OFFERED REVIEW

REVIEW ON BROWN ROT (*RALSTONIA SOLANACEARUM* RACE 3, BIOVAR 2, PHYLOTYPE IIB) EPIDEMIOLOGY AND CONTROL IN THE NETHERLANDS SINCE 1995: A SUCCESS STORY OF INTEGRATED PEST MANAGEMENT*

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SUMMARY

The disease brown rot of potato, caused by the bacterium *Ralstonia* (*Pseudomonas*) *solanacearum* (*Rsol*), Race 3, biovar 2 (R3b2), was found for the first time in 1992 in a potato field in The Netherlands and caused an outbreak in the warm summer of 1995 that appeared to be connected to use of contaminated irrigation water as in other outbreaks in western Europe at that time. The Dutch Plant Protection Service (PPS) immediately took action and started to test all traded seed for (latent) infections, applied strict control measures upon positive detections, and started an intensive survey of surface water contamination. In later years the control measures and testing procedures for *Rsol* in different substrates, laid down in an EU Directive, were followed. The PPS also conducted intensive applied research (in cooperation with the University of Wageningen, Plant Research International, and foreign research Institutions, also in the framework of an EU-SMT project on the EU brown rot testing method to unravel the complex epidemiology of the pathogen and to improve its detection and identification. These actions have led over a *ca.* 15-year period to a drastic reduction, actually a functional eradication (only one single finding in 2009/2010 and 2010/2011), of the disease from the production system. A main factor in the combat was a nation-wide irrigation ban for seed potatoes since 2005. The PPS coordinated two EU-funded projects, where the UK, The Netherlands, Belgium and France assisted Egypt in implementing a brown rot safe potato production system. In this project substantial epidemiological research was conducted. This article wants to be a reflection on the work done and the results obtained by the PPS and has a glance into the future, where it will be indicated that the persisting presence of *Rsol* in surface water necessitates an enduring alert and actively maintained control and survey system. The main lessons learned are: stay away, if possible, from surface water; use disease-free (tested) and certified seed; apply strict hygiene; handle/grade and store seed and ware/industry potatoes separately; compensate growers or enable them to insure against the disease; invest pro-actively in emergency plans and in up to date diagnostic expertise, education and advice; maintain an active and statistically meaningful survey and control system; perform a regular survey in ware and industry potatoes, greenhouse host crops and surface water.

INTRODUCTION

Brown rot of potato, a quarantine disease caused by the bacterium *Ralstonia* (earlier: *Pseudomonas*) *solanacearum* (biovar 2, race 3, phylotype IIB), was diagnosed with certainty for the first time in The Netherlands in 1992. It was an isolated case in the south of the country, that later appeared to be connected with earlier infection of surface water and potato in Belgium, reported there in 1989. Due to undetected surface water contaminations, the disease almost certainly slumbered at the end of the 1980s leading to an outbreak in 1995. In the same year surface water contamination was actually determined in the northern Netherlands. Since than, thanks to extensive efforts of the Dutch Plant Protection Service (PPS), Dutch General Inspection Service (NAK), industry and research [Plant Research International or PRI (earlier IPO) and the University of Wageningen (WU)] much has been achieved in tracing and eradication/control of the bacterium by increased knowledge of diagnostics and epidemiology of the disease it causes. This article presents an overview of the measures adopted and the investigations conducted since the 1995 brown rot outbreak and highlights some future policy aspects and developments.

THE BACTERIUM *RALSTONIA SOLANACEARUM*

At the end of the 19th century a severe wilting disease (called 'slime disease') was described in sub-tropical regions on tomato, tobacco, potato (Fig. 1A and B), ba-

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* Paper presented at the 5th International Bacterial Wilt Symposium, Wuhan, China, 2011

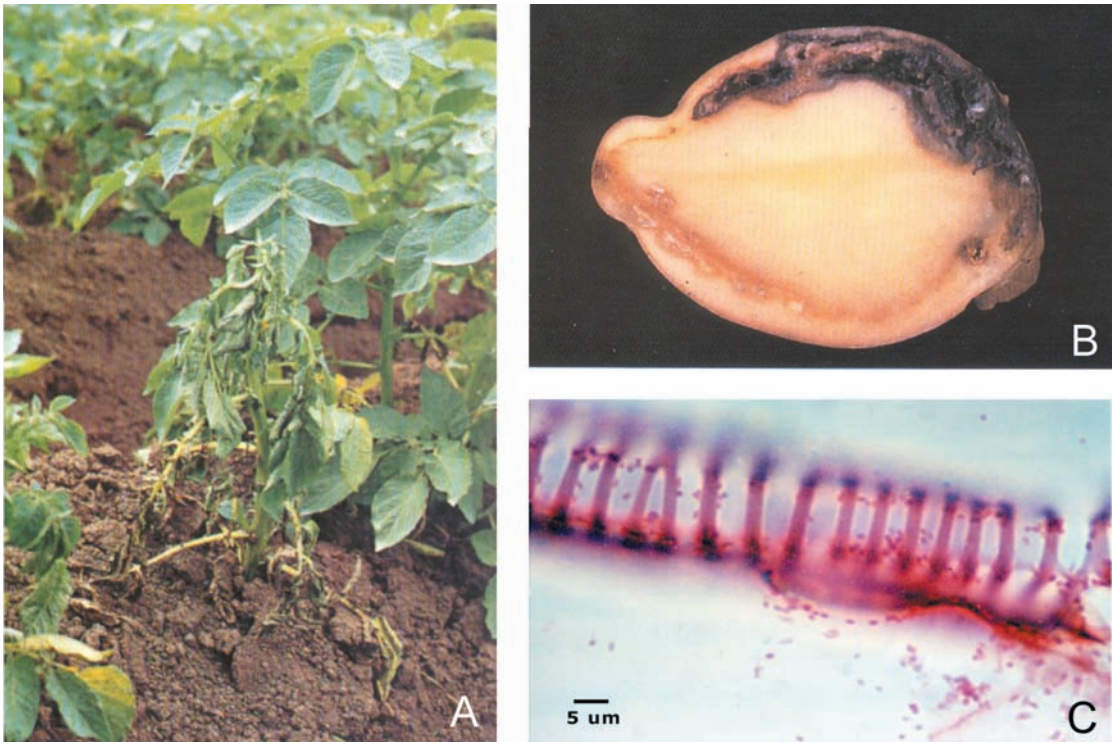


Fig. 1a. Symptoms of brown rot caused by *Ralstonia solanacearum* in potato: rapid wilting due to blocking and destruction of vascular tissues (Copyright: Janse, 2006). **1b.** Symptoms of brown rot caused by *Ralstonia solanacearum* in a potato tuber: light brown vascular discoloration, from the infected vascular tissues cream-white bacterial slime oozes spontaneously. The black tissues are caused by secondary rotting micro-organisms (Copyright: Janse, 2006). **1c.** Small, red colored cells (Gram stain) of *Ralstonia solanacearum* in a spiral vessel (smallest element in vascular tissue) of a potato tuber (Copyright: Janse, 2006).

