

Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms

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Survival strategies exhibited over 4 years by *Ralstonia solanacearum* phylotype (ph) II biovar (bv) 2 in environmental water microcosms were examined. The bacterium is a devastating phytopathogen whose ph II bv 2 causes bacterial wilt in solanaceous crops and ornamental plants. Outbreaks of the disease may originate from dissemination of the pathogen in watercourses, where it has to cope with prolonged nutrient limitation. To ascertain the effect of long-term starvation on survival and pathogenicity of *R. solanacearum* in natural water microcosms, survival experiments were conducted. Microcosms were prepared from different sterile river water samples, inoculated separately with two European strains of ph II at 10^6 c.f.u. ml⁻¹ and maintained at 24 °C for 4 years. In all assayed waters, starved *R. solanacearum* remained in a non-growing but culturable state during the first year, maintaining approximately the initial numbers. Thereafter, part of the population of *R. solanacearum* progressively lost the ability to form colonies, and non-culturable but metabolically active cells appeared. During the whole period, the bacterium remained pathogenic on host plants and underwent a transition from typical bacilli to small cocci which tended to aggregate. Some starved *R. solanacearum* cells filamented and formed buds. Starvation response, viable but non-culturable state, morphological changes and aggregation have not previously been reported for this pathogen as survival mechanisms induced in oligotrophic conditions. The potential existence of long-starved pathogenic cells in environmental waters may raise new concerns about the epidemiology of bacterial wilt disease.

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INTRODUCTION

Bacterial wilt affects numerous crops and some ornamental plants worldwide (Elphinstone, 2005; Hayward, 1994). The causative agent is the *Ralstonia solanacearum* species complex (Fegan & Prior, 2005), classified into phylotypes and biovars according to molecular and biochemical characteristics respectively (Fegan & Prior, 2005; Hayward, 1991). It is considered a quarantine organism in the European Union (Anonymous, 2000) and a potential bioterrorism agent in the USA (Lambert, 2002). The bacterium is soil and water borne, waterways being major dissemination routes in the environment (Elphinstone, 2005). It enters the plant through the roots and colonizes the vascular system, producing severe disease (Hayward, 1991; Kelman, 1953). After destruction of the plant, an inter-host life period has been proposed for this pathogen (Denny *et al.*, 1994), where it would have to cope with the

stress of oligotrophic habitats. Survival studies in sterile water have reported persistence of the bacterium for variable periods (Kelman, 1956; van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004; Wakimoto *et al.*, 1982), and occasional capacity to wilt susceptible hosts (Caruso *et al.*, 2005; van Overbeek *et al.*, 2004). Although some environmental factors affecting *R. solanacearum* physiology have been reviewed (Hayward, 1991; Kelman, 1953; van Elsas *et al.*, 2005), knowledge of the survival strategies used by this pathogen under prevailing starvation in aquatic habitats is still scarce (López & Biosca, 2005).

Micro-organisms can resort to diverse mechanisms when confronted with nutrient deprivation (Morita, 1997). Several starvation-survival responses have been described, with starved bacterial populations maintaining their numbers over time in a non-growing but culturable state at different levels depending on the species (Morita, 1997). This starvation-induced state for survival proved to be distinctly different from that of active growth (Heim *et al.*, 2002). Under prolonged oligotrophy, reduction in cell size

Abbreviations: bv, biovar; DVC, direct viable count; ph, phylotype; VBNC, viable but non-culturable.

and change to a round cell shape are also considered as bacterial strategies to survive in the environment (Novitsky & Morita, 1976; Rollins & Colwell, 1986; Ruiz *et al.*, 2001), as well as the induction of a viable but non-culturable (VBNC) state (Oliver, 2005; Roszak & Colwell, 1987) that constitutes a distinct physiological state from that of the starvation response (Heim *et al.*, 2002). *R. solanacearum* becomes VBNC by exposure to copper (Grey & Steck, 2001) and after incubation at low temperature in soil and water (Caruso *et al.*, 2005; van Elsas *et al.*, 2000, 2001; van Overbeek *et al.*, 2004). Adaptations to oligotrophy have not previously been described for *R. solanacearum*.

The occurrence of disease outbreaks linked to the presence of *R. solanacearum* phylotype (ph) II biovar (bv) 2 in European waterways (Caruso *et al.*, 2005; van Elsas *et al.*, 2000, 2001), together with the existence of European Union (EU) Directives banning irrigation of host plants with contaminated water (Anonymous, 1998, 2000, 2006), makes the establishment of *R. solanacearum* in non-host environments a matter of concern. As the life of *R. solanacearum* in water is still little known, this work aimed to investigate the survival mechanisms exhibited by ph II bv 2 of this pathogen in response to nutrient deprivation in environmental water microcosms over 4 years.

