Bacterial Wilt of Potato

(*Ralstonia solanacearum* race 3, biovar 2):

Disease Management, Pathogen Survival and Possible Eradication

Nevein Anwar Shehata Messiha

Promotor:	Prof. Dr. Ir. Ariena H.C. van Bruggen Hoogleraar in Biologische Bedrijfssystemen Wageningen Universiteit
Co-promotoren:	Dr. Ir. Aad J. Termorshuizen Universitair Hoofddocent, leerstoelgroep Biologische Bedrijfssystemen Wageningen Universiteit
	Dr. Ir. Jaap D. Janse Hoofd Bacteriologie Groep Plantenziektenkundige Dienst, Wageningen
Promotiecommissie:	Prof. Dr. Willem M. de Vos (Wageningen Universiteit) Prof. Dr. Ir. Paul C. Struik (Wageningen Universiteit) Prof. Dr. Johannes A. van Veen (NIOO, Centrum voor Terrestrische Ecologie, Heteren) Dr. Jos M. Raaijmakers (Wageningen Universiteit)

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Nevein Anwar Shehata Messiha

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Abstract

Potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2 (Phylovar II, sequevar 1), is a serious endemic disease in the Nile Delta of Egypt. It is a quarantine disease in the EU, and export of potatoes from Egypt is restricted to pest-free areas in the desert. This thesis is directed at survival and eradication of the pathogen in areas that may become contaminated and at brown rot control in infested areas. *R. solanacearum* survival and disease suppression were studied for soils differing in origin (Dutch versus Egyptian soils), type (sand versus clay), and management (organic versus conventional). Effects of amendment of conventional soils with NPK and organic soils with compost or cow manure were compared with non-amended controls. The pathogen survived longer in Dutch than in Egyptian soils, and in clay than in sandy soils from both countries. Survival was never longer than 180 days and in many cases much shorter, especially in Egyptian sandy soil. Organic management reduced pathogen survival in Egyptian soils, especially in the sandy soil, but not in Dutch soils. Pathogen survival was positively correlated with dissolved organic carbon (DOC) in soil, negatively with organic matter (OM) content at similar DOC content, and negatively with bacterial diversity estimated from denaturing gradient gel electrophoresis (DGGE) of eubacterial 16S rDNA extracted from soil. NPK fertilization and cow manure amendment reduced pathogen survival in most soils while compost was not effective. There was a clear shift in microbial community after manure amendment, a weak shift by compost amendment, and no shift after NPK fertilization. Disease development was highest in Dutch sandy soils and similar in all other soil types. It was positively correlated with DOC (substrate availability), and negatively with K and Ca contents in soil, which may increase plant resistance. The disease was slightly suppressed in organic compared to conventional sandy soils from Egypt while organic management increased disease incidence in all Dutch soils. This was related to a high DOC content in organic Dutch soils. There were no differences in bacterial diversity and composition in different rhizosphere soils, and no correlations between disease development and bacterial diversity. Manure fertilization suppressed brown rot in most soils with a clear shift in rhizospere bacterial community. *Stenotrophomonas maltophilia*, isolated from the rhizosphere of eggplant in the Egyptian Delta, was antagonistic to *R. solanacearum in vitro*; its antagonistic activity was not Fe-siderophore dependent. A selected *S. maltophilia* strain survived longer and reduced *R. solanacearum* survival more in Egyptian than in Dutch clay soils, and suppressed potato brown rot in Egyptian soil (by at least 36%) but not in Dutch soil. Survival and suppressive activity of *S. maltophilia* were positively correlated with OM, which was highest in Egyptian organic clay soil. The effect of biological soil disinfestation (BSD) was tested in glass vessels, microplots, and in an accidentally infested commercial field. BSD is based on production of toxic organic acids through anaerobic digestion of fresh organic matter. BSD was accomplished by incorporating grass or potato haulms in soil and covering the soil with airtight plastic. Survival of *R. solanacearum* in soil and potato tubers was significantly reduced in the BSD treatment (>93%), and not in separate grass amendment -or plastic cover treatments. This thesis may contribute to regulatory decisions in the EU concerning the time required for keeping accidentally infested fields out of potato production and to recommendations for management of potato brown rot in areas where the disease is endemic.

For Mom, soul of Dad
George, Maria, Sarah
Ashraf and Bassem

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Chapter 1

Introduction and overview

Chapter 1. Introduction and overview

The pathogen

Ralstonia solanacearum, the causative agent of potato brown rot (bacterial wilt) disease, has been reported as an economic disease in hot and temperate regions of the world. *R. solanacearum* is subdivided into 5 races on the basis of host range and 5 biovars on the basis of catabolic properties (Schaad, 1988; Hayward, 1991). Strains belonging to race 1, biovars 1, 3, and 5 have a broad host range. Race 2 strains are pathogenic only to Musaceae. Race 3 strains are pathogenic to potato, some Solanaceous weeds such as black nightshade (*S. nigrum*), bittersweet (*S. dulcamara*) and *S. cinerea* (in Australia), and a number of non-solanaceous species, including *Pelargonium zonale* (Janse et al., 2004). Race 4 is pathogenic to ginger and race 5 to mulberry (Hayward, 1964). Biovars are defined by the ability of strains to oxidize three sugar alcohols and three disaccharides (Hayward, 1964). In more recent taxonomic studies using restriction fragment length polymorphism (RFLP) analysis and sequence analysis of various regions of the genome, race 3, biovar 2 strains belong to RFLP Division II (where strains primarily from America are grouped; Cook et al., 1989) and Phylotype II (mainly sequevar 1, Prior and Fegan, 2005). Race 3 biovar 2 is the dominant race in mountainous regions in S. America (Andes), where the potato and possibly this race of *R. solanacearum* originate, at higher altitudes in other tropical areas and also in the Mediterranean basin, including Egypt and more recently even in NW Europe. Potato losses up to 75% due to the bacterial wilt have been recorded in many countries (Cook and Sequeira, 1994).

Risks of brown rot for Egypt and the European Union potato production and export

For the European Union potato brown rot is a quarantine disease with zero tolerance requirements. Because of the frequent occurrence of brown rot in Egyptian potatoes exported to European markets, its endemic presence in the Nile Delta area and a possible threat via contamination of irrigation water that became contaminated by waste of industries processing Egyptian potatoes, the EC took restrictive measures. In its Decision 98/503/EC (Commission EC, 1998) it demanded that potatoes should be produced in so-called Pest Free Areas (PFA's) according to FAO standards, where the disease should be known not to occur (via testing) (also see European Communities, 2005; Elphinstone et al., 1998; Janse, 1996). Furthermore the export crop should be lab-tested immediately prior to export and infested fields should be quarantined. In Europe isolated outbreaks of brown rot were recorded in the last ten years in many countries, with successful eradication in most cases (De Guenin, 1998; Persson, 1998; Turco et al., 1998). In 1995, The Netherlands were confronted with an outbreak of the disease in potato and immediately an intensive program for eradication was started (Schans and Steeghs, 1998) and in season 2005/2006 only one case of brown rot was recorded (J.D. Janse, pers. comm.).

Survival

In most European findings, first introduction appeared not via seed but via surface water that became contaminated by industrial or household waste where infected potatoes were used. The pathogen can, however, also be introduced into an area by planting infected potato tubers, whence it can be disseminated, and survive in contaminated surface water (Janse, 1996; Persson, 1998; Schans and Steeghs, 1998; Wenneker et al., 1999) and weed hosts such as *Solanum dulcamara*, *S. nigrum*, *Portulaca oleracea* in Europe (Elphinstone et al., 1998) and *Rumex dentatus* and *Solanum nigrum* in Egypt (Farag et al., 2004). Volunteer plants or (in colder climates: perennial) weeds can be a reservoir, and responsible for transmission of the pathogen through successive seasons (Janse, 1996; Lopez and Biosca, 2004). The pathogen can persist for a long time in soil, in infected host plant debris or by colonizing potato volunteer plants, alternative hosts or even non-host plants (Graham et al., 1979; Granada and Sequeira, 1983; Akiew and Trevorrow, 1994). The disease cycle is shown in Figure 1 (Janse, 2006). When the disease establishes in a growing area in temperate zones it can survive for periods between 12 months to 3 years in the absence of a potato crop as shown for New South Wales in Australia (Graham et al., 1979). It is therefore crucial to understand the ecology of the organism and the factors that affect its survival and suppression in different soil types.

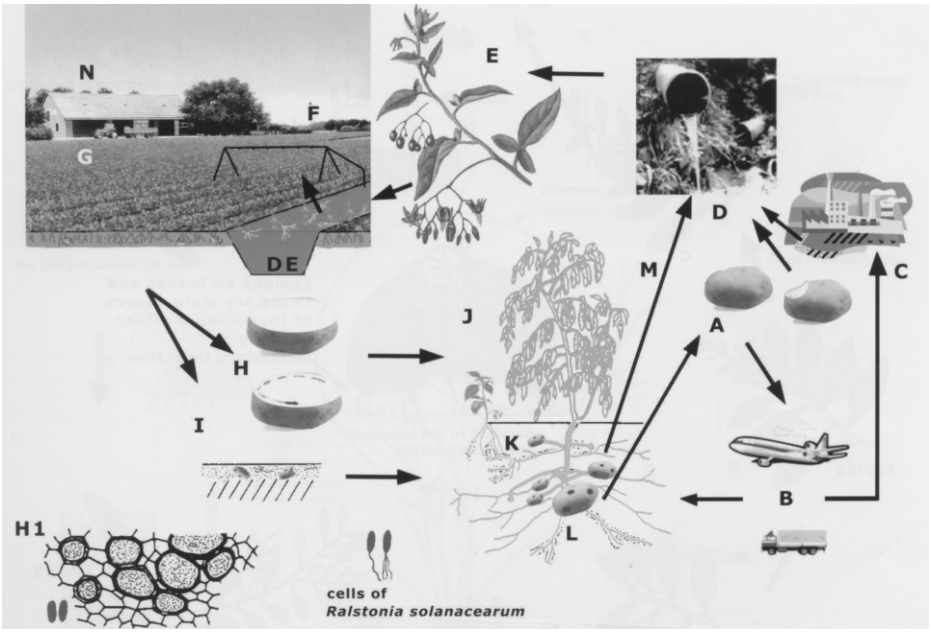

Figure 1. Lifecycle of *Ralstonia solanacearum* race 2, biovar 3 in association with potato. (A) Seed potato as a primary source, (B, C, D) transport via airplane, truck or processing industry, and water to the field and plants, (DE) contaminated surface water, (E) latently infected bittersweet plants (*Solanum dulcamara*), (F) irrigation water, (G) contaminated machinery, (H, I) infected potato tubers, (H1) vascular tissue infected, (J) wilted potato plant, (K) latently infected daughter tubers which contaminate the soil, (L) infected rhizosphere, (M) transmission to surface water and eventually to bittersweet, (N) seed storage sheds, grading belts, packing materials and machines contaminated by infected tubers (Janse, 2006).

Few possibilities for control

Many trials have been carried out all over the world to control the disease without much success. No promising control of brown rot was achieved using antibiotics (Habashy et al., 1993), soil fumigants (Weingartner and Shumaker, 1988), chemical control (Murakoshi and Takahashi, 1984) or breeding of resistant varieties (Hartman and Elphinstone, 1994; Mendoza, 1994; Fock et al., 2001; Lopez and Biosca, 2004). Moreover chemical control becomes much less of an option due to the increasing demand for low-input and organically produced products since the last ten years because of fear of the hazardous effects of pesticides and chemical residues both in Europe and Egypt (Sylvander and Le Floc'h-Wadel, 2000; Parrott and Kalibwani, 2004).

Disease suppression

Soil-borne plant pathogens are frequently suppressed in organically compared to conventionally farmed land (van Bruggen and Termorshuizen, 2003). This phenomenon has not been investigated for *Ralstonia solanacearum*. Survival of *R. solanacearum* was found to be affected by soil type (texture and organic matter), temperature and moisture content (van Elsas et al., 2000). Despite reports on suppression of various pathogens in organically managed soils (van Bruggen and Termorshuizen, 2003), it was found that *R. solanacearum* survived least in loamy soil with relatively high organic matter content (4%) whilst survival was highest in soil with lower organic matter content of 2.0-2.5% (van Elsas et al., 2000). Yet, it was stated that efficient soil management would be related to composition and/or activity of the soil microbiota by enhancing natural biological control capacity (van Elsas et al., 2005). Soil amendment with organic materials or NPK fertilizers or with different combination of these organic and inorganic amendments significantly affected bacterial wilt incidence and increased potato yields (Lemaga et al., 2005). Also the addition of household compost was found to increase disease suppression (Schönfeld et al., 2003). Pig slurry had a similar suppressive effect (Gorissen et al., 2004). It was found that amendment with chicken manure resulted in an increase of the bacterial population during the first two days followed by a decline of the pathogen population due to the accumulation of ammonia or the increase in pH (Himathongkham and Riemann, 1999). Ammonium is known to reduce the growth of *R. solanacearum*, but was not found to be suppressive and its effect was found to be pH dependent (Michel and Mew, 1998). The mechanism of suppression of *R. solanacearum* is unknown but it was hypothesized that the effect is related to a shift in the soil microbial community towards a community with enhanced antagonism against *R. solanacearum.* However, the suppressive effect of soil amendment on the survival of *R. solanacearum* can depend on soil type (Michel and Mew, 1998).

Biocontrol using selected antagonists

Biocontrol agents such as *Pseudomonas fluorescens*, *P. glumae*, *Burkholderia cepacia*,

Bacillus sp., *Erwinia* sp. and a Hrp⁻mutant of *R. solanacearum* were found to be relatively ineffective in the control of *R. solanacearum* populations under natural conditions (Lopez and Biosca, 2004; Ran et al., 2005). The failure probably results from the inability of the introduced biocontrol agent to establish itself and to produce the desired compound under the stress of competition by the native microbial community. Use of fluorescent pseudomonads as biocontrol agents, for example, sometimes succeeded *in vitro* or under controlled conditions but failed under field conditions or even in pots (Ran et al., 2005). Iron availability in soil may partially explain this failure. Hoitink and Boehm (1999) postulated that manipulation of natural communities of antagonistic microorganisms in soil through organic amendments provides a potentially effective form of biological control of soil-borne plant pathogens.

Several effective biocontrol bacteria have been isolated from the rhizosphere of healthy plants grown in infected areas in Egypt. They include *Pseudomonas fluorescens*, *Streptomyces griseus*, *Bacillus subtilis* and *Trichoderma harzianum* (Tolba, 1998). More potential antagonists against *R. solanacearum* have been identified and characterized by Messiha (2001). The potentially most active antagonist (up to 75% disease control) was recovered from eggplant rhizosphere and was identified as *Stenotrophomonas maltophilia*. The antagonistic potential of this organism was found not to be affected by incorporation of iron to the medium used for testing the antagonistic properties. Ferric chloride caused a drastic deterioration in the antagonistic activity of *P. fluorescens* that may be attributed to a possible effect of iron on siderophore pigments. (Messiha, 2001). It was found that *S. maltophilia* was able to colonize and persist in plant tissue from soil or dipped roots (Garbeva et al., 2001). Three antifungal compounds, designated xanthobaccines A, B and C, were isolated from the culture fluid of *Stenotrophomonas* sp. (Nakayama et al., 1999). *S. maltophilia* produces other antibiotics, for example, maltophilin, but this compound was inactive against Gram-positive and Gram-negative bacteria (Berg et al., 1996; Jakobi et al., 1996). Lytic enzymes were also found to be excreted by *S. maltophilia* (Berg et al., 1996; Dunne et al., 2000; Zhang and Yuen, 1999).

Biological soil disinfestation as a control possibility

A non-chemical approach to improve the control of soil-borne pests involves biological soil disinfestation (BSD) by anaerobic decomposition of fresh organic matter added to soil and covered by plastic. BSD is very effective at controlling various fungal plant pathogens (Blok et al., 2000). The effects of this technique on bacterial plant pathogens are not clear. It may have an effect on the microbial community as well as on the survival of *R. solanacearum*. BSD could possibly be an option for control of brown rot on larger farms in the Egyptian desert (in case of accidental introduction) or the Nile Delta area (where the disease is endemic).

Goal of this research

For controlling potato brown rot caused by *R. solanacearum* in dry desert and Nile delta areas in Egypt it is appropriate to look at the possibilities of relatively cheap biological control and non-chemical approaches. In both areas in Egypt there are farms using organic management techniques next to conventional farms. Application of cow manure on organically managed soils or NPK on conventionally managed soils may be a natural way to reduce *R. solanacearum* infestation, but these possibilities have not been extensively investigated yet. A promising biological control agent for potato brown rot in Egypt could be the naturally occurring *S. maltophilia* strains, which showed clear antagonistic activity in in-vitro studies.

Based on what has been elaborated above the objectives of this research were:

1. to determine survival of *R. solanacearum* in organically versus conventionally farmed soils from Egypt and the Netherlands (chapter 2).
2. to determine if disease suppression of potato brown rot is greater in organically than conventionally managed soils, and if there is a connection between disease suppression and nutrient availability or the complexity of the microbial community (chapter 3)
3. to determine effects of NPK, compost and cow manure on survival of *R. solanacearum* and brown rot development in potatoes, and if there is a relation between survival of the pathogen and soil chemical and biological characteristics (chapters 2 and 3).
4. to determine the survival and effectiveness of a selected biocontrol agent namely *S. maltophilia* on reduction of brown rot in Egyptian and Dutch soils (chapter 4).
5. to determine the effect of biological soil disinfestation on survival of *R. solanacearum* and development of brown rot disease on potato (chapter 5).

For the first three objectives four pairs of soils (organic versus conventional) from different soil types (sandy versus clayey) from different countries (Egypt versus the Netherlands) were studied. For the biological control experiments clayey soils from both countries were included in the study. For biological soil disinfestation studies Dutch sandy soil was used

This thesis will be concluded with a general discussion (chapter 6) on the possibilities, advantages and disadvantages of the various control options examined in chapters 2-5.

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Chapter 2

Survival of *Ralstonia solanacearum* is affected more by soil type than by management

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Messiha NAS [a,b,c], van Diepeningen AD [a], Franz E [a], Janse JD [b], Schoeman-Weerdesteijn ME [b], Termorshuizen AJ [a], and van Bruggen AHC [a,*] (2006) Survival of *Ralstonia solanacearum* is affected more by soil type than by management. Soil Biology and Biochemistry.

[a] *Wageningen University, Biological Farming Systems Group, Marijkeweg 22, 6709 PG Wageningen, the Netherlands*
[b] *Plant Protection Service, Dept. of Bacteriology, Geertjesweg 15, 6706 EA Wageningen, the Netherlands*
c Potato Brown Rot Project (PBRP), 3 El-Amira Fatma Ismail Street, Dokki, Cairo, Egypt
***** *Wageningen University, Biological Farming Systems Group, Marijkeweg 22, 6709 PG Wageningen, the Netherlands. E-mail: ariena.vanbruggen@wur.nl, tel +31 317 478201, fax +31 317 478213.*

Chapter 2. Survival of *Ralstonia solanacearum* is affected more by soil type than by management

Abstract

Potato brown rot disease (*Ralstonia solanacearum*) is a serious problem in Egypt. This is partly due to an EU requirement that potatoes for export to the European Union should be grown in so-called Pest Free Area's (PFA's), where fields are tested and found to be free from the pathogen and potential infested fields are put under quarantine measures. To investigate pathogen extinction and to determine time required to release infested fields from quarantine, we tested the survival of *R. solanacearum* race 3 biovar 2 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 (15ºC) or warm temperatures (28ºC). We also tested the additional effects of conventional (artificial fertilizer) and organic (compost and cow manure) amendments on survival of *R. solanacearum*. In all soils, with and without amendments, the pathogen was below detection limits (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 soils with a Dutch origin. The decline in colony forming units of *R. solanacearum* per g of soil was faster in sandy soils from either country than in clay soils from both countries. On the other hand, 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 of the fitted curve for pathogen survival than conventional management, while for other soils the differences were not significant. 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 as estimated from results of denaturing gradient gel electrophoresis (DGGE) of eubacterial 16S rDNA directly extracted from soil. 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. Thus, it appears that the Egyptian strains of *R. solanacearum* used in this study were better adapted to compete with the native microbial community when the soil was incubated at its usual temperature, namely 28°C for the Egyptian soils.

Introduction

Potato brown rot is an important disease, occurring world-wide and is caused by the bacterial pathogen *Ralstonia solanacearum* (Smith) 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 yield losses and economic problems. From the Mediterranean area the pathogen spread in the late 1980s to temperate regions of North-western Europe, where contamination of surface water led to introduction into potato production (Janse, 1996; Persson, 1998; Schans and Steeghs, 1998; Wenneker et al., 1999). Since *R. solanacearum* is under eradication measures in the European Union (European Communities, 1998) and only sporadically occurs in potato, it is still considered a quarantine pathogen. Therefore the EU has set strict importation requirements for Egypt (European Communities, 2005), including the exclusion of the Nile Delta area for exports, production of potatoes destined for the EU in approved Pest Free Area's (PFA's), mainly in the desert, and an import ban after 5 interceptions of brown rot. Therefore potato brown rot became an important economic problem for Egypt.

R. solanacearum can be soil-borne. Especially race 1, prevalent in the tropics, has a broad host range and is very persistent. Race 3, biovar 2 has a more narrow host range (Yu et al., 2003), but it can survive for up to 3 years, as shown for New South Wales in Australia (Graham et al., 1979), under European conditions survival of at least one season was substantiated (van Elsas et al., 2000). *Solanum dulcamara*, *S. nigrum*, *Portulaca oleracea* in Europe (Elphinstone et al., 1998) and *Rumex dentatus* and *Solanum nigrum* in Egypt (Farag et al., 2004) have been found so far as alternative hosts of importance for the pathogen. Survival of the pathogen in various substrates under laboratory conditions varied from 4 to 87 days (Janse et al., 1998). Both survival *in vitro* and virulence of *R. solanacearum* are optimal at temperatures between 24 and 35°C (Pradhanang and Elphinstone, 1996; Pradhanang et al., 2000). Lower temperatures decrease both survival and virulence. 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).

Since the last ten years, there has been an increasing demand for organically produced products, without pesticide or chemical residues, both in Europe and Egypt (Sylvander and Le Floc'h-Wadel, 2000; and Parrot and Kalibwani, 2004). Under organic management, traditional conservation-minded farming methods are combined with modern farming techniques but conventional inputs such as synthetic pesticides and fertilizers are excluded. Instead of synthetic inputs, 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 compared to conventionally farmed land (van Bruggen, 1995; van Bruggen and Termorshuizen, 2003). However, the persistence and virulence of *R. solanacearum* in organically managed soils is not known.

Several simple and cost-effective management strategies have been tested for their effect on survival of *R. solanacearum*: soil amendment with compost or manure had a potential positive effect on decline rates and disease suppression of soil-borne plant diseases, including brown rot (Hoitink and Boehm, 1999; Schönfeld et al., 2003). Gorissen et al. (2004) showed a

similar suppressive effect of pig slurry added to soil on the survival of *R. solanacearum*. Michel and Mew (1998) reported that the suppressive effect of soil amendment on the survival of *R. solanacearum* depends on soil type.

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. For that purpose survival of *R. solanacearum* was monitored in several conventionally and organically managed soils from both European and Egyptian origin. Both sandy and clay soils were investigated and survival was tested 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.

Materials and Methods

Soils

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. The soils were sampled three times, in June and July 2003, and in March 2004. Twenty-five kilogram samples were collected from random sampling points from 100 m^2 plots from 15 to 25 cm vertical depth with an augur 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 plant parts and earthworms were removed. The soils were stored at 4°C till the start of the experiment. Egyptian soils were sent to the Netherlands in plastic bags in Styrofoam boxes by overnight express airmail.

Soil inoculation, amendments, and incubation

Three replicate 100-g samples from each soil were inoculated to contain 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). These strains can all use nitrate as electron acceptor under anaerobic conditions. The cultures used for soil inoculation 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}). 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 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} (0.1 g N kg^{-1} soil), the maximum amount 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°C 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.

Table 1. Origin of soil samples.

Sample	Code	Country	Soil type	Management	Cover crop at time of sampling	Location
1	ESC[1]	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

[1] 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).

Sampling

The pathogen was monitored twice a week for the first two weeks, starting at the day of inoculation (T_0), once a week for the next two weeks and then once a month for a total of five 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 rev min^{-1} for 2 h at 20°C, 10-fold serial dilutions were made on modified SMSA (Selective Medium South Africa) agar plates (Anonymous, 1998) in triplicate and incubated at 28°C for 5-7 d. Per sample 3 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 g 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.

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 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 sulphuricacid and the produced carbon dioxide was driven out of the system by N_2-gas. The extract 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 laboratory BLGG (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 versus conventional soil, whereas $N\text{-}NO_3$ and $N\text{-}NH_4$ 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.

Table 2. Physical and chemical composition of the different amended and non-amended soils (experiment 3).

	Soil composition[1]			Nutrients mg/kg									
Soil + amendment	Clay%	Silt%	Sand%	K	Ca	Na	P	N	DOC[2]	N-NO_3	N-NH_4	pH-KCl	% OM[3]
ESC[4]	0.1	0.6	99.3	601	9615	168	123	315	63.1	14	5.4	7.9	<0.2
ESC+NPK				976	11220	439	177	321	52.6	37	12.8		
ESO	0.1	0.6	99.3	964	24458	398	112	286	54.4	10	0.62	8.2	0.3
ESO+compost				969	24818	387	131	349	72.8	10	0.82		
ESO+manure				943	25354	307	118	282	54.2	10	0.21		
ECC	9.4	54.5	36.1	3827	4444	914	612	1080	133.2	128	19.2	7.6	4.9
ECC+NPK				3995	4639	966	664	1119	125.7	192	17.5		
ECO	13.9	65.7	20.4	4684	10506	1557	1031	1208	79.9	62	16.3	7.6	6.2
ECO+compost				4835	8270	1387	1192	1246	82.1	62	13.7		
ECO+manure				5316	23924	1051	1186	1259	66.1	62	11.0		
NSC	3.2	32.3	64.5	3118	9113	1403	426	924	131.3	10	12.2	7.4	1.5
NSC+NPK				3125	8894	1166	476	905	128.6	92	24.2		
NSO	3.2	33.3	63.5	3125	12620	1022	567	1375	137.3	17	8.5	7.1	4.1
NSO+compost				3053	12087	662	653	1622	175.4	22	9.3		
NSO+manure				3250	12500	863	592	1386	196.7	31	6.9		
NCC	7.7	51.9	40.4	6888	21259	1093	935	1107	122.1	10	13.9	7.4	2.2
NCC+NPK				7005	21618	954	1003	1144	122.3	94	14.5		
NCO	8.3	54.5	37.2	6281	22044	1034	712	1058	125.7	7	15.8	7.3	2.3
NCO+compost				6388	22482	1057	764	1182	135.1	13	11.7		
NCO+manure				6522	21739	870	735	1201	155.3	5	9.9		

[1] Clay particles size <2 µm, silt 2-50 µm, sand 50-2000 µm; [2] Dissolvable Organic Carbon; [3] Organic Matter; [4] See table 1 for the soil codes.

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 65ºC 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 968 f-GC and 1401 r (Heuer and Smalla, 1997). Two ng 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% (wt/vol) 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 we could extract up to 500 ng of DNA from 500 mg of soil.

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 twice per sample using the Dcode system (BIOrad Laboratories, Hercules, CA, USA). We used 6% acrylamide gels (ration 37.5 acrylamide: 1 bisacrylamide) with a 45 to 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 µl of the PCR products were 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 Centre, the Netherlands. All samples were analyzed at least in duplicate.

Gels were stained with BIOrad's Silver Stain (BIOrad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol, but using the protocol for gels > 1mm 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 dots per inch.

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 to 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-Wiener 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-Wiener diversity index was calculated as $H' = -P_i \log P_i$ 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.

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)

$$C_t = a_m / (1+\exp(-d*(t-c)))$$

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 $[a_m*d]/2$) 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 [$C(0)=a_m/2$], we used t=0.1 instead of t=0 as first point in the time series so that c can 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, Illinois, 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 between 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 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 v12. 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'.

Results

Survival in organic and conventional soils

Only the results of the first experiment are presented, because those of the second experiment were very similar. In all four pairs of soils (organic and conventional pairs of Egyptian and Dutch clay and sand) the log-transformed numbers of CFUs of *R. solanacearum* declined immediately after inoculation at both incubation temperatures. No temporary increases in numbers were observed. The log-transformed observed values fit well to the logistic decay model with asymptote, with R^2 values between 0.84 and 0.99 (Figure 1).

At both temperatures, the pathogen survived better in the Dutch than in the Egyptian soils (MANOVA for 28ºC: Wilk's Lambda = 0.049, $P < 0.001$; for 15ºC: Wilk's Lambda < 0.001; $P < 0.001$). In Dutch soils, the 50%-reduction-time was longer (ANOVA for 28ºC: F = 308, $P < 0.001$; for 15ºC: F = 1339, $P < 0.001$) and the decline rate was lower (ANOVA for 28ºC: F = 30, $P < 0.001$; for 15ºC: F = 78, $P < 0.001$) than in the Egyptian soils. Among the Egyptian soils, the pathogen survived longer in clay than in sandy soils (MANOVA at 28ºC: Wilk's Lambda = 0.024, $P < 0.001$; at 15ºC: Wilk's Lambda = 0.007, $P = 0.002$). In Egyptian clay, the 50%-reduction-time was longer (ANOVA at 28ºC: F = 5, $P < 0.06$; at 15ºC: F = 21, $P = 0.002$) and the decline rate lower compared to sandy soil (ANOVA at 28ºC: F = 275, $P < 0.001$; at 15ºC: F = 19, $P = 0.002$). Similarly, the pathogen survived longer in Dutch clay than sandy soils at 15ºC (Wilk's lambda < 0.001, $P < 0.001$), but not at 28ºC. At 15ºC, 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.

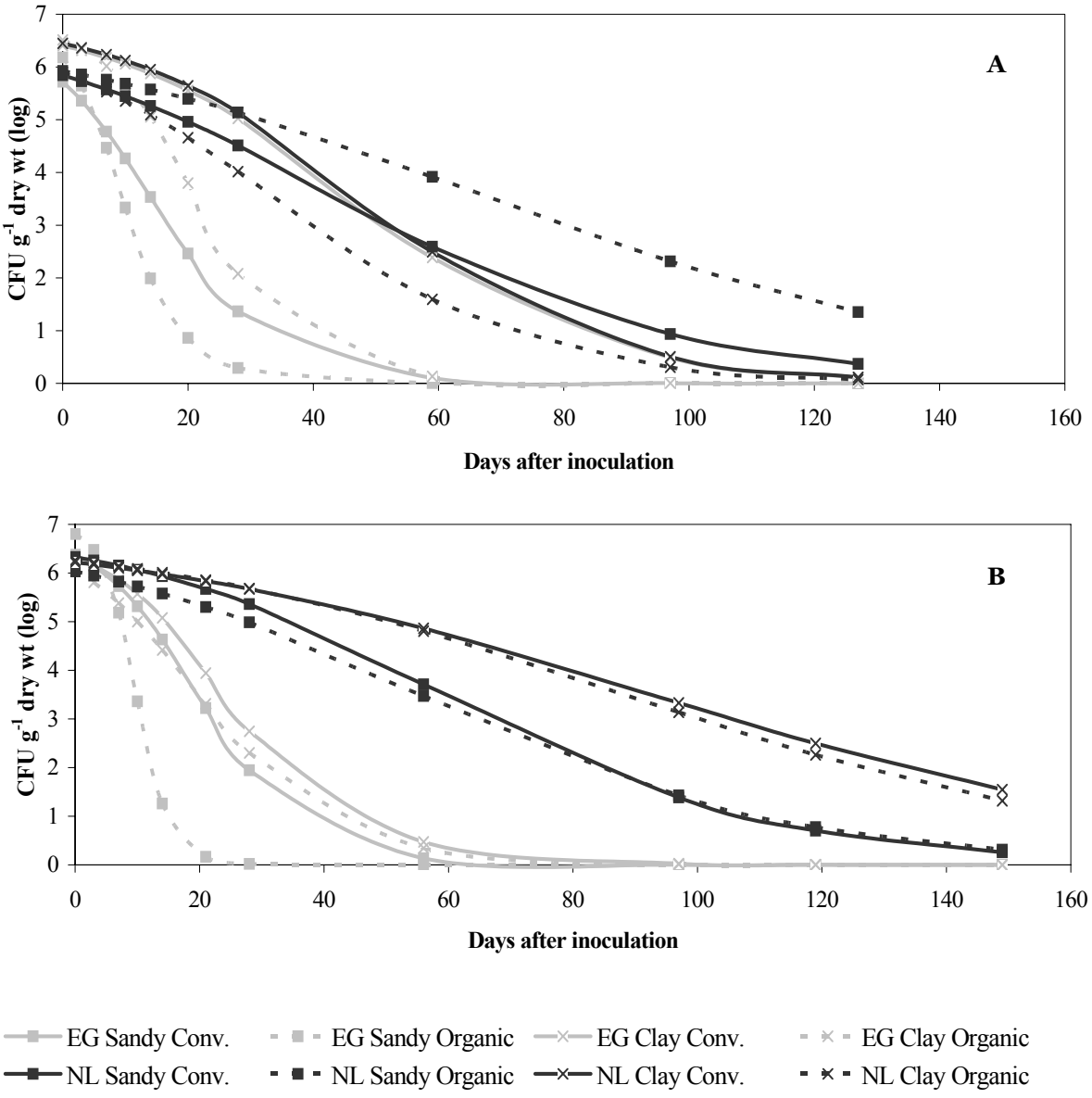


Figure 1. Decline in density (CFUs g^{-1} dry soil) of *Ralstonia solanacearum* in different soil types with different management regimes at 28°C (A) and 15°C (B), experiment 1. The lines are the predicted values from the logistic decline model:

$C_t = a_m / (1+\exp(-d*(t-c)))$. 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 = 50%-reduction-time in days (Franz et al., 2005).

The R^2 for the fit of the data to the logistic 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).

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 28ºC (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 15ºC, 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 15ºC, whereas at 28ºC, 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 (Wilk's lambda = 0.005, $P < 0.001$), with a shorter 50%-reduction-time (F = 31, $P = 0.005$) and a greater decline rate (F = 11, $P = 0.03$). In general, there was at least a tendency at both temperatures for a faster decline of *R. solanacearum* in organically than in conventionally managed soils, revealed by both a shorter 50%-reduction-time and a stronger decline rate, although the Dutch soils formed sometimes an exception to this rule at higher temperatures (Figure 1).

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

Average biodiversity measures S and *H'* of all soils incubated at 15°C or 28°C are given in Table 3 (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 (Figure 2). The sandy soils had lower biodiversity (*H'*) and species richness (*S*) than the clayey soils (t-test, $P < 0.001$). The Dutch soils had a higher (*H'*) and (*S*) than the Egyptian soils (t-test, $P < 0.001$). 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 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, $P = 0.003$). In addition, *S* was higher in organic than in conventional Egyptian clay soil at the lower incubation temperature (t-test, $P = 0.005$). *S* was also higher in organically managed Dutch clay soil than in its conventional counterpart at the higher incubation temperature (t-test, $P = 0.031$).

