ELSEVIER

Journal of Microbiological Methods 51 (2002) 349–359

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates

Stéphane Poussier [a,b], Jean-Jacques Chéron [a], Annie Couteau [a], Jacques Luisetti [a,*]

[a] *Pôle de Protection des Plantes, CIRAD, 7, chemin de l'IRAT, 97455 St. Pierre, La Réunion, France*
[b] *Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, INRA/CNRS, UMR215, chemin de Borde-Rouge, 31326 Castanet-Tolosan, France*

Received 7 January 2002; received in revised form 22 February 2002; accepted 14 May 2002

Abstract

Several procedures were compared for reliable PCR detection of *Ralstonia solanacearum* in common substrates (plant, seed, water and soil). In order to prevent the inhibition of PCR by substances contained in crude extracts, numerous DNA extraction procedures as well as additives to buffers or PCR mixtures were checked. Our results showed that the efficiency of these methods or compounds depended greatly upon the nature of the sample. Consequently, preparation of samples prior to PCR depended upon sample origin. Simple methods such as a combined PVPP/BSA treatment or the association of filtration and centrifugation for detecting the bacterium in plant or water samples were very powerful. DNA capture also efficiently overcame PCR inhibition problems and ensured the detection of *R. solanacearum* in environmental samples. However, the commercial DNA extraction QIAamp® kit appeared to be the most effective tool to guarantee the accurate PCR detection of the pathogen whatever the origin of the sample; this was particularly true for soil samples where the commonly used methods for the detection of *R. solanacearum* were inefficient. This study demonstrates that using an appropriate procedure, PCR is a useful and powerful tool for detecting low levels of *R. solanacearum* populations in their natural habitats.

Keywords: DNA capture; DNA extraction; Environmental samples; Methodology; PCR detection; *Ralstonia solanacearum*

1. Introduction

Bacterial wilt, caused by *Ralstonia solanacearum*, is responsible for severe losses to many important crops, mainly Solanaceous plants and bananas, in tropical and subtropical regions (Hayward, 1991). In Western Europe, where several outbreaks of the disease were recently reported, the disease represents a serious threat (Janse, 1996; Stead et al., 1996). Despite the use of many different long-term control strategies, bacterial wilt is still a very devastating disease and control measures often remain ineffective. Adapted prophylactic measures combined with the use of resistant cultivars is, up to now, the most effective way to reduce the incidence of the disease. In order to optimise the efficiency of prophylactic measures, powerful tools for the identification and detection of the bacterium in diverse substrates (plant, seed, water, soil) are required. However, the com-

* Corresponding author. Tel.: +33-262-49-92-24; fax: +33-262-49-92-93.
E-mail address: luisetti@cirad.fr (J. Luisetti).

0167-7012/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0167-7012(02)00111-2

monly used methods [such as isolation on semi-selective medium (Kelman, 1954; Nesmith and Jenkins, 1979; Engelbrecht, 1994), serological methods (ELISA or immunofluorescence, see Janse, 1988; Robinson-Smith et al., 1995), or pathogenicity tests on host plants (McCarter et al., 1969; Graham and Lloyd, 1978)] for the diagnosis of bacterial wilt are often inadequate in terms of specificity, sensitivity or response time, especially for detecting the bacterium in soil. Indeed, it is often difficult to isolate *R. solanacearum* from soil due to overgrowth by abundant saprophytic bacterial colonies. Moreover, serological methods commonly give false positive results since anti-sera are not totally specific and consequently tedious and time-consuming confirmation bioassays are often required.

DNA amplification pathogen offers many advantages over classical techniques; neither purification nor cultivation of the pathogen are required and the specificity, sensitivity and response time of tests are improved. Nevertheless, the PCR method has not yet become a routine diagnostic tool for many laboratories, mainly because of the inhibition of the amplification reaction by compounds contained in crude bacterial extracts which give false negative results or low detection sensitivity. Although a wide range of inhibiting substances have been reported, the identity and mode of action of most of them remain unclear (Wilson, 1997). Some compounds may inhibit the DNA amplification by denaturing or binding to the thermostable DNA polymerase (Young et al., 1993; McGregor et al., 1996), by chelating the Mg^{2+} cofactor for *Taq* polymerase (Tsai and Olson, 1992) or by binding to target DNA (Steffan et al., 1988). Therefore, the purpose of this study was to develop and compare several procedures to overcome PCR inhibition problems and to propose a set of standard protocols for reliable detection of *R. solanacearum* whatever the origin of the sample.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two *R. solanacearum* strains originating from Reunion island were used: JT516 isolated from potato and identified as a biovar 2, and JT519 from geranium rosat (*Pelargonium asperum*) and belonging to biovar 3. These strains, selected for their natural resistance to certain antibiotics, were cultivated on a modified Granada and Sequeira (GS) medium, as described previously (Poussier et al., 1999), supplemented by two antibiotics: nalidixic acid (50 mg/l) and streptomycin (65 mg/l) for strain JT516, rifamycin (120 mg/l) and streptomycin (65 mg/l) for strain JT519.

