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A new and sensitive Co-operational polymerase chain reaction for rapid detection of *Ralstonia solanacearum* in water

Paola Caruso[1], Edson Bertolini[1], Mariano Cambra, María M. López*

Departamento de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, 46113 Moncada, Valencia, Spain

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Abstract

Three primers from 16S rRNA were successfully assayed simultaneously in one reaction for sensitive detection of *Ralstonia solanacearum* in watercourses. The protocol is a modification of the Co-operational polymerase chain reaction (Co-PCR), which allows the simultaneous and co-operational action of the primers. It specifically amplified *R. solanacearum* strains belonging to biovars 1, 2 and 4. No products were obtained from any of the 162 unidentified isolates from river water. The sensitivity of the assay was <1 cfu/ml as determined by analysis of heat-treated water samples spiked with *R. solanacearum*, also containing indigenous microbiota up to 10^5 cfu/ml. The developed Co-PCR assay was more sensitive than other standard PCR assays in the analysis of 51 Spanish environmental water samples. Namely 31.3% of the samples were positive using the newly developed assay, whereas 13.7% or less positive samples were found with the other protocols. The Co-PCR improves the detection sensitivity of *R. solanacearum* and provides an important tool for its routine detection from environmental water samples and for epidemiological studies.

Keywords: Bacterial wilt; Brown rot; Diagnosis; Identification; 16S rRNA gene

1. Introduction

The incidence of the bacterium *Ralstonia solanacearum* (E.F. Smith) (Yabuuchi et al., 1995) in the European Union has increased since the first reported European outbreak in potato (Olson, 1976) and particularly in the last decade (Janse, 1996; Hayward et al., 1998; Elphinstone et al., 1998; Wenneker et al., 1999; Timms-Wilson et al., 2001). This microorganism is the causal agent of potato brown rot and bacterial wilt in Europe and bacterial wilt in many crops in other parts of the world (Hayward, 1994), and it is responsible for losses of up to 75% of the potato crop in several countries (Cook and Sequeira, 1994). Most of the European outbreaks seem to be related to the irrigation of crops with *R. solanacearum*-contaminated water. However, little is known about its distribution and persistence in natural reservoirs such as water or the molecular or physiological bases of survival strategies for this bacterium (Van Elsas et al., 2000).

* Corresponding author. Tel.: +34-96-342-4000; fax: +34-96-342-4001.
E-mail address: mlopez@ivia.es (M.M. López).
[1] Equal first authors.

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Epidemiological and ecological studies on pathogen distribution in the environment are seriously hindered by the lack of efficient detection methods (Janse, 1988, 1996), especially when the concentration of the pathogen is either very low or is present in a latent, dormant or nonculturable state (Grey and Steck, 2001; Van Elsas et al., 2000). Until now there is not a validated PCR protocol recommended for the detection of *R. solanacearum* in water. Most of the detection protocols for this quarantine organism in water are time-consuming and too laborious for its routine identification in a large number of samples. Selective isolation is the method employed in most EU countries, but it requires specific identification of the suspected colonies by different techniques (Anonymous, 2001). To increase the sensitivity, concentration by filtration or centrifugation of variable volumes of water is recommended (Elphinstone et al., 1998; Janse and Schans, 1998; Poussier et al., 2002). A previous enrichment of the sample may also improve the number of *R. solanacearum* (Elphinstone et al., 1998) before applying serological or molecular detection methods (Anonymous, 1998). Besides, the detection results are frequently negative, especially in cooler months when the bacterial population is very low (Janse et al., 1998; Biosca et al., in press). Enrichment DASI-ELISA with specific monoclonal antibodies is used to detect latent infections in potato tubers (Caruso et al., 2002) but the method may need modification for routine detection of *R. solanacearum* in water.

The design of a specific PCR test for the molecular identification of *R. solanacearum* is complex due to its genetic diversity (Cook et al., 1989; Li et al., 1993; Seal et al., 1993, 1999; Tagahavi et al., 1996; Fegan et al., 1998; Timms-Wilson et al., 2001) and to its close relationship with other bacterial species (Roberts et al., 1990; Hayward, 1991; Yabuuchi et al., 1995) such as *Pseudomonas syzygii* (Roberts et al., 1990), *Ralstonia pickettii* (Ralston et al., 1973) and the Blood Disease Bacterium (BDB) (Gäumann, 1923; Eden-Green and Sastraatmadja, 1990). *R. solanacearum*, in fact, represents a heterogeneous group that was conventionally subdivided into five races (Buddenhagen et al., 1962; He et al., 1983) and five biovars (Hayward, 1964; Hayward et al., 1990; He et al., 1983) based on pathogenicity and biochemical characteristics, respectively. Until now all strains isolated in Europe from water were classified as biovar 2 (Olson, 1976; Janse, 1996; Elphinstone et al., 1998; Janse et al., 1998; Janse and Schans, 1998; Person, 1998; Wenneker et al., 1999; Palomo et al., 2000; Wood et al., 2002; Biosca et al., in press). The pathogen was also subdivided in two divisions (“Americanum” and “Asiaticum”) by using restriction fragment length polymorphism (RFLP) (Cook et al., 1989) and phylogenetic studies based on sequence data (Tagahavi et al., 1996). In addition, amplified fragment length polymorphism (AFLP) and PCR-RFLP revealed a further subdivision containing African strains (Poussier et al., 2000). A recent study of European strains, based on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), fatty acid methyl esters (FAME) analysis, exopolysaccharide (EPS) production, 16S rRNA RFLP, amplified ribosomal DNA restriction analysis (ARDRA) and sequence analysis of 16S–23S rRNA, has proposed the possible selection of a *R. solanacearum* “European” variant (Timms-Wilson et al., 2001). Several PCR protocols and different specific oligonucleotides from 16S and 23S rRNA have been described for diagnose and/or identification of *R. solanacearum* (Seal et al., 1993; Elphinstone et al., 1996; Opina et al., 1997; Fegan et al., 1998; Boudazin et al., 1999; Pastrik and Maiss, 2000; Poussier and Luisetti, 2000; Weller et al., 2000). These protocols, however, are mainly devoted to its detection in plants or soil. Although highly sensitive in vitro, routine PCR application for bacterial detection is currently limited because of the presence of PCR-inhibitory compounds in environmental samples (Picard et al., 1992; Tsai and Olson, 1992; Maes et al., 1996). A two-stage nested PCR developed by Elphinstone et al. (1996) improved the detection level (up to 10 cfu/ml) of a previously described PCR protocol (Seal et al., 1993). However, the introduction of a second amplification step seriously increases the risk of contamination during the subsequent manipulation of the previously amplified material (Elphinstone et al., 1996; Niepold and Schöber-Butin, 1997; Llop et al., 2000; Bertolini et al., 2002).

A new method called Co-operational polymerase chain reaction (Co-PCR) (Spanish patent 31 October 2000; P20002613) has recently been described (Olmos et al., 2002) for the sensitive detection of RNA viruses. This technique can be carried out easily in a simple reaction based on the simultaneous action of three or more primers that produce three or more

amplicons by the combination of the primers and the co-operational action of amplicons for the production of the largest fragment amplified by the external primers. Co-PCR allows as sensitive detection as the nested-PCR technique and does not require two different annealing temperatures (Olmos et al., 2002). This prompted this study to develop a sensitive Co-PCR method to improve the detection limits of described PCR assays for *R. solanacearum* in water. We selected the previously reported primers (OLI1; OLI2; Y2 and JE-2) for their sensitivity and specificity and four or three of them were assayed simultaneously in one reaction for sensitive detection of the bacterium in watercourses by a modification of the Co-PCR. In addition, a colorimetric detection using an internal probe was also employed to further improve the detection and facilitate routine applications.

2. Material and methods

2.1. Bacterial strains and growth conditions

Biovars and origins of the bacterial strains and isolates used are listed in Table 2, including 39 *R. solanacearum* strains from the five biovars, reference strains of the other *Ralstonia* species and related genera, as well as 162 unidentified bacterial isolates from different watercourses in Spain. *R. solanacearum* strains were grown on YPGA medium (Lelliot and Stead, 1987) at 29 °C while the water isolates were cultured at 25 °C for 48 h. The optical density at 600 nm (OD_{600}) of a culture containing approximately 1×10^9 cells/ml of *R. solanacearum* was 0.5.

