® and Vydate® (Moens et al., 2004). Our results are in agreement with the statements by Smelt and Leistra (1992), who concluded that nematicides are vulnerable to accelerated degradation by adapted microorganisms, whenever regular and frequent applications of the same compound occur and Stirling et al. (1992), who postulated that repeated exposure to a particular pesticide can lead to enhanced microbial degradation. Furthermore, indigenous bacteria and a microbial mat were shown to be responsible for mineralization of carbofuran in the soils of banana plantations in St. Vincent, in the West Indies (Murray et al., 1997). In banana plantations of the Ivory Coast where fenamiphos was applied every 4 months, a step-by-step build up of microbial

populations was detected which was responsible for the highly accelerated degradation of the nematicide (Anderson and Lafuerza, 1992). In laboratory tests and in commercial banana plantations, treatment of soils every 4 months with 3 grams of active ingredient per plant caused the gradual build-up of bacteria and fungi which could degrade fenamiphos (Anderson, 1989). The results in the present investigation proved that terbufos is no exception to the biodegradation rule.

In the present study microbial degradation of terbufos was absent in the nematicide treated and non-autoclaved soils from F1, F2 and F3. The effectiveness of the nematicide in these soils was stable for the entire 60 day evaluation period in the autoclaved and the non-autoclaved treatments. Previous to the present research, the F1 soil had never been treated with a nematicide. The F2 soil was treated once a year with oxamyl, a carbamate nematicide. Soil F3 had only been treated previously twice with terbufos, but one year had passed since the last application. These results suggested that the low level of nematicide used in these fields prevented or delayed the selection of microorganisms responsible for the biodegradation of terbufos. Similar results were obtained by Pattison et al. (2000) who found high efficacy of fenamiphos against *R. similis* for up to 8 weeks after nematicide treatment on a sandy loam soil with no previous nematicide use history as compared to increased biodegradation after 2 weeks on a silty loam soil with 15 previous fenamiphos treatments. Additionally, in one field cultivated with banana where alternative nematicide applications were used Moens et al. (2004) did not find enhanced biodegradation of Counter® after 5 consecutively applications.

The reduction in the number of nematodes in F4 in the non-autoclaved terbufos treatment 45 days after nematicide application was inconsistent with the preceding and successive sampling dates. This suggests that an experimental error at treatment establishment or during nematode inoculation had occurred. However, the data obtained in the four other sampling dates were consistent, thus supporting the conclusion that biodegradation is a very important factor in reducing terbufos efficacy.

Terbufos had no negative side effects on banana root growth. Under green house conditions terbufos reduced seedling emergence to pearl millet (Kennedy, 2002), diminished carrot seedlings emergence, decreased onion and carrot seedling size and delayed emergence of both crops (Sinclair et al., 1992). In contrast to the above results, terbufos treatment to

autoclaved soil in the present study increased root weight of banana plants on three occasions. This effect was found 45 days after terbufos application in soils F1 and F4, and 30 days after nematicide treatment in F2. On these dates *R. similis* was highly controlled in the autoclaved soils with a terbufos application history. This suggests that high invasion of the nematodes negatively affected root weight in case the nematicide was not applied in the bioassay. The root weight of *in-vitro* propagated bananas planted in virgin soils with 5 consecutive applications of Counter® was higher than from non-treated soil (Araya and Lakhi, 2004). Moens and Araya (2002) also found that *R. similis* reduced root weight by 66% when compared to non-nematode inoculated controls, supporting the results obtained in the present work. However, the lack of root phytotoxicity in banana could be related to the limited exposure time of 7 days, the amount of active ingredient, and the size of the plant at treatment time. Terbufos probably did not negatively affect root development in our assays because of the resilient root system of the banana plant. Therefore phytotoxic effects in the field are not to be expected.

All banana plants grew for 7 days in the treated soils. However there were differences in growth between evaluation periods, such as 60 days after terbufos treatment in F2 and F3, where a decrease in the overall root weight could be observed. The original bioassay described by Pattison et al. (2000) uses corn seeds, which grow more uniformly than the young *in-vitro* propagated bananas used in this study. The variation in growth may thus be due to a lack of uniformity of the *in-vitro* plants used for each set of experiments. These plants were received from different banana production batches and differed in size. However for each period of evaluation all banana plants came from the same batch, thus making a comparison among them is possible.

Radopholus similis was found in all banana fields, accounting over 70% of the total plant parasitic nematode population in roots. Comparable high *R. similis* densities were found by Araya et al. (1995) in the same Atlantic region of Costa Rica. Both studies confirm the presence of economical important populations of the burrowing nematode in this area. This work reveals that even with several chemical treatments on a year basis, *R. similis* continues to be the main nematode problem in banana cultivation, as it has been for decades. Furthermore, Menjivar (2005) showed that *R. similis* numbers increased in the banana roots, even when non-fumigant nematicides were applied in the field. Consequently, research that is focussed on controlling this nematode will remain of prime importance.

Terbufos should only be used in an integrated nematode management program to retain high efficacy, which will be lost with frequent application as demonstrated in this study. Application of nematicides having different active ingredients can reduce and in some cases prevent the onset of increased biodegradation (Anderson and Lafuerza, 1992). Such chemical rotations are, however, becoming difficult to maintain, due to the use-restriction of many nematicides that have been on the market for a long time and the lack of availability regarding new compounds that can be used for nematode control in banana production. Increasing the time interval between chemical applications, e.g. by utilizing alternative biocontrol agents (Pocasangre et al., 2000; Mendoza et al. 2004/2008; zum Felde et al., 2006) could reduce the emergence of enhanced microbial nematicide degradation through sustaining a non-biased and diverse microbial population over time and thus support the maintenance of nematicide efficacy.

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4. Involvement of microorganisms other than Pseudomonads on fenamiphos enhanced degradation

4.1. Introduction

Fenamiphos is a broad spectrum, non-volatile, systemic, organophosphorus nematicide extensively used throughout the world to control plant-parasitic nematodes in fruit and vegetable crops (Stirling et al., 1992). The activity tends to be nematostatic rather than nematotoxic, inhibiting movement of nematodes and delaying egg hatching (Van Gundy and Mckenry, 1977).

A result of continuous or repeated exposure to a particular pesticide, microbial populations in the soil are capable of developing degradative traits, which leads to an accelerated degradation of the active components as shown in the previous chapter of this thesis. Such events have also been demonstrated in tomato cultivation, in which the soil was frequently treated with fenamiphos (Stirling et al., 1992).

Several microorganisms such as fungi, yeasts and bacteria are capable to produce surface-active molecules, known as biosurfactants, which can facilitate the uptake of an insoluble substrate (Fiechter, 1992; De Souza, 2002). Biosurfactant producing bacteria have, for example, been shown to rapidly degrade xenobiotics (Johnsen et al., 1996). Among the bacteria genera, *Pseudomonas* sp. was reported to produce biosurfactants (Fiechter, 1992; Ron and Rosenber, 2001; De Souza, 2002). The genus *Pseudomonas* is a common inhabitant of the rhizosphere and has received considerable attention in bioremediation of xenobiotics (De Souza, 2002). Arino et al. (1996) and Deziel et al. (1996) have shown the implications of surfactant-producing *Pseudomonas* in facilitating degradation of pollutants such as polycyclic aromatic hydrocarbons and *n*-alkanes.

To date, however, little is known about the influence of *Pseudomonas* species on the enhanced biodegradation of non-fumigant nematicides and its importance on supplying a key emulsifier to the soil for starting the rapid microbial degradation process. For these reasons, the objectives of the research in this chapter were to:

1. Determine the *Pseudomonas* population and growth in soil amended with different concentrations of organic matter.
2. Investigate the enhanced biodegradation of fenamiphos in soil with high densities of *Pseudomonas* spp. after several consecutive fenamiphos treatments.
3. Establish the production of biosurfactants by *Pseudomonas* spp.
4. Study the presence of *Pseudomonas* spp. in the degrading microorganisms of fenamiphos.

4.2. Materials and Methods

4.2.1. Effect of compost on total soil bacteria and *Pseudomonas* spp.

Soil 4, which had no nematicide treatment history, was used to determine the *Pseudomonas* population density and evaluate the effect of the addition of different compost concentrations. One day prior to the addition of compost, all culturable soil bacteria and *Pseudomonas* spp. were extracted and cultivated on TSA^{+} and KBM^{+}, respectively, as described in section 2.4. After adding 1, 4 or 7% (w/w) green compost, the soil was thoroughly mixed by hand to obtain a homogenized mixture. Tap water was added to increase the moisture content and the soil was mixed again. Soil bacteria were isolated 1 and 3 weeks after compost amendment. S4 with no addition of compost served as control. Each treatment had 4 replicates.

4.2.2. Efficacy of fenamiphos after three consecutive treatments

Soil 4-composte mixtures were split into three subsamples and each subsample was (i) not treated any further (control), (ii) treated with fenamiphos or (iii) autoclaved and subsequently treated with fenamiphos. Autoclaving was performed twice for 60 min at 121 °C in two consecutive days. In case of nematicide treatment, 10 µg a.i. of Nemacur 5GR per gram of soil was applied three times with 6 weeks interval. To all samples, water was added to field capacity after each nematicide treatment. After the first nematicide treatment, four 250 ml soil samples were transferred to plastic pots from each soil every two weeks until 6 weeks after nematicide application. Three holes were made per pot in the soil using a plastic 1 ml pipette tip and a suspension containing 2500 *M. incongita* eggs was inoculated. Then, 50 seeds of lettuce cv. Milan were sown per pot and covered with a thin layer of autoclaved sand to enhance germination. Lettuce plants were regularly irrigated with tap water as needed and

30 days after sowing the seeds the lettuce plants were harvested. The roots were gently washed with tap water and percentage of galled roots was determined. Efficacy of Nemacur 5GR was calculated according to the formula described by Abbott (1925).

4.2.3. Analysis of biosurfactant production

4.2.3.1. Drop collapse test

A modification of the drop collapse test described by Jain et al. (1991) was performed to evaluate biosurfactant production capacity of bacteria. Therefore, 100 bacterial single colonies were isolated from S4 amended with 7% compost and grown on KBM^+ plates. A full loop of each colony was transferred into autoclaved distilled water in a 2 ml Eppendorf tube. The tube with the bacterial suspension was vortexed vigorously for 15 s and 20 µL of the bacterial suspension were carefully pipetted on Parafilm paper (Parafilm M, Laboratory Film, Pechiney, Plastic Packaging, Chicago, IL). Collapse of the drop was evaluated up to one minute after application on the Parafilm and compared to the drops of autoclaved distilled water, autoclaved distilled water mixed with the biosurfanctant producer, *Pseudomonas* SS101 (positive control), and autoclaved distilled water mixed with *Pseudomonas* 10.24 (negative control) which does not produce the biosurfactant.

4.2.3.2. Blue media test

Since biosurfactant production may be media dependent, a second analysis was performed to verify biosurfactant production. Therefore, every single bacterial colony used in the drop collapse test was further grown on KBM^+ for 48 h at 28 °C in the dark. Subsequently a full loop containing the bacterial colony was transferred onto 'blue media' (Siegmund and Wagner, 1991). Petri dishes were incubated at 28 °C in the dark and the halo formation was evaluated after 5 days.

4.2.4. Identification of *Pseudomonas* spp.

A total of 16 well separated randomly selected bacterial single colonies, from the 100 isolates tested for biosurfactant production, were identified to verify whether the bacteria growing on KBM^+ were *Pseudomonas* species. Every isolate was transferred from KBM^+ onto CG-TSB with a sterile 4 mm loop under laminar flow. After 48 h of incubation in the dark at 28 °C, the bacteria were identified by GC-FAME analysis as described in section 2.7.1.

4.2.5. Fenamiphos metabolization and presence of Pseudomonads in the degrading microorganisms

To investigate the role of *Pseudomonas* spp. on the biodegradation of fenamiphos, two different tests were performed. In the first test, two of the GC-FAME identified soil bacteria were analyzed for their ability to metabolize fenamiphos. One full loop containing *Pseudomonas syringae* or *P. putida* was inoculated on a 10 cm diameter plate containing SEAM supplemented with 100 or 200 µg of fenamiphos ml^{-1}. As control a fenamiphos-SEAM plate was incubated without bacteria. The plates were sealed with Parafilm and all treatments were incubated for 10 days in the dark at 28 °C. Then, one triangular shaped agar block was transferred to a reaction tube using a sterile scalpel. Fenamiphos extraction and quantification was performed by HPLC analysis as described in section 2.8. Each treatment had 4 replicates. Analysis of Variance (ANOVA, $P < 0.05$) was performed to compare the amounts of remaining fenamiphos in the plates. For statistical analysis the program GenStat Discovery Edition 3 was used.

In the second test, a mixture of all the different microorganisms present in S1, S2, S3 or S4 were used. To obtain these microorganisms, 10 g of each soil was transferred to 100 ml of autoclaved distilled water and incubated in the dark for 16 h at 28 °C on an orbital shaker set at 120 rpm. Then, 1 ml of the suspension was transferred to 19 ml soil extract liquid medium supplemented with fenamiphos (analytical grade) to a final concentration of 100 µg ml^{-1} in a 115 X 28 mm sterile plastic tube (Sarstedt). SELM treated with fenamiphos without bacterial inoculation served as control. All plastic tubes were tightly capped, placed vertically in an orbital shaker set at 60 rpm and incubated in the dark at 28 °C. After 0, 1, 6, 10 and 14 days of incubation, a 400 µl sample of each treatment was transferred and mixed with 1600 µL of methanol. This mixture was filter-sterilized (0.4 µm) and analyzed by HPLC as described section 2.8. Each treatment was done in triplicate. The Standard Error of the Mean was calculated for every treatment.

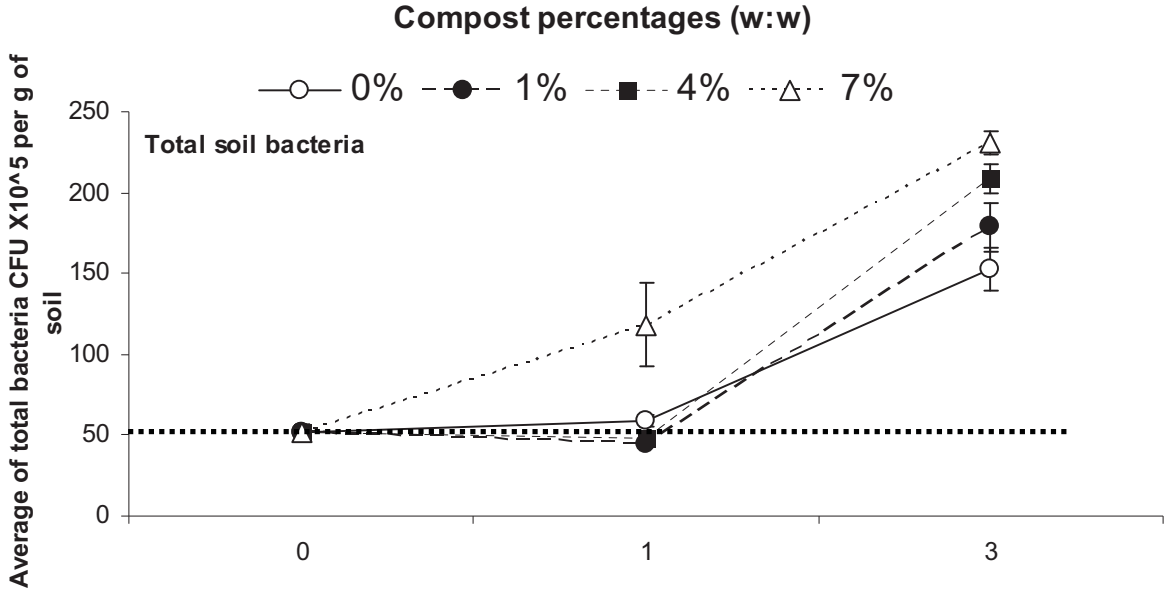
In addition, to investigate the total soil bacterial populations and verify the presence of Pseudomonads in the fenamiphos-degrading microorganisms, 50 µl suspension of each of the four German soils was plated on TSA^{+} or KBM^{+} 14 days after nematicide treatment. The plates were incubated in the dark for 48 h at 28 °C and subsequently the number of CFU was determined. Every treatment was repeated six times.

4.3. Results

4.3.1. Effect of compost on total soil bacteria and *Pseudomonas* spp.

The number of total bacteria and *Pseudomonas* spp. before the addition of the compost treatments was 51 × 10^5 CFU and 7 × 10^5 CFU per gram of soil, respectively. The addition of compost to S4 resulted in an increase of total soil bacteria compared to the untreated control (Figure 1). In particular for S4 supplemented with 7% compost the bacterial population density had increased. In this mixture 118 × 10^5 total CFU per gram of soil were found after the first week, whereas the other two soil compost mixtures and the S4 soil control showed significant increase when compared to the initial total bacterial population density. After 3 weeks S4 with 7% compost contained 231 × 10^5 CFU per gram of soil. The application of 1 or 4% compost were not significantly different than the untreated soil control. Between one and three weeks the population density in all soils had significantly increased when compared to the initial densities, with S4 supplemented with 4% compost ranking second and S4 supplemented with 1% compost ranking third.

The number of Pseudomonads isolated on King B medium, did not increase after 1 week of compost treatments. However, after three weeks the S4 supplemented with 7% compost contained significantly more Pseudomonads than the other soils and had increased 33 × 10^5 CFU per gram of soil.



