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Recovery of *Ralstonia solanacearum* from canal water in traditional potato-growing areas of Egypt but not from designated Pest-Free Areas (PFAs)

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Abstract Surveys over three seasons of irrigation, drainage and artesian well water throughout the major potato-growing areas of Egypt indicated that *Ralstonia solanacearum* bv. 2 race 3 (phylotype II sequevar 1), cause of potato brown rot, was limited to the canals of the traditional potato-growing areas in the Nile Delta region, with positive findings more commonly associated with the network of smaller irrigation canals flowing through potato-growing areas. Pathogen populations in the canals of the Delta (~100–200 cfu l^{-1}) were generally variable throughout the year with presence linked to potato cultivation in the immediate area. The pathogen was not detected in irrigation or drainage water associated with potato cultivation in the newly reclaimed desert areas (designated as Pest-Free Areas, PFAs) or in the main branches of the Nile upstream from these areas. *In vitro* studies showed that temperature and microbial activity were the main factors affecting survival of the pathogen in canal water. In experiments at temperatures of 4, 15, 28 and 35°C, survival was longest at 15°C and shortest at 35°C. Survival at 4 and 28°C tended to be intermediate between these extremes as was survival when the bacterium was grown at fluctuating temperatures. Aeration, solarisation and pH variation between 4 and 9 appeared to have little effect on survival. Survival in autoclaved or filter-sterilised canal water was longer than in untreated water irrespective of other factors with survival times exceeding 300 days at 15°C in some experiments. Evidence is presented indicating that survival in water-saturated sediment may be longer than in the overlying water suggesting that sediment may provide a protective niche for the pathogen in some circumstances. The maximum survival time in non-sterile Egyptian canal water at high inoculum pressure was estimated to be up to 300 days at optimum temperature for survival (15–30°C) suggesting the potential for long-distance spread in Egyptian surface waters from sources of contamination.

Keywords Brown rot · Canal water · Survival

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Introduction

The presence of *Ralstonia solanacearum* bv. 2 race 3 (phylotype II, sequevar 1), cause of potato brown rot,

in Egypt has been a major concern affecting Egypt's potato export trade with the European Union. The disease is common in parts of the traditional potato-growing areas in the Nile Delta and potatoes grown in this area are now not accepted for import into EU countries because of the risk of spreading this quarantine-listed organism to European potato crops.

Egypt produces approximately 2 million tonnes of potato annually, of which approximately 400,000 tonnes are currently exported to EU countries. Egypt's entire export crop is now grown outside the traditional potato-growing areas in designated Pest-Free Areas (PFAs). The PFAs are declared free from *R. solanacearum* on the basis of knowledge of past cultivation practices and the results of specific surveys of potato crops, soil, weeds and both surface and artesian water, carried out according to FAO recommendations (Anon. 2007). The PFAs are established and reviewed annually, in agreement with the EU, and are almost exclusively located in desert areas on the border of the Nile Delta, where potatoes have never previously been grown.

Following relocation to the PFAs of all Egyptian potato production for the EU market, interceptions of *R. solanacearum* in potatoes exported to the EU decreased significantly although occasional interceptions of potatoes infected with *R. solanacearum* continued at EU ports. Further investigation was therefore needed to minimise any risk that the pathogen was contaminating the PFA production areas.

With a projected increase in demand for Egyptian potatoes from Europe and sustained interest from the potato growers in the Nile Delta areas of Egypt to export to Europe, a need was identified for further information on distribution of the pathogen in existing and potential Egyptian potato-growing areas as well as on the survival of the pathogen under Egyptian conditions.

Contamination of European waterways with *R. solanacearum* has been associated with infection and overwintering in the wild riparian host *Solanum dulcamara* (Olsson 1976a; Elphinstone et al. 1998). However, findings of *R. solanacearum* in Egyptian canal water were first reported in the Nile Delta of Egypt in 1997 (Farag et al. 1999) despite the absence of this or any other identified alternative host.

A programme was therefore initiated to both map the distribution of the pathogen throughout the delta region and to carry out research to assess its potential for survival in Egyptian waterways as well as to assess the risks of contamination of potato crops through irrigation. Whilst several studies have been carried out on the survival of the pathogen in European conditions (Olsson 1976a, b, Elphinstone et al. 1998, Janse et al. 1998, van Elsas et al. 2001, Wenneker et al. 1998, 1999) this is the first study on survival of the pathogen in irrigation water under the range of environmental conditions typical for Egypt.

In this study, the effects of temperature, pH, aeration, solarisation and biological activity on the *in vitro* survival of *R. solanacearum* were investigated. Population levels of the pathogen in a canal in one of the major potato-growing areas were also monitored throughout the study to provide information on the natural seasonal fluctuations in populations of the bacterium.

Materials and methods

All sampling and testing methodology followed, as far as possible, protocols adopted by the EU (Anon. 1998 and amendments).

Surveys of irrigation water

Surveys were carried out at potato-growing sites in the PFAs and at selected sites in the traditional potato-growing regions of the Nile Delta over a three-year period. Sampling sites were reviewed annually to reflect changes in cultivation in the PFAs and to ensure wide coverage of potato-growing areas in the Delta. GPS coordinates of all sites were recorded to ensure specific sampling points could be relocated.

Surface and artesian water used to irrigate potato crops within the PFAs was monitored throughout the potato-growing season for the presence of *R. solanacearum*, in addition to the routine crop inspections and tuber sampling and testing prior to export. An average of sixty-four sampling sites was visited three to four times during each cropping season.