2.2. DNA extraction

A simple DNA extraction protocol was used (Llop et al., 1999) with the environmental and spiked water samples. Briefly, 1 ml of each sample was centrifuged at 13,000 × *g* for 5 min. The pellet was resuspended in 500 μl of extraction buffer [200 mM Tris–HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, 2% polyvinylpyrrolidone], vortexed and left for 1 h at room temperature with continuous shaking. After centrifugation, 450 μl of the supernatant was taken, mixed gently with 450 μl of isopropanol, and left for 1 h at room temperature. The mixture was centrifuged, the supernatant was discarded, and the dried pellet was resuspended in 100 μl of sterile water. Five microlitres of each DNA extract was used for conventional PCR assays and for Co-PCR.

2.3. Conventional PCR

The analysis of the environmental and spiked water samples was performed comparing the previously described conventional PCR assays (Seal et al., 1993; Boudazin et al., 1999) with the Co-PCR. The conventional PCR assays evaluated were those described by Seal et al. (1993) and Boudazin et al. (1999) with small modifications. In the first, primers OLI1 and Y2 were used for amplification of a 288-bp fragment. The reaction was performed in a final volume of 25 μl by adding the following reagents: 10 × *Taq* buffer (GIBCO-BRL), 1.5 mM $MgCl_2$, 0.1 mM concentrations of each deoxynucleoside triphosphate (Pharmacia LKB), 0.1 μM of primer OLI1 and Y2, 1 U of *Taq* DNA polymerase (GIBCO-BRL) and 5 μl of each heat-treated water sample. Amplification conditions included a denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min and then one cycle of 72 °C for 10 min in a MasterCycler Gradient (Eppendorf) thermal cycler. Primers OLI1 and Z (Boudazin et al., 1999), were used for amplification of a 403-bp fragment. The reaction was performed in a final volume of 25 μl with the following reagents: 10 × *Taq* buffer (GIBCO-BRL), 2.5 mM $MgCl_2$, 0.1 mM concentrations of each deoxynucleoside triphosphate (Pharmacia LKB), 0.1 μM each of primers, 1 U of *Taq* DNA polymerase (GIBCO-BRL) and 5 μl of each heat-treated water sample. Reaction mixtures were cycled as above, but the annealing temperature was 60 °C. A further PCR assay using the primers OLI1 (Seal et al., 1993; Elphinstone et al., 1996) and OLI2 (Elphinstone et al., 1996) was also tested. The reaction was performed in a final volume of 25 μl by combining the aforesaid reagents and adding 5 μl of each heat-treated water sample. Reaction mixtures were cycled as described above, but an annealing temperature of 65 °C was used. In addition to the amplification of heat-treated water samples, 5 μl of DNA from each sample after the described extraction protocol was also analysed by all PCR methods. Amplified products (12 μl) were separated by elec-

trophoresis (100 V) on 2% agarose gels. One microlitre was used for subsequent dot-blot hybridization (see below). The amplifications were performed in duplicate.

2.4. Co-operational PCR (Co-PCR)

A first attempt to apply the Co-PCR method described by Olmos et al. (2002) was done using primers OLI1, OLI2, Y2 (Seal et al., 1993; Elphinstone et al., 1996; Boudazin et al., 1999) and JE-2 (Elphinstone et al., 1996). The reaction was performed in a final volume of 25 μl with the following reagents: 10× *Taq* buffer (GIBCO-BRL), 4 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate (Pharmacia LKB), 5% of dimethyl sulfoxide (DMSO), 0.15 μM of external primers, 0.075 μM of internal primers, 1 U of *Taq* DNA polymerase (GIBCO-BRL) and 5 μl of each heat-treated water sample. The amplification was performed by a denaturation phase at 95 °C for 3 min followed by 55 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 45 s, and then one cycle at 72 °C for 10 min, in a MasterCycler Gradient (Eppendorf) thermal cycler. The PCR products were visualised after electrophoresis in 2% agarose and 1 μl was dispensed onto nylon membranes for subsequent colorimetric detection. In a second attempt, some changes were made to improve the specificity of the detection. Only primers OLI1, OLI2 (Seal et al., 1993; Elphinstone et al., 1996; Boudazin et al., 1999) and JE-2 (Elphinstone et al., 1996) were used (Fig. 3). The reaction was performed in a final volume of 25 μl with the aforesaid reagents, adding 3% of formamide and 5 μl of each heat-treated water sample. The amplification, electrophoresis and the colorimetric detection were performed in duplicate as described above.

2.5. Sensitivity evaluation of the different PCR protocols in spiked water samples

R. solanacearum strain IVIA 2528.55 (biovar 2) was used to prepare spiked water samples to test the sensitivity of the different PCR protocols. Appropriate bacterial concentrations in the spiked samples were achieved in either sterile (PBS: NaCl, 8 g/l; $PO_4H_2Na{\times}2H_2O$, 0.4 g/l; $PO_4HNa_2{\times}12H_2O$, 2.7 g/l) or autoclaved river water from a watercourse bordering potato fields adding serial 10-fold dilutions. Inoculum densities were optically adjusted to 0.5 OD at 600 nm (approximately 1×10^9 cfu/ml) and verified by standard plate viable counts. As negative control, non-spiked samples (only PBS or autoclaved river water) were included. For sensitivity determination, 5 μl of each heat-treated water sample was analysed by conventional PCR and by Co-PCR as described above. Aliquots of 1 ml from every dilution were transferred to Eppendorf tubes to perform the simple DNA extraction (see above) and then analysed by conventional PCR and by Co-PCR as indicated. In order to assess the threshold sensitivity of the detection more accurately, total cells count of the samples and their serial 10-fold dilutions was performed by using immunofluorescent antibody staining assay with commercially available antiserum IVIA 1546 (Plant Print Diagnòstics, Spain) using the protocol as previously described (De Boer, 1990). All the samples were analysed in duplicate.

2.6. Specificity of the PCR assays

In order to investigate the specificity of the methods, a panel of 39 *R. solanacearum* strains representing each of the five biovars of this species was checked (Table 2). In addition, different *Ralstonia* species and other related genera as well as 162 unidentified bacterial isolates from different water sources were tested by conventional PCR and by Co-PCR (Table 2). All the amplifications were performed in duplicate.

2.7. Probe design, hybridization and colorimetric detection

The probe design was performed as described by Bertolini et al. (2001). Briefly, sequenced regions of *R. solanacearum* 16S rRNA gene were recovered from public databases. The alignment view was performed as master-slave with identities, by using the similarity search tool Advanced BLAST 2.0 program (Altschul et al., 1997). An internal probe EPRS (5′-CCGATGTCTGATTGCTAGTTGGTGGGG-3′), was designed among nucleotides 217 and 244 of the 16S rRNA of *R. solanacearum* ATCC 11696 (type strain) by Yabuuchi et al. (1992) (Genbank accession no. S55002). For colorimetric detection, positively charged nylon mem-

brane (Roche Molecular Biochemicals) was used to bind the amplicons (1 μl) with UV light (4 min, 254 nm). Standard buffer consisting of 5 × SSC, 0.1% (w/v) *N*-lauroyl-sarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS) and 1% blocking reagent (Roche) was used for prehybridization (50 °C, 1 h) and hybridization (50 °C, 2 h; probe at 10 pmol/ml). Colorimetric detection with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolil phosphate (BCIP) substrate was performed using the DIG Nucleic Acid Detection Kit and DIG Wash and Block Buffer Set (Roche) according to the manufacturer's instructions.

2.8. Restriction fragment polymorphism and sequencing

The restriction pattern of the amplification products obtained from bacterial suspensions (strain IVIA 2528.55) was examined with *Ava*II (MBI Fermentas) to confirm its identity. The fragments amplified from this strain by conventional PCR and Co-PCR were purified using the QIAquick gel extraction kit (Qiagen) and sequenced with the primers OLI1, Y2, OLI2 and JE-2. The resulting sequences were then compared to the corresponding sequence obtained from strain ATCC 11696 using the program CLUSTALW, version 1.5 (Thompson et al., 1994).

