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Short communication

Ralstonia (*Pseudomonas*) *solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (*Urtica dioica*)

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Abstract

The population dynamics of the brown rot bacterium *Ralstonia* (*Pseudomonas*) *solanacearum* in surface water of two selected water-areas were monitored over a two-year period. In some cases during summer, high bacterial numbers (up to 10^6 cfu l^{-1}) were observed. In a host plant survey a few plants of stinging nettle (*Urtica dioica*) were found to be a natural host of the bacterium when plants were growing with their roots in contaminated water. The significance of *U. dioica* in the epidemiology of the brown rot bacterium is not yet known and subject to further investigation. Pathogenicity of *R. solanacearum* to stinging nettle (*U. dioica*) and bittersweet (*Solanum dulcamara*) was demonstrated in a greenhouse experiment.

Introduction

Since 1976 (Olsson, 1976) outbreaks of brown rot or bacterial wilt of potato caused by the bacterium *Ralstonia* (*Pseudomonas*) *solanacearum* race 3, biovar 2 have been reported in several Western European countries (Janse, 1996; Stead et al., 1996).

R. solanacearum is a heterogeneous species; five races have been described according to the hosts affected, and five biovars according to their ability to utilize and/or oxidize several hexose alcohols and disaccharides (Hayward, 1991). Race 1 (biovars 1, 3 and 4) is pathogenic to potato, and has a broad host range and is restricted to tropical areas. *R. solanacearum* race 3 has a lower optimum temperature than race 1 and occurs in cool upland areas of tropical regions. In general race 3, the potato race, and biovar 2 are equivalent. Due to its low temperature optimum, race 3 is considered a threat to potato culture in Europe. In comparison with race 1, race 3 strains have a narrow host range; causing disease mainly in potato and tomato. The bacterium also infects natural solanaceous weed hosts like *Solanum dulcamara* (Olsson, 1976), *S. nigrum* (Hayward, 1975), *S. cinereum* (Graham and Lloyd, 1978). Tusiime et al. (1998) reported a number of latently infected non-solanaceous weeds such as *Amaranthus* spp., *Bidens pilosa*, *Galinsoga perviflora*, *Oxalis latifolia*, *Spergula arvensis*, *Rumex abyssinicum*, *Tagetes minuta*, and *Stellaria sennii* in highland Uganda.

The bacterium is often considered a soil-borne vascular pathogen, but is, however, reported to have poor survival ability in soil (Graham et al., 1979). It seems likely, therefore, that the bacterium survives mainly in sheltered sites such as the roots of alternate hosts, infested plant debris, volunteer tubers from earlier crops and perhaps, in some cases, in deeper soil layers protected from antagonism by other micro-organisms (Graham et al., 1979). Experiments of Granada and Sequeira (1983) indicated that *R. solanacearum* can infect the roots of many plants previously considered as non-hosts. In nature the bacterium may survive by continually infecting susceptible plants or by colonizing the rhizospheres of non-host plants.

Under temperate conditions in Australia, Kenya, Sweden and the UK, the bacterium could be detected in field soil for no longer than two years after harvest of potato crops affected by brown rot (Shamsuddin et al., 1979; Harris, 1976; Olsson, 1976; Elphinstone, 1996). Survival and overwintering of *R. solanacearum* in temperate regions appears, therefore, to be more attributed to infection of perennial host plants than with persistence in soil. European weed spp. of several plant families have been identified as potential hosts following artificial inoculation (e.g. *Eupatorium cannabinum*, *Tussilago farfara* and *Ranunculus sceleratus*); although no evidence has been found that they are infected in their natural habitat (Elphinstone, 1996).

In Europe, only two (solanaceous) natural weed hosts of *R. solanacearum* have been reported: the perennial bittersweet (*Solanum dulcamara*) and the annual black nightshade (*Solanum nigrum*). Bittersweet commonly inhabits the edges of waterways. It was reported as a host for the first time in Sweden (Olsson, 1976). An ecological study carried out in the north of the Netherlands along 20 main waterways revealed varying incidences of bittersweet: from few plants to several hundreds of plants per 100 m of waterway (Kempenaar et al., 1998). In the Netherlands and England the presence of the brown rot bacterium in roots and vascular tissue of bittersweet has also been demonstrated (Janse et al., 1997; Elphinstone et al., 1998).