nana and peanut. The founder of phytobacteriology, the American scientist Erwin F. Smith, proved already in 1896 that the causal organism was a bacterium (named by him as *Bacillus solanacearum*). In 1914, Smith placed this non-spore forming, Gram-negative bacterium (Fig. 1C) in the genus *Pseudomonas*. In the following years, slime disease was found in many different hosts in subtropical regions. Moraes (1947), however, described a variant of the bacterium in Portugal that was better adapted to temperate climatic regions (growth optimum of 27ºC instead of 35ºC). This variant was later found also in other Mediterranean countries, especially Egypt, and in mountainous areas in the tropics. This 'cold' variant and the variant on banana could easily be discriminated on the basis of pathogenicity to different hosts (classification into races) and the use of carbon sources in the laboratory (biochemical varieties or biovars). The tropical variant with a very broad host range was classified by Buddenhagen (1962) as race 1 (from which later race 4 and 5 were separated), the variant specialised on banana and the related *Heliconia* spp. as race 2, and the 'cold' variant with a restricted host range (mainly solanaceae) as race 3. The 'cold' variant (race 3) appeared to belong to biovar 2 (later also named 2A) in the biochemical classification of Hayward (1994), whereas the other races contained biovars 1 and 3-5. A particular biovar (2T or 2N) of race 3 occurs in the Andes, an area in which resistance against race 3 was found in wild potato. This led to the presumption that race 3 was introduced with potatoes from the Andes to the Mediterranean, perhaps during the second world war by allied troops. Race 3, biovar 2 appears to be genetically very homogeneous. Further molecular, biological and taxonomic investigations have enabled a more refined classification of the potato brown rot agent based on restriction fragment length polymorphism (RFLP) and 16S rRNA analysis, and sequence analysis of the endonuclease (*egl*) gene and other so-called household genes (Castillo and Greenberg, 2007; Cook and Sequeira, 1994; Fegan and Prior, 2005, Gabriel *et al*., 2006; Pinghsheng *et al*., 2007; Poussier *et al*., 2000; Saddler *et al*., 1998; Timms *et al*., 2001; van der Wolf *et al*., 1998) following which, the bacterium was placed in the genus *Ralstonia* (Yabuuchi *et al*., 1995).

Based on sequence analysis, four so-called phylotypes can be discriminated: (i) phylotype I, containing strains from Asia; (ii) phylotype II, containing strains from the American continent (IIB holds biovar 2 strains of race 3, whereas IIA comprises other strains, some also from Africa); (iii) phylotype III, holding biovars 1 and 2T from Africa; (iv) phylotype IV, comprising biovars 1,2, and 2T and the closely related *R. syzygii*, a pathogen causing Sumatra disease of clove tree (*Syzygium aromaticum*) in Indonesia and the blood disease bacterium

(BDB), a pathogen of banana in Indonesia. Strains of this phylotype originate from Indonesia, Japan and Australia (Fegan and Prior, 2005).

A new, aggressive variant (phylotype II/4NPB) with many hosts, including *Anthurium*, cucurbitaceae and tomato, was recently described from Martinique (Wicker *et al.*, 2007; see also Wicker *et al.*, 2009; Cellier and Prior, 2010). This phylotype is a threat for European greenhouse cultivations and has already been found in France (Cellier and Prior, 2010). The 'cold' form (Race 3, biovar 2 or R3b2), that occurred/occurs in The Netherlands and many western European countries (Janse, 1996; EPPO, 2012), was shown by recent typing studies to be genetically very homogenous and has been classified up till now as *Ralstonia solanacearum* (*Rsol*) R3b2, phylotype IIB (sequevar 1 and 2). This homogeneity was recently confirmed also from China (Xue *et al.*, 2011).

An overview of the present classification and the complexity of *R. solanacearum* is presented in Table 1 and Fig. 2.

In a recent taxonomic study based on whole genome sequencing (Remenant *et al.*, 2011), the following important taxonomic and nomenclatorial changes have been proposed:

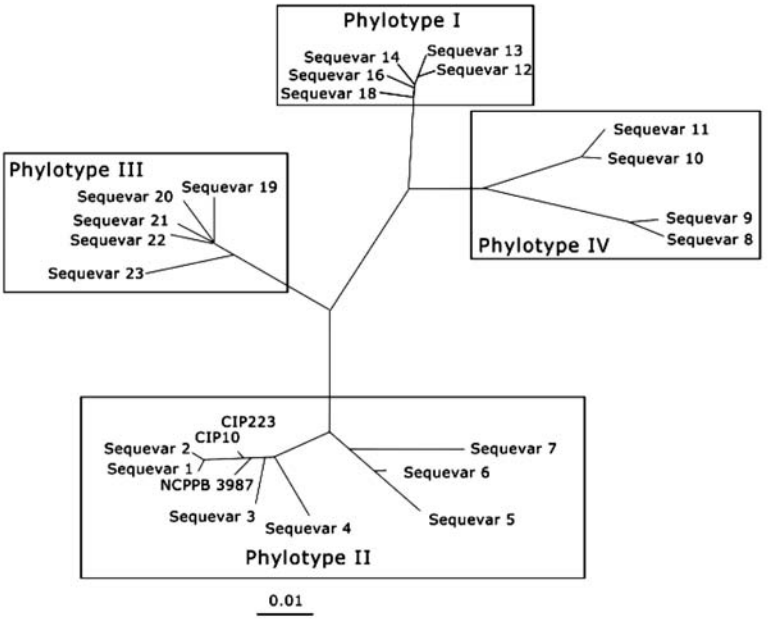


Fig. 2. Classification of *Ralstonia solanacearum* in so-called phylotypes and sequevars (sequence variants) on the basis of partial sequence analysis of the endoglucanase gene. The bar represents 100 nucleotide positions. After Fegan and Prior (2005).

Table 1. Subspecific diversity of *Ralstonia solanacearum* [a]

Race	Biovar	RFLP pattern[b]	Phylotype[c]	Host range	Geographical distribution
1	1	1-7	IIB	broad	South America, USA
1	3	8-14	I	broad	Mainly south-east Asia, South America, Australia, China, some in the USA
1	1 and 2T (N)		III	broad	Africa, Indian Ocean
1	1, 2, 2T (N) and *R. syzygii* of clove tree and Blood disease bacterium (BDB) of banana		IV	broad	Indonesia, Japan, 1 strain in Australia
1	4	11, 15-18, 21-23	I	broad	south-east Asia, China, Australia, some in the USA
1	5	19, 20	I	*Morus alba* (mulberry)	China
2	1	24, 25, 28	IIA and B (banana variant on Philippines IV)	Banana (*Musa* spp.), *Heliconia*	Moko and Bugtok disease strains, S. and Central America, Philippines
3	2 or 2A	26A, B	IIB	restricted	All inhabited continents
3	2 or 2A	27A,B,C	IIB	restricted	West of Andes: Chile, Colombia
Intermediate 1 and 3	2T (2N)	29-33	IIB	restricted	East of Andes: lowlands Brazil, Peru
4	4		I	ginger	Australia, China, Hawaii, India, Japan, Mauritius, south Asia, India

[a] For confirming AFLP and PCR-RFLP of these subdivisions, see Poussier *et al.* (2000) and Horita *et al.* (2005). [b] After Cook and Sequiera (1994). [c] For subdivision in phylotypes and sequevars, see Fig. 2 and Fegan and Prior (2005, 2006), Castillo and Greenberg (2007) (in this paper phylotype IIB is called phylotype IIa), Villa *et al.* (2007), Wicker *et al.* (2007, 2009, 2011), Toukam *et al.* (2009).

Table 2. Number of potato samples tested and number of samples found to be contaminated from 1996-2011.

Category	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Integral sampling*	57.500	67.150	59.700	66.800	66.775	63.091	60.928	62.817	60.700	32.524	28.338	22.472	24.823	22.407	25.193
Number of contaminated samples	9	30	5	62	34	16	14	7	1	1	1	1	1	2	0
% contaminated samples	0,01	0,04	0,01	0,09	0,05	0,03	0,02	0,01	0,002	0,002	0,004	0,004	0,004	0.008	0
Surveys (inc. tracing)	2.850	4.300	4100	5.300	4.573	3463	4339	2682	4965	3339	2222	2880	3081	3000	1273
Number of contaminated samples	15	17	137	60	19	10	40	6	1	1	3	1	14	0	1
% contamniated samples	0,53	0,39	3,34	1,13	0,4	0,3	0,9	0,22	0,02	0,03	0,14	0,03	0,45	0	0.08
Total % contaminated samples	0,04	0,07	0,22	0,17	0,07	0,04	0,08	0,02	0,003	0,006	0,01	0,008	0,05	0.004	0.003

* Integral sampling = NAK certified seed + industrial potatoes certified seed + new clones.