2.2. Preparation of samples

A schematic summary of the methods tested is shown in Fig. 1.

2.2.1. Plants

Samples from plants (tomato, sweet pepper, eggplant, tobacco, geranium rosat and pepper), cultivated on a field naturally contaminated by *R. solanacearum*, were collected. Stem fragments of 3-cm length were superficially disinfected with ethanol, sliced and ground in 5 ml of Tris buffer or TENPP buffer [50 mM Tris, 20 mM EDTA pH 8.0, 100 mM NaCl, 5% polyvinylpolypyrrolidone (PVPP)], and allowed to steep for 30 min at room temperature.

2.2.2. Seeds

Commercial tomato and eggplant seeds were first washed in Tris buffer for 10 min in order to dissolve the fungicidal coating. Then, 1 g of washed seeds (~ 500 seeds) was artificially contaminated by soaking for 3 h at 4 °C in 20 ml of bacterial suspensions (10^2–10^8 cfu/ml) prepared from 1-day-old cultures (strains JT516 or JT519), and then dried under air flow at room temperature. Bacterial populations associated with seeds were estimated by steeping 45 seeds in 4.5 ml of Tris buffer (10 mM Tris base, pH 7.2) overnight at 4 °C, then half of each sample was homogenised (Ultra-Turrax blender for 30 s). Different maceration buffers were also checked to overcome any inhibitory effect on DNA amplification: PP buffer [8.5 mM K_2HPO_4, 7.5 mM KH_2PO_4, pH 7.0 5% polyvinylpyrrolidone (PVP)], PPP buffer (same content as PP buffer except PVP replaced by PVPP), TENPP buffer (see above), SCPAPP buffer (4.5 mM succinate, 4 mM citrate, 9 mM K_2HPO_4, 11 mM KH_2PO_4, 100 mM ascorbic acid, 5% PVPP) and SCPNAPP (same content as SCPAPP buffer except ascorbic acid replaced by sodium ascorbate).

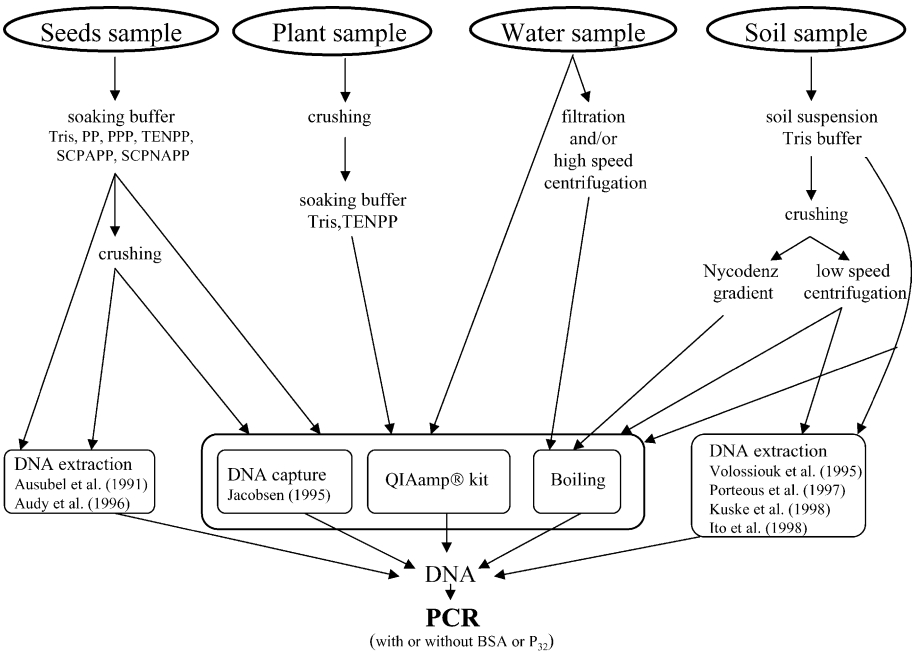

Fig. 1. Procedures assayed for detecting *R. solanacearum* by PCR in common natural substrates.