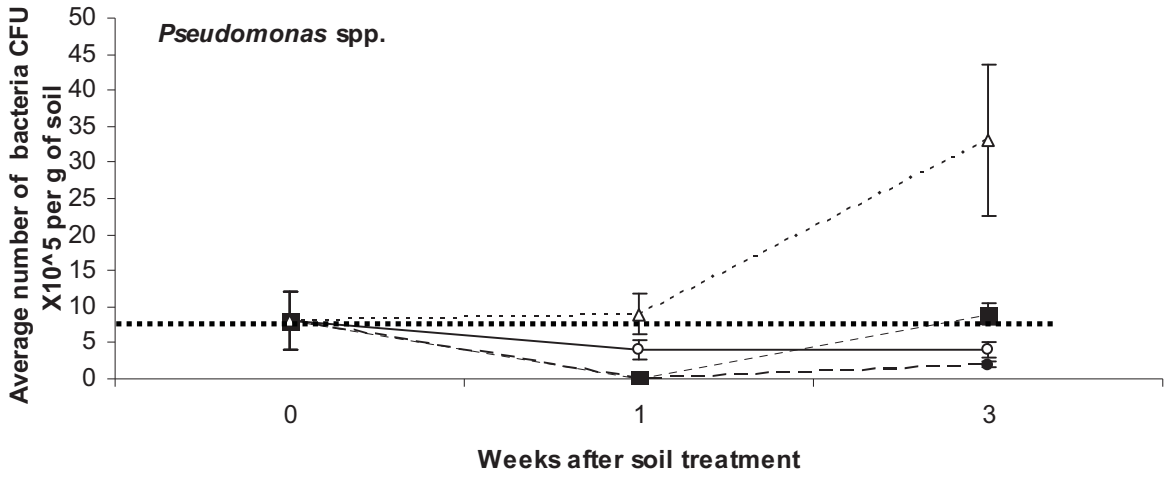


Figure 1. Effect of compost application at different rates to S4 on growth of total soil bacteria or *Pseudomonas* spp. 0, 1 and 3 weeks after soil treatment. Transversal line indicates the original amount of bacteria before compost application (■■■). N=4. Bars indicate the Standard Error of the Mean.

4.3.2. Efficacy of fenamiphos on nematode control

Overall, the efficacy of fenamiphos in nematode control remained above 60% against *M. incognita* in all treatments and was independent from both autoclaving the soil prior to nematicide treatment and the consecutive nematicide applications (Figure 2). However, the nematicide treatments were in some cases less effective in the non-autoclaved soils when compared to the autoclaved ones, in particular S4 amended with 7% compost 0 and 6 weeks after the second nematicide application. Despite this variation, the efficacy remained relatively high and therefore no enhanced biodegradation of fenamiphos could be observed, as is illustrated in figure 2.

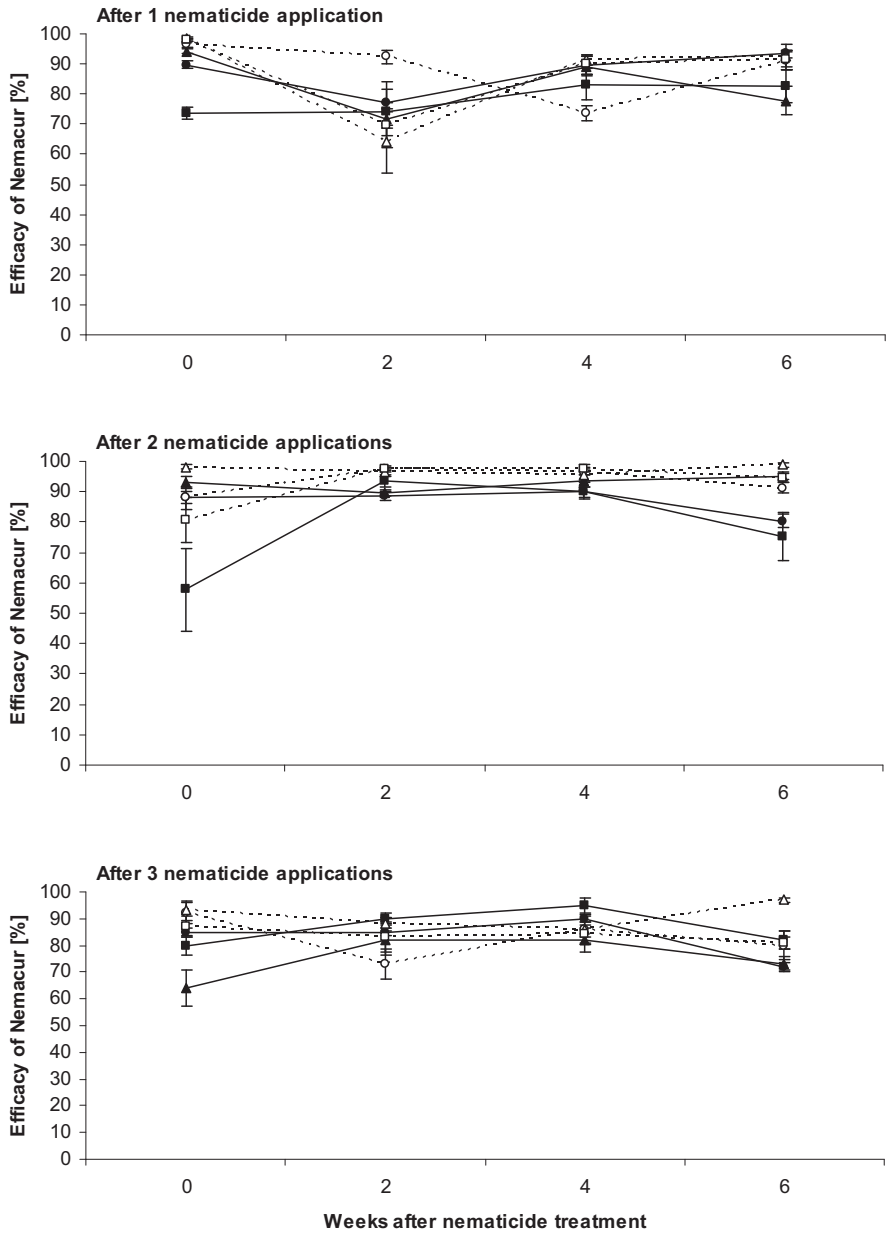


Figure 2. Efficacy of Nemacur GR on root galling of lettuce cv. Milan 0, 2, 4 and 6 weeks after one, two or three consecutive nematicide treatments. Autoclaved (----) or non-autoclaved (—) soil amended with 1% (●), 4% (▲) or 7% (■) compost. N = 4. Bars indicate the Standard Error of the Mean.

4.3.3. Biosurfactant production tests

One hundred single colonies isolated from S4 and grown on KBM^{+} were tested for biosurfactant production. Besides the positive biosurfactant control *Pseudomonas* SS101, none of the one hundred single colonies was able to collapse the water drop on the Parafilm paper (Figure 3).

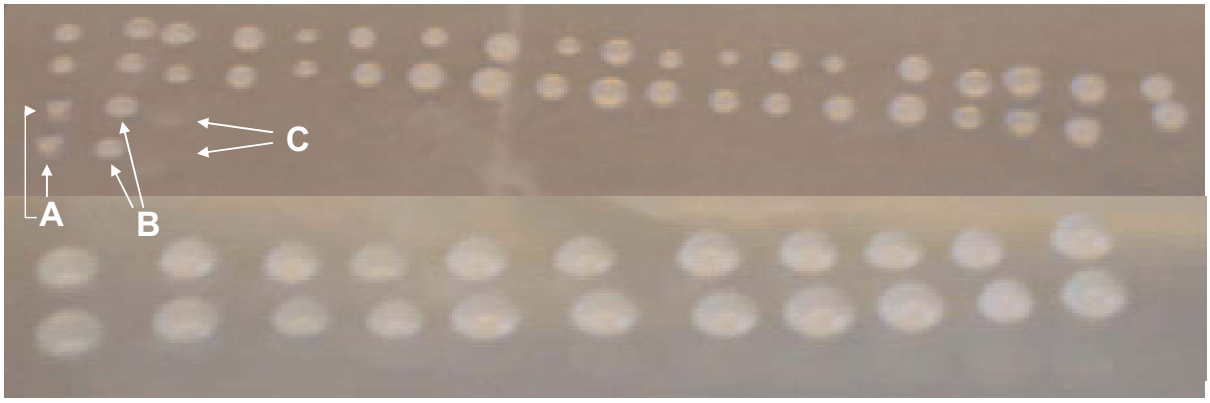


Figure 3. Drop collapse test on Parafilm paper after 1 minute of placing the drops. Drop caused by autoclaved distilled water alone (A). Drop caused by the mixture of autoclaved distilled water and the negative biosurfactant producing control *Pseudomonas* 10.24 (B). Drop collapse caused by the mixture of autoclaved distilled water and the biosurfactant producing control *Pseudomonas* SS101 (C). The rest of the drops were caused by the mixture of individual bacterial single colonies isolated on KBM^{+} from S4, a soil with no fenamiphos application history, and autoclaved distilled water.

The same one hundred bacterial isolates used in the drop collapse test were further tested on 'blue media' for biosurfactant production. During the 5 days of incubation none of the isolates was able to produce a halo, confirming that all bacterial single colonies were not capable of producing biosurfactants (Figure 4).

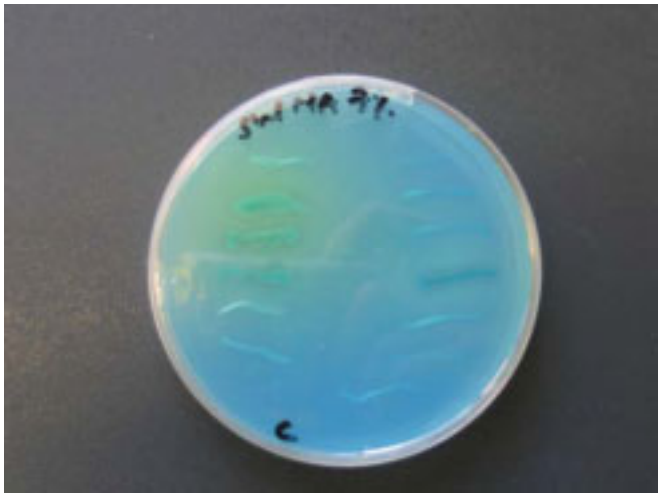


Figure 4. Plate with 'blue medium' containing 13 bacterial single colonies isolated on KBM^{+} from S4, a soil with no fenamiphos application history, with no halo formation after 5 days of incubation.

4.3.4. Identification of single bacterial colonies

From the 100 isolates that were tested for biosurfactant production grown on King B medium, 16% were identified by their fatty acid composition. GC-FAME analysis

demonstrated that all isolates belonged to the genus *Pseudomonas*. Fifty percent of the isolates were identified as *Pseudomonas putida*, 31% corresponded to *P. fluorescens*, 13% were identified as *P. syringae* and 6% were *P. chlororaphis* (Figure 5).

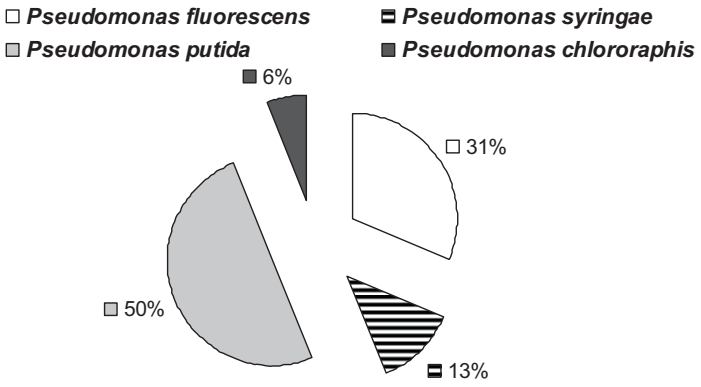


Figure 5. Diversity of different non-biosurfactant *Pseudomonas* isolated on KBM$^+$ from S4, a soil with no fenamiphos application history, amended with 7% compost and identified by GC-FAME.

4.3.5. Pseudomonads and the conversion of fenamiphos

In the first *in-vitro* nematicide assay, individual bacteria were tested for their fenamiphos metabolizing abilities on culture plates supplemented with 100 or 200 μg μl^{-1}. No significant differences ($P < 0.05$) were observed with respect to the remaining amounts of fenamiphos between SEAM inoculated with bacteria and the not inoculated control for both concentrations 10 days after incubation (Figure 6). The final fenamiphos concentration in agar medium with initially applied at 100 μg ml^{-1} fenamiphos was of 99 and 98 μg ml^{-1} when inoculated with *Pseudomonas syringae* and *P. putida*, respectively, whereas the non-inoculated control had reached 99 μg ml^{-1}. When the initial fenamiphos concentration was of 200 μg ml^{-1} the final concentrations were 198, 193 and 200 μg ml^{-1}, respectively. Thus, both Pseudomonads were not capable of converting fenamiphos over a period of 10 days.

In the second assay, all microorganisms were tested together for the ability to convert fenamiphos in liquid cultures. HPLC analysis showed that in the presence of microorganisms, extracted from the soils S2 and S3, fenamiphos was completely degraded after incubating for 14 or 6 days, respectively (Figure 7). In the same assay the microorganisms from the soils S1 and S4 were not able to convert fenamiphos, even after incubating for 14 days. In S1 and S2 no Pseudomonads were found (Figure 8). In contrast, in S3 and S4 17 × 10^4 and 32 × 10^4

CFU of *Pseudomonas* were found, respectively. Thus, the results obtained from soil S2 suggest that bacterial genera, other than *Pseudomonas* are capable of degrading fenamiphos.

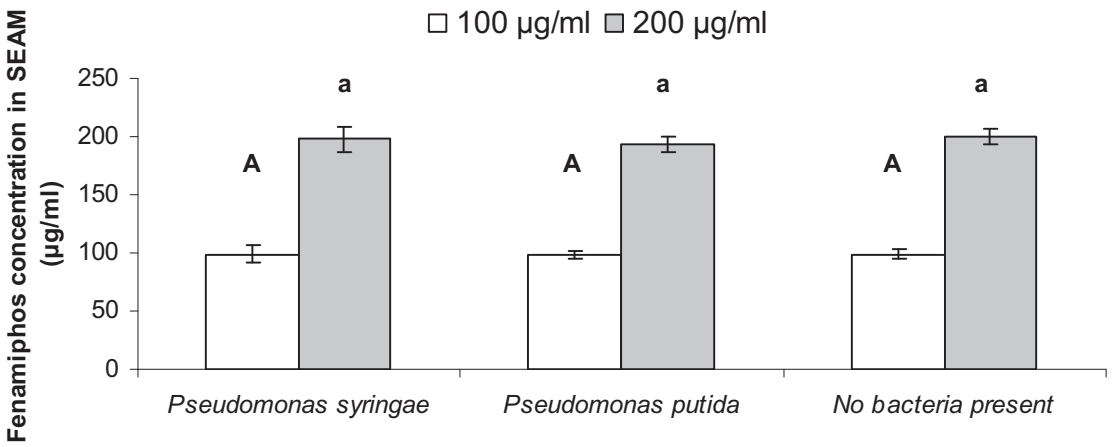


Figure 6. Fenamiphos concentrations in soil extract agar medium (SEAM) after 10 days of incubation which was initially applied at 100 or 200 µg ml^{-1} and inoculated with *Pseudomonas syringae*, *P. putida* or not inoculated. These bacteria were isolated on KBM^{+} from S4, a soil with no fenamiphos application history, and were not able to produce surfactants. N = 4. Bars indicate the Standard Error of the Mean.

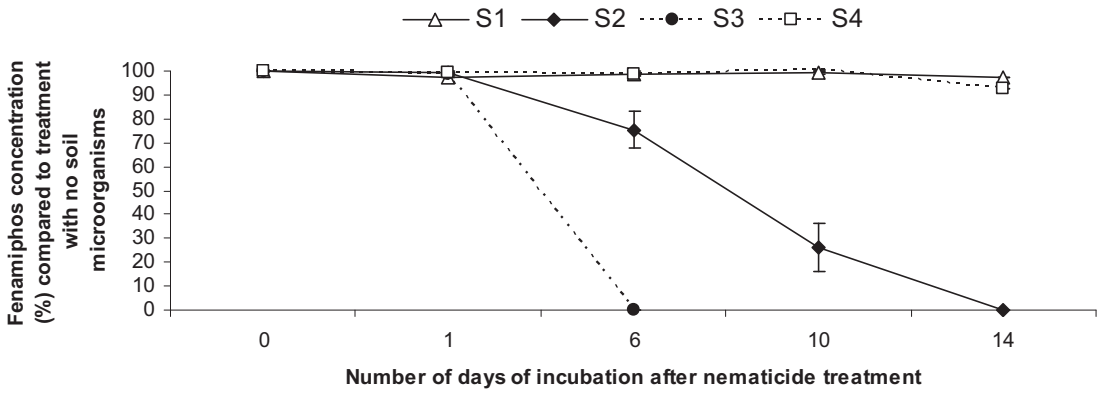


Figure 7. Metabolization of fenamiphos by microorganisms obtained from the German soils S1, S2, S3 and S4, in samples taken 0, 1, 6, 10 or 14 days of incubation after nematicide treatment. Different than soils S1, S2, and S3, soil S4 had no fenamiphos application history. N = 3. Bars indicate the Standard Error of the Mean.