In the Netherlands, brown rot outbreaks appear to be mainly caused by contaminated surface (irrigation) water or by (latently) infected seed potato tubers. Surface water contamination may be associated with effluents from the potato processing industry and municipal water purification plants that handle diseased potatoes (Elphinstone, 1996; Janse, 1996). This is followed by establishment and multiplication of the pathogen in aquatic *S. dulcamara* and subsequent leaching into waterways which could be used for irrigation. Most of the known brown rot cases in the Netherlands could probably be explained by the use of contaminated water for irrigation (Janse et al., 1998). Monitoring over four years in the UK showed that bittersweet plants remained infected from year to year and the viable pathogen could be isolated all through the winter months (Elphinstone et al., 1998).

R. solanacearum is considered a quarantaine organism within the European Union. After the recent outbreaks of brown rot (1995) in the Netherlands, the Dutch Plant Protection Service (PPS) has tested all seed potato lots. In addition to testing potato tubers the PPS conducted an extensive survey of surface water (20,000 duplicated-samples in 1996; 10,000 duplicated-samples in 1997). Sometimes high numbers of bacteria (up to 10^6 cfu l^{-1}) were recorded in surface water but on average, population densities were about 10^3–10^4 cfu l^{-1}. The survey revealed that the bacterium was present in different parts of the country. The areas where the bacterium was present were designated as risk areas and surface water irrigation in those areas is prohibited (Janse et al., 1998).

The objectives of this article are (1) to report the results of surface water surveys in the Netherlands for monitoring ecology and population dynamics of *R. solanacearum* and a weed host survey along contaminated waterways, (2) to report the effect of inoculation of bittersweet and stinging nettle with the brown rot bacterium under controlled conditions, and (3) to describe *U. dioica* as an occasional natural host for *R. solanacearum* race 3.

Materials and methods

Isolation and identification of R. solanacearum

Culturing on a modified semi-selective medium, SMSA (Engelbrecht, 1994; modified by Elphinstone *et al*., 1996), was used for detecting viable cells in soil, surface water, and plant tissue. On this medium the brown rot bacterium formed typical colonies (reddish, irregular and fluidal) which were easily distinguished from other types of bacteria which grew on SMSA. Final colony counts were made after 5–6 days of incubation at 28 °C. Colony-morphology determination was confirmed by a serological test, immunofluorescence microscopy (IF; Anon., 1990) or a polymerase chain reaction (PCR) DNA-test, (Seal et al., 1993). To confirm pathogenicity, tomato seedlings were inoculated with a suspension of a 48 h Nutrient Agar (NA) culture, 10^6 cells ml^{-1} in sterile water (Anon., 1990). Race determination was performed by fatty acid analysis, using a reference library created at the PPS that allows discrimination between races. To determine biovar of *R. solanacearum*, strains were checked for the ability to oxidize three disaccharides (cellobiose, lactose, maltose) and three hexose alcohols (dulcitol, mannitol, sorbitol) (Janse, 1991).

Standard water sampling

For surface water sampling, two sample tubes of 40 ml were filled at each location (at a depth of c. 30 cm), delivered within 24 h (stored in an icebox) to the laboratory, and concentrated by centrifugation (15 min at 7,000*g*). The resulting pellets were resuspended in 0.01 M sterile phosphate buffer (total volume 1.0 ml). Aliquots of 100 µl per sample were spread onto the semi-selective SMSA medium.

Soil sampling

Soil samples were collected by harvesting plants with surrounding soil (c. 500 g). To extract the bacterium from rhizosphere soil, samples of 10 g of soil (without roots) were suspended in 100 ml phosphate buffer and shaken for 1–2 h at 100 rpm (20 °C). Aliquots of 100 µl per sample were spread onto the SMSA-medium and further procedures were followed as described for analyzing water samples.

Population dynamics of R. solanacearum *in surface water*

Two water regions (with heavily and moderately contaminated surface water) selected from three earlier studied areas (Janse et al., 1998) were used to further monitor population dynamics of *R. solanacearum*. Starting in week no. 19 of 1996, the heavily contaminated water region was sampled weekly at 20 defined sample locations (locations were chosen near bittersweet plants). The moderately contaminated water region was sampled weekly until week no. 31 and then every third week at 10 sample locations. Surface water temperature at the time of sampling was also recorded.