2.2.3. Water

Samples (500 ml) of irrigation water were artificially contaminated by serial dilutions of strain JT519, giving a final concentration of $10-10^7$ cfu/ml. Then, different methods were applied to samples to concentrate the bacteria. (i) Ten milliliters were centrifuged at $13{,}000 \times g$ for 10 min; the pellet was suspended in 1 ml of Tris buffer; the resulting suspension was centrifuged again at $13{,}000 \times g$ for 10 min; and finally, the pellet was suspended in 300 μl of Tris buffer. (ii) Ten milliliters were filtered through a 0.2-μm nitrocellulose membrane and the membrane was washed for 5 min in 1 ml of Tris buffer. (iii) Ten-milliliter samples were treated by a combination of both methods: filtration as described above then centrifugation at $13{,}000 \times g$ for 10 min; the pellet was finally suspended in 300 μl of Tris buffer.

2.2.4. Soils

Four natural soil types (brown soil, ferrallitic soil, andosol and vertisol), representative of the main soil types described in Reunion island, were collected (Table 1). *R. solanacearum* was not detected in these soils as assessed by plating onto modified GS medium followed by PCR amplification (Poussier and Luisetti, 2000). Samples (500 g) were artificially contaminated by known amounts of bacterial suspensions giving a final concentration of 2×10^3, 2×10^5 or 2×10^7 cfu/g of soil. Twenty grams of soil were suspended in 100 ml of Tris buffer and direct or indirect DNA extraction procedures were applied. For the indirect approach, DNA extraction was preceded by extraction of bacterial cells. First, soil suspensions were crushed using Waring-Blender for 1 min or Ultra-Turrax T25 (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) apparatus for 30 s. Then, from the collected supernatant, a bacterial

Table 1
Main characteristics of the four soil types used in this study

Soil type	Clay content (%)	Organic matter content (%) (0–20 cm)	pH
Andosol	50	22	4.5
Ferrallitic soil	67	6	5
Brown soil	45	6	6.5
Vertisol	67	4	7

fraction was separated from other soil components either by Nycodenz (Life Technologies, Cergy Pontoise, France) gradient as recommended by Bakken and Lindahl (1995) or by centrifugation at $1000 \times g$ for 5 min.

2.3. DNA capture and DNA extraction methods

Aliquots (1 ml) from all of the samples, prepared according to the above protocols (Section 2.2), were simply boiled for 5 min and then cooled on ice to release DNA from cells before performing PCR amplification. In a comparison assay with boiled samples, two rapid and simple methods were also assayed in all samples whatever their origin. The first method is based on DNA capture through binding of a biotynilated specific probe to streptavidin-coated magnetic micro-beads (M-280, Dynal®, Compiègne, France). DNA capture (Jacobsen, 1995) was done using the *R. solanacearum*-specific biotynilated primers RS30 and RS31 (Poussier and Luisetti, 2000). The second method was based on DNA extraction with mini spin columns from the QIAamp® DNA mini kit (Qiagen®, Courtaboeuf, France). In both cases, the protocols were modified by the addition of 5% of PVPP to the recommended lysis buffer.

Further DNA extraction methods for seed samples (Audy et al., 1996; Ausubel et al., 1991) or for soil samples (Volossiouk et al., 1995; Porteous et al., 1997; Ito et al., 1998; Kuske et al., 1998) were also performed. However, the latter protocols were modified by the addition of 5% of PVPP to the recommended lysis buffer.

All samples were also plated onto modified GS medium (Poussier et al., 1999) for estimation of bacterial population sizes and comparison with PCR results. All assays were repeated at least three times.

2.4. PCR amplification

PCRs were performed using either primers OLI1-Y2 as described by Seal et al. (1993), or primer pairs RS30–RS31 followed by RS30a–RS31a and RS30b–RS31b according to the nested PCR procedure detailed by Poussier and Luisetti (2000). In all experiments, 1-μl aliquots of template DNA or water, as negative control, were used. Moreover, 500 ng/μl of bovine serum albumin (BSA) or protein 32 of T4 phage (P_{32}) were added to PCR mixtures to prevent any inhibition effect on PCR. PCR products were analyzed by electrophoresis on 1% agarose gels at 5 V/cm and visualized with UV light after ethidium bromide staining.

