Applied Soil Ecology 43 (2009) 206–215

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Effects of soil type, management type and soil amendments on the survival of the potato brown rot bacterium *Ralstonia solanacearum*

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ARTICLE INFO

Article history:
Received 5 June 2009
Received in revised form 23 July 2009
Accepted 27 July 2009

Keywords:
Organic
Conventional
NPK
Compost
Cow manure
Biodiversity

ABSTRACT

Potato brown rot disease (*Ralstonia solanacearum*) is a serious economic problem in Egypt, partly due to an European Union requirement that potatoes for export to the EU should be grown in so-called pest free area's (PFA's), where fields are tested and infested fields are put under quarantine measures. To investigate pathogen survival and to determine the time required to keep infested fields in quarantine, the survival of *R. solanacearum* race 3 biovar 2 was tested in soils differing in origin (Dutch versus Egyptian soils), soil type (sand versus clay), and management type (organic versus conventional). All eight soils were tested at moderate (15 °C) and elevated temperatures (28 °C). Also the effects of artificial fertilizer and organic (compost and cow manure) amendments on survival of *R. solanacearum* were tested.

In all soils, with and without amendments, the pathogen dropped below the detection limit (10^2 CFU g^{-1} d.w. soil) within 5 months. At both temperatures, all Egyptian soils showed a significantly faster decline in pathogen density than the Dutch soils. The decline in colony forming units of *R. solanacearum* per gram of soil was faster in sandy soils than in clay soils from both countries. Management effects on decline of *R. solanacearum* were smaller and less consistent: for some soils, organic management resulted in a significantly shorter 50%-reduction-time and/or greater decline rate than conventional management, for other soils the differences were not significant. Survival periods at 15 °C were longer than at 28 °C in Dutch soils, but not in Egyptian soils, where survival was slightly shorter at the lower temperature. Amendments with NPK fertilizer to the conventional soils and with cow manure to the organic soils enhanced the decline rate of *R. solanacearum* in these soils. The decline rate of the pathogen was negatively correlated with total soluble organic matter and positively with bacterial diversity. In conclusion, the overriding factors determining survival of *R. solanacearum* in soil may be the production of toxic concentrations of ammonia on the one hand, and availability of substrate in combination with microbial competition on the other hand. The sandy desert soils of Egypt are very suitable for production of export potatoes because the pathogen would survive for only a relatively short period in those soils, if it were accidentally introduced. Addition of ammonia-producing amendments can reduce populations of *R. solanacearum*, whereas compost addition and organic management do not necessarily result in an enhanced decline of the pathogen.

1. Introduction

Potato brown rot is an important bacterial disease world-wide, caused by *Ralstonia solanacearum* (Smith) Yabuuchi (Yabuuchi et al., 1995). In temperate climates and in cooler, mountainous areas in the tropics, race 3 biovar 2 is the most prevalent race of *R. solanacearum* infecting potato. This race was reported for the first time from the Mediterranean area in the 1940s, and became established in the Nile Delta area of Egypt, causing severe yield losses. From the Mediterranean area the pathogen spread in the late 1980s to temperate regions of North-western Europe, where

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doi:10.1016/j.apsoil.2009.07.008

contamination of surface water led to introduction into the production of potato (Janse, 1996; Persson, 1998; Schans and Steeghs, 1998; Wenneker et al., 1999). *R. solanacearum* is under eradication measures in the European Union (European Communities, 1998) and nowadays occurs in the EU sporadically in potato. Nevertheless, it is still considered a quarantine pathogen. Therefore the EU has set strict importation requirements: for example Egypt (European Communities, 2005) these encompass the exclusion of the Nile Delta area where *R. solanacearum* is endemic for exports, production of potatoes destined for the EU only in approved pest free area's (PFA's) – mainly in the desert – and an import ban after 5 interceptions of brown rot. Thus, potato brown rot became an important economic problem for Egypt.

Since the last 10 years, there has been an increasing demand for organically produced products (Sylvander and Le Floc'h-Wadel, 2000; Parrott and Kalibwani, 2004). Under organic management, conventional inputs such as synthetic pesticides and fertilizers are excluded, while compost and animal and green manures are used to build up soil fertility (Reganold et al., 2001). Organic soils generally have a higher biological activity and diversity (van Diepeningen et al., 2006) and soil-borne plant pathogens are frequently suppressed in organically managed soils compared to conventionally farmed land (van Bruggen, 1995; van Bruggen and Termorshuizen, 2003).

Messiha et al. (2007a) have shown that the incidence of *R. solanacearum* infections starting with an inoculum of 10^7 CFU g^{-1} is the highest in soils high in dissolved organic carbon (DOC), whereas higher K and Ca contents seem to make the plants more resistant. In Egyptian organically managed sandy soils a slight suppression of the disease was observed when compared to conventional managed soils. In contrast with this, in the relatively rich organic soils from the Netherlands, disease incidence was higher in organic compared to conventional management. Amendment with NPK fertilizer of conventionally managed soils or with cow manure (but not with compost) of organically managed soils suppressed the disease in most soils (Messiha et al., 2007a). However, about the persistence of *R. solanacearum* in different soils and the effects of different management treatments on pathogen survival little is known, while for a farmer facing quarantine measures reducing persistence of the pathogen through optimal management is crucial.

In New South Wales in Australia survival of *R. solanacearum* race 3 biovar 2 in soil was shown to be up to 3 years (Graham et al., 1979); in Europe its survival appeared to last at least 1 year (van Elsas et al., 2000). Under laboratory conditions, survival of *R. solanacearum* in and on various substrates other than soil varies from only 4 to 87 days (Janse et al., 1998). Survival *in vitro* and virulence of *R. solanacearum* is optimal at temperatures between 24 and 35 °C (Pradhanang and Elphinstone, 1996; Pradhanang et al., 2000). Severe drought negatively affects *R. solanacearum* survival and thus, its survival period positively correlates with wet and poorly drained soils (van Elsas et al., 2000). As soil quality, including waterinfiltration, is often better in soils under organic compared to conventional management (van Bruggen, 1995; van Bruggen and Termorshuizen, 2003), it would be of interest to compare pathogen survival in organic and conventional soils.

Some simple and cost-effective management strategies have been tested for their effect on survival of *R. solanacearum*: soil amendment with compost, pig slurry or manure had a positive effect on pathogen decline and disease suppression (Gorissen et al., 2004; Hoitink and Boehm, 1999; Schönfeld et al., 2003). However, the suppressive effect of soil amendment on the survival of *R. solanacearum* may depend on soil type (Michel and Mew, 1998).

The aim of the current study was to test the suppressive ability of differently managed soils on the survival of *R. solanacearum* race 3 biovar 2 and to investigate the effects of fertilizer and different organic amendments on this decline. Therefore, survival of *R. solanacearum* was monitored in several conventionally and organically managed soils from both European and Egyptian origin. Furthermore, two types of soils were investigated per country: sandy and clayey soils, and all experiments were done both under moderate (15 °C) and high (28 °C) ambient temperatures. Different amendments were applied to the conventional and organic soils and their effects on the survival of *R. solanacearum* were investigated. Finally, the relationship was investigated between pathogen survival and chemical characteristics and biodiversity of the different soils.