METHODS

Bacterial strains and culture conditions. Strain IVIA-1602.1 of *R. solanacearum* ph II bv 2, isolated from potatoes with brown rot symptoms in Spain, was used in all experiments, and the Dutch strain IPO-1609 (also ph II bv 2) (van Elsas *et al.*, 2001), kindly provided by J. D. van Elsas, was included for comparison. Strains were kept at $-80\text{ }^{\circ}\text{C}$ in a 30% (v/v) glycerol medium and were grown on non-selective Yeast Peptone Glucose Agar (YPGA) (Rid , 1969) with filter-sterilized glucose, for 72 h at $29\text{ }^{\circ}\text{C}$.

Characteristics of water samples. Four river water samples were collected from different sites in Spain (Caruso *et al.*, 2005), and each of them was separately used for survival experiments. Mineral nutrient concentrations ranged as follows, depending on the water sample (values per litre): Na, 5.7–12.2 mg; K, 1.4–1.6 mg; Mg, 3.5–3.8 mg; Ca, 10.3–12.2 mg; Fe, 0.21–0.25 mg; Mn, 0.04–0.06 mg; NO_3^- , 4.5–6.8 mg, and P_2O_5 , 0.24–0.36 mg. Organic matter contents estimated by biochemical oxygen demand were $<1.8\text{ mg O}_2\text{ l}^{-1}$; pH values of the different water samples were around neutrality, from 6.5 to 7.3.

Preparation of water microcosms. Survival experiments were conducted similarly to Caruso *et al.* (2005). For each of the four water samples, three microcosms were prepared. Each microcosm was composed of 200 ml river water filtered through 0.22 μm pore-size filters, autoclaved, and then inoculated with strain IVIA-1602.1 or IPO-1609 in PBS at pH 7.2, to give a final concentration of 0.5% (5×10^6 c.f.u. ml^{-1}). After inoculation, the microcosms were incubated for 4 years at $24 \pm 1\text{ }^{\circ}\text{C}$. This temperature was selected because it was the nearest to the optimum for *R. solanacearum* growth that had been registered in some Spanish rivers in warm months (Caruso *et al.*, 2005).

Bacterial cell counts. Microscopic counts for total and viable *R. solanacearum* cells and plate counts for culturability were performed from each microcosm as described previously ( lvarez *et al.*, 2007) at inoculation time (day 0) and days 1, 2, 4, 8, 14, 28 and 40; also at 2, 3

and 6 months, and every 6 months up to 4 years of incubation. For total and viable cell counts, aliquots from the microcosms were incubated with yeast extract and nalidixic acid according to a direct viable count (DVC) method (Kogure *et al.*, 1979), for 16 h (van Elsas *et al.*, 2001) except with long-starved cells, for which the incubation was prolonged up to 72 h. Cells were fixed with 2% (v/v) formaldehyde and stained with 0.1% (w/v) acridine orange (Oliver, 1987). Long-starved cells were additionally stained by indirect immunofluorescence (Anonymous, 1998, 2006), with the polyclonal antiserum 1546-H IVIA against *R. solanacearum* (Caruso *et al.*, 2005), to determine possible changes in surface antigens. Viable cells were also measured by the Live/Dead BacLight Bacterial Viability kit (Molecular Probes) in some of the assays. After mixing cells and dyes (SYTO 9 and propidium iodide) bacterial cells can be counted as alive or dead in terms of membrane integrity (Boulos *et al.*, 1999). Mixtures were incubated in the dark at $24 \pm 1\text{ }^{\circ}\text{C}$ for 15 min, and up to 1 h in the case of long-starved cells. Bacterial cells were visualized with a Leica epifluorescence microscope at a magnification of $\times 1250$ and counted in at least 20 random fields. For culturability, plate counts on YPGA were done after at least 72 h at $29\text{ }^{\circ}\text{C}$.

Cell size and shape. Microcosm aliquots were taken at some sampling times and cells were directly fixed and stained either by immunofluorescence or with acridine orange as described above, without being incubated for viability tests. *R. solanacearum* cell morphology was observed with a Nikon Eclipse E800 microscope at a magnification of $\times 1000$. Pictures were taken with an adapted digital camera using ACT-1 for DXM1200 software, and no processing of the images was performed. Cell size was determined according to the software Image-Pro Plus (Media Cybernetics). For each sample, at least 300 cells were measured in length and width, and cell volumes were calculated.