2.9. Environmental water samples

Fifty-one water samples were taken from September 2001 to May 2002 from various sources in Spain (Table 3). They were collected by filling sterile containers at a depth of 30–40 cm and within 2 m from the bank and kept at 4 °C until processed. Total volume of each sample was in the range of 500 to 1500 ml. They were tested for the presence of *R. solanacearum* by spreading 100 or 1000 μl in duplicate, directly and two decreasing dilutions (1:10 and 1:100) onto YPGA (Lelliot and Stead, 1987) and modified SMSA (Elphinstone et al., 1996). Enumeration of viable aerobic bacteria from water samples was performed by standard plate count procedure on YPGA and SMSA after 48–72 h at 29 °C. Water samples were also analysed by conventional PCR (Seal et al., 1993; Boudazin et al., 1999) and modified Co-PCR, as described below. One millilitre of each water sample was heat-treated (100 °C for 10 min), diluted (range of 1:10–1:100) in sterile 10 mM phosphate buffered saline solution, pH 7.2 (PBS: NaCl, 8 g/l; $PO_4H_2Na \times 2\ H_2O$, 0.4 g/l; $PO_4HNa_2 \times 12\ H_2O$, 2,7 g/l), and 5 μl was added to the PCR reaction mix. The population of indigenous microbiota in the water sample was evaluated in the same way as indicated in nonselective YPGA medium (Lelliot and Stead, 1987). All the analyses were performed in duplicate.

3. Results

3.1. Sensitivity of the different PCR protocols with pure culture and spiked water samples

The sensitivity threshold of pure *R. solanacearum* suspensions in PBS using all assayed amplification protocols is shown in Table 1. The sensitivity level reached by Co-PCR amplification was higher than the one obtained using any of the other methods (Seal et

Table 1
Sensitivity threshold of boiled pure culture suspensions of *Ralstonia solanacearum* in PBS and spiked samples in autoclaved river water, employing conventional PCR methods and Co-PCR

PCR assay	Sensitivity in PBS		Sensitivity in river water	
	Cfu/ml[a]	Total cells/ml[b]	Cfu/ml[a]	Total cells/ml[b]
OLI1/Y2 (Seal et al., 1993)	10^2–10	10^4–10^3	10^3–10^2	10^5–10^4
OLI1/Z (Boudazin et al., 1999)	10–1	10^3–10^2	10	10^3
OLI1/OLI2 (Seal et al., 1993; Elphinstone et al., 1996)	10^4	10^6	10^5	10^7
Co-PCR (this work)	10^{-2a}	1	10^{-2a}	1

[a] Cfu/ml determined by plate count in SMSA and YPGA media.
[b] Total cell count determined by immunofluorescence with polyclonal antibodies according to De Boer (1990) corresponding to culturable, nonculturable and dead cells.

al., 1993; Elphinstone et al., 1996; Boudazin et al., 1999). When spiked water samples prepared with autoclaved river water were analysed, similar results were found (Table 1). All spiked samples prepared with autoclaved river water were positive by Co-PCR assay down to the 10^{-11} dilution from a suspension of 10^9 cfu/ml (Table 1 and Fig. 1). Therefore, detection limit was about 1 cell/ml corresponding to 10^{-2} cfu/ml, and nonculturable plus dead cells. In fact, the results obtained by the total cell counts revealed that the cfu/ml count underestimated the real number of the *R. solanacearum* cells. The number of total cells, indeed, was at least one or two orders higher than the standard plate viable counts as shown in Table 1.

The sensitivity of detection obtained with the other methods in spiked water samples was lower, being 10^5 to 10 cfu/ml with the protocols of Seal et al. (1993), Elphinstone et al. (1996) and Boudazin et al. (1999). DNA extraction from the spiked autoclaved water samples did not appear to improve the detection limit of the method (Fig. 1). When spiked autoclaved river water samples were amplified after DNA extraction by all the methods the results demonstrated that the best detection limit was obtained with Co-PCR reaching again a sensitivity of 10^{-2} cfu/ml (Fig. 2). Uninoculated control samples were always negative.

3.2. Specificity of the assays

The Co-PCR was developed using previously described primers whose specificity was already well known. Consequently, as expected, the primers described by Seal et al. (1993) enable the amplification of all 39 *R. solanacearum* strains assayed as well as the Blood Disease Bacterium (BDB) and *P. syzygii* (Table 2). The primers described by Boudazin et al. (1999) enabled the specific amplification of only the strains of *R. solanacearum* division 2 (18 *R. solanacearum* strains from biovar 2 and 7 from biovar 1), as well as the BDB and *P. syzygii* (Table 2) and they produced the 403-bp band expected. In addition, the analysis of 162 unidentified bacterial isolates from different water sources revealed that a total of six isolates generated a PCR product of the size expected for *R. solanacearum* when amplified with the OLI1/Y2 primers. The Co-PCR performed using the OLI1/OLI2/Y2/JE-2 primers also produced the 408-bp band with those strains (Table 4). They were identified by fatty acid profile analysis using the Microbial Identification System (MIS, Hewlett-Packard) as *Acinetobacter johnsonii* and *Aeromonas salmonicida* (Table 4). When the Co-PCR was carried out using only the three primers OLI1, OLI2 and JE-2, no amplification product was observed (Table 4). However, the primers OLI1, OLI2 and JE-2 generated a single amplification band of 408 bp with DNA from 31 out of the 39 *R. solanacearum* strains listed in Table 2 as well as the BDB and *P. syzygii* strains, keeping the sensitivity above indicated. Five isolates of biovar 3 and three of biovar 5 tested negative (Table 2). The fragments obtained digesting the IVIA 2528.55 strain and all positive environmental samples after Co-PCR (OLI1/

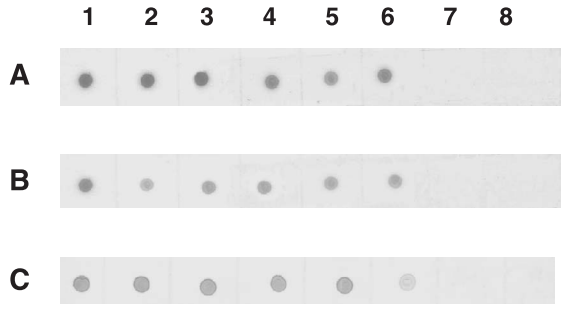

Fig. 1. Sensitivity of Co-PCR assay in PBS suspensions of *R. solanacearum* (A), and in autoclaved river water with (B) and without (C) DNA extraction; lanes 1 to 6: decimal dilutions from 10^3 to 10^{-2} cfu/ml; lane 7: sterile PBS or autoclaved river water without bacteria; lane 8: Co-PCR negative control.

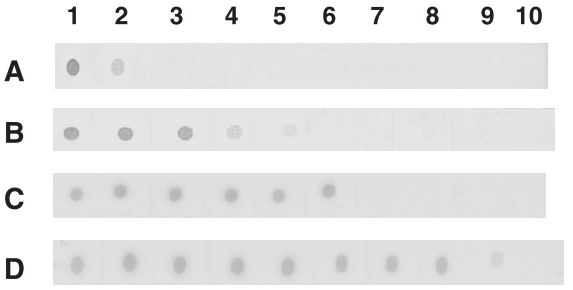

Fig. 2. Sensitivity of the pure cultures of *R. solanacearum* in the conventional PCR assays [(A) OLI1-OLI2 primers; (B) OLI1-Y2 primers; (C) OLI1-Z primers] and Co-PCR assay (D) in spiked autoclaved river water without DNA extraction. Lanes 1 to 9: decimal dilutions from 10^6 to 10^{-2} cfu/ml, in autoclaved river water; lane 10: autoclaved river water without bacteria.