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.

Table 3. Average species richness *S* and Shannon-Wiener diversity index *H'* and their standard deviations for differently managed soils (experiments 1 and 2).

Tem-per-ature	Diver-sity index	Soil[1]							
		ESC	ESO	ECC	ECO	NSC	NSO	NCC	NCO
28 °C	S	10.3[2]±2.1[3]	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

[1] See table 1 for the soil codes; [2] Sample mean (4 measurements), [3] SD

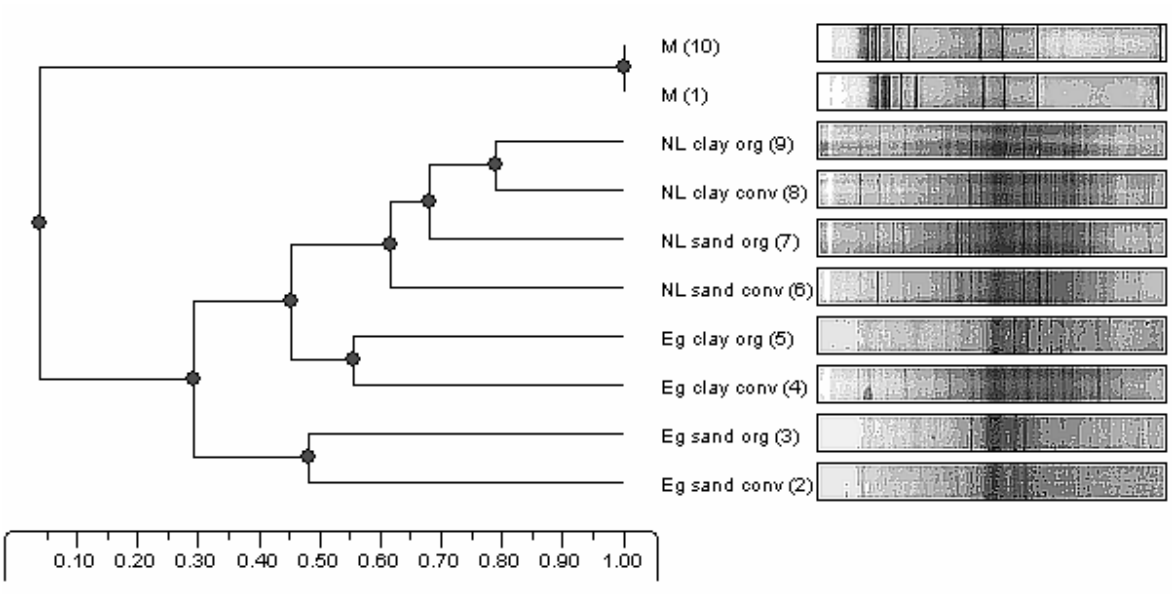


Figure 2. Cluster analysis for 16S-rDNA PCR-DGGE products from different soils, experiment 1 (Phoretix 1D, NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). The scale on the X-axis depicts similarity. The numbers between brackets indicate the lane numbers on the gel.

Negative correlations were found between S and c[1] and d[1] (r = -0.8, $P = 0.04$ and r = -0.9 $P = 0.03$, respectively), within Egyptian sandy soils at 15°C. 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 clayey soils at 28°C. There were no significant correlations in all other cases (Table 4). Significant positive correlations were found between the 50%-reduction-time (c) and decline rate (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).

Effects of soil amendments on pathogen survival

Contrast analysis revealed significantly shorter survival of the pathogen in organic soils compared to conventional soils for the 50%-reduction-time ($P = 0.005$) only and not for the decline rate (Figure 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 1.7% compost did not significantly increase the decline rate in the organic soils; in Egyptian clay soil the compost addition even seemed conducive for the survival of *R. solanacearum* (Figure 3B) ($P = 0.023$ and $P = 0.034$ for c and d, respectively). Compost addition also gave a clear shift in microbial community in that soil (Figure 4B). 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) (Figure 3). The clearest differences between different amended soils were found for Egyptian sandy soils (Figure 3A) and Dutch clay soils (Figure 3D).

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-Wiener 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 (Figure 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 (Figure 4A).

[1] 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).

Table 4. Correlations between the decline of the *R. solanacearum* (50%-reduction-time c and decline rate d) and the bacterial species richness S and diversity *H* (experiments 1 and 3).

	Egyptian sand				Egyptian clay				Dutch sand				Dutch clay			
	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[1]	-[2]0.59	-0.40	-0.83*[3]	-0.7	+0.40	+0.44	+0.64	+0.47	+0.31	+0.45	-0.10	-0.10	-0.87*	-0.06	-0.62	-0.66
d[4]	-0.60	-0.55	-0.98*	-0.77	-0.08	-0.01	+0.64	+0.81	-0.10	+0.18	-0.09	+0.08	-1*	-1*	+0.15	+0.40

[1] c = 50%-reduction-time; [2] - = a negative correlation means a faster decline at higher biodiversity; [3] * = significant correlation, *P*- value <0.05; [4] d = decline rate (negative values).

Table 5. Correlations between the decline of the *R. solanacearum* (50%-reduction-time c and decline rate d) and soil nutrient concentrations, sand content, and pH (experiment 3).

Survival	Nutrient, sand content and pH									
	K	Ca	Na	P	N	DOC	$N\text{-}NO_3$	$N\text{-}NH_4$	Sand content	pH
c[1]	+[2]0.72*[3]	+0.15	+0.45*	+0.42	+0.60*	+0.69*	-0.30	+0.29	-0.40	-0.74*
d[4]	+0.60*	-0.28	+0.60*	+0.59*	+0.72*	+0.62*	+0.18	+0.49*	-0.59	-0.89*

[1] c = 50%-reduction-time; [2] + = a positive correlation means a slower decline at higher nutrient contents, sand content or pH; [3] * = significant correlation, *P*-value < 0.05; [4] d =decline rate (negative values).

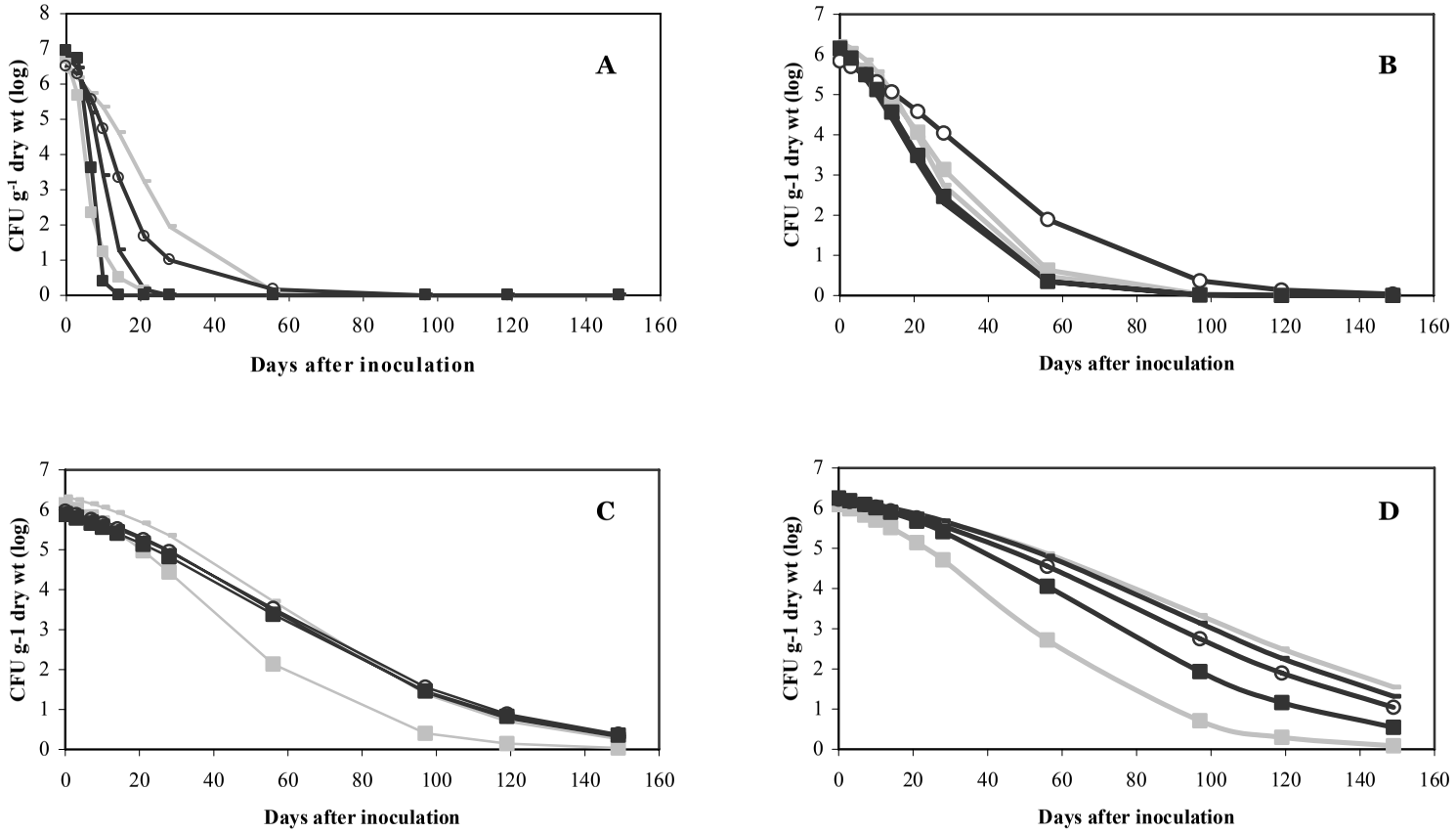
Also, for the Egyptian clay soils (Figure 4B) there was a clear distinction between the five classes with 86% correct classification according to canonical variable 1 and Wilk's lambda of 0.03 ($P < 0.0001$). In this group of soils the largest differences were among the organic, organic with manure and 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 (Figure 4C). There was little difference between compost amended soil and non-amended organic soil. 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 nonamended organic soil being grouped closest together (Figure 4D).

Significant negative correlations were found between diversity (S and *H*) and survival (c and d) only for conventional sandy soils from both countries ($P < 0.05$, 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). There was no significant correlation in all the other cases.

Discussion

Soil type and origin as strong determinants for survival of the pathogen

The main findings of this research were that (1) survival of *R. solanacearum* race 3 biovar 2 strains (PD 5239, PD 5240 and PD 5241) used in this study was affected more by soil type and soil origin than by management or incubation temperature, (2) decline was faster in sandy than in clayey soils from either country, (3) decline was faster in all soils from Egypt than in soils with a Dutch origin regardless of incubation temperature, (4) 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 soil origin were found to be stronger determinants for pathogen suppression than soil management. This was contrary to the expectation, as soil-borne plant pathogens were found to be more frequently suppressed in organically farmed land as compared to conventionally farmed land (van Bruggen, 1995; van Bruggen and Termorshuizen, 2003). The minimal 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



- Conventional, ■ Conventional amended with NPK, - Organic, ○ Organic amended with compost and ■ Organic amended with cow manure

Figure 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, experiment 3.

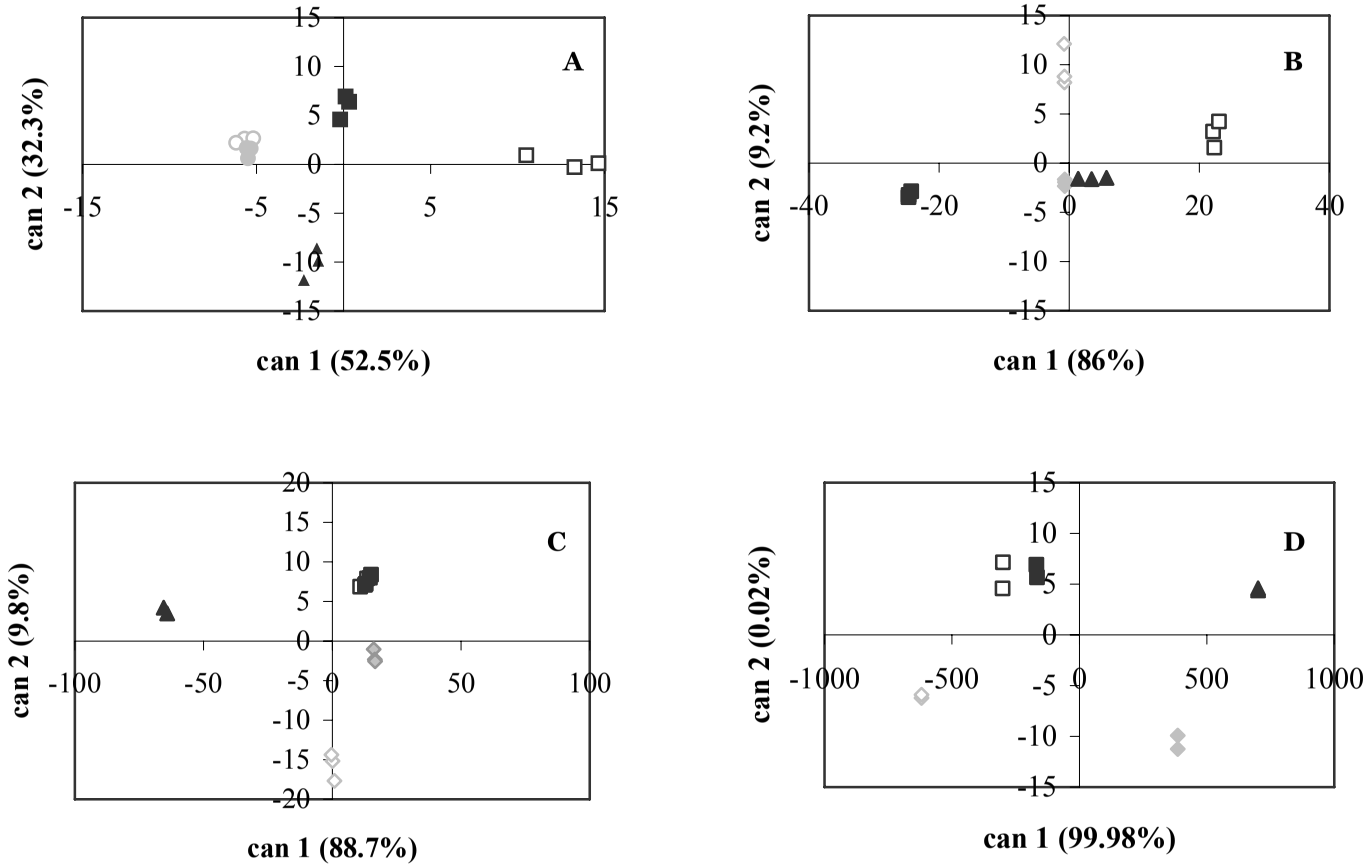


Figure 4. CDA-plots of canonical coefficients 1 and 2 for discrimination between the five classes of non-amended and amended soils based on eubacterial DGGE data, experiment 3. A. Egyptian sand, B. Egyptian clay, C. Dutch sand, and D. Dutch clay.

Termorshuizen, 2003). Nevertheless, significant pathogen suppression was found for organic versus conventional soils in case of Egyptian sandy soil kept at a moderate temperature and for the Dutch clayey and Egyptian clayey soils kept at a high temperature.

Survival periods of the pathogen under different conditions

In our controlled environment tests, CFUs of *R. solanacearum* detected on selective medium SMSA dropped below 10^2 per g d.w. soil within 5 months for all soils tested. On the other hand, survival under field conditions has been reported for up to 3 growing seasons (Graham et al., 1979; Gorissen et al., 2004). Lower temperatures, different microbial populations and temperature fluctuations under field conditions may be partially debit to this (Semenov et al., submitted).

We observed slightly longer survival periods at 15°C than at 28°C in Dutch soils, but not in Egyptian soils, where survival was slightly shorter at the lower temperature. Perhaps competition of *R. solanacearum* with the native microbial community was impaired/diminished when the soil was incubated at low temperature in Dutch soils (15°C, more normal for the Dutch soils) or high temperature in Egyptian soils (28°C, more normal for the Egyptian soils). This notion is supported by a sharp drop in bacterial biodiversity 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 15ºC. Thus, the Egyptian bacterial communities seemed to be better adapted to temperature changes in the range from 15-28ºC than the Dutch soil bacteria were. This is understandable considering the large variations in soil temperatures in Egypt (arid climate) compared to the Netherlands (temperate climate) for the temperatures included in our experiments. Our data confirm that race 3 biovar II is adapted to the lower soil temperatures occurring in temperate regions (Moraes, 1947; Elphinstone, 1996). Unfortunately, microbial activity was not measured.

Another 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.

The faster decline rate of *R. solanacearum* in sand as compared to clay soils may also be related to the soil texture. It is well documented that the finer the soil texture the less the decline of a bacterial inoculum is, 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.

Effect of different amendments on the survival of the pathogen

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. It is documented that in some cases the disease suppression effect of the compost could be seen only after several years of addition (van Bruggen and Termorshuizen, 2003). Also, a suppressive effect of compost may be clearer in the presence of plants. Composts may enhance the plant defense mechanism by improving soil structure and water holding capacity and hence protecting the plant from being susceptible to pathogens when there would be drought stress in non-amended soil (Termorshuizen et al., 2004).

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, such as *Verticillium dahliae* (Conn et al., 2005).

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 we used 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* strain, which could use nitrate as electron acceptor at relatively low oxygen concentrations (which probably occurred in vitro even though the intention was to avoid anoxic conditions). 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). Thus the higher the pH the higher the concentration of toxic ammonia can be after fertilizer application (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) may have a deleterious effect on *R. solanacearum.* In addition, soil pH affects the availability of soil ions: a higher pH restricts the availability of many soil nutrients. Positive correlations were found between the availability of potassium, sodium, phosphorus, and nitrogen respectively and the 50%-reduction time and negative rate of decline of the pathogen, implying a slower decline at higher concentrations of these nutrients. Indeed, the soil with the highest (most negative) decline rate had the lowest available nutrient contents and the highest pH. However, these correlations may simply be coincidental, and are difficult to interpret.

Bacterial biodiversity and pathogen survival

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 generally poorer Egyptian soils than in their Dutch counterparts. The biodiversity was also lower in the sandy than in the clayey soils from both countries. This was opposite to the finding of van Diepeningen et al. (2006), who found, on average, a higher diversity in sandy than clayey soils in the Netherlands. 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 with 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: Egyptian sandy soils were low in biodiversity and also had the worst conditions for the survival of *R. solanacearum*. 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).

Conclusions

Thus, 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, we suggest to quantify soluble sugars and amino acids 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.

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Chapter 3

Potato brown rot incidence and severity under different management and amendment regimes in different soil types

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Messiha NAS [1,2,3], van Bruggen AHC [1], van Diepeningen AD [1], de Vos OJ [1], Termorshuizen AJ [1], Tjou-Tam-Sin NNA [2], and Janse JD [2] (2006) Potato brown rot incidence and severity under different management and amendment regimes in different soil types. European Journal of Plant Pathology

[1] *Biological Farming Systems Group, Marijkeweg 22, 6709 PG Wageningen, the Netherlands*
[2] *Plant Protection Service, Dept. of Bacteriology, Geertjesweg 15, 6706 EA Wageningen, the Netherlands*
[3] *Potato brown rot project, Agricultural Research Centre, Cairo, Egypt*

Plant protection service (PD), Dept. of Bacteriology, P.O. Box 9102, 6700 HC Wageningen, the Netherlands. E-mail: j.d.janse@minlnv.nl, tel +31 317-496810, fax +31 317-421701.

Chapter 3. Potato brown rot incidence and severity under different management and amendment regimes in different soil types

Abstract

Ralstonia solanacearum race 3 biovar 2, the causative agent of potato brown rot (bacterial wilt) disease, is an economically important disease in hot and temperate regions of the world. In view of previous reports on suppression of the disease by organic amendments, and the expansion of organic agriculture, it was timely to investigate the effects of organic and conventional management and various amendments on brown rot development in different soils. Soil characteristics that may be involved in disease development or suppression were also studied. Disease incidence and severity were highest in Dutch sandy soils where Dissolved Organic Carbon (DOC) content was relatively high, and were likely related to substrate availability. The disease was reduced in the Dutch clayey soils where both the K and Ca contents were high, which may have made the plants more resistant. Brown rot infection was only slightly suppressed in organically compared to conventionally managed sandy soils from Egypt, but organic management significantly increased disease incidence in Dutch sandy and clay soils. This was again related to a high DOC content in organic Dutch soils. There was no correlation between disease incidence or severity and bacterial diversity of 16S rDNA extracted from the potato rhizosphere in differently managed soils. The effect of various soil amendments, NPK fertilizer for conventional soils and compost or manure for organic soils was also investigated. NPK fertilization suppressed brown rot incidence and severity in Egyptian soils but not in Dutch soils. Cow manure fertilization suppressed the disease in all soils except the Dutch clayey soils, while amendment with compost did not have a suppressive effect in any soil type. The absence of a disease suppressive effect of fertilization in Dutch clayey soils may be related to the already high availability of inorganic and organic nutrients in those soils.

Introduction

Ralstonia solanacearum (Smith) Yabuuchi et al. causes a vascular disease in many different host plants, including many economically important crops. Three races are distinguished, race 1 having a broad host range, race 2 affecting bananas, and race 3 infecting primarily solanaceous crops. The disease is generally called ‘bacterial wilt’, but in potato it is also called ‘brown rot’. Bacterial wilt causes economic problems for about three million farm families in 80 countries causing a devastating annual loss exceeding $ 950 million (Walker and Collion, 1998).

In some areas, naturally suppressive soils have been located (Nesmith and Jenkins, 1983; Nesmith and Jenkins, 1985; Ho et al., 1988; Hayward, 1991; Shiomi et al., 1999). An

influence of soil type on disease severity has been repeatedly established (Moffett et al., 1983; van Elsas et al., 2000), but the actual effects are controversial and depend on geographical location, strains (race, biovar) of the pathogen, and crop. In some cases, bacterial wilt was most severe on well-drained sandy loams (French, 1994; Graham and Lloyd, 1979; Hayward, 1991; Ho et al., 1988; Kelman, 1953; López and Biosca, 2004, Nesmith and Jenkins, 1985), and the decline rate of the pathogen was higher in clay loam than in sandy loam (Moffett et al., 1983). In other cases, the disease was most severe on heavy clay-loam soil (Kelman, 1953). Thus, suppressiveness of soil towards bacterial wilt may be related to other factors than soil type *per se*, such as pH, organic matter content and microbial communities (van Elsas et al., 2005). Disease suppression was lost after soil treatment with methyl bromide or heat, indicating that a biological principle was the main effector (Nesmith and Jenkins, 1976; Nesmith and Jenkins, 1985).

Control of bacterial wilt in areas conducive to the disease is generally very difficult. There are no curative chemicals. Resistance breeding has only been reasonably successful against race 1 of the pathogen in tropical crops like eggplant, tomato, peanut, pepper, and to a very small extent potato in South America. Cultural measures such as the use of healthy (tested) seed, a wide crop rotation, use of certain rotation crops such as corn or rice, rouging and burning of diseased plants, and careful water management also have had only limited success (Kelman, 1953; Hartman and Elphinstone, 1994; López and Biosca, 2004).

Inorganic soil amendments have sometimes shown promising results both in pot experiments and in the field, but in other cases there was little effect. An increase or decrease in pH reduced wilt in tobacco, tomato and eggplant when sulphur (acidification) or lime (neutralization) were added, especially in relatively acid, fine sandy soils (Kelman, 1953; Michel and Mew, 1998). However, for potato on sandy loam soils with high organic matter content this was not effective. A high nitrogen dose reduced bacterial wilt in sandy soils; nitrates were more effective than ammoniacal compounds (Kelman, 1953; Michel and Mew, 1998). Soil amendment with NPK at 100 kg ha^{-1} significantly decreased bacterial wilt incidence on potato and increased potato yield (Lemaga et al., 2005). Amendment of soil with high inputs of urea and CaO or MgO also significantly decreased the bacterial wilt population (Elphinstone and Aley, 1993; Michel et al., 1997).

Besides inorganic amendments, organic amendments can affect both survival of the pathogen in soil and infection of the host. Amendment of soil with compost or manure had a positive effect on decline rates of the pathogen in soil (Hoitink and Boehm, 1999; Schönfeld et al., 2003). Pig slurry had a similar suppressive effect (Gorissen et al., 2004). However, the suppressive effect of soil amendment on the survival of *R. solanacearum* can depend on soil type (Michel and Mew, 1998). Disease suppressive effects of organic amendments were attributed to abundance of antagonistic microbial populations (Mahmoud et al., 1978; Schönfeld et al., 2003; van Elsas et al., 2005). Suppression of bacterial wilt by pig slurry was associated with a microbial community shift (Gorissen et al., 2004), but ammonia toxicity

could have been involved as well (Michel and Mew, 1998). Reduction of the disease by mustard green manure was considered to be due to the release of volatile glucosinolates (Akiew et al., 1996). However, compost, manure and cow dung were sometimes not effective at controlling bacterial wilt in impoverished soil in South Africa and India (Bandara, 1984; Kelman, 1953).

Nevertheless, soil amendments have reduced the inoculum and bacterial wilt development in many cases, and thus, diminished yield losses. This is especially important for small farmers in tropical countries, where the disease is endemic. This holds for race 1 in many (food) crops all over the world, but also for the narrow host range or 'potato' race 3, biovar 2 in many mountainous areas in South America and Asia as well as in the Nile Delta area in Egypt.

This last area has heavy clay soils deposited by overflowing of the river Nile. Vegetables, including potatoes, have been grown there for centuries and *R. solanacearum* race 3 biovar 2 is widespread since the 1940's, also in the surface and irrigation water (Farag et al., 1999). The common occurrence of potato brown rot in this area has prompted the EU to ban importation of Egyptian potatoes from the Nile Delta area, as *R. solanacearum* is still a quarantine organism for the EU (Anonymous, 1998). Export of Egyptian potatoes to the EU is allowed from so-called pest-free areas, newly developed desert areas with sandy soils, which are pivot-irrigated, for a large part with ancient water from a large aquifer, so that contamination by irrigation water in this case is unlikely. However, in case of accidental introduction of *R. solanacearum* into the desert area, it would be important to know what the disease risk would be and what farmers could do to reduce this risk.

In view of the potential suppressive effects of soil amendments, it would be interesting to know if organic management could lead to bacterial wilt suppression. Root diseases and wilts caused by fungi are frequently suppressed in organically managed soils (van Bruggen and Termorshuizen, 2003), but effects of organic versus conventional management have not been studied for bacterial diseases. Organic crop production is increasing in Egypt, including potato production for export, and it would be important to know if the risks of brown rot are reduced by organic compared to conventional crop management.

The effects of organic versus conventional management, different soil types, and amendment with NPK fertilizer or cow manure on survival of *R. solanacearum* race 3 biovar 2 were studied by us previously (Messiha et al., accepted). The decline in *R. solanacearum* was faster in sandy than in clay soils; management effects were much smaller than effects of soil type. Amendments with NPK fertilizer or cow manure enhanced the decline rate of *R. solanacearum*. The decline rate of the pathogen was negatively correlated with total soluble organic matter and positively with bacterial diversity as estimated from results of denaturing gradient gel electrophoresis (DGGE) of eubacterial 16S rDNA directly extracted from soil (Messiha et al., accepted).

The main aim of this study was to investigate if soil management could affect potato brown rot development. Both whole management types (organic versus conventional) and individual management components (amendment of conventional soil with NPK and of organic soil with manure or compost) were compared. Sandy and clayey soils both from Egypt and the Netherlands were used. Various factors that could possibly explain differences in potato brown rot development in the different soils were measured, namely soil physical and chemical characteristics and bacterial diversity in the bulk soil and rhizosphere of healthy potato plants grown in the various soils. As bacterial wilt development is partially dependent on the ability of the pathogen to grow inside the xylem vessels (Buddenhagen and Kelman, 1964), and the composition of xylem sap may vary with nutrient availability in the soil (Persson and Näsholm, 2002), xylem sap of potato plants grown in the various soils was analyzed for amino acid and ammonium content and checked for suitability as a growth substrate for *R. solanacearum*.

Materials and methods

Soils and amendments

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 two others were from the Netherlands (Table 1). For repetition of the experiments the soils were sampled three times, in July 2003, March 2004, and February 2005 (experiments 1, 2 and 3, respectively). Twenty-five kilogram soil was collected at random sampling points in 100 m^2 plots down to 15-25 cm vertical depth with an augur and mixed. Samples were transported in plastic bags in containers with ice. Upon arrival in the laboratory each soil sample was thoroughly mixed and plant parts and earthworms were removed. The soils were stored at 4°C till the start of the experiment. Egyptian soils were sent to the Netherlands in plastic bags in styrofoam boxes by overnight express airmail.

To test the effect of different soil amendments (experiments 2 and 3), soils were left non-amended or supplemented to 170 kg N ha^{-1} (0.1 g N kg^{-1} soil), the maximum amount that could be applied in manure according to EU regulations. Conventional soils were amended with NPK fertilizer (12% N = 7% ammonium + 5% nitrate; 10% P; 18% K; 0.09 g/100 g soil) or left non-amended. Organic soils were amended with compost (Compost 8.1S, made from 88% wood chips, 2.5% manure and 10% clay with 0.61% of total N content; 1.7 g compost/100 g soil) or manure (organic cow manure with 0.48% of total N content; 2.17 g/100 g soil) or left non-amended.

Table 1. Origin of soil samples.

Sample	Code	Country	Soil type	Management	Previous and actual crops at time of sampling	Location
1	ESC[a]	Egypt	Sand	Conventional	Potatoes[b,c,d,e]	Nubaria, desert area
2	ESO	Egypt	Sand	Organic	Potatoes[b,c,d,e]	Nubaria, desert area
3	ECC	Egypt	Clay	Conventional	Beans[b], potatoes[c,d,e]	Behera, Delta area,
4	ECO	Egypt	Clay	Organic	Potatoes[b,c,d,e]	Kaliobia, Delta area,
5	NSC	NL	Sand	Conventional	Grass[b], unplanted[c,d,]	Marknesse, Northeast polder
6	NSO	NL	Sand	Organic	Grass[b,c,d]	Marknesse, Northeast polder
7	NCC	NL	Clay	Conventional	Winter wheat[b], seed potatoes[c], sugar beet[d]	Ens, Northeast polder
8	NCO	NL	Clay	Organic	Grains[b], onions[c], beans[d]	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).
[b] In 2003, [c] in 2004, [d] in 2005.
[e] In the Egyptian fields, the potatoes was still there or had just been harvested.

Soil characterization

Physical characterization

For physical analysis, 100 g samples of non-amended soils were dried to air-dry at room temperature. Fractions of different soil particle sizes were determined at the Laboratory of Soil Science and Geology of Wageningen University and Research Centre, the Netherlands, with a Coulter LS230 particle size analyzer (Beckman Coulter, Inc., Fullerton CA, USA). The method used was obtained from the manual of the Coulter grain-sizer type of 230. Particle sizes of <2 µm were considered clay, 2-50 µm was considered silt and 50-2000 µm was considered sand (Table 2).

Table 2. Physical and chemical composition, and rhizosphere biodiversity for organic and conventional sandy and clayey soils with and without different amendments (1 sample for soil texture, organic matter and pH in experiment 1; averages of experiments 2 and 3 (1 sample each) for the rest).

Soil + Amendments	Soil composition[a] mg / kg soil													Bulk soil biodiversity[b]		Rhizosphere biodiversity[b]	
	Clay%	Silt%	Sand%	OM%[c]	pH-KCl	Ca	Na	K	P	N	$N\text{-}NO_3$	$N\text{-}NH_4$	DOC[d]	S[e]	H[f]	S[d]	H[e]
ESC[j]	0.1	0.6	99.3	<0.2	7.9	15531	211	572	131	223	9.6	5	50	27	1.25	27.5	1.31
ESC+NPK						16536	326	755	166	251	36	11.9	47			27	1.3
ESO	0.1	0.6	99.3	0.3	8.2	22970	347	821	101	217	9.2	1.8	54	31	1.27	30	1.31
ESO+compost						27495	428	1069	127	300	17.1	1.6	80			29	1.33
ESO+manure						24782	354	861	106	228	9.2	1.9	53			18.5	1.19
ECC	9.4	54.5	36.1	4.9	7.6	4326	958	3925	786	1022	90.6	15.6	113	35	1.45	36.5	1.45
ECC+NPK						5512	1013	4076	796	1055	135.8	25.5	113			33.5	1.45
ECO	13.9	65.7	20.4	6.2	7.6	11292	1382	4848	1070	1242	36.8	15.6	89	30	1.43	31	1.32
ECO+compost						8029	1315	4961	1171	1313	40.6	15	96			31.5	1.36
ECO+manure						24034	879	5480	1224	1292	38.6	11.9	78			37.5	1.43
NSC	3.2	32.3	64.5	1.5	7.4	16419	1046	5035	744	1114	9.1	13.4	138	28	0.94	27.5	1.3
NSC+NPK						16105	1026	5147	763	1149	60.5	19.6	153			26.5	1.32
NSO	3.2	33.3	63.5	4.1	7.1	17092	973	4791	653	1260	18.3	12.2	170	28	0.99	32.5	1.39
NSO+compost						18418	993	4933	694	1381	23.4	12.2	196			28.5	1.3
NSO+manure						13462	1255	3632	708	1571	22.7	9.3	197			31	1.29
NCC	7.7	51.9	40.4	2.2	7.4	21556	653	3711	545	644	7.6	15.7	127	39	1.43	27	0.94
NCC+NPK						25377	878	6969	1086	1251	72.2	31.6	133			25.5	0.65
NCO	8.3	54.5	37.2	2.3	7.3	26108	751	3725	417	654	9.9	12.8	130	30	1.26	30.5	1.23
NCO+compost						23616	1191	6600	750	1161	19.3	16	135			29.5	1.22
NCO+manure						24401	963	6562	732	1210	9.9	15.6	152			24.5	1.52
LSD ($\alpha = 0.05$)						**11076**	**598**	**3721**	**475**	**549**	**62.4**	**14.6**	**40**			**8**	**0.3**

[a] Particle sizes: clay <2 µm, silt 2-50 µm, sand 50-2000 µm.

[b] Bacterial diversity determined by 16S rDNA PCR – DGGE, only in experiment 3 (2 replicates).

[c] Total Organic Carbon, [d] Total Organic Carbon, [e] Species richness, [f] Shannon-Wiener diversity index, [j] See table 1 for the soil codes.