2. Materials and methods

2.1. Soils

The International Federation of Organic Agriculture Movements (IFOAM), defines "Organic agriculture is a production system that sustains the health of soils, ecosystems and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved". Under organic management, conventional inputs such as synthetic pesticides and fertilizers are excluded, while compost and animal and green manures are used to build up soil fertility (Reganold et al., 2001), here we define our soils being under conventional management as (occasionally) synthetic pesticides and fertilizers are used. Four pairs of soil, half from organically managed arable farms and the other half from conventionally managed arable farms were used in this study. Two pairs were sandy soils; the other pairs were clayey soils. Two pairs originated from Egypt, the others were from the Netherlands (Table 1). All Egyptian soils were from known potato fields, while the Dutch soils were from fields that were either fallow, under grass, planted with seed potato or onions. These soils were sampled three times, in June and July 2003, and in March 2004,

Table 1
Origin of soil samples.

Sample	Code[a]	Country	Soil type	Management	Cover crop at time of sampling	Location
1	ESC	Egypt	Sand	Conventional	Potatoes were just harvested	Nubaria, Desert area
2	ESO	Egypt	Sand	Organic	Potato	Nubaria, Desert area
3	ECC	Egypt	Clay	Conventional	Beans after the potatoes	Behera, Delta area
4	ECO	Egypt	Clay	Organic	Potatoes	Kaliobia, Delta area
5	NSC	NL	Sand	Conventional	Unplanted	Marknesse, Northeast polder
6	NSO	NL	Sand	Organic	Grass	Marknesse, Northeast polder
7	NCC	NL	Clay	Conventional	Seed potato	Ens, Northeast polder
8	NCO	NL	Clay	Organic	Onions	Ens, Northeast polder

[a] The first letter stands for the country of origin (Egypt or the Netherlands), the second for soil type (clay or sand) and the third for management type (conventional or organic).

respectively. Each time 25 kg samples were collected from 10 random sampling points in 100 m^2 plots from 15 to 25 cm vertical depth with an auger and mixed. Samples were transported in plastic bags in containers with ice. Upon arrival in the laboratory each soil sample was thoroughly mixed and sieved through a ∅ 0.5 cm mesh sieve and any plant parts or earthworms were removed. The soils were stored at 4 °C till the start of the experiment. The Egyptian soils were sent to the Netherlands in plastic bags in Styrofoam boxes by overnight express airmail (Messiha et al., 2007a).

2.2. Soil inoculation, amendments, and incubation

Three replicate 100 g samples from each soil were infested to a final concentration of 5×10^7 CFU g^{-1} d.w. soil with a mixture of equal amounts of three virulent strains of *R. solanacearum* race 3 biovar 2, originally isolated from infected potato tubers from Egypt: PD 5239, PD 5240 and PD 5241 (culture collection Plant Protection Service, Wageningen, the Netherlands) (Messiha et al., 2007b). These strains can all use nitrate as electron acceptor under anaerobic conditions. The cultures used for soil infestation were grown for 48 h on nutrient agar (NA) plates at 28 °C. The bacterial culture was suspended in 0.01 M phosphate buffer (PB) and the bacterial density was adjusted using a spectrophotometer (OD_{600} = 2.1 equals 5×10^9 CFU ml^{-1}) (Messiha et al., 2007a). Soil samples were kept in 50 ml Greiner tubes with loosely closed lids to allow the exchange of air. The moisture content varied for each soil, but was kept at about field capacity: water loss was checked weekly and any lost water was replaced to keep a constant moisture level.

The effect of different soil amendments on survival of *R. solanacearum* was tested in one experiment with freshly collected soil (March 2004). Soils were left non-amended or supplemented with about 170 kg N ha^{-1} (0.05 g N kg^{-1} soil), the maximum amount allowed according to EU regulations. Conventional soils were amended with NPK fertilizer (12% N = 7% ammonium + 5% nitrate; 10% P; 18% K; 0.9 g kg^{-1} soil). Organic soils were amended with compost (of which the parent material consisted of wood chips (88%), manure (2.5%) and clay (10%), 17 g kg^{-1} soil, with 0.61% of total N content) or manure (organic cow manure, 21.7 g kg^{-1} soil with 0.48% of total N content) or left non-amended.

The survival experiments with the different soils were conducted twice at two different temperatures, 28 and 15 °C, to approximate temperature conditions in subtropical and temperate climates, respectively. In a separate set of experiments the effects of amendments were tested at 15 °C only.

2.3. Sampling

The pathogen was monitored twice a week for the first 2 weeks, starting at the day of inoculation (T_0), once a week for the next 2 weeks and then once a month for a total of 5 months until the pathogen population was below the detection limit (100 CFU g^{-1} d.w.) in all soils.

For bacterial counts, 1 g samples of the soils (1 per soil replicate) were suspended in 9 ml of sterile 0.05 M phosphate buffer. After shaking at 100 rpm for 2 h at 20 °C, 10-fold serial dilutions were made on modified SMSA (Selective Medium South Africa) agar plates (Anon., 1998) in triplicate and incubated at 28 °C for 5–7 days. Per sample three colonies typical for *R. solanacearum* (colony with irregular shape, diffuse white or purple centers and luxuriant slime) were tested using Immunofluorescence Antibody Staining, IFAS (Janse, 1988). Total CFUs were calculated per gram dry soil.

For DNA analysis, 1 g soil samples were taken at the beginning and end of each experiment. These samples were stored at −20 °C for DNA extraction within 6 months.

2.4. Physical and chemical analysis

For physical and chemical analysis 100 g samples of non-amended and amended soils were dried to air-dry at room temperature. Fractions of different soil particle sizes were assessed in the Laboratory of Soil Science and Geology of Wageningen University. Particle sizes of <2 μm were considered clay, 2–50 μm was considered silt and 50–2000 μm was considered sand.

The soil samples were dried and chemically analyzed. $N\text{-}NO_3$ and $N\text{-}NH_4$, were extracted in 0.01 M $CaCl_2$ and total P was extracted in 0.5 M H_2SO_4 before spectrophotometric analysis with

Table 2
Physical and chemical composition of the different amended and non-amended soils.

Soil + amendment	Soil composition[a]			Nutrients (mg/kg)								pH-KCl	%OM[b]
	Clay%	Silt%	Sand%	K	Ca	Na	P	N	DOC[c]	$N\text{-}NO_3$	$N\text{-}NH_4$		
ESC[d]	0.1	0.6	99.3	601	9,615	168	123	315	63.1	14	5.4	7.9	<0.2
ESC + NPK				976	11,220	439	177	321	52.6	37	12.8		
ESO	0.1	0.6	99.3	964	24,458	398	112	286	54.4	10	0.62	8.2	0.3
ESO + compost				969	24,818	387	131	349	72.8	10	0.82		
ESO + manure				943	25,354	307	118	282	54.2	10	0.21		
ECC	9.4	54.5	36.1	3827	4,444	914	612	1080	133.2	128	19.2	7.6	4.9
ECC + NPK				3995	4,639	966	664	1119	125.7	192	17.5		
ECO	13.9	65.7	20.4	4684	10,506	1557	1031	1208	79.9	62	16.3	7.6	6.2
ECO + compost				4835	8,270	1387	1192	1246	82.1	62	13.7		
ECO + manure				5316	23,924	1051	1186	1259	66.1	62	11.0		
NSC	3.2	32.3	64.5	3118	9,113	1403	426	924	131.3	10	12.2	7.4	1.5
NSC + NPK				3125	8,894	1166	476	905	128.6	92	24.2		
NSO	3.2	33.3	63.5	3125	12,620	1022	567	1375	137.3	17	8.5	7.1	4.1
NSO + compost				3053	12,087	662	653	1622	175.4	22	9.3		
NSO + manure				3250	12,500	863	592	1386	196.7	31	6.9		
NCC	7.7	51.9	40.4	6888	21,259	1093	935	1107	122.1	10	13.9	7.4	2.2
NCC + NPK				7005	21,618	954	1003	1144	122.3	94	14.5		
NCO	8.3	54.5	37.2	6281	22,044	1034	712	1058	125.7	7	15.8	7.3	2.3
NCO + compost				6388	22,482	1057	764	1182	135.1	13	11.7		
NCO + manure				6522	21,739	870	735	1201	155.3	5	9.9		