Table 2
Amplification of *Ralstonia solanacearum* strains, related bacteria, other plant pathogenic bacteria, and 162 indigenous isolates from river water

Strains	Collection[a]	Biovar	Geographic origin	Host or source	PCR amplification		
					OLI 1/Z	OLI1/Y2	Co-PCR
Ralstonia solanacearum	NCPPB 325 (Type strain)	1	USA	*Lycopersicon esculentum*	+	+	+
	PD 1446	1	Costa Rica	*Heliconia* spp.	+	+	+
	PD 511	1	USA	*Lycopersicon esculentum*	+	+	+
	PD 1608	1	Trinidad	*Musa* sp.	+	+	+
	PD 1940	1	Reunion Island	*Pelargonium capitatum*	+	+	+
	PD 1946	1	Reunion Island	*Pelargonium capitatum*	+	+	+
	42 SMT	1	Costa Rica	*Solanum tuberosum*	+	+	+
	IVIA 2528.55	2	Spain	River water	+	+	+
	IVIA 2167	2	Spain	River water	+	+	+
	IVIA 1602.1	2	Spain	*Solanum tuberosum*	+	+	+
	14 SMT	2	UK	*Solanum dulcamara*	+	+	+
	17 SMT	2	UK	River water	+	+	+
	18 SMT	2	UK	River water	+	+	+
	19 SMT	2	UK	River water	+	+	+
	20 SMT	2	UK	River water	+	+	+
	21 SMT	2	UK	River water	+	+	+
	25 SMT	2	Sweden	*Solanum dulcamara*	+	+	+
	27 SMT	2	Belgium	*Solanum dulcamara*	+	+	+
	31 SMT	2	Belgium	River water	+	+	+
	43 SMT	2A	Colombia	*Solanum tuberosum*	+	+	+
	45 SMT	2T	Brazil	*Solanum tuberosum*	+	+	+
	74 SMT	2	The Netherlands	River water	+	+	+
	PD 2762	2	The Netherlands	*Solanum tuberosum*	+	+	+
	PD 2763	2	The Netherlands	*Solanum tuberosum*	+	+	+
	CIP 309	2	Colombia	*Solanum tuberosum*	+	+	+
	PD 1434	3	Australia	*Lycopersicon esculentum*	–	+	–
	46 SMT	3	Peru	*Lycopersicon esculentum*	–	+	–
	47 SMT	3	Australia	*Solanum tuberosum*	–	+	–
	CIP 365	3	Philippines	*Solanum tuberosum*	–	+	–
	CIP 65	3	Costa Rica	*Capsicum* spp.	–	+	–
	ACH 0647	3	Australia	*Lycopersicon esculentum*	–	+	–
	PD 1454	4	Philippines	*Capsicum* spp.	–	+	+
	PD 1410	4	Sri Lanka	*Solanum tuberosum*	–	+	+
	PD 1424	4	Japan	*Arachis hypogae*	–	+	+
	PD 1455	4	USA	*Capsicum* spp.	–	+	+
	49 SMT	4	Philippines	*Zingiber officinale*	–	+	+
	JT 661	5	China	*Morus alba*	–	+	–
	JT 659	5	China	*Morus alba*	–	+	–
Ralstonia syzygii	NCPPB 3791		n.a.	n.a.	+	+	+
Ralstonia pickettii	58 SMT		n.a.	n.a.	–	–	–
Ralstonia sp.	PD 2778		n.a.	n.a.	–	–	–
Pseudomonas chlororaphis	65 SMT		n.a.	n.a.	–	–	–
Blood Disease Bacterium (BDB)	NCPPB 3726		n.a.	n.a.	+	+	+
Pseudomonas marginalis pv. *marginalis*	CFBP 1538		USA	*Solanum tuberosum*	–	–	–
Burkholderia andropogonis	NCPPB 1127		Zimbabwe	*Bougainvillea* sp.	–	–	–
Burkholderia caryophylli	NCPPB 353		USA	*Dianthus* sp.	–	–	–
Burkholderia cepacia	NCPPB 945		n.a.	n.a.	–	–	–
Burkholderia glumae	NCPPB 3708		n.a.	n.a.	–	–	–
Burkholderia plantarii	NCPPB 3590		n.a.	n.a.	–	–	–

(continued on next page)

Table 2 (*continued*)

Strains	Collection[a]	Biovar	Geographic origin	Host or source	PCR amplification		
					OLI 1/Z	OLI1/Y2	Co-PCR
Enterobacteriaceae	61 SMT		n.a.	n.a.	–	–	–
Rahnella aquatilis	62 SMT		n.a.	n.a.	–	–	–
Ochrobactrum anthropi	63 SMT		n.a.	n.a.	–	–	–
Coryneforme bacterium	64 SMT		n.a.	n.a.	–	–	–
Bacillus polymyxa	CFBP 1954		France	*Solanum tuberosum*	–	–	–
162 indigenous isolates from river water	IVIA		Spain	Different water courses	0/162	6/162	0/162

n.a.: not available.

[a] SMT, Collection of EC-SMT-4-CT97-2179 project (DGXII-EGAA); PD, Collection of Plant Protection Service, Wageningen, The Netherlands; IVIA, Collection of Plant Pathogenic Bacteria, Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain; NCPPB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, Sand Hutton, York, United Kingdom; CFBP, Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; CIP, International Collection, Centro Internacional de la Papa, Lima, Perú; ACH, A.C. Hayward, University of Queensland, Australia; JT, Stéphane Poussier, INRA Toulouse.

OLI2/JE-2) were of 127 and 281 bp, as expected. The restriction fragments obtained from the six indigenous water strains, which generated a PCR product of the same size as *R. solanacearum*, differed from the *R. solanacearum* restriction fragments or generated no product at all (data not shown).

To confirm the identification of *R. solanacearum*, the Co-PCR (OLI1/OLI2/JE-2) amplification product from IVIA 2528.55 strain was sequenced. To investigate the homology between *R. solanacearum* and the six strains from water, the nucleotide sequence of their amplicons after Co-PCR with OLI1/OLI2/Y2/JE-2 primers was obtained and compared with those of *R. solanacearum* strains IVIA 2528.55 and ATCC 11696. The strain IVIA 2528.55 gave a nucleotide sequence highly similar to strain ATCC 11696 (Yabuuchi et al., 1992). There were only two variations: an A at position 139 instead of a G, and a C at position 243 instead of a T. Nevertheless, the strain IVIA 2.3 seemed to be quite different from the ATCC 11696 strain, showing a variation percentage of 18.46% (48 base variations); while 43 differences appeared when we compared this sequence with all other five strains from water corresponding to a variation percentage of 16.53%. The isolates IVIA 2.1, 5.2, 5.6, 15.1 and 15.2 have the same nucleotide sequence among them, but show 45 nucleotide variations in their sequences compared to ATCC 11696 strain (Fig. 4). In every case, the sequence from Y2 primer was present and it appeared conserved and identical in the six analyzed strains.

3.3. Hybridization and colorimetric detection

The usefulness of the designed probe EPRS for *R. solanacearum* detection was tested using pure cultures and spiked water samples. Specific hybridization and high signal were observed in the suspensions of pure cultures and with spiked water samples (Fig. 1). The results from the 51 environmental water samples (Table 3) were also examined. In cases where a very weak band was obtained in gel electrophoresis, a strong signal was obtained after colorimetric analysis (data not shown) demonstrating that the use of the EPRS probe improved ease of recognition of a positive result. All samples from which an intense amplification band was obtained also yielded a clear positive color reaction. Negative control (without DNA) was always negative. No colorimetric signal was observed when *R. solanacearum*-negative isolates IVIA 2.1, 2.3, 5.2, 5.6, 15.1 and 15.2 from water were tested by Co-PCR using primers OLI1/OLI2/JE-2, while a positive reaction was obtained when the same isolates were tested by Co-PCR using the four-primer combination (OLI1/OLI2/Y2/JE-2) (Table 4).