In planta pathogenicity assays

Stem inoculation. Microcosm aliquots were stem-inoculated in non-axenic tomato plants (*Lycopersicon esculentum* ‘Roma’) susceptible to *R. solanacearum*, at time zero, 1, 2, 3 and 6 months, 1 year, and then every 6 months up to 4 years of microcosm incubation. Inoculations were performed by injecting into the stem 10 μl directly taken from the water microcosms during the first year, and thereafter with 50 μl . Groups of 14 plants grown in greenhouse conditions from seeds tested to be *R. solanacearum*-free were used. All stem inoculations were done with aliquots from strain IVIA-1602.1-inoculated microcosms from the four water samples, in triplicate. For comparative purposes, assays with aliquots from strain IPO-1609-inoculated microcosms were also performed. In all cases, PBS and a fresh bacterial suspension of either strain IVIA-1602.1 or IPO-1609 as negative and positive controls were used. During incubation in a growth chamber ($26\text{ }^{\circ}\text{C}$, 16 h day; $22\text{ }^{\circ}\text{C}$, 8 h night; 70% relative humidity) under quarantine conditions, inoculated plants were periodically monitored for up to 6 weeks for appearance of symptoms and disease development. Stems from wilted plants were cut at different levels above the inoculation point and segments of 2–3 cm were analysed for presence of *R. solanacearum*. Extracts were plated onto a modified semiselective medium South Africa (SMSA) agar (Elphinstone *et al.*, 1996) and colonies were PCR-tested (OL11 Y2 primers) as described in EU Directives (Anonymous, 1998, 2006). Stems from inoculated non-wilted plants were processed in the same way after 6 weeks.

Watering inoculation. Volumes of 20 ml from the microcosms were used after one-fold dilution, to water groups of 10 ‘Roma’ tomato plants at time zero and after 2 and 4 years of microcosm incubation. Assays were performed with water microcosms inoculated with either strain IVIA-1602.1 or IPO-1609. Controls, monitoring and analyses of plants were as described above.

Statistical analysis. Each survival and pathogenicity assay was done independently with *R. solanacearum*-inoculated microcosms from four different water samples in triplicate. For survival experiments, log-transformed data of total, viable and culturable *R. solanacearum* cell counts were analysed by using mean values. Significant differences were assessed by variance analysis (ANOVA). Factors considered for the analysis were: water sample, time of incubation and bacterial strain. For pathogenicity experiments, results were analysed by the Fisher's exact test for categorical data to study possible differences between the two inoculation methods, the two bacterial strains and among the four water samples. In all cases, differences were recorded as significant at $P < 0.05$.

RESULTS

Effect of long-term starvation on *R. solanacearum* survival in environmental water microcosms

Within 1, 2, 3 and 6 months, and approximately by the first year post-inoculation in river water microcosms at 24 °C, total *R. solanacearum* strain IVIA-1602.1 populations remained at approximately $2\text{--}6 \times 10^7$ cells ml^{-1} (Fig. 1). In this period, metabolically active cells measured by the DVC method remained at around $1\text{--}2 \times 10^7$ cells ml^{-1} (Fig. 1), with comparable values obtained by the Live/Dead kit, and culturability ranged between 3 and 8×10^6 c.f.u. ml^{-1} , depending on the water sample (Fig. 1). During the second,

third and fourth year, the numbers of total bacterial cells slightly increased up to values from 9×10^7 to 2×10^8 cells ml^{-1} (Fig. 1). In this period and until 30–36 months post-inoculation, viable *R. solanacearum* cells measured by the DVC method and Live/Dead kit also increased, remaining at levels around 0.5–1 log units below the total cell numbers. From 30–36 to 48 months, decreases in nutrient-responsive cells to values around $1\text{--}3 \times 10^6$ cells ml^{-1} were observed (Fig. 1), while Live/Dead counts revealed roughly $3\text{--}6 \times 10^7$ cells ml^{-1} retaining intact membranes, depending on the water sample. Culturable *R. solanacearum* populations declined from approximately the first year post-inoculation to the end of the experiments, reaching values of 8×10^3 to 4×10^4 c.f.u. ml^{-1} (Fig. 1). For each water sample, significant differences ($P < 0.05$) were observed among total, viable and culturable counts, although trends in total, viable and culturable populations among the four water samples remained similar ($P > 0.05$) (Fig. 1). The stronger decreases in culturability with respect to viability ($P < 0.05$) in all waters assayed indicated the entry of some of the bacterial population into the VBNC state. On plates, the colonies of *R. solanacearum* that were still culturable had a typical smooth morphology with a normal size.