[a] Clay particles size < 2 μm, silt 2–50 μm, sand 50–2000 μm.
[b] Organic matter.
[c] Dissolvable organic carbon.
[d] See Table 1 for the soil codes.

a Segmented Flow Analyzer (Skalar Analytical BV, Breda, the Netherlands). Total N and dissolvable organic C (DOC) contents were determined with a CHN1110 Element Analyzer (CEInstruments, Milan, Italy). Inorganic carbon is excluded from the measurements by acidification of the extract with sulphuric acid and the produced carbon dioxide was driven out of the system by N_2. The extract was then digested under UV light in persulphate and tetraborate. The organic carbon was acidified into carbon dioxide then mixed with H_2 (Houba et al., 1999). The pH-KCl of the different soils was determined as well. Percent organic matter, K, Ca, and Na contents of the soils were determined at the BLGG laboratory (Oosterbeek, the Netherlands).

The Egyptian soils had higher pH levels than the comparable Dutch soils (Table 2). In general, the Ca content was higher in organic than in the conventional soils, whereas N-NO_3 and N-NH_4 tended to be higher in the conventional soils. The Egyptian sandy soils were the poorest in soluble total organic carbon (DOC), potassium, sodium, total phosphorous and total nitrogen. The DOC in Dutch soils was approximately two times as high as that in the Egyptian soils.

2.5. DNA extraction and PCR amplification

Total DNA was extracted from 0.5 g (wet weight) soil samples with the Bio101 FastDNA® SPIN Kit for Soil, according to the manufacturer's specifications (Bio101, Carlsbad, CA, USA), a 20 min incubation time at 65 °C was added to enhance the elution. DNA quality and quantity was checked on 1.2% (w v^{-1}) agarose gel in 0.5 × Tris–borate (TBE) buffer (0.045 M Tris–borate, 0.001 M EDTA) (Sambrook et al., 1989), stained with ethidium bromide and visualized by UV trans-illumination.

For DGGE analysis of the eubacterial soil population, the V6 to V8 region of the 16S rRNA gene was amplified from total soil DNA with the primers 968f-GC and 1401r (Heuer and Smalla, 1997). Two nanograms of DNA was added to 50 μl PCR reactions and amplified using the touchdown scheme of (Rosado et al., 1998) with small modifications (Hiddink et al., 2005). The PCR products were examined by standard gel electrophoresis using 1.2% (w v^{-1}) agarose in 0.5 TBE, ethidium bromide staining and UV trans-illumination, to confirm product integrity and to estimate yield. The expected product size was approximately 450 bp (Duineveld et al., 2001). Depending on the soil type and temperature up to 500 ng of DNA was extracted from 500 mg of soil.

2.6. DGGE and bacterial diversity

Per survival experiment, the microbial biodiversity in each soil was determined for two soil samples per treatment (at the beginning and end of each experiment), each sample on duplicate gels. Thus, per non-amended or amended soil two soil samples were taken for DNA extraction and subsequent denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes amplified from 2 ng of the extracted DNA. DGGE was performed using the DCode system (Bio-Rad Laboratories, Hercules, CA, USA). Gels were prepared with 6% acrylamide (ratio 37.5 acrylamide:1 bisacrylamide) with a 45–60% denaturing gradient (Muyzer et al., 1996) to separate the amplicons generated (100% denaturant is 7 M urea and 40% formamide) and an 8% acrylamide stack without denaturing agents. The gels were poured from the top in the Dcode template, prepared with Gelbond PAG film (Amersham Pharmacia Biotech AG, Uppsala, Sweden) to one side, using a gradient maker and a Heidolph Pumpdrive (Heidolph, Schwabach, Germany) set at 4 ml min^{-1}. Electrophoresis was performed in 0.5 × TAE buffer for 16 h at 100 V at a constant temperature of 60 °C. Twenty microliters of the PCR products was used for the DGGE. As DGGE marker a mixture of V6–V8 fragments of 9 different bacteria isolated from the human gut was used. This marker was provided by Dr. Hans Heilig, Department of Microbiology, Wageningen University and Research Center, the Netherlands. All samples were analyzed at least in duplicate.

Gels were stained with Bio-Rad's Silver Stain (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol, but using the protocol for gels > 1 mm thick instead of 0.5–1 mm, in order to compensate for the barrier formed by the Gelbond. After staining, the gels were preserved for at least 1 h in Cairn's preservation solution of 25% ethanol (v v^{-1}) and 10% glycerol (v v^{-1}), covered by a permeable cellophane sheet (Amersham Pharmacia Biotech Ag, Uppsala, Sweden) and dried overnight at 60 °C. The gels were scanned using ScanSoft Omnipage, pro. 14 at a resolution of 300 dpi.

Scanned gels were analyzed with Phoretix 1D (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). Only bands with pixel intensity above one were included in the analysis. Data of different DGGE gels were standardized by referring to the DGGE marker. The 16S rDNA fragments detected by DGGE were considered to represent dominant bacterial groups, making up at least 0.1–1% of the total community (Muyzer et al., 1996).

The bacterial diversity in the samples was estimated in two ways: as species richness S, and as the Shannon–Weaver index of bacterial diversity, H'. Species richness S was determined by the number of DGGE detected bands per soil type (van Diepeningen et al., 2006). The Shannon–Weaver diversity index was calculated as $H' = -\sum\{P_i \log P_i\}$ based on the relative band intensities as formulated by Eichner et al. (1999). P_i is defined as n_i/N where n_i is the area of the peak in intensity and N the sum of all peak areas in the lane profile. Both biodiversity indices are given as averages of the two samples of each experiment (beginning and end of the experiment), with two gels per sample.