3.4. Environmental water samples

Fifty-one environmental water samples from various Spanish sources were examined. All the samples were plated and tested before and after DNA extraction by the conventional PCR amplification assays,

Table 3
Detection of *Ralstonia solanacearum* in Spanish water samples comparing conventional PCR assays, Co-PCR and cultural methods

Sample	Geographical origin	Source	PCR amplification						Culturable *R. solanacearum* on SMSA (cfu/ml)	Average total culturable bacteria on YPGA (cfu/ml)
			OLI1/Z		OLI1/Y2		Co-PCR (OLI1/OLI2/JE2)			
			Non-E.[a]	E.[b]	Non-E.[a]	E.[b]	Non-E.[a]	E.[b]		
1	Andalucía	Irrigation channel	–	–	–	–	–	–	–	4.7×10^2
2	Andalucía	Irrigation channel	–	–	–	–	–	–	–	60
3	Andalucía	Irrigation channel	–	–	–	–	–	–	–	1.8×10^5
4	Andalucía	Irrigation channel	–	–	–	–	–	–	–	1.6×10^4
5	Aragón	River water	–	–	–	–	–	–	–	1.2×10^5
6	Aragón	River water	–	–	–	–	–	–	–	1.3×10^5
7	Aragón	River water	–	–	–	–	–	–	–	2.2×10^4
8	Aragón	River water	–	–	–	–	–	–	–	2.6×10^4
9	Aragón	River water	–	–	–	–	–	–	–	1.9×10^4
10	Aragón	River water	–	–	–	–	–	–	–	1.4×10^4
11	Aragón	River water	–	–	–	–	–	–	–	9.6×10^4
12	Aragón	River water	–	–	–	–	–	–	–	1×10^5
13	C. Valenciana	Spring	–	–	–	–	–	–	–	2×10^2
14	C. Valenciana	Stream	–	–	–	–	–	–	–	6×10^2
15	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	1×10^3
16	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	1×10^3
17	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	7×10^4
18	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	3.5×10^4
19	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	3.4×10^4
20	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	1.9×10^4
21	C. Valenciana	Well	–	–	–	–	–	–	–	1×10^2
22	C. Valenciana	Well	–	–	–	–	–	–	–	2.7×10^3
23	C. Valenciana	Well	–	–	–	–	–	–	–	1.4×10^3
24	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	1×10^4
25	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	1.3×10^5
26	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	5.3×10^2
27	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	1.5×10^4
28	C. Valenciana	Irrigation channel	–	–	–	–	–	–	–	1.8×10^4
29	Castilla-La Mancha	Stream	–	–	–	–	–	–	–	5.7×10^3
30	Castilla-La Mancha	Spring	–	–	–	–	–	–	–	6.1×10^3
31	Castilla-León	River water	–	–	–	–	+	–	< 100	1.6×10^3
32	Castilla-León	River water	–	–	–	–	+	+	< 100	4.1×10^3
33[c]	Castilla-León	River water	+	–	+	+	+	+	–	2.2×10^3
34	Castilla-León	River water	+	–	+	–	+	+	–	7.7×10^2
35[c]	Castilla-León	River water	+	+	+	–	+	+	–	1.6×10^3
36	Castilla-León	River water	+	–	+	+	+	+	–	3.5×10^3
37[c]	Castilla-León	River water	–	–	–	–	+	+	–	1.4×10^3
38	Castilla-León	River water	–	–	–	–	+	–	< 100	2×10^3
39[c]	Castilla-León	River water	–	+	–	–	+	+	–	6.7×10^2
40	Castilla-León	River water	–	–	–	–	+	+	< 100	2.4×10^3
41	Castilla-León	River water	–	–	–	+	+	+	< 100	2.6×10^3
42	Castilla-León	River water	–	–	–	–	+	+	< 100	3.5×10^3
43[c]	Castilla-León	River water	–	–	–	+	+	+	–	1.8×10^3
44[c]	Castilla-León	River water	–	–	–	–	+	–	–	2.5×10^3
45	Castilla-León	River water	–	–	–	–	+	–	< 100	1.6×10^3
46[c]	Castilla-León	River water	+	–	+	–	+	+	–	4.8×10^2
47	Galicia	River water	–	–	–	–	–	–	–	5×10^3
48	Galicia	River water	–	–	–	–	–	–	–	4×10^2

(continued on next page)

Table 3 (*continued*)

Sample	Geographical origin	Source	PCR amplification						Culturable *R. solanacearum* on SMSA (cfu/ml)	Average total culturable bacteria on YPGA (cfu/ml)
			OLI1/Z		OLI1/Y2		Co-PCR (OLI1/OLI2/JE2)			
			Non-E.[a]	E.[b]	Non-E.[a]	E.[b]	Non-E.[a]	E.[b]		
49	Galicia	River water	–	–	–	–	–	–	–	1.6×10^3
50	Galicia	River water	–	–	–	–	–	–	–	4×10^2
51	Galicia	River water	–	–	–	–	–	–	–	2×10^2

[a] Without DNA extraction.
[b] With DNA extraction.
[c] Sample confirmed positive by modified protocol based on the enrichment DASI-ELISA described by Caruso et al. (2002).

and by Co-PCR. As shown in Table 3, 16 out of 51 water samples tested positive by Co-PCR assay before the DNA extraction, but only 12 of these positive samples were also positive after DNA extraction. Seven samples (five positive before the DNA extraction and two positive only after the extraction) were positive by using OLI1/Y2 primers, and six (five positive before the DNA extraction and one positive only after the extraction) using OLI1/Z primers (Table 3). The indigenous microbiota of the water samples varied from 6×10 to 1.8×105 cfu/ml in nonselective YPGA medium (Lelliot and Stead, 1987).

The attempts to isolate *R. solanacearum* by culturing methods using a general YPGA (Lelliot and Stead, 1987) and a selective modified SMSA medium (Elphinstone et al., 1996) provided positive results for only 7 of the 51 water samples analysed, with *R. solanacearum* populations ranging below 10^2 cfu/ml (Table 3).

In the PCR assays, similar results were obtained using OLI1/Y2 or OLI1/Z primers (13.7% positive samples) and being inferior to the number of the positive samples using the developed Co-PCR assay (31.3%). Seven of the environmental samples (33, 35, 37, 39, 43, 44 and 46 in Table 3) that were negative by plating were confirmed positive by enrichment DASI-ELISA. Two Co-PCR positive environmental samples (34 and 36 in Table 3) were not confirmed by the isolation or the serological technique but were confirmed by other PCR methods.

4. Discussion

The application of Co-PCR to detect low populations of *R. solanacearum* in watercourses showed greater sensitivity and specificity than conventional PCR protocols. As previously described for RNA viruses (Olmos et al., 2002), we observed that the reaction of the three primers OLI1/OLI2/JE-2 (Fig. 3A) allows their concomitant action in a single amplification reaction giving origin to two specific products (Fig. 3B). We suppose, according to the hypothesis of Olmos et al. (2002), that the reverse strand of the shortest amplicon obtained by the action of primers OLI2 and JE-2 is used as an additional primer to

Table 4
Amplification of six strains from different water sources that generated a 408-pb product

Strain	PCR amplifications				
	OLI1/Z	OLI1/Y2	Co-PCR OLI1/OLI2/Y2/JE2	Co-PCR OLI1/OLI2/JE2	Fatty acid identification[a]
IVIA 2.1	–	+	+	–	*Acinetobacter johnsonii*
IVIA 2.3	–	+	+	–	*Aeromonas salmonicida*
IVIA 5.2	–	+	+	–	*Acinetobacter johnsonii*
IVIA 5.6	–	+	+	–	*Acinetobacter johnsonii*
IVIA 15.1	–	+	+	–	*Acinetobacter johnsonii*
IVIA 15.2	–	+	+	–	*Acinetobacter johnsonii*

[a] Analysis of fatty acid profile by MIS system.