Chemical characterization

For chemical analysis, 100 g samples of non-amended and amended soils were dried to air-dry at room temperature. $N\text{-}NO_3$ and $N\text{-}NH_4$, were extracted with 0.01M $CaCl_2$ and total P was extracted with 0.01 M H_2SO_4 before spectrophotometrical analyses with a Segmented Flow Analyzer (Skalar Analytical BV, Breda, the Netherlands). Total N and organic C contents were determined with a CHN1110 Element Analyzer (CEInstruments, Milan, Italy). The dissolved Na and K were determined after sample extraction with 0.01 M $CaCl_2$, vaporized, and analyzed by flame emission spectrophotometer at a wavelength of 589.0 nm for Na and 766.5 nm for potassium. Since the Ca can interfere with Na analysis, the Ca content was suppressed by forming a difficult to dissociate Ca-Al complex by adding aluminum salt (Houba et al., 1989b). The pH was measured after 0.01 M $CaCl_2$ extraction using a pH/mV meter and combined electrode (Houba et al., 1989a; Houba and Novozamsky, 1998). Organic matter was determined by loss-on ignition i.e. by dry combustion of the organic material in a furnace at 500-550°C and the loss in the weight indicated the content of organic matter in the sample (Houba et al., 1997).

Microbial characterization-DGGE analysis

Total DNA was extracted from 0.5 g (wet weight) soil samples from bulk soil (at the beginning of experiment 3) or rhizosphere soil (at the end of experiment 3) 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. The DNA quality and quantity were checked (Sambrook et al., 1989) on 1.2% agarose gels with ethidium bromide and visualized by UV trans-illumination..

For DGGE analysis of the eubacterial soil population, PCR amplification of the V6 to V8 region of the 16S rRNA gene from total soil DNA was conducted as described by (Heuer and Smalla, 1997; Rosado et al., 1998; Hiddink et al., 2005). In general, DNA extracts from rhizosphere were diluted ten times more than for bulk soil to have the same amount of extracted DNA (about 2 ng). Extracted DNA samples of different managements and amendments within the same soil type from the same country of origin were loaded on the same gel at least twice. DGGE was carried out using the Dcode system (BIOrad Laboratories, Hercules, CA, USA), as mentioned in (Messiha et al., submitted). The scanned gels were analysed with Phoretix 1D (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK) as mentioned in (Messiha et al., submitted).

The bacterial diversity in the samples was estimated in two ways: as species richness *S*, and as the Shannon-Wiener index of bacterial diversity, *H'* as described by (Eichner et al., 1999; van Diepeningen et al., 2006) and (Messiha et al., submitted).

Bacterial strains and inoculation

A mixture of equal amounts of three virulent *R. solanacearum* strains: PD5239, PD5240 and PD5241 (culture collection, Plant Protection Service, Wageningen, the Netherlands) were used in this study. These strains are from Egyptian origin, isolated from infected potato tubers. All three strains can use nitrate as electron acceptor under anaerobic conditions and belong to race 3 biovar 2. The cultures used for soil inoculation 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}). The inoculum was mixed with the soil to have a final bacterial concentration of 10^7 CFU g^{-1} dry soils.

Host plants and greenhouse conditions

The susceptible potato variety Nicola was used in the disease suppression experiments. In each experiment, six replicate pots were used per treatment, and two tuber eyepieces were planted per pot one day after inoculation of the soil. Two pots with non-inoculated soil per treatment were included as controls. The experiment was conducted three times for differently managed soils (experiments 1, 2 and 3) and two times (experiments 2 an 3) for differently amended soils in the quarantine greenhouse of the Plant Protection Service (PD) in Wageningen, The Netherlands. The conditions of the greenhouse were adjusted to be 25°C during the day and 20°C during the night, with a RH of 75 to 80% and a total of 14 h light per day.

Monitoring the disease

Disease development was monitored daily. Wilt was scored according to the percentage of wilted leaves relative to the total number of leaves per pot. Latent infection in the lower stem area (crown area) of each plant was checked at the end of the experiment (35 days). This latency was tested by plating the cut, weighed, and surface sterilized plant tissues from the crown area (in phosphate buffer saline PBS 0.01 M) on Selective Media South Africa (SMSA) plates (Anonymous, 1998). From all plant samples three ten-fold serial dilutions were plated starting from 100 µl per plate and the recovered *R. solanacearum* bacteria were counted after 5-7 days of incubation at 28°C.

Population densities of the pathogen were determined in rhizosphere soil of potato plants and in bulk soil at the end of each experiment. In case of rhizospere soil, 1 g was added to 99 ml of sterile phosphate buffer (0.05 M). Bulk soil suspensions were made by adding 10 g soil to 90 ml of sterile phosphate buffer. After shaking for 2 h at 15°C, 100 µl was spread on the surface of SMSA agar plates and a further one-tenth dilution in another set of plates. Plates were incubated at 28°C for 5-7 days. Putative *R. solanacearum* colonies (one from each treatment) were randomly tested for identity using Immunofluorescence Antibody Staining

(Janse, 1988).

Extraction of xylem sap and apoplastic fluid

Another greenhouse experiment was conducted at the same time as experiment 3 using soils from the same sources and under the same greenhouse conditions but without inoculating the soils. There were 3 replications per treatment (management and soil types). The aim of the experiment was to assess the amino acid and the ammonium content in the xylem and apoplastic fluid of plants grown in different soils and to find their relation with disease severity in the parallel experiment. Xylem exudates were collected from each sample (a 5-cm stem section of 10 g) by centrifugation at 4°C, starting with a centrifugation force of 100 g for 15 min, increasing it by 100 g up to 600 g (every time for 15 min) until about 100 µl fluid was obtained per sample. Fluid was extracted from about 10 stem sections per plant. Samples with <100 µl fluid from the same treatments were pooled, so that mostly 2 replicates per treatment remained. Cytoplasmic contamination of all samples was assessed comparing the MDH (malate dehydrogenase E.C.1.1.1.137) activity in the extracted sap with that of the total leaf homogenate. Samples with MDH contamination less than 5% were stored at -20°C for further analysis (24 samples in total) and others were discarded (Dannel et al., 1995; Husted and Schjoerring, 1995; López-Millán et al., 2000). Apoplastic and xylem fluids collected from each (pooled) sample were divided into three parts, one part for assessment of total amino acid content, one for ammonium content and the third for testing the survival of *R. solanacearum* in the extracted fluid.

The ammonium content of the extracted fluid was determined as described above for soil extracts. Total amino acids content was quantified using ninhydrin color reagent and a spectrophotometer at wave length of 570 nm (Jones et al., 2002). As ninhydrin detects the NH_4^+ with equal sensitivity as amino acids, amino acids content was determined as follows:

$$\text{Amino acids } (\mu M) = ((O_N - B_N - A_N)\, S_N^{-1}) \times 100 \qquad (1)$$

O_N = spectrometer reading of the sample mixed with the ninhydrin reagent

B_N = spectrometer reading of the blank (if in most samples B_N=0, this factor can, therefore, be ignored)

S_N = spectrophotometer reading of 100 µM amino acids standard

A_N = $AC_N \times AR_N\, AS_N^{-1}$ (accounts for the interference of NH_4^+ in the procedure)

AC_N = ammonium concentration in the sample as determined by autoanalyser-based spectrophotometry (µM)

AS_N = ammonium concentration of aminoacids standard (µM)

AR_N = spectrophotometer reading of the ammonium standard using the ninhydrin colorimetric procedure (the ammonium standard is the same standard as used for the NH_4^+-determination with the autoanalyser).

Growth rates of *R. solanacearum* in extracted fluid from potato plants grown in differently managed soils and different soil types were determined. Two samples each of 100 µl from different soils were inoculated with *R. solanacearum* to have a final concentration of 10^4 cfu. $100\mu l^{-1}$. Control samples were included by inoculating sterile distilled water. Inoculated samples were incubated at 28°C for 48 h. Cells of *R. solanacearum* were counted under a UV microscope after IFAS staining (Janse 1988).

Statistical analysis

Wilt severity was plotted over time and area under the disease progress curve (AUDPC) was calculated for each pot. AUDPC values and log transformed (log (x+1)) densities of *R. solanacearum* in bulk soil, in rhizosphere soil and in plant tissue were tested for normality. As these variables were generally not normally distributed, non-parametric analysis (NPar-Tests, Mann-Whitney Test) was conducted for AUDPC, log(cfu+1) in soil, log(cfu+1) in rhizosphere soil, and log(cfu+1) in plant tissue using SPSS v 12 (SPSS Inc., Chicago, Illinois, USA) to test the effects of soil origin (countries), type, management, and amendment. Chi-square (χ^2) tests were conducted on contingency tables with two categories of wilted or infected plants per pot (wilted / infected or not) and soils from different origin, type, management, and amendment using Microsoft Excel 2003 (Microsoft Corporation, Seattle, WA, USA).

All soil characteristics were subjected to ANOVA (post-hoc, LSD) to test for differences between soil and management types and amendments, using SPSS v 12. Correlation analyses between AUDPCs (or population densities of *R. solanacearum* in soil, rhizosphere or plant tissues) and chemical characteristics of the soils were also conducted in SPSS v 12.

The amino acid content, ammonium content, and the ratio of amino acid and ammonium content of the xylem and apoplastic fluids were compared for organically and conventionally managed soils, and for clay and sandy soils, using independent t-tests. The mean AUDPC, *R. solanacearum* density in soil, rhizosphere and plant tissue and the concentration of *R. solanacearum* in extracted fluids per treatment were plotted versus the mean amino acid content and the ratio of amino acid to ammonium content in the extracts, and correlation coefficients were determined.

Cluster analysis was carried out on the DGGE band intensities of all lanes on individual gels, representing the bacterial composition for different managements or amendments within the same soil type from the same country of origin, using the Phoretix software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK).

Results

Disease incidence and severity in organic and conventional non-amended soils

Pathogen behavior was assessed using six indicators for each pot: percentage of wilted and infected plants, AUDPC (based on disease severity), CFU in bulk soil, in rhizosphere, and inside plant tissue (lower stem).

Disease incidence and severity were not normally distributed, so that analyses of variance could not be carried out. Chi-square tests on incidence classes showed that more plants were wilted and infected by *R. solanacearum* ($\chi^2 = 22.38 > 16.27$, $\alpha = 0.001$) when growing on Dutch sandy soils ($\chi^2 = 24.33 > 16.27$, $\alpha = 0.001$) than plants growing on any of the other soils (Table 3 and 4). The number of wilted plants per pot was higher in organic than in conventional Dutch soils. This difference was just not significant in the sandy soil ($\chi^2 = 3.00 < 3.84$, $\alpha = 0.05$), but was significant in the clayey soil ($\chi^2 = 4.05 > 3.84$, $\alpha = 0.05$). The number of plants from which *R. solanacearum* was isolated was higher in the conventional Egyptian sandy soil than in its organic counterpart, while it was the reverse for the pair of Dutch sandy soils. However, the differences were just not significant (Table 4). No management effects on disease incidence were observed for the other soil pairs.

Table 3. Contingency table for the effect of country of origin (Egypt or the Netherlands) and soil type (clay or sand) on the number of pots distributed over 3 categories of bacterial wilt incidence on potato and number of plants from which *R. solanacearum* was isolated, 35 days after planting in 3 greenhouse experiments combined.

Country and soil type	No. of wilted plants per pot			No. of infected plants per pot		
	0	**1**	**>1**[a]	**0**	**1**	**>1**
ES	26	5	5	20	6	10
EC	24	5	7	17	6	13
NS[b]	9	9	18	7	2	27
NC	26	4	6	21	4	11
χ^2[c]	**24.3**			**22.4**		

[a] 2 or 3 wilted or infected plants per pot; [b] significantly different from the three other soil types; [c] significant when $\chi^2 > 16.27$, α=0.001.

Log transformed AUDPC and CFUs were still not normally distributed, so that Mann-Whitney non-parametric tests were carried out. The median AUDPC for the plants growing

on Egyptian soils and for those on Dutch conventional clay soils was 0 although disease did occur in some of the plants (Table 5). Most disease occurred in plants growing on Dutch sandy soils (Figure 1), irrespective of their type of management. AUDPC of inoculated plants growing on Dutch sandy soils were significantly higher than of those growing on Dutch clay soils or Egyptian soils ($P < 0.001$). Similarly, CFUs were higher in soil, rhizosphere, and potato stems ($P = 0.001$, in all cases). This is in agreement with the higher percentage of wilt and higher percentage of infection in Dutch sandy soils, as described above. Disease severity (AUDPC) and population densities of *R. solanacearum* in soil, rhizosphere and plant were slightly lower in the organic than in the conventional Egyptian sandy soil (just not significant), but the reverse was true for the Dutch sandy and clayey soils (only significant for CFU g^{-1} soil). Thus, there was a tendency that the pathogen and disease development were suppressed in organic Egyptian sandy soil and increased in Dutch organic and conventional soils (Table 5).

Table 4. Contingency table to the effect of management (conventional or organic) of sandy and clay soils on the number of pots distributed over 3 categories of bacterial wilt incidence on potato and number of plants from which *R. solanacearum* was isolated, 35 days after planting in 3 greenhouse experiments combined.

Country Soil type	No. of wilted plants per pot			No. of infected plants per pot		
Management[a]	0	1	>1[b]	0	1	>1
ESC[c]	12	3	3	7	4	7
ESO	14	2	2	13	2	3
χ^2 [d]	**0.35**			**3.40**		
ECC	11	2	5	8	3	7
ECO	13	3	2	9	3	6
χ^2	**1.45**			**0.14**		
NSC	6	6	6	5	2	11
NSO	3	3	12	2	0	16
χ^2	**3.00**			**2.21**		
NCC	16	1	1	13	0	5
NCO	10	3	5	8	4	6
χ^2	**4.05**			**1.28**		

[a] Conventional (C) versus organic (O); [b] 2 or 3 wilted or infected plants per pot; [c] For soil codes see Table 1; [d] Not significant when $\chi^2 < 3.84$, $\alpha<0.05$), a trend when $\chi^2 > 2.71$, $\alpha<0.10$.

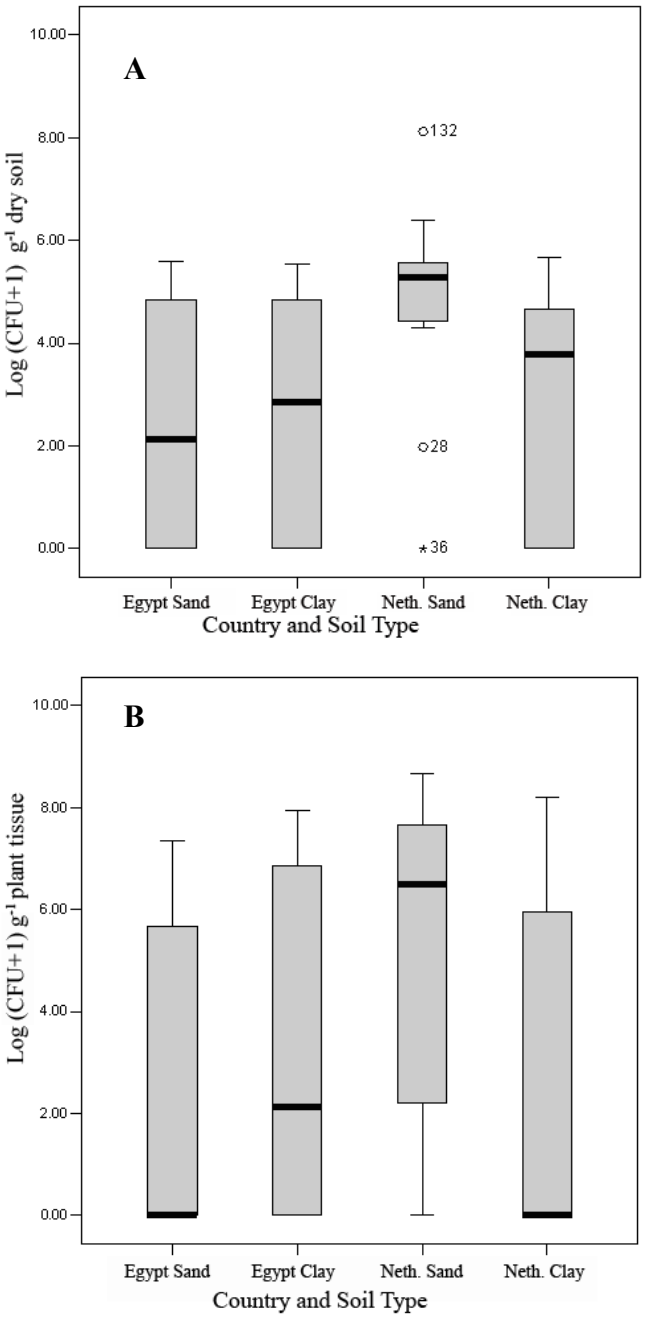

Figure 1. Median and range of log transformed (CFU + 1) *of Ralstonia solanacearum* per g dry soil (A) or per g plant tissue (B), isolated from different inoculated soil types from Egypt and the Netherlands, 35 days after planting of potatoes (average of three experiments with 6 replicates each).

Table 5. Effect of organic versus conventional management on potato brown rot, AUDPC and CFU per g of soil, rhizosphere soil and per plant (log transformed + 1), 35 days after planting of potato in 3 greenhouse experiments (data combined).

	AUDPC			**CFU g^{-1} soil**			**CFU g^{-1} rhizosphere**			**CFU g^{-1} plant**		
Soil type	Median	5% Trimmed Mean	Mean rank	Median	5% Trimmed Mean	Mean rank	Median	5% Trimmed Mean	Mean rank	Median	5% Trimmed Mean	Mean rank
ESC	0	83.06	67.28	4.14	2.83	67.64	5.97	4.65	64.58	2.79	3.13	70.64
ESO	0	17.78	55.03	0.39	1.78	52.83	2.78	3.12	49.81	0	1.57	53.44
ECC	0	63.98	68.08	4.05	2.93	70.81	6.18	4.65	71.47	3.46	3.26	73.03
ECO	0	59.07	62.94	1.38	2.02	55.22	5.61	4.94	71.58	3.12	3.22	74.11
NSC	133.5	177.25	91.17	4.84	3.67	86.36[a]	6.81	6.33	88.56	4.92	4.43	88.81
NSO	348.5	375.56	109.92	5.45	5.08	111.94[a]	7.67	7	105.11	7.06	5.48	100.11
NCC	0	11.79	50.22	2.55	2.30	55.50[b]	5.31	4.08	55.67	0	1.41	52.83
NCO	12.25	91.16	75.36	4.30	3.68	79.69[b]	5.95	5.32	73.22	0	2.57	67.03

[a] Significant difference between organic and conventional soils at $\alpha = 0.05$ Same as under [a].

Physical and chemical soil characteristics for non-amended soils

The clayey soils from Egypt and the Netherlands did not differ in their clay, silt, and sand content, while the sandy soils from the two countries had clearly different textures. The Egyptian sand was 99% pure sand while the Dutch sandy soils contained 32-33% of silt (Table 2). The Egyptian soils had a slightly higher pH than the comparable Dutch soils ($P < 0.001$), but all soils were alkaline. Dutch sandy soils had generally higher nutrient and DOC contents ($P \leq 0.001$) than Egyptian sandy soils. Dutch clayey soils had a higher Ca and DOC content ($P < 0.001$ and 0.04, respectively), but lower OM and nitrate content ($P<0.001$ and 0.006, respectively). Egyptian clay soils had higher OM, DOC and general nutrient contents ($P \leq 0.001$) than Egyptian sandy soils, while Egyptian sandy soils contained more Ca ($P = 0.008$) than Egyptian clay soils. The Dutch clayey soil had a higher Ca content ($P = 0.018$) compared to the Dutch sandy soil. Organic soils from Egypt had higher Ca, Na and K contents ($P < 0.05$ to < 0.001) than their conventional counterparts, while the conventional sandy soil contained more ammonium ($P = 0.012$) than its organic counterpart. For Dutch soils, the nitrate content was significantly higher in the organic than in the conventional sandy soil ($P = 0.002$).

Overall, there were positive correlations between on the one hand DOC and on the other hand AUDPC, final disease severity, percentage of wilted and infected plants (r = 0.48, $P = 0.009$; r = 0.46, $P = 0.012$; r = 0.38, $P = 0.034$; r = 0.39, $P = 0.029$, respectively). There were negative correlations between pH or AUDPC on the one hand and disease severity, wilt incidence, number of infected plants and *R. solanacearum* density in soil, on the other hand, at the end of the experiment (r = -0.53, $P = 0.004$, r = -0.47, $P = 0.010$, r = -0.40, $P = 0.028$, r = -0.39, $P = 0.031$, r = -0.36, $P = 0.043$ respectively).

For Dutch soils, where the disease was lower in the clayey than in the sandy soils (Table 3), availability of Ca and K in non-amended soils were negatively correlated with the AUDPC (r = -0.51, $P = 0.045$ and r = -0.50, $P = 0.051$, respectively) and wilt incidence (r = -0.68, $P = 0.008$ and r = -0.44, $P = 0.075$, respectively). There were positive correlations between Na and percentage of infection, CFU in soil and CFU in rhizosphere (r = 0.51, $P = 0.046$; r = 0.64, $P = 0.013$; r = 0.61, $P = 0.018$, respectively), and negative correlations between ammonium and AUDPC, disease severity, and incidence of wilt and infection (r = -0.723, $P = 0.004$; r = -0.61, $P = 0.019$; r = -0.60, $P = 0.020$; r = -0.50, $P = 0.05$, respectively). On the other hand, there were positive correlations of nitrate with AUDPC, disease severity, and incidence of wilt and infection (r = 0.74, $P = 0.003$; r = 0.64, $P = 0.013$; r = -0.61, $P = 0.018$; r = -0.62, $P = 0.016$, respectively).

For Egyptian soils, fewer correlations were found, namely a positive correlation between nitrate and CFU in plant tissue (r = 0.53, $P = 0.037$) and a positive correlation between DOC and disease severity (r = 0.50, $P = 0.047$).

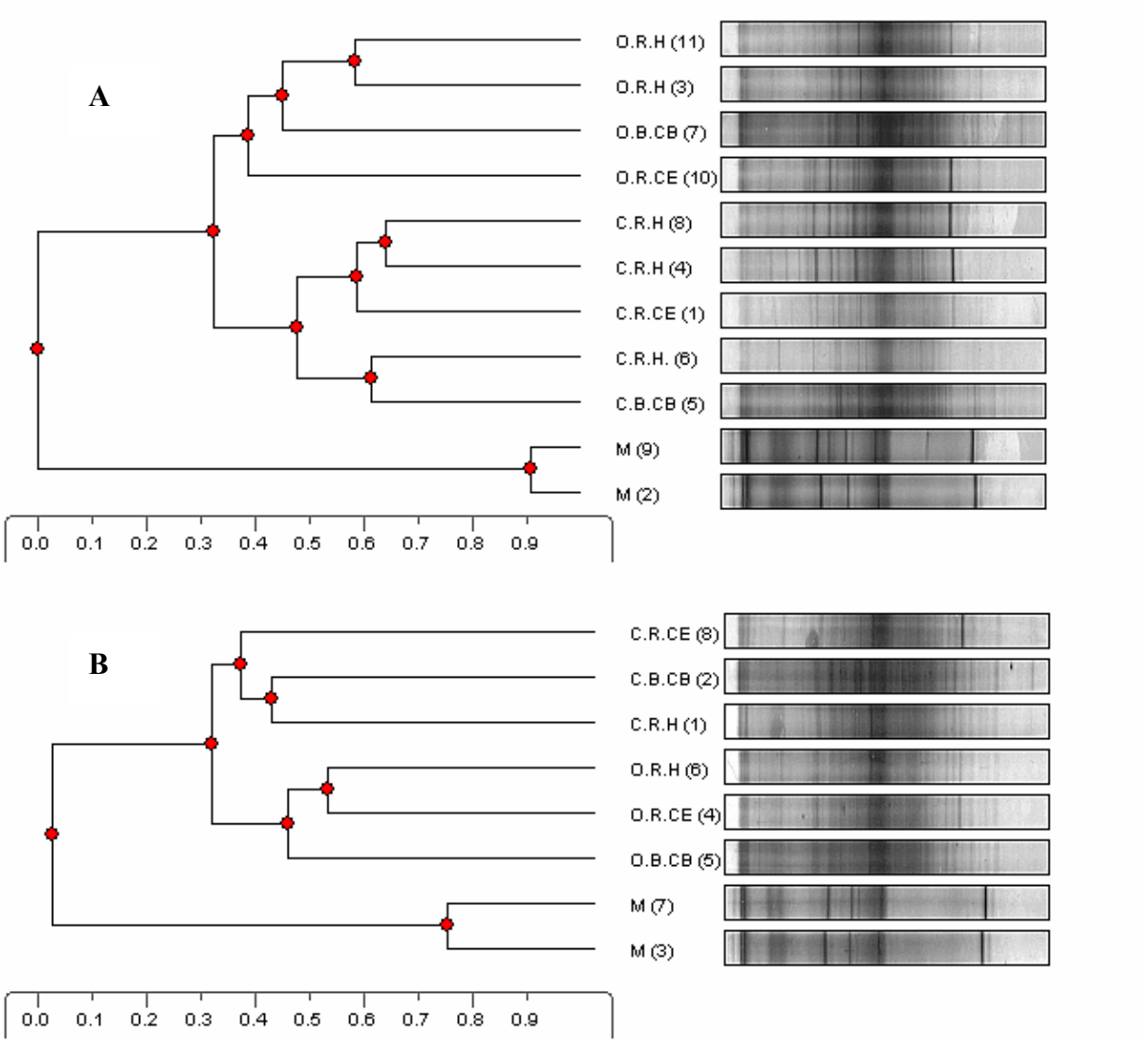

Figure 2. Cluster analysis (Phoretix 1D, NonLinear Dynamics Ltd., Newcastle upon Tyne, UK) for PCR-DGGE products from the rhizosphere of differently managed clayey soils, from Egypt (A) and the Netherlands (B). The scale on the X-axis depicts similarity. The first letters indicate management (O = organic and C = conventional) or marker (M), the second letters stand for rhizosphere (R) or Bulk soil (B), the third letters for treatment (H = inoculated but healthy and C = noninoculated control), and the fourth letters for sampling time (B = beginning and E = end). The numbers between brackets indicate the lane number on the gel.

Biological characteristics of non-amended soils

Only one bulk soil sample of each of the different soils from each country was included in the DGGE analysis. In general, [S] and [*H'*] had higher values in clayey than in sandy soils in both countries (Table 2). The organic Egyptian sandy soil had a higher diversity compared to the conventional counterpart, while the organic Egyptian clay soil had lower diversity than its

conventional counterpart. There were no clear differences between organic and conventional Dutch sandy soils, while the organic Dutch clay soil had less biodiversity compared to the conventional counterpart. On average, no clear difference in biodiversity was found between soils from different countries, management types or soil types (Table 2). These results were by and large in agreement with the biodiversity data for the same soils used in experiments on survival of *R. solanacearum* (Messiha et al., submitted), except that in the latter study the organic clay Dutch soil had higher diversity compared to the conventional counter part.

Cluster analysis of DGGE banding patterns of 16S-rDNA extracted from the rhizosphere of potatoes grown in the different soils resulted in a clustering according to organic or conventional management (Figure 2). There were no significant differences in microbial community composition between rhizosphere and bulk soil for any of the soils as detected with DGGE.

Disease incidence and severity in amended soils

Fertilization of conventional soils with NPK suppressed the disease in some cases. NPK-amendment to Egyptian sandy soil reduced wilt severity at the end of the experiment by 100% (from 18.8% to 0%; $\chi^2 = 3.8$, $\alpha = 0.05$) (Table 6), *R. solanacearum* density in plant tissues by 67.6% and AUDPC by 100% ($P = 0.02$ and $P = 0.033$, respectively) (Table 7). NPK-amendment to Egyptian clayey soil reduced percentage of infection by 84% ($\chi^2 = 5.04 > 4.02$, $\alpha = 0.025$) (Table 6) and CFU of *R. solanacearum* in bulk soil by 92.61%, in the rhizosphere by 80%, and in plant tissues by 90.12% ($P < 0.05$), but not AUDPC ($P = 0.07$) (Table 7). On the other hand, NPK amendment to the Dutch sandy soils had no effect on disease development and NPK significantly increased the percentage of wilted plants from 8.3% to 28.4% ($\chi^2 = 14.19 > 10.83$, $\alpha = 0.001$) (Table 6) and final wilt severity from 4.3% to 27.1% (data not shown) in Dutch clayey soils.

Amendment of organic soils with cow manure also suppressed brown rot in some cases. Manure amendment reduced soil CFU counts by 100% ($P = 0.031$) in Egyptian sandy soil and rhizosphere CFU counts by 61.5% ($P = 0.045$) in Egyptian clay soil (Table 7). Amendment of Dutch sandy soil with manure decreased the percentage of infected plants from 83.3% to 33.3% ($\chi^2 = 6.17 > 5.02$, $\alpha = 0.025$) (Table 6) as well as the AUDPC by 52% ($P = 0.05$) (Table 6 and 7) and final wilt severity from 47% to 20% (data not shown). Amendment of organic soils with compost had no effects on CFU of *R solanacearum* nor on brown rot incidence or severity (data not shown).

Physical and chemical soil characteristics for amended soils

Amendment of Egyptian conventional sandy soil with NPK significantly increased both the nitrate and the ammonium contents ($P = 0.028$ and $P = 0.019$, respectively). Amendment of Egyptian organic sandy soil with compost and cow manure significantly increased the DOC

Table 6. Contingency table for the effect of amendment on the number of pots distributed over 3 categories of bacterial wilt incidence on potato and number of plants from which *R. solanacearum* was isolated, 35 days after planting in two greenhouse experiments (data of experiments 2 and 3 combined).

Country Soil type[a]	No. of wilted plants per pot			No. of infected plants per pot		
Amendment [b]	0	1	>1[c]	0	1	>1
ESC	8	1	3	5	0	7
ESCN	12	0	0	9	0	3
$\chi^{2,d}$	**3.8**			**2.74**		
ESO	10	1	1	10	0	2
ESOM	11	0	1	10	0	2
χ^2	**0.05**			**0**		
ECC	9	2	1	6	0	6
ECCN	12	0	0	11	0	1
χ^2	**1.43**			**5.04**		
ECO	7	4	1	7	1	4
ECOM	10	2	0	10	0	2
χ^2	**1.53**			**1.2**		
NSC	6	3	3	5	0	7
NSCN	7	1	4	9	0	3
χ^2	**0.22**			**2.74**		
NSO	3	3	6	2	0	10
NSOM	8	0	4	8	0	4
χ^2	**2.67**			**6.17**		
NCC	11	0	1	8	0	4
NCCN	7	2	3	7	2	3
χ^2	**14.19**			**0.44**		
NCO	8	4	0	7	1	4
NCOM	11	0	1	10	0	2
χ^2	**1.47**			**1.2**		

[a] For soil codes see table 1

[b] Amendment with NPK (N) or manure (M): the last letter of the soil code

[b] 2 or 3 wilted or infected plants per pot

[c] $\chi^2 < 3.84$ not significant, $\chi^2 > 2.71$ is a trend $\alpha = 0.10$

Table 7. Effect of amendment with NPK to conventional soils and manure to organic soils on AUDPC, CFUs in bulk soil, rhizosphere soil and inside plant tissues (stem base), 35 days after planting potatoes in two greenhouse experiments (data of experiments 2 and 3 combined).

	AUDPC			**CFU g^{-1} soil**			**CFU g^{-1} rhizosphere**			**CFU g^{-1} plant**		
Soil type	Median	5% Trimmed Mean	Mean rank	Median	5% Trimmed Mean	Mean rank	Median	5% Trimmed Mean	Mean rank	Median	5% Trimmed Mean	Mean rank
E[a]S[b]C[c]	0	134.7	14.50*[e]	14.14	3.03	14.58	6.44	4.97	15.08	2.79	3.21	14.92*
ESCN[d]	0	0.0	10.50*	0.00	1.06	10.42	1.51	2.71	9.92	0.00	1.04	10.08*
ESO	0	11.6	12.92	0.00	1.85	15.00*	2.78	3.07	14.17	0.00	0.91	12.67
ESOM	0	19.6	12.08	0.00	0.00	10.00*	0.00	1.11	10.83	0.00	0.64	12.33
ECC	0	55.9	14.00	2.54	2.57	14.92*	3.08	3.50	14.92*	1.71	2.42	15.00*
ECCN	0	0.0	11.00	0.00	0.19	10.08*	0.00	0.70	10.08*	0.00	0.22	10.00*
ECO	0	59.1	13.00	0.00	1.69	13.58	5.61	4.57	15.25*	0.00	2.06	14.00
ECOM	0	28.2	12.00	0.00	0.72	11.42	0.00	1.76	9.75*	0.00	0.62	11.00
NSC	15	169.0	11.13	3.56	2.74	13.17	5.52	5.39	12.79	2.58	3.14	13.17
NSCN	0	142.7	13.88	3.17	3.50	11.83	5.61	4.25	12.21	0.00	2.65	11.83
NSO	495	386.8	15.17*	5.17	4.60	10.08	7.18	6.03	13.25	4.56	4.33	13.21
NSOM	0	144.9	9.83*	5.95	5.72	14.92	5.05	4.74	11.75	3.60	3.44	11.79
NCC	0	10.7	10.46	3.81	2.99	12.00	5.63	4.75	12.33	0.00	1.64	10.46
NCCN	0	247.1	14.54	3.60	3.69	13.00	5.83	4.02	12.67	1.63	2.69	14.54
NCO	0	114.0	13.92	3.96	3.38	13.92	5.60	4.61	13.75	0.00	2.57	13.79
NCOM	0	11.5	11.08	3.01	2.56	11.08	3.97	3.83	11.25	0.00	0.94	11.21

[a] Country of origin, E = Egypt and N = NL; [b] Soil type, S = sandy and C = clay; [c] Soil management, C = conventional and O = organic; [d] Soil amendment, N = NPK and M = cow manure; [e] *Asymptotic significant difference between amended and unamended soils (2-tailed): ($P \leq 0.05$).

($P = 0.035$ and $P = 0.023$ respectively). Amendment of the Egyptian organic clay soil with cow manure significantly increased the Ca content ($P = 0.028$). Amendment of Dutch organic clay soil with cow manure significantly increased the DOC ($P = 0.015$). Amendments of the other soils did not affect the studied chemical characteristics of those soils. Correlations between soil characteristics and disease measurements or pathogen populations were similar for the data sets with and without amendments.

Biological characteristics of amended soils

Amendment of conventional soils with NPK and organic soils with compost did not affect the bacterial diversity in the rhizosphere. On the other hand, amendment of organic soils with manure affected bacterial diversity in various ways. In the poor Egyptian sandy soils, rhizosphere bacterial diversity [S] decreased from 30.00 ± 1.00 to 18.50 ± 0.50 ($P = 0.001$), and [*H'*] decreased from 1.31 ± 0.05 to 1.19 ± 0.01 ($P = 0.064$), indicating that some species grew at the cost of others thanks to the substrate added with the manure. In the richer clay soils from Egypt, bacterial diversity [S] in the rhizosphere increased after manure amendment from 31.00 ± 3.00 to 37.50 ± 0.50 ($P = 0.082$), and [*H'*] from 1.32 ± 0.04 to 1.43 ± 0.10 ($P = 0.061$). In the Dutch clay soils, manure amendment decreased ($P = 0.003$) the rhizosphere [S] from (30.5 ± 0.50) to (24.50 ± 0.50), and increased rhizosphere [*H'*] from (1.23 ± 0.3) to (1.52 ± 0.02) ($P = 0.035$). In Dutch sandy soils, the amendments did not affect bacterial diversity in the rhizosphere.