2.7. Statistical analysis

Microbial data (the CFU counts) of the pathogen were log-transformed and each replicate per soil and treatment fitted to a standard logistic model with an asymptote as described by (Franz et al., 2005)

$$C_t = \frac{a_m}{1 + e^{(-d \times (t-c))}}$$

where C_t = log-transformed number of bacteria at time t (days), a_m = upper asymptote (level of inoculation), d = the slope at the inflection point ($days^{-1}$) which is referred to as decline rate (the absolute decline rate is given by $[a_m \times d]/2$) and c = the position of the inflection point (days) which is referred to as 50%-reduction-time. To avoid the situation of a standard 50% decline in initial pathogen count when $c = 0[C(0) = a_m/2]$, $t = 0.1$ instead of $t = 0$ was used as first point in the time series (so that c could never be 0). The lower asymptote of this model is zero. Where parameter d gives only information on the maximum decline rate (slope at point of 50% reduction), parameter c gives information on the overall survival time. The value of c depends on the decline rate and the presence of an initial shoulder. The goodness of the data fitting to the model (R^2) was calculated according to Franz et al. (2005). The estimated parameter values c and d for the different managed soils were subjected to multivariate analysis of variance (MANOVA, using SPSS v 12, SPSS Inc., Chicago, IL, USA) to detect differences between soil types and management strategies. Next, effects of soil types and origin on c and d separately were tested with ANOVA.

To determine the effect of different amendments and management on the survival of the pathogen, contrast analyses were conducted using SAS v. 9.1 (SAS Institute Inc., Cary, NC, USA).

Correlations between the chemical composition and the survival of the pathogen in different soils were tested with correlation analyses using SPSS v. 12.

For comparison of microbial communities among the 5 different amended soil classes (non-amended conventional, NPK amended conventional, non-amended organic, compost amended organic and manure-amended organic) within each soil type in each country, discriminant analyses were conducted per gel using SAS v. 9.1. For these analyses the ^{10}log-transformed band intensities were used. Analyses were done with the DISCRIM, CANDISC and STEPDICS procedures. Each data set was first split into small subgroups of 9 variables and the most significant discriminating variables were combined and subjected to the analyses again (van Diepeningen et al., 2006). A similar discriminant analysis was done to compare the four classes of soil: Egyptian sand, Egyptian clay, Dutch sand and Dutch clay. In addition, cluster analysis was carried out on the DGGE bands per gel (for example with samples from organic and conventional sandy and clayey soils from Egypt and the Netherlands) using the Phoretix software to compare the bacterial composition in the different soils.

For comparison of the biodiversity between soils from different origins and soil type, a paired *t*-test was conducted using SPSS v. 12. Difference in biodiversity for soils from different origins under the two different temperature regimes was studied also, using a paired *t*-test.

Correlation analyses were conducted between survival (*c* and *d*) and bacterial community (species richness *S* and bacterial diversity *H*) using SPSS v. 12, as described under 'DGGE and bacterial diversity'.

3. Results

3.1. Survival in organic and conventional soils at different temperatures

In all four pairs of soils (organic and conventional pairs of Egyptian and Dutch clays and sands) the log-transformed numbers of CFUs of *R. solanacearum* declined immediately after inoculation at both incubation temperatures. The log-transformed observed values fit well to the logistic decay model with asymptote, with R^2 values between 0.84 and 0.99 (Fig. 1). Here only the results of the first experiment are presented, because those of the second experiment were very similar.

Comparing *R. solanacearum* survival between Dutch and Egyptian soils, at both temperatures, the pathogen survived for longer period in the Dutch than in the Egyptian soils (MANOVA for 28 °C: Wilk's Lambda = 0.049, $P < 0.001$; for 15 °C: Wilk's Lambda < 0.001, $P < 0.001$). Looking at the fitted survival curves, in the Dutch soils, the 50%-reduction-time was longer (ANOVA for 28 °C: $F = 308$, $P < 0.001$; for 15 °C: $F = 1339$, $P < 0.001$) and the decline rate was lower (ANOVA for 28 °C: $F = 30$, $P < 0.001$; for 15 °C: $F = 78$, $P < 0.001$) than in the Egyptian soils. Among the Egyptian soils, the pathogen survived for a longer period in clay than in sandy soils (MANOVA at 28 °C: Wilk's Lambda = 0.024, $P < 0.001$; at 15 °C: Wilk's Lambda = 0.007, $P = 0.002$). Looking at the survival curves in Egyptian clay, the 50%-reduction-time was longer (ANOVA at 28 °C: $F = 5$, $P < 0.06$; at 15 °C: $F = 21$, $P = 0.002$) and the decline rate lower compared to sandy soil (ANOVA at 28 °C: $F = 275$, $P < 0.001$; at 15 °C: $F = 19$, $P = 0.002$). Similarly, the pathogen survived longer in Dutch clay than sandy soils at 15 °C (MANOVA: Wilk's Lambda < 0.001, $P < 0.001$), but not at 28 °C. At 15 °C, the 50%-reduction-time was longer (ANOVA: $F = 175$, $P < 0.001$) and the decline rate slower (ANOVA: $F = 41$, $P < 0.001$) in Dutch clay compared to sandy soil. In general, the survival periods at 15 °C were longer than at 28 °C in Dutch soils, but not in Egyptian soils, where survival was slightly shorter at the lower temperature.

Comparing management types, there was a significantly faster decline in log-transformed CFU of *R. solanacearum* in organically managed Egyptian sand and clay soils at 28 °C (Wilk's Lambda = 0.057, $P < 0.001$) than in conventionally managed soils, with a shorter 50%-reduction-time and faster decline rate (ANOVA for 50%-reduction-time: $F = 123$, $P < 0.001$ for decline rate: $F = 9$, $P = 0.017$). At 15 °C, the difference between management types was only significant for the Egyptian sandy soils (Wilk's Lambda = 0.017, $P = 0.002$), the 50%-reduction-time being shorter and decline rate faster in the organically managed sandy soil (ANOVA for 50%-reduction-time: $F = 55$, $P = 0.002$; for decline rate: $F = 21$, $P = 0.01$). For the Dutch soils, no significant differences between the management types were found at 15 °C, whereas at 28 °C, the log-transformed numbers of *R. solanacearum* declined more slowly in the Dutch organic sandy soil than in its conventionally managed counterpart (Wilk's Lambda = 0.008, $P = 0.047$), with a longer 50%-reduction-time and a lower decline rate (ANOVA for 50%-reduction-time: $F = 27$, $P = 0.007$; for decline rate: $F = 8$, $P = 0.047$). On the other hand, the pathogen population declined faster in the organic than in the conventional clayey soil from the Netherlands (MANOVA: Wilk's Lambda = 0.005, $P > 0.001$), with a shorter 50%-reduction-time (ANOVA: $F = 31$, $P = 0.005$) and a greater decline rate ($F = 11$, $P = 0.03$).