(i) Phylotype I and III strains form a unique genomic species, for which the name *Ralstonia sequeirae* is proposed, with type strain GM I1000,

(ii) Phylotype II strains, including the original *Rsol* type strain K60^T, are maintained as *R. solanacearum*.

(iii) Phylotype IV strains, including those of *R. syzygii* and the BLDB strains, form a genomic species denoted *Ralstonia haywardii* with type strain PSI07. Within this species, the broad host range strains are designated *R. haywardii* subspecies *solanacearum*, with type strain PSI07; the BLDB strains are denoted *R. haywardii* subspecies *celebensis*, with type strain R229; the *R. syzygii* strains, that are insect-transmitted by *Hindola* spp. cercopoids, are designated *R. haywardii* subspecies *syzygii*.

In this article, however, the earlier described taxonomic configuration is maintained.

FIRST FINDINGS AND OUTBREAK IN POTATO IN THE NETHERLANDS IN 1995

In the 1960s, early potatoes were already exported to western Europe from the Mediterranean area, especially Malta, Cyprus and Egypt. In 1961, the UK and Germany reported the presence of brown rot in potatoes originating from these countries, but nobody worried. In 1972, it was demonstrated in Sweden that brown rot infections developed downstream of two potato factories that processed potatoes of Mediterranean origin and dumped untreated potato waste into a river that was used to irrigate potatoes. Research in Sweden also showed that the perennial weed bittersweet (*Solanum dulcamara*) growing along and in waterways, kept the bacterium in the area. In a four-year control program (purification of processing waste, control of bittersweet along the river, no potato cropping in contaminated fields and destruction of infected lots) both disease and bacterium were eradicated (Janse, 1996; Persson, 2008). In the meantime, there was a strong increase in the export of early potatoes from Egypt, in the number of brown rot interceptions in these potatoes, and in irrigatated potato fields (irrigation gives higher yields and controls the potato scab bacterium *Streptomyces scabiei*) in western Europe. In 1989, this caused in Belgium, like in Sweden, an outbreak alongside a canal, downstream of a potato processing factory, and outbreaks were also registerd in The Netherlands, in the middle of the export season of 1995, and in many western European countries (Elphinstone *et al.*, 1998; Janse, 1996). The emerging problems with brown rot, led the Dutch PPS to develop pro-actively a method for detecting latent *R. solanacearum* infections in potato, that was accepted by the European and Mediterranean Plant Protection Organisation (EPPO) and the EU (Janse, 1988), and to investigate the taxonomy of the organism (Janse, 1991). The 1995 outbreak in The Netherlands was confined to a large extent to a heavy infection in some clonal lines of

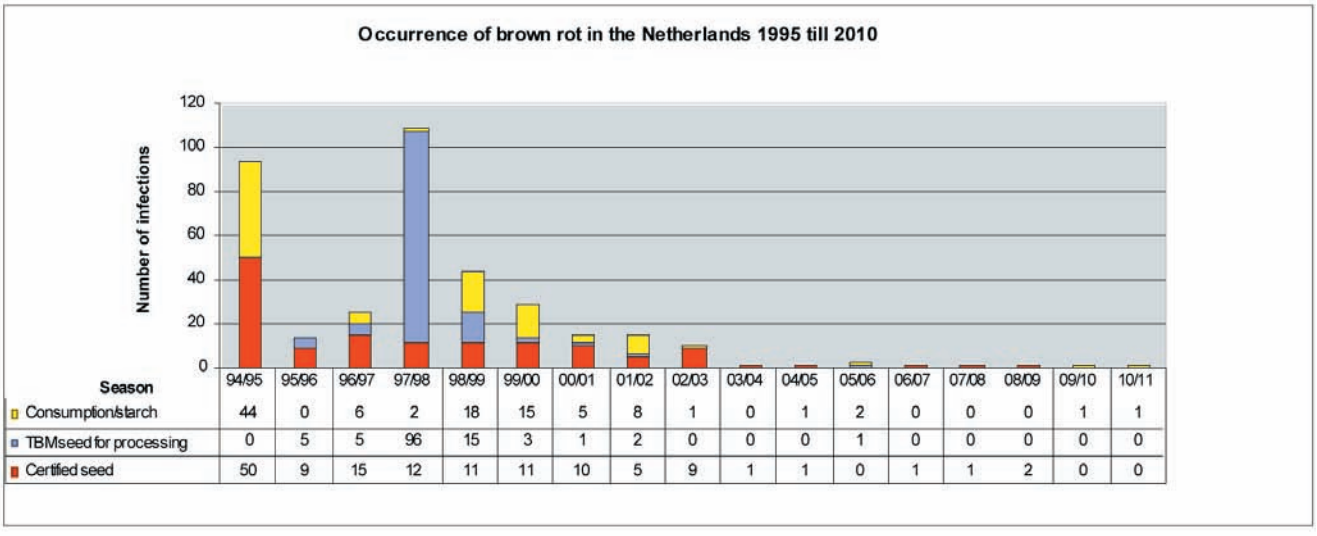
the local cv. Bildtstar, that spread via these clonal lines (seed lots) and contact (agricultural machinery, etc.). The Dutch Government decided, also under pressure of EU member states and obligation by the EU Standing Committee on Plant Health (SCPH), for integral testing of all traded seed. Therefore, in 1995, PPS, the Dutch General Inspection Services (NAK and Naktuinbouw) and The Dutch Technology Development Organisation (TNO) tested *ca*. 55.000 samples (200 tuber samples per each 25 tons of potatoes) in a few months time, discovering 99 infections in 24 different cultivars (Table 2). Since a number of infections could not be traced back to contact or seed, the involvement of surface water was suspected and proven true by the end of 1995 in surface water and bittersweet in different parts of the country (Janse, 1996; Janse *et al.*, 1998).

INTEGRAL TESTING AND CONTROL MEASURES

In order to force back the disease (elimination of infected potato lots/fields from the production column) and keep the export position, all potato seeds, including farm-saved seeds, were tested for a number of years integrally (200 tubers/25 tons). Surveys of consumption and industrial potatoes and monitoring (follow-up of infections found) took also place. This operation was conducted since 1996 by the PPS and NAK. Furthermore, extensive monitoring of surface water was organised and executed. In 2004, when the disease level had decreased substantially, it was decided to test one 200-tuber sample per seed lot, independently of the size of the lot. In 2006, integral testing of seed was outsourced from the PPS to NAK completely, but the PPS still carried out monitoring, surveys, water testing and final testing of bacterial cultures from water and tuber samples. These latter activities (although still under responsibility of the PPS, except for final testing of cultures from tuber samples), have also been outsourced to NAK in 2008.