2.5. Statistical analysis

The arcsine transformation was applied to the frequencies (x) of detection according to the formula $y = \arcsin \sqrt{x/100}$, before performing a variance analysis. Duncan's multiple comparison test was used to compare means when applicable. Otherwise the frequencies were compared using a GSTAT test (Sokhal and Rohlf, 1969).

3. Results

3.1. Plant samples

Plating onto modified GS medium and nested PCR, using PVPP and BSA as additives to maceration buffer and PCR mixture, respectively (Poussier and Luisetti, 2000), allowed the presence of *R. solanacearum* to be detected in 86 samples, while 27 samples were negative (Table 2). Ten of the twenty-four remaining samples (five from geranium rosat, three from tobacco and two from eggplant) were positive only after plating, and 14 of them (nine from tobacco, four from tomato and one from eggplant) were positive only by nested PCR.

DNA capture and DNA extraction using the QIAamp® kit were compared to the combined PVPP/BSA treatment on 24 plant samples (Table 3). DNA capture and the combined PVPP/BSA treatment identified 16 positive samples, and the QIAamp® kit gave 21 positive samples. These differences are not significant due to the low number of samples. However, detection of *R. solanacearum* from geranium rosat was greatly improved by using the QIAamp® kit.

3.2. Seed samples

Without additives to buffers or PCR mixtures, detection of *R. solanacearum* by PCR or nested

Table 2
Comparison between plating onto semiselective culture medium and nested PCR for detecting *R. solanacearum* in plant tissues

Plant	No. of samples	Estimated ranges of bacterial population densities [log(cfu/ml)]	Detection of *R. solanacearum* by nested PCR[a]
Tomato	35	3.32–10.41	+
	4	0	+
	1	0	–
Sweet pepper	33	3.32–10.14	+
Eggplant	12	2.64–9.83	+
	2	2.08–3.83	–
	1	0	+
	22	0	–
Tobacco	1	3.61	+
	3	2.15–3.43	–
	9	0	+
	2	0	–
Geranium rosat	4	4.34–9.08	+
	5	3.04–4.38	–
	2	0	–
Pepper	1	3.04	+

[a] The combined treatment PVPP, added to the maceration buffer, and BSA, added to the PCR mixture, was used as described by Poussier and Luisetti (2000).

PCR was not possible from crushed seeds. PCR products were observed from soaked seeds but only when they were highly contaminated ($>10^6$ cfu/seed). Different maceration buffers did not improve detection, but addition of BSA or P_{32} to the PCR reaction, enhanced the intensity of bands and allowed detection of the bacterium in tomato seeds.

Five methods were compared for extraction or capture of DNA from soaked or crushed seeds. The detection frequency appeared to be highly variable, ranging from 0% to 100%, depending on the method used (Table 4). Ausubel's (1991) protocol did not work. DNA capture was very effective, allowing detection of the pathogen from all samples of soaked seeds. Detection frequency was reduced significantly in crushed seeds (70%). The overall sensitivity was excellent since the detection was still positive for seeds previously contaminated with bacterial suspension calibrated at 10^2 cfu/ml (Fig. 2). DNA extraction according to Audy et al. (1996) or using the QIAamp® kit also gave excellent and consistent results with a high detection sensitivity on either crushed or soaked seeds (Fig. 2).

3.3. Water samples

Without performing any particular method to concentrate bacteria or to extract or capture DNA, the detection of *R. solanacearum* by nested PCR was possible only in highly contaminated irrigation water (10^7 cfu/ml) (Table 5). Filtration or centrifugation increased the sensitivity of detection to 10^4–10^5 cfu/ml but when applied successively, they allowed the detection of the pathogen in minimally contaminated water (10 cfu/ml). Both DNA capture and the QIAamp® kit were highly sensitive since detection was positive in irrigation water contaminated with 10 and 10^4 cfu/ml, respectively (Table 5).