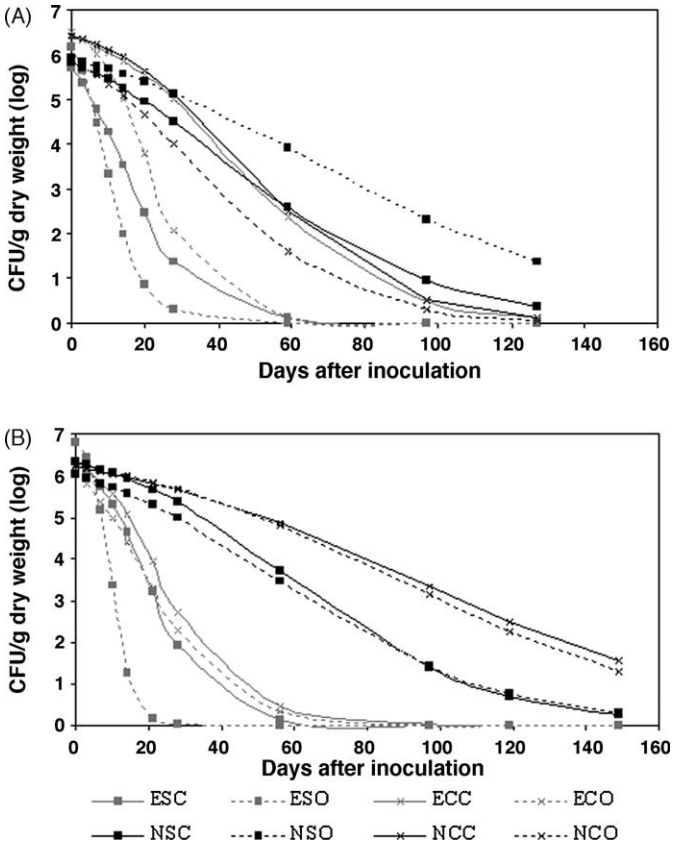

Fig. 1. Decline in density (CFUs g^{-1} dry soil) of *Ralstonia solanacearum* in different soil types with different management regimes at 28 °C (A) and 15 °C (B). The lines are the predicted values from the exponential decline model: $C_t = a_m/(1+e^{(-d\times(t-c))})$. Where C_t = log-transformed number of bacteria, a_m = initial count of the pathogen (asymptote), d = decline rate (days^{-1}), and t = time (days) and c = length of the lag phase in days (Franz et al., 2005). The R^2 for the fit of the data to the exponential model with asymptote were for the 28 °C curves: ESC = 0.96, ESO = 0.89, ECC = 0.99, ECO = 0.88, NSC = 0.96, NSO = 0.93, NCC = 0.99, NCO = 0.84 and for the 15 °C: ESC = 0.99, ESO = 0.98, ECC = 0.95, ECO = 0.97, NSC = 0.96, NSO = 0.93, NCC = 0.93, NCO = 0.89. For soil codes see Table 1.

Table 3
Average species richness S and Shannon–Wiener diversity index H' based on 4 measurements and their standard deviations for differently managed soils.

Temperature	Diversity index	Soil[a]							
		ESC	ESO	ECC	ECO	NSC	NSO	NCC	NCO
28 °C	S	10.3 ± 2.1	12 ± 2.9	21.3 ± 5.5	18.3 ± 5.5	14.8 ± 1.9	19.3 ± 9.5	18.8 ± 2.8	24.5 ± 0.7
	H'	0.94 ± 0.07	0.98 ± 0.09	1.23 ± 0.11	1.19 ± 0.13	1.07 ± 0.12	1.15 ± 0.12	1.11 ± 0.07	1.13 ± 0.01
15 °C	S	8.8 ± 2.6	14.3 ± 2.1	10.3 ± 2.1	21.5 ± 4.8	23.8 ± 6.9	24.5 ± 4.7	38.7 ± 7.3	37.3 ± 6.6
	H'	0.83 ± 0.17	1.02 ± 0.11	1.20 ± 0.16	1.18 ± 0.1	1.31 ± 0.12	1.32 ± 0.08	1.27 ± 0.28	1.74 ± 0.30

[a] See Table 1 for the soil codes.

3.2. Relation between microbial diversity and pathogen decline in organic and conventional soils

Based on DGGE measurements we calculated two values for the microbial diversity, average species richness S and Shannon–Weaver diversity H'. For all soils, incubated at 15 °C or 28 °C, the values for average species richness S and Shannon–Weaver diversity H' are given in Table 3 (again results from the first experiment only, as results obtained in the second experiment were very similar). The DGGE profiles of the organic and conventional soils from the different countries on which these indices were based clustered together (Fig. 2). The sandy soils had lower S and H' values than the clay soils (t-test, $P < 0.001$). Over all soils and temperatures the Dutch soils had higher S and H' values than the Egyptian soils (t-test, $P < 0.001$), though the ECC soil at 28 °C is clearly an exception. But, when the Dutch soils were incubated at higher temperatures (28 °C), they showed a remarkable drop in H' (t-test, $P < 0.001$) and S (t-test, $P = 0.005$), whereas the Egyptian soils did not show a significant difference for these two parameters between the two incubation temperatures. The organically managed Egyptian sandy soil had a higher H' than the conventionally managed soil incubated at 15 °C (t-test, $P = 0.038$). S was also significantly higher in organic than in conventional Egyptian sandy soil at the lower incubation temperature (t-test, $P = 0.003$), and in the organic than conventional Egyptian and Dutch clay soils at the higher incubation temperature (t-test, $P = 0.005$ and $P = 0.031$, respectively).

Canonical discriminant analysis (CDA) of the DGGE data resulted in a clear separation of the four soil types: Egyptian sand, Egyptian clay, Dutch sand and Dutch clay (data not shown). The analysis gave 100% correct classification according to canonical variable 1, with a Wilk's Lambda of 0.02 ($P = 0.0008$). The Dutch clay soils showed the largest statistical distance from the other soil types, while organic and conventional management were again grouped together per soil type and origin just like in the cluster analysis.

Correlating microbial diversity and the survival of *R. solanacearum*, negative correlations were found between S and the 50%-reduction-time (c) and the decline rate (d) ($r = -0.8$, $P = 0.04$ and $r = -0.9$, $P = 0.03$, respectively), within Egyptian sandy soils at 15 °C. A negative correlation between biodiversity and 50%-reduction-time (c) or decline rate (d) means that higher biodiversity is associated with a shorter shoulder (faster decline) or a more negative decline rate (also a faster decline because d has a negative value). Negative correlations were also found between S and c and d ($r = -0.9$, $P = 0.02$ and $r = -1$, $P < 0.001$, respectively) and between H and d ($r = -1$, $P < 0.001$) within Dutch clay soils at 28 °C. There were no significant correlations in all other cases (Table 4). Significant positive correlations were found between c and d with K, Na, total P, total N and DOC contents of the soils, respectively, meaning that with higher contents of those nutrients the decline of the pathogen was slower (Table 5).

3.3. Effects of soil amendments on pathogen survival

Comparing the survival of *R. solanacearum* under the different management regimes, contrast analysis revealed significantly shorter survival of the pathogen in organic than in conventional soils for the 50%-reduction-time ($P = 0.005$) but not for the decline rate (Fig. 3). The pathogen survived significantly shorter in NPK amended than in non-amended conventional soils ($P < 0.000$ and $P = 0.02$ for c and d, respectively). Addition of compost did not significantly increase the decline rate in the organic soils; in the Egyptian clay soil addition of the compost reduced the rate of decline of *R. solanacearum* (Fig. 3B) ($P = 0.023$ and $P = 0.034$ for c and d, respectively). The amendment with cow manure, however, did increase the decline rates in the organically managed soils significantly ($P = 0.003$ and $P = 0.005$ for c and d, respectively) (Fig. 3). The clearest differences between different amended soils were found for Egyptian sandy soils (Fig. 3A) and Dutch clay soils (Fig. 3D).