Surface water was also collected on at least four occasions in the potato-cropping season from selected sites in the Nile Delta, where potatoes were produced for the local and non-EU export markets. Some of these sites were changed each year in an attempt to plot the distribution of the pathogen in the delta region. A total of 20–30 sampling sites was visited

four times per year. A more detailed survey of the surface water was carried out at three sites along the El-Nagaar Canal flowing through a potato-growing area in Minufiya governorate over a two-year period. Water samples were collected fortnightly and populations of *R. solanacearum* estimated using the sampling and identification procedures detailed below.

At each selected sampling site, three duplicate 50 ml water samples in plastic centrifuge tubes were collected at approximately 2–3 m spacing at a depth of around 30 cm. The capped centrifuge tubes were transported to the laboratory in refrigerated cool boxes (5–10°C) and refrigerated overnight before testing (Anon. 1998). *Ralstonia solanacearum* populations in water samples were concentrated by centrifuging each 50 ml sample at 7,000*g* for 15 min. After discarding the supernatant, the pellet was re-suspended in 1.0 ml sterile 10 mM phosphate buffer, pH 7.2 ($NA_2HPO_4.12H_2O$:2.7 g, $NaH_2PO_4.2H_2O$: 0.4 g, distilled water: 1.0l). Ten and 100-fold serial dilutions were made of this suspension and duplicate 100 µl aliquots from the original suspension and the dilutions were uniformly spread onto plates of SMSA (Englebrecht 1994 as modified by Elphinstone et al. 1996) agar using a sterile plastic spreader. All plates were incubated at a temperature of 28°C in the dark in controlled temperature cabinets and colonies with typical fluidal consistency and pink centres characteristic of *R. solanacearum* counted after 48 or 72 h. Average counts from the replicate plates were used to assess *R. solanacearum* populations in the water at sample sites.

Colonies were confirmed as *R. solanacearum* both directly using lateral flow devices (Danks and Barker 2000), or by plating selected colonies onto SMSA basal medium without antibiotics or sucrose peptone agar and testing discrete colonies from these plates by immunofluorescence antibody staining (IFAS; Janse 1988), real-time PCR (Weller et al. 2000) and bioassay in tomato (Janse 1988); 500 µl suspensions of *R. solanacearum* containing approximately 10^6 cfu ml^{-1}, in 20% sterile glycerol were stored at −30°C for future reference.

Preparation of inoculum

Ralstonia solanacearum bv. 2 race 3 was freshly isolated from Egyptian irrigation canals on semi-selective SMSA medium. Irrigation water for all experiments was collected from either the El Nagaar canal, Minufiya governorate or the Gharfariya canal, El Gharbiya governorate, adjacent to a potato-growing area, and used within 72 h of collection. The pH of the water varied between 7.2 and 8.8 depending on the time and place of collection. Cultures were identified to species by PCR, IFAS and bioassay in tomato. The biovar was confirmed by biochemical tests (Hayward 1964) and by real-time PCR (Weller et al. 2000). Isolates were stored either in sterile distilled water at room temperature or in 20% glycerol at −30°C. For all experiments inoculum was prepared from 48 to 72 h cultures of the bacterium grown on SMSA basal agar medium without antibiotics. Bacterial colonies were dispersed in 2–5 ml sterile canal water added to the Petri dishes using sterile spreaders. This suspension was then aseptically pipetted into 80–100 ml natural or sterilised canal water, to give a final concentration of approximately 10^8 colony forming units per millilitre (cfu ml^{-1}). After mixing, 15 ml volumes of the suspension were aseptically dispensed into triplicate sterile 22 ml capacity screw-cap bottles.

In vitro survival of *R. solanacearum* in canal water at different temperatures

To obtain sterilised water, freshly collected irrigation water was autoclaved at 121°C for 15 min. All experiments were carried out using triplicate suspensions incubated in controlled temperature cabinets at 4, 15, 28 and 35°C to study the effect of temperature on survival, and at 15 and 28°C to study the effect of pH and background organisms. To study the effect of temperature fluctuation on survival, triplicate samples were switched between 4 and 15°C, 15 and 28°C and 28 and 35°C respectively at approximately 24 h intervals.

Samples (100 µl each) were collected from replicate bottles after gentle mixing at times zero, 1, 2 and 3 days. Thereafter, samples were collected at twice weekly or weekly intervals as appropriate, and 10-fold serial dilutions uniformly spread on duplicate SMSA plates. Typical fluidal colonies with pink centres appearing after 48 to 72 h were counted and means of replicates taken to calculate population levels in the water. Identity of selected colonies was checked using lateral flow device test kits (Danks and Barker 2000), IF and PCR. Plates showing heavy growth of background organisms were ignored and means taken of colony counts from the remaining plates.

Effect of natural microflora on survival of *R. solanacearum* in canal water

Up to 10 l of freshly collected canal water was filtered through 0.22 µm membranes under vacuum. The membranes were then placed in 50 ml volumes of filter-sterilised canal water in conical flasks and shaken on a rotary shaker to resuspend the trapped microflora. Experiments were then carried out in the same way as above using zero, normal and increased (100-fold) background microflora levels and a concentration of *R. solanacearum* of around 10^8 cfu ml^{-1}.