In the comparative experiments using the IPO-1609 strain, survival curves showed trends similar to those of strain IVIA-1602.1 ($P > 0.05$) (data not shown).

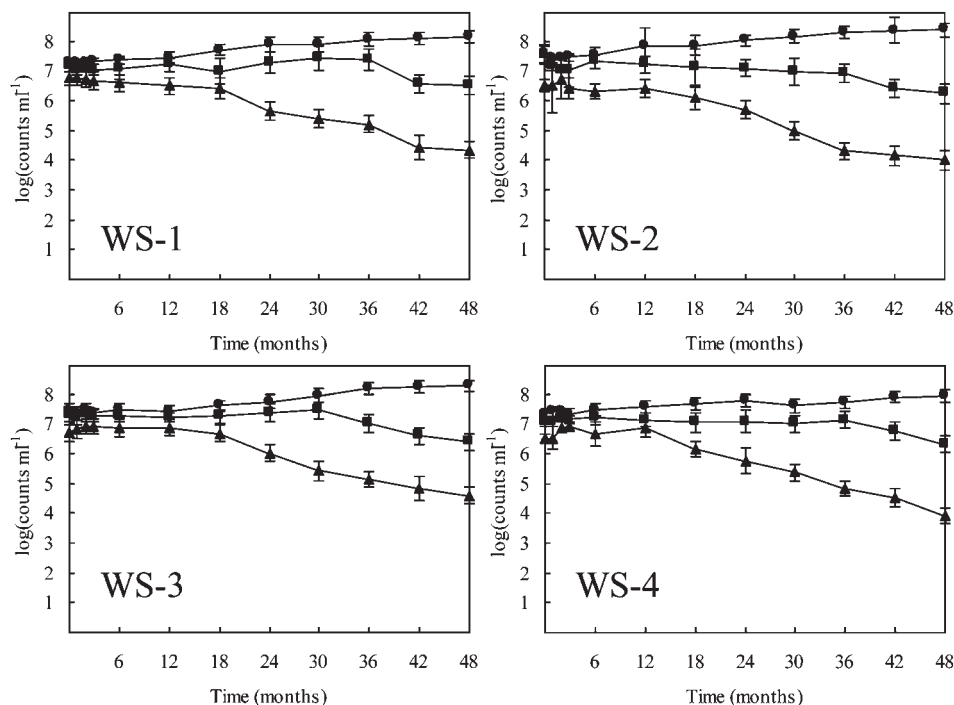


Fig. 1. Survival curves of *R. solanacearum* ph II bv 2 strain IVIA-1602.1 over 4 years in river water microcosms at 24 °C: WS-1, water sample 1; WS-2, water sample 2; WS-3, water sample 3; WS-4, water sample 4. The graphs show total (●) and viable (■) cells, and colony-forming units on YPGA (▲). Points represent means \pm SD.