As an addition to integral testing, following national considerations and obligations stemming from the EU Control Directive for potato brown rot that was issued meanwhile (Anonymous, 1998, 2006), the following general and specific measures were/are taken whenever brown rot is found in a potato lot or field:

- intensive testing for latent infections of all seed produced by the contaminated place of production;
- surveys of industrial and consumption potatoes (including imports);
- destruction of contaminated potato lots by heat (steam) and subsequent feeding to cattle, bio-fermentation with a category 3 fermenter, tunnel composting, processing in PPS-licensed potato processing industry or (incidentally) deep burial;
- lots tested free from a contaminated place of production can only be traded in small (maximum 10 kg) packaging directly to the consumer market or used for industrial processing;
- disinfection of contaminated places of production and strict hygiene;
- control of volunteer potatoes in contaminated fields and no potato growing for 4 years (when the following crop will be for consumption potatoes) or 5 years (when the following crop will be for seed);
- reporting of new records to SCPH in Brussels and, if applicable, to individual member states;
- irrigation ban in areas with known contaminated surface water, as delineated by the PPS:
- tracing of infection sources (clonal relationships between seedlots, contact, irrigation).



Season	94/95	95/96	96/97	97/98	98/99	99/00	00/01	01/02	02/03	03/04	04/05	05/06	06/07	07/08	08/09	09/10	10/11
Consumption/starch	44	0	6	2	18	15	5	8	1	0	1	2	0	0	0	1	1
TBM seed for processing	0	5	5	96	15	3	1	2	0	0	0	1	0	0	0	0	0
Certified seed	50	9	15	12	11	11	10	5	9	1	1	0	1	1	2	0	0

Fig. 3. Number of potato samples found to be contaminated with *Ralstonia solanacearum* in three different categories of potatoes from 1996-2011 (Source: Dutch PPS).

DEVELOPMENT IN THE NUMBER OF BROWN ROT CASES IN THE NETHERLANDS FOLLOWING CONTROL MEASURES ENFORCED

Table 2 and Fig. 3 show the testing activities and brown rot cases found in The Netherlands over the years. From the Table 2 it is clear that in recent years the disease occurred very sporadically (thus justifying the use of the term "functionally eradicated"). The recurrent finding of one or two cases per year, however, also indicates that cultivation of seed potatoes near contaminated surface water is still risky because the bacterium is widespread in surface water from which is most probably impossible to eradicate. This has also been the reason for the integral irrigation ban of surface water for seed potatoes in 2005. Detection of some brown rot cases with very low incidence in recent years, indicate that other factors [e.g. failure of water household management around fields (flooding), drift of irrigation water from neighbouring fields, birds (possibly), tourists, etc.] present a (small) risk of reintroduction of the pathogen and disease. On the other hand, it has never been demonstrated with the PPS monitoring programs (sampling at outlets of drains of contaminated fields), that bacteria are transported by drainage water to surface water, although this is theoretically possible. The package of preventive measures proved to be very effective. In fields where a (heavy) contamination was found since 1995, already one to several subsequent potato crops have been grown. In none of these cases (where literally hundreds of thousands of host plants have been grown, that could have caught the bacteria, especially the supposed so-called "viable but non-culturable cells" of *Rsol* or VBNC's) renewed infection was found (contaminated places of production are included in yearly survey programs of the PPS). This is a strong indication against the existence of these VBNC's (Grey and Steck, 2001; Caruso *et al.*, 2005; Imazaki and Nakaho, 2010) that would be present everywhere in the environment, waiting for their chance. If they do exist at all, these cells are apparently part of a dying population, that can occasionally be 'revived' by our manipulations, but they are not a functional, dormant, and virulent form of the bacterium that survives forever.

Not immediately perceveable from Table 2 is the tremendous amount of work done by the PPS inspectors. Apart from inspections and sampling (Fig. 4), there were the difficult discussions with severely hit growers, many visits to places of production in the framework of tracing, monitoring and surveying, with many extra samples that had to be taken, the cumbersome tracing of sources of infections that, apart from hard confirmations, were often only suspicions and non-proved hypotheses. Note- and praiseworthy, and substantially contributing to the success in controlling the disease, is also the extensive support from the industry. The irrigation ban (with its large impact, especially in dry summers) needed extensive discussion and persuasion. There was also support during tracing from growers and trade, support when expertise visits abroad had to be made, willingness to take supplementary measures, when the instruments of the government were insufficient (e.g. removing lots from trade) and the actions to create an insurance against brown rot.

MONITORING OF SURFACE WATER

Testing for the presence of *Rsol* in surface water is performed on an artificial agar medium, called SMSA, that is very selective for the brown rot bacterium on the basis of certain nutrients and a panel of antibiotics (Fig. 5). Very

Fig. 4. Sampling of standard 200 tuber sample for testing on latent brown rot infection by an inspector of the Dutch Plant Protection Service. The inspector wears a disposable overall, gloves and overshoes to avoid possible spread of bacteria (Copyright: NIVAP, the Netherlands).

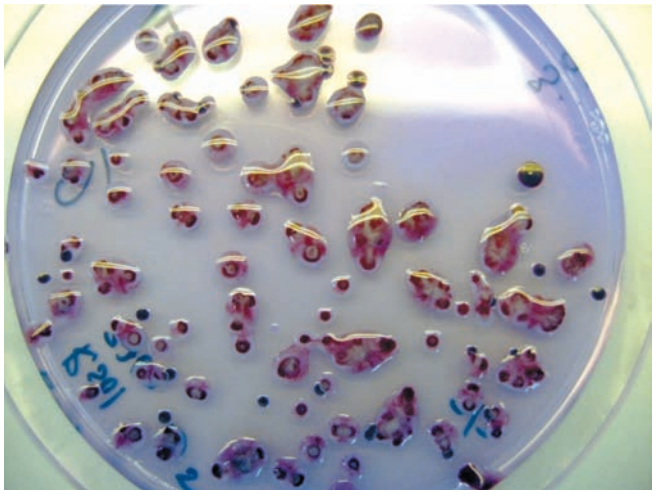


Fig. 5. Typical colonies of *Ralstonia solanacearum* (slimy with a diffuse red nucleus, by uptake of the dye tetrazoliumchloride, entire margin, slightly concave, diameter 3-7 mm) on modified selective SMSA medium, 5 days after plating at 27°C (Copyright: J.D. Janse).

few bacteria other than *Rsol* will grow on this medium enabling the detection of very low numbers (1-10 cells/ml) in surface water and other substrates (Wenneker *et al.*, 1999). Over the years the bacterium was shown to be present in an ever increasing area in all kinds of surface water (channels, rivers, ditches, polder reservoirs) and was also detected in bittersweet growing along these waterways. Since 2005 sampling takes place at the margin of contaminated areas and in non-contaminated areas and, therefore, the total number of samples has decreased since then (Table 3). The maps of The Netherlands (Fig. 6) clearly demonstrate how the bacterium has spread in a large area. Sampling also took place in potato processing plants and municipality purification plants (Fig. 7). *Rsol* has never been found in the effluent of these processing or purification plants.

Table 3. Surface water and bittersweet (*Solanum dulcamara*) sampling 1997-2010.