Effect of solarisation and aeration on survival of *R. solanacearum* in canal water

Light and aeration experiments were carried out in open tanks at room temperature using 15 l of inoculated canal water (10^3–10^5 cfu ml^{-1}). In the light-dark experiments, light was provided by two 18 W (~900 lumens) Activa 172 fluorescent full spectrum daylight tubes with a colour temperature of 6,500 K (Lightbulbs Direct Ltd., Amersham, Bucks, UK). Experiments were carried out both under continuous light and under a regime of 8–10 h light/14–16 h dark per day. Light was excluded from the dark treatment by covering one of the tanks with black polyurethane sheeting. In the aeration experiments, 15 l water was aerated and circulated using a standard aquarium pump fitted with an aerator and results compared with a non-aerated control. The daily water temperature in these experiments varied between 18 and 30°C.

Effect of pH on survival of *R. solanacearum* in canal water

In pH experiments, the pH of freshly collected canal water was adjusted by drop-wise addition of 0.1 M HCL or NaOH to produce pH levels of 4, 5, 6, 7, 8 and 9 and either used directly or after autoclaving. The pH was rechecked after autoclaving and readjusted using sterile 0.1 M HCL or NaOH as necessary.

Survival of *R. solanacearum* in canal water sediment

Canal water and sediment were collected from the same site and mixed to produce a uniform suspension. A *R. solanacearum* suspension in canal water from a 48 h culture of the bacterium on SMSA basal medium was then added to give a final concentration of around 10^7–10^8 cfu ml^{-1}; 50 ml aliquots of this mixture were then decanted into twelve sterile 120 ml capacity screw-cap plastic beakers and 100 µl volumes of serial dilutions of the sediment-water mixture in phosphate buffer were immediately spread on SMSA agar. The resultant colonies were counted after 48 to 72 h. Three beakers were then each placed in incubators at temperatures of 4, 15, 28 and 35°C and sampling commenced immediately and continued during settling of the sediment portion. At this stage of the experiment, samples were collected both just below the surface of the sediment-water mixture (water phase) and at the bottom of the container (sediment phase). Further samples were collected from the water and sediment layers respectively at regular intervals, taking care to avoid remixing of the sediment and water layers.

Statistical analysis

Data from all experiments were transformed logarithmically (log10 x+1) to stabilise the variance. Data points on all graphs represent the mean of at least three observations with the relevant standard error bars. The area under the population curves (AUPC) was calculated using the trapezoidal method from time zero to the time when bacterial populations fell to non-detectable levels in one or more treatments. AUPCs were compared in all experiments by conducting an ANOVAR using Genstat 8.1 (VSN International Ltd) on the AUPC data.

Results

Surveys

Sampling sites visited in both the PFAs and the delta region during the 2003–2005 potato seasons are shown in Fig. 1. Over a three-year period, an average of 262 samples was tested per year from the PFA sites. In none of these samples was *R. solanacearum* detected, indicating that neither surface nor artesian water used to irrigate the potato crops was a source of contamination for exported potato consignments. The corresponding number of samples collected

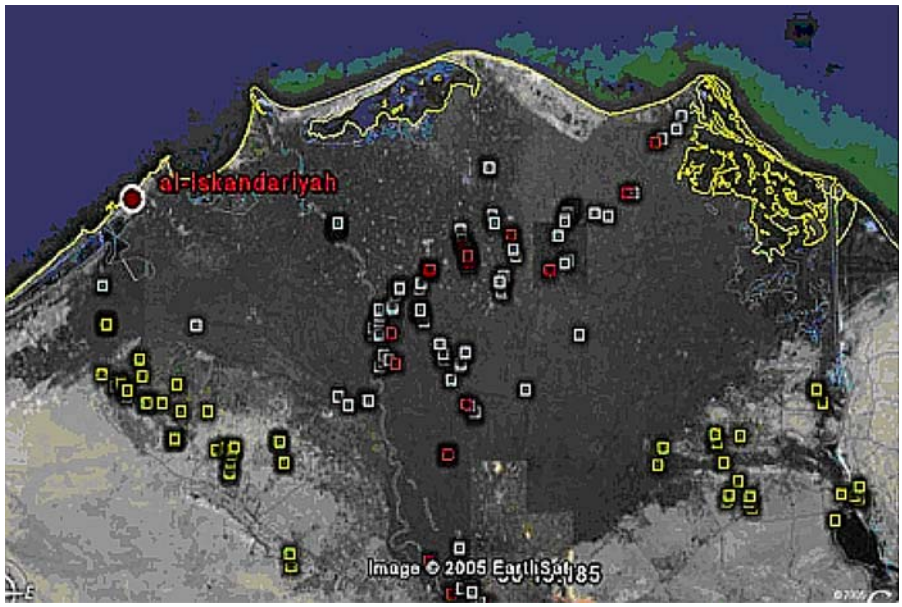

Fig. 1 Water sampling sites in the Nile Delta and Pest-Free Areas of Egypt. Yellow symbols represent PFA sites, blue symbols Nile Delta sites and red symbols all sites from which *R. solanacearum* was isolated on at least one occasion. (Source: Google Earth. 2005)

from sites in the Nile Delta each year was 140 with contamination rates varying from 4.7 (2004/05) to 23.3% (2003/04) over a three-year period and with an average contamination level over the three seasons of 16%.

Most sampling sites in the Nile Delta were areas where potatoes were grown throughout the year. Nevertheless, the pathogen could not always be detected in the surface waters supplying these areas and was only consistently isolated from the smaller canals immediately adjacent to fields in which potatoes were under cultivation. At sampling sites in areas where potatoes were not under cultivation, the pathogen was rarely isolated.