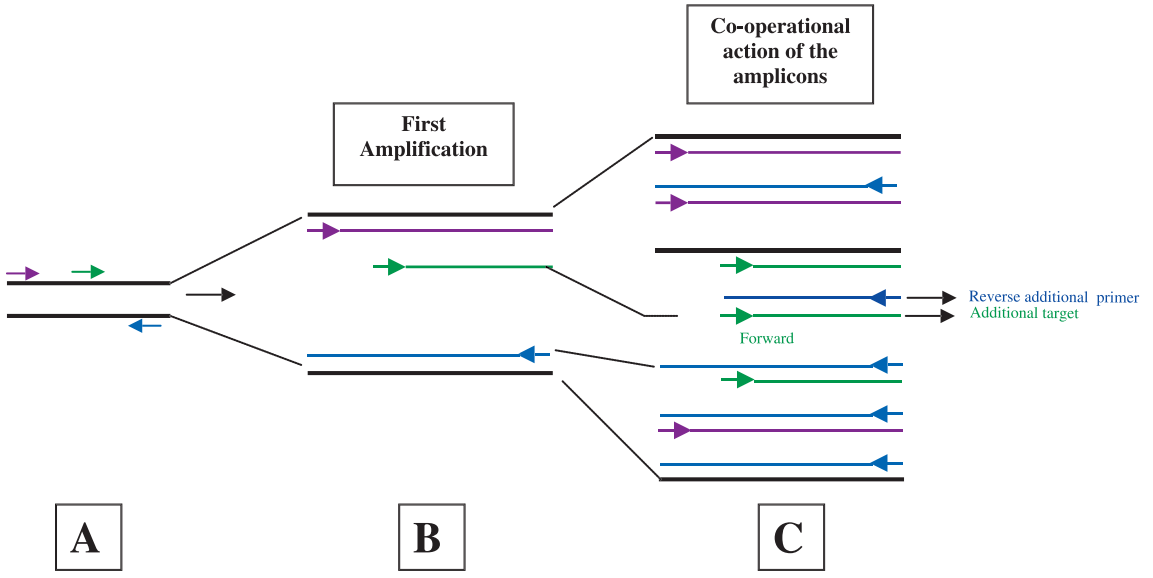

Fig. 3. Scheme of the modified Co-operational amplification (Co-PCR) procedure. (A) Annealing of the three different primers; (B) concomitant action of the primers; (C) synthesis of the new additional primer and co-operational action of the amplicons and primers.

increase the yield of the largest amplicon, which is the final product visualized in the agarose gel. The forward strand of the shortest amplicon is used as additional target for OLI2 to produce new reverse primer, which could be incorporated to the reaction as an auxiliary primer (Fig. 3C). The internal primer was added at a smaller concentration than the external primers to promote the production of the largest amplicon. This is an explanation of a process that highly enhances the PCR sensitivity allowing the detection of < 1 cfu/ml of *R. solanacearum* from water without the need for a DNA extraction of the sample. This procedure is as sensitive as the nested technique, but it is performed employing only one tube and, in this way, is able to decrease the risks of contamination due to the previous amplification. Moreover, the close annealing temperatures of the primers used in this study would not permit to perform a nested-PCR in a single tube. The primers used were based on the 16S rDNA consensus sequence of *R. solanacearum* species from all strains of subdivision 2a sequenced by Tagahavi et al. (1996). Unsurprisingly, these primers identify the *P. syzygii* and the BDB since their 16S rRNA sequences have a similarity around 99% to *R. solanacearum* (Seal et al., 1993). The amplification product generated from *P. syzygii* and the BDB using these primers should not diminish the interest of this PCR as a tool to detect *R. solanacearum* since *P. syzygii* and the BDB have only been recorded in clove and in banana in Indonesia (Gäumann, 1923; Roberts et al., 1990) and never from water samples.

The amplification of DNA target from several unexpected water samples taken from *R. solanacearum*-free areas suggested that the initially selected four-primer combination amplified sequences of the indigenous microbial populations other than *R. solanacearum*. This was confirmed by their isolation and sequencing of their amplicons. To overcome this problem, formamide was added to the PCR cocktail, but it did not prevent the appearance of the unspecific PCR products. Only after removing primer Y2 from the PCR mix of Co-PCR did none of the 162 unidentified isolates from water generate any PCR product. The nucleotide sequence of the fragments amplified from strains IVIA 2.1, 2.3, 5.2, 5.6, 15.1 and 15.2 with OLI1/Y2 PCR and/or OLI1/OLI2/Y2/JE-2 Co-PCR assays demonstrated that the production of this fragment was related to the action of the nonspecific primer Y2 (Fig. 4). This result reduces the usefulness of the OLI1/Y2 primers as a tool to detect *R. solanacearum* in watercourses due to a risk of false positive results. Moreover, the lack of ampli-

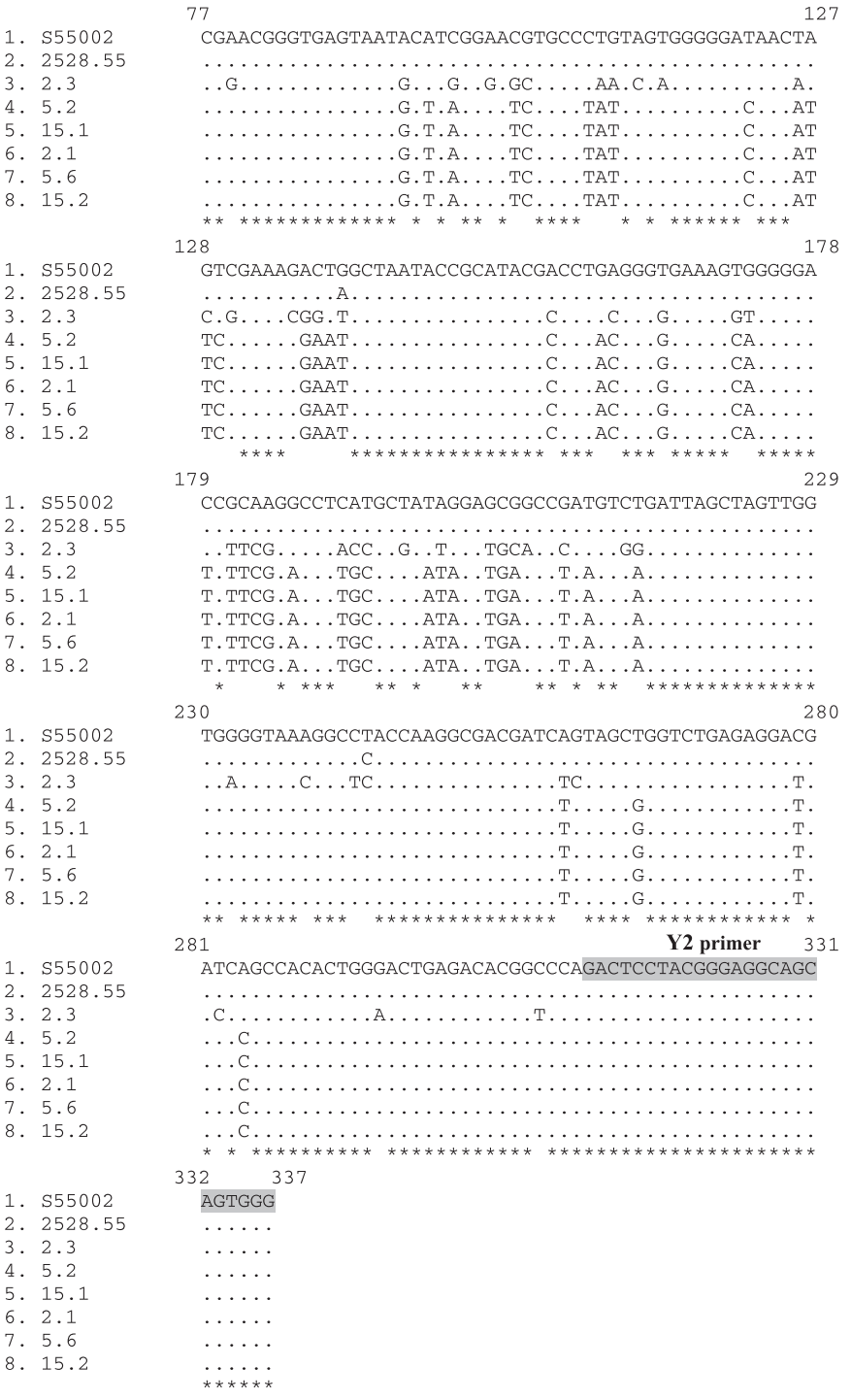

Fig. 4. Multiple sequence alignment by CLUSTALW comparing the *R. solanacearum* strain ATCC 11696 (GenBank accession no. S55002) with *R. solanacearum* IVIA 2528.55 and six strains from different water sources that generated a 408-bp product when amplified with OLI1/OLI2/Y2/JE2 primers. The sequence of the Y2 primer is indicated.

fication of target from biovar 3 and 5 isolates was probably due to the removal of primer Y2. Nevertheless, this did not represent an obstacle in the use of the developed Co-PCR to detect *R. solanacearum* from water sources because biovars 3 and 5 have never been recorded from water samples and biovar 5 has only been recorded from *Morus alba* in China (Roberts et al., 1990; Gäumann, 1923; Eden-Green and Sastraatmadja, 1990). As Co-PCR performed with only OLI1/OLI2/JE-2 primers and 3% formamide showed a good specificity, the protocol was considered appropriate for routine use.

