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.

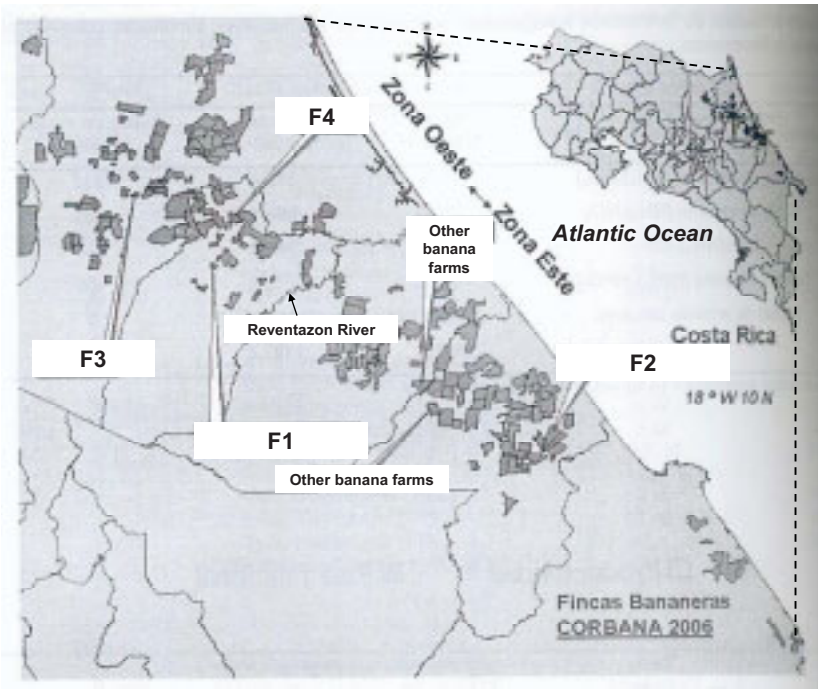

Figure 1. Enlargement of the Atlantic region of Costa Rica showing fields with banana cultivation in the area (dark gray blocks). Collection of soil and roots was performed in the four commercial banana plantations indicated as F1, F2, F3 and F4. Map of Costa Rica (above right). This map was adapted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