Survey of *R. solanacearum* populations in the El-Nagaar Canal in the Nile delta

Populations of *R. solanacearum* detected in the smaller canals of the Nile Delta (0–200 cfu l^{-1}) were generally variable throughout the year as shown in a series of fortnightly sample results from three sites along the El-Nagaar Canal in Minufiya governate (Fig. 2). *Ralstonia solanacearum* populations were typically <200 cfu l^{-1} of canal water with the exception of one sample taken at around the mid-point of the canal in late May 2004 which contained approximately 700 cfu l^{-1}.

Over the two-year period, the water temperature at a depth of around 15–20 cm varied from a minimum of 14°C to a maximum of 29°C depending on the season, the water level in the canal, and whether the water was stagnant or free-flowing. Detection of *R. solanacearum* did not appear to be correlated with water temperature over this range as the bacterium was detectable throughout the year, although less frequently and in lower numbers during the winter months (October–January). In 2004 the *R. solanacearum* population levels in the El-Nagaar canal peaked between April and June, coinciding with the main harvest period for both imported certified seed potatoes and farmer-saved seed potatoes planted between late November and January.

Factors affecting survival of *R. solanacearum*

Effect of temperature on survival of R. solanacearum in autoclaved and natural canal water

Temperature and competition from competing micro-organisms were shown to have a marked effect on survival of *R. solanacearum* (Fig. 3). Whilst there was a degree of variability in survival time of *R. solanacearum* in different experiments, survival was significantly longer in autoclaved than in natural canal water except at 4ºC. Survival was also always longer

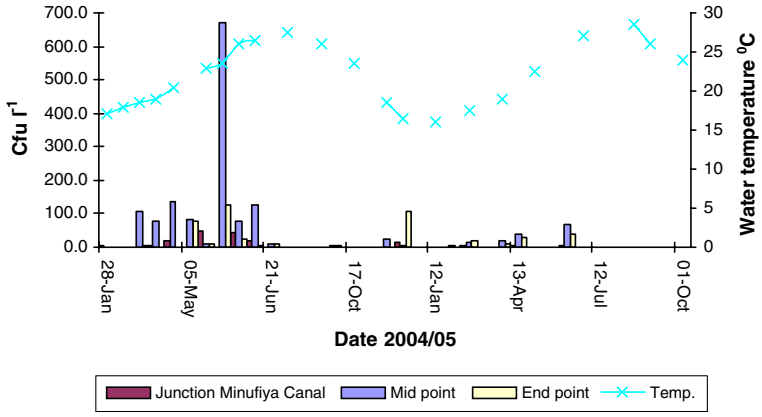

Fig. 2 *R. solanacearum* populations detected at three sites on the El-Nagaar Canal, Minufiya governate between January 2004 and October 2005

at 15ºC in both autoclaved and non-sterile canal water than at other temperatures and with one exception, shortest at 35ºC. Survival at 28ºC was generally intermediate between survival at 15 and 35ºC whilst survival at 4°C was always shorter than at 15 and 28ºC. but occasionally longer than at 35ºC. In experiments to simulate daily temperature fluctuations in autoclaved and natural canal water, respectively, the profiles of the population curves at fluctuating temperatures tended to fall between those of the constant temperatures.

Effect of background microflora on survival of R. solanacearum in canal water

Ralstonia solanacearum declined significantly more slowly at a temperature of 28ºC in filter-sterilised canal water than in canal water with normal or augmented populations of background microflora (Fig. 4). Means of AUPC (arbitrary units) for survival of *R. solanacearum* with zero, normal and high levels of background microflora were 310, 224 and 100 respectively. Differences in the AUPC between the

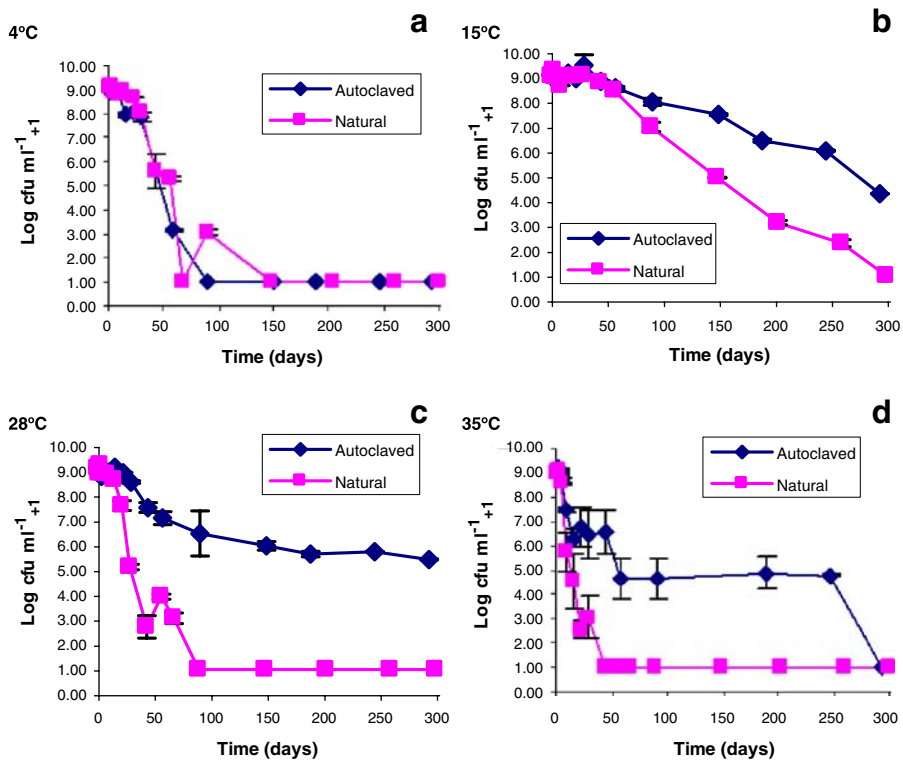

Fig. 3 Survival of *R. solanacearum* in autoclaved and natural canal water (pH 8.4) at temperatures of 4, 15, 28 and 35ºC. Effects of temperature ($F=88.16$; df= 6,70; $P<0.001$), water source ($F=266.19$; df=1,70; $P<0.001$); interaction between temperature and water source ($F=30.69$; df= 6,70; $P<0.001$) on AUPC