One of the major obstacles to the use of PCR on environmental water samples is the frequent presence of organic matter such as humic substances, which are strong inhibitors of PCR, possibly by interacting with the DNA polymerase or the binding of the primers to the template, and thereby greatly reducing the detection limit (Tsai and Olson, 1992). In this study, sometimes a dilution of the samples was necessary to increase the efficiency of detection. Undiluted samples that showed a weak product by Co-PCR gave a strong signal when diluted in a range of 1:10–1:100 (data not shown).

DNA extraction of the samples is often required to concentrate the DNA target to increase the sensitivity. However, the DNA extraction protocol used could be not efficient enough for water samples and/or the DNA extract results in a reduction of the amount of the target sequence and, consequently, in a reduction in the detection sensitivity (Bej and Mahbubani, 1992). In our assays with low numbers of pathogen, the results of amplification after DNA extraction were erratic as shown in Table 3. A simple cell lysis by heat treatment was generally more efficient than a DNA extraction. On the other hand, in exceptional situations, e.g. when the presence of strong inhibitors is suspected, DNA extraction of the sample could be advised. This step should be optimised and/or other protocols assayed before routinely used for water samples.

The developed Co-PCR protocol allowed detection of <1 cfu/ml of *R. solanacearum* from both natural and spiked water without the need of a DNA extraction step and only within a few hours. The sampling methodology applied was defined in an EC funded project but obviously the analysis of a fraction of the original sample could be a limiting factor for the final sensitivity. The amount of indigenous water microbiota (from 10^2 to 10^5 cfu/ml) did not interfere with the detection of *R. solanacearum* present at low levels. All samples positive by Co-PCR were collected from river water at points where *R. solanacearum* had been isolated previously (Palomo et al., 2000), while the remaining 35 negative samples were taken from localities where *R. solanacearum* was not reported. The attempt to isolate *R. solanacearum* by traditional culturing methods was successful only in 7 out of 51 analysed samples. It is not surprising that the other samples positive by Co-PCR were undetected by the other PCR methods because they showed inferior sensitivity in the comparative assays.

The described method provides a useful tool for the detection of *R. solanacearum* from environmental water samples. In fact, the ability displayed by *R. solanacearum* in surviving in water for long periods, even under adverse conditions, implies an epidemiological threat, irrigation water being an important carrier of dissemination of this quarantine pathogen (Olson, 1976; Janse et al., 1998; Hayward et al., 1998; Person, 1998; Van Elsas et al., 2000; Timms-Wilson et al., 2001; López and Biosca, in press). In conclusion, we have set up a sensitive detection method for this microorganism, taking into consideration that the impaired detection of the pathogen, under unfavorable conditions, could lead to an underestimation of its occurrence, with negative economic repercussions on agriculture.

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References

Anonymous, 1998. Council Directive 98/57/EC of 20 July 1998 on the control of *Ralstonia solanacearum* (Smith) Yabuuchi et al. Official Journal of the European Communities, No. L235/1, 21.08.98.

Anonymous, 2001. Optimised protocols for diagnosis of potato brown rot and bacterial wilt and detection of the causal bacterium (*Ralstonia solanacearum*) in infected plants, surface water, soil, industrial potato waste and sewage. EC-funded SMT Project CT97-2179. Final report. Appendix 1.

Altschul, S.F., Madden, T.L., Schäfer, A.A., Zhang, J., Zhang, Z., Miller, W., Myers, E.W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bej, A.K., Mahbubani, M.H., 1992. Application of polymerase chain reaction in environmental microbiology. PCR Methods Appl. 1, 151–159.

Bertolini, E., Olmos, A., Martínez, M.C., Gorris, M.T., Cambra, M., 2001. Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA viruses in olive trees. J. Virol. Methods 96, 33–41.

Bertolini, E., Olmos, A., López, M.M., Cambra, M., 2002. Multiplex nested RT-PCR in a single tube for sensitive and simultaneous detection of four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in olive trees. Phytopathology 93, 286–292.

Biosca, E.G., Caruso, P., Bertolini, E., Álvarez, B., Palomo, J.L., Gorris, M.T., López, M.M., 2003. Improved detection of *Ralstonia solanacearum* in culturable and VBNC state from water samples at low temperature. 3rd International Bacterial Wilt Symposium. In press.

Boudazin, G., Le Roux, A.C., Josi, K., Labarre, P., Jouan, B., 1999. Design of division specific primers of *Ralstonia solanacearum* and application to the identification of European isolates. Eur. J. Plant Pathol. 105, 373–380.

Buddenhagen, I.W., Sequeira, L., Kelman, A., 1962. Designation of races in *Pseudomonas solanacearum*. Phytopathology 52, 726.

Caruso, P., Gorris, M.T., Cambra, M., Palomo, J.L., Collar, J., López, M.M., 2002. Enrichment-DASI-ELISA for sensitive detection of *Ralstonia solanacearum* in asymptomatic potato tubers using a specific monoclonal antibody. Appl. Environ. Microbiol. 68, 3634–3638.

Cook, D., Sequeira, L., 1994. Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. In: Hayward, A.C., Hartman, G.L. (Eds.), Bacterial Wilt: The Disease and Its Causative Agent, *Pseudomonas solanacearum*. CAB International Mycological Institute, Wallingford, UK, pp. 137–142.

Cook, D., Barlow, E., Sequeira, L., 1989. Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment length polymorphisms that specify virulence and the hypersensitive response. Mol. Plant-Microb. Interact. 2, 113–121.

De Boer, S.H., 1990. Immunofluorescence. Bacteria. In: Hampton, R., Ball, E., De Boer, S. (Eds.), Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. APS Press, St. Paul, MN, pp. 295–298.

Eden-Green, S.J., Sastraatmadja, H., 1990. Blood disease present in Java. FAO Plant Prot. Bull. 38, 49–50.

Elphinstone, J.G., Hennessy, J., Wilson, J.K., Stead, D.E., 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tubers extracts. Bull. OEPP 26, 663–678.

Elphinstone, J.G., Stanford, H.M., Stead, D.E., 1998. Detection of *Ralstonia solanacearum* in potato tubers, *Solanum dulcamara* and associated irrigation water extracts. In: Prior, P., Allen, C., Elphinstone, J. (Eds.), Bacterial Wilt Disease. Molecular and Ecological Aspects Springer Verlag editions, Heidelberg, Germany, pp. 133–139.

Fegan, M., Holoway, G., Hayward, A.C., Timmis, J., 1998. Development of a diagnostic test based on the polymerase chain reaction (PCR) to identify strains of *Ralstonia solanacearum* exhibiting the biovar 2 genotype. In: Prior, P., Allen, C., Elphinstone, J. (Eds.), Bacterial Wilt Disease. Molecular and Ecological Aspects Springer Verlag editions, Heidelberg, Germany, pp. 34–43.

Gäumann, E., 1923. Onderzoekeningen over de bloedziekte der bananen op Celebes II. Mededeelingen van het Institut voor Plantenziekten 59, 1–47.

Grey, B.E., Steck, T.R., 2001. The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. Appl. Environ. Microbiol. 67, 3866–3872.

Hayward, A.C., 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol. 27, 265–277.

Hayward, A.C., 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29, 65–89.