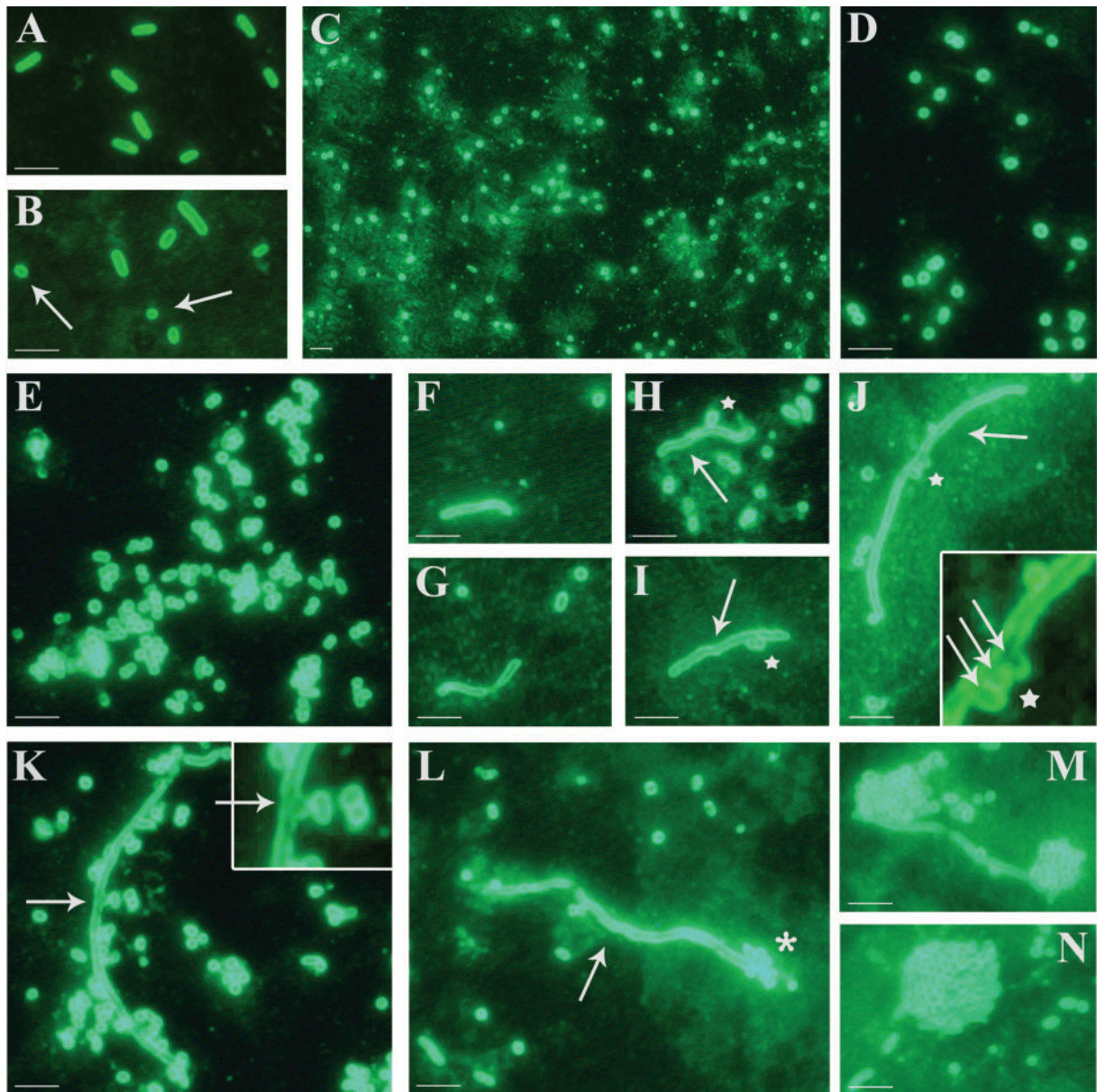


Fig. 2. Microscopic observations of the changes in morphology of *R. solanacearum* ph II bv 2 strain IVIA-1602.1 under starvation in river water microcosms at 24 °C. (A) Freshly grown cells, with typical bacillar form. (B) One-month starved cells; co-existence of bacillar and coccoid forms (arrows). (C) Four-year starved cells; majority of coccoid forms. (D) Detail of coccoid forms in the fourth year. (E) Aggregates of coccoid forms. (F) Filamentous cells formed individually or (G) in pairs. (H, I, J) Suspensor cells (arrows) with buds (stars), either one (H), two (I) or more (J) per cell. (K) Suspensor cell developing a bud (arrow) surrounded by coccoid forms and small aggregates. The insets in J and K (higher magnifications of the arrowed areas) show basal parts of developing buds lacking a cell wall (arrows). (L) Coccoid forms at the extremities of a suspensor cell (arrow), either as incipient aggregates (asterisk) or (M) developing aggregates. (N) Detail of a well-formed aggregate. Bacteria were visualized at a magnification of $\times 1000$ after specific immunofluorescence staining. Scale bars, 2.5 μm .

Effect of long-term starvation on *R. solanacearum* cell morphology in environmental water microcosms

Freshly growing cells of *R. solanacearum* strain IVIA-1602.1 have a bacillar form (Fig. 2A). However, from the first days

of starvation and depending on the water sample, coccoid forms were observed up to 1.7 %, progressively increasing to 7–10 % by the first week and to 25–30 % by 1 month (Fig. 2B). By 1 year, coccoid forms had increased to about 40–50 %, by the second year to 60–70 %, and after 4 years

to 80–90% (Fig. 2C, D), with a general tendency to aggregate (Fig. 2E). At inoculation time cells had the following sizes (mean \pm SD): length, $1.5 \pm 0.3 \mu\text{m}$; width, $0.5 \pm 0.1 \mu\text{m}$; and volume, $0.20 \pm 0.07 \mu\text{m}^3$. After 4 years the cells had reduced in size, showing a mean diameter of $0.65 \pm 0.2 \mu\text{m}$ and a mean volume of $0.14 \pm 0.08 \mu\text{m}^3$. Volume reduction ranged from 18 to 77%.