Host plant identification

To detect possible natural infection of host plants by *R. solanacearum*, plants were collected and roots were washed in tap water. To determine systemic root infection, root samples and (basal) stem samples of individual plants were surface disinfected with alcohol (70%) and dried on tissue paper. Stem pieces and roots (crushed) were left for at least 30 min in 5 ml sterile phosphate buffer. Aliquots of 100 µl per extract were spread onto the SMSA-medium and further procedures were followed as described for analyzing water samples.

In autumn 1997 a project was initiated to identify possible aquatic and riparian host plants of *R. solanacearum* (see Table 1). Several aquatic and riparian weed species were collected from a single location with contaminated surface water. These weeds, *Bidens frondosa* ($n = 15$), *Lycopus europaeus* ($n = 12$), *Mentha aquatica* ($n = 15$), *Solanum dulcamara* ($n = 12$) and *Urtica dioica* ($n = 14$), had been continuously exposed to the surface water. Water samples were taken to monitor actual presence and level of the brown rot bacterium.

Greenhouse experiments

Bittersweet

Bittersweet stem cuttings (c. 30 cm in length) were rooted for several weeks in tap water. Plastic containers were filled with 25 l of tap water. The container was closed with a lid containing 32 holes. Per container eight rooted stemcuttings were placed individually in a hole, secured with foam rubber. Each container was continuously aerated and fertilized several times (Steiners' solution, Steiner, 1984) during the experimental period.

Table 1. Detection of natural infection of *R. solanacearum* in aquatic and riparian weeds

Weed species	Presence of *R. solanacearum* in	
	Non-sterile roots	Surface sterilized roots or stems
Bidens frondosa ($n = 15$)	0	0
Solanum dulcamara ($n = 53$)	12*	9
Lycopus europaeus ($n = 13$)	0	0
Mentha aquatica ($n = 15$)	0	0
Urtica dioica ($n = 39$)	13	2

**S. dulcamara* plants were mainly tested for systemic infections (see text).

Isolates of *R. solanacearum* from potato (strain PD 2762) and bittersweet (strain PD 3101) were routinely maintained in sterile tap water at room temperature and also lyophilized. To obtain fresh cultures, stock suspensions (from a culture stock maintained in sterile tap water) were grown for 48–72 h at 28 °C on Yeast Peptone Glucose agar (YPG). Also, naturally infested surface water was collected from the heavily contaminated waterway. Stem inoculation of the cuttings was carried out by injecting c. 10–50 µl of a 10^7 cfu ml^{-1} suspension of a 48 h NA culture in sterile 0.01 M phosphate buffer. Inoculations were made in the woody parts of the cutting and in the lowest freshly grown green shoot. Root inoculation was carried out by pouring 25 ml of a 10^{10} cfu ml^{-1} suspension of a 48 h NA culture in sterile 0.01M phosphate buffer per container. In addition, containers of naturally infested surface water (10^4 cfu l^{-1}) were ten-fold diluted in tap water. Each treatment consisted of two replications; eight stem cuttings per treatment were used. The experiment was carried out at 75% RH; 25–23 °C day/night air temperature during a 14 h photoperiod. At the end of the experimental period bittersweet plants were checked for the presence of *R. solanacearum* infection.

Stinging nettle

An exploratory experiment was conducted to examine pathogenicity of *R. solanacearum* to *U. dioica* plants under controlled conditions. Dormant stinging nettle plants (free from *R. solanacearum*) were collected in January 1998 from natural habitats. In a quarantaine greenhouse of the PPS, plants were grown from rhizome cuttings in potting soil at 23–25 °C (75% RH). Inoculation was carried out by pouring 10 ml of a suspension containing 10^7 cfu ml^{-1} of a 48 h NA culture (strain PD 2762) per plant with ($n = 6$) and without ($n = 6$) root injury. Roots were injured by inserting a disinfected knife five times into the soil around the stem.