Area	Year	Total number of samples	Contaminated samples (No.)	Contaminated samples (No.)
Within prohibition zone	1997	1,860	650	35
	1998	2,198	725	33
	1999	1,617	437	27
	2000	2,119	1.421	33
	2001	1,780	526	30
	2002	1,236*	452	37
	2003	250*	17	7
	2004	213*	5	2
	2005	158*	0	0
	2006	206*	4	2
	2007	175	5	2.9
	2008	310	9	2.9**
	2009	234	6	2.6**
	2010	272	22	8.1**
Outside prohibition zone	1997	2,890	85	3
	1998	2,495	102	4
	1999	3,042	95	3
	2000	5,586	154	3
	2001	3,128	90	3
	2002	3,114*	97	3
	2003	1,867*	21	1
	2004	2,034*	28	1
	2005	1,915*	2	0.1
	2006	1,959*	4	0.2
	2007	2,076	8	0.4
	2008	1,906	10	0.5
	2009	1,961	30	1.5
	2010	1866	16	0.9

* = excluding bittersweet samples
** = intentional sampling in prohibition zone

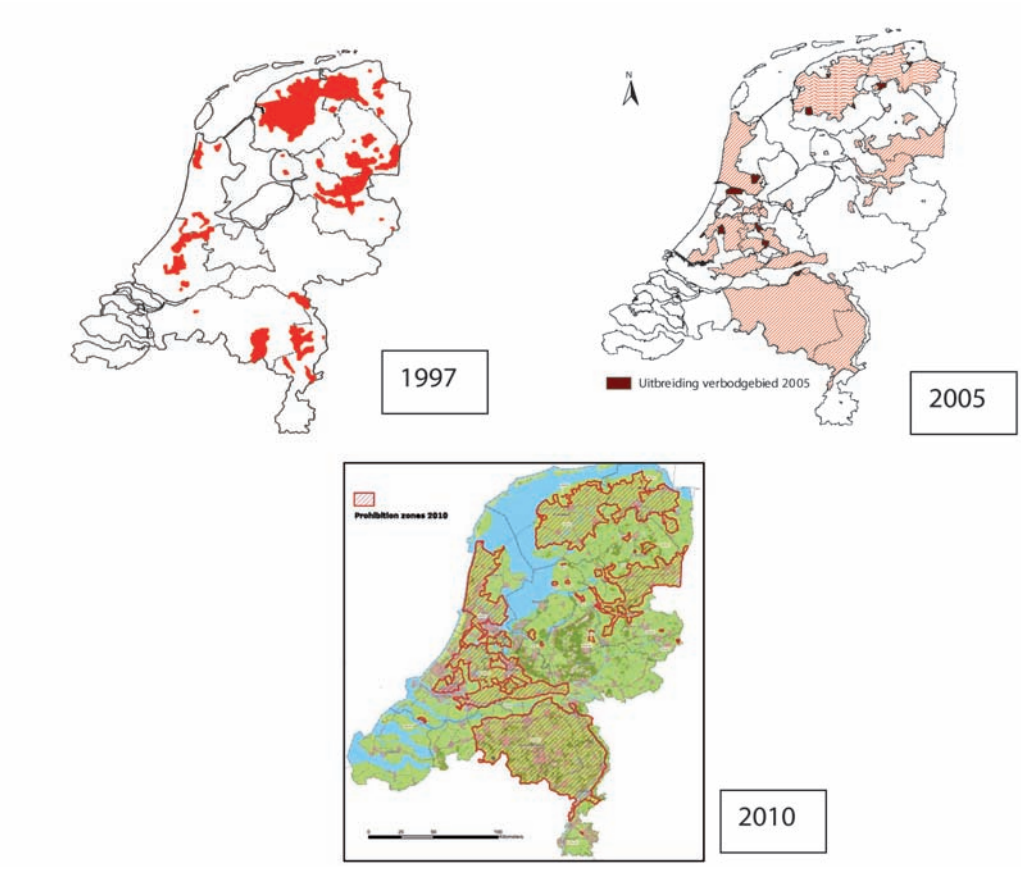


Fig. 6. Distribution of *Ralstonia solanacearum* in surface water, as determined over the years by intensive monitoring of the Dutch Plant protection Service and Dutch General Inspection Service (NAK). (Copyright: PPS, The Netherlands).

DIAGNOSIS

Screening for latent infections and identification of bacteria obtained from latent of visually infected material, water, soil and waste, is performed according to the EU Control Directive for potato brown rot (Anonymous, 1998, 2006). The basic screening test is still a serological assay using fluorescence microscopy, the so-called immuno-fluorescence test (IF). When a positive IF test is obtained, a compulsory second screening based on a different principle is applied, i.e. polymerase chain reaction (PCR) or fluorescent *in-situ* hybridisation (FISH). If the second screening test is positive, the sample becomes brown rot-suspected and is subjected to isolation on SM-SA to obtain a bacterial culture. When typical colonies are observed, one colony is further purified on a new agar plate and the resulting culture tested with biochemical (fatty acid analysis, biovar determination), serological (IF) and DNA methods (rep-PCR and FISH), and checked for pathogenicity through artificial inoculation to young tomato plants. The definitive, confirmatory test is pathogenicity, because all other assays (including the molecular ones) may give false-positive reactions with other micro-organisms (so-called cross-reactions). The testing and decision scheme of the standard EU-method that is applied by PPS and NAK is shown in Fig. 8.

Fig. 7. Sampling of waste water at a sewage plant of a potato processing industry in the Netherlands by an inspector of the Plant Protection Service (Copyright: PPS, the Netherlands).

SOME RESULTS OBTAINED IN THE EPIDEMIOLOGICAL RESEARCH OF THE DUTCH PPS

During 1996-2006 the Dutch PPS has also contributed to the national and international epidemiological research on the brown rot bacterium and has published the results

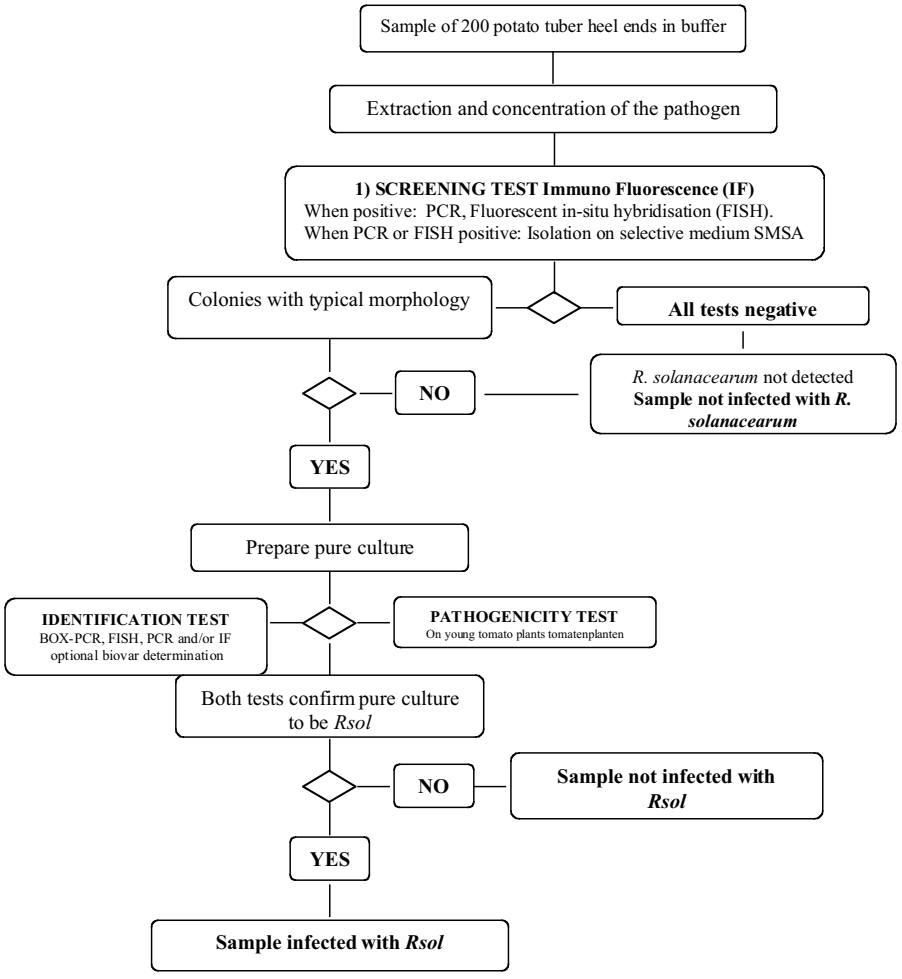


Fig. 8. Scheme of testing and diagnosis of a potato sample for (latent) infections of potato brown rot according to the EU Control Directive 2006/63/EC. This Directive also provides testing schemes for samples of water, soil, sewage, bittersweet and some other hosts like tomato.