During the long-term starvation, filamentous, elongated forms of *R. solanacearum* were observed (Fig. 2F, G). Some longer filamentous cells showed localized expansions or buds which maintained a cell wall, as evidenced by immunofluorescence (Fig. 2H–J). Cells of this type have been referred to as suspensor cells (Thomas *et al.*, 1999). Whereas individual filamentous cells were observed to form buds along the cell (Fig. 2H–J), sometimes surrounded by coccoid aggregates (Fig. 2K), filamentous cells in pairs had the budding points at their extremities (Fig. 2L, M). Well-formed aggregates were, at

least superficially, composed of coccoid forms (Fig. 2N). Acridine orange staining showed that filamentous cells, coccoid forms, buds and aggregates contained nucleic acid. In comparative experiments, strain IPO-1609 similarly changed shape, reduced size and filamented.

Effect of long-term starvation in environmental water microcosms on *R. solanacearum* pathogenicity and water-borne transmission

Cells of *R. solanacearum* strain IVIA-1602.1 starved in microcosms from different water samples retained disease-inducing capacity up to the fourth year, as shown in Table 1. As expected, wilting percentages were significantly ($P < 0.05$) higher by stem inoculation than by watering, except at time zero (Table 1). For stem inoculation, in general and regardless of the time period for which cells were under starvation, tomato plants started wilting at around 1 week, with a similar pace of symptom

Table 1. *In planta* pathogenicity assays of *R. solanacearum* ph II by 2 strain IVIA-1602.1 long-starved in river water microcosms, on tomato plants 'Roma'

Water microcosms were from: WS-1, water sample 1; WS-2, water sample 2; WS-3, water sample 3; and WS-4, water sample 4. Control plants inoculated with *R. solanacearum* strain IVIA-1602.1 yielded 100% wilting in stem inoculation and 93–100% wilting in watering inoculation. Plants inoculated with PBS were negative. Data represent mean values from triplicate assays. Standard deviation intervals: for *R. solanacearum* plant inocula $\pm 0.16 \log(\text{c.f.u. per plant})$ and $\pm 0.05 \log(\text{c.f.u. per pot})$; and for wilting $\pm 3.34\%$ in stem inoculation and $\pm 5.95\%$ in watering inoculation.

Starvation period (months)	<i>R. solanacearum</i> -inoculated river water microcosms from:							
	WS-1		WS-2		WS-3		WS-4	
	Stem inoculation							
	Inoculum (c.f.u. per plant)	Wilting (%) [*]	Inoculum (c.f.u. per plant)	Wilting (%) [*]	Inoculum (c.f.u. per plant)	Wilting (%) [*]	Inoculum (c.f.u. per plant)	Wilting (%) [*]
0	5.50×10^4	100	2.09×10^4	100	5.13×10^4	100	3.24×10^4	100
1	5.62×10^4	100	2.00×10^4	100	6.76×10^4	100	3.39×10^4	100
2	5.37×10^4	98	3.16×10^4	100	8.13×10^4	100	7.76×10^4	100
3	4.47×10^4	95	1.59×10^4	98	8.51×10^4	98	7.94×10^4	98
6	3.98×10^4	98	1.26×10^4	100	7.59×10^4	98	4.47×10^4	98
12	3.16×10^4	95	1.59×10^4	100	7.76×10^4	98	6.92×10^4	93
18	1.26×10^5	100	5.00×10^4	91	2.34×10^5	100	6.90×10^4	88
24	2.19×10^4	95	2.51×10^4	83	5.25×10^4	93	2.95×10^4	91
30	1.20×10^4	93	5.00×10^3	88	1.35×10^4	86	1.20×10^4	86
36	8.10×10^3	86	1.00×10^3	93	4.16×10^3	95	3.31×10^3	81
42	1.32×10^3	88	7.05×10^2	93	2.40×10^3	88	1.62×10^3	83
48	1.10×10^3	83	5.00×10^2	95	2.00×10^3	91	4.00×10^2	81
	Watering inoculation							
	Inoculum (c.f.u. per pot)	Wilting (%) [†]	Inoculum (c.f.u. per pot)	Wilting (%) [†]	Inoculum (c.f.u. per pot)	Wilting (%) [†]	Inoculum (c.f.u. per pot)	Wilting (%) [†]
0	1.10×10^7	93	4.18×10^6	87	1.03×10^7	93	6.48×10^6	90
24	8.74×10^5	73	1.00×10^6	70	2.10×10^6	53	1.18×10^6	67
48	4.38×10^4	47	2.00×10^4	40	8.00×10^4	20	1.60×10^4	17

^{*}Value corresponding to 100% = 42 tomato plants (14 \times 3 sets of plants).