Xylem and apoplastic extracts

Generally, 100 µl sap could be extracted from 10 g of stems. The amino acid content, ammonium content, and the ratio between amino acids and ammonium content were higher in xylem juice from plants grown in organic than in conventional soils, but the difference was not significant. However, growth of *R. solanacearum* (48 hrs) was significantly ($P = 0.052$) greater in the xylem and apoplastic extracts of potato plants grown in organic versus conventional soils (Table 8), and was positively correlated ($r = 0.58$, $P = 0.05$) with the ratio of the amino acid and ammonium contents (Figure 4). Differences in amino acid and ammonium contents between soil types and soil origin were not significant, except that the amino acids content was significantly higher in Dutch clay soils (159.89 ± 13.65; µM ± SEM) compared to Egyptian clay soils (114.84 ± 4.18; $P = 0.02$).

Discussion

The main objective of this study was to investigate if organic management could reduce infection of potato by *R. solanacearum* and potato brown rot development, since root diseases caused by fungi are generally suppressed in organically managed soils (van Bruggen and

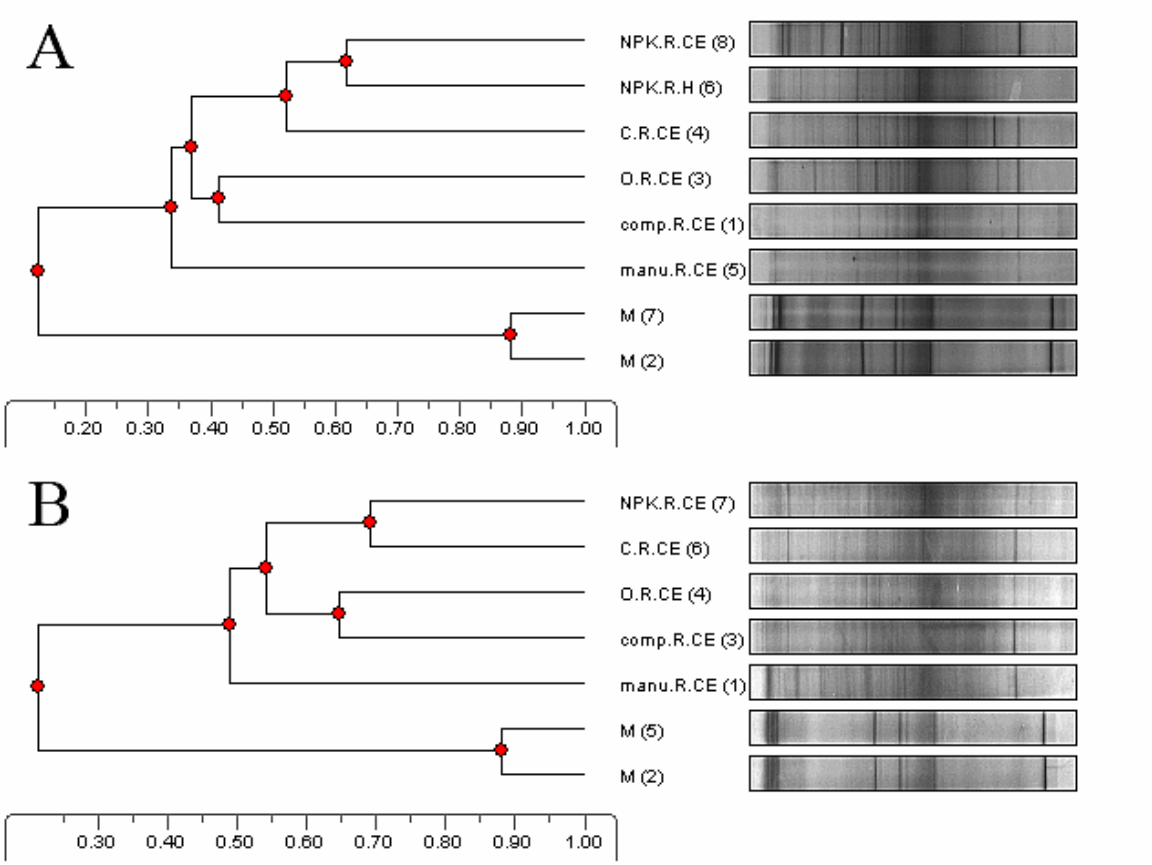

Figure 3. Cluster analysis (Phoretix 1D, NonLinear Dynamics Ltd., Newcastle upon Tyne, UK) for PCR-DGGE products from the rhizosphere of different amended sandy soils from Egypt (A) or the Netherlands (B). The scale on the X-axis depicts similarity. The first set of letters indicate amendment (NPK = fertilizer, comp = compost, manu = cow manure) O = organic and C = conventional) or marker (M), the second letters stand for rhizosphere (R) or Bulk soil (B), the third letters for treatment (H = inoculated but healthy and C = noninoculated control), and the fourth letters for sampling time (B = beginning and E = end). The numbers between brackets indicate the lane number on the gel.

Termorshuizen, 2003), and various bacterial wilt diseases are suppressed by certain organic amendments in some soils (Gorissen et al., 2004; Islam and Toyota, 2004; Satoh and Toyota, 2004). Moreover, in our previous study on survival of *R. solanacearum* in various soils from Egypt and the Netherlands, the pathogen declined faster in organic than in conventional sandy soil from Egypt, although the reverse was found for Dutch sandy soils (Messiha et al., accepted). However, in this study, there was only a tendency for reduced infection by *R. solanacearum* in organic sandy soil from the Egyptian desert ($\chi^2 = 3.4$ instead of the required 3.8 for significance at $\alpha = 0.05$). The biodiversity in these poor desert soils was higher in the organically managed soil, possibly leading to more competition for the limited substrate compared to the conventional soil (Messiha et al., accepted). Availability of nutrients in potato root exudates may have overcome this competition, so that the difference between

organically and conventionally managed soils was just not significant any more in the rhizosphere.

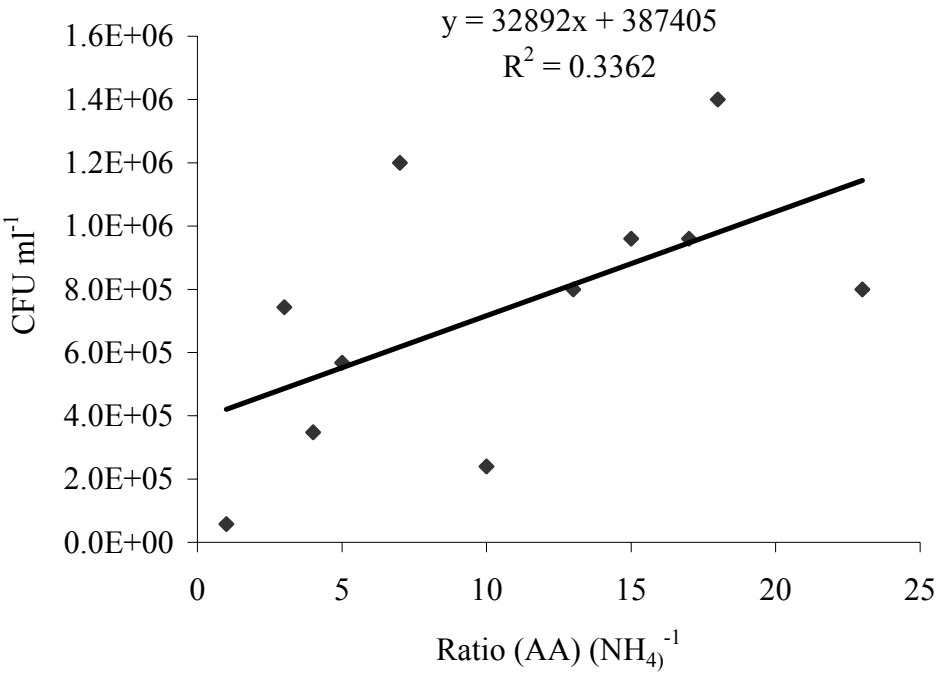

Figure 4. The relation between density of *R. solanacearum* (CFU ml^{-1} of extracted xylem sap) and the ratio of amino acids and ammonium (µM $µM^{-1}$) in the xylem sap of potato plants.

Table 8. Characterization of xylem sap extracted from potato plants grown in different managed soils 35 days after planting, and density of *R. solanacearum* grown for 48 hrs in extracted xylem sap.

Management	Amino acids (AA) (µM)	NH_4 (µM)	AA NH_4^{-1}	Log (no. of cells)
Conventional	142.4 ± 9.97	139.6 ± 20.00	1.19 ± 0.26	5.57 ± 0.19
Organic	172.0 ± 15.52	144.0 ± 14.72	1.24 ± 0.14	6.00 ± 0.03
T-test	**0.124**	**0.864**	**0.869**	**0.052**

[a] Log (no. of cells) of *R. solanacearum* / 100 µl of xylem sap 48 hrs after inoculation with pathogen suspension of 10^4 cfu L^{-1} distilled water. The control treatment in distilled water: Log (cells 100 $µl^{-1}$) = 4.84 ± 0.07.

[b] Standard error of the mean.

Similar to our previous results on survival of the pathogen, both the density of *R. solanacearum* in bulk soil and disease incidence (proportion wilted plants) were higher in organic than in conventional sandy and clay soils from the Netherlands. This may be related to the high availability of substrate in the Dutch soils, especially the organically managed soils, where large quantities of manure were commonly used (van Diepeningen et al., 2006). Indeed, in this study the DOC content in soil was positively correlated with AUDPC, and was higher in the organic than in the conventional Dutch sandy soil (not the clay soil). Cluster analysis for the DGGE bands of DNA samples from Dutch soils indicated that there was a rhizosphere effect in organic clay soil and not in conventional clay soil. This is the reverse of previous results, when a larger rhizosphere effect in terms of microbial communities was observed in soil from a conventional farm than in that from an organic farm (van Diepeningen et al., 2005). The lower disease incidence and severity in the conventional than in the organic soils in the current experiments are in agreement with the notion that a reduced rhizosphere effect, called "root camouflage", would lead to less pathogen attraction and root disease (Gilbert et al., 1994). A second objective was to determine if *R. solanacearum* and potato brown rot could be suppressed by various amendments in organically and conventionally managed soils. Amendment of organically managed soil with cow manure gave significant disease suppression in the sandy soil from the Netherlands and a significant reduction in density of *R. solanacearum* in the organic sandy soil from Egypt. No significant effects were found for the clay soils. Satoh and Toyota (2004) also found that the effect of repeated manure amendments on bacterial wilt development (on tomato) varied from soil to soil. The suppressive effects of manure added to organic sandy soils from the Netherlands and Egypt could have been due to the immediate release of ammonia which is known to be toxic to microorganisms (Michel and Mew, 1998) or to a shift in the microbial community (Gorissen et al., 2004). The bacterial diversity in the rhizosphere was not changed by manure amendment of Dutch sandy soil, but bacterial composition was different between the non-amended and manure-amended Dutch sandy soil (Figure 3b). Effects of bacterial composition rather than diversity on root disease suppression had been documented earlier (Hoitink and Boehm, 1999). In the poor Egyptian sandy soils, rhizosphere bacterial composition also changed after manure amendment (Figure 3a), and diversity decreased, indicating that some, likely fast-growing species, increased at the cost of others thanks to the substrate added with the manure. In clay soils both from Egypt and the Netherlands, the Shannon-Wiener index increased after manure amendment, and no significant effects on potato brown rot were found. Thus, the effects of manure on *R. solanacearum* may be through effects on survival (reduced CFU in Egyptian sandy soil), due to ammonia volatilisation or higher microbial activity by a limited number of fast-growing bacteria (Islam and Toyota, 2004), or through effects on growth in the rhizosphere and infection (reduced incidence and severity in Dutch sandy soil), again through ammonia production or specific inhibition by rhizosphere organisms.

Amendment of soils with wood chip compost was not effective in potato brown rot suppression. Similarly, compost from tree bark or coffee residues did not suppress, but even enhanced bacterial wilt of tomato, while compost from farmyard manure plus rice straw and

of poultry manure suppressed *R. solanacearum* in soil and reduced disease development (Islam and Toyota, 2004). The suppressive effects of composted farmyard manure and poultry manure was attributed to high substrate availability with high N content (high DOC content, high available N content, and a low C/N ratio), and high microbial activity (Islam and Toyota, 2004). Others also showed that the disease suppressive or enhancing effects of compost depend on their chemical and biological composition (Litterick et al., 2004), as well as on the pathogen involved (Termorshuizen et al., 2006). In all these studies, compost was added to conventional field soil or greenhouse soil, while we added compost to organic field soil, which was already relatively suppressive in the case of organic sandy soil from Egypt. Also, relatively small amounts of compost were added, in accordance with the Dutch regulations, compared to other studies (Islam and Toyota, 2004; Termorshuizen et al., 2006).

Amendment of conventional soils with NPK gave remarkable disease suppression only in Egyptian sandy soil and a reduction in *R. solanacearum* populations in soil, rhizosphere and potato plants in Egyptian clay soil. The pH of Egyptian soils was slightly, but significantly higher (7.6-7.9) than that of Dutch soils (7.4). The observed disease suppression may be a result of high ammonia concentrations which are toxic to *R. solanacearum* (Michel and Mew, 1998). The NPK we used contained 7% of ammonium. The conversion of ammonium to ammonia depends on the pH of the soil, ranging from 1% conversion at pH 7.3 to 10% at pH 8.3 (Kissel et al., 1985). The effect of NPK amendment may be a combination between the effect of ammonia and potassium (K). Amendment of soil with potassium phosphate resulted in decreasing the severity of bacterial spot and speck disease by increasing the resistance of the plant (Abbasi et al., 2002). Fertilization of potato plants with superphosphate (15% P_2O_5) or potassium sulphate (48-58% K_2O) decreased incidence of brown rot disease (Fahmy and Mohamed, 1990). Mixing NPK fertilizers with compost was most effective in suppressing bacterial wilt of banana (Roy et al., 1999). On the other hand, amendment of conventional clay soil from the Netherlands with NPK increased the percentage of wilted potato plants. It is not clear what the reason for this increase could be. The available nutrients and DOC were already high in the non-amended Dutch conventional clay soil, but this was also the case for the Egyptian conventional clay soil.

Another objective of this study was to relate brown rot development to various characteristics of different soil types from different locations, to find factors that might be associated with disease suppression. Disease severity was highest in Dutch sandy soils compared to all other soils. Chemical analysis revealed that the Total Organic Carbon (DOC) was the highest in those soils and was positively correlated with disease incidence, suggesting high substrate availability and better survival chances for *R. solanacearum* (Messiah et al., submitted).

Compared to Dutch sandy soils, the disease was suppressed in Dutch clay soils, where the DOC was also relatively high, but both the Ca and K contents were remarkably higher in the Dutch clayey compared to the sandy soils and were negatively correlated with disease incidence. Both K and Ca are known for enhancing plant defences (Flego et al., 1997;

Romeis, 2001; Abbasi et al., 2002). As mentioned above, fertilization with potassium phosphate and potassium sulphate reduced the severity of bacterial leaf spot diseases on tomato (Abbasi et al., 2002) and the incidence of brown rot on potato (Fahmy and Mohamed, 1990), respectively. In our study, the Ca and K contents were also higher in organic than in conventional Egyptian sandy soil, and the proportion of *R. solanacearum* infected plants was reduced in the organic soil, supporting previous findings about enhanced resistance in plants high in Ca and/or K.

The Na contents were significantly higher in the Egyptian clay (especially the organic clays soil) and Dutch soils than in the sandy soils from Egypt, which also had low *R. solanacearum* populations and infection, particularly in the organic sandy soil from Egypt. Thus, a positive correlation was found between Na availability and percentage of infected plants, CFU in the rhizosphere and CFU in the plant, which may explain the absence of disease suppression in the organic clay soil from Egypt even when both Ca and K concentrations were high. It is well known that root exudation is increased in sodic soils, enhancing the attraction for and susceptibility to various root diseases (Snapp et al., 1991).

As mentioned above, brown rot incidence was highest in the Dutch sandy soils. In our previous study, the survival of *R. solanacearum* was also best in the Dutch soils, but there was no difference between Dutch sandy and clayey soils. Also, bacterial diversity was higher in Dutch than in Egyptian soils, and higher in clayey than in sandy soils; it was the lowest in Egyptian sandy soils (Messiah et al., submitted). In the current study, the Shannon Wiener index H' was again higher in clay than in sandy soils, but there was no difference between the countries of origin. No significant difference was found in bacterial diversity in the rhizosphere of plants grown in different soil types from the different countries, indicating that the effect of root exudates exceeded the effect of soil type in determining the microbial diversity, and there was no correlation between rhizosphere biodiversity and brown rot incidence or severity.

The amino acid content in the xylem and apoplastic fluid was significantly higher in plants grown on Dutch than on Egyptian clay soils, and slightly higher in plants grown on the organic compared to the conventional soils. This is understandable, as the direct uptake of amino compounds and also uptake of ammonium (commonly converted to amino acids in the roots) by plants is reduced at high nitrate concentrations in soil (Persson and Näsholm, 2002). Growth of *R. solanacearum* was significantly faster in the extracted fluids from plants on organic than on conventional soils and was positively correlated with the ratio of amino acids and ammonium contents in the fluids.

Thus, contrary to our original expectation of disease suppression in organically managed soils (van Bruggen and Termorshuizen, 2003), populations of *R. solanacearum* were actually higher in the bulk soil and rhizosphere of potato plants grown in organic soils from the Netherlands, in particular organic sandy soil. Also disease incidence was higher in these

organically managed soils. This was probably related to the availability of substrate for growth of the pathogen in soil, the rhizosphere and even inside the plants (xylem and apoplastic fuids). Lack of substrate in the organically managed Egyptian sandy soil due to high competition may have been responsible for the suppression in that soil compared to its conventional counterpart. When Ca and K contents of soils were high, potato brown rot incidence or severity was generally low, both in soils naturally high in these elements and in soils amended with manure. However, the Ca and K contents of the plants were not determined. Application of NPK fertilizer reduced the disease, especially at high soil pH in Egyptian soils, probably due to ammonia volatilization. However, high fertilizer applications cannot be recommended for control of brown rot, as these may enhance susceptibility to other diseases, and would not be sustainable in the long run. Farmers could apply cow manure to reduce the disease, but only in moderation to limit the availability of substrate for growth of *R. solanacearum*. Application of composted poultry manure would be an interesting option to be explored for the Nile Delta of Egypt (Islam and Toyota, 2004), but for the Egyptian desert and the Netherlands, prevention of spread and quarantine remain important tools to combat potato brown rot.

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Chapter 4

Stenotrophomonas maltophilia: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot

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Messiha NAS [a,b,c], van Diepeningen AD [d], Farag NS [e], Abdallah SA [f], Janse JD [b]✉ and van Bruggen AHC [a] (2006) *Stenotrophomonas maltophilia*: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot. European Journal of Plant Pathology.

[a] *Wageningen University, Biological Farming Systems Group, Marijkeweg 22, 6709 PG Wageningen, the Netherlands*
[b] *Plant Protection Service, Dept. of Bacteriology, Geertjesweg 15, 6706 EA Wageningen, the Netherlands*
[c] *Potato Brown Rot Project (PBRP), 3 El-Amira Fatma Ismail Street, Dokki, Cairo, Egypt*
[d] *Wageningen University, Laboratory of Genetics, Arboretumlaan 4, 6703BD Wageningen, the Netherlands.*
[e] *Plant Pathology Research Institute(PPRI), 9 Gamaa Street. Giza, Cairo, Egypt*
[f] *Faculty of Science, Dept of Botany, Benha Univ., Egypt*

✉ *Plant protection service (PD), Dept. of Bacteriology, P.O. Box 9102, 6700 HC Wageningen, the Netherlands. E-mail: j.d.janse@minlnv.nl, tel +31 317-496810, fax +31 317-421701.*

Chapter 4. *Stenotrophomonas maltophilia*: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot

Abstract

Stenotrophomonas maltophilia was isolated from the rhizosphere of eggplant in the Nile Delta of Egypt, and its antagonistic potential against *Ralstonia solanacearum* race 3 biovar 2, the causal agent of potato brown rot, was *in vitro* evaluated on KB agar medium and *in vivo* on potato plants. *In vitro*, four isolates of *S. maltophilia* (PD3531, PD3532, PD3533, and PD3534) appeared antagonistic. The isolate (PD3533) was screened as the most promising antagonist for the *in vivo* tests. In the greenhouse, the antagonist was applied directly to soil or by bacterization of potato eyepieces. *S. maltophilia* significantly suppressed potato brown rot in Egyptian clay soil but not in Dutch clay soil. Survival of a rifampicin and chloramphenicol resistant *S. maltophilia* strain (PD4560) was investigated in two pairs of clay soils, conventionally and organically managed, from Egypt and the Netherlands. The survival of *S. maltophilia* was significantly less in Dutch than in Egyptian soils, while this was the reverse for *R. solanacearum*. These results are in agreement with those obtained in the *in vivo* biocontrol tests. In conclusion, *S. maltophilia* may be useful for control of brown rot in the area where it was originally isolated, the Nile Delta in Egypt.

Introduction

Potato brown rot is a systemic bacterial wilt disease caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al. (Yabuuchi et al., 1995). *R. solanacearum* is subdivided into 5 races on the basis of host range and 5 biovars on the basis of catabolic properties (Schaad, 1988; Hayward, 1991). The potato race 3 biovar 2, more recently also typed on the basis of genetic sequencing as Phylotype II, sequevar 1 (Prior and Fegan, 2005), is the dominant race in both Egypt and Europe. Potato losses up to 75% due to the bacterial wilt were recorded in many countries (Cook and Sequeira, 1994).

Potato is one of the most important vegetable crops in Egypt, both for local consumption and for export. Brown rot was first recorded in Egypt by Briton Jones (1925) in the Nile Delta area, based on symptoms only. The disease became endemic after the Second World War, being serious along the river Nile from the 1970s onwards. The pathogen has also been found in irrigation water (Mickail et al., 1974; Mickail et al., 1985; Farag et al., 1999). Poor drainage and consequently high soil moisture levels in the heavy textured soils were associated with the high disease incidence in this area (Mickail et al., 1985). As potato brown rot was declared a quarantine disease of concern in the EU, Egypt moved the main potato

production area to the virgin desert adjacent to the traditional agricultural lands, because these areas were still free from *R. solanacearum*.

Since 1989, potato brown rot was also observed in several north-western EU countries, where clear links were established with surface water contamination in almost all cases. It was first found in the Netherlands in 1992 in an isolated case and again in 1995, more wide-spread due to contaminations via irrigation water. An intensive programme for eradication was started and control measures taken according to European legislation (EC Control Directive 98/57/EC (Janse, 1996; Janse et al., 1998; Schans and Steeghs, 1998).

Control of potato brown rot has proven to be a serious, very difficult and puzzling task.. Some resistant cultivars are available but these are not adapted to different agro-ecological zones and are not effective against all strains of the pathogen (Hartman and Elphinstone, 1994; Mendoza, 1994; Lopez and Biosca, 2004). Breeding for disease tolerance is not desirable because of a possible correlation between the earliness of a cultivar and low disease tolerance (Farag, 1970 and 1976). Tolerant varieties could harbour virulent bacteria in a latent form (Priou et al., 1999). Chemical control by soil fumigants, antibiotics, and copper compounds was tried without much success (Farag et al., 1982; Hartman and Elphinstone, 1994; Murakoshi and Takahashi, 1984). In addition, most chemical pesticides have hazardous effects on the environment, non-target beneficial organisms and human health. Therefore, cultural and biological control of bacterial diseases was tried by many investigators as an alternative solution with some promising success (Michel and Mew, 1998; Ran et al., 2005a and b; Rhodes and Logan, 1987; Xu and Gross, 1986a and b).

R. solanacearum race 3, biovar 2 can survive in latently infected potato tubers, volunteer potatoes, tomatoes and weed hosts (such as woody nightshade, *Solanum dulcamara*), but also in soil for one up to several years (Graham et al., 1979; van Elsas et al., 2000) and a number of weeks in surface water, depending on temperature (Janse et al., 1998; van Elsas et al., 2001) . Thus, the most commonly recommended cultural practices to control potato brown rot are: use long crop rotations with non-hosts, do not use surface water for irrigation if possible, remove susceptible weeds, and only use healthy, tested seed tubers. In addition, fertilization practices can be adjusted so that the pH is lowered to less than 5 or raised to more than 8 (Michel and Mew, 1998; Sturz et al., 2004), provided that the crop can tolerate this. Application of certain composted animal manures or pig slurry can also reduce *R. solanacearum* populations and bacterial wilt disease of tomato and potato (Gorissen et al., 2004; Islam and Toyota, 2004). Similarly, amendment of organically managed soils with cow manure can reduce survival of *R. solanacearum* in soil and suppress brown rot development on potato plants (Messiha et al., unpublished). One of the mechanisms of disease suppression by manure or slurry may be a shift in microbial community towards greater antagonism against *R. solanacearum* (Gorissen et al., 2004; Messiha et al., unpublished). Repeated applications of organic materials like manure or compost can ultimately result in higher substrate availability for competitors, reduced growth of the pathogen in the rhizosphere, and

reduced infection (Hoitink and Boehm, 1999; Satoh and Toyota, 2004). Thus, soil amendment with manure or compost may in fact be an indirect way of stimulating biological control.

Apart from indirect biological control, limited research has been carried out to find directly applicable biocontrol agents for management of bacterial wilt. Microorganisms which are able to grow in the rhizosphere are ideal for controlling root-borne pathogens (Bapat and Shah, 2000; Ran et al., 2005b). Brown rot infection was reduced significantly after bacterization of healthy seed potatoes with *Bacillus cereus*, *B. subtilis* and with an avirulent strain of *R. solanacearum* under field conditions (Sunaina et al., 1997; Wagih, 1991). *Pseudomonas fluorescens* was highly effective in reducing bacterial wilt in tomato and potato under experimental conditions by aggressively colonizing the roots of young plants and pre-empting entry of *R. solanacearum* (Aspiras and De-la Cruz, 1986). *P. fluorescens* was less effective at controlling bacterial wilt of Eucalyptus trees (Ran et al., 2005b). Iinhibition of the pathogen by *P. fluorescens* was attributed to the siderophore pseudobactin, depriving root-colonizing microorganisms, including plant pathogens, of Fe^{3+} and inducing systemic resistance, ISR (Buyer and Leong, 1986; Ran et al., 2005a and b).

S. maltophilia is a common microorganism in the rhizosphere of cruciferous plants, and has also been found in association with corn and beets (Debette and Blondeau, 1980). Excretion of sulfur-containing amino acids such as methionine by roots of cruciferous plants may favor the growth of this species. However, *S. maltophilia* is also quite dominant in the rhizosphere of cereal crops (Lambert and Joos, 1989). *S. maltophilia* can even colonize and persist inside tissues of potato plants (Garbeva et al., 2001). However, *S. maltophilia* has not been evaluated yet for its potential to control potato brown rot. Yet, it was proven to be an effective biocontrol agent for the control of various fungal and oomycetous plant pathogens (Berg et al., 2005; Dal Bello et al., 2002; Dunne et al., 1997; Nakayama et al., 1999; Zhang and Yuen, 1999). *S. maltophilia* inhibited the growth of *Rhizoctonia solani* and *Verticillium dahliae in vitro*, possibly as a result of antibiosis and production of lytic enzymes (Berg et al., 1996). Three antifungal compounds, designated xanthobaccins A, B and C were isolated from the culture filtrate of a strain of *Stenotrophomonas* isolated from sugar beet that suppressed damping-off of beet seedlings caused by *Pythium* spp. (Nakayama et al., 1999). Xanthobaccins were *in vitro* not effective against three bacterial species (*R. solanacearum* was not included) (Nakayama et al., 1999). *S. maltophilia* can take up iron (to a limited extent) from the siderophore pseudobactin (Jurkevitch et al., 1992), but it is unknown if it produces a siderophore itself.

This research was undertaken to investigate the antagonistic potential of *S. maltophilia* against *R. solanacearum* under laboratory and greenhouse conditions. Survival of the antagonist was tested in two pairs of clay soils, conventional and organic from Egyptian and Dutch origins.

Materials and Methods

Isolation of antagonists and in vitro inhibition bioassay

Selection of potential antagonists was made from rhizosphere soils of eggplant (*Solanum melongena* L. 'Black Beauty') and sweet pepper (*Capsicum annuum*) at the late fruiting stage from two locations of vegetable growing areas in Kafr El-zayat, Gharbia governorate, Delta, Egypt. Soil was gently shaken from the roots, and remaining soil plus roots were considered as rhizosphere soil. About 1 g of rhizosphere soil was sampled and suspended in sterile phosphate buffer (PB 0.05 M: Na_2HPO_4. $12H_2O$, 4.26 g; KH_2PO_4, 2.72g; 1 L dist. water; pH 7.2). Samples were shaken for 1 hour. Six serial 10-fold dilutions were made for each sample and spread on soil extract agar media (SEA: glucose, 1 g; K_2HPO_4, 0.5 g; yeast extract, 1.5 g; soil extract (1 kg in 1 L water), 500 ml; agar, 20 g; tap water, 500 ml; pH 7.2) (Allen, 1957). Colonies developed on isolation plates were washed with sterile water (2 ml/isolate), purified, and streaked over the middle of the surface of King's B agar plates (KB: proteose peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4$, 1.5 g; agar, 20 g; glycerol, 15 ml; dist. water; 1000 ml). Plates were incubated at 28°C for 48 hours. A mixture of three previously identified *R. solanacearum* strains that had been isolated from potato tubers in Egypt (marked as PD5239, PD5240 and PD5241 in the culture collection of the Plant Protection Service, Wageningen, the Netherlands) was streaked in six lines, perpendicular to the antagonist streak, and incubated again at 28°C for 48 hours. The distance free from *R. solanacearum*, near the antagonist line was determined. In another trial both the antagonist and the pathogen were streaked parallel to each other, 0.5 cm apart then incubated side by side and the plates were kept under the same conditions. The absence of *R. solanacearum* growth was scored positive for suppression by the antagonist.

In a third trial, ferric ions were incorporated in King's B medium in the form of ferric sulphate [$Fe_2 (SO_4)_3 .H_2O$] with a final concentration of 100 µM of Fe^{+3}. KB medium plates without supplemental iron served as controls. One hundred µl of a *S. maltophilia* suspension (10^9 CFU ml^{-1}) was spread onto the plates to determine the effect of iron availability on the antagonistic potential of *S. maltophilia*. A similar suspension of an antagonistic *P. fluorescens* strain (PD3340), spread onto iron-amended and non-amended plates, served as control.

Identification of the antagonist

Whole cell fatty acid methyl esters (FAME) were prepared according to standard protocols (Janse 1991; Stead 1992), and fatty acid analysis (FAA) was carried out using a gas chromatograph coupled to the Microbial Identification System with a commercially available database and software (MIDI, Newark DE 19711, USA). The method is briefly described in the following paragraph.

Four antagonistic bacterial isolates were grown on trypticase soy broth agar (TSBA) at 28°C for 24 hours and harvested in glass tubes. For saponification, 1 ml of reagent 1 (NaOH, 45 g; methanol, 150 ml; distilled water 150 ml) was added to each sample and heated in a water bath for 30 minutes. For methylation, 2 ml of reagent 2 (certified 6.0 N HCl, 325 ml; methanol, 275 ml) was added to the cooled tubes and heated for 10 ± 1 minutes at 80 ± 1°C, dropping the pH below 1.5. FAME was extracted in 1.25 ml of organic solvent (reagent 3: 200 ml; methyl tetra-butyl ether, 200 ml) per cooled tube. The aqueous phase was removed by pipette and the organic phase was washed with 3 ml of reagent 4 (NaOH, 10.8 g; distilled water 9000 ml) to reduce contamination of the injection port liner, the column, and of the detector of the gas chromatograph. A standard calibration mixture (MIDI) was used, consisting of straight chain saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0), with five hydroxy fatty acids. All bacterial strains were identified based on a comparison of the fatty acid compositions with those in the database of the MIDI system (TSBA).

The strains were also physiologically and biochemically characterized according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) to confirm the results of FAA. The following tests were carried out: Gram-staining, motility, oxidative/fermentative metabolism of glucose (O/F), gelatine hydrolysis, levan formation from on 5% sucrose agar utilization of H_2S, utilization of nitrate as nitrogen source, acid production from D-glucose, trehalose and inositol (1%) in peptone-free basal medium with bromothymol blue (Dowson, 1957). The media were inoculated at a density of 10^6 bacteria. ml^{-1}, and incubated at 28°C for 14 days. Acid production from the three sugars was recorded at 3-day intervals. A colour change from green to yellow was considered positive.

Suppression of R. solanacearum and brown rot control

Biological control potential of *S. maltophilia* against *R. solanacearum* was evaluated under greenhouse conditions for Egyptian and Dutch soils separately. Conventionally managed clay soil was used in Egypt, while both conventionally and organically managed clay soil was used in the Netherlands. Two potato cultivars were used: medium-early maturing disease-susceptible cv. Nicola and medium-late maturing moderately tolerant cv. Diamant (for details on their use, see under the individual experiments). Potato eyepieces were immersed for 5 min in 0.05% (aq.) NaOCl, washed 5 times in sterile distilled water, and air dried for 48 hours. Soil was infested with a mixture of three virulent Egyptian strains of race 3 biovar 2 of *R. solanacearum*: PD5239, PD5240 and PD5241 (culture collection, Plant Protection Service, Wageningen, the Netherlands). The bacterial strains were grown for 48 h on nutrient agar (NA) plates at 28°C. The bacterial cultures were suspended in 0.01 M phosphate buffer (PB) and the bacterial density was adjusted to 5×10^9 CFU ml^{-1} using a spectrophotometer. The inoculum was mixed with the soil to have a final bacterial concentration of 10^7 CFU g^{-1} dry soils.

One strain of *S. maltophilia* (PD3533) that could use nitrate as nitrogen source was selected

for the biocontrol tests, as potato brown rot is especially severe in soils containing high nitrate concentrations (Messiha et al., unpublished). The antagonist was applied both by soil inoculation and bacterization of potato eyepieces. *S. maltophilia* was propagated on trypticase soy broth (TSB) and incubated for 18 hours (logarithmic phase) at 28°C. The bacteria were harvested by centrifugation, washed with sterile distilled water, centrifuged again and re-suspended in water to the needed concentration (Campbell et al., 1986). The inoculation suspension was adjusted to give 10^8 CFU per g soil (Ciampi-Panno et al*.,* 1989). For bacterization of eyepieces, the antagonist was resuspended in sterile 1% carboxymethyl cellulose (CMC) at 10^{10} CFU ml^{-1} (Bapat and Shah, 2000). Eyepieces were immersed in the bacterial suspension in CMC for 15 min and left to dry for two days before planting.