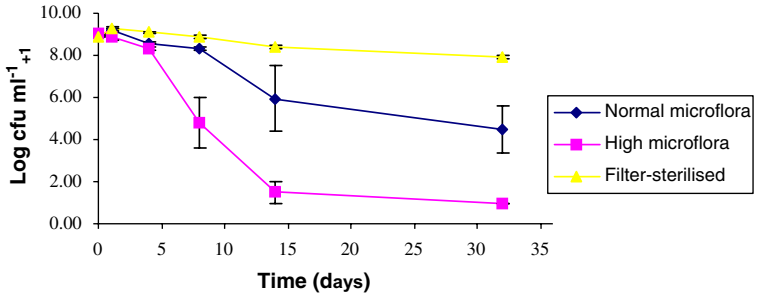

Fig. 4 Survival of *R. solanacearum* in canal water (pH 7.8) in the presence of a high concentration of background microflora at a temperature of 28ºC

three treatments were highly significant (f=19.81; df= 2,15; P<0.001) with an LSD (0.05) of 71.6.

Effect of pH on survival of R. solanacearum in autoclaved and natural canal water

Ralstonia solanacearum was capable of surviving for prolonged periods (>=240 days) in autoclaved canal water at pH levels between 4 and 9 and at temperatures of 15 and 28ºC irrespective of the pH level (Fig. 5 a,b), indicating that pH *per se* was not a major determinant of survival of the pathogen. However, in non-autoclaved canal water at 15 and 28ºC, the bacterium remained culturable for longer at pH 4 (Fig. 5 c,d).

Effect of aeration and solarisation on survival of R. solanacearum in natural canal water

No significant effect was observed, over a number of experiments, of the effect of increased oxygen levels and solarisation on survival of *R. solanacearum*.

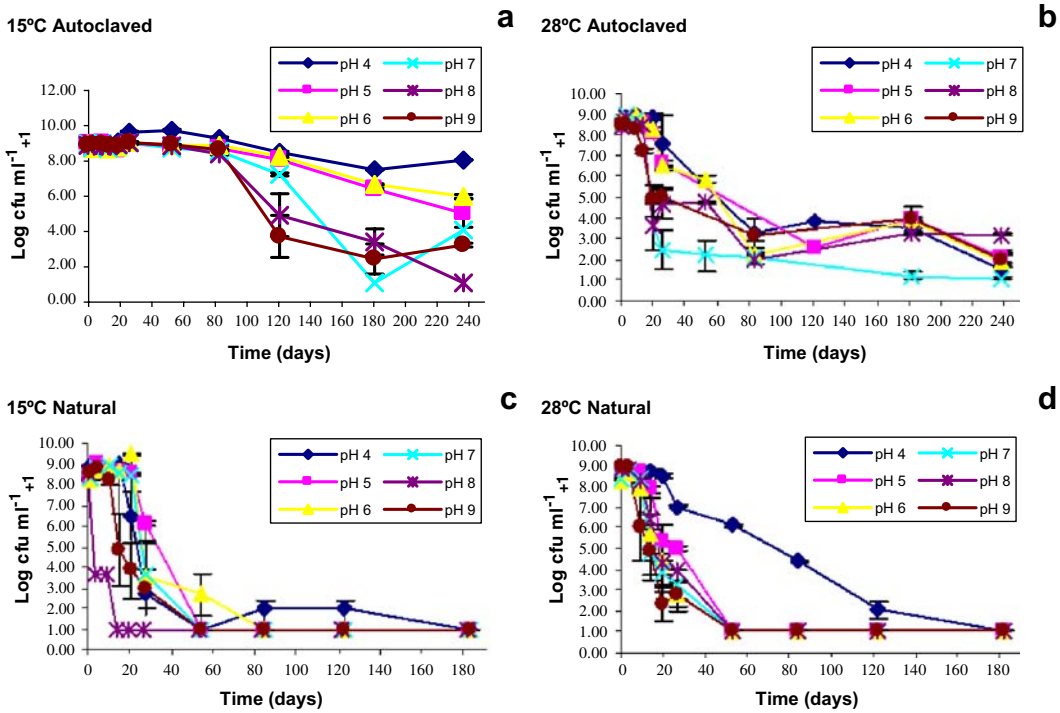

Fig. 5 Survival of *R. solanacearum* in autoclaved and natural canal water at temperatures of 15 and 28ºC and pH values of 4, 5, 6, 7, 8 and 9. Differences in the AUPC between the source water (f=1,871.71; df=1,120), pH (f=51.67; df=5,120) and temperature (f=237.61; df=1,120) were all highly significant (P<0.001). Interactions between pH and temperature (f=8.96; df=5,120), source and pH (f=12.25; df=5,120) and between water source and temperature (f=345.20; df=1,120) were also highly significant (P<0.001)