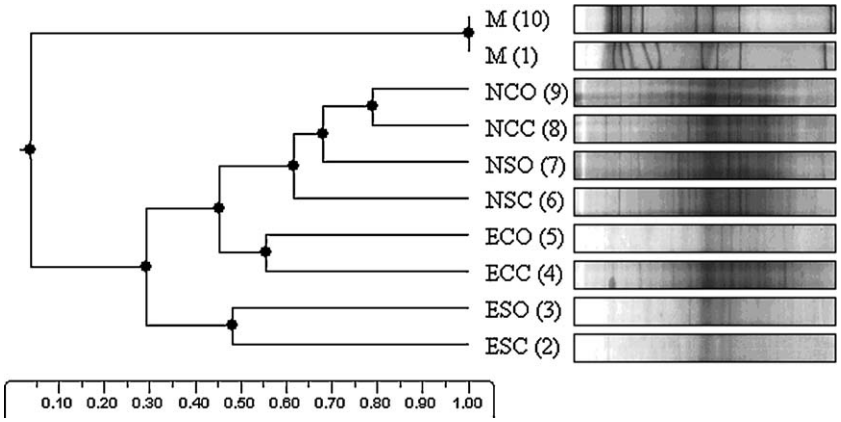

Fig. 2. Cluster analysis based on similarity (UPGMA) for 16S-rDNA PCR-DGGE products from different soils (Phoretix 1D, NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). The scale on the X-axis depicts similarity. The numbers between brackets indicate the lane numbers on the gel. For soil codes see Table 1.

Table 4
Correlations between the decline of *R. solanacearum* (lag phase *c* and decline rate *d*) and the bacterial species richness *S* and diversity *H*. A negative correlation means a faster decline at higher biodiversity. Significant correlations with a *P*-value < 0.05 are marked with *.

Decline	Egyptian sand (ES)				Egyptian clay (EC)				Dutch sand (NS)				Dutch clay (NC)			
	28 °C		15 °C		28 °C		15 °C		28 °C		15 °C		28 °C		15 °C	
	S	*H′*	*S*	*H′*	*S*	*H′*	*S*	*H′*	*S*	*H′*	*S*	*H′*	*S*	*H′*	*S*	*H′*
c	−0.59	−0.40	−0.83*	−0.7	+0.40	+0.44	+0.64	+0.47	+0.31	+0.45	−0.1	−0.1	−0.87*	−0.06	−0.62	−0.66
d	−0.60	−0.55	−0.98*	−0.77	−0.08	−0.01	+0.64	+0.81	−0.1	+0.18	−0.09	+0.08	−1*	−1*	+0.15	+0.40

Table 5
Correlations between the decline of the *R. solanacearum* (lag phase *c* and decline rate *d*) and soil nutrient concentrations, sand content, and pH, respectively. A positive correlation means a slower decline at higher nutrient content, sand content or pH. Significant correlations with a *P*-value < 0.05 are marked with *.

Decline	Nutrient, sand content and pH									
	K	Ca	Na	P	N	TOC	$N\text{-}NO_3$	$N\text{-}NH_4$	Sand content	pH
c	+0.72*	+0.15	+0.45*	+0.42	+0.60*	+0.69*	−0.30	+0.29	−0.40	−0.74*
d	+0.60*	−0.28	+0.60*	+0.59*	0.72*	+0.62*	+0.18	+0.49*	−0.59	−0.89*

3.4. Relation between microbial diversity and pathogen decline in (non) amended soils

To determine the effect of soil amendments on bacterial biodiversity (Species Richness and Shannon–Weaver Index), all non-amended and amended soils were again subjected to DGGE analyses. Canonical discriminant analysis of the bacterial community for the five treatment classes for Egyptian sandy soils (non-amended conventional, NPK amended conventional, organic, compost amended organic and manure-amended organic) revealed a clear distinction between the five classes (Fig. 4A). The analyses resulted in a 52% correct classification according to canonical variable 1, with a Wilk's Lambda of 0.046 ($P < 0.0001$). The differences between the non-amended and NPK fertilized conventional soils were smallest, but organic, organic with compost, organic with manure, and the set of conventional soils were clearly distinct groups (Fig. 4A).

Also, for the Egyptian clay soils (Fig. 4B) there was a clear distinction between the five classes with 86% correct classification according to canonical variable 1 and Wilk's Lambda of 0.03 ($P < 0.0001$). In this group of soils the largest differences were among the organic, organic with manure and organic with compost soils. Again the amendment of conventional soil with NPK hardly affected the microbial community. The microbial composition of organic soil with manure shifted towards that of the conventional soils. For the Dutch sandy soils, there was 89% correct classification according to canonical variable 1, with Wilk's Lambda of 0.008 ($P < 0.0001$), with a clear distinction of manure-amended organic soil from the other soils (Fig. 4C). There was little difference between compost amended soil and non-amended organic soil.

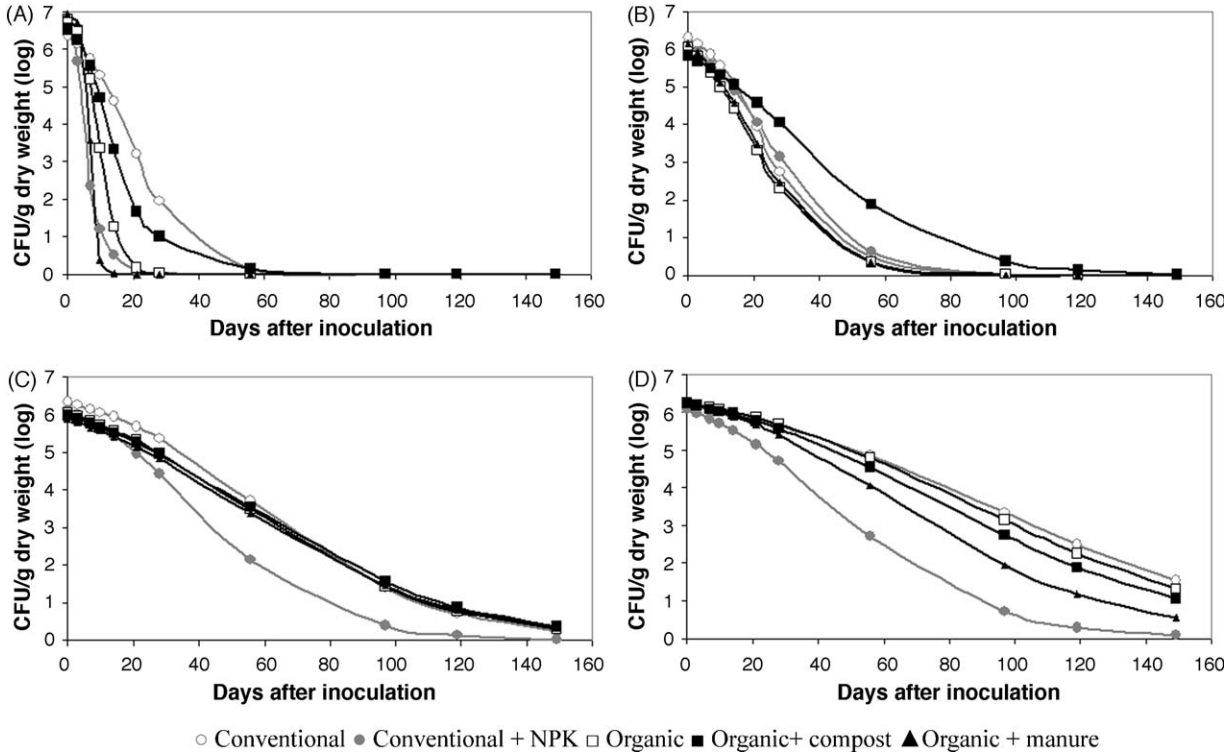

Fig. 3. The effects of different amendments to the soils on the decline of *R. solanacearum* at 15 °C (CFU g^{-1} dry soil). (A) Egyptian sand, (B) Egyptian clay, (C) Dutch sand, and (D) Dutch clay.