The conditions of the greenhouse were adjusted to 25°C during the day and 20°C during the night, with a RH of 75 to 80% and a total of 14 hours light. Three experiments were conducted as follows:

Experiment 1

Non-sterilized Egyptian conventional clay soil was used for this experiment. *S. maltophilia* was applied by soil inoculation or by bacterization of the eyepieces of potato cv. Nicola and cv. Diamant. Soil inoculated with the pathogen only was added to 48 pots (5 kg each), 24 to be planted with non-treated potato eye pieces and 24 to be planted with eye pieces treated with *S. maltophilia.* Soil inoculated with the pathogen and antagonist was added to 24 pots, and non-inoculated soil to 6 pots as negative controls. Two eyepieces of either of the potato cultivars (with or without *S. maltophilia*) were planted in each of 12 pots (replications). The 78 pots were placed on a greenhouse bench in a completely randomized design. Potted plants were not fertilized since the soil came from fertilized potato fields. Plants were carefully irrigated overhead to prevent splashing. The percentage infected plants was determined 90 days after planting. Rhizosphere samples were collected from each pot as mentioned above. Three ten-fold serial dilutions were made from each sample and 100 µl was plated onto Selective Media South Africa (SMSA) (Anonymous, 1998). Latent infection in the lower stem area (crown area) of each plant was checked by plating 5-cm long, weighed sections of surface-sterilized and macerated plant tissues on SMSA plates. Maceration by mortar and pestle was done in phosphate buffer saline (PBS 0.01 M), (Na_2HPO_4. $12H_2O$, 2.7 g; NaH_2PO_4. $2H_2O$, 0.4 g; NaCl, 8 g; 1 L dist. water; pH 7.2), and the extract was left for 1 hour. About 1% of all colonies with a morphology typical for *R. solanacearum* (colony with irregular shape, diffuse white or purple centers and luxuriant slime) were randomly selected and tested using Immunofluorescence Antibody Staining, IFAS, (Janse, 1988). The number of *R. solanacearum* CFUs were calculated per g dry rhizosphere soil.

Experiment 2

Eyepieces of cv. Diamant were planted in non-sterilized and sterilized Egyptian conventional

clay soil. The pathogen was mixed into soil as mentioned before, keeping some of the soil non-inoculated. The antagonist was applied by eyepiece bacterization only; half of the eyepieces remained non-treated. There were 2×2 = 4 treatments with 20 replications (pots of 5 kg) each in a completely randomized design. All 80 pots contained soil with *R. solanacearum*; in addition, there were 4 negative control pots without the pathogen. No fertilizer was added, and irrigation was carefully applied overhead. Wilt development was scored after 60, 75 and 85 days. A 0-5 scoring scale was used: 0 = no symptoms, 1 = 1-2 leaves wilted per plant (about 20%), 2 = 3-4 leaves wilted per plant (about 50%), 3 = most of the leaves wilted (about 80%), 4 = all leaves wilted, and 5 = dead plant. A wilt index was calculated as the sum of the [number of plants in each category times the category number]. Before carrying out Chi-square tests, plants in the categories 1, 2 and 3 were combined and the categories 4, and 5 were combined, so that the following classes resulted: 0 = no symptoms, 1 = slight to severe wilt, and 2 = completely wilted or dead. Visible and latent infection by *R. solanaceearum* were tested at the end of the experiment (after 85 days), by plating surface–sterilized and macerated stem sections on SMSA as mentioned above. The identity of putative *R. solanacearum* colonies was confirmed by IFAS. The experiment was conducted twice.

Experiment 3

Eyepieces of cv. Nicola were planted in non-sterilized Dutch conventional and organic clay soil. The pathogen and the antagonist were applied as in experiment 2, resulting again in 4 treatments. Instead of pots, trays were used, containing 5 kg of soil. Eight eye pieces were planted per tray. There were 4 trays (replications) per treatment, plus 4 trays with non-inoculated soil as negative controls. Wilt incidence and severity were observed daily for 45 days as described under experiment 2. Latent infection was tested by making isolations from the crown areas of each plant onto SMSA plates. The experiment was carried out twice.

Survival of S. maltophilia in soil and its effect on survival of R. solanacearum

A spontaneous rifampicin and chloramphenicol resistant mutant of *S. maltophilia* strain PD3533 was selected and adapted in the lab for testing its survival in different soils. The strain was selected from the wild type strain by growing it in TSBA amended with increasing concentrations of rifampicin and chloramphenicol (0, 20, 40, 60, 80 and 100 μg ml^{-1} for each antibiotic). The antibiotic-resistant strain (PD4560) was subjected again to FAA to confirm its identity as *S. maltophilia*, and was tested *in vitro* for antagonistic potential against *R. solanacearum*. The same strain mixture of *R. solanacearum* was used as in the previous experiment.

Two pairs of clay soil, half from organically managed arable farms and the other half from conventionally managed arable farms were used in this study. One pair originated from Egypt, the other was from the Netherlands (Table 1). The soils were the same as those used in the greenhouse experiments. The antibiotic resistant *S. maltophilia* was grown for 48 h on

trypticase soy broth agar (TSBA) plates at 28°C, and *R. solanacearum* was grown on NA plates under the same conditions. The bacterial cultures were suspended in 0.01 M phosphate buffer (PB) separately. Suspensions of *R. solanacearum*, *S. maltophilia* or both were mixed with 100 g of each soil in plastic bags so that a final concentration of 5×10^7 CFU g^{-1} dry soil was attained for each strain. Inoculated samples were divided over five 50-ml Greiner tubes with loosely closed lids to allow air exchange, and incubated at 28°C. The moisture content was 22.6% of the soil fresh weight, water loss was checked weekly and any lost water was replaced to keep a constant moisture level. Both the pathogen and antagonist populations were monitored in all treatments twice a week for the first two weeks, starting at the day of inoculation (T_0), once a week for the next two weeks and then once a month for a total of five months until the pathogen population was below the detection limit (100 CFU g^{-1} dry soil) in all soils.

For bacterial counts, 1-g samples of the soils (1 sample per soil replicate) were suspended in 9 ml of sterile 0.05 M phosphate buffer. After shaking at 100 rev min^{-1} for 2 h at 20°C, 10-fold serial dilutions were made, and spread on 3 plates each of TSBA amended with 100 mg chloramphenicol and 100 mg rifampicin per liter for *S. maltophilia* detection and on SMSA plates for *R. solanacearum* detection. *S. maltophilia* colonies were counted after 3 days and *R. solanacearum* colonies after 5-7 days of incubation at 28°C. IFAS was used to confirm putative colonies of the pathogen. The experiment was conducted twice.

Soil analyses

For physical and chemical analysis 100-g samples of all soils were air dried at room temperature. Soil textures were assessed in the Laboratory of Soil Science and Geology of Wageningen University (Table 1). Particle sizes of < 2 µm were considered clay, 2-50 µm was considered silt and 50-2000 µm was considered sand. Organic matter content was determined using the loss on ignition method according to Houba et al. (1997). Dissolved organic carbon (DOC), nitrate and ammonium content and pH were measured after Houba and Novozamsky (1998).

PCR and DGGE analysis

Duplicate soil samples used for PCR and DGGE were stored at -20°C. 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 65ºC was added to enhance the elution. 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 968 f-GC and 1401 r (Heuer and Smalla, 1997). Two ng of DNA was added to 50 µl PCR reactions and amplified using a touchdown scheme (Hiddink et al., 2005).

Table 1. Physical, chemical and biological composition of the different soils. Shannon-Wiener diversity index *H'* .

				Soil composition[a]				Nutrients mg/kg				Diversity index[b]	
Soil code[c]	Country	Management	Location	Clay %	Silt %	Sand %	OM %	TOC [d]	N-NO3	N-NH4	pH-KCl	S	*H'*
ECC	Egypt	Conventional	Behera, Delta area,	9.4	54.5	36.1	4.9	133	128	19.2	7.6	21.3	1.23
ECO	Egypt	Organic	Kaliobia, Delta area,	13.9	65.7	20.4	6.2	080	62	16.3	7.6	18.3	1.19
NCC	NL	Conventional	Ens, Northeast polder	7.7	51.9	40.4	2.2	122	10	13.9	7.4	18.8	1.11
NCO	NL	Organic	Ens, Northeast polder	8.3	54.5	37.2	2.3	125	7	15.8	7.3	24.5	1.13

[a] Particles size: clay < 2 µm, silt 2-50 µm, sand 50-2000 µm; [b] Average species richness *S* and [c] The 1st letter stands for country of origin (Egypt or the Netherlands), the 2nd for soil type (clay) and the 3rd for management (conventional or organic); [d] Total soluble organic carbon.

DGGE was performed using the Dcode system (BIOrad Laboratories, Hercules, CA, USA) according to Hiddink et al. (2005). All samples were analyzed at least in duplicate. Gels were stained with BIOrad's Silver Stain (BIOrad Laboratories, Hercules, CA, USA), preserved 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 than scanned using ScanSoft Omnipage, program 14 at a resolution of 300 dots per inch and analyzed with Phoretix 1D (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK), including only bands with pixel intensity above one.

Statistical analyses

To evaluate the disease incidence in the first two biocontrol experiments, chi-square tests were conducted on contingency tables with classes of number of plants infected or wilted versus the various treatments to be compared (soil origin, soil treatment and antagonist amendment) using Microsoft Excel 2003 (Microsoft Corporation, Seattle, WA, USA).

For the first experiment, ANOVA was conducted to compare the density of *R. solanacearum* in the rhizosphere for the two different cultivars and *S. maltophilia* treatments (control, soil amendment with the antagonist and bacterization of eyepieces with the antagonist). Interaction between cultivar and antagonist treatment was also determined. Fisher's LSD test was used to compare each pair of treatments. A t-test was conducted to compare the densities of *R. solanacearum* in the rhizosphere of the two different cultivars in the treatment without *S. maltophilia*. All analyses were conducted using SPSS v 12 (SPSS Inc., Chicago, Illinois, USA).

For the third experiment, areas under the disease progress curves (AUDPC) were calculated based on percentage of leaves wilted per plant over time. Non-parametric Mann-Whitney tests were carried out on AUDPC and percent of stem pieces infected for the two management types (organic and conventional) and *S. maltophilia* treatments (inoculated and non-inoculated eyepieces).

Survival curves of the pathogen and the antagonist were fitted to colony forming units over time as follows. Log (CFU+1 g^{-1} dry soil) was calculated for each sample. Log transformed data (for each replicate, soil and treatment separately) were fitted to an logistic decay model as described by (Franz et al., 2005)

$$C_t = a_m / (1+\exp(-d*(t-c)))$$

Where C_t = log transformed bacterial density, a_m = initial density, d = decline rate ($days^{-1}$), and t = time (days) and c = length of the 50%-reduction-time in days. The estimated parameter values c and d for the different soils were subjected to multivariate analysis of variance (MANOVA, using SPSS v 12, SPSS Inc., Chicago, Illinois, USA) to determine the effect of soil origin and management on the survival of *S. maltophilia* and *R. solanacearum*, and to detect the effect of *S. maltophilia* on the survival of *R. solanacearum* in different soils. Next, effects of different treatments on c and d separately for both the antagonist and the pathogen were tested with ANOVA.

The bacterial diversity in the soil samples was estimated in two ways: as species richness S and as the Shannon-Wiener index of bacterial diversity, *H'*. Species richness S was determined by the number of detected DGGE bands per soil (van Diepeningen et al., 2006). The Shannon-Wiener diversity index was calculated as $H' = -Pi\log Pi$ based on the relative band intensities as formulated by Eichner et al. (1999). *Pi* is defined as *ni*/N where *ni* 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 with standard deviations based on multiple samples on replicate DGGE gels. A t-test was conducted to compare S and *H'* from conventional versus organic soil for each country.

Results

Isolation and identification of the antagonists

Total bacterial densities in rhizosphere soil from eggplant were 3.5×10^7 cfu g^{-1} at the first and 1.8×10^7 cfu g^{-1} at the second location. Bacterial densities from sweet pepper rhizosphere soil were lower: 9.4×10^6 cfu g^{-1} and 2.4×10^7 cfu g^{-1}, respectively. From the rhizosphere of eggplant, three out of sixteen (18.8%) and one out of twenty-six (3.8%) randomly selected colonies on the countable plates showed *in-vitro* antagonistic properties against *R. solanacearum* from the first and second location, respectively. All four isolates showed a strong inhibition zone of about 9 mm. The rhizosphere of pepper did not yield any

antagonistic bacteria against the pathogen at similar dilutions.

For all four antagonistic strains, the majority of FAs were branched and were identified as C15:0 ISO, C15:0 ANTEISO, C16:1 ω7c/15 ISO 2OH, C16:0 and C15:0 ISO 2OH/ 16: 1ω7c. Three isolates (PD3531, PD3533, and PD3534) were identified as *Stenotrophomonas maltophilia* (Hugh 1981) Palleroni and Bradbury 1993 (previously known as *Pseudomonas maltophilia*, then *Xanthomonas maltophilia*), with a high degree of similarity (>79%) to the reference strain, while PD3532 showed only 41% of similarity to the *S. maltophilia* reference strain using the TSBA database of the MIDI system.

All four strains produced a non-diffusible yellow colour on TSBA medium, and were aerobic Gram-negative, motile rods. All isolates were able to liquefy gelatine, produced acid from D-glucose and trehalose, and utilized H_2S. Only PD3533 and PD3534 were able to use nitrate as nitrogen source. All isolates were negative for arginine dihydrolase production, and were unable to form levan on 5% sucrose agar and acid from inositol. The results of the biochemical and physiological tests were in agreement with Holt et al. (1994) and with the FAA identification.

The four *S. maltophilia* isolates from eggplant rhizospheres were tested for their ability to suppress *R. solanacearum* in the absence and presence of 100 μM Fe^{+3} in the medium. The additional iron did not affect the antagonistic effect of *S. maltophilia,* while that of *P. fluorescens* disappeared on iron-amended media (data not shown).

Suppression of R. solanacearum and brown rot control

Experiment 1

There was a significant interaction ($P = 0.001$) between *S. maltophilia* treatment and cultivar with respect to disease development in *R. solanacearum* inoculated soil. Potato plants in non-inoculated soil remained healthy. The percentage of plants infected by *R. solanacearum* was higher for Nicola compared to Diamant in the control treatment without *S. maltophilia* (100% and 58.3%, respectively; $P = 0.012$). For Nicola, inoculation of soil or eyepieces with *S. maltophilia* decreased the percentage of infected plants from 100% to 25% or 16.7% ($P < 0.001$) in case of soil or eyepiece amendment, respectively. For Diamant, inoculation with *S. maltophilia* decreased the percentage of infected plants from 58.3% to 16.7% ($P < 0.001$) in case of soil amendment, and to 25% ($P = 0.008$) in case of eyepiece inoculation (Figure 1).

There was also a significant interaction ($P = 0.004$) between cultivar × *S. maltophilia* treatment with respect to density of *R. solanacearum* in the rhizosphere of potato plants grown in inoculated soil. No significant difference in pathogen density was found between the two cultivars grown without *S. maltophilia*. For Nicola, application of *S. maltophilia* significantly decreased the pathogen density from 2.0×10^5 g^{-1} in the non-amended control to

undetectable levels for both *S. maltophilia* treatments ($P < 0.001$). For Diamant, application of *S. maltophilia* decreased the pathogen density from 2.3×10^5 g^{-1} to 2.2×10^5 g^{-1} in case of soil amendment (not significant) and to an undetectable level in case of eyepiece amendment ($P = 0.002$).

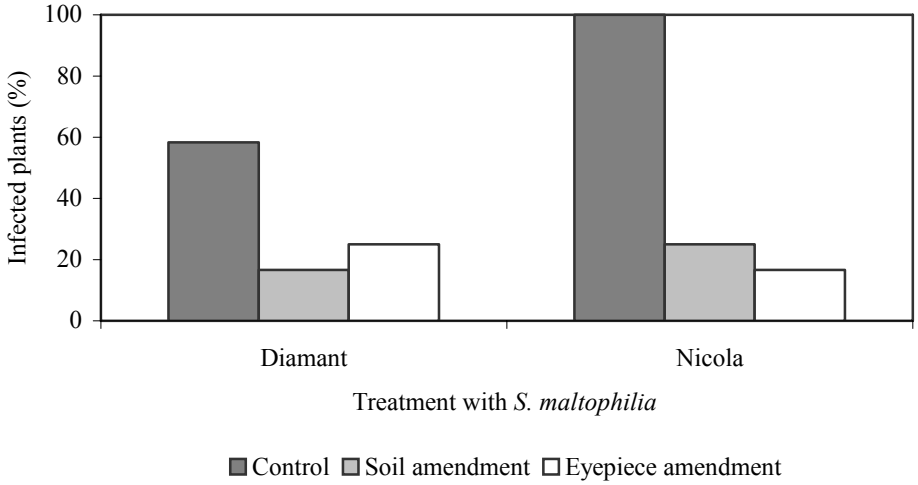

Figure 1. Effect of soil amendment and eyepiece treatment with *S. maltophilia* on percentage potato plants infected by *R. solanacearum* 90 days after planting, for the medium-late, brown rot tolerant cultivar Diamant and the early, susceptible cultivar Nicola.

Experiment 2

Wilt incidence was higher in sterilized soil compared to non-sterilized soil at all three sampling times: the χ^2 value was 13.89 ($P < 0.001$), 8.72 ($P < 0.01$), and 15.09 ($P < 0.001$) at 60, 75, and 85 days, respectively. The percentage of infection at the end of the experiment was also significantly higher in sterilized than in non-sterilized soil ($P = 0.03$). For sterilized soil, wilt incidence was significantly less in antagonist amended soil compared to non-amended soil at the three time intervals: the χ^2 value was 12.73, 20.83, and 13.36 ($P < 0.001$) for 60, 75 and 85 days, respectively. There was 50% reduction in percentage of infected plants in antagonist amended soil compared to non-amended soil ($P = 0.002$) (Table 2). In non-sterilized soil, wilt incidence was also significantly lower in antagonist-amended than in non-amended soil, but only 85 days after planting ($\chi^2 = 11.51$, $P < 0.001$). There was 36.4% reduction in percentage of infected plants in antagonist-amended soil compared to non-amended non-sterilized soil ($P = 0.015$) (Table 2).

Experiment 3

Disease incidence and severity were generally low in the non-sterilized Dutch clay soils. On average, 37.5% plants were infected and 12.5% wilted 45 days after planting. No significant

differences were found in wilt incidence and percentage of infection between conventional versus organic, nor between *S. maltophilia* amended and non-amended soil (Data not shown).

Survival of S. maltophilia in soil and its effect on survival of R. solanacearum

Survival of *S. maltophilia* was significantly higher in Egyptian soils compared to Dutch soils (Figure 2), MANOVA: Wilk's Lambda = 0.075, $P = 0.021$, ANOVA for the 50%-reduction-time (c): F = 5.2, $P = 0.084$ while for the decline rate (d): F = 34.3, $P = 0.004$. No significant difference in survival of *S. maltophilia* was found between the different management types for soils from both countries. Soil pH and organic matter content (OM) were both positively correlated to survival of *S. maltophilia*: for pH, r = 0.965 ($P = 0.035$) for c and r = 0.968 ($P = 0.032$) for d; and for OM, r = 0.855 ($P = 0.146$) for c and r = 0.953 ($P = 0.047$) for d. There were no significant correlations with other soil characteristics.

Table 2. Effect of application of *S. maltophilia* on wilt development of potato caused by *Ralstonia solanacearum* race 3 biovar 2 in sterilized and non sterilized Egyptian conventionally managed clay soil [a]

		Days after planting			IF[b]
Inoculation treatment	Soil treatment	60	75	85	(%)
Control	Sterilized	0	0	0	0
	Non-sterilized	0	0	0	0
Sterilized	*R. solanacearum*	10.0	22.0	46.4	70
	R. solanacearum + *S. maltophilia*	0	0	6.3	35
X^2		**12.7**	**20.8**	**13.4**	**10.4**
Non-sterilized	*R. solanacearum*	0	6.5	13.3	55
	R. solanacearum + *S. maltophilia*	0	0	0	35
X^2		**0**	**0.6**	**11.5**	**5.4**

[a] Values represent the average of two experiments, with 20 replicates each and 2 seed tubers per pot; [b] Immunofluorescence antibody staining test; [c] $\chi^2 \geq 5.02$ represent $P \leq 0.025$, $\chi^2 \geq 6.63$ represent $P \leq 0.01$, and $\chi^2 \geq 10.83$ represent $P \leq 0.001$.

Contrary to *S. maltophilia*, survival of *R. solanacearum* was significantly higher in Dutch soils than in Egyptian soils (Fig 3), MANOVA: Wilk's Lambda = 0.46, $P = 0.003$, ANOVA for the 50%-reduction-time (c): F = 8.92, $P = 0.009$ while for the decline rate (d): F = 18.16, $P = 0.001$. The pathogen generally survived longer in conventional than in organic soils, especially in the Dutch soils. For Egyptian soils, the difference between organic and conventional management was due to a higher decline rate in the organic soil (MANOVA: Wilk's Lambda = 0.03, $P = 0.005$, ANOVA for the 50%-reduction-time (c): F = 1.88, $P =$

0.243; for the decline rate (d): F = 119.02, $P < 0.001$). For Dutch soils, the difference was due to a shorter 50%-reduction-time and a higher decline rate in the organic soil (MANOVA: Wilk's Lambda = 0.10, $P = 0.032$, ANOVA for the 50%-reduction-time (c): F = 34.15, $P = 0.004$; and for the decline rate (d): F = 26.56, $P = 0.007$). There were negative correlations between the survival of *R. solanacearum* and NO3 (r = -0.86, $P = 0.069$; r = -0.88, $P = 0.060$, for 50%-reduction-time and decline phase, respectively) and pH (r = -0.95, $P = 0.025$; r = -0.96, $P = 0.019$, respectively).

Amendment of conventional soils from both countries with *S. maltophilia* did not affect the survival of *R. solanacaerum*. Amendment of Egyptian organic soil with *S. maltophilia* suppressed the survival of *R. solanacearum*, MANOVA: Wilk's Lambda = 0.098, $P = 0.03$, ANOVA for the 50%-reduction-time (c): F = 23.36, $P = 0.008$ while for the decline rate (d): F = 5.64, $P = 0.076$. On the other hand, amendment of Dutch organic soil with *S. maltophilia* enhanced the survival of *R. solanacearum*, MANOVA: Wilk's Lambda = 0.145, $P = 0.055$, ANOVA for the 50%-reduction-time (c): F = 3.81, $P = 0.123$ and for the decline rate (d): F = 23.58, $P = 0.008$ (Figure 3).

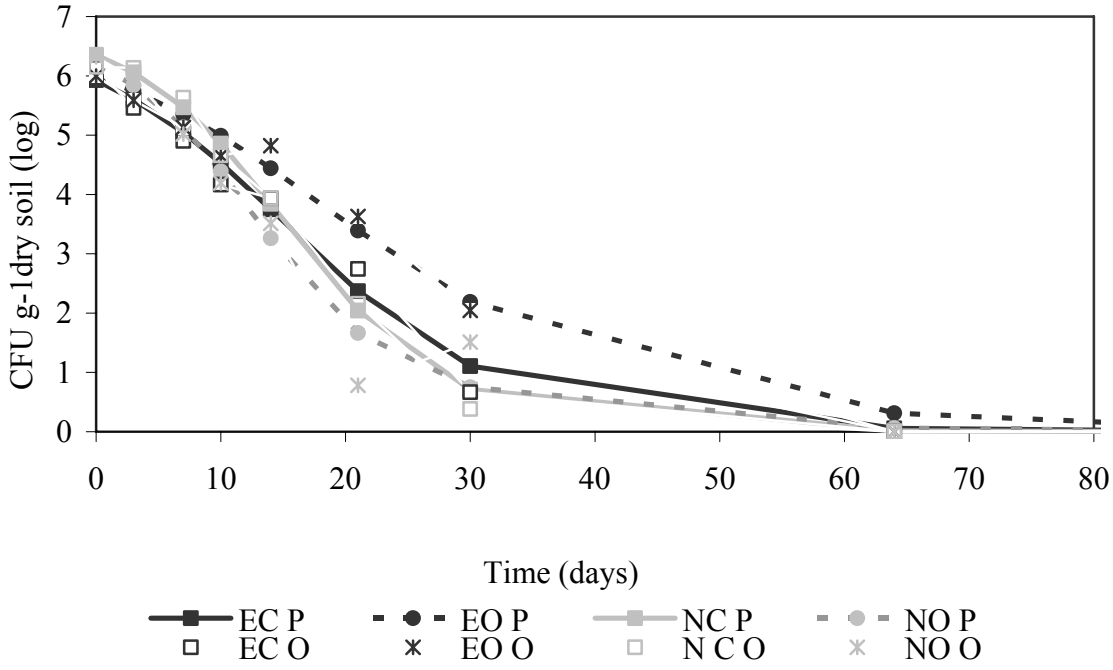

Figure 2. Decline in density (CFUs g^{-1} dry soil) of *S. maltophilia* in different soil types with different management regimes at 28°C. The dots are the observed values. The lines are the predicted values from the logistic decline model:
$C_t = a_m / (1+\exp(-d*(t-c)))$. Where C_t = log transformed number of bacteria, a_m = initial density of the pathogen, d = decline rate ($days^{-1}$), and t = time (days) and c = length of the 50%-reduction-time in days (Franz et al., 2005). The first letter represents country, E = Egypt, N = NL; the second letter represents management, C = conventional, O = organic; and the last letter represents the type of values, P = predicted and O = observed values.

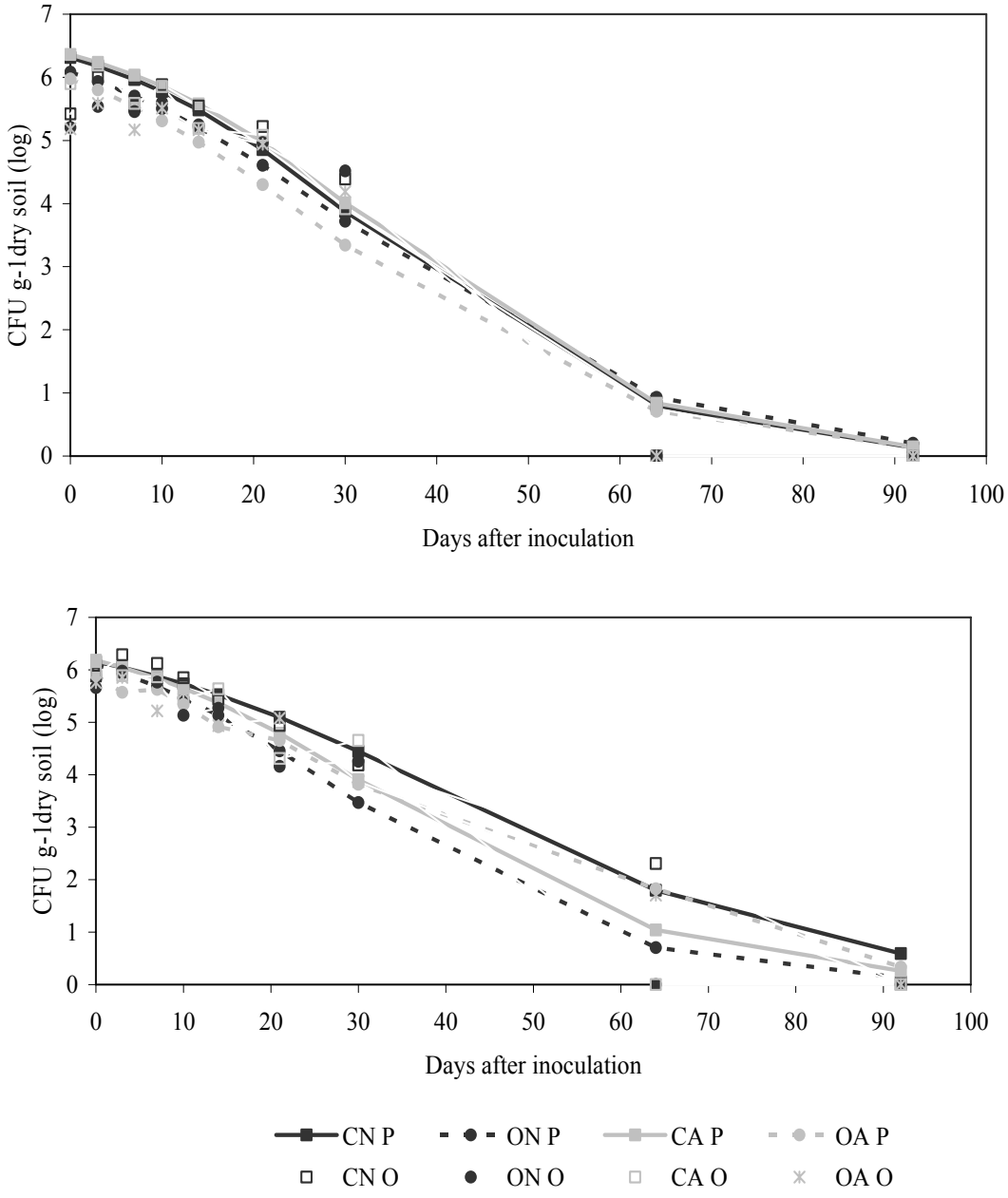

Figure 3. The effects soil amendment with *S. maltophilia* on the decline of *R. solanacearum* at 28°C (cfu g^{-1} dry soil): (a) soil from Egypt and (b) soil from the Netherlands. The first letter represents management, C = conventional, O = organic; the second letter represents amendment, N = non-amended, A = Amended with *S. maltophilia*, and P = predicted values and O = observed values.

Bacterial diversity in different soils

The Bacterial diversity *S* was significantly lower (t-test; $P = 0.005$) in Egyptian organic than in the conventional soil while the Shannon-Wiener index *H*' did not significantly differ (Table

1). The reverse was true for the Dutch soils: *S* was significantly higher in organic than in the conventional soil (t-test, $P = 0.031$). There was no difference in *H'* between the Dutch soils. There was no significant correlation between *S* and the 50%-reduction-time c or the decline rate d of either of the decline curves for *S. maltophilia* and *R. solanacearum*. However, there was a significant negative correlation between *H'* and the decline rate d for *R. solanacearum* ($r = -0.66$, $P = 0.019$).

Discussion

For the first time, strains of *S. maltophilia* were found that inhibited *R. solanacearum in vitro* on agar plates and *in vivo* on potato plants grown in soil. The *in vitro* test revealed a possible production of antibiotics responsible for such inhibition. *S. maltophilia* produces various antibiotics, for example, maltophilin, a macrocyclic lactam antibiotic, which has antifungal activity, but is inactive against Gram-positive and Gram-negative bacteria (Berg et al., 1996; Jakobi et al., 1996). Three other antibiotics produced by *S. maltophilia* have been characterized, namely xanthobaccin A, B, and C, which have the same plain structure as maltophilin (Nakayama et al., 1999). These were effective against fungi and oomycetes, but not against the bacteria tested (Nakayama et al., 1999). Unfortunately *R. solanacearum* was not included in those tests. Apart from antibiotics, *S. maltophilia* produces lytic enzymes that have been implicated in biological control activity against fungi (Berg et al., 1996), for example chitinase (Zhang and Yuen, 2000) and an extracellular proteolytic enzyme (Dunne et al., 2000). This last enzyme may also be active against *R. solanacearum*, as Gram-negative bacteria contain, next to lipopolysaccharide, Braun lipoprotein in their cell walls.

The inhibitory effect against *R. solanacearum* by the *S. maltophilia* strains used in this study was proven not to be based on siderophore production like that by *P. fluorescens* (Ran et al., 2005b), as the availability of iron affected only the antagonistic activity of *P. fluorescens* but not that of *S. maltophilia*. The antibacterial efficiency of *P. fluorescens* depends at least partially on the production of the siderophore pseudobactin which can efficiently form complexes with iron in soils making it unavailable to plant pathogens, thus inhibiting their growth (Buyer and Leong, 1986; Ciampi-Panno et al., 1989; Ran et al., 2005b). Jurkevich et al. (1992) found one isolate of *S. maltophilia* that was able to utilize Fe^{3+} in the siderophore pseudobactin as sole iron source, but only to a limited extent. It is not known if *S. maltophilia* produces its own siderophore.

The antagonistic isolates of *S. maltophilia* used in this work were easily recovered from the rhizosphere of eggplant but not from that of sweet pepper. These two solanaceous crops have been listed among the plant species susceptible to race 1 of *R. solancearum* (Kelman, 1953). Race 3 of the pathogen, used in this work, is known to affect potatoes and to some extent tobacco (Buddenhagen and Kelman, 1964). This race may variably infect other host plants under greenhouse conditions, especially less compatible hosts under high soil infestation

levels. Thus, *S. maltophilia* probably did not evolve as a specific antagonist of race 3 of *R. solanacearum* on eggplant. However, *S. maltophilia* is known to be a good rhizosphere colonizer (Juhnke et al., 1987), and is a common member in the rhizosphere of many plants (Berg et al., 1996), especially of plants with high concentrations of sulphur-containing compounds such as methionine in their rhizosphere (Debette and Blondeau, 1980). Our *S. maltophilia* strains were able to produce H_2S, which would be beneficial in rhizospheres rich in sulfur-containing compounds, especially under moist, reducing conditions.

The antagonistic *S. maltophilia* strains were much more effective in controlling *R. solanacearum* in Egyptian clay soils than in Dutch clay soils, where they did not have significant suppressive effects on brown rot development. Thus, the antagonistic strain was effective in the soil from which it was isolated, probably because it co-evolved with the plants that were grown in the Nile Delta for centuries. Moreover, *S. maltophilia* survived significantly better in Egyptian than in Dutch clay soils, while *R. solanacearum* survived better in Dutch than in Egyptian soils, which may be related to clay and organic matter content, which were both higher in the Egyptian soils. High organic matter content was shown to be detrimental to *R. solanacearum* (Balabel et al., 2005). Moreover, *S. maltophilia* may not have survived long enough to be able to control *R. solanacearum* in the Dutch soils. A similar conclusion had been drawn previously about the failure of other potential biocontrol agents in controlling root pathogens in soil under greenhouse conditions (Waller, 1988). Biocontrol agents selected in the laboratory often fail under field conditions (Fravel, 1988). Factors affecting direct antagonism on agar media are usually not known, and the conditions that allow *in vitro* activity may not be present in nature (Wagih, 1991). Thus, it was quite fortuitous to find strains that were effective in field soil (at least in Egyptian soil), although relatively few strains were originally tested in the laboratory.