[†]Value corresponding to 100% = 30 tomato plants (10 \times 3 sets of plants).

development with respect to the positive controls. At approximately 2 weeks post-inoculation, about 25–40 % of the inoculated tomato plants were wilting and by 3 weeks 80–100 % showed severe disease (Table 1); the pathogen was reisolated from all of them on SMSA, and PCR-identified. On plates, colonies of *R. solanacearum* were typically smooth. The inoculated tomato plants remaining nonwilted after 6 weeks (up to 20 %) were also processed, and the bacterium was detected in about 70 % of them at distances 2–16 cm from the inoculation point, proving the infectivity and spread of the inoculum. Differences observed among the four water samples were not significant ($P > 0.05$) (Table 1).

In watering inoculation assays, *R. solanacearum* strain IVIA-1602.1 starved cells caused wilting of the tomato plants within 6 weeks after plant inoculation, even 4-year-starved cells (17–47 %; Table 1). Appearance of symptoms was roughly at 2 weeks post-inoculation, with an irregular pace of disease spread in the sets of plants inoculated with the different microcosm samples (data not shown). The pathogen was reisolated and identified from the wilted plants, and colonies grown on SMSA were smooth. In both stem and watering inoculations, negative control plants did not show any disease symptoms.

Comparative pathogenicity assays performed with the Dutch IPO-1609 strain yielded similar results in stem inoculation ($P > 0.05$) (76–91 % of the tomato plants wilted when inoculated with 4-year-starved cells, depending on the water sample). This strain was also capable of water-borne transmission of the disease, although to a lesser extent (10–23 % of the plants by the fourth year, depending on the water sample).

DISCUSSION

R. solanacearum ph II by 2 survival strategies during long-term exposure to oligotrophic conditions were investigated in environmental water microcosms. To our knowledge, this is the longest monitoring of *R. solanacearum* behaviour in a non-host environment, and the results support the great potential for persistence of this pathogen, retaining its disease-inducing capacity.

R. solanacearum was able to survive under conditions of extended nutrient limitation in sterile river waters, exhibiting diverse survival mechanisms. One of them was a starvation response consisting of maintaining populations in a non-growing but culturable state, as commonly described for other bacterial species (Morita, 1997) but not for *R. solanacearum* as such up to now. Previous studies on survival of the pathogen were performed in sterile pure water (Kelman, 1956; van Overbeek *et al.*, 2004; Wakimoto *et al.*, 1982) or sterile natural water only for several months (Kelman, 1956; van Elsas *et al.*, 2001) to a maximum of 1 year (Tanaka & Noda, 1973). During the starvation period in river water microcosms, proportions of the viable *R. solanacearum* populations progressively lost the ability to

form colonies on plates, as already observed in starved bacteria (Besnard *et al.*, 2000; Colwell & Huq, 1994), becoming VBNC as another survival strategy under prolonged oligotrophy. Nutrient deprivation has been recognized as an important stimulus to induce the VBNC state, which represents a common strategy for bacterial survival in energy-deficient environments (Colwell & Huq, 1994; Oliver, 2005). Starvation as a VBNC-inducing factor has not been reported previously for *R. solanacearum*.

Increase in the total and viable *R. solanacearum* population during long starvation could be due to the occurrence of cell division, observed for other bacterial species under nutrient deprivation (Byrd, 2000) and considered a survival strategy for bacteria facing adverse environmental conditions (Byrd, 2000; Morita, 1997; Roszak & Colwell, 1987). Multiplying the number of individuals raises the probability of one surviving cell, enabling dissemination and colonization of new ecological niches, which implies a selective advantage for diversification of a bacterial species (Morita, 1997). These cell divisions might have been produced by cryptic growth (Roszak & Colwell, 1987) at the expense of cell debris resulting from the degradation of non-viable cells in the water microcosms. For most of the 4 years of our study, many starved *R. solanacearum* cells retained membrane permeability and proved to be nutrient-responsive, being capable of protein and nucleic acid synthesis as reported for other bacteria (Kogure *et al.*, 1979). Only towards the end of the period, the appearance of starved cells retaining intact membranes (according to Live/Dead staining) but being unable to metabolize the added substrate (according to DVC determination) might indicate that metabolic response is likely to be a more stringent viability criterion than membrane integrity, as suggested by Boulos *et al.* (1999).