Results

Identification of isolated R. solanacearum *strains*

R. solanacearum strains isolated from surface water, bittersweet, and stinging nettle proved always to be race 3, biovar 2 using fatty acid analysis and biochemical tests.

Population dynamics of R. solanacearum *in surface water*

In the heavily contaminated water region an increase in contaminated sample locations as well as in total bacterial numbers was recorded at the end of spring (Figures 1a and b). An especially sharp increase was observed when water temperatures rose above 15 °C. This occurred over the three year period in the first weeks of May. During midsummer the bacterium was present, usually in high numbers (on average 10^3–10^4 cfu l^{-1}), at all 20 sample locations. However, the level of contamination varied between the locations. At some of the sample locations considerably high pathogen densities were recorded. These locations will be the focus of future research. During winter the number of brown rot bacteria detected in surface water declined close to or below the limits of detection; although at a few sample locations the bacterium was found in very low numbers until ice formation, at the end of December 1996, and again directly after the ice melted, at the end of January 1997. In the summer of 1997 bacterial numbers decreased sharply during two short periods (week no. 35, at the end of August and week no. 40, at the end of September). At 18 and 15 of the 20 sample locations (for the first and second period, respectively) the presence of the brown rot bacterium declined to undetectable levels (Figure 1a). During the first period, massive blooming of cyanobacteria (mainly *Oscillatoria* spp.) and algae (mainly *Euglena* and *Scenedesmus* spp.) was observed. During the second period, lysis of algal and cyanobacterial cells occured. Similar observations concerning contamination levels were made in the moderately contaminated water region (Figures 2a and b).

Natural host plant identification

At the time of sampling (first week of October 1997) populations of *R. solanacearum* in the surface water had declined to undetectable levels. The bacterium could not be isolated from non-sterile roots of *Bidens frondosa* and *Lycopus europaeus* (Table 1). The pathogen was isolated from all extracts of non-sterile bittersweet roots; in two of these plants a systemic infection was confirmed. No symptoms were observed in any of the infected plants. Furthermore, the brown rot bacterium was detected in nearly all the extracts of non-sterile *Urtica dioica* roots (c. 10^2–10^3 cfu g^{-1} roots). Systemic infections were not found in these