Table 4a. Survival (in days) of *Ralstonia solanacearum* R 3b2, in different substrates at room temperature in daylight and at 4ºC in the dark. Bacterium spiked in concentrations of *ca.* 10^7 cells ml^{-1} (after Janse *et al.*, 1998).

Substrate	Survival in days	
	Room temperature, daylight	Low temperature (4ºC), dark
Surface water	17	33
Ditch mud	6	24
Sewage sludge processing industry	23	53
Chicken manure	23	30
Cow manure	7	11

Table 4b. Survival (in days) of *Ralstonia solanacearum* R3b2, on different surfaces when the bacterium was added in suspension from an agar culture or present in naturally infected potato tissue, subsequetly dried, in concentration of c. 10^7 cells ml^{-1} (after Janse *et al.*, 1998).

Substrate	Survival in days	
	Pure culture in sterile water, dried	Smear of naturally infected tissue, dried
Metal	14	14
Wood	4	4
Rubber	55	87

in technical and scientific journals. Primary investigations concerned the survival of *Rsol* on and in diverse substrates (Table 4a and 4b) (Janse *et al.*, 1998; Wenneker *et al.*, 1999). Survival in naturally contaminated soil was determined from several fields in cooperation with PPS inspectors. Survival in soil for at least one year was definitively demonstrated. In these experiments it was also determined that, when growing in the field with roots and stem strictly in the soil and not in water, bittersweet shows the classical wilting symptoms which are usually absent when the plant grows partly in water. The fodder corn cv. LG11 did not show micro-infections as reported for sweet corn varieties (Janse *et al.*, 1998; van Beuningen *et al.*, 1999). Most of these data harmonised with those obtained by Plant Research International (PRI, Wageningen), in microcosm experiments (van Elsas *et al.*, 2000, 2001). *Rsol* was isolated for the first time from naturally infected stinging nettle (*Urtica dioica*) plants that were growing as riparian plants, with their roots partly in water (Wenneker *et al.*, 1999). All these experimental findings were important for the development of advice to growers on what to use in their crop rotation in an infested field. Furthermore, in cooperation with the Biological Farming Systems (BFS) Group of the University of Wageningen, it was investigated if *Rsol* could be eliminated from infected plant material by a 6-week anaerobic mesophylic fermentation in a special tank, which appeared to be successful (Termorshuijzen *et al.*, 2003).

In cooperation with the Dutch starch production industry the fate (survival) of *Rsol* in the waste treatment process of factories was determined and monitored for many years. The bacterium was found in the untreated wash water and in the first (aerobic) steps of the waste treatment process, but never in the final liquid or solid effluent. Again in cooperation with BFS and N. Messiha, an Egyptian PhD student, successful experiments were conducted for the semi-anaerobic elimination of *Rsol* in the field under plastic (so-called biological soil disinfestations), where 93% killing of bacterial cells was determined (Messiha *et al.*, 2007b). With another PhD research, using a pot-greenhouse system, it was established that *Rsol* survives for at least 180 days in natural soil, longer in Dutch than in Egyptian soil types, and longer in clayey than in sandy soils. Survival was shortest in the Egyptian acidic sandy soils. Addition of ammonia-producing amendments reduced *Rsol* populations, whereas compost addition and organic management did not necessarily result in an enhanced decline of the pathogen (Messiha, 2006; Messiha *et al.*, 2007a, 2007b, 2009). Experiments with biological control, using the antagonistic bacterium *Stenotrophomonas maltophilia* were also very promising (Messiha *et al.*, 2007c). *Rsol* survival in surface waters was exhaustively mapped for three years in three regions that were light-

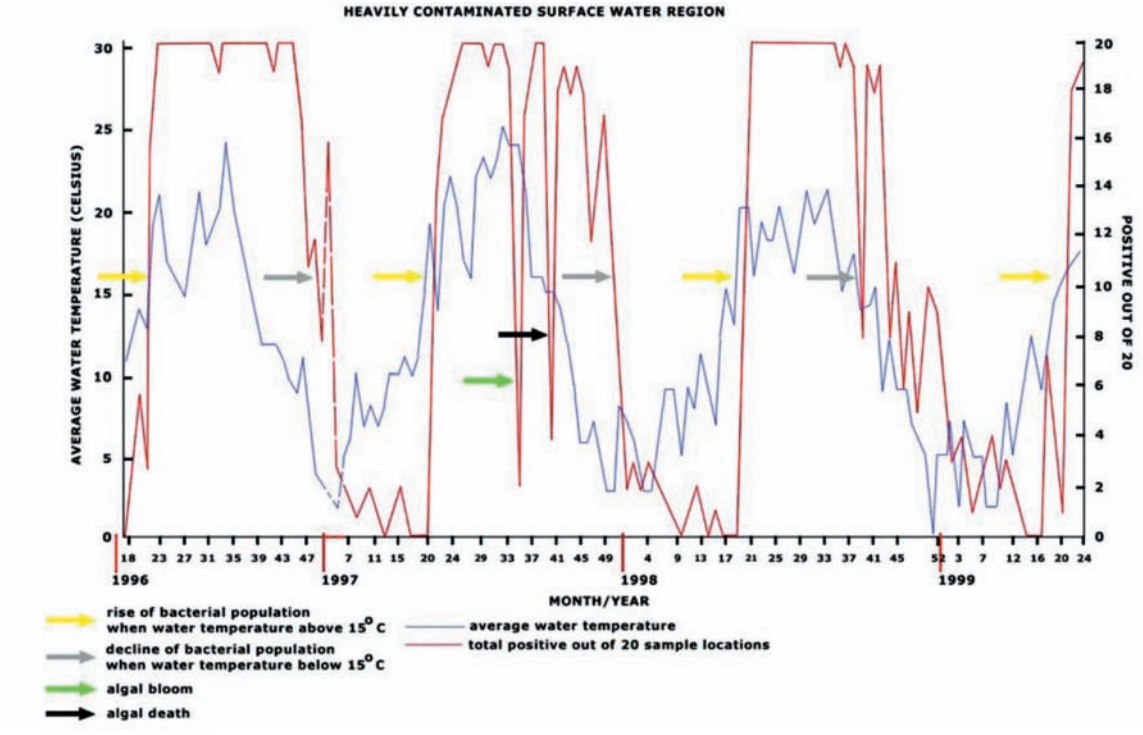


Fig. 9. Numbers of colony forming units (CVFU) of *Ralstonia solanacearum* in a heavily contaminated surface water area during three seasons. The bacterium was shown to be present up till ice formation in winter, although in very low numbers. Numbers increase or decrease strongly in spring respectively autumn when temperature reaches *ca.* 15°C. In the year 1997/98 a strong decline in cell population took place two times in summer, most probably related to a strong algal and cyanobacterial bloom and massive simultaneous death of them a few weeks later (Copyright Janse, 2006).

ly, moderately and heavily contaminated (Fig. 9). *Rsol* appeared to survive till ice formation in winter, although in very low numbers. A steep increase or decrease in numbers was always observed when the temperature came above or below 15°C (Wenneker *et al.*, 1999).

Extensive research was also carried out on the statistical reliability of potato lot sampling for detection of latent infections under practical conditions of harvesting, storing and sampling. The conclusion was that sampling practice as currently operated (given a random distribution of latently infected tubers and random sampling) approaches very reproducibly and reliably the statistical chance of finding latent infection (Janse and Wenneker, 2002).