Table 3
Comparison of three methods for detecting *R. solanacearum* in plant tissues by nested PCR: combined treatment PVPP/BSA, QIAamp® kit and DNA capture

Plant	Estimated bacterial population densities [log(cfu/ml)]	Method used for the detection of *R. solanacearum* by nested PCR		
		PVPP and BSA	QIAamp® kit	DNA capture
Tomato	10.41	+[a]	+	+
	9.75	+	+	+
	9.56	+	+	(+)
	9.56	+	+	(+)
	9.51	+	+	+
	4.96	+	+	(+)
	3.71	+	+	+
Sweet pepper	3.32	+	+	+
Eggplant	3.79	(+)	(+)	(+)
	3.04	(+)	(+)	–
	2.64	(+)	(+)	(+)
	0	+	+	+
Tobacco	2.38	–	–	–
	2.15	–	–	–
	0	–	–	–
	0	–	+	(+)
Geranium rosat	9.08	+	+	+
	8.18	+	+	+
	7.15	+	+	–
	4.34	(+)	(+)	–
	4.00	–	+	+
	3.85	–	(+)	–
	3.32	–	+	–
	3.04	–	+	(+)

[a] –, no amplification signal; +, strong intensity band; (+), weak intensity band.

Table 4
Influence of DNA extraction procedure on the frequency of PCR detection of *R. solanacearum* in soaked or crushed tomato seeds

DNA extraction procedure adapted from:	Soaked seeds[a]		Crushed seeds[a]	
	No. of samples	Frequency of detection (%)	No. of samples	Frequency of detection (%)
Ausubel et al. (1991)	17	6 **c**[b]	17	6 **c**
Audy et al. (1996)	26	85 **ab**	7	100 **ab**
DNA capture	20	100 **a**	20	70 **b**
QIAamp® kit	18	94 **ab**	18	94 **ab**

[a] Bacterial populations associated with seeds were estimated to be between 10^1 and 10^7 cfu/seed. For each DNA extraction procedure, samples contained the same proportions of highly, moderately and weakly contaminated seeds.
[b] Values followed by the same letter (in bold) are not significantly different ($P = 0.05$).

3.4. Soil samples

3.4.1. Direct DNA extraction

DNA was successfully extracted directly from soil samples whatever the method used. However, the efficient extraction of high molecular weight DNA (>12 kb), as visualized on agarose gels (not shown), appeared to be highly variable according to the DNA extraction method and the soil type. Protocols by Porteous et al. (1997) or Kuske et al. (1998) gave stronger intensity DNA bands from ferralitic soil and vertisol than those from other soils and/or other protocols. This suggests that DNA from different soil sources is present in different relative concentrations. When used for PCR, the DNA did not give any amplification product even with the addition of BSA

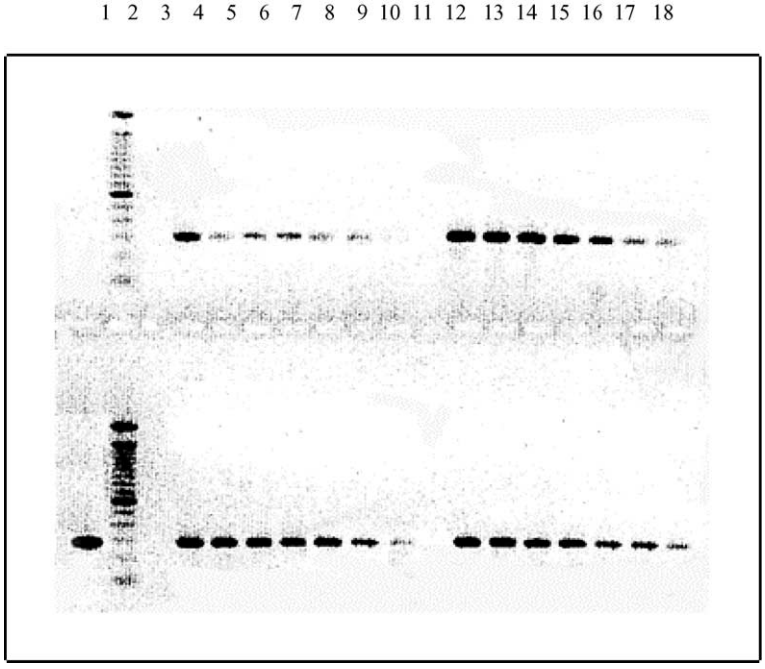

Fig. 2. Detection by PCR using OLI1-Y2 primers pairs of *R. solanacearum* in crushed (left part of gel) and soaked (right part of gel) tomato seeds following DNA capture (upper part of gel) and the QIAamp® kit (lower part of gel) protocol. Lane 1: negative control (upper part of gel), positive control (lower part of gel). Lane 2, 100-bp ladder, Life Technologies. Lanes 3 and 11, empty. Lanes 4–10 and 12–18, estimated bacterial concentrations (cfu/ml) used for contaminating tomato seeds: $\sim 10^8$, $\sim 10^7$, $\sim 10^6$, $\sim 10^5$, $\sim 10^4$, $\sim 10^3$ and $\sim 10^2$, respectively.