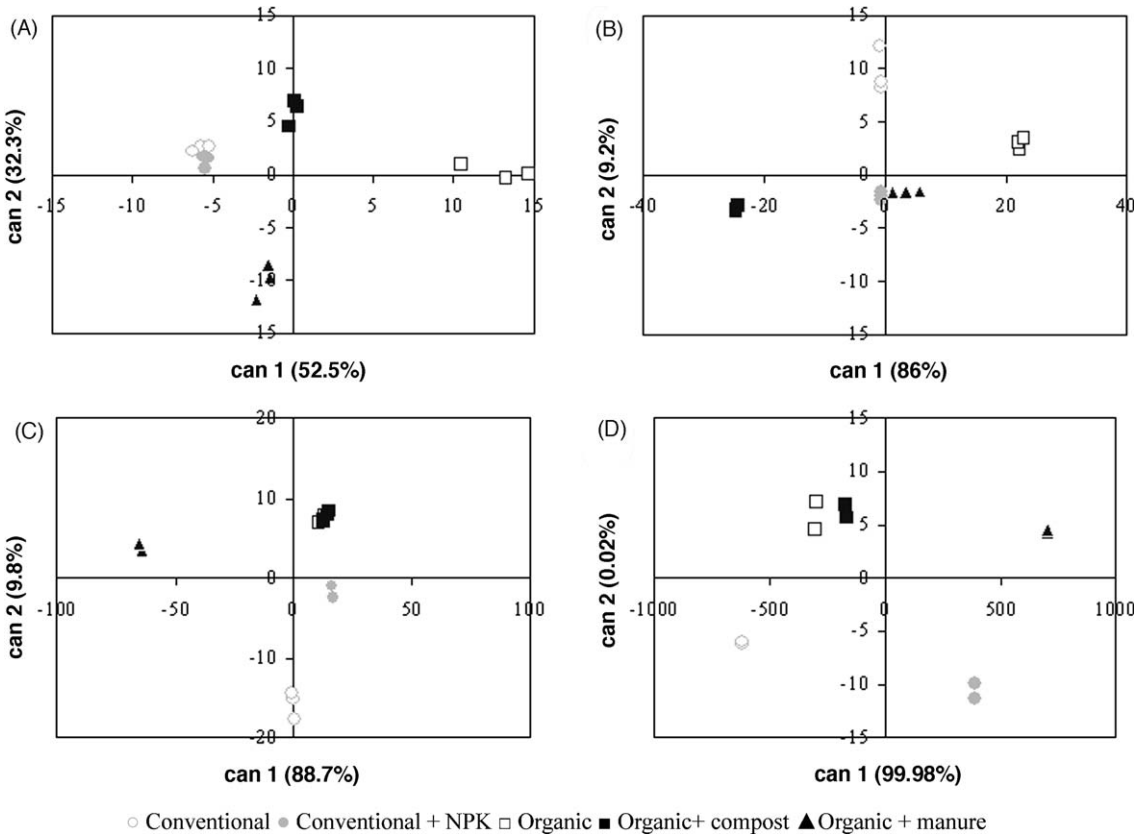

Fig. 4. CDA-plots of canonical coefficients 1 and 2 for discrimination between the five classes of non-amended and amended soils based on eubacterial DGGE data. (A) Egyptian sand, (B) Egyptian clay, (C) Dutch sand, and (D) Dutch clay.

The Dutch clay soils had 99% correct classification according to canonical variable 1, with Wilk's Lambda of 0.008 ($P < 0.0001$). There was a very large difference between each of the five treatments, compost amended and non-amended organic soil being grouped closest together (Fig. 4D).

Only for conventional sandy soils from both countries significant negative correlations were found between diversity (S and H) and survival (c and d) ($P < 0.05$, data not shown). Amendment of the conventional sandy soils from both countries with NPK slightly increased S and H, which might have had a negative effect on the survival of the pathogen. For Dutch organic sandy soil, a significant positive correlation was found between S and d ($P < 0.05$, data not shown). In the other cases there was no significant correlation.

4. Discussion

The main findings of this research were that (1) the survival of *R. solanacearum* race 3 biovar 2 was affected more by soil type and soil origin than by management or incubation temperature, (2) the decline of *R. solanacearum* was faster in sandy than in clay soils from either country, (3) the decline was faster in all soils from Egypt than in soils with a Dutch origin regardless of incubation temperature, (4) the decline of *R. solanacearum* was sometimes faster in organically managed than in conventionally managed soil, while for other soil pairs the difference was not significant, and (5) one-time amendment with NPK fertilizer to conventional soil and with cow manure to organic soil enhanced the decline rate of *R. solanacearum* more than longer-term organic management did in all soil types.

Thus, in this study soil type and origin were stronger determinants for pathogen suppression than soil management. This was contrary to the expectation, as soil-borne plant pathogens generally are suppressed more in organically than in conventionally farmed land (van Bruggen, 1995; van Bruggen and Termorshuizen, 2003). The small management effects found here may be related to the controlled moisture conditions, which would not occur in the field, where organic management usually has a pronounced effect on water-holding capacity (van Bruggen and Termorshuizen, 2003). Nevertheless, significant pathogen suppression was found for organic Egyptian sandy soil at a moderate temperature and for organic clay soils from both countries kept at a high temperature.

In our controlled environment tests, CFUs of *R. solanacearum* dropped below the detection limit of 10^2 g^{-1} d.w. soil within 5 months for all soils tested. On the other hand, in a few cases survival under field conditions has been reported for up to three growing seasons (Graham et al., 1979; Gorissen et al., 2004). Lower temperatures and different microbial populations may have contributed to the longer survival of *R. solanacearum* under these field conditions.

The survival periods were slightly longer at 15 °C than at 28 °C in Dutch soils. Álvarez et al. (2007) studied the survival of *R. solanacearum* in Spanish water microcosms and ascribed the observed longer survival at 14 °C in comparison to 24 °C to the slower biotic interactions at the lower temperature. However, in our Egyptian soils, the decline was slightly faster at the lower temperature in the majority of the soils (75%). Perhaps competition of the native microbial community with *R. solanacearum* was impaired and/or diminished when the soil was incubated at a temperature different from its normal ambient temperatures (15 °C, more normal for the Dutch soils; 28 °C, more normal for the Egyptian soils). This notion is supported by a sharp drop in bacterial diversity in Dutch soils when incubated at 28 °C,

indicating that few species grew fast at the cost of other species, while the biodiversity of the Egyptian soils did not change as much when they were kept at 15 °C. Thus, the Egyptian bacterial communities seemed to be better adapted to temperature changes in the range from 15 to 28 °C than the Dutch soil bacteria were. Our data confirm further that race 3 biovar 2 has adapted to the lower soil temperatures occurring in temperate regions (Moraes, 1947; Elphinstone, 1996).