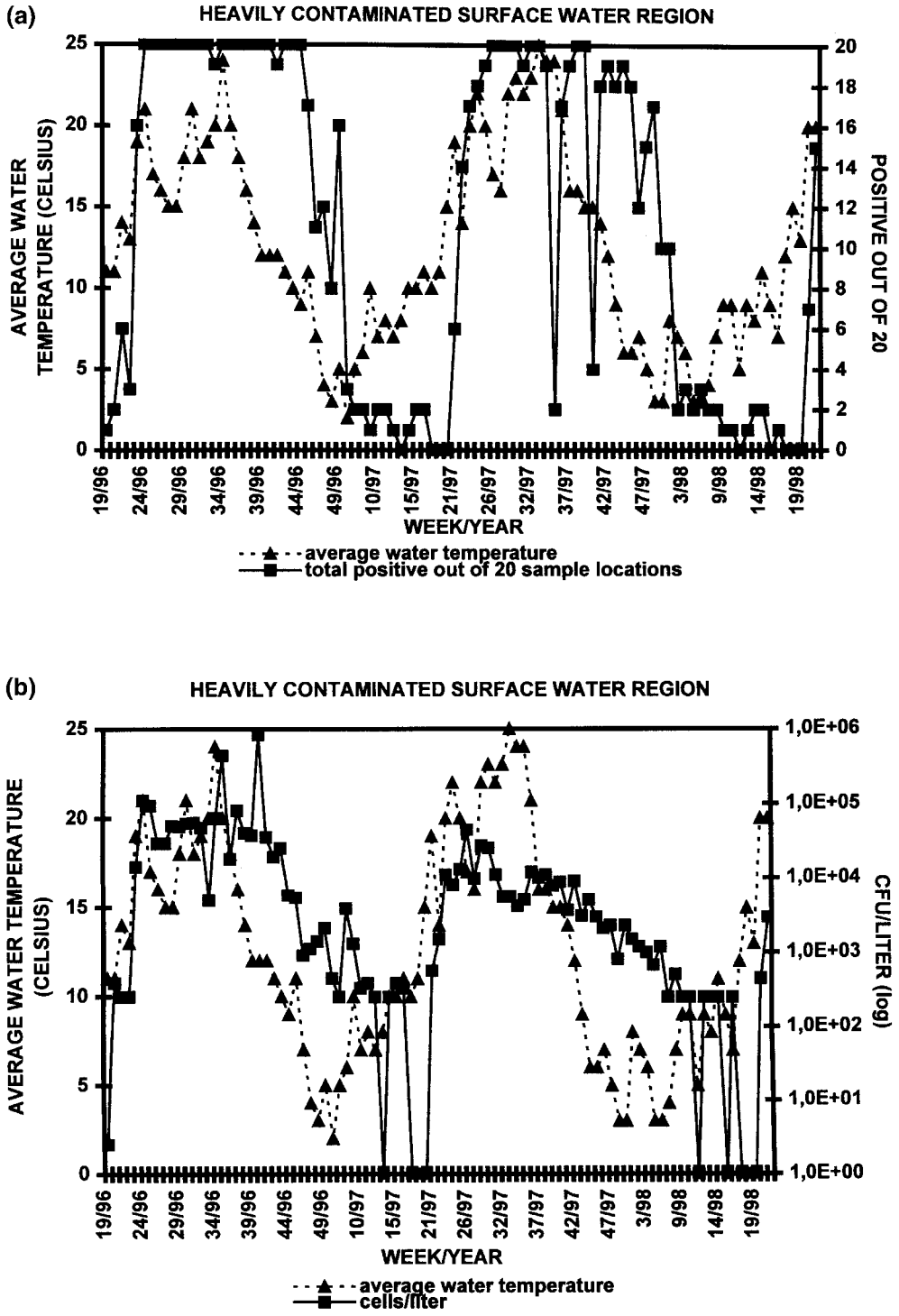

Figure 1. (a) Graph showing number of contaminated surface water locations (out of 20) in a heavily contaminated waterway in relation to the water temperature. (b) Graph showing number of bacterial cells per liter in a heavily contaminated waterway in relation to the water temperature.