Table 5
Influence of bacterial concentration and DNA extraction methods on the frequency of nested PCR detection of *R. solanacearum* (strain JT519) in irrigation water

Method	Frequency of detection (%) according to inoculum concentration (cfu/ml)						
	10^1	10^2	10^3	10^4	10^5	10^6	10^7
Control	b[a] 0 **b**[b]	b 0 **b**	b 0 **b**	b 0 **b**	b 0 **b**	b 0 **b**	a 100 **a**
Filtration	b 0 **b**	b 0 **b**	b 22 **b**	b 44 **bc**	a 100 **a**	a 100 **a**	a 100 **a**
Centrifugation	b 0 **b**	b 0 **b**	b 0 **b**	a 89 **ab**	a 100 **a**	a 100 **a**	a 100 **a**
Filtration and centrifugation	a 100 **a**	a 100 **a**	a 100 **a**	a 100 **a**	a 100 **a**	a 100 **a**	a 100 **a**
DNA capture	a 67 **a**	a 100 **a**	a 100 **a**	a 100 **a**	a 100 **a**	a 100 **a**	a 100 **a**
QIAamp® kit	d 0 **b**	cd 33 **b**	abc 56 **b**	ab 89 **ab**	abc 78 **a**	abc 67 **a**	a 100 **a**

[a] For each method, frequencies preceded by the same letter are not significantly different ($P=0.05$).
[b] For each inoculum concentration, frequencies followed by the same letter (in bold) are not significantly different ($P=0.05$).

or P_{32} to PCR mixtures. However, after purification of the DNA from an agarose gel through a Qiagen® mini-column, amplification signals were obtained, but only from highly contaminated soil samples (2×10^7 cfu/g of soil) and only if BSA or P_{32} was added to the PCR mixture.

3.4.2. Indirect DNA extraction

Use of either a Waring-Blender or a Ultra-Turrax blender for dispersing soil particles gave equivalent bacterial cell extraction yields, and about 30–50% of inoculated bacteria were recovered. Increasing the duration or the number of grinding steps did not improve the yield. Low-speed centrifugation or addition of Nycodenz to the soil suspension led to similar yields, but the purity of the fraction containing bacteria was greatly improved on the Nycodenz gradient. Indeed, PCR detection was positive only for bacteria recovered on a Nycodenz gradient when BSA or P_{32} was used. DNA was then successfully extracted from the recovered bacterial fraction of soil samples even though the extraction yield was lower than that issued from a direct approach.

Various DNA extraction methods and PCR additives were tested to overcome the inhibition of PCR in soil-derived samples (Table 6). Some methods (Volos-

Table 6
Efficiency of indirect DNA extraction procedures for detecting *R. solanacearum* by PCR in different soil types

DNA extraction protocol adapted from[a]:	Inoculum concentration (cfu/g of soil)											
	2×10^3	2×10^5	2×10^7	2×10^3	2×10^5	2×10^7	2×10^3	2×10^5	2×10^7	2×10^3	2×10^5	2×10^7
	Brown soil			Ferrallitic soil			Andosol			Vertisol		
Porteous et al. (1997)	–[b]	–	+	–	–	–	–	±	±	–	–	(+)
+BSA[c]	+	+	+	–	–	–	–	–	+	–	–	+
+P_{32}[d]	+	+	+	–	–	–	–	–	+	–	–	+
Kuske et al. (1998)	–	–	(+)	–	–	–	–	–	+	–	–	(+)
+BSA	+	+	+	–	–	–	–	–	+	–	–	+
+P_{32}	+	+	+	–	–	–	–	–	+	–	–	+
DNA capture	+	+	+	–	–	(+)	(+)/–	(+)/–	+	–	–	–
+BSA	+	+	+	±	±	+	+	+	+	+	+	+
+P_{32}	+	+	+	–	–	+	+	+	+	+	+	+
QIAamp® kit	–	–	+	+	+	+	+	+	+	+	+	+
+BSA	+	+	+	+	+	+	+	+	+	+	+	+
+P_{32}	+	+	+	+	+	+	+	+	+	+	+	+