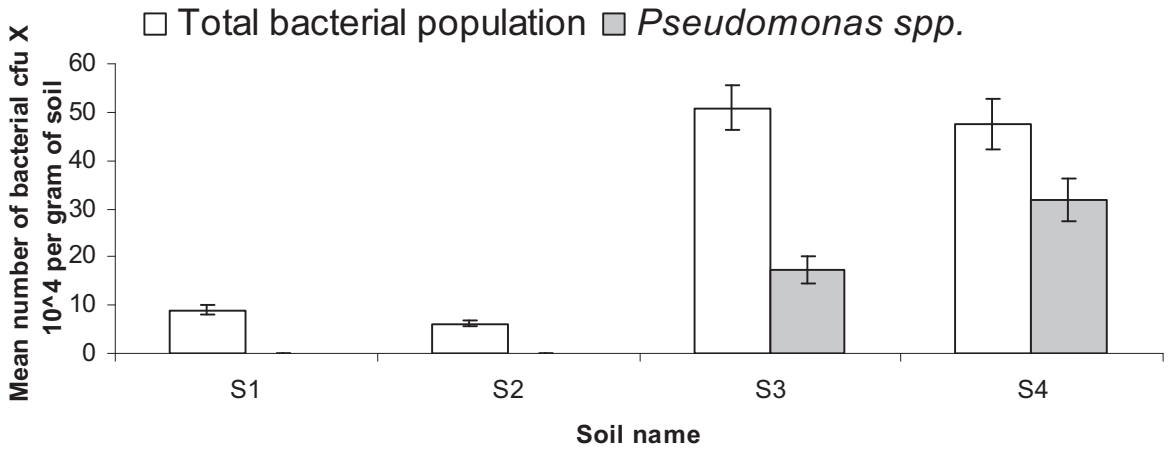


Figure 8. Mean number of bacterial colony forming units per gram of soil isolated from the four German soils, S1, S2, S3 and S4, all with different nematicide histories. Different than soils S1, S2, and S3, soil S4 had no fenamiphos application history. N = 6. Bars indicate standard error of the mean.

4.4. Discussion

Bacterial population densities increased when the German S4 soil was amended with compost. However, this effect was not significant until the amount of compost had reached 7%. This confirms the observation that even thought the response of soil microorganisms to compost applications may vary, bacterial populations generally increase in density since composts are an important source of nutrients (Pérez-Piqueres et al., 2006). In a greenhouse bioassay, it was demonstrated that compost amendments did not show evidence of an onset or increase in biodegradation of fenamiphos, even after repetitive nematicide application in the presence of 7% compost and the presence of a high percentage of Pseudomonads. The biodegradability of another non-fumigant organophosphate nematicide, ethoprophos, was reported to be slow in a soil with 8.5% organic matter, suggesting that the availability of the pesticide was reduced by the high content of organic matter and therefore did not result in an enhanced biodegraded (Karpouzas and Walker, 2000). Greer and Shelton (1992) obtained similar results and attributed this phenomenon to the decreased amounts of soluble pesticide available for metabolism by the degrading bacteria due to the relatively high organic matter content.

Soil S4 had no history of nematicide application, which could explain the high fenamiphos efficacy. Similar results were obtained by Pattison et al. (2000) who found high efficacy of fenamiphos towards *R. similis* for up to 8 weeks after nematicide treatment on a sandy loam

soil with no previous nematicide application history. Pesticide pre-treatment history is a main factor need to detect the expression of the biodegradation processes of non-fumigant nematicides (Karpouzas et al., 1999). Apparently a prolonged application over a substantial period is essential for the soil microorganisms to adapt and start degrading a nematicide.

Smelt and Leistra (1992) stated that the most commonly used nematicides are all vulnerable to conversion by adapted microorganisms, which arise by frequent applications. However, the rate at which the microorganisms adapt to the nematicide cannot be predicted. This unpredictability is illustrated by the present findings on the fenamiphos biodegradation by microorganisms from soils with different fenamiphos application history. In the soil with the longest fenamiphos application history, S1, no fenamiphos biodegradation could be observed, whereas, the other two soils with a less fenamiphos application history, S2 and S3, did contain biodegradation activity.

It is difficult to explain why microorganisms of S1 and S4 were not able to degrade fenamiphos. It may be important to consider that S1 had the lowest pH of all soils (4.0), which is regarded unfavourable for bacterial growth (Smelt et al., 1996). This could be the reason why, even with a significant fenamiphos application history, the microorganisms living in this soil were not able to transform into biodegrading isolates. It has been previously demonstrated that soils with high pH are more likely to adapt and build-up large numbers of bacteria that quickly degrade nematicides when compared to soils with a low pH (Smelt et al., 1996). Suett et al. (1996) found that carbofuran degrading microorganisms were present in all soils previously treated with the nematicide, but that the degrading microbes were predominant in soils with the highest pH. More specific, Singh et al. (2003) found that biodegradation of fenamiphos developed mainly in soils with high pH and a slow degradation process developed in soil with a pH value of less than 5. Based on pH, soil S4 was optimal to build-up to high populations of nematicide biodegrading isolates. It maybe that further nematicide treatments are required to build up such microbial populations. However, next to the pH, other biotic and abiotic factors may also play an important role, like the competition or collaboration with other microorganisms, the availability of minerals and the composition of root exudates. Soil S4 contained the highest percentage of Pseudomonads, of which *Pseudomonas putida* was the most dominant species, representing half of all isolates characterized. The other three species identified were *P. fluorescens*, *P. syringe* and *P. chlororaphis*. All these four *Pseudomonas* species have been previously reported to degrade organophosphate and/or carbamate non-fumigant nematicides. For example, *Pseudomonas*

putida, P. fluorescens and *P. syringae* were capable of degrading carbofuran (Karpouzas et al., 2000b). *P. putida* was responsible for ethoprophos degradation (Karpouzas et al., 2000a). In addition, *P. chlororaphis* has shown to degrade 1,2,3,4-tetrachlorobenzene (Portawfke et al., 1998). As demonstrated by the biotest, none of the *Pseudomonas* species in S4 were capable of degrading and this capability did not developed with fenamiphos application. Also, none of the individually tested *Pseudomonas* isolates did produced surfactants, which are surface-active molecules that may facilitate the uptake of an insoluble substrate (Fiechter, 1992) and/or may facilitate degradation of certain chemicals by emulsifying them (Guerra-Santos et al., 1984; Zhang and Miller, 1992/1994). Biosurfactant producing Pseudomonads, are known to be present in the rhizosphere at relatively low densities, ranging between 5 to 14% of the total Pseudomonads population (De Souza et al., 2003). Furthermore not all soils contain biosurfactant Pseudomonads at detectable levels (Raaijmakers et al., 1997). The lack of surfactant producing Pseudomonads present in S4 may make this soil intrinsically incapable of developing a fenamiphos degrading microbial population.

According to HPLC analysis in the first metabolization test, the two bacterial isolates *P. syringae* and *P. putida* were not able to degrade fenamiphos. The absence of degrading capability could thus be related to the lack of biosurfactant production. Furthermore, the results obtained in this investigation can also be related to the lack of degradation ability of *Pseudomonas* when it is grown in the absence of a suitable helper bacterium. In a previous study with a bacterial consortium where *Pseudomonas* spp. was present, a rapid degradation of fenamiphos occurred (Singh et al., 2003). However, the Pseudomonads themselves were not able to degrade fenamiphos without the presence of two other bacteria of the genus *Flavobacterium* and *Caulobacter*. Perhaps *Pseudomonas* strains that can not produce surfactants can still profit from the surfactant production of other bacteria. *Flavobacterium*, for example, is known to produce surfactants supporting this theory (Bodour et al., 2003/2004).

Overall, the exact role of neither the Pseudomonads nor the surfactants in the facilitation of fenamiphos degradation could be demonstrated in the present studies. Further characterization of the *Pseudomonas* population, the nematicide biodegradation ability and the presence of surfactant producing isolates in S3, a soil with demonstrated fenamiphos degrading abilities and containing Pseudomonads, may reveal more information on this matter.

Nevertheless, the results obtained with S2, a soil with no detectable numbers of Pseudomonads, do demonstrate that microorganisms other than Pseudomonads can be involved in the efficient degradation of fenamiphos. Until now, few microorganisms have been identified that can degrade fenamiphos, e.g. *Brevibacterium* sp. MM1 (Megharaj et al., 2003). Perhaps soils S2 and S3 contain significant numbers of this type of bacteria resulting in efficient nematicide degradation. Furthermore, since in this study all microorganisms of the soil were tested, other microorganisms than bacteria may be involved in the degradation of fenamiphos. Fungi, for example, have also been reported to be involved in the fenamiphos degradation process (Anderson, 1989).

In conclusion, this study demonstrated that a prolonged nematicide application does not necessarily lead to an increase in biodegradation of the active compound and that microorganisms other that Pseudomonads can be important factors in the in the degradation of the nematicides. These microorganisms are the focus of the next chapter.

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5. Isolation and characterization of fenamiphos-degrading microorganisms

5.1. Introduction

Microbial breakdown has been reported for several soil-applied herbicides, fungicides, insecticides (Somasundram et al., 1989) and, in particular, for a large number of different non-fumigant nematicides (Somasundram et al., 1989; Pattison et al., 2000; Mclean and Lawrence, 2003; Moens et al., 2004). Fenamiphos, an organophosphate non-fumigant nematicide, can be degraded by soil microorganisms into non-nematoxic compounds (Johnson, 1998). Several microorganisms, either in pure or mixed culture, have been isolated from soils exhibiting the biodegradation of organophosphorus nematicides, like *Flavobacterium* spp., *Penicillium waksmani* Zaleski, *Trichoderma viride* Persoon, *Pseudomonas alcaligenes* and *Pseudomonas putida* (Sethunthan and Yoshida, 1973; Rao and Sethunathan, 1974; Sheela and Pai, 1983; Matsumura and Boush, 1986; Karpouzas et al., 2000a).

In most cases, degrading microorganisms have been characterized using standard microbiological taxonomic techniques, which are based on morphological and biochemical characteristics (Ramanand et al., 1988). More recently, molecular based methods have been added to the process of characterization (Feng et al., 1997). Information about the DNA sequence of certain genomic regions is particularly valuable since there are extensive sequence databases available, which can be used for taxonomic purposes. In this way, Karpouzas et al. (2000b) molecularly characterized 23 carbofuran-degrading bacteria from soils collected in UK and Greece.

Repetitive fenamiphos treatments accumulated bacteria and fungi capable of degrading the active compound (Anderson, 1989; Anderson and Lafuerza, 1992). However, the microorganisms involved in the degradation process were not identified. The degradation of fenamiphos seems to be a complex process, since it was not possible to isolate one microorganism which was solely responsible of the degradation activity. Fenamiphos degradation could only be achieved by a mixed bacterial culture (Ou, 1991). Singh et al. (2003) also found a bacterial consortium capable to rapidly degrade fenamiphos and, again, the individual isolates were not capable of degrading the nematicide. *Brevibacterium* sp.

MM1 was the first reported individual bacterium capable of rapidly hydrolyzing fenamiphos, but the bacterium was not molecularly characterized (Megharaj et al., 2003). To date, there is little information about fenamiphos degrading microorganisms especially of those that can perform the metabolization process individually. Knowledge about the different species that can be responsible for the degradation of fenamiphos and nematicides in general, will facilitate prediction of how successful nematicides can behave in certain soils and how a rapid break down of the nematicidal activity can be avoided. In the previous chapter it was demonstrated that soils were found containing microbes, other than Pseudomonads, which could degrade fenamiphos. Therefore, the objectives of this study were to:

1. Determine which type of microorganism is primarily involved in the fenamiphos metabolization, bacteria or fungi.
2. Analyze the fenamiphos biodegrading capacity of individual purified isolates.
3. Elucidate if adherent compounds in the formulation, Nemacur GR, enhance or interfere with fenamiphos metabolization.
4. Investigate if individual bacterial colonies utilize fenamiphos as sole source of carbon to grow.
5. Characterize and identify fenamiphos degrading bacteria.

5.2. Materials and Methods

5.2.1. Determination of microorganisms responsible for enhanced fenamiphos degradation

To determine whether or not bacteria and fungi were involved in fenamiphos metabolization an *in-vitro* degradation assay was set up. Since microorganisms living in soil S3 were able to rapidly metabolize fenamiphos, as demonstrated in the previous chapter, this soil was selected for this assay. 10 g of S3 were mixed with 100 ml of autoclaved distilled water. This mixture was incubated in dark conditions for 16 h at 28 °C in an orbital shaker at 120 rpm. Subsequently, 1 ml of the water soil mixture containing all soil microorganisms of S3 was transferred to 19 ml SELM in sterile plastic tubes, supplemented with a stock solution of fenamiphos analytical grade to a final concentration of 100 µg ml^{-1}. This mixture was further treated with Cycloheximide (150 µg ml^{-1}), Streptomycin sulfate (150 µg ml^{-1}) and

Chloramphenicol (150 µg ml^{-1}) or untreated. SELM containing fenamiphos and no microorganisms from S3 was used as control. All treatments were maintained vertically in dark conditions at 28 °C in an orbital shaker at 60 rpm. At day 0, 3, 6, 9, 12, 15 and 20 after nematicide treatment, a 400 µl sample was taken from each treatment, mixed with 1600 µl of methanol, filter-sterilized and analyzed by HPLC as described in section 2.8. Each treatment was done in triplicate.

To determine whether fungi and bacteria had been affected by the fungicide and antibiotic applications, respectively, 50 µl of each treatment was transferred onto TSA^+ or PDA^+ in 10 cm diameter Petri dishes 20 days after nematicide application. The plates were incubated for 24 h in dark conditions at 28 °C and subsequently the number of CFU were determined. Each treatment had three replicates.

5.2.2. Metabolization of fenamiphos by bacterial single colonies

From the previous treatments, 50 µl SELM supplemented with fenamiphos, with and without Cycloheximide and inoculated with the microorganisms obtained from soil S3, were plated onto 10 cm TSA^+. The plates were sealed with Parafilm and incubated for 48 h in the dark at 28 °C. A total of 20 well separated single colonies obtained from each of the two treatments were picked with a sterile 4 mm loop and transferred to fresh TSA^+ and further incubated at 28 °C for 48 h in the dark. One full 4 mm loop of each purified colony was taken under laminar flow and transferred onto SEAM supplemented with fenamiphos at 100 µg ml^{-1} in 5 cm diameter Petri dishes. Samples from the agar medium were taken 0, 7 and 14 days after bacterial inoculation as shown in Figure 1. To determine the remaining fenamiphos concentration the agar samples were extracted by adding methanol to the agar samples in a 1:1 ratio (v:w) and analyzed by HPLC as described in section 2.8. After every sampling date, the plates were sealed again and further incubated at 28 °C in the dark until the next sampling moment. Each sampling was done in triplicate.

The single colonies that were able to metabolize fenamiphos were further cultured on TSA^+, grown in the dark at 28 °C and used in a second metabolization assay, similar as the one described above, but now using the formulation Nemacur 5GR instead of fenamiphos analytical grade. Plates contained Nemacur 5 GR with an equivalent of 100 µg ml^{-1} of active ingredient. The incubation, sampling and fenamiphos extraction was identical to the plate assay with the purified fenamiphos. Each sampling was done in triplicate.

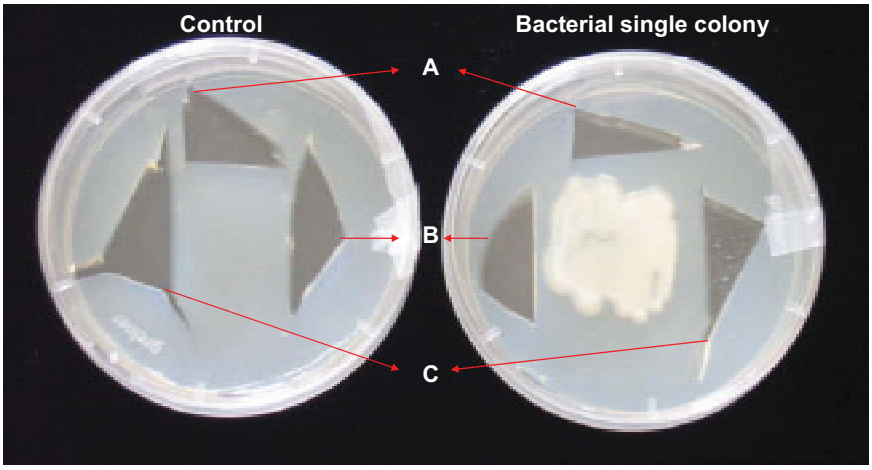


Figure 1. Bioassay for determining nematicide degradation in agar medium. Petri dishes containing soil extract agar medium amended with 100 µg ml^{-1} of fenamiphos without bacteria (Control) or with a bacteria single colony growing in the center. Arrows show the 3 samples taken for nematicide quantification at 0 (A), 7 (B) and 14 (C) days after nematicide application. Nematicides were extracted from the agar by adding methanol to the agar sub-samples in a 1:1 ratio (v:w) and analyzed by HPLC.

5.2.3. Use of fenamiphos as sole carbon source

To investigate whether soil bacteria of S3 can use fenamiphos as sole carbon source, one bacterium of those previously isolated was grown in the minimal SELM in the presence and absence of fenamiphos. A bacterium isolated from the non-degrading S4 was used as a negative degrading control. A single colony of each bacterium was slected and grown in 10 cm diameter Petri dishes for 72 h in dark conditions at 28 °C. From these plates, 0.1 g of each bacterial colony was transferred with a sterile 4 mm loop to 10 ml of sterile SELM in a plastic tube and mixed. From this suspension, 1 ml of the mixture was transferred to 19 ml of SELM with and without 100 µg ml^{-1} fenamiphos analytical grade. SELM with and without fenamiphos served as controls. The medium was incubated in the dark at 28 °C, in an orbital shaker set at 60 rpm. From each treatment two 1.6 ml samples were taken at 0, 4, and 72 h after inoculation. For the first sample the optical density (OD) of the medium was determined using a Ultrospect III Spectrophotometer (Pharmacia Biosystems) set at 600 nm, in which the OD_{600} of the non inoculated medium without fenamiphos was set at zero. Each treatment had 3 replicates.