During long starvation, *R. solanacearum* bacilli underwent a progressive transition to coccoid forms as observed in other bacteria (Chaiyanan *et al.*, 2007; Novitsky & Morita, 1976; Rollins & Colwell, 1986; Ruiz *et al.*, 2001). Cell volume progressively decreased without significant antigenic change, since cells were readily immunostained, similarly to 2-year-starved *Vibrio cholerae* (Chaiyanan *et al.*, 2007). A round cell shape and size reduction are assumed to be survival responses as a result of cell divisions, to minimize cell maintenance and better capture scarce nutrients (Chaiyanan *et al.*, 2007; Morita, 1997; Roszak & Colwell, 1987). In *R. solanacearum*, size reduction and rise in total and viable cell populations could have been caused by reductive divisions, as commonly reported under long starvation (Byrd, 2000; Morita, 1997; Roszak & Colwell, 1987). That represents a survival advantage, since a higher number of small and round cells may penetrate narrower and deeper sites looking for food and protecting themselves from predators (Byrd, 2000; Morita, 1997), favouring species perpetuation. The increase in numbers might have been also produced by filamentation and budding phenomena. *R. solanacearum* filamented under starvation conditions,

as reported for *Escherichia coli* (Wainwright *et al.*, 1999) and *Pseudomonas oleovorans* (Ruiz *et al.*, 2001). Some of the *R. solanacearum* filamentous cells were observed to have cell wall evaginations or buds, similarly to starved *Campylobacter jejuni* (Lázaro *et al.*, 1999; Thomas *et al.*, 1999) and *V. cholerae* (Chaiyanan *et al.*, 2007); these budded cells are considered as transients in coccoid formation (Thomas *et al.*, 1999). *R. solanacearum* filamentous cells were visualized by immunostaining as well as bacilli and coccoid cells, as hypothesized by Thomas *et al.* (1999). This appears to be the first report of filamentation and budding phenomena occurring in a plant-pathogenic bacterium.

Many of the coccoid forms were viable, although non-viable cells were also observed. In our belief and in agreement with Shleeva *et al.* (2002), in *R. solanacearum* VBNC populations there could be a co-existence of cells viable enough to be resuscitated and of cells just dying off. Like other bacterial species (Colwell & Huq, 1994; Chaiyanan *et al.*, 2007; Morita, 1997), *R. solanacearum* coccoid forms showed a tendency to aggregate under starvation, which can be considered a survival mechanism, since cell aggregation enables utilization of the content of lysing cells and protects the cells from environmental hazards such as free radicals, toxins and predators (Blat & Eisenbach, 1995).

R. solanacearum retained virulence during the 4 years of nutrient scarcity in river water microcosms. It had previously been reported to be able to wilt host plants of 132 days of incubation in ultrapure water (van Overbeek *et al.*, 2004). The similar disease incidence on plants stem-inoculated with *R. solanacearum* either starved (from microcosms) or freshly grown (from controls) during the 4 years demonstrated the great capacity of this pathogen to cause disease. Interestingly, bacterial wilt was also observed in host plants after watering with long-starved *R. solanacearum*, which constitutes a more natural way of transmission of this pathogen. Therefore, a hypothetical water-borne dissemination of disease-causing *R. solanacearum* after extended periods of starvation in environmental waters should not be ruled out, as suggested by field studies (Caruso *et al.*, 2005; Hong *et al.*, 2005).

Oligotrophy is the fundamental factor limiting bacterial survival in open aquatic systems (Morita, 1997), but there are other environmental factors playing a role. The influence of temperature, incident light, presence of sediment, seawater salts and aquatic microbiota on *R. solanacearum* populations has been monitored, also in water microcosms (Álvarez *et al.*, 2007; van Elsas *et al.*, 2001; van Overbeek *et al.*, 2004). Among these factors, only temperature has been assessed in natural water systems, causing a seasonal variation of *R. solanacearum* populations in temperate areas (Caruso *et al.*, 2005; Hong *et al.*, 2005) which did not prevent the survival of the pathogen, even in the presence of aquatic micro-organisms.

Although this work was performed under natural oligotrophic conditions in a confined system, the great

ability observed for *R. solanacearum* to persist and cause disease is in agreement with studies in open natural waters, where the bacterium was detected for several consecutive years (Caruso *et al.*, 2005; Hong *et al.*, 2005). Prior to our study, the strategies used by starved *R. solanacearum* ph II bv 2 to survive until contact with the next host were quite unknown. This work revealed a high capacity of this pathogen to adapt itself to prolonged nutrient scarcity in natural water microcosms. It displayed an efficient starvation response, maintaining a non-growing but culturable population over 4 years, retaining pathogenicity, but also going into a VBNC state. It increased the number of individuals to favour dispersion, reduced its size to reach smaller places, changed from bacilli to cocci to better capture scarce nutrients and aggregated for protection. Overall, this work provides a broader understanding of the long-term survival of *R. solanacearum* under the oligotrophic conditions characteristic of natural waters, which eventually may improve measures to prevent the dissemination of this devastating pathogen in water systems.

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