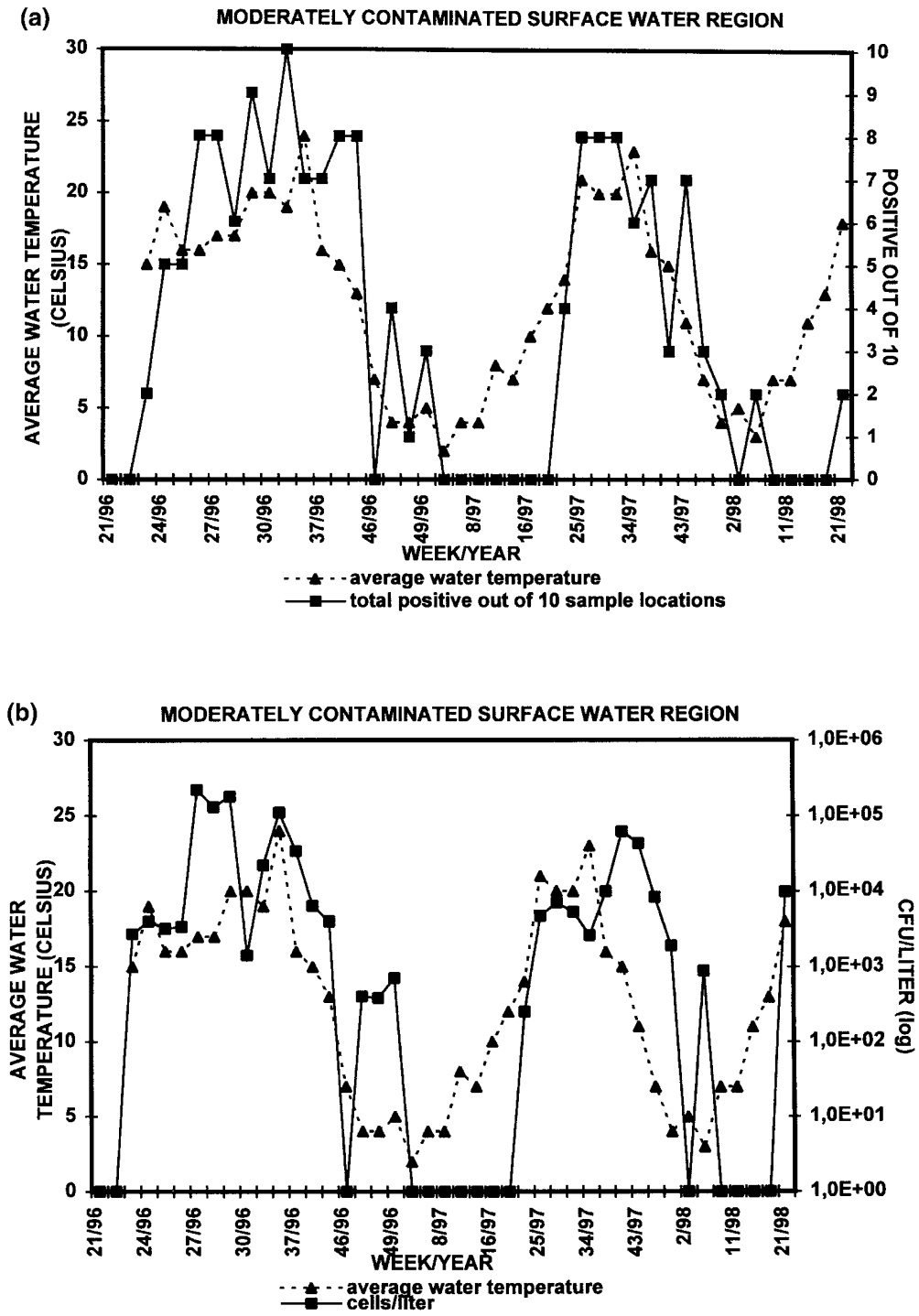

Figure 2. (a) Graph showing number of contaminated surface water locations (out of 10) in a moderately contaminated waterway in relation to the water temperature. (b) Graph showing number of bacterial cells per liter in a moderately contaminated waterway in relation to the water temperature.

plants at that time. The results of this survey indicated that *R. solanacearum* would not infect *U. dioica* roots but may survive in the rhizosphere. However, upon further analysis *U. dioica* plants collected from the heavily contaminated waterway, systemic root infection was detected in one of 15 stinging nettle plants (c. 10^4–10^5 cfu g^{-1} roots). In 1997, seventeen bittersweet plants from this heavily contaminated waterway were tested; in five plants a systemic infection was found.

From three other locations in total 10 stinging nettle plants were collected, growing near or just above the waterline. All plants were collected with rhizosphere soil. In one case the bacterium was found in rhizosphere soil and was also isolated from systemically infected roots of this stinging nettle plant (c. 10^4–10^5 cfu g^{-1} roots). Bittersweet plants ($n = 24$) were also collected from these locations. Two of the bittersweet plants were systemically infected.

Greenhouse experiments

Bittersweet

Stem inoculation. Green shoots inoculated with *R. solanacearum* potato-strain PD 2762 or bittersweet-strain PD 3101 showed severe wilting within one week, eventually resulting in dead shoots. Most of the plants inoculated with strain PD 3101, however, had more than one shoot; shoots not inoculated showed no symptoms and the pathogen was only isolated from these shoots in a few cases, indicating slow or no spreading of the bacterium. In all cases the bacterium could be isolated from the woody stemcutting parts inoculated.

Root inoculation. From the cuttings inoculated with strain PD 2762, two plants showed severe wilting and eventually died. In one cutting a latent infection was found. In the treatments with strain PD 3101 one plant with symptoms and one plant with a latent infection was recorded. No infections were found in the treatments with naturally contaminated surface water (Table 2). Control plants showed normal growth and upon isolation no bacterial infection was found.

Stinging nettle

Within two weeks after treatment all stinging nettle plants with injured roots showed severe wilting; starting with a few leaves with typical wilting symptoms and followed by complete wilting. In addition two plants without root injury wilted. Old wounds from the rhizome cuttings or lateral root formation may have served as infection courts for the brown rot bacterium in these cases. All control plants showed normal growth. Root samples and basal, middle and top stem segments from the wilted plants were analyzed as described previously. Bacterial oozing was observed in buffer solutions containing basal stem parts. The bacterium was isolated from all selected parts of the wilted plants.