Parallel to the *in-vitro* assay described above, a second SELM sample was taken from every treatment to quantify the amount of fenamiphos present in the medium according to the procedure as described in the second test in section 4.2.5. HPLC analysis was performed to every sample as described in section 2.8. Each sampling was done in triplicate.

5.2.4. Molecular characterization and identification of fenamiphos degrading bacteria

To molecularly characterize and identify the fenamiphos-degrading bacterial single colonies, each isolate was grown individually on TSA^+ in 10 cm Petri dishes for 48 h at 28 °C in the dark. Restriction Fragment Length Polymorphism (RFLP) patterns of the 16S rRNA gene and partial 16S rRNA coding sequence analysis was performed as described in section 2.7.2. The RFLP analysis was performed as described in section 2.7.2.5. using *Cfo*1 as restriction endonuclease. Chromatographic sequencing data was visually/manually inspected for sequence quality using BioEdit (Hall, 1999). The DNA sequences were analyzed using a Blast search on the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/).

5.2.5. Phylogenetic analysis and sequence comparison

The phylogenetic analysis was performed as described by Kurtz (2009). The analysed dataset consisted of a total of 25 bacterial strains. The 17 sequences of fenamiphos-degrading bacteria were aligned with NCBI sequences of *Microbacterium, Sinorhizobium, Brevundimona, Ralstonia* and *Cupriavidus* species resembling the taxonomic groups identified by the score of highest identity at NCBI Blast. The sequence of *Pseudomonas fluorescence* was used as reference. *Staphylococcus aureus* was used as outgroup. Chromatographic sequencing data was visually/manually inspected for sequence quality using BioEdit. Assembling of sequences and transformation into fasta format was performed with Vector NTI Advance 10.0 (Invitrogen). Aligning, editing and converting of fasta files into nexus format was performed with ClustalX 1.81 (Thomson et al., 1997).

To identify the best fitting model for nucleotide substitution of 16S rRNA datasets MrModeltest was used. According to AIC (-lnL = 4693.9512; AIC = 9405.9023) implemented in MrModeltest general time reversible model of nucleotide substitution with gamma rates (GTR+G) and base frequencies freq A = 0.2353; freq C = 0.2597; freq G = 0.2791; freq T = 0.2260 was selected for the Baysian inference and analysis settings for Paup and MrBayes. Baysian inference started from a random tree defining *Staphylococcus* as for outgroup. The burn period started after 25% of the 5.000.000 cycles performed, discarding all trees generated prior burnin. Paup started from a random tree using *Staphylococcus* as outgroup. Convergence amongst chains was followed by log-likelihood values using Tracer

v.1.3. Bayesian semi-strict 50% majority-rule consensus tree was generated in Paup*. A Parsimony Jackknife analysis was performed with default settings, except for gaps, which were handled as a new fifth state. Jackknife was performed with 37% deletion of data, 10.000 replicates searching under fast stepwise addition.

5.3. Results

5.3.1. Determination of microorganism responsible for enhanced fenamiphos degradation

To determine what type of microorganisms were involved in fenamiphos degradation an *in-vitro* bioassay was set up in which the degradation of the nematicide was followed over time. In parallel assays the development of either fungi or bacteria was repressed by Cycloheximide or antibiotics, respectively. In the absence of Cycloheximide and antibiotics the degradation of fenamiphos started 3 days post inoculation (d.p.i.) and was completed 12 d.p.i. (Figure 2). In the presence of the fungicide Cycloheximide, the degradation of fenamiphos started 12 d.p.i. and was almost completed 20 d.p.i. In the presence of the antibiotics Streptomycin sulfate and Chloramphenicol, fenamiphos was not degraded at all during the 20 days incubation period. These results indicated that the metabolization of fenamiphos was primarily mediated by soil bacteria and that fungi are less involved.

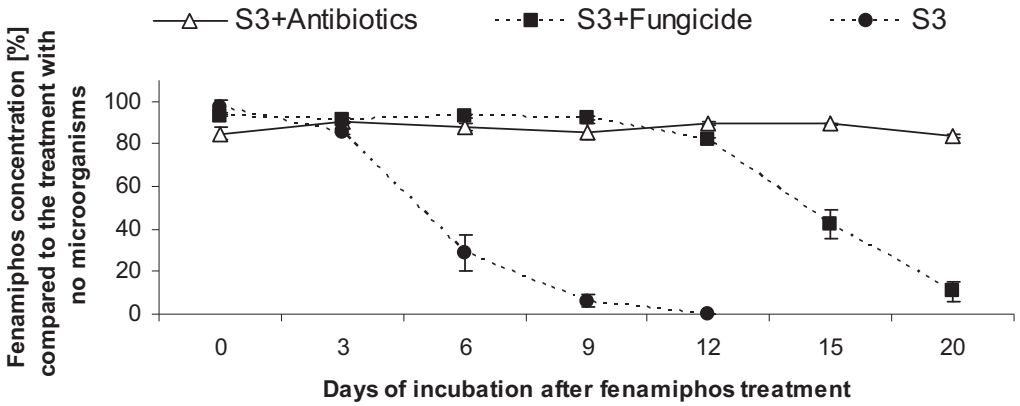


Figure 2. The remaining amount of fenamiphos during incubation in the presence of microorganisms isolated from the German soil S3. Samples from medium plates, initially containing 100 µg ml^{-1} fenamiphos analytical grade from plates supplemented with antibiotics (Streptomycin sulfate at 150 µg ml^{-1} and Chloramphenicol at 150 µg ml^{-1}), fungicide (Cycloheximide at 150 µg ml^{-1}) or untreated (S3), were taken 0, 3, 6, 9, 12, 15 and 20 days after inoculating the microorganisms from S3. N = 3. Bars indicate the Standard Error of the Mean.

Samples from the fungicide, antibiotic and control treatments were taken 20 days after inoculating the microorganisms of S3 and plated on TSA^+ and PDA. Although the treatments were supplemented with either antibiotics or a fungicide, bacteria and fungi were isolated from both treatments (Figure 3). However, the amount of bacteria in the treatment with antibiotics was significantly lower than in the fungicide treated and untreated control, being 120×10^4, 963×10^4 and 1071×10^4 CFU per gram of soil, respectively. Thus, the number of bacteria in the fungicide treatment was not significantly different from the untreated control. Similarly to what happened with soil bacteria, the number of fungi in the treatment with fungicide was significantly lower than in the treatment with antibiotics and the untreated control with 42×10^4, 823×10^4 and 830×10^4 CFU per gram of soil, respectively. Here, the number of fungi in the antibiotic treatment was not significant different that the untreated control.

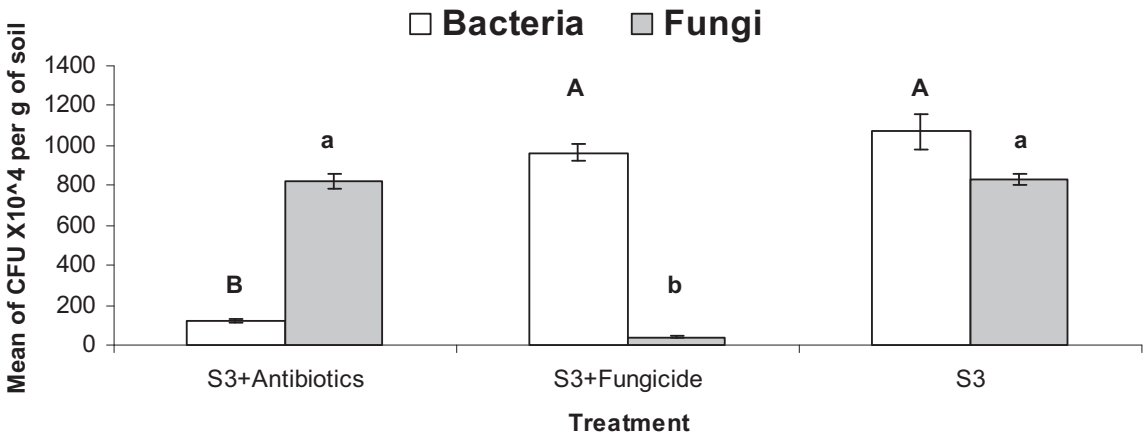


Figure 3. Mean number of bacteria and fungi colony forming units (CFU) per gram of soil growing after 24 h of incubation on TSA^+ or PDA^+ and isolated 20 days after nematicide application from SELM amended with antibiotics (Streptomycin sulfate at 150 µg ml^{-1} and Chloramphenicol at 150 µg ml^{-1}), fungicide (Cycloheximide at 150 µg ml^{-1}) or untreated (S3), inoculated with microorganisms from Soil 3. N = 3. Bars indicate the Standard Error of the Mean.

5.3.2. Metabolization of fenamiphos by bacterial single colonies

From the 40 bacterial single colonies tested 17 isolates with the numbers 1, 2, 3, 4, 6, 10, 12, 15, 17, 20, 22, 23, 24, 32, 34, 35 and 38 were able to metabolize 100 µg ml^{-1} fenamiphos analytical grade almost to completion in a plate assay with the nematicide as sole carbon source after 14 days of incubation (Figure 4). After 7 days all these isolates had degraded fenamiphos by more that 50%. The bacterial isolates 1 to 20 were isolated from the treatment

with Cycloheximide and isolates number 21 to 40 came from the untreated control. Isolates number 5, 7, 8, 9, 11, 13, 14, 15, 18, 19, 21, 25, 26, 27, 28, 29, 30, 31, 33, 36, 37, 39 and 40 were not able to significantly metabolize fenamiphos after 14 days of incubation.

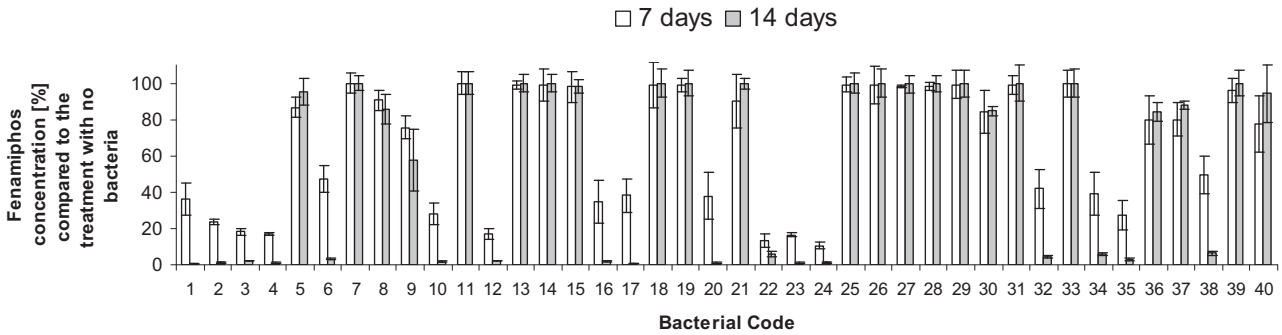


Figure 4. Metabolization of fenamiphos in soil extract agar medium originally amended with 100 µg ml^{-1} fenamiphos analytical grade by 40 bacterial single colonies isolated from S3 after 7 and 14 days of incubation. N =3. Bars indicate the Standard Error of the Mean.

All 17 fenamiphos analytical grade degrading isolates were further tested for their ability to metabolize fenamiphos in the formulation Nemacur 5GR. With the exception of isolate number 3 all isolates degraded the active ingredient, although degradation was not as efficient compared to the analytic grade (Figure 5). After 7 days some isolates had not yet degraded at all (isolate 38) or only degraded fenamiphos by 30 to 40%. However, after 14 days most isolates had degraded 80% of fenamiphos. The isolate number 38 was able to metabolize only half of the nematicide concentration 14 days after incubation. Apparently, components present in the formulation affect the development of the bacteria or their degradation efficacy.

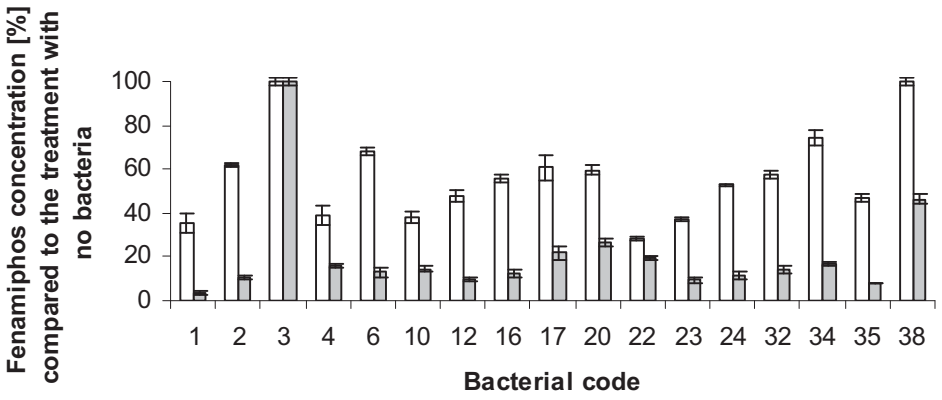


Figure 5. Metabolization of fenamiphos in soil extract agar medium originally amended with Nemacur 5 GR applied at 100 µg ml^{-1} active ingredient by 17 fenamiphos-analytical grade degrading bacterial single colonies isolated from S3 after 7 and 14 days of incubation. N =3. Bars indicate the Standard Error of the Mean.

5.3.3. Use of fenamiphos as sole carbon source

The fenamiphos degrading bacterium isolate 32 was not able to grow in SELM that was not amended with fenamiphos, resulting in no increase in bacterial density (Figure 6). When 100 µg ml^{-1} fenamiphos was present the OD_{600} increased from 0.12 to 0.16 nm at 4 hours after application, indicating bacterial growth. However, after this optical density increment, the OD_{600} of the culture decreased to 0.08 nm after 72 hours. The bacterium that was isolated from the fenamiphos non-degrading soil S4 did not show an increase in density, both in the absence and presence of fenamiphos. Apparently this isolate was not able to use fenamiphos as a carbon source and no alternative carbon sources were available for this bacterium.

In the same assay the fate of the fenamiphos was monitored by HPLC analysis. This showed that the fenamiphos degrading isolate 32 had already converted 95% of the total fenamiphos after 4 hours of incubation (Figure 7). As expected, in the medium containing the non-degrading isolate no fenamiphos degradation was observed, thus remaining at the same level as the non inoculated control. The almost complete conversion of fenamiphos 4 hours after the start of the incubation explains the observation that the bacterial biomass only increases in these first 4 hours.

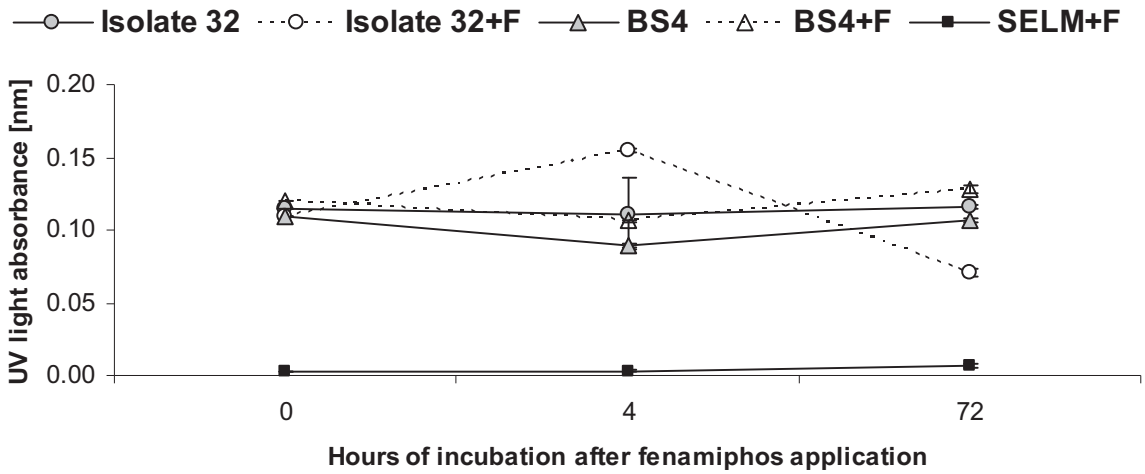


Figure 6. Optical density analysis at 600 nm wave length to determine growth behaviour of the fenamiphos-degrading isolate number 32 isolated from S3 and a bacterium isolated from non-degrading S4 (BS4) in soil extract liquid media (SELM) in the presence (+F) or absence of fenamiphos. N = 3. Bars indicate the Standard Error of the Mean.

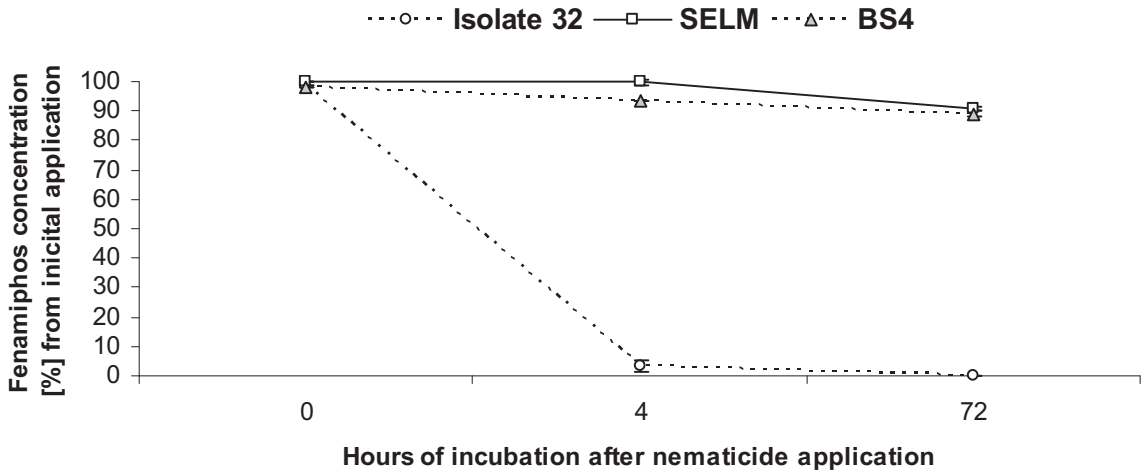


Figure 7. Metabolization of fenamiphos initially applied at 100 µg ml^{-1} in soil extract liquid media (SELM) by fenamiphos-degrading isolate number 32 isolated from S3 or a bacterium isolated from non-degrading S4 (BS4). N = 3. Bars indicate the Standard Error of the Mean.