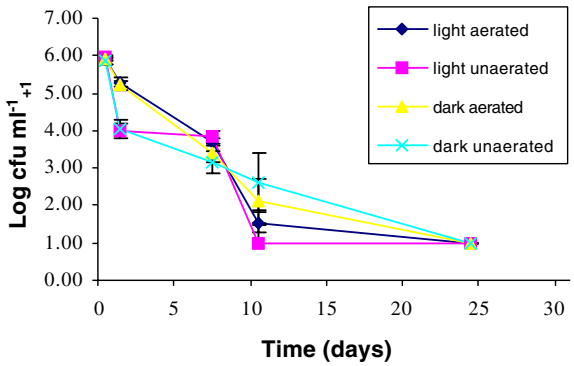

Fig. 6 Survival of *R. solanacearum* in canal water at ambient temperature in the light and dark with and without aeration (pH 8.2)

Figure 6 shows the results of one of several experiments on survival of the pathogen in aerated and unaerated canal water in light and dark conditions. Results of all experiments were similar. The short survival time in this and similar experiments (results not shown) is probably a reflection of the low initial inoculum concentrations used.

Isolation of R. solanacearum from a sediment water mixture

At lower temperatures (4 and 15ºC) *R. solanacearum* appeared to persist in a culturable state for longer in the sediment than in the aqueous phase (Fig. 7). This was not the case at higher temperatures where culturable populations generally declined at a faster rate.

Discussion

Ralstonia solanacearum has been described as a species complex (Gillings and Fahy 1994, Fegan and Prior 2005) to reflect the high degree of heterogeneity between strains of the pathogen from different hosts and geographical regions. Classification of the organism has evolved from separation of the strains into five races based on host range (Buddenhagen et al. 1962, He et al. 1983,), six biovars based on biochemical properties (Hayward 1964, 1991, 1994), two divisions (Cook and Sequeira 1994) and four phylotypes comprising a number of sequevars (Fegan and Prior 2005) based on a range of molecular techniques. The four phylotypes broadly comprise isolates of the pathogen from different geographical regions. Thus strains primarily from Asia group in phylotype I; those from the Americas in phylotype II; those from Africa and offshore islands in phylotype III and strains primarily from Indonesia in phylotype IV.

Strains from all four phylotypes have been recorded on potato and phylotype III potato strains are known from a number of African countries. However, representative isolates from this study all proved to belong to phylotype II sequevar 1, the strain commonly associated with commercially grown potatoes. Nevertheless, it is conceivable that the genetic diversity of *R. solanacearum* strains on Egyptian potatoes may be greater than that reported here.

The phylotype II sequevar 1 strain of *R. solanacearum* (race 3 biovar 2) is endemic in the Nile delta of Egypt, where it appears to be closely associated with traditional areas of potato production. Previous surveys of soil and weeds have found the pathogen only in weeds and soil in fields where potatoes were being cultivated or had recently been harvested, suggesting that the pathogen might not survive for long periods in the absence of potatoes (F. Fawzi, PBRP, Egypt, pers. observation)

In the survey of the River Nile and irrigation canals of the Nile Delta reported here, contamination of surface waters by transient populations of the pathogen was only observed in the vicinity of traditional potato-production areas. In practice, this means that a large proportion of the Nile Delta surface waters are susceptible to contamination for most of the year as potatoes are cropped almost continuously in these areas. *Ralstonia solanacearum* could routinely be isolated from small canals adjacent to potato fields. In these canals, the water is slow flowing or stationary for much of the time and the volume is often relatively low, so that contaminated water may persist for some time with relatively high, localised, concentrations of the pathogen. Work carried out as part of this project (results not shown) indicated that persistence of the pathogen was related to its initial inoculum concentration in the water. When higher initial inoculum concentrations were used in experiments, culturable populations of the pathogen could subsequently be recovered over a greater time span than when lower inoculum concentrations were used. This was also found in a three-year study of

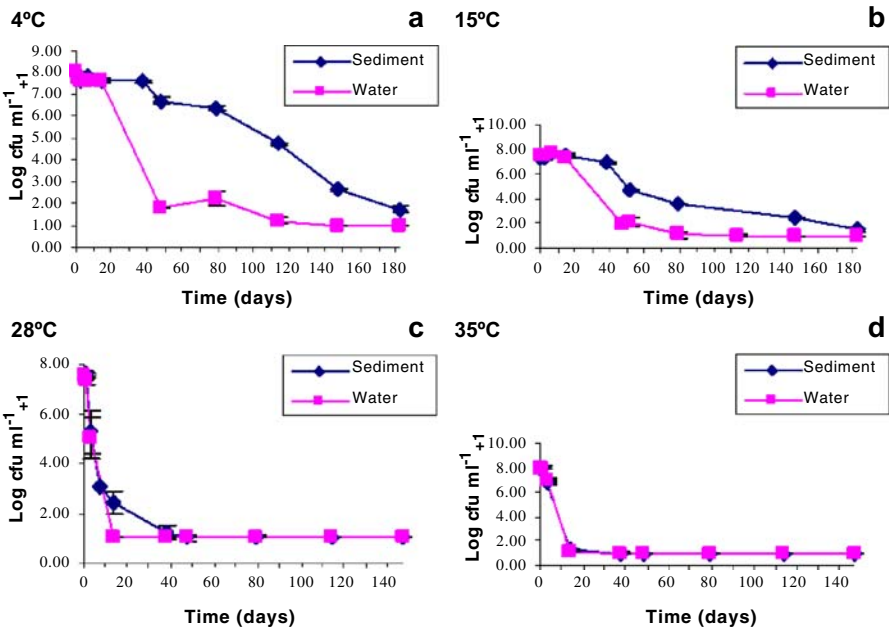

Fig. 7 Isolation of *R. solanacearum* from the sediment and water phases of a sediment water mix at temperatures of 4, 15, 28 and 35ºC during settling of the sediment. Effects of source ($F=2,071.14$;df=1,40) and temperature ($F=2,673.83$; df=3,40) were both highly significant ($P<0.001$). There is also evidence of a strong interaction between source and temperature ($F=562.02$;df=3,40; $P<0.001$)