Hayward, A.C., 1994. The hosts of *Pseudomonas solanacearum*. In: Hayward, A.C., Hartman, G.L. (Eds.), Bacterial Wilt: The Disease and Its Causative Agent, *Pseudomonas solanacearum*. CAB International Mycological Institute, Wallingford, UK, pp. 9–24.

Hayward, A.C., El-Nashaar, H.M., Nydegger, U., De Lindo, L., 1990. Variation in nitrate metabolism in biovars of *Pseudomonas solanacearum*. J. Appl. Bacteriol. 69, 269–280.

Hayward, A.C., Elphistone, J.G., Caffier, D., Janse, J., Stefani, E., French, E.R., Wright, A.J., 1998. Round table on bacterial wilt (brown rot) of potato. In: Prior, P., Allen, C., Elphinstone, J. (Eds.), Bacterial Wilt Disease. Molecular and Ecological Aspects Springer Verlag editions, Heidelberg, Germany, pp. 420–430.

He, L.Y., Sequeira, L.L., Kelman, A., 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis. 67, 1357–1361.

Janse, J.D., 1988. A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. Bull. OEPP 18, 343–351.

Janse, J.D., 1996. Potato brown rot in Western Europe: history, present occurrence and some remarks on possible origin, epidemiology and specificity. Bull. OEPP 26, 679–695.

Janse, J.D., Schans, J., 1998. Experience with the diagnosis and epidemiology of bacterial brown rot (*Ralstonia solanacearum*) in The Netherlands. Bull. OEPP 28, 65–67.

Janse, J.D., Araluppan, F.A.X., Schans, J., Wenneker, M., Westerhuis, W., 1998. Experiences with bacterial brown rot *Ralstonia solanacearum* biovar 2, race 3 in the Netherlands. In: Prior, P., Allen, C., Elphinstone, J. (Eds.), Bacterial Wilt Disease. Molecular and Ecological Aspects Springer Verlag editions, Heidelberg, Germany, pp. 146–152.

Lelliot, R.A., Stead, D.E., 1987. Methods for the diagnosis of bacterial diseases of plants. In: Preece, T.F. (Ed.), Methods in Plant Pathology, vol. 2, p. 216.

Li, X., Dorsch, M., Del Dot, T., Sly, L.I., Stackebrandt, E., Hayward, A.C., 1993. Phylogenetic studies of the rRNA group II pseudomonas based on 16S rRNA gene sequences. J. Appl. Bacteriol. 74, 324–329.

Llop, P., Caruso, P., Cubero, J., Morente, C., López, M.M., 1999. A simple extraction procedure for efficient routine detection of plant pathogenic bacteria in plant material by polymerase chain reaction. J. Microbiol. Methods 37, 23–31.

Llop, P., Bonaterra, A., Peñalver, J., López, M.M., 2000. Development of highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. Appl. Environ. Microbiol. 66, 2071–2078.

López, M.M., Biosca, E.G., 2003. Potato wilt management: new prospects for an old problem. 3rd International Bacterial Wilt Symposium. In press.

Maes, M., Garbeva, P., Crepel, C., 1996. Identification and sensitive endophytic detection of the fire blight pathogen *Erwinia amylovora* with 23S ribosomal DNA sequences and the polymerase chain reaction. Plant Pathol. 45, 1139–1149.

Niepold, F., Schöber-Butin, B., 1997. Application of the one-tube PCR technique in combination with a fast DNA extraction procedure for detecting *Phytophthora infestans* in infected potato tubers. Microbiol. Res. 152, 345–351.

Olmos, A., Bertolini, E., Cambra, M., 2002. Simultaneous and Cooperational amplification (Co-PCR): a new concept for detection of plant viruses. J. Virol. Methods 106, 15–23.

Olson, K., 1976. Experiences of brown rot caused by *Pseudomonas solanacearum* (Smith) Smith in Sweden. Bull. OEPP 6, 199–207.

Opina, N., Tavner, F., Holloway, G., Wang, J.-F., Li, T.H., Maghirang, R., Fegan, M., Hayward, A.C., Krishnapillai, V., Hong, W.F., Holloway, B.W., Timmis, J.N., 1997. A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia Pac. J. Mol. Biol. Biotechnol. 5, 19–33.

Palomo, J.L., Caruso, P., Gorris, M.T., López, M.M., García-Benavides, P., 2000. Comparación de métodos de detección de *Ralstonia solanacearum* en aguas superficiales. X Congreso de la Ed. Sociedad Española de Fitopatología, Spain, p. 120.

Pastrik, K.H., Maiss, E., 2000. Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. J. Phytopathol. 148, 619–626.

Person, P., 1998. Successful eradication of *Ralstonia solanacearum* from Sweden. Bull. OEPP 28, 113–119.

Picard, C., Ponsonnet, C., Paget, E., Nesme, X., Simonet, P., 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. 58, 2717–2722.

Poussier, S., Luisetti, J., 2000. Specific detection of biovars of *Ralstonia solanacearum* in plant tissue by nested PCR. Eur. J. Plant Pathol. 106, 255–265.

Poussier, S., Trigalet-Demery, D., Vandewalle, P., Goffinet, B., Luisetti, J., Trigalet, A., 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the hrp gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146, 1679–1692.

Poussier, S., Chéron, J.-J., Couteau, A., Luisetti, J., 2002. Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates. J. Microbiol. Methods 51, 349–359.

Ralston, E., Palleroni, N.J., Doudoroff, M., 1973. *Pseudomonas pickettii*, a new species of clinical origin related to *Pseudomonas solanacearum*. Int. J. Syst. Bacteriol. 23, 15–19.

Roberts, S.J., Eden-Green, S.J., Jones, P., Ambler, D.J., 1990. *Pseudomonas syzygii* sp. nov., the cause of Sumatra disease of cloves. Syst. Appl. Microbiol. 13, 34–43.

Seal, S.E., Jakson, L.A., Young, J.P.W., Daniels, M.J., 1993. Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii*, and blood disease bacterium by partial 16S sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J. Gen. Microbiol. 139, 1587–1594.

Seal, S.E., Taghavi, M., Fegan, N., Hayward, A.C., Fegan, M., 1999. Determination of *Ralstonia (Pseudomonas) solanacearum* rRNA subgroups by PCR tests. Plant Pathol. 48, 115–120.

Tagahavi, M., Hayward, C., Sly, L.I., Fegan, M., 1996. Analysis of the phylogenetic relationship of strains of *Burkholderia solanacearm*, *Pseudomonas syzygii*, and the blood disease bacterium of banana based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. 46, 10–15.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Timms-Wilson, T.M., Bryant, K., Bailey, M.J., 2001. Strain characterization and 16S–23S probe development for differentiating geographically dispersed isolates of the phytopathogen *Ralstonia solanacearum*. Environ. Microbiol. 3, 785–797.

Tsai, Y.L., Olson, B.H., 1992. Detection of low numbers of bacterial cells in soil and sediments by polymerase chain reaction. Appl. Environ. Microbiol. 58, 754–757.

Van Elsas, J.D., Kastelein, P., van Bekkum, P., van der Wolf, J.M., de Vries, P.M., van Overbeek, L.S., 2000. Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathology 90, 1358–1366.

Weller, S.A., Elphinstone, J.G., Smith, N.C., Boonham, N., Stead, D.E., 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl. Environ. Microbiol. 66, 2853–2858.

Wenneker, M., Verdel, M.S.W., Groeneveld, R.M.W., Kempenaar, C., van Beuningen, A.R, Janse, J.D., 1999. *Ralstonia (Pseudomonas) solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: first report on stinging nettle (*Urtica dioica*). Eur. J. Plant. Pathol. 105, 307–315.

Wood, J.R., Breckenridge, K., Chard, J.M., 2002. Eradicating *Ralstonia solanacearum* from Scottish rivers. In: Terblanche, J. (Ed.), Abstracts of the 3rd International Bacterial Wilt Symposium, Tobacco and Coton Research Institute, Rustenburg, South Africa, p. 31.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., Arakawa, M., 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 36 (12), 1251–1275.

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., Nishiuchi, Y., 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov. Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov.: *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. 39, 897–904.