The reasons for the differences in survival of *S. maltophilia* in the various soils are not clear. The nitrate and ammonium contents and the pH of the Egyptian soils were higher than those of the Dutch soils. There was a positive correlation between survival of *S. maltophilia* and pH, but not the available nitrogen content. On the other hand, there was a negative correlation between *R. solanacearum* survival and pH, and also with NO_3. There could have been indirect effects, with a greater sensitivity of *R. solanacearum* than of *S. maltophilia* towards ammonia, which could have been higher in the high-pH Egyptian soil. The percentages of clay and organic matter were also higher in the Egyptian soils, especially the organically managed Egyptian soil, than in the Dutch soils, and there was a positive correlation between the organic matter content and survival of *S. maltophilia*. Also in this case, there might have been an indirect effect, as the availability of organic matter can increase the activity of biocontrol agents (Hiddink et al., 2005; Hoitink and Boehm, 1999), which may explain the better survival of the antagonist and the suppression of the pathogen by the antagonist in the Egyptian organic clay soil. The bacterial diversity in the Egyptian organic clay soil was less than in the conventional soil, which may explain the higher efficiency of the biocontrol agent in the Egyptian organic versus conventional soil (Hiddink et al., 2005). On the other hand, the

antagonist enhanced the survival of the pathogen in the Dutch organic soil by delaying its decline phase. The biodiversity was higher in the organic than in the conventional Dutch soil, reducing the efficacy of *S. maltophilia* to suppress *R. solanacearum,* so that the pathogen could survive longer. Similarly, the biocontrol agent *P. fluorescens* declined faster and was less effective in controlling take-all disease, when added to organically managed soil with a diverse microbial community than in a biologically impoverished conventionally managed soil (Hiddink et al., 2005).

S. maltophilia was more efficient at controlling the wilt disease for the early maturing susceptible cultivar, Nicola, compared to the late maturing moderately tolerant Diamant (only tested in Egyptian soil). The relation between susceptibility to brown rot and maturity of the potato variety was addressed by Farag (1976). Root exudates of early maturing, disease susceptible potato varieties are known to be rich in amino acid content compared to late maturing, disease tolerant varieties, resulting in larger microbial populations in the rhizosphere of susceptible varieties compared to tolerant varieties (Farag et al., 1986). The quality and quantity of substrate available in the rhizosphere of plants greatly affect the establishment of biocontrol agents on plant roots (Hoitink and Boehm, 1999), suggesting that roots of Nicola may have been better colonized by *S. maltophilia* than those of Diamant.

The practical implication of this work is that *S. maltophilia* could be used as biological control agent in the Nile Delta of Egypt where the disease is endemic. This could be accomplished either by stimulating naturally occurring populations, for example by crop rotation or intercropping with crops that have high concentrations of sulphur-containing amino acids in their root exudates, or by application of selected antagonistic *S. maltophilia* strains, for example by bacterization of potato tubers. However, the production of *S. maltophilia* for biological control would need to be approved by the appropriate regulatory agencies, as certain strains of *S. maltophilia* have been associated with various illnesses in immuno-depressed human patients (Berg et al., 2005).

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Chapter 5

Biological Soil Disinfestation, a new control method for potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2

Submitted as:
Nevein A.S. Messiha[a,b,c], Anne D. van Diepeningen[a], Marcel Wenneker[b,d], Alexander A.R. van Beuningen[b], Jaap D. Janse[b], Trudie G.C. Coenen[a], Aad J. Termorshuizen[a], Ariena H.C. van Bruggen[a,] and Wim J. Blok[a]✉ (2006) Biological Soil Disinfestation, a new control method for potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2. European Journal of Plant Pathology.

[a] *Wageningen University, Biological Farming Systems Group, Marijkeweg 22, 6709 PG Wageningen, the Netherlands*
[b] *Plant Protection Service, Dept. of Bacteriology, Geertjesweg 15, 6706 EA Wageningen, the Netherlands*
c Potato Brown Rot Project (PBRP), 3 El-Amira Fatma Ismail Street, Dokki, Cairo, Egypt
[d] Current address: *Applied Plant Research, Wageningen UR, P.O. Box 200, 6670 AE Zetten, the Netherlands*
✉ *Wageningen University, Biological Farming Systems Group, Marijkeweg 22, 6709 PG Wageningen, the Netherlands. E-mail: wim.blok@wur.nl, tel +31 317 483124, fax +31 317 478213.*

Chapter 5. Biological Soil Disinfestation, a new control method for potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2

Abstract

The potential of Biological Soil Disinfestation (BSD) to control potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2*,* was investigated. BSD involves the induction of anaerobic soil conditions by increasing microbial respiration through incorporation of fresh organic amendments (here: grass or potato haulms) and by reducing re-supply of oxygen by covering with airtight plastic sheets. Control treatments were left without cover and amendment, or amended without covering or only covering without amendment. The effect of BSD on survival of *R. solanacearum* was tested at three different scales: in 1-L glass mesocosms under laboratory conditions, in 1.2 m-diameter microplots that were positioned in an outdoor quarantine field, and in a naturally infested commercial field. Within a few days, anaerobic conditions developed in the BSD-treated soils. In the mesocosm and microplot experiment, anaerobic conditions persisted till the end of the 4-wk experimental period. In the field experiment, the period of anaerobiosis was shorter due to birds damaging the plastic cover. In all three experiments, BSD reduced soil populations of *R. solanacearum* significantly by 92.5% to >99.9% compared to the non-amended and uncovered control treatment. In the field experiment, BSD also resulted in a significant reduction of *R. solanacearum* survival in potato tubers buried at 15 or 35 cm and in the rapid decomposition of superficially buried potatoes left after harvesting, thus destroying an important inoculum reservoir of *R. solanacearum*. The treatments with only grass amendment or only covering with plastic did not result in anaerobic conditions and did not decrease *R. solanacearum* populations during the experimental period. PCR-DGGE analyses of 16S-rDNA isolated from soil samples of the various treatments in the mesocosm and microplot experiments revealed that BSD hardly affected bacterial diversity but it did result in clear shifts in the composition of the bacterial community. The possible implications of these shifts are discussed. It is concluded that BSD has the potential to strongly decrease soil infestation levels of *R. solanacearum* and to become an important element in a sustainable and effective management strategy for potato brown rot, especially in areas where the disease is endemic.

Additional keywords: anaerobiosis, *Ralstonia solanacearum*, soilborne pathogens, non-chemical control, fresh organic matter

Introduction

Potato brown rot caused by the bacterium *Ralstonia solanacearum* race 3 biovar 2 is a quarantine disease with zero tolerance in the Netherlands. The pathogen is soil-borne and can persist in soil for a long time in infected host plant debris or by colonizing potato volunteer

plants, alternative hosts or even non-host plants (Graham et al., 1979; Granada and Sequeira, 1983; Akiew and Trevorrow, 1994). In absence of potato, the pathogen was found to survive up to 3 years in soil (Graham et al., 1979). Cultural control through crop rotation is commonly used but implies long periods without host crops, and must be combined with strict control of volunteer host plants. Two seasons of nonhost crops reduced wilt from 81% to only 22-49% (Lemaga et al., 2001). Completely resistant potato cultivars are not commercially available yet (Zimnoch-Guzowska et al., 2005). Most cultivars that seem to show (partial) resistance, appear to be tolerant. The level of tolerance that is expressed can be negatively affected by environmental conditions (Boshou, 2004), such as high temperature (French and De Lindo, 1982), low light intensity and short photoperiods (Sequeira and Rowe, 1969). Chemical control of potato brown rot with currently available antibiotics or fungicides is not effective (Hartman and Elphinstone, 1994; Lopez and Biosca, 2004). Development of more effective chemical controls is not stimulated due to the general awareness about negative impacts of synthetic crop protectants on human health and the environment, leading to the phase out of an increasing number of crop protectants. The latter is exemplified by the widely used and very effective soil fumigant methyl bromide (MB), which is prohibited for agricultural use in developed countries since 2005 and will be prohibited in developing countries by 2015 (Duniway, 2002). Some countries, for example the Netherlands, started to restrict its use even earlier because of its toxicity (Braun and Supkoff, 1994).

In the Netherlands, potato brown rot is currently effectively managed by enforcing the statutory measures of the Brown Rot Control Directive 98/57/EC, which implies not growing potato or alternative host crops for a minimum period of four years in infested fields and applying strict control of volunteer plants in this period. However, a four-year period without host crops of *R. solanacearum* is a severe restriction for specialized potato farmers and costly and time-consuming for inspection services. Therefore, there is a clear need to develop alternative practical, safe and effective management strategies that can shorten the time that no host plants can be grown. Candidate methods include solarization (Katan, 1981; Gamliel and Stapleton, 1993), organic amendments (Lazarovits et al., 2001; Gorissen et al., 2004; Conn et al., 2005) and Biological Soil Disinfestation (BSD). BSD is a new method, first described by Blok et al. (2000). With BSD, anaerobic soil conditions are induced by increasing microbial respiration through incorporation of fresh organic amendments (e.g. grass) and by reducing re-supply of oxygen by covering with airtight plastic sheets (Blok et al., 2000). Under these conditions the survival of a number of persistent soilborne pathogenic fungi and nematodes, including *Fusarium oxysporum*, *Meloidogyne* spp., *Rhizoctonia solani*, *R. tuliparum*, *Sclerotinia sclerotiorum* and *Verticillium dahliae,* was strongly reduced or completely eradicated in repeated field experiments over the last ten years (Blok et al., 2000; Blok et al., unpublished; Goud et al., 2004, Lamers et al., 2004). The effect of BSD on survival of *R. solanacearum* was not tested so far. However, data obtained by Termorshuizen et al. (2003) indicated that BSD could also be effective against *R. solanacearum*. They studied the effect of anaerobic mesophilic digestion of vegetable, fruit and garden waste on the

survival of a number of pathogens including *R. solanacearum* and found that *R. solanacearum* was readily inactivated under these conditions.

The aim of the present study was to investigate the effect of BSD on survival of *R. solanacearum* in order to develop a practical, effective and sustainable management strategy for this important quarantine pathogen. As many soil disinfestation methods have also a more or less drastic effect on non-target soil organisms, an attempt was made to also evaluate the effect of BSD on the soil bacterial community. The fact that *R. solanacearum* is a quarantine pathogen brings about limitations for the experiments that can be carried out. The effect of BSD on survival of *R. solanacearum* was first tested in a field experiment. This was done in 1999 when potato brown rot had been recently detected in the Netherlands and a few heavily infested fields were available. In later years, the disease was under control and infested fields that could be used for further field experiments were not available anymore. To further explore the potential of BSD two additional experiments were carried out in which field application of BSD was simulated, first using glass mesocosms under laboratory conditions and then using microplots situated in an outdoor quarantine area. The experiments will, however, be described in what seems to be the logical order, starting with the mesocosm experiment, then the microplot experiment and then the field experiment.

Materials and Methods

General set-up

A field experiment was performed on a commercial field of industrial potato at Dalerveen (province of Drenthe, the Netherlands) that had a high natural infestation with *R. solanacearum*. The effect of Biological Soil Disinfestation (BSD) was further studied using glass mesocosms under laboratory conditions and then in microplots consisting of concrete rings (1.2 m diameter) filled with soil, which were positioned in the outdoor quarantine field of the Plant Protection Service and had a cage of metal wires around and above it. Anaerobic soil conditions were induced by increasing microbial respiration through incorporation of fresh organic amendments (grass or potato haulms) and by reducing re-supply of oxygen by covering with airtight plastic sheets (Blok et al., 2000). The soil was covered with airtight plastic tarps (Hermetix, a 3-layered coextruded plastic ensilage film, 0.135 mm thick, produced by Klerks Plastic Industry, Noordwijkerhout, the Netherlands). Hermetix has a green and a white side, and the oxygen diffusion rate is 1400 ml O_2 m^{-2} 24 h^{-1}. The tarp was applied with the green side up.

Inoculation and isolation of the pathogen

In this study a mixture of equal amounts of three virulent *Ralstonia solanacearum* race 3 biovar 2 strains (PD5239, PD5240, and PD5241; culture collection, Plant Protection Service,

Wageningen, the Netherlands) was used as inoculum. These strains were originally isolated from infected potato tubers from Egypt and all were found to be able to reduce nitrate. Nitrate reduction was tested following the protocol of Fahy and Persley (1983).

The cultures used for soil inoculation were grown for 48 h on nutrient agar (NA; Difco, Detroit, MI, USA) plates at 28°C. The inoculum was suspended in 0.01 M phosphate buffer (PB: $Na_2HPO_4.12H_2O$, 2.7g; $NaH_2PO_4.2H_2O$, 0.4 g; 1 L distilled water; pH 7.2). The bacterial density was adjusted to 0.5×10^9 CFU ml^{-1} using a spectrophotometer (OD_{600} = 0.3 equals 0.5×10^9 CFU ml^{-1}). For isolation of the pathogen, 10 g of soil was suspended in 90 ml of sterile 0.05 M PB ($Na_2HPO_4.12H_2O$, 4.26 g; KH_2PO_4, 2.72 g; 1 L distilled water; pH 7.2). After shaking at 100 rpm for 2 h at 20°C, 10-fold serial dilutions were made and 100-µl aliquots were plated in triplicate onto modified SMSA (Selective Medium South Africa) agar plates (Anonymous, 1998) and incubated at 28°C for 5-7 d. Per sample, the identity of 3 colonies with a morphology typical for *R. solanacearum* (colonies with irregular shape, diffuse white or purple centres and luxuriant slime) was checked using Immunofluorescence Antibody Staining (IFAS; Janse, 1988). Plate counts were expressed as colony forming units (CFU) per g dry soil.

Physical, chemical and biological soil characteristics

The soil used for the mesocosm and microplot experiments was a sandy soil (1.4% clay, 5.9% silt and 92.7% sand) with pH-$CaCl_2$ 7.3, 1.4% organic matter, 7.2 g C, 0.8 g N and 36.0 mg NO_3^-N, all per kg soil. The area where the field experiment was located had a mixed sand-peat soil that was not analysed in detail.

For DNA analyses, 1-2 g samples of sandy soil were collected and stored at -20°C for DNA extraction within 6 months. Soil bacterial diversity was studied using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). For DNA extraction, PCR and DGGE analysis, the methods described by Hiddink et al. (2005) were followed, the main steps of which are described shortly below. Total DNA was extracted from 0.5-g soil samples using the Bio101 FastDNA® SPIN Kit for Soil according to the manufacturer's specifications (Bio101, Vista, CA, USA) except that before the final centrifugation step the DNA extract was incubated for 20 min at 65°C for better DNA elution. The DNA quality and quantity were checked on a 1.2% agarose gel stained with ethidium bromide and visualized by UV trans-illumination (Sambrook et al., 1989). The PCR used the U968 (40-bp GC clamp) and L1401 universal eubacterial primers and primers FR1 (58-bp BC-clamp) and FF390 for amplification of fungal small-subunit (SSU) rDNA. The amount and quality of the obtained PCR products were again checked as described before. DGGE was performed using the Dcode Universal Mutation Detection System (BIO-Rad Laboratories, Hercules, CA, USA) with a vertical denaturing gradient of 45 to 60% (100% denaturant is defined as 7 M urea plus 40% formamide; the gel was topped with a 8% acrylamide stack without denaturing agents). Gels were run at 60°C at 100 V for 16 h and bands were visualized using the Bio-Rad Silver

Stain Kit. After scanning, gels were analysed with Phoretix 1D Advanced version 4.00 (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). The bacterial diversity in the samples was estimated in two ways: as species richness *S*, and as the Shannon-Wiener index of bacterial diversity, *H'* (Eichner et al., 1999; van Diepeningen et al., 2006).

Mesocosm experiment

Mesocosms consisted of 1-L glass beakers of 18 cm height and 9 cm diameter with one short glass tube halfway. A porous rubber tube of 10 cm length was placed inside the glass tube and extended into the beaker. This tube provided a space from which the soil atmosphere could be sampled by piercing the septum with a syringe needle. At the start of the experiment soil was infested with *R. solanacearum* by adding a bacterial suspension that resulted in a density of 5×10^7 CFU g^{-1} d. w. soil. Soil for the grass-amended treatments was mixed with grass leaves that were cut into pieces of 1-2 cm in a 0.1% ratio (d. w./d. w.). The mesocosms were filled with soil in layers that were compacted after adding a new layer to ensure a homogeneous porosity over the whole profile. The amount of soil and water and the compaction were such that the volumetric proportion of solids, water and air was 55%, 25% and 20%, respectively, which are values that are representative for field experiments with BSD performed earlier. Mesocosms that were covered received a Vaseline layer on the edge to ensure an airtight seal between the mesocosm and the Hermetix plastic cover. The latter was kept in place with rubber bands and tape. The following treatments were used in this experiment: (1) non-amended and uncovered (positive control; 15 mesocosms); (2) grass-amended and covered (BSD treatment; 15 mesocosms); (3) grass-amended and uncovered (5 mesocosms) (4) non-amended and covered (5 mesocosms). The mesocosms were placed completely randomized in an incubator at 28°C. A mesocosm filled with noninfested, non-amended soil was left uncovered and was incubated under the same conditions to serve as a negative control for detection of *R. solanacearum*. The mesocosms that were not covered with Hermetix were covered with parafilm, which allowed for air exchange but reduced evaporation.

One-ml soil atmosphere samples were collected from each mesocosm twice a week and oxygen and carbon dioxide concentrations were determined with a gas chromatograph (Chrompack Micro-GC 2002).

The population density of the pathogen in the soil was determined at the start of the experiment for all the mesocosms. After 17 days, five mesocosms of the positive control and five mesocosms of the BSD treatment were opened and sampled for pathogen quantification. After 31 days all remaining mesocosms were opened and samples were collected for pathogen quantification and for DNA extraction and PCR-DGGE. The latter samples were stored at -20^oC. Soil from three BSD-treated mesocosms were aerobically incubated in loosely capped, 50-ml centrifuge tubes at 28°C for an extra period of 3 wk to study the changes in the composition of the bacterial community upon return to aerobic conditions. PCR-DGGE patterns of the following treatments (with number of samples per treatment given in brackets)

were compared: non-amended, uncovered control (3), grass-amended, uncovered (1), non-amended, covered (1), BSD, harvested after 17 d (1), BSD, harvested after 31 d (2) and BSD, harvested after 31 d and stored aerobically for an additional 3 wk (2).

Microplot experiment

This experiment was conducted in the quarantine field at the Plant Protection Service (Wageningen, the Netherlands). In this field 12 microplots were available for the experiment. The microplots consisted of concrete rings with internal diameter of 120 cm and height of 40 cm. The rings were open at the bottom and buried almost completely into the soil. The following four treatments were compared: (1) non-amended and uncovered (3 microplots); (2) grass-amended and covered (BSD treatment; 3 microplots); (3) grass-amended and uncovered (2 microplots); (4) non-amended and covered (2 microplots). The microplots were filled with sandy soil similar to the soil used for the mesocosm experiment. For the grass-amended treatments, the soil was first mixed thoroughly with mown grass in a 0.1% ratio (d. w./d. w.). After filling the microplots with soil, three inoculum samples were buried in each microplot. These inoculum samples consisted of nylon bags filled with 2.5 kg soil from that microplot, infested with *R. solanacearum* race 3 biovar 2 to a density of 10^6 CFU g^{-1} d. w. soil. The remaining two microplots were filled with non-amended soil and left uncovered and received three nylon bags with soil to which noninfested PB buffer was added; these samples served as negative controls. All microplots were then irrigated to field capacity to stimulate microbial activity. Finally the plastic covers were applied. Because of the small diameter of the microplots oxygen supplied from the borders with untreated soil could have a relatively large effect on soil oxygen levels in the microplot and could prevent reaching anaerobiosis. To reduce this border effect, the plastic covers were buried to 50 cm depth to cover the outside of the concrete ring. In addition, a shallow ditch was made around the microplot that was frequently filled with water to create an oxygen barrier layer of water-saturated soil around the covered microplot. The experiment was finished by removing the plastic covers after 31 d.

The oxygen level of the soil atmosphere was measured twice a week using an oxygen analyzer (570 A; Servomex, Crowborough, East Sussex, UK.). Soil atmosphere samples were extracted with this analyzer from three gas diffusion chambers that were buried in each microplot at 15 cm depth. The gas diffusion chamber consisted of a piece of PVC tubing (diameter 1.6 cm, length 5 cm, volume 10 ml) that was open at the bottom and closed with a butyl rubber stopper at the top. The rubber stopper was pierced by copper tubing (internal diameter 1.0 mm) extending about 5 cm above the soil surface. The copper tube was fitted at the top with a copper head closed with a rubber septum and crimp cap. The soil temperature was measured every hour using thermocouples buried at 15 cm depth in one microplot for each treatment, and a data logger (DL2e, Delta-T Devices Ltd, Burwell, and Cambridge, United Kingdom). Pathogen population densities for each inoculum sample were determined as described earlier at the beginning and at the end of the experiment. Three sub-samples were tested from each inoculum sample.

For PCR-DGGE analyses, soil samples were collected from four grass-amended microplots at the start of the experiment. At the end of the experiment, two samples were collected from BSD microplots, two samples from grass-amended, uncovered microplots and two samples were collected from non-amended, covered microplots. These samples were stored at -20°C for DNA extraction at a later time.

Field experiment

This experiment was conducted on a commercial field of a mixed sand-peat soil at Dalerveen in the province of Drenthe, the Netherlands. In this field a potato crop had been grown in which during the summer of 1999 a heavy infection of brown rot was detected. The foliage was cut using a rotary cultivator in mid August. Three days later the crop residues were incorporated to a depth of 25 cm using a rototiller and six plots of 7×8 m were laid out. The amount of incorporated potato material was about 30 tons fresh weight ha^{-1}. In each plot, nylon bags, each containing one *R. solanacearum*-infected potato tuber, were buried, three bags at a depth of 15 cm and three at 35 cm. After the soil was compacted with tractor wheels, gas diffusion chambers, as described earlier, were installed in one uncovered and one covered plot (three chambers in each plot at 15 cm). Fifty soil cores were collected from 0-15 cm depth and mixed into one composite sample for each of the six plots. Finally, three randomly selected plots were covered with Hermetix plastic sheets, burying the edges to a depth of 10 cm.

Oxygen and methane concentration of the soil atmosphere was measured after 7 and 35 days from the start of the experiment. Samples were extracted using evacuated blood collection tubes and analysed at the laboratory using a Varian 3200 cx gas chromatograph. After 6 wk, the plastic covers were removed, the nylon bags were retrieved and one composite soil sample was collected for 0-15 cm and one for 30-45 cm from each plot using 50 and 20 randomly collected soil cores, respectively.

The potato tubers buried were tubers collected from plants that had grown in the experimental field and had shown typical brown rot symptoms. *R. solanacearum* infection of the tubers was confirmed by slime exudation from eyes and isolation of *R. solanacearum* from vascular tuber tissue plated onto SMSA plates (Anonymous, 1998). After retrieval of the nylon bags, pieces of potato vascular tissue from different places in the tuber were suspended and shaken for one hour in phosphate buffer, and then 100-µl aliquots were plated onto SMSA. Soil adhering to the tuber was also plated onto SMSA to test for spread of *R. solanacearum* from the tuber or tuber residues into the surrounding soil and survival therein. The density of *R. solanacearum* in the soil samples collected at the start and end of the experiment was determined as described earlier. In addition, pathogencity testing, PCR and fatty acid analysis was used to confirm the identity of colonies with a morphology characteristic for *R. solanacearum* (Janse, 1988, 1991; Roy, 1988; Pastrik and Maiss, 2000).

Statistical analyses

Data of *R. solanacearum* population density of the mesocosm experiment were analysed by analysis of variance on log-transformed data (log (CFU + 1)) followed by Tukey's mean separation test using SPSS, version 12 (SPSS Inc., Chicago, Illinois, USA). For the microplot and the field experiment the means for *R. solanacearum* population densities of the control and BSD treatment were compared with the nonparametric Mann-Whitney U test using SPSS.

The PCR-DGGE banding patterns were first analysed by hierarchical cluster analysis of the band intensity data using the Dice coefficient and the UPGMA algorithm with the Phoretix software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). The banding patterns were further analysed with Principal Component Analysis (PCA) using the PRINCOMP procedure of SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA) on log-transformed band intensities and with Discriminant Analyis (DA) using the DISCRIM, CANDISC, and STEPDICS procedures of SAS. For the DA, each data set was first split into small subgroups of 5 variables and the most discriminating variables were then combined and subjected to the final analyses (van Diepeningen et al., 2006). The banding patterns compared in one PCA or DA analysis were all from the same gel. The values of the diversity indices calculated from the PCR-DGGE banding patterns for the mesocosm and microplot experiment were analysed together using ANOVA and mean separation by a protected LSD test using PROC GLM of SAS.

Results

Mesocosm experiment

Three days after starting the experiment the oxygen percentage in the BSD mesocosms had dropped to 1.7 ± 0.21%, indicating almost complete anaerobiosis. The oxygen levels for the control and the grass-amended, uncovered treatments were 16.8 ± 0.97% and 14.9 ± 1.4%, respectively. The non-amended, covered mesocosms had an oxygen level of 3.7 ± 0.88%, which indicates that also in these mesocosms a large part of the soil volume experienced anaerobic conditions in the first period after covering. As from one week after the start of the experiment, oxygen levels of all non-BSD treatments were similar (>16.0%), whereas the oxygen levels in the BSD mesocosms were <1.0%.

At the start of the experiment the *R. solanacearum* population density was equal in all mesocosms: 6.50 ± 0.30 (mean ± SEM of log (CFU g^{-1} d. w. soil + 1)). After 17 days a trend of more inactivation by BSD was found with a density of 4.49 ± 0.01 and 3.30 ± 0.52 in the control and BSD mesocosms, respectively. After 31 days, *R. solanacearum* was almost completely eradicated from the BSD mesocosms, leaving only 0.59 ± 0.31, while its population density was still above 3.60 in the other treatments (Table 1).

Table 1. Effect of the different treatments applied in the mesocosm experiment on survival of *R. solanacearum* after 17 and 31 days of incubation. Values are means ± SEM of log-transformed numbers (log(CFU+ 1)).

Treatment	17 days incubation	31 days incubation
Non-amended, uncovered (control)	4.49 ± 0.01 a [1]	3.73 ± 0.02 a
Grass-amended, covered (BSD)	3.30 ± 0.52 a	0.59 ± 0.31 b
Grass–amended, uncovered		4.07 ± 0.15 a
Non-amended, covered		3.60 ± 0.05 a

[1] Means with a different letter are significantly different according to the Tukey test ($P < 0.05$).

The species richness (S) and Shannon-Wiener diversity index (*H*) derived from the PCR-DGGE banding patterns of the 16S rDNA were quite similar for the four treatments. There were not sufficient values to perform proper statistical tests and, therefore, the data were combined with similar data from the microplot experiment for comparing treatment means (Table 2). The composition of the bacterial microflora was different among the treatments as shown by cluster analysis of the banding patterns. These analyses show that the BSD samples are clearly separated from the other samples and that the samples from the non-BSD treatment cluster together. The BSD samples that were incubated for 3 wk after the end of the experiment still cluster with the BSD samples collected immediately after the end of the experiment indicating that there was not a fast return to the original bacterial community composition (Figure 1). The groupings revealed by the cluster analysis were confirmed by those of a principal component analysis (Figure 2). DA identified 8 bands as the most discriminative ones (11% from the total number of detected bands). Four of these bands were found only in samples from the control treatment, two bands were found only in the BSD (including the BSD samples stored for an extra 3 wk), one band was found in higher density in BSD soil samples compared to control samples and one band was found only in the BSD samples stored for an extra 3 wk.

Microplot experiment

The oxygen levels of the soil atmosphere showed roughly the same trend as found in the mesocosm experiment with >15% in the non-BSD microplots and low values in the BSD microplots. In contrast to what was found in the mesocosm experiment, the non-amended, covered treatment did not show a decrease in oxygen content in the first period after covering. For the BSD treatment, two of the three microplots had oxygen levels <1% during the whole experimental period, in the third microplot the level was between 1 and 3%. The daily mean soil temperature of the uncovered plots during the experiment ranged from 14.9-26.4°C, the

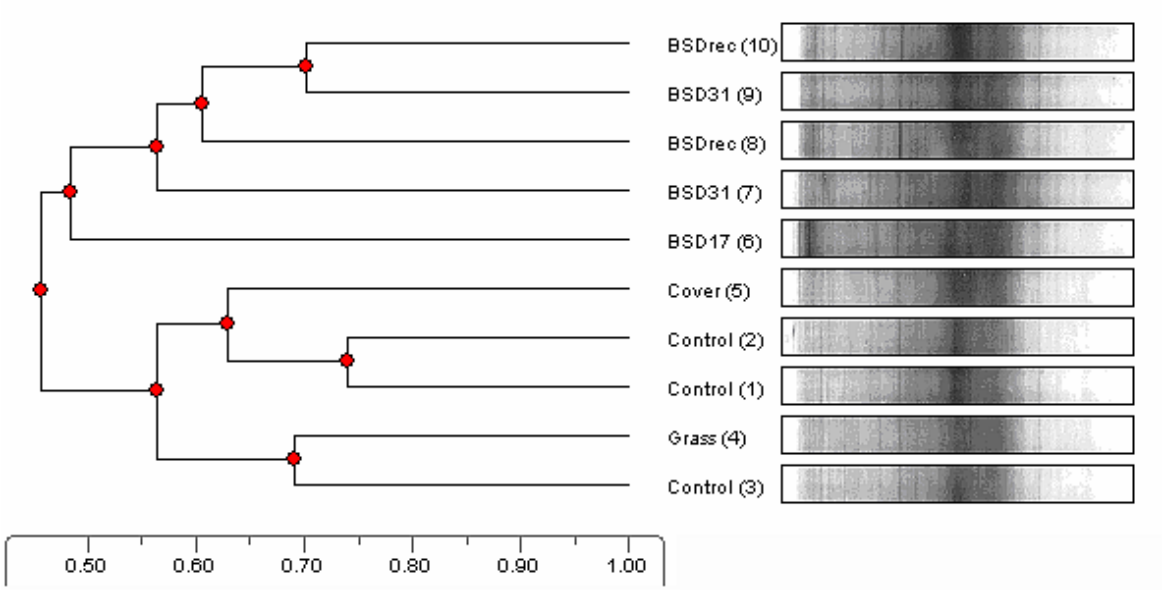

Figure 1. UPGMA dendrograms based on Dice coefficient matrices for PCR-DGGE banding patterns of 16S-rDNA for samples of the mesocosm experiment. The meaning of the codes is as follows: Control – non-amended, uncovered; Grass – grass-amended, uncovered; Cover – non-amended, covered; BSD – grass-amended, covered. The additional codes for the BSD samples indicate the incubation period (17 or 31 d) or samples that were incubated for an additional 3-wk period after the end of the experiment (rec). The scale on the X-axis depicts similarity.

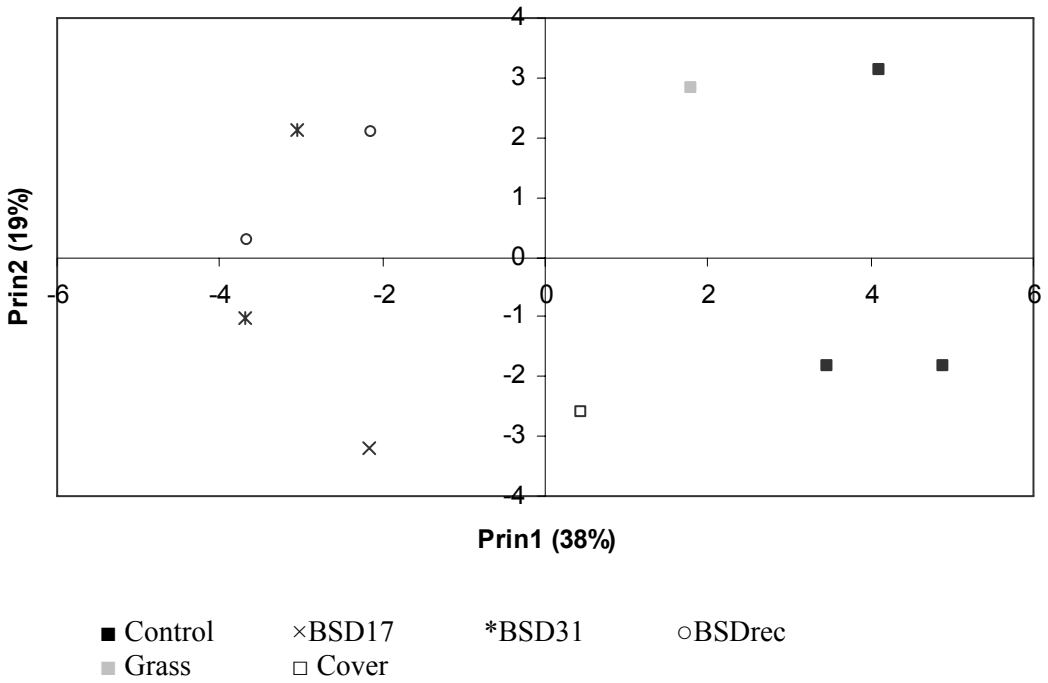

Figure 2. Scatter plots of the scores for principal components 1 and 2 of the PCR-DGGE banding patterns for samples of the mesocosms experiment. Principal components 1 and 2 explain 38% and 19% of the variation, respectively.

Table 2. Species richness (S) and Shannon-Wiener index of bacterial diversity (H) (means ± SEM) calculated from PCR-DGGE banding patterns for eubacterial 16S-rDNA extracted from differently treated soil collected at the end of the mesocosm and microplot experiment

Treatment	*S*	*H*
Non-amended, uncovered (Control)	30.5 ± 0.5 a[1]	1.43 ± 0.01 a[1]
Grass-amended, covered (BSD)	29.0 ± 1.0 a	1.26 ± 0.05 c
Grass –amended, uncovered	30.5 ± 1.2 a	1.41 ± 0.02 ab
Non-amended, covered	28.0 ± 2.0 a	1.36 ± 0.04 abc

[1] Means with a letter in common do not differ according to the protected LSD-test ($P < 0.05$).

daily maximum temperature from 16.8-32.8°C. Covering the soil raised the daily mean and the daily maximum temperature with 0.1-4.4 and 0.0-4.3°C, respectively.