5.3.4. Molecular characterization and identification of fenamiphos degrading bacteria

Of all 17 fenamiphos-degrading bacterial isolates the genomic 16S rRNA coding regions were amplified by PCR and analyzed by restriction length polymorphism and DNA sequence analysis. The isolated bacteria produced a variety of 16S rRNA RFLP profiles (Figure 8). The

restriction enzyme analysis provided an initial clustering of strains which divided into two groups with three or more members within the same profile. The first group of isolates, or group A, comprised eleven members; 1, 3, 4, 10, 12, 22, 23, 24, 34, 35 and 38. All these isolates showed high homology to the internal fragment of the 16S rRNA gene. A second group, group D, was conformed by three isolates; 16, 17 and 20. There were three individual isolates with unique fragmentation patterns, been number 2, 6 and 32.

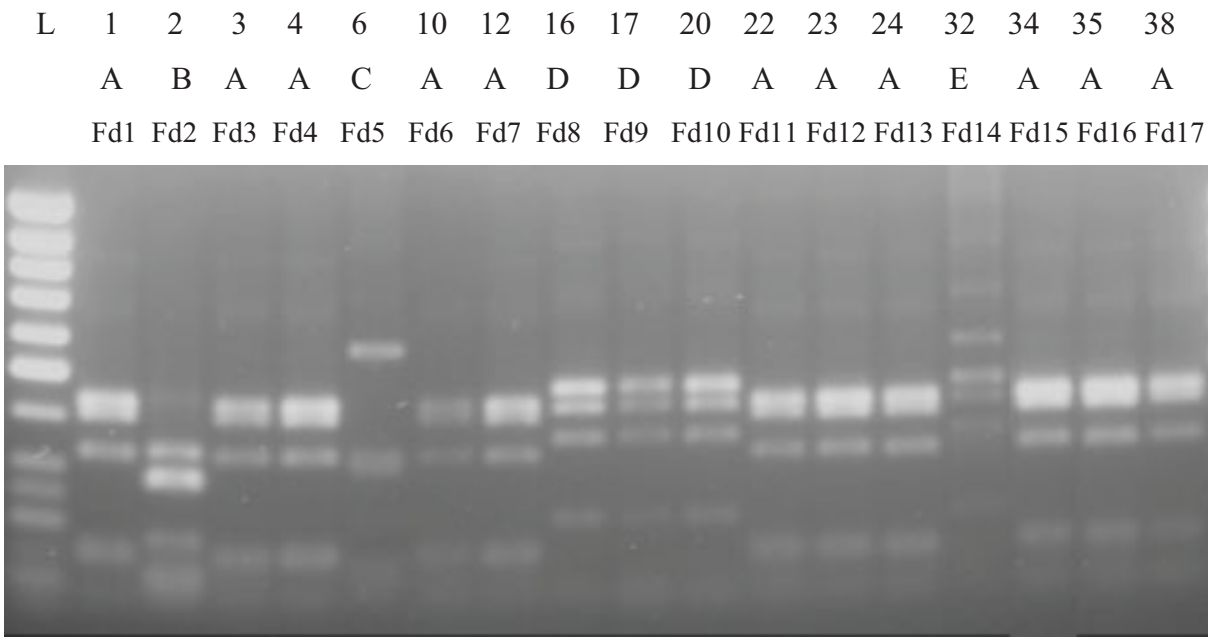


Figure 8. RFLP patterns of the 1.5 kb PCR amplified fragments of the 16S rRNA gene of 17 fenamiphos-degrading bacterial isolates, digested with the restriction endonuclease *Cfo*1. Lane L is the 50bp molecular size marker (Promega). Numbers in the upper line indicate the number of the isolate in the previous fenamiphos metabolization tests. Letters in the middle line show the group to which each isolate correspond according to its RFLP type. Fd in the lower line shows the new code assigned for its capacity of fenamiphos degradation.

Almost complete 16S rRNA sequences of 17 fenamiphos-degrading bacterial strains were obtained, ranging in length from 1080 to 1397 nucleotides. Blast analysis revealed that isolates 1, 3, 4, 10, 12, 22, 23, 24, 34, 35 and 38 had high similarities, ranging from 95 to 99% of match identity, with the sequences of two *Microbacterium* sp. from the NCBI library (Table 1). The isolates 16 and 20 had a match identity of 95 and 96% with the sequence of *Ralstonia* sp., respectively. The partial sequence of isolate 2 was similar to *Sinorhizobium* spp. with 93% identity match. The sequence similarity between isolate 6 and *Brevundimonas mediterranea* was of 95%. Isolates 17 and 32 had similar sequences as *Cupriavidus* sp. and *C. necator* with 95 and 92% identity match, respectively.

Table 1. RFLP patterns and identification of 17 fenamiphos-degrading (Fd) bacterial single colonies according to their partial sequence of the 16S rRNA gene.

Isolate[w]	RFLP [x]	New code[y]	Genus	Species	Genebank No.[Z]	Identities (%)
1	A	Fd1	*Microbacterium*	n.d.	EU037292	96
2	B	Fd2	*Sinorhizobium*	n.d.	EU399910	93
3	A	Fd3	*Microbacterium*	n.d.	AY040877	95
4	A	Fd4	*Microbacterium*	n.d.	AY040877	95
6	C	Fd5	*Brevundimonas*	*mediterranea*	AJ244706	95
10	A	Fd6	*Microbacterium*	n.d.	AY040877	97
12	A	Fd7	*Microbacterium*	n.d.	EU037292	96
16	D	Fd8	*Ralstonia*	n.d.	AB167214	95
17	D	Fd9	*Cupriavidus*	n.d.	AB266612	95
20	D	Fd10	*Ralstonia*	n.d.	AB167214	96
22	A	Fd11	*Microbacterium*	n.d.	EU037292	96
23	A	Fd12	*Microbacterium*	n.d.	EU037292	97
24	A	Fd13	*Microbacterium*	n.d.	EU037292	96
32	E	Fd14	*Cupriavidus*	*necator*	AB167205	92
34	A	Fd15	*Microbacterium*	n.d.	EU037292	99
35	A	Fd16	*Microbacterium*	n.d.	EU037292	99
38	A	Fd17	*Microbacterium*	n.d.	EU037292	99

[W] = number of the isolate in the previous fenamiphos metabolization tests. [X] = Restriction Fragment Length Polymorphism group to which every isolate belong. [Y] = New code FD indicates fenamiphos degrading. n.d. = Not defined species at the identification library. [Z] = gene bank number according to the partial sequence of the 16S rRNA gene at the NCBI database.

5.3.5. Phylogenetic analysis and sequence comparison

The phylogenetic analysis of the fenamiphos-degrading bacterial strains resulted in a fully resolved tree showing sister lineage between the Fd isolates and the bacteria on the NCBI library thus confirming their identification (Figure 9). The outgroup *Staphylococcus aureus* and the reference *Pseudomonas fluorescence* were largely distant from the Fd isolates obtained from S3 and proved to be a suitable choice for this set of bacteria supporting a common evolutional origin of the fenamiphos-degrading genera. The tree topology is strongly supported by high significant scores for Jackknife analysis, except for 2 braches in the *Microbacterium* Fd isolates, as well as there is strong support for clade credibility by Posterior Probabilities, ranging from 0.90 to 1.00, with two exceptions (0.67 and 0.54). These results corroborate that the fenamiphos-degrading bacteria have been precisely identified by the blast analysis.

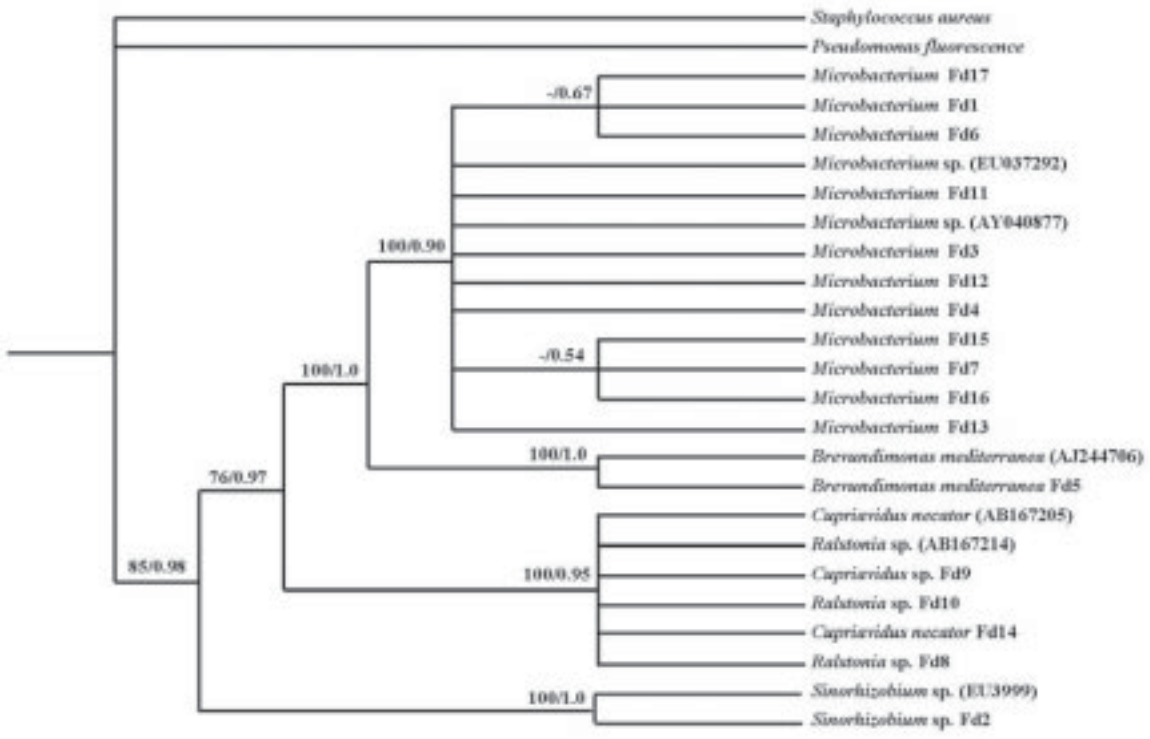
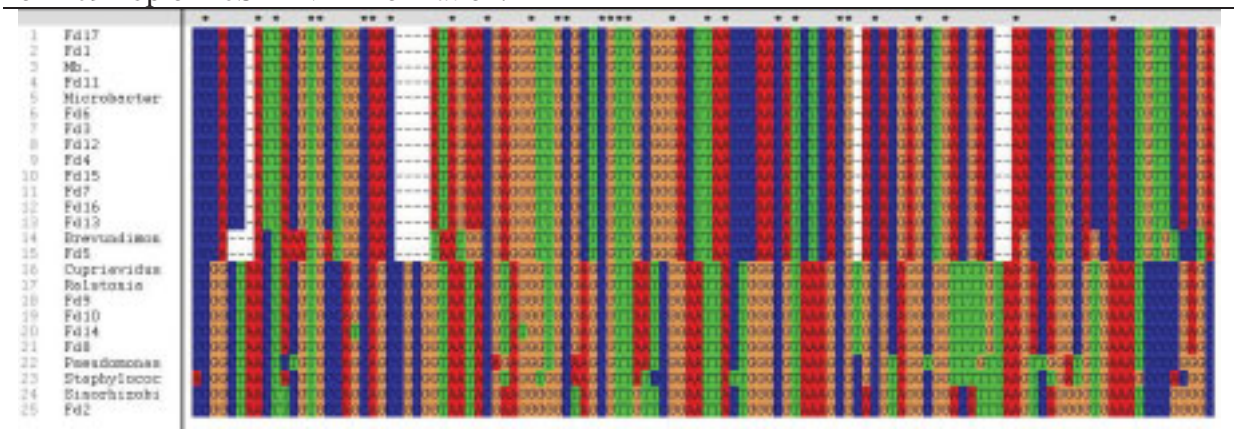


Figure 9. Phylogenetic analysis of the partial 16S rRNA of fenamiphos-degrading and closely related bacteria using *Staphylococcus aureus* (FJ609418) as outgroup and *Pseudomonas fluorescence* (EU73092) as reference. 50% majority rule semi-strict consensus tree generated in MrBayes is displayed as dendrogram. Model of nucleotide substitution was GTR+G. Values annotated to each node indicate: Jackknife (37% deletion of data) / Posterior probabilities. Nodes with Jackknife values below 67 are generally considered as weak supported (-). Bars indicate the relative number of substitutions per site. The accession number of the 16S rRNA sequences obtained from NCBI database showing a high identity with Fd bacteria are shown in parenthesis.

The sequence alignment of 17 fenamiphos-degrading and other 8 closely related bacteria, showed different regions of similarity among isolates (Table 2). Based on 1092 bp of the 16S rRNA coding sequence information this study revealed that there were 29 similar nucleotide regions among all the isolates compared. The Fd sequences were divided in two groups according to their sequence homology. The first group was composed by the *Microbacterium* sp. and *Brevundimonas mediterranea* isolates and the second group by the rest of bacteria. However, every sequence of the Fd isolates had more similar nucleotide regions with their respective identified genera than with the rest of bacteria. These results support the blast identification and the phylogenetic dendrogram and re-confirm the high similarity between the Fd isolates from S3 and the bacteria reported on the NCBI library.

Table 2. Sequence alignment of 17 fenamiphos-degrading and closely related bacteria based on 1092 bp of 16S rRNA information.



Microbacterium sp. EU037292 (Mb.), *Microbacterium* sp. AY040877 (Microbacter), *Brevundimonas mediterranea* AJ244706 (Brevundimon), *Cupriavidus necator* AB167205 (Cupriavidus), *Ralstonia* sp. AB167214 (Ralstonia), *Pseudomonas fluorescence* EU73092 (Pseudomonas), *Staphylococcus aureus* FJ609418 (Staphylococ), and *Sinorhizobium* sp. EU399910 (Sinorhizobi). *Identical nucleotide position in all bacteria. Bacteria were aligned according to similarity. Different colours highlight the homology and changes in the nucleotide.

5.4. Discussion

In this study, it is further demonstrated that metabolization of fenamiphos was performed by microorganisms of S3, a soil with a 12 year long fenamiphos application history. In contrast, there was no nematicide metabolization where antibiotics were applied. These results strongly suggest the involvement of bacteria rather than fungi in fenamiphos degradation. However, microorganisms in the treatment where neither antibiotics nor fungicide were applied degraded fenamiphos at the highest rate suggesting that the combination of bacteria and fungi accelerate even more fenamiphos degradation than bacteria alone. Similar results were reported for another organophosphate non-fumigant nematicide ethoprophos. Karpouzas et al. (1999) found that an untreated degrading-soil metabolized more rapidly the nematicide than soil treated with Cycloheximide and this degraded faster than soil treated with Chloramphenicol. Both studies are in agreement that bacteria are the main microbes involved in the rapid degradation process of organophosphate nematicides in soil. Additionally, bacteria have been reported to be capable of degrading other organophosphate pesticides such as parathion, fensulfothion and diazinon (Sheela and Pai, 1983; Serdar et al., 1982; Sethunthan and Yoshida, 1973). Furthermore, Rosenberg and Alexander (1979) demonstrated that the breakdown of organophosphate pesticides result from the synthesis of

an enzyme or enzyme complex which support the organism in using the organophosphates as phosphorus sources for growth and development.

Even though the soil extract liquid medium was treated with either fungicide or antibiotics, it was still possible to isolate fungi and bacteria from the corresponding amended treatments. However, the amount of bacteria growing on TSA^+ obtained from the antibiotic amended treatment and the number of fungi growing on PDA^+ obtained from the fungicide amended treatment were 10 times lower than those obtained from the opposite treatment or the untreated control. It is well known that Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms. Streptomycin sulfate inhibits protein synthesis leading to death of microbial cells and Chloramphenicol is a bacteriostatic antimicrobial compound, explaining the results herein. These results illustrated that fungi and bacteria suffered and their growth and development was strongly inhibited by the application of Cycloheximide or Streptomycin sulfate together with Chloramphenicol, respectively. Thus this confirms the conclusion that bacteria are the dominating factor in the fenamiphos degradation process.

From the 40 bacterial single colonies tested, obtained from the biodegrading soil S3, 17 were able to metabolize fenamiphos analytical grade at an accelerated rate during the 14 days of incubation period. In addition, these 17 isolates were capable to metabolize fenamiphos as active ingredient of Nemacur 5GR, with the exception of isolate number 3 (Fd3), confirming their biodegrading capability. This conclusion agrees with other studies that have reported bacteria as principal factors for fenamiphos enhanced degradation in soil (Ou, 1991; Megharaj et al., 2003; Singh et al., 2003).

Isolate number 3 (Fd3) was the only bacteria not capable of degrading fenamiphos in Nemacur 5GR. Additionally, most of the isolates were not as efficient in degrading fenamiphos in the commercial product as in the analytical grade, although the amount of fenamiphos in both tests was identical (100 $\mu g\ ml^{-1}$). Nemacur 5GR formulation contains 5% of the active ingredient and 95% of other unspecified aggregate compounds and it may have been that these additives directly interfered with the degradation process or supplied an alternative nutrient source for the bacteria, thus reducing the metabolization of fenamiphos.