naturally-contaminated watercourses in The Netherlands when comparisons were made between heavily and moderately-contaminated surface waters (Wenneker et al. 1999). The pathogen was never isolated from the River Nile itself and only sporadically from the main waterways passing through the Nile Delta from which the networks of small canals branch to provide irrigation water to the farmland. This may indicate infrequent contamination of the larger canals. It may also indicate relative short survival times of *R. solanacearum* when present in low numbers relative to the natural biota. Alternatively, it may be a result of the dilution effect of a large body of water reducing the relative concentration of the pathogen to below detectable levels.

Water flowing through the agricultural areas is likely to become contaminated either at source from one of the main branches of the Nile or by contamination by household or agricultural waste as it flows close to farms and villages. There is also the possibility of contamination from reservoirs of the pathogen in the roots of semi-aquatic weeds lining the canals, similar to the situation in Europe, where the submerged roots of the riparian solanaceous weed *Solanum dulcamara* provide a protected niche for the pathogen in some rivers (Elphinstone et al. 1998, Janse 1996, Olsson 1976 a, b, Persson 1998). Other weeds may also play a role in persistence of the pathogen in waterways (Wenneker et al. 1999). However, such weeds have not, so far, been found along Egyptian waterways despite a number of limited surveys.

The PFAs bordering desert areas are irrigated either by water from artesian wells or by Nile water from artificial canals constructed to provide irrigation to the farming areas. Over the three-year survey period, *R. solanacearum* was never isolated from artesian well water, the irrigation canals, or the waters of the River Nile upstream from the main irrigation canals supplying the PFAs. Many of the irrigation canals feeding the PFAs are concrete-lined and relatively free from plant growth thus reducing the possibility of brown rot-susceptible riparian weeds becoming infected and providing a source of contamination for these waterways. This strongly suggests that the source of the periodic infection of potatoes exported to EU countries is not the canal or artesian water used to irrigate the potato crops. The relatively low number of interceptions of *R. solanacearum* in exported potatoes despite a strict testing regime for Egyptian potatoes coupled with the failure to detect the pathogen in the field during multiplication of the seed or cultivation of the export crop also suggests that contaminated irrigation water is probably not the source of infection of export potatoes from the PFA areas. However, in some areas, land is cultivated with a variety of crops, including potatoes, on the banks of major rivers, principally in the Delta regions but also including those supplying water to irrigation canals

feeding some PFAs. This presents a risk that contamination of these waterways by the brown rot pathogen may occasionally occur with a concomitant risk of infection of potato crops in the PFAs.

Although *in vitro* studies showed that the bacterium declined relatively quickly in non-sterilised canal water under a range of typical Egyptian conditions, the bacterium can potentially remain viable in non-sterile water under ideal conditions (high initial inoculum concentration and a temperature of ~15°C), for up to around 300 days, long enough for long distance transmission in water.

The transient nature of the contamination of canal water was demonstrated by repeatedly sampling three sites along a single canal flowing through an area of intensive potato production and relatively high population density in Minufiya Governate in the Nile Delta (Fig. 2). In this canal the peak in *R. solanacearum* numbers in 2004 more or less coincided with the main harvest period in April and May. It is during this period when canals are most likely to receive contaminated potato waste. Smaller peaks were observed during 2005, which were also coincident with potato harvests. In between the harvesting of one potato crop and the planting of another, isolation of the pathogen from the canals was less frequent, implying either that the pathogen does not survive for long periods in these canals despite the water temperature being suitable throughout the year (15–30°C) or that after each episode of contamination, it is rapidly diluted to below detectable levels. This also suggests that the presence of the pathogen in these canals is dependent on repeated contamination by potato waste rather than long-term survival in the water column or the roots of any unidentified susceptible weeds that may be growing along the canal banks.

In vitro studies suggest that *R. solanacearum* has the potential to survive for extended periods (~300 days) in Egyptian canal water, but probably does not do so, as demonstrated by the frequent inability to detect the pathogen in sequential water samples from the same sites. Van Elsas et al. (2001, 2005) and other workers (Grey and Steck 2001) have proposed that the pathogen may persist in a viable but non-culturable state induced by certain environmental parameters (e.g. exposure to a temperature of 4°C), followed by revival and renewed ability to initiate disease in susceptible hosts, when the conditions change. They propose that this may explain the over-wintering of the bacterium in the apparent absence of host plants or host plant debris in European soils. *In vitro* experiments carried out in Egypt as part of this study have provided no evidence for this effect. In these experiments at each of the four temperatures used, (4, 15, 28 and 35°C) *R. solanacearum* was never subsequently isolated after populations had declined to below detectable levels. The pathogen population dynamics in Egyptian surface water also suggest repeated contamination with the bacterium rather than either survival of the pathogen in numbers below the limits of detection or its persistence in a viable but non-culturable state.