At the start of the experiment the population density of *R. solanacearum* was similar for all microplots: 4.99 ± 0.04 (mean ± SEM of log (CFU g^{-1} d. w. soil + 1)). At the end of the experiment (after 31 d) population density was 2.69 ± 0.14 for the control, 3.00 ± 0.19 for the grass-amended, uncovered treatment and 3.18 ± 0.13 for the non-amended, covered treatment indicating that grass amendment alone or covering alone does not result in control of *R. solanacearum*. The BSD treatment resulted in a mean density of 1.02 ± 0.60, which is significantly lower than the mean of the control treatment ($P = 0.050$; Mann-Whitney U test). The pathogen survival in the BSD microplots showed, however, a relatively high variation. The log (CFU g^{-1} d. w. soil + 1)-values for the individual inoculum samples were as follows: microplot 1 - 0.00, 0.00, 0.00; microplot 2 - 0.00, 0.00, 2.96; microplot 3 - 0.85, 1.99, 3.40. As mentioned above, microplot 3 showed higher oxygen levels than microplots 1 and 2, which likely explains the higher survival of the pathogen in this microplot. *R. solanacearum* was not detected in any of the noninfested samples

PCR-DGGE banding patterns of the BSD samples were clearly different from the non-BSD samples (Figure 3). Strikingly, the grass-amended, uncovered samples were more similar to the non-amended, covered samples than to the grass-amended samples collected at the start of the experiment indicating that the effect of incubation time on composition of the bacterial microflora was stronger than that of grass amendment. PCA of the DGGE data resulted in a clear separation of soil samples: grass-amended, uncovered sampled at the start (coded Control), BSD, and grass-amended, uncovered and non-amended, covered (Figure 4) and thus confirm the results of the cluster analyses. DA revealed 9 bands as the most discriminative ones (11% from the total number of detected bands). Five bands were found only in the BSD soil samples, two bands were much less intense in the BSD samples as compared to the samples from all other treatments, one band was found only in the control treatment and one

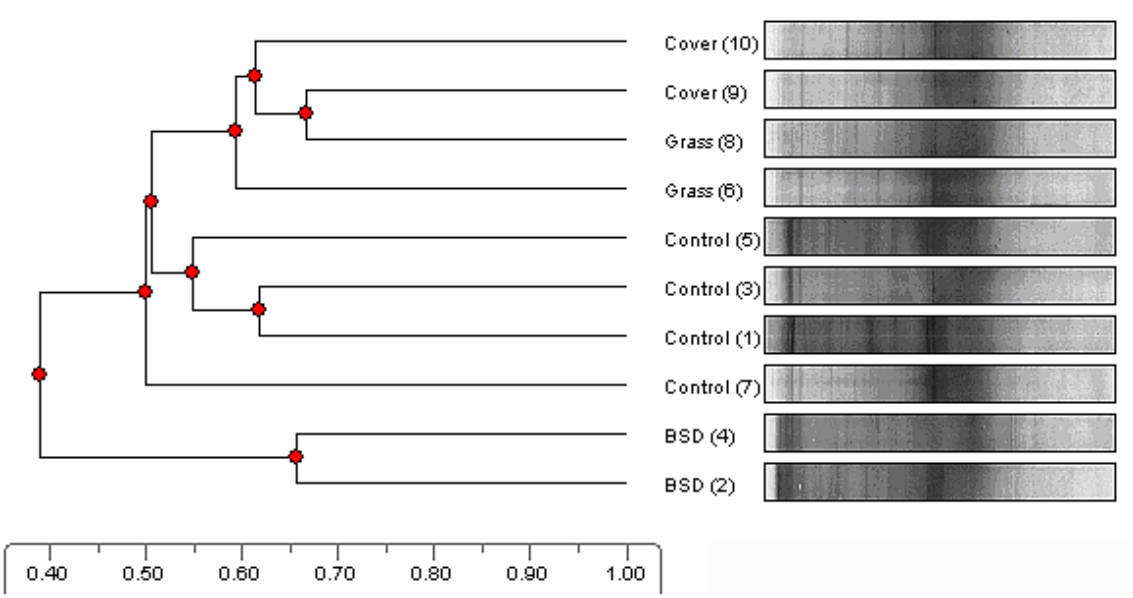

Figure 3. UPGMA dendrograms based on Dice coefficient matrices for PCR-DGGE banding patterns of 16S-rDNA for samples of the microplot experiment. The meaning of the codes is as follows: Control – grass-amended plots sampled at the start of the experiment (before covering of plots); Grass – grass-amended, uncovered, sampled at end of experiment; Cover – non-amended, covered, sampled at end of experiment; BSD – grass-amended, covered, sampled at end of experiment. The scale on the X-axis depicts similarity.

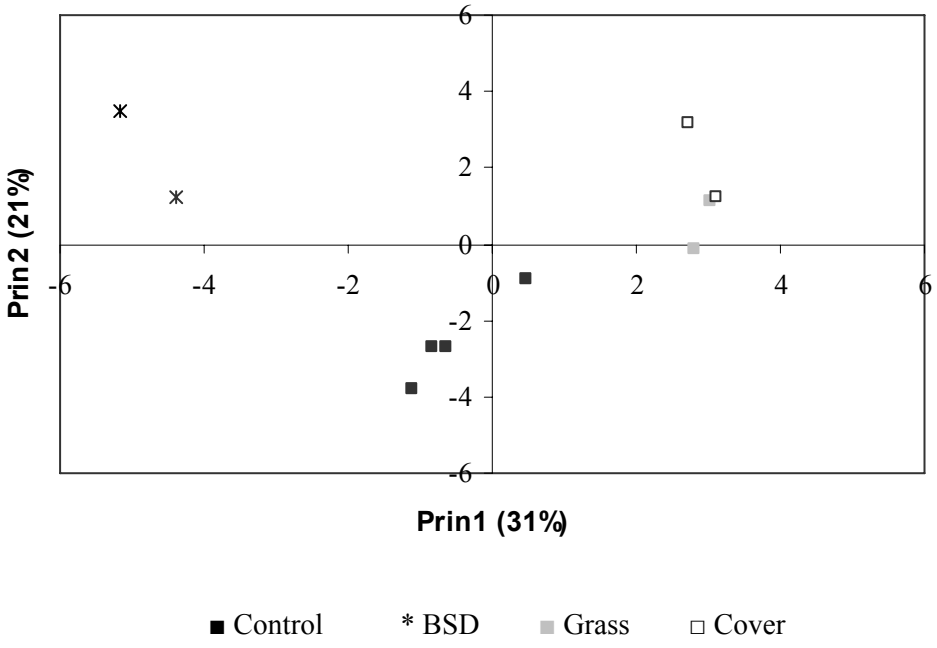

Figure 4. Scatter plots of principal components 1 and 2 of the PCR-DGGE banding patterns for samples of the microplot experiment. Principal components 1 and 2 explain 31% and 21% of the variation, respectively.

band was found only in the grass–amended, uncovered treatment and in the non-amended, covered treatment.

Field experiment

The field could be visited only a few times during the experiment. During the first visit, 7 days after the start, the plastic cover was still almost completely intact. However, after 2-3 wk birds started to make holes in the plastic tarps and after 5 wk the tarps were severely damaged. The soil atmosphere samples collected after 7 days from the BSD plot contained very low levels of oxygen (0.03 ± 0.03 vol %; mean ± SEM) compared to the control plots (15.28 ± 1.63 vol %) and traces of methane (0.13 ± 0.04 vol %), indicating anaerobic, reducing conditions in most of the soil volume. After 35 days, however, soil oxygen levels were similar for the control and the BSD plot (9.44 ± 2.48 and 13.47 ± 1.49 vol %, respectively) and methane was no longer present in the BSD plot.

After removal of the plastic cover from the BSD plots, the soil surface was completely free of weeds and no potato tubers were present on the surface. On the control plots numerous weeds and firm potato tubers were present. Apparently, the BSD treatment resulted in fast decomposition of the potato tubers. Thirty potato tubers were collected from the surface of the untreated plots and tested for presence of *R. solanacearum*. Thirty % of these tubers were infected by *R. solanacearum. R. solanacearum* was detected in 55% of infected tubers that had been buried in the control plots, but was not detected in any of the tubers retrieved from BSD plots, from either 15 or 35 cm depth. In soil adhering to potato tubers buried on purpose, *R. solanacearum* was detected in 75% of samples collected from the control plots, while in only 5% of samples collected from BSD plots ($P = 0.001$). The infestation of the soil with *R. solanacearum* at the beginning of the experiment was found to be very similar for the six plots with a population density of 5.06 ± 0.09 (log (CFU g^{-1} d. w. soil + 1)). At the end of the experiment, after 6 weeks, the density of *R. solanacearum* in the BSD plots was significantly lower than that in the control plots for both the 0-15 cm and the 30-45 cm soil layer (Table 3), with reduction percentages of 92.5% and 96.7 % for the 0-15 cm and the 30-45 cm soil layer, respectively.

Table 3. Soil population density of *R. solanacearum* (log (CFU+1)) at the end of the field experiment.

Treatment	0-15 cm		30-45 cm	
	Mean	P^1	Mean	P^1
Control	4.55	0.025	3.83	0.066
BSD	3.24		1.76	

[1] *P*-value of the nonparametric Mann-Whitney U test comparing population densities of the BSD treatment and the control treatment for each depth separately.

Discussion

In this study, Biological Soil Disinfestation (BSD), the combination of incorporation of easily decomposable organic matter and application of an airtight soil cover, was found to strongly reduce soil population densities of *R. solanacearum*, both under controlled laboratory conditions and under (semi-)field conditions. Incorporation of easily decomposable organic matter, grass or potato haulms, alone or covering the soil with airtight plastic without incorporation of organic amendment did not result in reduction of *R. solanacearum* soil populations.

The microplot experiment showed for the nine inoculum samples buried in the BSD microplots complete eradication of the pathogen in five samples, significant reduction in two samples and no reduction in the remaining two samples. This large variation in pathogen inactivation was not found for the glass mesocosms, for which anaerobic conditions could easily be obtained and maintained, but also not for the field plots. It can most probably be explained by local oxygen leakage into the microplots leading to prevention or nullification of the anaerobic conditions. Oxygen leakage will occur more easily in these microplots than in field plots due to the larger border length relative to the plot's surface area.

In the field experiment anaerobic conditions were not maintained for a long period due to birds damaging the plastic tarp. Nevertheless, pathogen populations were reduced significantly by more than 95%. If this bird damage can be prevented, and farmers have various effective methods available for this, anaerobic conditions can be maintained for a longer period resulting in an even stronger decline of *R. solanacearum* populations (Termorshuizen et al., 2003). Especially relevant is the observation that after the BSD treatment potato tubers were completely decomposed and weed growth was absent. Potato tubers are a major reservoir of *R. solanacearum* where it normally can survive better than in soil (Graham et al., 1979). Also various weed species such as *Portulaca oleracea, Rumex dentatus* and *Solanum nigrum*, have been found to be important reservoirs for the pathogen (Elphinstone et al., 1998; Farag et al., 2004). In this study we did not determine if weeds were really destroyed or only showed delayed emergence in the BSD plots. However, from other BSD studies we got strong indications that weed populations are indeed reduced. Destruction of these 'hiding' places for the pathogen by BSD will greatly contribute to a decrease of soil infestation levels.

The mechanism of inactivation of *R. solanacearum* was not studied in detail in this study. Most probably, toxic compounds produced during fermentation of the fresh organic matter under the anaerobic soil conditions will have played a major role in the decline of *R. solanacearum* populations. It has been well documented that a vast array of potentially toxic compounds, including alcohols, aldehydes and organic acids, is produced when organic matter is decomposed under anaerobic soil conditions (Ponnamperuma, 1972; Strandberg, 1987). In earlier BSD experiments we also found a temporary, strong build-up of fermentation

products (Blok et al., unpublished). In addition to the toxic products produced during organic matter decomposition under anaerobic conditions, biocontrol by facultative anaerobic and strictly anaerobic bacteria such as *Bacillus* and *Clostridium* spp., respectively, may have contributed to pathogen inactivation (Cook and Baker, 1983). The small increase in soil temperature induced by the plastic can not explain the BSD effect found in this study as the plots with only plastic covering without incorporation of fresh organic matter, which have experienced the same temperature as the BSD plots, did not show a decline in *R. solanacearum* populations. This does not exclude, however, that in other situations increased soil temperatures can contribute to pathogen inactivation. If solar radiation levels are higher, such as often in Mediterranean and tropical countries, the soil temperatures can reach sublethal temperatures that will make soilborne pathogens more vulnerable to all kinds of stress factors including toxic fermentation products (Katan, 1981).

The results of this study clearly show the potential of BSD to strongly reduce the level of soil infestation by *R. solanacearum*. The levels of reduction are similar to those obtained using chemical soil disinfestations against other soilborne pathogens and pests. If BSD becomes part of the management strategy currently used in many European countries following the Brown Rot Control Directive 98/57/EC that aims at eradication of potato brown rot, the period for which statutory measures are needed can probably be reduced considerably. Application of BSD is rather expensive because of the costs of the plastic tarp, however, if statutory measures can be lifted earlier, application of BSD will be economically attractive. But, also in countries where potato brown rot is endemic application of BSD will often be economically feasible and attractive as the losses due to potato brown rot can be high. The method might be further optimized, possibly resulting in lower costs, by using different types of plastic tarp (Lamers et al., 2004). For example, transparent, thin but oxygen tight barrier films can be used that increase soil temperature more than the standard plastic thus combining soil solarisation and BSD. The advantage of using thinner tarps would also be lower environmental costs because lower amounts of plastics are used. Environmental costs are further restricted by setting up a collection and recycling scheme for the tarps.

The results of the PCR-DGGE analyses revealed that although BSD did hardly affect bacterial diversity it induced clear changes in the composition of the soil bacterial community. The observation that a 3-wk aerobic incubation period following BSD did not result in a return to the pre-BSD community patterns indicates that the BSD-induced changes are relatively persistent. The effect of the changes in bacterial community composition on soil functioning cannot be deduced from our data and needs further research. Both possible negative and positive effects can be envisaged. If the altered microbial composition results in a lower natural suppressiveness of the soil against potato brown rot, this will result in increased rates of re-infestation by the pathogen, which will partially or wholly nullify the initial decline in soil population brought about by BSD. Such effects have been reported for soil flooding and for soil fumigation (Kreutzer, 1965; Stover, 1979). However, we have obtained indications that for BSD, in contrast to soil flooding and chemical disinfestation, the risk of a significant

decrease in disease suppressiveness of the soil is limited (Blok et al., 2000; Goud et al., 2004). In a study in which we tested disease suppressiveness against *Fusarium oxysporum* f.sp. *asparagi* immediately after BSD, so at the most vulnerable stage, we found similar suppressiveness levels for the BSD and the untreated soil (Blok et al., 2000). A possible explanation for this result is that, unlike broad spectrum soil fumigants, BSD does not create a temporary biological vacuum but a gradual shift from an aerobes-dominated microbial community to an anaerobes-dominated microbial community and vice versa upon return to aerobic conditions. A possible positive effect of the changes in microbial composition brought about by BSD is that this disturbance could offer a possibility for introduced biocontrol organisms to establish in the soil, something very hard to achieve in an undisturbed soil.

We conclude that the data presented clearly show the potential of BSD to strongly decrease soil infestation levels of *R. solanacearum* and to become an important element in a sustainable, practical and effective management strategy for potato brown rot.

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Chapter 6

General Discussion

Chapter 6. General Discussion

Potato brown rot, caused by *Ralstonia solanacearum* race 3 biovar 2 (Phylovar II, sequevar 1), is an important and widespread disease in the Nile Delta of Egypt, affecting yields and income of poor farmers (Janse, 1996; Wenneker et al., 1999). Egyptian potatoes are not only consumed locally but are also exported to Europe in winter and early spring, when the demand and prices for fresh consumption potatoes are high. *R. solanacearum* did not occur in Europe until the 1980's, when it appeared occasionally in different EU countries. This led to the establishment of eradication measures in the European Union (European Communities, 1998), and *R. solanacearum* is still considered a quarantine pathogen. Therefore, the EU has set strict importation requirements for Egypt (European Communities, 2005), including the exclusion of the Nile Delta area for exports, production of potatoes destined for the EU 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 not only a local problem in the Nile Delta, but also an economic problem for Egypt as a whole.

Effect of different soil characteristics on survival and disease suppression of potato brown rot

Potatoes are basically grown on two soil types in Egypt: clay soil in the Nile Delta, and sandy soil in the desert. Although the desert soils are still generally free from *R. solanacearum*, PFA's became occasionally contaminated and it is possible that the pathogen gets introduced there too. It was therefore relevant to study pathogen survival, disease development and disease control for both types of Egyptian soils. As the Netherlands is one of the countries where *R. solanacearum* was found in the past, and where it is still a quarantine organism, it was worthwhile to compare pathogen survival, disease development and disease control also for Dutch clay and sandy soils.

Pathogen survival was poorest in the Egyptian sandy soils, followed by the Egyptian clay soils, the Dutch sandy soils, and it was the best in the Dutch clay soils (Chapter 2). Thus, in both countries, the decline in *R. solanacearum* density was faster in sandy soils than in clay soils. It is well known that bacterial cells can be protected in heavy textured soils, for example from predation by nematodes and protozoa (England et al., 1993; Hassink et al., 1993; Heijnen et al., 1991; Rutherford and Juma, 1992). Disease incidence and severity were highest in Dutch sandy soils and was similar in all other soils, (Chapter 3). An influence of soil type on survival of *R. solanacearum* and bacterial wilt severity has been repeatedly established (Moffett et al., 1983; van Elsas et al., 2000), but the actual effects are controversial and depend on geographical location, strains (race, biovar) of the pathogen, and crop. In some cases, the decline rate of the pathogen was higher in clay loam than in sandy loam (Moffett et al., 1983), in other cases it was the reverse (Kelman, 1953). Similarly, in some areas bacterial wilt was most severe on well-drained sandy loams (French, 1994; Graham and Lloyd, 1979; Hayward, 1991; Ho et al., 1988; Kelman, 1953; Lopez and Biosca, 2004; Nesmith and Jenkins, 1985), and in other cases, the disease was most severe on heavy

clay-loam soil (Kelman, 1953). Thus, conduciveness of soil towards bacterial wilt may be related to other factors than soil type *per se*, such as pH, organic matter content and microbial communities (van Elsas et al., 2005).

Two main factors were found to affect the survival of the pathogen in the various soil types, namely the total organic carbon (DOC) and organic matter (OM) contents. The DOC content was the highest in the Dutch sandy soils and the lowest in Egyptian sandy soils while it was similar in clayey soils from both countries, indicating that higher nutrient availability resulted in better survival chances for *R. solanacearum* (although DOC also contains humic acids, which are not easily available to microorganisms). The percentage of OM was also higher in the Egyptian soils, particularly for the clayey soils from Egypt compared to those from the Netherlands and was negatively correlated with the survival of the pathogen, when the DOC content was similar. This may partially explain the longer survival of the pathogen in Dutch clayey than in Egyptian clayey soils. The relation between survival of *R. solanacearum* and high OM content was addressed by Balabel et al. (2005) and van Elsas et al. (2000), and can work in two directions: enhanced survival due to the higher substrate availability (Maloney et al., 1997) or reduced survival due to greater competition reducing nutrient availability to the pathogen (Hoitink and Boehm, 1999).

The soil pH was negatively correlated with both survival and disease suppression as the disease was less severe in Egyptian soils with higher pH compared to Dutch soils. The pH affects the availability of soil ions: a higher pH restricts the availability of many soil nutrients and minerals, for example Fe and Zn, while Ca, Mg and P can be restricted at low pH (Sangha et al., 2005). In our study, potassium and calcium contents of soils were positively correlated with disease suppression in the Egyptian soils with a high pH. Both potassium and calcium are known for enhancing plant defences (Flego et al., 1997; Romeis, 2001; Abbasi et al., 2002). Fertilization with potassium phosphate and potassium sulphate reduced the severity of bacterial leaf spot diseases on tomato (Abbasi et al., 2002) and the incidence of brown rot on potato (Fahmy and Mohamed, 1990). On the other hand, a positive correlation was found between Na availability and disease incidence, which may explain the absence of disease suppression in the organic clay soil from Egypt even when both calcium and potassium concentrations were relatively high. It is well known that root exudation is increased in sodic soils, enhancing the attraction for and susceptibility to various root diseases (Snapp et al., 1991).

In conclusion, *R. solanacearum* survives better and induces more severe disease in clay soils, especially when they are high in available carbon. However, when the available carbon is high in sandy soil (as found for the Dutch sandy soil), brown rot can be very severe, while survival of and infection by the pathogen is strongly restricted in sandy soil low in available carbon sources (Egyptian sandy soil). This implies that growing potatoes in the Egyptian desert is an excellent alternative for production in the Nile Delta, as the pathogen would decline rapidly even if it were introduced accidentally. Disease development seemed to be suppressed in soils

with high Ca and K contents, so that amendment of soils with Ca or K containing fertilizers may be beneficial, while using amendments which increase soil acidity and salinity should be avoided.

Management

Organic management of Egyptian sandy soil decreased the survival of the pathogen with a tendency to suppress disease development under greenhouse conditions. The Egyptian desert soils contained limited nutrients and substrate for microorganisms and supported only a low biodiversity. Organic management increased the biodiversity and hence probably the competition for the limited nutrients and substrate. Microbial activity was not studied, but it is generally higher in organic than in conventional soils (Mäder et al., 2002; van Diepeningen et al., 2006). Availability of nutrients in potato root exudates may have overcome this competition, so that the difference between organically and conventionally managed soils was reduced in the rhizosphere. On the other hand organic management of Egyptian clay soil under greenhouse conditions was effective in decreasing the survival of the pathogen but was not suppressive. In contrast, the pathogen survived longer in Dutch organic sandy soil where the DOC was higher compared to the conventional sandy soil even when the biodiversity was higher in the organic soil versus its conventional counterpart. This indicates that nutrient availability may be more important than evasion from predators (Hoitink and Boehm, 1999). The effect of management was not clear for Dutch clay soils even when the biodiversity was also higher in the organic than in the conventional soil.

In conclusion, organic management is recommended particularly for the Egyptian soils. For these soils the management effect was clear in decreasing the survival of the pathogen in the soil even if the effect was not significant for the disease suppression.

Amendments

Inorganic and organic soil amendments are primarily used to fertilize a crop but they could also be used concomitantly to influence plant disease development (Marschner, 1995). Altered plant disease development can be achieved through changes in the resistance or tolerance of the host plant, in the pathogen or in the microbial community influencing the pathogen. Notably the form of nitrogen fertilization can have substantial effects on disease incidence (Velazquez and Formento, 2000). This can be related to preferences of the plant for one form of nitrogen but it can also deal with toxic effects on the pathogens by formation of ammonia from ammonium or by formation of nitrite from urea.

Amendment of Egyptian conventional soils with NPK and organic soils with manure reduced disease development remarkably. The suppressive effect of NPK in conventional soils appeared to exceed the suppressive effect of manure amendment of the organic soils. Amendment of Dutch conventional soils with NPK reduced the survival of *R. solanacearum*

in both soil types, but appeared to enhance the conduciveness of the clayey soil for disease development under greenhouse conditions. Amendment of organically managed soils with manure was effective at decreasing survival of the pathogen in case of the clay soil, and suppressed the disease in organic sandy soil only slightly (not significantly) in the organic clay soil. In a previous study, the effect of various soil amendments on survival of R. solanacearum was also dependent on soil type (Michel and Mew, 1998).

The mechanism underlying the suppressive effect of NPK fertilization against potato brown was not studied, but may be due to a gradual assimilation of nitrate and conversion to nitrite (Michel and Mew, 1998) and ammonium by various microbes, including the *R. solanacearum* strain we used. The decline was likely also partly due to a toxic effect of ammonia after conversion of ammonium into ammonia. The ratio of ammonia/ammonium depends on the pH of the soil: from 1% conversion at pH 7.3 to 10% at pH 8.3 (Kissel et al., 1985). Although the *R. solanacearum* strains used in this research were all able to utilize nitrate, they may still have been sensitive to nitrite and certainly to ammonia. 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, such as *Verticillium dahliae* (Conn et al., 2005). The clearest negative effect of NPK amendment on the pathogen decline rate and disease development was found for the Egyptian conventional sandy soil (pH = 7.9) followed by the Egyptian conventional clay soil (pH = 7.6), where only disease suppression was found. NPK fertilization also increased decline rate of the pathogen for Dutch sandy and clayey conventional soils, but there was either no effect on disease development or even increased disease severity. The N content in these soils was similar to that of the Egyptian clayey soil but the pH was somewhat lower (pH = 7.4) for the Dutch soils. The amount of ammonia released probably was not sufficient to suppress the pathogen effectively while the excessive nitrogen may have resulted in a higher level of disease (van Bruggen, 1995). It can be concluded that exaggeration in application of fertilizers especially when the soil nitrogen content is already high may result in reverse effect.

Amendment of the organic soils with cow manure accelerated the decline of *R. solanacearum* in Egyptian sandy soil and Dutch clay soil. It also resulted in significant disease suppression in the sandy soil from the Netherlands and a significant reduction in density of *R. solanacearum* in the organic sandy soil from Egypt. No significant effects were found for the clay soils. Satoh and Toyota (2004) also found that the effect of repeated manure amendments on bacterial wilt development (on tomato) varied from soil to soil. Amendment with cow manure generally resulted in a clear shift in microbial community which may have had an effect on the disease suppression. This suppressive effect of manure may have been partly due to the immediate release of ammonia as a result of microbial decomposition and partly to a shift in microbial community (Gorissen et al., 2004; Lazarovits et al., 2001).

Amendment of organic soil with wood chip compost was ineffective in controlling potato brown rot and even seemed to be conducive, as for the survival in Egyptian clay soil.

Similarly, Islam and Toyota (2004) found that compost from tree bark or coffee residues did not suppress or even enhanced bacterial wilt of tomato, while compost from farmyard manure plus rice straw and of poultry manure suppressed *R. solanacearum* in soil and reduced disease development. This effect of manure in compost was attributed to high substrate availability with high N content (high DOC content, high available N content, and a low C/N ratio), combined with high microbial activity (Islam and Toyota, 2004). In our experiment a relatively small amount of compost was added, equivalent to the maximum amount allowed per ha in the EU. However, higher doses could be applied by concentrating the maximum amount in the planting holes. Thus, it would be worthwhile to investigate the effects of higher doses of compost on survival of and infection by *R. solanacearum*.

In conclusion, organic amendment with manure (fresh or composted) or a limited amount of NPK is recommended to maximize disease suppression. Amendment with manure may have additional benefits, as various organic amendments are known to improve soil health, soil structure, water penetration and drainage and increase the complexity of microbial community (Bulluck et al., 2002; van Bruggen, 1995).

Biological control

Various bacterial species have been tested for biological control of *R. solanacearum* (Trigalet et al., 1994). Many of the strains were only effective *in vitro* or in potting media *in vivo*, but not or only moderately in field soil. In this study, 4 strains of the common soil saprophyte *Stenotrophomonas maltophilia* were isolated from the rhizosphere of eggplants growing in the Nile Delta. One strain tested for biological control was quite effective at suppressing pathogen populations and disease in Egyptian soil, but not in the Dutch soils.

The activity of biocontrol agents is determined by one or more of four mechanisms: competition for nutrients (mostly C and/or Fe); antibiosis; hyperparasitism; or induced protection (Hornby, 1990). In our studies the activity of the biocontrol agent used was not dependent on competition for Fe, as the availability of ferric ions *in vitro* did not affect biocontrol activity of the strains tested. Also, the DOC content in soil had no correlation with the survival of *S. maltophilia* in different soils. Nevertheless, the activity of the biocontrol agent was positively correlated with the organic matter content, similar to the finding of Hoitink and Boehm (1999), which was higher in the Egyptian soils than in the Dutch soils used in this study. The survival of *S. maltophilia* was better in Egyptian organic soil than its conventional counterpart, possibly as a result of higher OM content. In contrast it survived less in Dutch organic compared to conventional soil, perhaps as a result of higher biodiversity and possibly activity in the organic soil. Similarly, the biocontrol agent *P. fluorescens* declined faster and was less effective at controlling take-all disease, when added to the same Dutch organically managed soil with a diverse microbial community than in the same biologically impoverished conventionally managed soil as used in this study (Hiddink et al., 2005). The efficacy of the biocontrol agent selected here may be enhanced by adding a food

base before application to soil. In this study, the wood chip compost used was not effective by itself, but amendment of compost with a biocontrol agent can increase its suppressions effect (Litterick et al., 2004). Enrichment of the compost with *S. maltophilia* might have increased the suppressive capability of the compost (Termorshuizen et al., 2004).

Biological soil disinfestation (BSD)

BSD was developed by Blok et al. (2000). It is a nonchemical control method aimed at imposing a general soil anaerobiosis by incorporating green plant material into moist soil and covering the soil with thick ensilage plastic for at least 6 weeks. This resulted in a reduction in inoculum density by at least 95% of a number of soilborne pathogens including *Fusarium oxysporum* f.sp. *asparagi*, *Verticillium dahliae*, *Pratylenchus penetrans*, *Meloidogyne chitwoodi* and *Globodera pallida*. This study demonstrated for the first time that BSD resulted in a strong decline of *R. solanacearum* in infested soil. Weeds were also practically eliminated. This is an additional benefit, because *R. solanacearum* could be eradicated from its "hiding" reservoirs such as volunteer infected potato tubers (Graham and Lloyd, 1979) and weed hosts, such as *Portulaca oleracea*, *Rumex dentatus* and *Solanum nigrum* (Elphinstone et al., 1998; Farag et al., 2004).

The mechanism of inactivation of *R. solanacearum* was not studied in detail in our study. Incorporation of easily decomposable organic matter, grass or potato shoots, alone or covering the soil with airtight plastic without incorporation of organic amendment did not result in reduction of *R. solanacearum* soil populations. Most probably, toxic compounds produced during fermentation of the fresh organic matter under the anaerobic soil conditions have played a major role in the decline of *R. solanacearum* populations (Blok et al., 2000). A vast array of potentially toxic compounds, including alcohols, aldehydes and organic acids, is produced when organic matter is decomposed under anaerobic soil conditions (Ponnamperuma, 1972; Strandberg, 1987). Also biocontrol by facultative anaerobic and anaerobic bacteria (e.g. *Bacillus* and *Clostridium* spp., respectively) may have contributed to pathogen inactivation (Cook and Baker, 1983). If solar radiation levels are higher than tested in our study, such as often in Mediterranean and tropical countries, the soil temperatures can reach sublethal temperatures that will make soilborne pathogens more vulnerable to all kinds of damaging effects including toxic fermentation products.

There was a clear shift in microbial composition after the BSD treatment. A change in soil microbial composition may affect natural suppressiveness of the soil against potato brown rot. On the one hand, surviving microorganisms may enhance natural suppressiveness. On the other hand, the removal of a large part of the microbial community may enhance re-establishment of plant pathogens. Such effects have been reported for soil flooding and for soil fumigation (Kreutzer, 1965; Stover, 1979), where an initial strong control effect was followed by disease that was more severe than that occurring in the control. However, such a risk seems limited for BSD (Blok et al., 2000; Goud et al., 2004) although more research with

respect to suppression of brown rot after BSD treatment is needed. Introduction and establishment of a biocontrol agent after BSD may also limit the risk of re-establishment of a pathogen (Blok et al., 2000). In our study *S. maltophilia* was found to be more effective in sterilized soil. Application of *S. maltophilia* directly after BSD may help for long term control of the pathogen, as the biocontrol agent will be able to get established much easier than if it were applied directly to a balanced ecosystem.

Oxygen leakage likely occurred in our microplots as well as in the field, where the plastic had been damaged by picking birds (possibly because of the green color of the plastic chosen). Still, pathogen populations were reduced significantly by more than 95%. If bird damage can be prevented, and farmers have various effective methods available for this, anaerobic conditions can be maintained for a longer period resulting in an even stronger decline of *R. solanacearum* populations. Especially important was the observation that potato tubers were completely decomposed after the BSD treatment, because potato tubers are a major reservoir of *R. solanacearum* where they normally can survive better than in soil (Graham and Lloyd, 1979). When these reservoirs are destroyed during BSD this will greatly contribute to a decrease of soil infestation levels.

BSD is currently applicable only for high-value crops because the plastic is expensive and the land needs to stay fallow for some time in late summer. Although consumption and starch potatoes are not considered high-value crop, seed potatoes certainly are. However, for all types of potato incidence of brown rot brings about great damage, as potatoes are not allowed to be cultivated for multiple years. As BSD can result in a fast and (almost) complete decline of *R. solanacearum*, the prohibition of growing potatoes may be lifted sooner if BSD has been performed.

In conclusion, this study clearly shows the potential of BSD to strongly decrease soil infestation levels of *R. solanacearum* and to become an important element in a sustainable and effective management strategy for potato brown rot.

Conclusions and prospective

The results obtained in the studies described in this thesis have different implications and prospective for the control of potato brown rot depending on disease incidence and its statutory status in a particular area. Therefore a distinction will be made between (1) Europe in general and in Pest Free Areas (PFA's) in Egypt, where *R. solanacearum* is a quarantine pathogen with zero tolerance and eradication is the aim according to EU regulations (European Communities, 1998; European Communities, 2005) and (2) areas such as the Nile Delta in Egypt, where the disease and pathogen are endemic and where disease suppression is the aim.

Areas with a quarantine status for *R. solanacearum*:

- Survival in European sandy soils is longer than in clay soils, and they pose therefore a potentially higher risk in rotation schemes, where infested fields are taken out of potato production for a number of years. There is little disease suppression in these soils, so when present it will show up easily.
- Survival in all European soils is longer than in Egyptian soils, which have a higher pH. Survival, however, is no longer than c. 160-200 days, confirming earlier observations (Janse et al., 1998, J. van Vaerenbergh, Institute for Research in Agriculture and Fisheries, Merelbeke, pers. comm.) with more scientific data. This would mean that the obligatory measure under the EU Control Directive 98/57/EC of taking infested fields for 4 or 5 years (depending on whether consumption or seed potatoes will be planted) out of potato production could be reduced to 3-4 years. When growers have the normal 3-year rotation, control volunteers and weeds and do not irrigate with surface water, the organism has little survival chances in potato fields depending on weather conditions.
- The NW-European climate with relatively low temperatures in the growing season has a negative effect on population decline. This temperature effect was also established in other research (Tomlinson et al., 2005).
- Since there is no clear difference between conventional and organic European soils in these studies no preference on this basis can be given. Adequate supply with manure in the case of organic soils and of NPK on conventional soils will decrease the survival chances of the bacterium. Compost addition to organic soils did not result in an enhanced decline of the pathogen, at least when the maximum quantity allowed under European legislation would be evenly spread out over the field. Higher concentrations could be tested to simulate concentrating the same amount of compost in the planting holes.
- For Egypt (or probably generally for reclaimed desert sandy soil) the situation is quite different from that in Europe: survival is relatively short in desert sandy soils (25-60 days) and it is advisable to plant potatoes in these soils when zero tolerance is the aim. Thus, PFA's have been created in the past in the right place. Possibly rotation schemes in sandy Egyptian PFA's can be much shorter (1 full season) than in Europe.
- Survival in Egyptian sand and soil under natural conditions was found to be close to that estimated in this study (generally 60-90 days, but could be as short as 15 days under high temperature conditions In greenhouse experiments in Egypt, more or less similar survival times as in this study were found, namely 120-180 days both for clay and sand soils of the Nile Delta area (Tomlinson et al., 2005).
- Organic management was shown to be beneficial in sandy desert soils in Egypt, meaning that soil of organic farms in PFA's if they were to become infested, would have less problems with surviving bacteria than infested soil of conventional farms.
- A strain of the common soil saprophyte *Stenotrophomonas maltophilia* was isolated that appeared to be quite effective in suppressing pathogen populations in Egyptian soil, but not in the Dutch soils. Anyhow, under zero tolerance conditions in Europe and in Egyptian PFA's its usefulness in control is limited.
- Biological soil disinfestation (BSD) has the potential to strongly decrease soil infestation

levels of *R. solanacearum*. This method could possibly help in reducing time necessary for statutory measures under the Brown Rot Control Directive 98/57/EC (leaving the field out of potato for 4-5 years) where eradication is the aim. It could also be helpful in desert areas, if *R. solanacearum* were to be introduced accidentally.