All fenamiphos-degrading bacteria were identified. According to the amplification of the 16S rRNA gene and blast analysis, eleven isolates from the seventeen fenamiphos-degrading

corresponded to *Microbacterium* spp. showing high similarity to *Microbacterium* species reported at the NCBI library. Restriction enzyme analysis with *Cfo*1 revealed that these bacteria had high homology of their internal DNA fragment. This first bacterial group represented 64.7% of all the fenamiphos-degrading isolates which composed the RFLP type A. The phylogenetic analysis confirmed this identification showing sister lineage between *Microbacterium* Fd isolates and the *Microbacterium* sp. from NCBI. Sequence comparison reconfirmed this result. Previous phylogenetic analyses of most *Microbacterium* species have shown that this genus has a common ancestor and that the different species are close relatives (Schippers et al., 2005: Takeuchi and Hatano, 1998). The present study is in accordance with a recent short communication which reported that *Microbacterium esteraromaticum* was able to hydrolyze fenamiphos and its toxic oxidation products (Cáceres et al., 2009). Furthermore *Microbacterium* species have been previously reported to degrade the carbamate non-fumigant nematicide carbofuran and the herbicide isoxaben at an accelerated rate (Karpouzas et al., 2000b; Arrault et al., 2002). For the congruence between the present work and the above cited, the genus *Microbacterium* appears to be an important component of the pesticide enhanced biodegradation process.

A second group of fenamiphos-degrading bacteria was composed by isolates 16 (Fd8), 17 (Fd9) and 20 (Fd10) which had high homology of the internal fragment RFLP type D. The partial sequence of this group was identified to belong to the genus *Ralstonia* or *Cupriavidus*. Isolate 32 (Fd14) was identified as *Cupriavidus necator* but its RFLP pattern was unique (type E). *Ralstonia eutropha* is currently considered *Cupriavidus necator* and therefore the sequence similarities exist (Manzano et al., 2007). However, the species of these two genera can significantly differ from one another thus encoding different DNA internal fraction. Nevertheless, the phylogenetic analysis and sequence comparison confirmed the identification of these bacteria as it occurred with the *Microbacterium* Fd species. *Cupriavidus* and *Ralstonia* species can degrade herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (Huong, et al., 2007; Manzano et al., 2007; Martineza et al., 2008; Nicolaisen et al., 2008). Until now, there have been described 15 genes that play a role in the 2,4-D degradation (Streber et al., 1987; Perkins et al., 1990; Matrubutham and Harker, 1994; You and Ghosal, 1995; van der Meer, 1997; Leveau et al., 1999; Laemmli et al., 2000; Perez-Pantoja et al., 2000). Perhaps these genes are also necessary for the degradation of fenamiphos, thus explaining the results obtained in this work. Moreover this is the first time that *Ralstonia* and *Cupriavidus* species are reported to metabolize a nematicide.

There were other two isolates with a unique RFLP pattern. The first one was isolate number 6 (Fd5) identified as *Brevundimonas mediterranea*. Its identification was also confirmed by phylogenetic analysis and sequence comparison. Bacteria from this genus have been previously found to degrade other organophosphate pesticides such as the insecticide chlorpyrifos and the herbicide trifluralin (Bellinaso et al., 2003; Li et al., 2008). *Brevundimonas diminuta* contains the bacterial organophosphorus-hydrolyzing enzyme, which is a zinc-containing homodimeric protein (Horne, 2002). Since *B. mediterranea* and *B. diminuta* are close related bacteria, it can be expected that the isolate 6 (Fd5) found in this research also posses an enzyme involved in organophosphates degradation and utilize it to degrade fenamiphos. This is the first report of *Brevundimonas mediterranea* rapidly degrading a nematicide.

The last bacteria with unique RFLP pattern was isolate 2 (Fd2) identified as *Sinorhizobium* sp. according to its partial sequence of the 16S rRNA gene. As the other 16 Fd isolates, this bacterium was able to metabolize fenamiphos in soil extract agar medium as sole source of carbon. The phylogenetic analysis and sequence alignment confirmed that this isolate belongs to the *Sinorhizobium* bacteria. *Sinorhizobium* has been reported to degrade the polycyclic aromatic hydrocarbon Phenanthrene (Seo et al., 2007) and posses the herbicide degrading genes which are able to mineralize atrazine (Devers et al., 2007). Perhaps this bacterium also uses these genes to rapidly degrade fenamiphos. The nematicide degradation ability of *Sinorhizobium* sp. was not previously reported.

One fenamiphos degrading isolate, *Cupriavidus necator* Fd14, revealed that the bacterium utilized fenamiphos as sole carbon source for growth. The bacterium seemed to respond very quickly to the availability of fenamiphos resulting in a rapid and complete degradation of the organophosphate. The fenamiphos molecule can be degraded through different pathways into several products. Fenamiphos can be oxidized into fenamiphos sulfoxide (FSO) which is further oxidized into fenamiphos sulfone (Singh and Walker, 2006). Fenamiphos can be degraded into FSO and then turned into FSO-phenol and subsequently mineralized into CO_2 (Chung and Ou, 1996). Fenamiphos can also be directly converted into fenamiphos phenol, where probably the first oxidation step is replaced by hydrolysis (Singh et al., 2003).

In conclusion, this study demonstrated for the firs time that gram-positive and gram-negative bacteria are the principal microorganisms responsible for fenamiphos degradation in soil. These bacteria belong to six different genera, namely *Microbacterium*, *Ralstonia*,

Brevundimonas, *Cupriavidus* and *Sinorhizobium.* Additionally, this study revealed that the combination of all soil microorganisms degraded the organophosphate nematicide faster than bacteria alone. Synthetic organophosphates contain three phosphoester linkages and the phosphorus is also linked by a double bond to either an oxygen in oxon-organophosphates or a sulfur in thion-organophosphates (Horne et al., 2002). Hydrolysis of one of the phosphoester bonds reduces the toxicity of an organophosphate therefore reducing its efficacy for agricultural purposes. However, despite the fact that enhanced biodegradation has a negative effect on the control of plant-parasitic nematodes, it is a beneficial process from an ecological point of view since it eliminates potentially dangerous products from soil, which otherwise could cause serious problems to animal life and human health through accumulation in the environment (Moens et al., 2004). Fenamiphos activity has been found to persist for 3–4 months or more in soil (Homeyer, 1971) and for several years in groundwater (Franzman et al., 2000) thus the fenamiphos-degrading bacteria isolated in this study are good candidates for bioremediation of fenamiphos in areas where this organophosphate nematicide persist.

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6. Fosthiazate cross-degradation and specificity of nematicide degrading bacteria

6.1. Introduction

Repetitive applications of the same nematicide in soil have been shown to enhance its biodegradation process, especially in monocultures systems, as shown in the previous chapters and elsewhere (Karpouzas et al., 1999a; Karpouzas et al., 1999b). Adapted microorganisms, such as soil bacteria, are able to use the nematicides' active ingredients as a carbon source (Karpouzas and Walker, 2000; Karpouzas et al., 2005). In agricultural production, the enhancement of biodegradation is a great concern since it directly reduces nematicide efficacy (Rich et al., 2004).

The biodegradation of pesticides is not always restricted to the applied pesticide, but can extend to other pesticides that were never used in the field before. There is already evidence for this "cross-conditioning" of microbial populations in the soil (Morel-Chevillet et al., 1996). The efficiency of pesticides such as benfuracarb, carbosulfan and furathiocarb was reduced by prior treatment of carbofuran (Suett, 1987). Pest control by aldicarb in the field was reduced by prior treatment with carbofuran rather than prior treatments with aldicarb itself (Suett, 1989). Laboratory experiments have shown that several successive treatments with carbofuran led to increased rates of degradation of other compounds (Harris et al., 1984). *Sphingomonas paucimobilis* isolated from a soil often treated with cadusafos was able to cross-degrade ethoprophos (Karpouzas et al., 2005).

Fosthiazate is a relatively new organophosphate non-fumigant nematicide and effective in the control of potato cyst and root-knot nematodes (Rich et al., 1994; Woods et al., 1999; Woods and Haydock, 2000; Hafez and Sundararaj, 2006; Tobin et al., 2008). It is considered an alternative to nematicides that have been on the market for quite some time. However, it was reported that soil biotic factors can degrade fosthiazate (Pantelelis et al., 2006). Fosthiazate and fenamiphos belong to the same organophosphate family and it may therefore be possible that fenamiphos-degrading microorganisms can cross-degrade fosthiazate. The cross-degradation process is of importance since it could make new pesticides immediately ineffective, thus offering no alternative for the farmer.

The objectives of this investigation were to:

1. Monitor the degradation of fosthiazate by fenamiphos-degrading microorganisms.
2. Elucidate whether the presence of another organophosphate affects the degradation process of fenamiphos.
3. Determine whether fenamiphos-degrading bacteria can quickly degrade fosthiazate.

6.2. Materials and Methods

6.2.1. Degradation of fosthiazate

A stock solution of Fosthiazate 150 EC was applied to SELM to a final concentration of 100 µg ml^{-1} and either not inoculated or inoculated with a mixture of fenamiphos-degrading microorganisms extracted from soil S3. The inoculation conditions, sampling process and HPLC analysis were performed as described in the second test of section 4.2.5. Samples were taken 0, 3, 6, 9, 13, 16, 20 and 30 days after inoculation. Each treatment was done in triplicate.

6.2.2. Cross-degradation essays

Two cross-degradation assays were set up to investigate whether fenamiphos-degrading microorganisms can also degrade fosthiazate. In the first assay, to elucidate if all the fenamiphos-degrading microorganisms isolated from S3 would still enhance degrade fenamiphos in the presence of fosthiazate, the following treatments were performed. SELM was supplemented with fenamiphos analytical grade to a final concentration of 100 µg ml^{-1}, and Nemathorin 10WG to a final concentration of 100 µg ml^{-1} a.i. SELM supplemented with fenamiphos was used as control treatment. The SELM was then not inoculated, inoculated with a mixture microorganisms extracted from soil S3 or inoculated with an autoclaved mixture of microorganisms extracted from soil S3. To obtain the microorganisms from soil S3, the same process as described in the second test section 4.2.5. was performed. Autoclaving was performed for 60 min at 121 °C in the respective treatments. The incubation conditions, sampling and HPLC analysis were performed as described in section 4.2.5. Samples were taken 0, 3, 6, 9, 13 and 20 days after inoculation. Each treatment was done in triplicate.

In the second essay, the 17 fenamiphos degrading bacteria that were isolated from soil S3 in chapter 5 were grown on TSA^{+} for 48 h in dark conditions and used for inoculation onto SEAM plates supplemented with Nemathorin 10WG to a final concentration of 100 µg ml^{-1} a.i. The incubation conditions, sampling and HPLC analysis were performed as described in section 5.2.2. Samples were taken 0, 7 and 14 days after incubation. All samples were compared to the treatment with Nemathorin 10WG and no single colony inoculation. Each treatment was done in triplicate.

6.3. Results

6.3.1. Degradation of fosthiazate

The microorganisms present in the fenamiphos-degrading soil S3 were tested for their fosthiazate degrading abilities in an assay with soil extract liquid medium, supplemented with 100 µg ml^{-1} fosthiazate. This showed that in non-inoculated medium the fosthiazate was degraded to approximately 50% of the initial concentration after a 30 days incubation period (Figure 1). Fosthiazate can also be degraded by abiotic factors. However, in the presence of the microorganisms obtained from S3, fosthiazate was reduced by 70% of the initial concentration after a 30 days incubation period. This accelerated degradation in the presence of microorganisms was observed throughout the whole incubation period. Apparently, biotic factors present in soil S3 are involved in the accelerated degradation of fosthiazate even though this is the first time that the microorganisms are in contact with this specific nematicide.

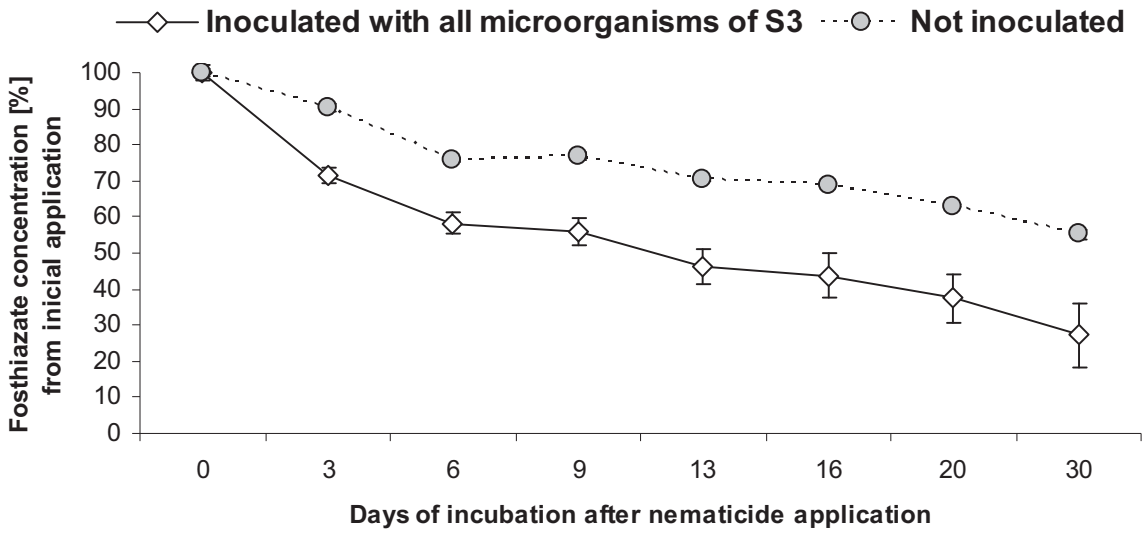

Figure 1. Degradation of fosthiazate applied to an initial concentration of 100 µg ml^{-1} in soil extract liquid medium alone or when inoculated with microorganisms of soil S3, which contained fenamiphos-degrading organisms. N = 3. Bars indicate the Standard Error of the Mean.

6.3.2. The effect of fosthiazate on fenamiphos degradation

To determine the effect on biodegradation when two organophosphates were present, the microorganisms extracted from S3 were incubated in the presence of both fenamiphos and fosthiazate and the degradation of fenamiphos was followed over time and compared to the S3 microbial extracts, incubated with fenamiphos alone. In the control treatments, in which no microorganisms were inoculated or the microbial extracts were autoclaved prior to inoculation, in the presence of only fenamiphos or both organophosphates, no fenamiphos metabolization could be observed during the 20 days incubation period (Figure 2). When fenamiphos was present alone, the degradation of the organophosphate was visible 6 days after the start of the incubation. At this time, the fenamiphos degradation was not yet visible when both organophosphates were present. However, for both treatments the fenamiphos was completely degraded after 13 days. Although, there was some delay in the initiation of the fenamiphos biodegradation in the presence of fosthiazate, this delay was apparently compensated by a higher biodegradation activity when finally initiated.

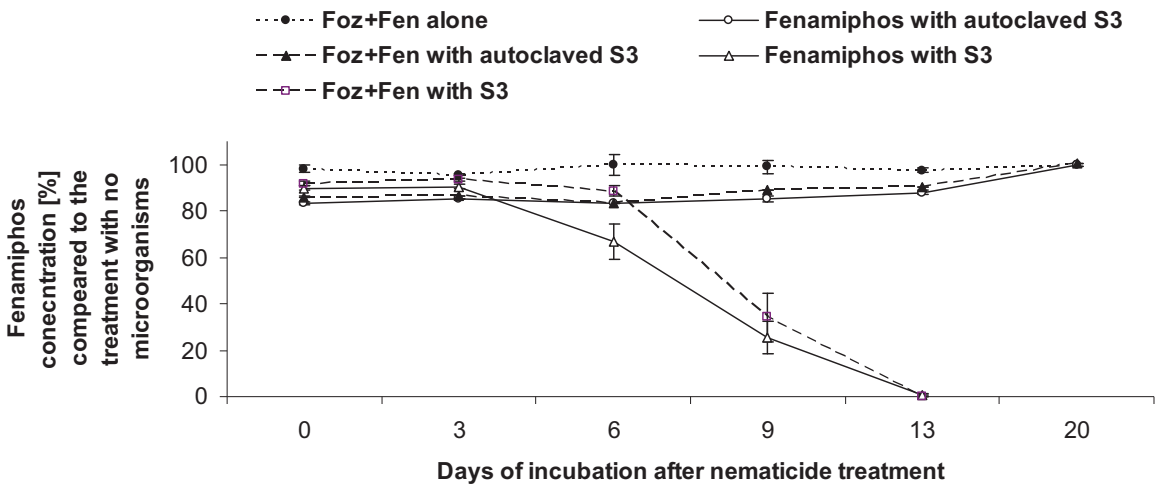

Figure 2. Metabolization of fenamiphos (Fen) in soil extract liquid medium, in the absence or presence of fosthiazate (Foz) and which was not inoculated with microorganisms, inoculated with microorganisms extracted from S3, a soil containing fenamiphos degrading microorganisms or inoculated with an autoclaved suspension containing the microorganisms extracted from S3. N = 3. Bars indicate the Standard Error of the Mean.

6.3.2. Degradation of fosthiazate by fenamiphos-degrading bacteria

The 17 fenamiphos degrading bacteria that were isolated from soil S3 in chapter 5 were tested for their ability to degrade fosthiazate in an assay with soil extract liquid medium at 100 µg ml^{-1}. HPLC analysis showed that throughout the 14 days incubation period none of these isolates was capable of degrading fosthiazate (Figure 3).