A reason for the relatively short survival periods in our study as compared to field studies may be the absence of growing roots or plant remains of hosts, as race 3 biovar 2 has been found to survive in and on roots (Pradhanang and Elphinstone, 1996; Swanepoel, 1996; Pradhanang et al., 2000; Janse et al., 2004) and can also be associated with plant debris (Graham et al., 1979). Low nutrient availability and high competition for nutrients in the soil samples used in this study may explain the failure of the pathogen to maintain itself for longer periods. This suggestion is supported by the longer survival periods in the Dutch soils used in this study, which had higher concentrations of dissolved organic carbon concentration (DOC) than Egyptian soils, indicative of higher nutrient availability and better survival chances for *R. solanacearum*. The DOC concentrations were particularly low in the Egyptian sandy soil where the pathogen dropped to undetectable levels within 1–2 months, especially in the organically managed soil with a relatively high microbial activity and diversity. A higher DOC concentration also appeared to correlate with a higher brown rot incidence, whereas for instance a higher K and Ca contents seemed to make the plants more resistant (Messiha et al., 2007a).

The faster decline rate of *R. solanacearum* in sand as compared to clay soils may also be related to soil texture. It is well documented that fine soil textures correlate with low levels of bacterial inoculum due to presence of more shielded sites for the bacteria to evade predation by protozoa (van Veen et al., 1997). Yet, the slower decline rate in the organic sandy soil than in the organic clay soil from the Netherlands indicates that nutrient availability may be more important than evasion from predators, as the Dutch organic sandy soil had higher DOC concentrations than the other Dutch soils.

Although the amendment with compost or manure generally increased the DOC concentrations compared to the respective non-amended organic soils, this did not necessarily lead to increased survival of *R. solanacearum*. Amendment of organic soils with 1.7% compost did increase survival of the pathogen in Egyptian sandy and clay soils, but not in Dutch soils, which were already relatively high in soil organic matter compared to Egyptian sandy soil. Michel and Mew (1998) also showed that the suppressive effect of soil amendment on the survival of *R. solanacearum* depends on a particular soil type. In other studies, soil amendment with compost frequently increased pathogen decline rates and disease suppression of several soil-borne plant diseases (Hoitink and Boehm, 1999; Schönfeld et al., 2003). In our study, amendment of the organic soils with compost obviously did not suppress the pathogen. This may be due to the relatively low amount of compost added.

Amendment of the organic soils with cow manure had an accelerating effect on the decline of *R. solanacearum* in Egyptian sandy soil and Dutch clay soil. This suppressive effect of manure was probably due to the immediate release of ammonia as a result of microbial decomposition (Lazarovits et al., 2001). Gorissen et al. (2004) showed a similar suppressive effect on the survival of *R. solanacearum* by pig slurry. The microbial toxicity of ammonia was recorded a long time ago (Warren, 1962) and has been reported to reduce populations of other soil-borne plant pathogens as well.

Amendment of the conventional soils with NPK gave a remarkable decrease in the survival of *R. solanacearum* in all soils tested except for the Egyptian clay soil, which was already extremely high in nitrate and ammonium without NPK amendment. The effect of NPK was clearest in the Egyptian sandy soil that had the highest pH (pH 7.9). The NPK fertilizer contained 7% of ammonium and 5% nitrate. The strong but gradual decline in *R. solanacearum* populations may be partly due to a gradual assimilation of nitrate and conversion to nitrite (a general toxin) and ammonium by various microbes, including the *R. solanacearum* strains. The decline was likely also partly due to a toxic effect of ammonia after conversion of ammonium into ammonia. The ratio of ammonia/ammonium conversion depends on the pH of the soil: from 1% conversion at pH 7.3 to 10% at pH 8.3 (Kissel et al., 1985). The differences in pH between Dutch and Egyptian soils may also have had a direct effect on the pathogen. Michel and Mew (1998) showed that a high pH (similar to the pH in the Egyptian soils) can have a deleterious effect on *R. solanacearum*. In Egyptian organically managed sandy soils a slight suppression of the disease in the plant was observed when compared to conventional managed soils. However, in the relatively rich organic soils from the Netherlands disease incidence rather increased compared to conventional treatment. Amendment with NPK fertilizer of conventionally managed soils or with cow manure (but not with compost) of organically managed soils suppressed the disease in most soils (Messiha et al., 2007a).

DGGE analysis is one of the most powerful tools currently available to study microbial communities, despite its limitations (van Diepeningen et al., 2006). The bacterial diversity, as determined by DGGE analysis was lower in the Egyptian than in the Dutch soils and lower in the sandy than in the clay soils. Organically managed soils generally have higher biological diversity than conventionally managed soils of the same soil type (Mäder et al., 2002; van Diepeningen et al., 2006), and our findings agree to that. Suppression of *R. solanacearum* was positively correlated with bacterial species richness for those soil types and conditions where the pathogen declined faster in the organic soil than in its conventional counterpart (Egyptian sand at 15 °C and Dutch clay at 28 °C). However, the higher bacterial biodiversity *per se* with possibly larger numbers of species of antagonists and competitors probably did not contribute always to the decline of *R. solanacearum* in soil. Considering the DOC concentrations in the various soils, the availability of substrate for *R. solanacearum* in combination with microbial competition was likely more important (Hoitink and Boehm, 1999).

In conclusion, the overriding factors determining survival of *R. solanacearum* in soil may be the production of toxic concentrations of ammonia on the one hand, and availability of substrate in combination with microbial competition on the other hand, considering the observed correlations with DOC. However, DOC also contains humic acids, which cannot be utilized directly by bacteria. Thus, for future research on survival of *R. solanacearum* in soil, soluble sugars and amino acids should be quantified in combination with microbial activity in soil.

The practical value of our research is that the sandy desert soils of Egypt are very suitable for production of export potatoes, in the first place, because most desert areas are still free from *R. solanacearum*, and in the second place, because the pathogen would survive for only a relatively short period in those soils, if it were accidentally introduced. Other practical results are that addition of ammonia-producing amendments (manure or fertilizer) can reduce populations of *R. solanacearum*, whereas compost addition and organic management do not necessarily result in an enhanced decline of the pathogen.

Acknowledgements

This project was funded by the EU through the EU-Egypt Potato Brown Rot Project Phase II (SEM03/220/51A/EGY1B/1999/0192). The advisory and technical help from the team at the Department

of Bacteriology of the Plant Protection Service (PD), Wageningen, is greatly appreciated; this holds specifically for B. Briaire, J.G.B. Voogd, J.L.J. van de Bilt, M.J.A.E. Villalón-Robbles, N.M. Landman, and S. Somovilla-carrasco. We thank O.J. de Vos at the Biological Farming Systems Group of Wageningen University for technical assistance and preparation of the DGGE gels. The help of G.A. Hiddink from the Biological Farming Systems Group with the DGGE analysis is also appreciated. H.D. Halm is thanked for conducting the chemical soil analyses.

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