Areas where *R. solanacearum* is endemic:

- In clay soils from the Egyptian Nile Delta, decline of *R. solanacearum* was much slower than in the sandy soils from the desert. In other studies it was shown that decline of *R. solanacearum* in Nile Delta sandy soils is not as rapid as that in the desert sands, probably because the former have higher organic matter content, similar to European sandy soils.
- Organic management did not affect pathogen survival or disease development in the clay soils from the Nile Delta, but the supply of adequate nutrients with manure in the case of organic soils and NPK in the case of conventional soils enhanced extinction of the pathogen.
- Selected *S. maltophilia* strains were quite effective in suppressing pathogen populations and disease in Egyptian soils. This biocontrol agent could therefore possibly be used to control potato brown rot and prevent further spread of the pathogen in heavily infected areas, such as the clay soils in the Egyptian Delta. Effective use of this biocontrol agent could be made by stimulating naturally occurring populations, for example by crop rotation or intercropping with crops that have high concentrations of sulphur-containing amino acids in their root exudates, or by application of selected antagonistic *S. maltophilia* strains, for example by bacterization of potato tubers. Production of *S. maltophilia* for biological control would need to be approved by the appropriate regulatory agencies, as certain strains of *S. maltophilia* have been associated with various illnesses in immuno-depressed human patients (Berg et al., 2005).
- BSD has the potential to become an important element in a sustainable and effective management strategy for potato brown rot in areas where the disease is endemic and cost of plastic are not a limiting factor. It could be a good control method for larger farmers in the Nile Delta that really suffer losses – these farmers can afford to buy the plastic and usually have or more easily obtain know-how to properly apply the method. Birds in the Delta could be problem, since pigeons are very commonly kept in the countryside and they may damage the plastic.

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Summary

Potato brown rot, caused by the bacterium *Ralstonia solanacearum* race 3 biovar 2, is an economic quarantine disease with zero tolerance according to EU regulations. In the EU, there is an increasing demand for early Egyptian potatoes, which can only be grown in what is called Pest Free Areas (PFAs), primarily in virgin desert soils. If fields in the EU become infested, they should be under quarantine measurements for at least 4 or 5 years. The pathogen can infect potato and some solanaceous and non-solanaceous weeds and can survive in surface water. The pathogen can survive for a long period in soil and can be sheltered in plant debris and volunteer potato tubers (Chapter 1). To develop more precise recommendations for the time period needed for infested fields to be free from the pathogen, survival of *R. solanacearum* was studied in various soils from Egypt and the Netherlands. In an attempt to develop control methods for areas in Egypt where the disease is endemic or recently introduced, different control strategies were investigated, namely organic management, conventional and organic amendments, application of a biocontrol agent and biological soil disinfestation. Soil-borne plant pathogens are frequently suppressed in organic versus conventional soil, but this phenomenon was not investigated for *R. solanacearum* while many organic amendments were found to suppress the disease when used separately or in combination. Many biocontrol agents were tested for controlling potato brown rot without much success in most of the cases. Finally, biological soil disinfestation (BSD) is a new effective new tool for controlling fungal and nematode diseases but this method had not been investigated for bacterial diseases.

Survival of the pathogen was studied in organic versus conventional soils with different soil types, sandy and clayey, and from different countries, Egypt and the Netherlands, under two temperature regimes, moderate (15ºC) and warm temperatures (28ºC) (Chapter 2). For some soils organic management resulted in shorter 50%-reduction-time and/or a greater decline rate of the fitted curve for pathogen populations over time, compared to similar soils with conventional management, while for other soil pairs there was no difference. The bacterial diversity, as estimated by denaturing gradient gel electrophoresis (DGGE) of eubacterial 16S rDNA directly extracted from soil, was higher in organic versus conventional soil for most of the soil types. In general there was a clear distinction between bacterial communities in organic and conventional soils. In Dutch soils the pathogen survived better at 15°C while in Egyptian soils the pathogen survived slightly better at 28°C. In general the pathogen survived longer in Dutch than in Egyptian soils, and in clay than in sandy soils. Survival was positively correlated with dissolved organic carbon and negatively correlated with total organic matter content. Effect of NPK fertilization for conventional soils and compost or fresh cow manure amendment for organic soils on the survival of *R. solanacearum* were tested at 15°C. Both NPK fertilization and cow manure amendment enhanced the decline rate of *R. solanacearum* in many but not in all soils, while wood chip compost was ineffective at reducing survival of the pathogen. In general, NPK amendment did not cause a shift in bacterial community except

in Dutch clay soil, while both the compost and cow manure caused a clear shift in bacterial diversity with a stronger shift in case of cow manure amendment. In general the survival of the pathogen was affected by soil type and origin more than by managements and amendments.

Disease incidence under different management and amendment regimes was also investigated using the same soils used for the survival experiment (Chapter 3). The disease was slightly suppressed in Egyptian sandy organic soil compared to the conventional counterpart. On the other hand, the disease was more severe in organic Dutch sandy and clayey soils than in their conventional counterparts. This was correlated with a higher total soluble organic carbon (DOC) content in the organic soils. The disease incidence was the highest in the Dutch sandy soils, which was again correlated with the high DOC content in those soils indicating that the disease severity is positively correlated with substrate availability. On the other hand, the disease was suppressed in the Dutch clay soils and that was related to high calcium and potassium contents in those soils, possibly due to enhanced resistance in the plants. There was no correlation between rhizosphere biodiversity and disease incidence or severity. Amino acid and ammonium content in xylem and apoplastic sap extracted from plants grown in differently managed soils were also estimated. In general both amino acid content and ammonium content were higher in organic than in conventional soils, but not significantly, while the growth rate of *R. solanacearum* was significantly higher in xylem and apoplastic sap from plants grown in organic than in that from plants grown in conventional soils. NPK fertilization suppressed the disease in Egyptian soils but did not affect the disease in Dutch clayey soil. Cow manure was suppressive towards potato brown rot in all soils except for the Dutch clay soil. A clear shift in bacterial diversity in response to manure amendment was shown, especially for sandy soils.

A new biocontrol agent against *R. solanacearum* was isolated intensively from the rhizosphere of eggplant grown in the Egyptian Nile Delta and identified as *Stenotrophomonas maltophilia* (Chapter 4). The antagonistic properties of the antagonist were not dependent on siderophore production. Under greenhouse conditions, the antagonist was applied either by soil inoculation or by potato eyepiece bacterization. The antagonist was effective in controlling the disease in Egyptian clay soils but not in Dutch clay soils. The survival of a chloramphenicol and rifampicin resistant mutant strain of *S. maltophilia* was studied in organic versus conventional clayey soils of Egyptian and Dutch origins. Its effect on the survival of *R. solanacearum* was also studied. The antagonist survived significantly shorter in Dutch than in Egyptian soils. This was the reverse for survival of the pathogen, which was in agreement with the greenhouse results. The survival of the antagonist was positively correlated with the OM content. *S. maltophilia* survived longer and was more efficient in reducing the survival of *R. solanacearum* in organic versus conventional Egyptian soil. The OM content was higher in the organic soil where the bacterial diversity was lower compared to the conventional soil. On the other hand, *S. maltohilia* survived shorter in organic than in conventional Dutch soil and even enhanced the survival of *R. solanacearum* in the former.

The bacterial biodiversity was higher in the organic than in the conventional Dutch soil. Thus, studies on survival of *S. maltophilia* and *R. solanacearum* confirmed results obtained from the biocontrol tests in the greenhouse.

Survival of *R. solanacearum* under anaerobic conditions using biological soil disinfestation (BSD) was investigated (Chapter 5). The anaerobic conditions were stimulated by increasing microbial respiration through incorporation of fresh organic matter (Italian rye grass) in the soil and avoiding resupply of oxygen by covering the soil with airtight plastic. Control treatments consisted of non-amended and non-covered, covered only and grass amended pots or plots. The effect of BSD on survival of *R. solanacearum* was tested at three different levels: in 1-L glass mesocosms under laboratory conditions, in 1.2 m-diameter microplots in an outdoor quarantine field, and in a naturally infested commercial field. BSD was effective in significantly reducing the populations of *R. solanacearum* and even almost eradicating the pathogen in many cases at all three levels of the experiment. BSD did not only highly reduce the CFU of the pathogen in the soil but also in buried infected potato tubers in the field experiment. There was no effect of grass only amendment or covered only treatment on the survival of the pathogen. There was a clear shift in bacterial community after BSD from all other treatments, which did not recover even after three weeks of uncovering the BSD soils.

This research gave insight in the effect of physical, chemical and biological soil characteristics on the survival of the pathogen and disease suppression, which may help in selecting the proper control strategies for different soils. The advantages and disadvantages of many control strategies were investigated. More research is needed to know the dominating microbial groups associated with soil suppressiveness towards the pathogen and to be able to enhance a shift in the microbial community towards suppressiveness in a natural way.

Samenvatting

Aardappelbruinrot wordt veroorzaakt door de bacterie *Ralstonia solanacearum* race 3 biovar 2 en is een quarantaineziekte die, als deze optreedt, grote economische gevolgen heeft. Binnen de EU is er een 'zero tolerance'-beleid ten aanzien van deze ziekte. In de EU is er een groeiende vraag naar vroege Egyptische aardappelen. Deze mogen alleen geteeld worden in ziektevrije gebieden, de zogenaamde 'Pest Free Areas'. Deze ziektevrije gebieden zijn voornamelijk gelegen in recent ontgonnen woestijngebieden. Als velden in de EU besmet raken met *R. solanacearum*, blijven deze gedurende minimaal 4 of 5 jaar onder quarantaine. De bruinrotbacterie infecteert naast aardappel ook verscheidene andere kruiden uit de nachtschade- en andere plantenfamilies en kan ook gedurende enige tijd overleven in oppervlaktewater. Daarnaast kan de bacterie gedurende lange tijd overleven in de bodem, in plantenresten en in wildgroeiende aardappelknollen (Hoofdstuk 1). Om betere aanbevelingen te kunnen doen over de periode die nodig is om geïnfecteerde velden weer helemaal ziektevrij te krijgen is de overleving van *R. solanacearum* onderzocht in verscheidene Egyptische en Nederlandse grondsoorten. In een poging bestrijdingsmaatregelen te ontwikkelen voor die gebieden in Egypte waar de ziekte voorkomt zijn diverse strategieën onderzocht: biologisch beheer, gangbare en biologische bemesting, biologische bestrijding en biologische grondontsmetting. Bodemgebonden plantenpathogenen worden in biologische gronden relatief sterk onderdrukt in vergelijking met gangbare gronden, maar het was nog niet duidelijk of dit fenomeen ook optrad bij *R. solanacearum*. Het is bekend dat diverse typen organische stof, alleen of in combinatie, de ziekte kunnen onderdrukken. Ook is uit de literatuur bekend dat een scala aan potentieel antagonistische bacteriën is onderzocht op hun vermogen aardappelbruinrot te onderdrukken, maar zonder veel succes. Tenslotte was bekend dat Biologische grondontsmetting een nieuw en effectief middel is ter bestrijding van schimmelziektes en nematoden, maar deze werkwijze was nog niet getest op de bruinrotbacterie.

De overleving van *R. solanacearum* is vergeleken in gronden onder biologische en gangbare teelt, in zand- en kleigronden, en in gronden uit verschillende landen (Egypte en Nederland) en bij twee verschillende temperaturen (een gematigde 15ºC en een warme 28ºC) (Hoofdstuk 2). In sommige gronden resulteerde biologisch beheer in een kortere halveringstijd en/of een grotere afnamesnelheid van de bacterie in de tijd, maar voor andere gronden was er geen verschil. De bacteriële diversiteit in de grond geschat op basis van 'denaturerende gradiënt gel electroforese' (DGGE) aan direct uit de grond geïsoleerd eubacterieel 16S rDNA, was hoger in biologische gronden dan in gangbare gronden in de meeste van de onderzochte grondmonsters. Over het algemeen waren er duidelijke verschillen tussen de bacteriële populaties in de biologische en gangbare gronden. In de Nederlandse gronden overleefden de bacteriën beter bij 15ºC, terwijl in de Egyptische gronden *R. solanacearum* beter overleefde bij 28ºC. De bacterie overleefde beter in de Nederlandse gronden dan in de Egyptische, en beter in klei- dan in zandgrond. De overleving was positief gecorreleerd met de concentratie opgeloste organische koolstof ('Dissolved Organic Carbon', DOC) en negatief gecorreleerd

met het gehalte aan organisch materiaal (OM) in de grond. Het effect van bemesting met NPK-kunstmest in gangbare gronden en toevoeging van compost of koemest aan biologische gronden op de overleving van *R. solanacearum* is onderzocht bij 15ºC. Zowel het toevoegen van NPK-kunstmest als dat van koemest versnelde de afname van *R. solanacearum* in veel van de gronden. Gebruik van een groencompost bleek daarentegen niet effectief. In het algemeen leidde NPK-kunstmest niet tot veranderingen in de bacteriële populaties, behalve in de Nederlandse kleigrond. Compost en vooral koemest leidden echter wel tot een duidelijke verschuiving in de bacteriepopulatie. De overleving van *R. solanacearum* werd vooral bepaald door soort en herkomst van de grond en in mindere mate door beheer en organische-stoftoevoegingen.

Het optreden van bruinrot als functie van beheer en verschillende organische-stoftoevoegingen werd onderzocht in dezelfde gronden als die gebruikt waren voor de overlevingsexperimenten (Hoofdstuk 3). De ziekte werd in lichte mate onderdrukt in de Egyptische biologische zandgrond vergeleken met de Egyptische gangbare grond. In de Nederlandse zand- en kleigronden was echter geen verschil tussen de gangbare en de biologische gronden. Dit hing samen met het hogere gehalte aan oplosbaar organisch koolstof (DOC) in deze biologische gronden. De ziekte kwam het meest prominent tot uiting in de Nederlandse zandgronden, wat opnieuw bleek samen te hangen met de hoge DOC-gehaltes in deze gronden. Dit geeft aan dat de mate van ziekteontwikkeling positief gecorreleerd is met de beschikbaarheid van voedingsstoffen. In de Nederlandse kleigrond werd de ziekte juist onderdrukt en dat was gecorreleerd met de hoge gehaltes aan calcium en kalium in deze gronden, die mogelijk werken via een verhoogde weerstand van de plant. Er bleek geen verband tussen de diversiteit van bacteriën in de rhizosfeer en de mate van optreden van de ziekte. De hoeveelheid aminozuren en ammonium in het xyleem en de apoplastische vloeistof was hoger in planten geteeld op biologische dan die op gangbare gronden, maar niet significant, terwijl de groeisnelheid van *R. solanacearum* in het xyleem en de apoplastische vloeistof van planten van biologische gronden wél significant hoger was dan die van gangbare gronden. Bemesting met NPK-kunstmest onderdrukte de ziekte in Egyptische gronden, maar had geen effect op de ziekte in Nederlandse kleigrond. Koemest onderdrukte bruinrot in alle geteste gronden, behalve in de Nederlandse kleigrond. De bacteriële diversiteit liet een duidelijke verschuiving zien als koemest werd toegevoegd, het sterkst in de zandgronden.

Een nieuwe antagonist van *R. solanacearum* werd meermalen geïsoleerd uit de rhizosfeer van aubergine in de Egyptische Nijldelta en werd geïdentificeerd als *Stenotrophomonas maltophilia* (Hoofdstuk 4). De werking van deze antagonist bleek niet afhankelijk te zijn van de productie van sideroforen. In kasexperimenten werd de antagonist toegevoegd aan de bodem of door het inoculeren van pootaardappelstukjes. De antagonist bleek effectief in het bestrijden van de ziekte in Egyptische maar niet in Nederlandse kleigrond. De overleving van een mutante *S. maltophilia* stam die resistent was tegen chloramfenicol en rifampicine is bestudeerd in biologische en gangbare kleigronden uit Egypte en Nederland. Het effect van de antagonist op de overleving van *R. solanacearum* is ook onderzocht. De antagonist overleefde

significant korter in de Nederlandse dan in de Egyptische gronden, en het omgekeerde was het geval voor het pathogeen, hetgeen in overeenstemming was met de resultaten van de kasexperimenten. Overleving van de antagonist bleek positief gecorreleerd met het organisch-stofgehalte. *S. maltophilia* overleefde langer en was effectiever tegen *R. solanacearum* in biologische dan in gangbare Egyptische gronden. Het organisch-stofgehalte was hoger in de biologische gronden waar de bacteriële diversiteit lager was dan in de gangbare gronden. Daarentegen overleefde *S. maltophilia* korter in de Nederlandse biologische gronden en stimuleerde daar zelfs de overleving van *R. solanacearum* in vergelijking met de gangbare gronden. De bacteriële biodiversiteit was hoger in de biologische dan in de gangbare Nederlandse grond. Al met al bevestigden de overlevingsexperimenten van *S. maltophilia* en *R. solanacearum* de resultaten van de kasexperimenten.

De overleving van *R. solanacearum* onder anaërobe omstandigheden werd onderzocht door middel van Biologische bodemontsmetting ('Biological Soil Disinfestation', BSD) (Hoofdstuk 5). De anaërobe omstandigheden werden geïnduceerd door verhoging van de microbiële respiratie na toevoegen van vers organisch materiaal (Italiaans raaigras) aan de grond en het verhinderen van zuurstoftoevoer aan de bodem door afdekken ervan met luchtdicht plastic. De controlebehandelingen bestonden uit potten of veldjes met onbedekte grond zonder toevoeging van Italiaans raaigras, bedekte grond zonder toevoeging van Italiaans raaigras en onbedekte grond met toevoeging van Italiaans raaigras. Het effect van BSD op de overleving van *R. solanacearum* is bepaald op drie verschillende niveaus: in glazen potten van 1 liter in het laboratorium, in kleine veldjes (diameter 1,2 m) en in de vollegrond in een van nature besmet commercieel perceel. BSD bleek populaties van *R. solanacearum* significant te reduceren en zelfs bijna uit te roeien. BSD verminderde niet alleen in hoge mate het aantal kolonievormende eenheden in de bodem maar ook in ondergronds geïnfecteerde aardappelknollen in het veldexperiment. Er was geen effect op de overleving van het pathogeen als alleen gras werd toegevoegd aan de bodem of als de bodem alleen werd afgedekt met plastic. BSD had een duidelijke verschuiving in de bacteriële bodemgemeenschap tot gevolg, die zelfs na drie weken zonder afdekken van de bodem met plastic niet hersteld was.

De resultaten van dit onderzoek hebben tot meer inzicht geleid in de effecten van verscheidene fysische, chemische en biologische bodemeigenschappen op de overleving en ziekteonderdrukking van *R. solanacearum.* De voor- en nadelen van een scala aan beheersingsmaatregelen zijn onderzocht. Meer onderzoek is nodig om vast te stellen welke groepen micro-organismen bepalend zijn om een grond ziektewerend te maken ten aanzien van bruinrot. De resultaten dragen bij tot een selectie van methoden ter beheersing van bruinrot.

الملخص العربى

يعتبر مرض العفن البنى للبطاطس, والناتج عن الإصابة ببكتريا الرالستونيا سولاناسيرم من السلالة الثالثة ذات الطراز الحيوى الثانى, من الأمراض الخاضعة لقوانين الحجر الاقتصادى بدون أى نسبة سماح مرورى, وذلك بمقتضى قوانين الحجر الخاصة بالاتحاد الأوربى. وفى القارة الأوربية تتزايد الحاجة إلى البطاطس المصرية المزروعة فى وقت مبكر من السنة فى المناطق المعروفة بأنها مناطق خالية من المرض, وخاصة المناطق ذات التربة الصحراوية والتى لم يسبق زراعتها. هذا مع الأخذ فى الاعتبار أنه إذا تعرضت أى منطقة زراعية أوربية للإصابة بالمرض فإنها تقع تحت الحجر لمدة نحو أربع أو خمس سنوات على الأقل. يمكن لبكتريا الرالستونيا أن تصيب البطاطس بالإضافة إلى بعض نباتات أخرى من الفصيلة الباذنجانية أو حتى من فصائل أخرى غير الباذنجانية, كما يمكن أن تعيش فى طبقات المياه السطحية. كما يمكن للميكروب أن يعيش لفترات طويلة فى التربة أو يستتر فى بقايا النباتات ودرنات البطاطس (الفصل الأول). ولتقدير أكثر دقة للفترة الزمنية اللازمة للتربة المصابة حتى تصبح خالية من المرض, تمت دراسة معدلات بقاء بكتريا الرالستونيا سولاناسيرم فى عينات مختلفة من التربة, بعضها من مصر وبعضها من هولندا. كما تمت دراسة آليات تحكم مختلفة على المرض فى محاولة لقمع الإصابات الحديثة فى بعض المناطق بمصر. ومن أمثلة هذه الآليات التحكم فى التركيب العضوى للتربة, وتنقيح التربة العادية أو العضوية, والمقاومة البيولوجية, واستخدام مطهرات بيولوجية للتربة. أحياناً يتم تثبيط نمو الميكروبات المُمْرِضة للنبات, والمنقولة خلال التربة, وذلك بواسطة استخدام التربة العضوية, ولكن تلك الظاهرة لم تُلاحَظ فى ميكروب الرالستونيا, بينما استطاع التنقيح العضوى فى أحيان كثيرة أن يثبط المرض, وذلك عندما طُبِّقت هذه الآلية وحدها أو مع السابقة. كما تم اختبار العديد من المقاومات البيولوجية للتحكم فى مرض العفن البنى, ولكن لم تُسفِر المحاولات عن مقاومة ناجحة فى معظم الأحيان. وأخيراً فإن استخدام المطهرات البيولوجية للتربة يُعد سلاحاً جديداً وفعالاً للتحكم فى نمو الفطريات والديدان الخيطية, ولكن لم تتم دراسة هذه الطريقة من قبل لمقاومة الأمراض البكتيرية.

تمت دراسة فترة بقاء الميكروب حياً فى التربة العضوية مقارنةً بالتربة التقليدية وذلك باستخدام أنواع مختلفة من التربة, رملية وطينية, وذلك من مواطن مختلفة, تحديداً من مصر وهولندا, تحت درجتى حرارة مختلفتين هما المتوسطة (15°م) والدافئة (28°م) (الفصل الثانى). نتج عن المعالجة العضوية لبعض أنواع التربة نقص فى فترة عمر النصف أو زيادة فى معدل هبوط منحنى التعداد الزمنى للميكروب, وذلك مقارنةً بنظائرها من عينات التربة المعاملة بالطريقة التقليدية. على الجانب الآخر, لم تؤدِّ المعالجة العضوية إلى حدوث تغير فى المعايير السابقة عندما تم استخدام أنواع أخرى من التربة. أما عن درجة التنوع البكتيرى, والتى تمت دراستها بواسطة الهجرة الكهربائية المنحدرة داخل الجيل للمُنتَج البكتيرى 16-إس للحامض النووى الريبوسومى والمعزول من التربة مباشرةً, فقد كانت أعلى فى التربة العضوية مقارنَةً بالتربة التقليدية وذلك فى معظم أنواع التربة. وبشكل عام, كان هناك تباين واضح بين أنواع البكتريا المتعايشة فى التربة العضوية مقارنةً بالتربة التقليدية. فى التربة الهولندية, استطاع الميكروب البقاء حياً بشكل أفضل فى درجة حرارة 15°م, بينما فى التربة المصرية استطاع الميكروب البقاء حياً عند درجة 28°م بشكل أفضل قليلاً, وبشكل عام فإن بقاء الميكروب حياً كان لفترات أطول فى عينات التربة الهولندية مقارنةً بالتربة المصرية, وفى عينات التربة الطينية مقارنةً بالتربة الرملية. تناسب بقاء الميكروب حياً بشكل طردى مع نسبة الكربون العضوى المذاب, وبشكل عكسى مع المحتوى العضوى الكلى فى التربة. تمت دراسة تأثير التخصيب بمادة إن.بى.كيه للتربة التقليدية, أو المعالجة بالسماد العضوى الصناعى أو بالروث البقرى الطرى للتربة العضوية, على فترة بقاء ميكروب الرالستونيا سولاناسيرم حياً عند درجة 15°م. نتج عن استخدام مادة إن.بى.كيه أو الروث البقرى الطرى تسارع فى معدل تناقص تعداد الميكروب فى العديد ولكن ليس فى كل أنواع التربة, بينما لم ينتج عن استخدام السماد الغضوى الصناعى تغير فى فترة بقاء الميكروب حياً. بشكل عام, فإن المعالجة بمادة إن.بى.كيه لم تغير من نوعية التباين البكتيرى ما عدا فى التربة الهولندية, بينما المعالجة بالسماد العضوى الصناعى أو بالروث البقرى الطرى نتج عنها تغير واضح فى التنوع البكتيرى, وكان ذلك بشكل أقوى عند استخدام الروث البقرى الطرى. بشكل عام, فإن بقاء الميكروب حياً كان متأثراً بنوع التربة ومصدرها أكثر من معالجتها بالمواد السابق ذكرها.

تمت أيضاً دراسة إمكانية حدوث المرض تحت المعاملات وأنظمة التنقيح المختلفة باستعمال نفس عينات التربة المستخدمة فى تجربة دراسة فترة بقاء الميكروب حياً (الفصل الثالث). تم تثبيط المرض بشكل طفيف فى التربة العضوية الرملية المصرية بالمقارنة بنظيرتها التقليدية. على الجانب الآخر, كانت شدة المرض أكبر فى التربة العضوية الهولندية, الرملية والطينية, مقارنةً بنظائرها التقليدية. كان ذلك متوافقاً مع المعدلات العالية من المحتوى الكلى من الكربون العضوى الذائب فى عينات التربة العضوية. بلغ حدوث المرض أعلى معدلاته فى التربة الهولندية الرملية, والذى كان أيضاً متناسباً مع المحتوى الكلى المرتفع من الكربون العضوى المذاب, مما يشير إلى أن شدة المرض تتناسب طردياً مع إتاحة المواد التى يحتاجها الميكروب. على الجانب الآخر تم تثبيط المرض فى التربة الهولندية الطينية بشكل متناسب مع ارتفاع محتوى الكالسيوم والبوتاسيوم فى مثل تلك النوعية من التربة, وربما يُعزَى ذلك إلى تزايد قدرة النباتات على مقاومة الميكروب. لم يكن هناك تناسب بين التنوع الحيوى فى العقد الجذرية وبين حدوث المرض أو شدته. أيضاً تم قياس محتوى الأحماض الأمينية والأمونيوم فى الجزء الخشبى من النبات وأيضاً فى النُّسغ المستخلص من النبات, وذلك فى النباتات المزروعة فى عينات التربة المعالجة بالمعاملات المختلفة. بشكل عام كان محتوى كلٍّ من الأحماض الأمينية والأمونيوم أعلى فى التربة العضوية عنه فى التربة التقليدية, ولكن بفرق غير واضح, بينما كان معدل نمو الرالستونيا أعلى فى الجزء الخشبى وفى النُّسغ المستخلص من النباتات المزروعة فى التربة العضوية أكثر منه فى النباتات المزروعة فى التربة التقليدية. التخصيب بمادة إن.بى.كيه نتج عنه تثبيط المرض فى عينات التربة المصرية ولكن لم يؤثر على المرض فى عينات التربة الطينية الهولندية. الروث البقرى كان له تأثير مثبط على العفن البنى فى البطاطس فى كل عينات التربة عدا التربة الطينية الهولندية. كما لوحظ تغير واضح فى التنوع البكتيرى استجابةً للمعالجة بالروث, خاصةً فى عينات التربة الرملية.

تم عزل ميكروب جديد له القدرة على المقاومة الحيوية ضد الرالستونيا, وذلك بكميات كبيرة من العقد الجذرية لنبات الباذنجان المزروع فى دلتا النيل بمصر, وتم تعريفه بأنه استينوتروفوموناس مالتوفيليا (الفصل الرابع). هذا ولا تعتمد قدرة هذا المقاوم البيولوجى على علاقته بإنتاج مواد تؤثر على أيض عنصر الحديد. تمت دراسة هذا المقاوم البيولوجى فى الصوبة النباتية, إما عن طريق حقنه فى التربة أو بواسطة تطعيم عينات من عيون البطاطس بهذا المقاوم البيولوجى. كان المقاوم البيولوجى فعالاً فى التحكم فى المرض فى التربة الطينية المصرية ولكن ليس فى التربة الطينية الهولندية. تمت دراسة قدرة بكتريا الاستينوتروفوموناس ذات السلالة المتحورة, المقاومة لكل من الكلورامفينيكول والريفامبيسين, على البقاء حيةً فى التربة العضوية مقارنةً بالتربة الطينية التقليدية, المأخوذة من مصر أو من هولندا. تمت أيضاً دراسة تأثيرها على بقاء ميكروب الرالستونيا حياً. لم يتمكن المقاوم البيولوجى من البقاء حياً فى التربة الهولندية إلا لفترات كانت أقصر بشكل واضح عنها فى التربة المصرية. ذلك عكس الحال بالنسبة للميكروب المسبب للمرض والذى أظهرت نتائجه توافقاً مع نتائج اختبار الصوبة. تناسبت فترة بقاء المقاوم البيولوجى حياً مع محتوى المادة العضوية. استطاع الاستينوتروفوموناس مالتوفيليا البقاء حياً لفترات أطول, وكان أيضاً أكثر تأثيراً على إنقاص فترة بقاء ميكروب الرالستونيا حياً, فى التربة العضوية مقارنةً بالتربة التقليدية المصرية. المحتوى العضوى كان أعلى فى التربة العضوية وكان تنوع البكتريا فيها أقل, وذلك مقارنةً بالتربة التقليدية. على الجانب الآخر, استطاعت بكتريا الاستينوتروفوموناس مالتوفيليا البقاء لفترات أقصر فى التربة العضوية مقارنةً بالتربة التقليدية الهولندية, بل وحتى نتج عن استخدامها زيادة فى فترة بقاء ميكروب الرالستونيا حياً فى التربة العضوية. كان التنوع الحيوى البكتيرى أعلى فى التربة العضوية عنه فى التربة التقليدية الهولندية. وهكذا, فإن الدراسة التى أُجريت على فترة بقاء الاستينوتروفوموناس أو الرالستونيا أكدت النتائج التى سبق الحصول عليها من اختبارات المقاومة الحيوية فى الصوبة.

تمت دراسة بقاء ميكروب الرالستونيا حياً تحت الظروف اللاهوائية وذلك باستخدام المطهرات البيولوجية للتربة (الفصل الخامس). الظروف اللاهوائية تم تفعيلها بواسطة زيادة معدل التنفس الهوائى الميكروبى خلال إضافة مادة عضوية حية (عشب الجاودار الإيطالى) مع تجنب إمداد التربة بالأكسجين وذلك خلال تغطيتها بعازل من البلاستيك المحكم. المعاملات التى أُجريت على مجموعات أخرى لمقارنة النتائج اشتملت على عينات تربة غير معالجة وغير مغطاة, أو مغطاة فقط, أو معالجة بالعشب فقط. تم اختبار تأثير المطهرات البيولوجية للتربة على بقاء ميكروب الرالستونيا حياً, وذلك على ثلاثة

مستويات؛ إما فى أوعية زجاجية خاصة سعة لتر واحد وتحت ظروف معملية, وإما فى مزارع وعائية مصغرة بقطر 1,2م فى حقول حجر خارج معملية, وإما فى حقول طبيعية مصابة. المطهرات البيولوجية للتربة كانت فعالة فى إنقاص تعداد الرالستونيا بشكل واضح بل وحتى فى إبادة الميكروب فى الكثير من الأحيان على مستويات التجربة الثلاثة. لم يقف تأثير المطهرات البيولوجية للتربة فقط على إنقاص وحدة قياس تعداد الميكروب بالتربة, بل تجاوزت ذلك إلى درنات البطاطس المصابة والمدفونة بالتربة الخاضعة للدراسة. لم تؤثر المعالجة بالعشب فقط أو تغطية التربة بالعازل البلاستيك فقط على مدة بقاء الميكروب حياً. كان هناك تغير واضح فى التنوع البكتيرى بعد المعالجة بالمطهرات البيولوجية للتربة وذلك مقارنةً بكل المعاملات الأخرى, ولم يعد هذا التغير للوضع الطبيعى حتى بعد مرور ثلاثة أسابيع من إزالة العازل البلاستيك عن التربة المعامَلة بالمطهرات البيولوجية للتربة.

إن هذه الدراسة تتيح رؤية عملية لتأثير الخواص الفيزيائية والكيميائية والبيولوجية للتربة على درجة بقاء الميكروب المسبب للمرض حياً أو على قَمع المرض, مما يمكنه أن يساعد فى اختيار الوسائل اللائقة للتحكم فى المرض فى أنواع التربة المختلفة. مزايا وعيوب الوسائل العديدة للتحكم فى المرض تمت دراستها, ولكن لا تزال هناك حاجة إلى أبحاث أكثر لمعرفة المجموعات الميكروبية السائدة عند معاملة التربة بوسائل القَمع المختلفة للميكروب المسبب للمرض, ولكى يتسنى تغيير التنوع الميكروبى للتربة بشكل يمهد للتحكم فى المرض بوسيلة طبيعية.

Biography

Nevein Anwar Shehata Messiha was born on February 4, 1971 in Bani Suef, Egypt. After high school she studied botany and got her BSc from the Faculty of Science, Cairo University in 1992. From 1994 till 2001 she conducted her MSc research at the Department of Microbiology at the Faculty of Science, Zagazig University in Egypt with the research subject "Biological control of potato brown rot disease".

On May 1993 she started her professional career working for the Ciba-Geigy Company, Kaliobia, Egypt for 3 months testing the efficiency of different chemical pesticides in controlling plant diseases. On January 1995 she worked temporarily in the Central Agricultural Pesticide Laboratory (CAPL), Agricultural Research Center in Cairo, Egypt, for one year.

Since 1996 she was employed temporarily as an assistant researcher and got her permanent position in 1999 at the Potato Brown Rot Project (PBRP) Institute in the Agricultural Research Center, Cairo. She got the chance to participate in the EU-Egypt Potato Brown Rot Project Phase I, which started by the end of 1996. In 1997 she received an intensive training course in detection and identification of plant pathogenic bacteria for 6 weeks at the Plant Protection Service (PD) in the Netherlands and at Central Science lab (CSL) in the United Kingdom in 1996. After finishing her MSc in 2001 she was employed as a researcher assistant (current position) at PBRP.

From February 2003 till December 2006 she conducted her PhD study in the framework of the EU-Egypt Potato Brown Rot Project Phase II (SEM03/220/51A / EGY 1B/1999/0192) both at the Plant Protection Service (PD) and at the "Biological Farming Systems" Group of the Plant Science Department at Wageningen University, Wageningen, the Netherlands.

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