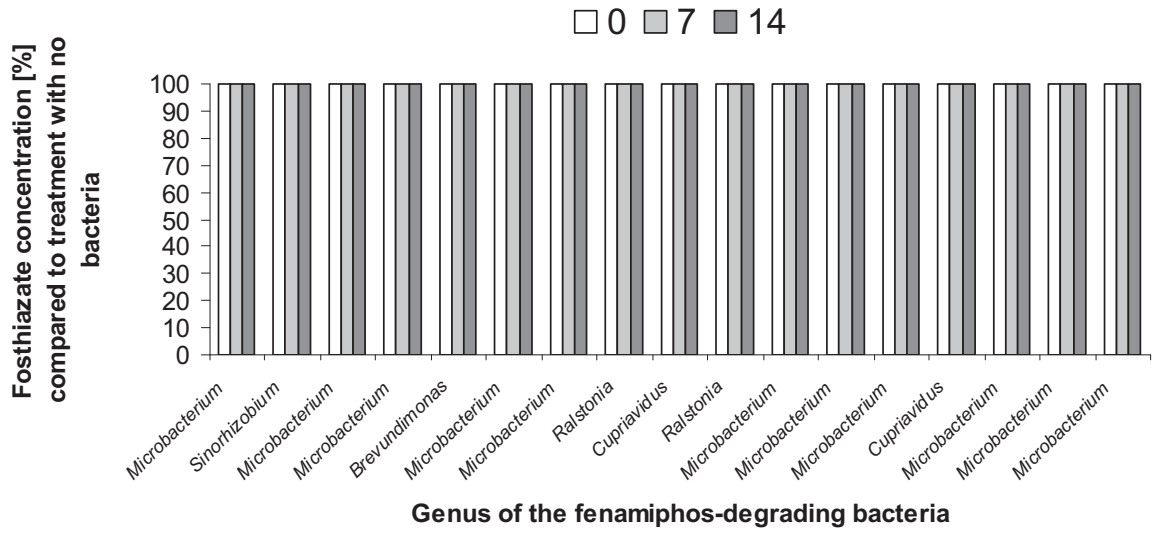

Figure 3. Percentage of remaining fosthiazate applied to an initial concentration of 100 µg ml^{-1} as detected by HPLC analysis in soil extract agar medium 0, 7 and 14 days after inoculation with one of the 17 fenamiphos-degrading bacteria isolated and identified in chapter 5. N = 3.

6.4. Discussion

Fosthiazate is a novel organophosphate which is also marketed in nematicidal formulation. The results in this investigation suggest that abiotic factors can cause degradation of fosthiazate. However, the degradation rate is not considered enhanced since the half-life of this organophosphate nematicide in soil has been reported to range between 0.5 to 1.5 months (Pantelelis et al., 2006). Furthermore it is also shown that fosthiazate may be degraded by biotic factors. These results are in accordance with other fosthiazate studies, since the presence of microorganisms has been shown to accelerate the degradation process. Elimination of microorganisms by sterilization resulted not only in an increase of fosthiazate persistence in soil but also in a decrease of its degradation rate (Qin et al., 2004). Additionally, soil autoclaving was reported to double the half-life of fosthiazate (Pantelelis et al., 2006).

The soil (S3) which was frequently exposed to fenamiphos and contains microorganisms that can degrade this organophosphate is also capable of biodegrading fosthiazate, although not as efficient. Even though currently these microorganisms may not be that important in the fosthiazate degradation, it is still remarkable that such organisms are already present in a soil that had never been exposed to this organophosphate. This observation may indicate that the application of fosthiazate will quickly lead to the accumulation of fosthiazate-degrading organisms and, consequently, to the accelerated biodegradation of the compound.

The bacteria that were found to be very effective in the degradation of fenamiphos could not degrade fosthiazate, thus no indications were found of cross-biodegradation. Apparently, fenamiphos degrading bacteria were rather specialized. Completely different microorganisms or types of microorganisms are involved and the degradation of each organophosphate may therefore require different enzymes (pathways). Bacteria from the genus *Enterobacter*, *Flavobacterium*, *Agrobacterium*, *Bacillus* and *Rhizobium*, among others, have been reported to degrade organophosphorus compounds (Singh and Walker, 2006). In addition, not only bacteria have been shown to rapidly degrade xenobiotics. Fungi from the genus *Phanerochaete*, *Hypholama*, *Coriolus*, *Spergillus*, *Trichoderma* and *Pencillium*, for example, have also bee reported to degrade organophosphorus pesticides (Singh and Walker, 2006). The results obtained in the present study suggest that the phenomena of cross-conditioning is not that important in the soil and therefore, the application history of one organophosphate nematicide for a soil is not informative regarding the development of biodegradation for

another organophosphate. This theory is supported by similar results of pesticide specificity degrading microorganisms reported with other organophosphate pesticides. Racke and Coats (1988) isolated *Artrhobacter* spp. from a soil with large history of isofenphos use. This bacterium was capable to degrade isofenphos at accelerated rates but could not degrade chlorpyrifos, fonofos, ethoprop, terbufos and phorate. In soils with large ethoprophos history this nematicide was rapidly biodegraded but degradation of fenamiphos, fonofos, cadusafos, isazofos, aldicarb and oxamyl did not occurred (Karpouzas and Walker, 2000). Furthermore, the evidence in all the above studies supports the hypothesis of bacterial adaptation to a specific compound. This theory would also explain why the presence of both organophosphates at the same time did not affect the degradation of fenamiphos, either positively or negatively, since different organisms are involved in the degradation process. Although some delay in the initiation of fenamiphos biodegradation was observed in the presence of fosthiazate, the complete biodegradation occurred in the same time frame. Fosthiazate may have some initial restrictive impact on the proliferation of the fenamiphos degrading microorganisms, but this effect disappears completely in the course of time.

It can be concluded from this study that fosthiazate biodegradation process can be developed by microorganisms even if they are exposed to this nematicide for the first time, that the fosthiazate-degrading microorganisms are different from those degrading fenamiphos and that the nematicide application history in soil of one nematicide does not necessarily influence the degradation rate of another one. From an agricultural point of view, these observations are of importance since it would suggest that the efficacy of nematicides with fosthiazate could rapidly decline even if it is applied for the first time. The emergence of new alternatives to slow down the enhanced biodegradation process is important and therefore is the focus of the next chapter of this thesis.

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7. Effect of natural plant enhancers on soil bacteria, *Meloidogyne incognita* and nematicide degradation

7.1. Introduction

The root-knot nematode *Meloidogyne incognita* is distributed worldwide (Sasser and Carter, 1985) and is known to be a major pest in a wide variety of crop plants. Among the host plants, lettuce is one of the vegetables that can be seriously affected by *M. incognita* (Sikora and Fernandez, 2005). To control plant-parasitic nematodes non-fumigant nematicides are presently preferred over the fumigant nematicides due to their lower environmental hazard (Hague and Gowen, 1987). However, the efficacy of non-fumigant nematicides has been reduced in recent times due to adapted soil microorganisms capable of degrading the nematicides, which is caused by the prolonged and intensive application (Karpouzas et al., 2000; Karpouzas and Giannakou, 2002) as described in previous chapters.

At present, there are some strategies available to maintain the efficacy of nematicides or to delay the onset of biodegradation. In soils of the Ivory Coast, the degradation rate of fenamiphos returned to the level found in previously untreated soils when the prolonged application of this pesticide were interrupted for 12 to 16 months (Anderson et al., 1992). Furthermore, to avoid redevelopment of accelerated degradation, fenamiphos application was alternated every second treatment with a different organophosphate nematicide. In Australia, a rotation in nematicide applications showed to delay the development of enhanced biodegradation in soils (Pattison and Cobon, 2003). Ample time intervals between application of the same nematicide, and repressing the disease incidence by crop rotation, could reduce the need for pesticide applications and thus further delay the development of an increased pesticide biodegradation (Smelt and Leistra, 1992). However, in intensive agriculture it may in some cases be unpractical to postpone nematicide applications for a long time, especially when the nematode numbers increase exponentially within time, thus drastically reducing crop yield. Additionally, in regions where farmers get attractive prices when buying products from the same chemical company, there is often little rotation in the application of pesticides.

Organic amendments have been used worldwide to positively affect soil properties and, ultimately, crop quality (Muller and Gooch, 1982). Among the organic amendments, plant

revitalizers, also known as plant enhancers, have been identified to stimulate microbial activity in soil and promote plant growth (Mulawarman et al., 2001). Additionally, it has been reported that the plant revitalizers promote growth of plant-beneficial microorganisms, such as mycorrhizal fungi, and reduce to some extent infection of *M. incognita* (Mulawarman et al., 2000). Soil amendments can thus reduce the presence of soil born pathogens such as plant-parasitic nematodes.

There is an increasing demand for plant enhancers with multiple beneficial effects, like to provide nutrients to the plant, increase the antagonistic potential of the soil and delay, stop or reverse the nematicide enhanced biodegradation process. Application of the plant revitalizers may be capable of shifting a nematicide-degrading microbial community into a non-degrading community and/or provide additional carbon sources, that could be used by the microorgasnisms in order to reduce the use of nematicides as carbon source. Therefore the objectives of this research were to:

1. Determine the effect of different inoculum densities of *Meloidogyne incognita* on lettuce cv. Milan.
2. Quantify the shifts in bacterial populations caused by the application of natural plant enhancers.
3. Establish the biocontrol activity of plant revitalizers on early root penetration of *M. incognita*.
4. Evaluate the effect of natural plant enhancers on the biodegradation of fenamiphos.

7.2. Materials and Methods

7.2.1. Effect of different *M. incognita* inoculum densities on lettuce cv. Milan

To determine the susceptibility of lettuce cv. Milan to *Meloidogyne incognita* a greenhouse bioassay was initiated, in which plastic pots with a diameter of 10 cm were filled with 250 ml of autoclaved mixture of soil S4 and sand, in a 1:1 (v:v) ratio and supplemented with 7% compost. Per pot 50 lettuce seeds cv. Milan were sown. After sowing, approximately 40 ml of autoclaved sand was put on top of the seeds for germination and the pots were gently watered. 1000, 3000, 5000 or 7000 *M. incongita* J2 were inoculated per pot through 3 cm

deep holes in the soil around the lettuce plants 1 week after sowing. Non infested pots were used as control. The lettuce plants were harvested 4 weeks past nematode inoculation and the shoot fresh weight was recorded. Roots were gently washed, weighted and the gall index (Zeck, 1971) with a scale from 0 (no galls) to 10 (completely galled root) was determined per root system. Each treatment had 6 replicates. All treatments were distributed in a randomized block design under greenhouse conditions at 26 ± 2 ºC. Plants were watered with tap water when necessary.

7.2.2. Effect of plant enhancers on soil bacteria, *Meloidogyne incognita* and biodegradation

7.2.2.1. Natural plant enhancers and nematicides

The tested plant commercial enhancers were MagicWet®, TerraPy® (Cognis Deutschland GmbH, Germany), Azet® (W. Neudorff GmbH KG, Germany), and Oscorna® (Oscorna-Dunger GmbH & Co. KG., Germany). MagicWet® is a liquid formulation of sugar based surfactants and fatty acid derivatives and TerraPy® is a liquid formulation composed of plant lipids, sugar based surfactants and organic bounds of nitrogen and phosphorous (Mulawarman et al., 2001). Azet® and Oscorna® are granular formulated plant enhancers. Azet® is a natural compost produced using animal and plant material. Oscorna® is composed of coral algae lime, clay minerals and extracts of primary rocks. Chicken manure and green compost were produced in the University of Bonn, Germany. As nematicides the biological DiTera® DF and the non-fumigant nematicide Nemacur® 5G were used in this research.

7.2.2.2. Soil treatment with plant enhancers and nematicides

Soil S4 was supplemented with 7% (w:w) organic matter and plastic bags were filled with 6 kg of soil each. Every bag was treated with Oscorna® (450 kg ha^{-1}), Azet® (300 kg $ha^{-}1$), Compost (5,000 kg ha^{-1}, Joshi et al., 2000), Chicken manure (5,000 kg ha^{-1}, Tüzel et al., 2003), Diterra® (112 kg ha^{-1}), Magic Wet® (200 kg ha^{-1}, Mulawarman et al., 2001), Terrapy® (200 kg ha^{-1} Mulawarman et al., 2001) or Nemacur® 5G (120 kg ha^{-1}) once at the beginning of the experiment. As a control, one plastic bag with soil was not treated. Tap water was added to all soils to reach field capacity immediately after treatment. After watering, all plastic bags containing the soil were vigorously mixed, thus distributing the amendments evenly and incubated in the green house at 27 ± 5 ºC until further use.

7.2.2.3. Effect on soil bacteria population densities

One day before the soil was treated, the bacteria present in the soil were isolated by spreading it onto TSA^{+} as described on section 2.4. in triplicate. One and three weeks after soil treatment, bacteria were isolated as described above. After 24 h of incubation the number of colony forming units was determined in every treatment.

7.2.2.4. Effect on *M. incognita* early root penetration

From every soil treatment 250 ml of soil was taken and transferred to 10 cm diameter plastic pots at 0, 2 and 4 weeks after soil treatment. Every pot was inoculated through three 3 cm deep holes with a suspension containing 2500 well developed *M. incongita* eggs. The holes were closed, approximately 50 seeds of lettuce cv. Milan were sown per pot and the seeds were covered with autoclaved sand to provide adequate germination conditions. Plants were watered with tap water when necessary. Plants were harvested 20 days after planting and the roots were gently washed with tap water, stained with lactic acid-fuchsin (768 ml lactic acid, 56 ml glycerol, 1 g acid fuchsin and 154 distilled water) and heated in a microwave for 2 min. Then roots were cut into 1 cm pieces and further fragmented in tap water using an Ultra-Turrax (IKA-Werk) at 11,000 rpm for 1.5 minutes. The suspension was adjusted to 100 ml and the number of penetrated nematodes per root system was determined. The experiment was arranged in a randomized complete block design with each treatment replicated four times and conducted under greenhouse conditions at 26 ± 5 °C.

7.2.2.5. Effect on enhanced biodegradation of fenamiphos

Soil S3, containing fenamiphos-degrading microorganisms, was used to determine whether the plant enhancers could delay or stop the biodegradation process. For this, 20 g soil samples of S3 were taken and transferred to sterile plastic beakers. Every sample was treated with one of the plant enhancers at the equivalent dosage described in section 7.2.2.2. with the exception of chicken manure which was not utilized in this test. A sample with no plant enhancer application was used as control. Sterile water was added to the soil samples up to holding capacity. The beakers were closed with a sterile cap, vigorously shaken and kept in the green house at 26 ± 5 ºC for one week. Then, 10 g of each sample were transferred to 100 ml of sterilized distilled water in Erlenmeyer flasks. The suspensions were incubated for 16

hours in the dark on a rotary shaker at 60 rpm at 28 ˚C. Subsequently, 1 ml suspension was transferred into 19 ml of SELM supplemented with 100 µg ml^{-1} fenamiphos analytical grade. From this medium samples were taken 0, 6, 10 and 14 days after inoculation. Incubating conditions, sampling and HPLC analysis were performed as described in the second test of section 4.2.5.

7.2.3. Statistical analysis

To determine the effect of each treatment in time the data obtained in the greenhouse and laboratory tests was analyzed by linear regression using Sigma Plot (version 8.02, SPSS, Inc., Chicago). The number of bacterial colony forming units was evaluated by one-way ANOVA (p<0.05). When significant differences were detected Fisher's Least Significant Difference (LSD) was performed.

7.3. Results

7.3.1. Effect of different *M. incognita* inoculum densities on lettuce cv. Milan

In a greenhouse bioassay, pots containing lettuce plants were inoculated with different number of *M. incognita* J2 and the gall index was determined 4 weeks after nematode inoculation. This assay revealed that the gall index made by *Meloidogyne incognita* increased from 4.0 when 1000 nematodes were inoculated to 6.8 when 7000 nematodes were inoculated (Figure 1). The non-linear regression analysis showed a significant relationship, with a correlation coefficient (r^2) of 0.97, between increasing nematode inoculum densities and a higher gall index.

Shoot fresh weight was significantly reduced from 36 to 29 g in the treatments with 0 and 7000 nematodes inoculated (Figure 2). The linear regression analysis showed a significant relationship with r^2 value of 0.91, between the increase nematode inoculum densities and the reduction in shoot fresh weight. In addition, root fresh weight was significantly reduced by the different inoculation treatments from 10 g in the non-inoculated control to 8.8 g when inoculated with 7000 nematodes. However, the root weight was less affected than the shoot weight and only showed an r^2 value of 0.51.

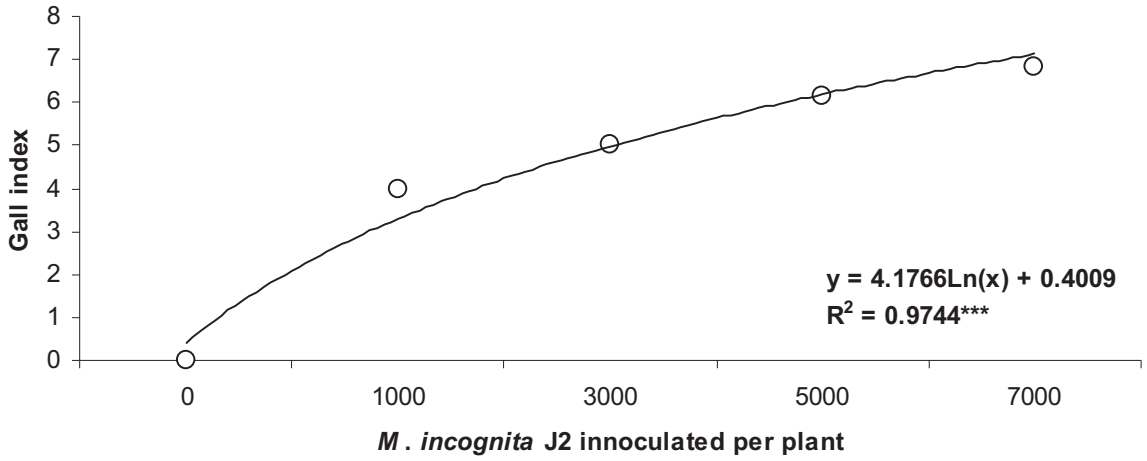

Figure 1. Effect of different amount of *Meloidogyne incognita* J2 inoculum on gall index of lettuce cv. Milan evaluated 4 weeks after nematode inoculation. N = 6. ***$P < 0.001$.