The effect of pesticides present in the spray tank on the survival of *Rsol* was also studied, as well as the efficacy of disinfectants such as chlorine compounds and hydrogenperoxide (H_2O_2) with peracetic acid. The latter also in the framework of possibilities to disinfect irrigation water. Water treated with an apparatus dosing hydrogen peroxide (Degaclean, manufactured by Brightspark, The Netherlands) was analyzed for the presence of the *Rsol*. Elimination of living bacteria was complete already at 100 ppm concentration, even in the irrigation hose (van Beuningen *et al.*, 2005). This study led to an authorisation by the Dutch Board for the Authorisation of Plant Protection Products and Biocides for the use of hydrogen peroxide as biocide disinfectant for irrigation water. Finally co-workers of the Dutch PPS delivered a lot of concrete expertise, samples and data for two PhD theses: (i) a bio-economic model, developed by A. Breukers at the University of Wageningen (Agricultural Economics and Rural Policy Group) for calculating the damage and losses of brown rot under different epidemiological regimes and the effect of the Dutch package of control measures, and for implementing a cost-effective strategy for the future. This study confirmed that a substantial level of monitoring, hygiene, use of healthy planting material and avoidance of contaminated surface water are the best and cheapest options for control in the long term (Breukers, 2006; Breukers *et al.*, 2007); (ii) a thesis by P. Stevens on the genetic variation and adaptation of Dutch *Rsol* R3b2 strains from surface water (see under 'Ongoing activities and future developments').

SOME RESULTS OF THE DIAGNOSTIC RESEARCH OF THE PLANT PROTECTION SERVICE

In a national cooperation program the Department of Bacteriology together with the Microbiology Group of the University of Wageningen developed a 16s rRNA probe that was adapted to and used for FISH (Wullings *et al.*, 1998). This test was incorporated in the international ring-tests in an EU SMT project that developed and validated the EU Standard testing method for potato brown rot and incorporated it in the final EU standard testing method (Anonymous, 2006; Elphinstone *et al.*, 2000). An isothermic RNA-based detection test (nucleic acid sequence based amplification or NASBA), adapted to *Rsol* by PRI was evaluated and validated, but found not to be reliable and sensitive enough. A real-time PCR developed by the Central Science Laboratory (CSL) [now Food and Environment Agency (FERA)] at York (UK) was also validated and is now further validated internationally in a EU-EUPHRESCO project together with NAK and EU memberstate laboratories. During the whole period, antisera produced by PRI and others were validated with a panel of cross-reactive bacteria and a large number of samples from practice, enabling their safe use in the integral testing of potato.

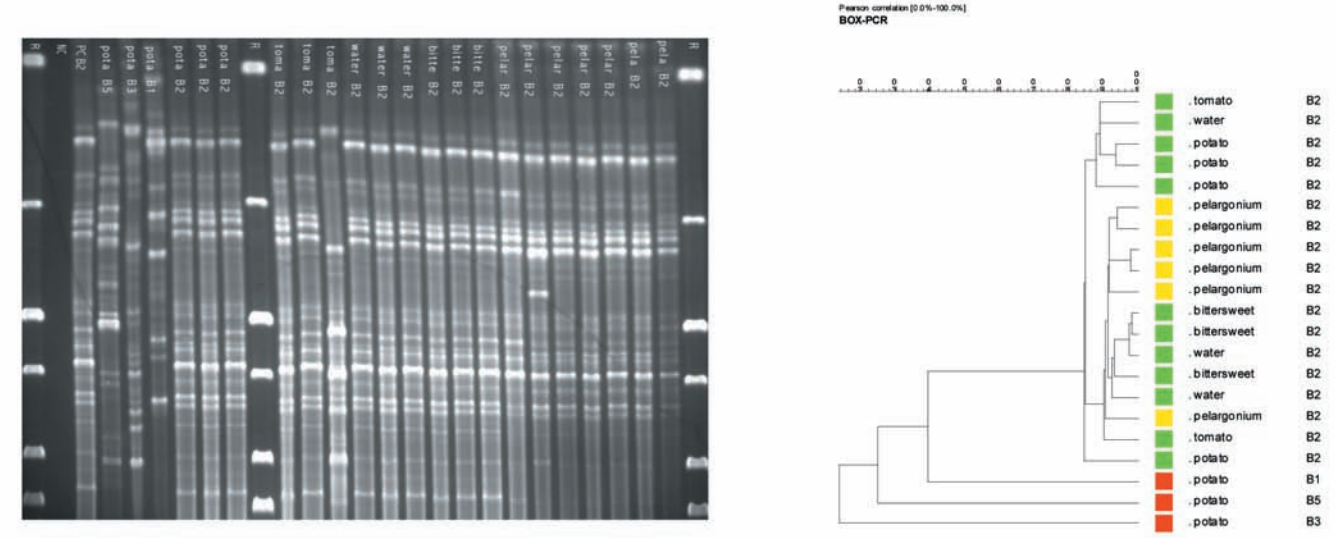


Fig. 10. Results of a BOX-PCR fingerprinting and resulting dendrogram to determine identity of *Ralstonia solanacearum* isolates from *Pelargonium*. In this investigation these isolates (green in dendrogram) appeared to belong to biovar 2, race 3, as confirmed also by other tests (Janse *et al.*, 2004). (Copyright: Janse, 2006).

FINDING OF *RALSTONIA SOLANACEARUM* RACE 3 BIOVAR 2, PHYLOTYPE 2B IN *PELARGONIUM* AND OF OTHER *RSOL* RACES/BIOVARS IN GREENHOUSE ORNAMENTALS

In 2002, the Dutch PPS received wilting plants of *Pelargonium* that were found to be infected by *Rsol* R3b2, phylotype IIB (Fig. 10). In all cases, the source proved to be cuttings produced in nurseries in Kenya that used contaminated surface water for irrigation. Measures taken by the PPS lead to successful eradication of the disease in *Pelargonium* and decontamination of the greenhouses involved. At the same time, this infection was also found in Germany, UK and Belgium, where it was successfully eradicated. Results of this research, with which it was also shown that the solanaceous ornamentals *Petunia* and *Calibrachoa* cv. Million bells can be latent hosts for *Rsol* R3b2 phylotype 2B, were presented at the 4th International Bacterial Wilt Symposium in Nelspruit, South Africa (Janse *et al.*, 2004b). Dutch PPS and the British CSL could advise the United State Department of Agriculture when comparable infections originating from Kenya in 2003 and, as early as 1999, from Costa Rica and Guatemala were encountered in the USA (Williamson *et al.*, 2002; Hamrick, 2004). Use of deep soil water for irrigation and strict hygiene completely solved the problems for nurseries (Janse *et al.*, 2004; Kim *et al.*, 2003; Sanchez-Peres *et al.*, 2008). A more or less similar finding and route of infection of *Rsol* R1b1 in cuttings of *Begonia eliator* imported from Costa Rica was reported by Janse *et al*. (2006). Occasional findings in Dutch greenhouses of *Rsol* race 1 biovars 3 and 4 in *Curcuma longa* and *C. anismatifolia* (Turmeric) rhizomes imported from Thailand, Indonesia, South Africa and Zimbabwe and their successful control, are registered in the Annual Reports of the Dutch PPS from 1996 (van der Tuin *et al.*, 1996).