Discussion

In the sampled water areas some heavily contaminated locations were observed. Apparently there are some foci of infection in bittersweet (*S. dulcamara*) along these waterways. Waterborne *R. solanacearum* population is expected to show a correlation with distance from infected bittersweet. It has been shown that infected bittersweet plants can release brown rot bacteria into surface water via aquatic roots (Olsson, 1976; Elphinstone et al., 1998); this was confirmed by

Table 2. Number of infected bittersweet plants after root and stem inoculation

	Symptoms*	Latent*	% Infected ($n = 16$)
Root inoculation			
PD 2762	2	1	19
PD 3101	1	1	13
Infested surface water	0	0	0
Stem inoculation			
PD 2762	16	—	100
PD 3101	15	1	100

*Total out of 16 cuttings (eight cuttings per replication).

greenhouse experiments carried out by the Dutch Plant Protection Service (data PPS, unpubl.).

In 1997 the incidence of infected bittersweet plants recorded in and along contaminated waterways was approximately 20%. Under quarantine glasshouse conditions in our experiment an infection rate of 15% was recorded for rooted bittersweet cuttings grown in heavily (artificially) contaminated water. No infections were found in the treatments with naturally contaminated surface water; however, these were at very low bacterium concentrations. Similar observations under natural conditions were made by Elphinstone et al. (1998) when three-week old *S. dulcamara* seedlings, floated in sand-filled polystene boxes in *R. solanacearum*-infested river water, in three different seasons, did not become infected. The overwintering of *R. solanacearum* in soil and bittersweet in the Netherlands is currently under investigation.

Decreasing numbers of contaminated sample locations in the summer of 1997 may have been correlated with algal blooms during the same periods, possibly due to production of toxins (active or after lysis of cells) or hyper oxygenation by microbes. The production of cyanobacterial toxins and antimicrobial substances by algae is well known (Jones, 1988; Codd and Poon, 1988). Interactions between *R. solanacearum* and water micro-organisms like algae, cyanobacteria and predatory protozoa, however, are still poorly understood.

The results of the field survey indicate that *R. solanacearum* can live on and may also systemically infect *U. dioica* roots under natural conditions. Stem infection of *U. dioica* under natural conditions was not proven. Based on these findings *U. dioica* should be regarded as a potential perennial host plant of the brown rot bacterium when its roots are in contact with contaminated surface water. This might have implications for the strategy for eradication of brown rot in the Netherlands. *U. dioica* is very common along arable fields and waterways. The incidence of infected stinging nettle plants in natural habitats has still to be determined, but the preliminary data indicate that this is very low. The role of stinging nettle in overwintering of the brown rot bacterium will be examined this season.

The results of the greenhouse experiments indicate that *R. solanacearum* is able to infect and multiply in *U. dioica* roots and stems. Further research with respect to root colonization, root penetration and latent infection is in progress. In addition, we have demonstrated pathogenicity of the brown rot bacterium for *U. dioica* and *S. dulcamara* under high infection pressure and high temperature.

As stated earlier, many weed species have been found infected with Race 3 after artificial stem inoculation (e.g. *Eupatorium cannabinum*, *Tussilago farfara* and *Ranunculus sceleratus*; Elphinstone, 1996), which implies that *R. solanacearum* Race 3 could act as an opportunistic plant pathogen in some hosts. Therefore the role of weeds other than bittersweet in the increase of *R. solanacearum* in surface water during summer may have been underestimated. In contaminated surface water, roots and stems of aquatic and riparian plants are exposed continuously to the brown rot bacterium during many months, which may increase the probability of opportunistic infections. As long as surface water contains high numbers of brown rot bacteria, this water should be regarded as a continuous source of inoculum. Total eradication of sources of inoculum (i.e. weed hosts) in farming areas will be difficult and in other areas (e.g. nature reserves) may be impossible. More feasible control of brown rot disease might be achieved by local eradication of (potential) hosts, and prohibiting the use of surface water for irrigation or in disinfection of contaminated surface water to be used for irrigation by chemical treatment (Janse, 1996; Wenneker et al., 1998).

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