Except at a temperature of 4ºC, *R. solanacearum* survived far longer in autoclaved canal water than in non-sterile canal water (Fig. 3). The maximum potential survival time in non-sterile canal water in laboratory experiments was around 300 days at a temperature of 15ºC. This did not appear to reflect the apparent survival time in the canals based on a fortnightly sampling programme. It was common for the *R. solanacearum* populations in these canals to vary between 100 cfu l^{-1} to below detectable levels over a two-week period. The differences in apparent survival times between the laboratory and field observations is quite likely the result of the much higher pathogen concentrations used in laboratory experiments compared to populations found in the canals. Other possibilities include the existence of varying levels of microbial competition in natural waters and the dilution and flushing out of the pathogen in flowing waterways leading to a rapid reduction in apparent population levels to below detectable levels in the absence of repeated contamination.

In Egypt, the normal daily surface water temperature varies from around 14ºC in winter to around 30ºC in summer, which coincides with the optimum temperature range for survival of *R. solanacearum.* It is likely, therefore, that the potential exists for the pathogen to persist in Egyptian surface waters for around 100–300 days. The fact that field observations (Fig. 2) do not appear to support prolonged survival in Egyptian surface waters, suggests that other factors also play a part.

Experiments on the effect of background microorganisms, pH, solarisation and aeration suggest that the concentrations of background microflora may have a marked effect on survival of *R. solanacearum* (Fig. 4) provided that the background levels are very

high (100-fold), suggesting that in natural ecosystems, survival may be lower in stagnant nutrient rich surface waters, where the microbial count would likely be high, than in free-flowing rivers. Survival times at pH levels between 4 and 9 do not appear to be a major factor in survival time (Fig. 5) although the effect of different pH levels in influencing the composition of the background flora may play a part in influencing the survival of *R. solanacearum*.

In vitro studies in which *R. solanacearum* was incubated in closed containers in a fixed volume of suspension at constant temperature (data not shown) indicated that under such conditions the pathogen could be detected over a longer period in containers with a high initial inoculum concentration than in containers with a low initial inoculum concentration. This effect was most marked at a temperature of 28°C. This suggests that detection of populations of the bacterium in closed bodies of water would be possible for longer when present at higher initial numbers.

Ralstonia solanacearum survived for a markedly longer time (>300 days) in autoclaved river/canal water than in natural water at four temperatures (Fig. 3). It is well known that a wide range of microbial species (Liao and Shollenberger 2003), including *R. solanacearum* (Kelman 1953, Wakimoto et al. 1982, Van Elsas et al. 2001) is able to survive for long periods in sterile distilled water, buffer, or surface water. This strongly suggests, that competition from other microflora, destroyed by autoclaving or removed by filtration, is a principal factor in limiting the survival of the brown rot pathogen in natural microcosms. Support for this is provided by Wenneker et al. (1998, 1999) and Alvarez et al. (2007), the latter reporting that lytic bacteriophages, indigenous protozoa and bacteria were all involved in the decline of *R. solanacearum* population densities in Spanish rivers.

No effect of either solarisation or aeration (Fig. 6) was observed on survival of *R. solanacearum* under laboratory conditions, suggesting that the role played by these factors may be limited. This contrasts with work by Van Elsas et al. (2001) who showed a clear effect of solarisation in reducing the survival time of the pathogen under field conditions. They concluded that the negative effect of light might be a result of one or both of two possible mechanisms. Firstly, they suggest that light may induce the formation of free radicals in the bacterial cells resulting in damage to cellular DNA and proteins leading to cell death. Secondly, they noticed a light-induced stimulation of the growth of cyanobacteria and green algae, which may have had a direct antagonistic effect on *R. solanacearum*, accelerating the decline in cell numbers observed in their study. In a three-year study of *R. solanacearum* contaminated waterways, Wenneker et al. (1999) also observed marked declines in *R. solanacearum* populations in summer seasons both during developing algal blooms and the subsequent death of these algae. In our study, we did not observe a marked difference in numbers of background microorganisms (as measured by dilution plating on SMSA basal medium) between the light and dark treatments either with or without aeration. In particular, there was no growth of cyanobacteria or green algae during the course of our experiments. This may partly explain the different results obtained.

In these experiments, in which we monitored the relative abundance of *R. solanacearum* in a sediment/water mixture at different water temperatures during the settling process at temperatures of 4, 15, 28 and 35ºC (Fig. 7), we found significantly higher pathogen populations in the sediment phase at temperatures of 4, 15 and 28ºC. Survival at 35ºC was too short (~15 days) in both the sediment and water phases to observe any difference in the two phases. Wenneker et al. (1998) also observed longer survival in sediment at 4ºC than at room temperature. The apparent differences in *R. solanacearum* cell numbers in the two phases was initially interpreted as a partitioning of the bacteria caused by adhesion to sediment particles. However, an equally plausible explanation would be that the pathogen was surviving for longer in the sediment rather than the water phase. Van Elsas et al. (2001) have similarly reported that *R. solanacearum* persists for a longer period in sediment than the overlying water at temperatures of 4, 12, 20 and 28ºC. This effect was also observed to a lesser extent in their experiments at temperatures of 36 and 44ºC although at these temperatures, survival in both sediment and sediment-free drainage water was always less than five days. They suggested that greater persistence in the sediment phase was possibly because sediment particles provide a protective niche against grazing by microfauna or competition from other microorganisms.

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