INTERNATIONAL CONTEXT AND COOPERATION

During the early years of the brown rot outbreak there was considerable fear abroad, especially in the EU member states surrounding The Netherlands, that quite a bit of Dutch potato seeds would be contaminated by *Rsol.* The more because extra irrigation in the warm summers of 1994 and 1995 led (as we now know) to contaminations in a number of those member states, where it was not yet fully discovered that contaminated surface water played such an important role. It was finally determined that import of infected consumption potatoes from the Mediterranean area and irrigation with contaminated surface water probably or actually formed the main cause of the infections, rather than the Dutch seed. The Dutch PPS put a lot of effort in explaining the Dutch situation and control system, nationally and internationally e.g. by clarification with the SCPH in Brussels and its expert group on bacterial diseases, lectures for growers, students, experts of sister institutes, lectures on symposia, reception of dozens of delegations from abroad, at the PPS and inside The Netherlands, but also in a wide number of countries, worldwide. In a large number of missions (more than 80 over the period) diagnostic expertise was delivered and the supposed infections in Dutch seed or its progeny were closely investigated, usually in excellent mutual cooperation.

Apart from these missions the acquired expertise was also used for setting up laboratories, developing diagnostics and training of staff in the framework of many bilateral or EU (Twinning) projects, e.g. in Croatia, Cuba, Estonia, Hungary, Morocco, Poland, Rumania, Slovenia, Czech Republic and Turkey. Furthermore, in 1996 the EU Commission asked a Dutch and a British expert to write a project proposal on the feasibility of controlling *Rsol* at the source (Egypt), so as to solve the problem of the increasing number of interceptions of potato brown rot in Egyptian early potatoes originating from the heavily contaminated Nile Delta area at that time. This resulted in the financing of two projects over the period 1996-1999 and 2002-2006, having the Department of Bacteriology of the Dutch PPS as project leader and scientific institutions from Belgium, France and UK as partners. Main aims of these projects were the creation of a durable control system for potato brown rot in Egypt, carrying out epidemiological research and training (Janse *et al.*, 2004a). In the first project a test laboratory with a capacity of *ca.* 12,000 samples per season and a quarantine greenhouse were installed and equipped, training was provided, and preliminary epidemiological investigations were carried out. A considerable effort was required by the creation and maintenance of so-called pest free areas (PFA's). During the lifetime of the two projects a steep decline was registered in the number of infections and interceptions in EU member states (Fig. 11). *Rsol* was detected in surface water in the Nile Delta and in certain weeds,

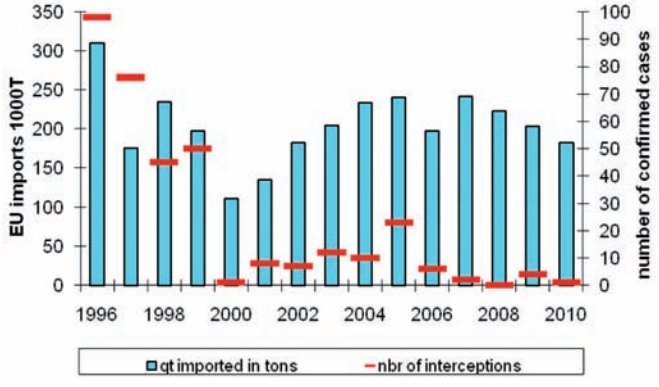


Fig. 11. Reduction in number of interceptions of EU-imported early potatoes from 1996-2010 as result of an intensive control program of the Egyptian Government supported by two EU-Egypt bilateral projects (1996/7-2000/01 and 2002/03-2005/06), where The Netherlands was appointed as project leader.

Fig. 12. Surface water sampling in the Nile Delta (Egypt) for detecting *Ralstonia solanacearum* contamination, done in the framework of an EU-Egypt cooperation project 1996-1999 and 2001-2006 (Copyright: Janse, 2006).

such as *Portulaca oleracea*, and extensive survival studies in soil and water were performed (Farag *et al.*, 1999; Tomlinson *et al.*, 2005, 2009) (Fig. 12). An extension package was developed, experimental and demonstration plots were installed and local- and EU-based short- and long-term trainings organised. Three MSc students in the UK and 1 PhD and 1 MSc student in the Netherlands successfully finished their studies. The PhD student turned out several scientific publications and a thesis (Messiha, 2006, 2007a, 2007b, 2007c, 2009).

ONGOING ACTIVITIES, FUTURE DEVELOPMENTS CONCERNING TESTING AND CONTROL POLICIES

The variability and adaptation of *Rsol* R3b2 phylotype II strains from Dutch waterways have recently been investigated by P. Stevens, a PhD student at the University of Groningen (Stevens and van Elzas, 2010). These studies confirmed the genetic homogeneity of the bacterial strains, although some showed deletions that could have some effect on their fitness and survival in the environment.

It is foreseen that the real-time PCR detection method, developed by colleagues from FERA (York, UK) (Weller *et al.*, 2000) will be a good alternative to immuno-fluorescence and classical PCR when screening potato extracts. The multiplication of *Rsol* DNA present in the sample is measured from the start using a fluorescent label. Compared with conventional PCR and IF, preparation and analysis takes less time, and the assay provides an equal it not better reliability. The sensitivity of the method was increased by optimizing DNA extraction. Two EU Euphresco projects in 2009-2011, involving 29 EU memberstate laboratories, where this method was ring-tested and validated in comparison with IF, selective plating, biotest and classical PCR, yielded quite promising results. Thus, a proposal will be formulated to the SCPH to incorporate real-time PCR in the European standard method for testing latent infections of potato brown rot (J. Vaerenbergh, personal communication and Table 5). Following real-time PCR adoption by the EU, it is expected that PPS and NAK will use this assay for routine testing of brown rot.

The future policy in the control strategy of potato brown rot by the Plant protection authorities will not only be active keep off and control, but also the creation of a scientific base to further define the critical processes in the production chain and nature that influence the survival and spread of *Rsol*. Both fundamental and applied research will remain important in the study of ecological and epidemiological constraints in order to develop balanced, cost-effective monitoring and control measures that can be implemented by government and industry.

Table 5. Results of an international EU-EUPHRESCO inter-laboratory ring-test and validation of diagnostic tests for detection of latent infections of potato brown rot, in 2010, from which it is clear that real-time PCR is a potential candidate to replace the present serological (IF) screening test. Every laboratory tested seven samples including controls (Source: Dr. J. Van Vaerenbergh, ILVO, Merelbeke, Belgium).

Test	IF	PCR	Real-time PCR	Selective plating
No. of labs participating	29	29	20	29
Consolidated positive agreement results*	0.960	0.975	0.885	0.793

* Test results that match the true status of the sample

CONCLUDING REMARKS

From all the work in research, monitoring, testing and tracing, the following 'golden rules' for the control of potato brown rot are:

- Do not use untreated (probably) contaminated surface water for irrigation if possible, use deep soil water or disinfected surface water instead;
- Use healthy, tested and certified potato seed or other propagating material;
- Separate production, grading and storage of consumption potatoes from those of seed potatoes;
- Store and grade potatoes on the original place of production, to avoid spread of the pathogen;
- Apply strict hygiene and exert effective control on the implementation of agreed hygienic protocols (e.g. cleaning of trucks, store facilities and packing materials);
- Pro-actively invest in ecological, epidemiological and diagnostic expertise in an international cooperation setting, a worst case action plan and also in advisory activities and education;
- Compensate for damage or create the possibility for growers to insure themselves;
- Maintain and execute regular surveys/monitoring programs in consumption, industrial and seed potato production and on alternative (wild) hosts, also in greenhouses.

ACKNOWLEDGEMENTS

The author wants to express his sincere thanks to the following colleagues that have given an invaluable contribution to the work and solutions in potato brown rot control in the Netherlands: Alex van Beuningen, Jeroen van de Bildt, Hans Derks, Peggy Gorkink, Henk Hendriks, Nico Horn, Frans Janssen, Jeroen Kavelaars, Marco Landman, Nevein A.S. Messiha, Mario van Sabben, Marjoleine Schoeman, Maarten Steeghs, Leon Tjou-Tam-Sin, Joris Voogd and Marcel Wenneker.

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