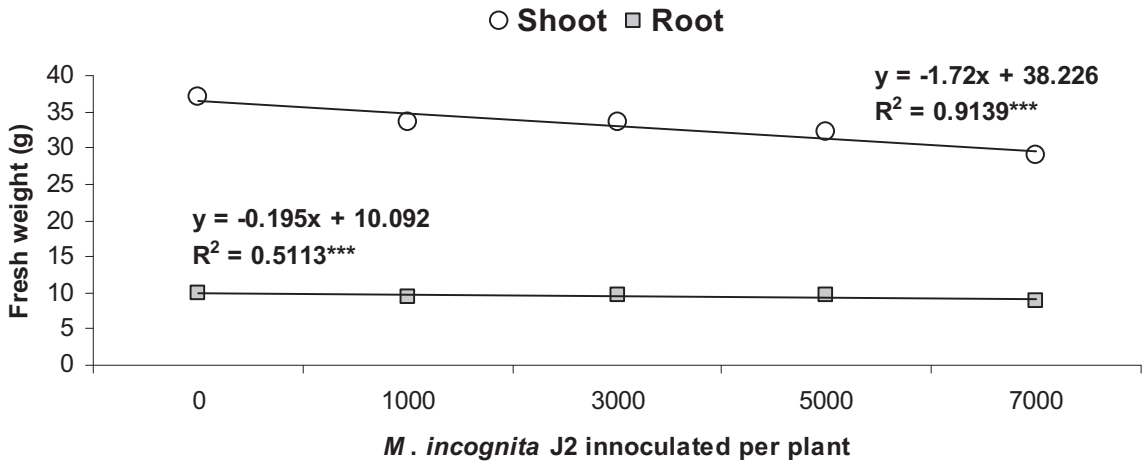

Figure 2. Effect of different amounts of *Meloidogyne incognita* J2 inoculum on shoot and root fresh weight of lettuce cv. Milan 4 weeks after nematode inoculation. N = 6.

7.3.2. Effect of plant enhancers on soil bacteria, *Meloidogyne incognita* and biodegradation

7.3.2.1. Effect on soil bacteria population densities

The amount of soil bacteria present in S4, a soil that does not contain fenamiphos degrading bacteria, was determined at 129 CFU × 10^5 per gram of soil previous the application of the plant enhancers or nematicides. One week after soil treatment, the enhancers MagicWet®, Oscorna®, Azet® and Diterra® had increased the bacterial population densities to over 1000

$\times 10^5$ CFU per gram of soil, which was significantly different from the untreated control soil with 222 $\times 10^5$ CFU per gram soil (Figure 3). The treatments with chicken manure and nematicides did not increase the bacterial population densities compared to the control. Three weeks after treatment, the bacterial populations had been reduced for all treatments including the control. Nevertheless, the plant enhancers Oscorna®, Azet® and Terrapy®, and the biological nematicide Diterra®, still resulted in higher bacterial densities, which were significantly different from the control. The bacterial populations densities in soil treated with chicken manure, compost and Nemacur® were never significantly different from in the untreated control.

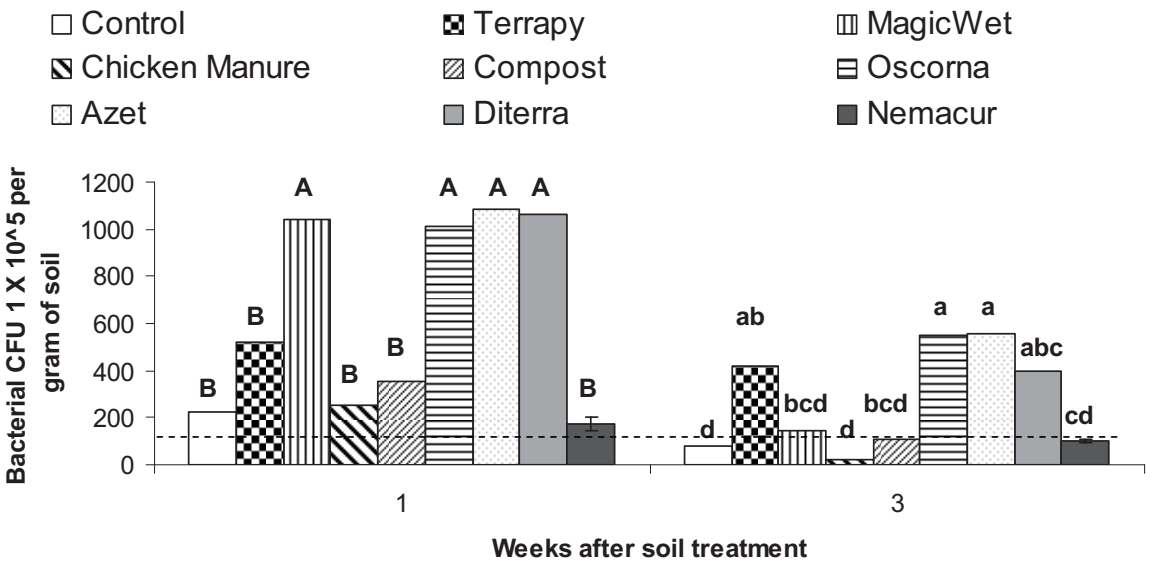

Figure 3. The average number of bacterial colony forming units (CFU) per gram soil present in S4, a soil with no fenamiphos degrading microorganisms, 1 and 3 weeks after application of one of the different liquid or granular plant enhancers or nematicide. Average number of CFU one day before soil treatment (---). N =3. ($p < 0.05$).

7.3.2.2. Effect on *M. incognita* early root penetration

The effect of plant enhancers on the penetration of *M. incognita* was determined. Therefore, pots containing lettuce plants were inoculated with different number of *M. incognita* eggs after the soil had been amended with the different treatments. The *M. incognita* early root penetration was determined and compared to the non-treated control. This evaluation showed that two plant revitalizers reduced root penetration of *M. incognita*. Linear regression analysis showed that Azet® application increasingly reduced ($r^2 = 0.94$) root penetration of the root-knot nematode from 5%, when the seeds were sown immediately after the soil treatment

(week 0), to over 50%, when the seeds were sown four weeks after soil treatment (week 4) Figure 4. MagicWet® application reduced ($r^2 = 0.99$) *M. incognita* penetration from 48%, at week 0, to 18% at week 4. The other plant enhancers showed no significant effect.

The treatment with chicken manure interfered with the germination of the lettuce seeds and therefore, no results were obtained. Two nematicide control treatments showed the highest nematode control. The chemical nematicide Nemacur gave the highest nematode control reducing over 70% the *M. incognita* root penetration at week 0, 1 and 4. The biological nematicide Diterra® gave a similar effect in nematode reduction when compared to Nemacur, at week 0, but the efficacy was reduced to 50% in week 4. In the fourth week of evaluation the plant enhancer Azet® gave similar nematode control effect as the biological nematicide Diterra®.

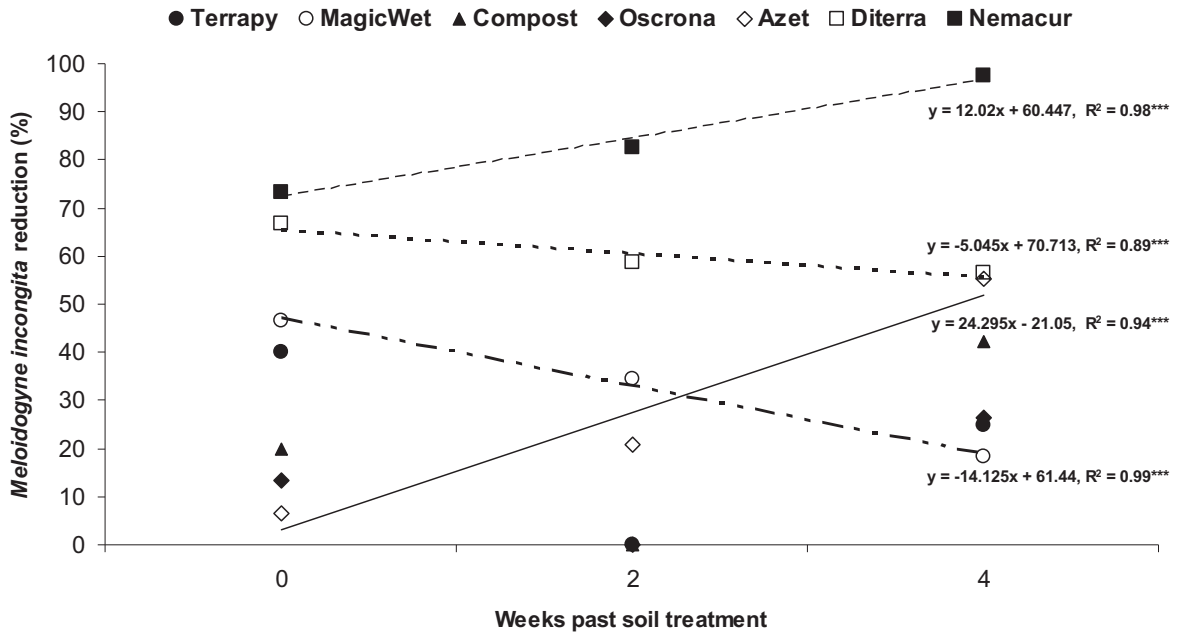

Figure 4. Effect of different liquid or granular plant enhancers at different time periods on *M. incognita* early root penetration in lettuce cv. Milan evaluated 20 days after nematode inoculation. N = 4. ***$P < 0.01$.

7.3.2.3. Effect on enhanced biodegradation of fenamiphos

Soil S3, a soil which contained fenamiphos-degrading microorganisms, was used to determine whether the plant enhancers could delay or stop the nematicide biodegradation process. Therefore, this soil was exposed to one of the plant enhancers or one of the nematicides for one week. Subsequently the microorganisms present in S3 were isolated and the overall fenamiphos degrading capabilities of the microorganisms was determined by HPLC analysis. This showed that the microorganisms of the untreated biodegrading-control

needed 6 days to totally metabolize fenamiphos in soil extract liquid medium (Figure 5). The biodegradation of fenamiphos took 10 days when the soils had been treated with Oscorna®, Azet®, Compost®, MagicWet® and Diterra®. The complete biodegradation of fenamiphos took 14 days when Terrapy® was applied. Apparently, the tested plant enhancers can affect the biodegradation capabilities of the microorganisms of S3.

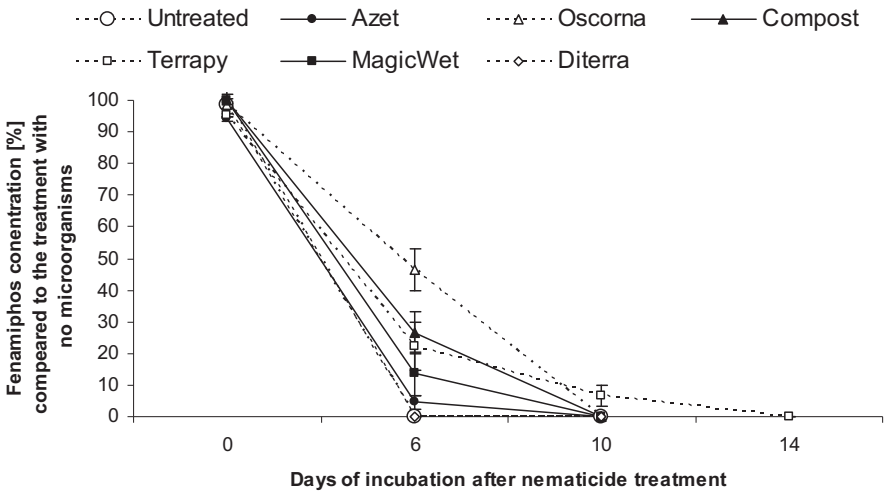

Figure 5. Effect of different the different liquid or granular plant enhancers, MagicWet®, Oscorna®, Azet® and Diterra®, compost, and the nematicides, Terrapy® and Diterra® on the fenamiphos biodegrading capabilities of S3, a soil containing fenamiphos degrading microorganisms, determined by HPLC analysis. N = 3. Bars indicate the Standard Error of the Mean.

7.4. Discussion

In this research, lettuce cv. Milan showed to be highly susceptible to *Meloidogyne incognita*. In addition, shoot and root growth parameters diminished with increasing nematode inoculum density. These results were expected since a similar susceptibility to root-knot nematodes has been reported for a large number of other lettuce cultivars (Wilcken et al., 2004) and similar amounts of root-knot J2 in soil have shown to reduce plant growth parameters in other lettuce varieties (Manoj and Pathak, 2005).

In this research, it is demonstrated that the plant enhancers MagicWet®, Oscorna® and Azet® and the biological nematicide Diterra® significantly increased bacterial population densities in the soil compared to the untreated control soil 1 week after application. This increase in bacterial population density was still visible three weeks after the application of

Oscorna® and Azet®. Also Terrapy® and Diterra® still showed a significant accumulation of bacteria. These results confirm that plant enhancers can increase the bacterial populations rapidly to about 5 times of its original amount, perhaps by providing large amounts of extra nutrients that soil microorganisms can use to proliferate. These results are in accordance with those of Mulawarman et al. (2001) who reported that some of these commercially available plant enhancers, such as Terrapy® and MagicWet®, stimulate soil microbial activity. In a previous study, Diterra® showed to stimulate, fungi and bacteria in soil and rhizosphere over time (Fernandez et al., 2001.). Moreover, even though the response of microbial communities depends on different factors, such as the amendment composition (Pereq-Piqueres et al., 2006), it could be expected that the effect of the plant revitalizers on microorganisms would be similar in different soils since the plant enhancers are uniformly produced.

The two plant enhancers that reduced the root infection of *M. incognita* to lettuce roots were Azet® and MagicWet®. Azet® gave an increasing nematode reduction, over time, that ended with similar levels of control as that given by the biological nematicide Diterra®. This suggests that Azet® can enhance the antagonistic potential of the soil for biological control of *M. incognita* by enhancing beneficial microorganisms over time. MagicWet® reduced *M. incognita* penetration to 50% immediately after treatment but its effectiveness was reduced significantly 2 and 4 weeks after application. These results confirm that MagicWet® application stimulates soil microbial activity and thereby the antagonistic potential in soils leading to a reduction in nematode infestation as previously reported by Mulawarman et al. (2001). However, its effect on root penetration is not maintained for a long period of time. This is the first time that Azet® is found to reduce nematode penetration and it is believed that this biocontrol activity is similar to that of MagicWet®. However, the biocontrol activity of Azet® appears to increase continuously over time and therefore it may be important to evaluate its effect over a longer time period. Moreover, it was believed that the other plant revitalizers would not affect nematode root penetration because the microorganisms involved in this process do not utilize or profit from their formulations.

The most effective treatment for controlling *M. incognita* penetration was still the chemical nematicide Nemacur, with fenamiphos as active ingredient. The high efficacy of fenamiphos can be explained, since it is well known that it inhibits nematode movement and suppresses the hatching of nematode eggs (Van Gundy and Mckenry, 1977). However, the efficacy of fenamiphos could rapidly deteriorate when the soil is frequently treated with fenamiphos for an extensive period since then adapted fenamiphos degrading microorganisms may

accumulate (Ou and Rao, 1986; Ou, 1991; Ou et al., 1994; Pattison et al., 2000; Stirling et al., 1992).

It was shown that the application of plant revitalizers delayed the biodegradation of fenamiphos. In particular, the application of the liquid plant enhancer Terrapy® delayed the complete metabolization of fenamiphos by a factor of 2.5. The previous two tests showed that plant revitalizers can stimulate specific bacterial population densities. Perhaps the non-biodegrading microorganisms living in S3 increased substantially with Terrapy® treatment while the degrading ones were suppressed. The actual mode of action that supports such a specific repression is however difficult to explain at the current moment, but it may be that fungi, with their more restricted biodegrading activity, become the more dominating group in the soil as a consequence of the Terrapy® treatment. Fungi from this soil lacks in fenamiphos degrading ability as shown in the Chapter 5 of this thesis supporting this theory. However, even though Terrapy® delayed the complete metabolization to 14 days, it is still not enough for managing the enhanced biodegradation process under field conditions. A nematicide should remain effective in soil for the first 6 weeks after application in order to provide adequate nematode control supporting plant development. From the current alternatives known today to slow down the biodegradation process one is to not apply nematicides in a period of over 12 months (Anderson et al., 1992). However, this is unpractical in intensive agriculture. Therefore, the alternative of nematicide rotation remains as an essential strategy for the management of enhanced nematicide biodegradation (Karpouzas and Walker, 2000b). Furthermore, the results in this thesis suggest that rotating the use of nematicides together with the application of natural plant enhancers, such as Azet, could also further suppress nematode root penetration and delay the onset of enhanced biodegradation.

In conclusion, lettuce cv. Milan was highly susceptible to *M. incognita* but the root-knot nematode damage can be reduced by the application of natural plant enhancers such as Azet® and MagicWet®. The plant enhancers increased soil bacteria populations providing a biocontrol activity and delayed the total metabolization process of fenamiphos.

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