[a] No amplification product was observed from two of the protocols tested (Volossiouk et al., 1995; Ito et al., 1998).
[b] –, no amplification signal; +, strong intensity band; (+), weak intensity band; ±, variable response according to the replicate.
[c] BSA was added to the PCR reaction mixture.
[d] P_{32} was added to the PCR reaction mixture.

siouk et al., 1995; Ito et al., 1998) were completely ineffective (not shown). In contrast, the QIAamp® kit combined with PCR additives enabled PCR detection of the bacterium whatever the soil type or the inoculum concentration. The other methods led to variable results. Both of the protocols described by Porteous et al. (1997) and Kuske et al. (1998) allowed PCR detection of *R. solanacearum*, but only when BSA or P_{32} was added, in brown soil whatever the inoculum concentration, or in highly contaminated andosol and vertisol. Using DNA capture and PCR additives, amplification was positive except for ferrallitic soil, where results were reproducible only for highly inoculated samples.

4. Discussion

We show that the nature of the sample is a key factor influencing the success of the PCR detection, suggesting that a great diversity of substances may inhibit DNA amplification, at qualitative as well as at quantitative levels, depending upon the origin of the sample. Thus, the requirements for sample preparation prior to PCR differ. For example, water samples could be prepared simply by filtration and centrifugation whereas soil samples required elimination of PCR inhibitors by extraction and/or purification of DNA or by using Nycodenz, an expensive but efficient product. However, several kinds of procedures were successful for each kind of sample. Thus, the choice of the most suitable protocol depends on different parameters such as the sample origin, the reliability and the cost (Table 7).

We tried numerous additives to buffers or PCR mixtures that are reported to inactivate or remove PCR inhibitors (Picard et al., 1992; Widjojoatmodjo et al., 1992; Tebbe and Vahjen, 1993; Minsavage et al., 1994; Leite et al., 1995; Kreader, 1996; McGregor et al., 1996; Wilson, 1997). Our results confirmed that some of these additives, particularly PVPP, BSA and P_{32}, were very effective in preventing inhibitory effects but others were completely ineffective. Furthermore, we showed that the effect of these compounds depended greatly on the nature of the sample.

We also compared several DNA extraction and/or purification methods that were reported to overcome inhibition of PCR. Although results were variable, we showed that certain methods are useful steps before PCR detection of *R. solanacearum* in plant or water samples when other methods failed, are highly recommended for reproducible results from seed samples, and are essential for soil samples. Among them, DNA capture and the commercial DNA extraction mini kit QIAamp® appeared very attractive compared to methods recommended by Ausubel et al. (1991), Volossiouk et al. (1995), Porteous et al. (1997), Kuske et al. (1998) or Ito et al. (1998). Both procedures require only few steps and simple handling without using hazardous chemicals such as phenol and chloroform. Moreover, DNA capture using specific DNA probes associated with magnetic micro-beads permitted the effective separation of target DNA from non target DNA and from PCR inhibitors. DNA capture is certainly more specific than a similar and more usual technique called immuno-capture. However, for samples of crushed seeds or for some plant samples, the experimental procedure requires improvement to guarantee highly reproducible results. We showed that the QIAamp® kit is the most effective and reliable method for separation of DNA from potential inhibitors and thus allows the detection of *R. solanacearum* in all environmental samples. In addition, as a confirmation of the efficiency of the QIAamp® kit, we successfully detected the bacterium in the soil of a naturally contaminated field (results not shown), where up to now, the most effective mean for detection of the pathogen was the use of bait plants. This result demonstrates that this kit, which is already used for clinical samples, can also be recommended for the accurate detection of *R. solanacearum* and likely of many other plant pathogenic bacteria.

The accurate and reliable PCR detection of *R. solanacearum* in soil samples we report here is important since, up to now, the commonly used methods for the detection of the pathogen are often inefficient mainly due to interactions with the abundant microbial flora in soil. Our next objective is the development and application of a PCR-based assay (TaqMan PCR, Most Probable Number-PCR or competitive PCR) to quantify *R. solanacearum* populations in environmental samples, and more particularly in soil samples, as already achieved for *R. solanacearum* (Weller et al., 2000) or for other microorganisms (Picard et al., 1992; Deng et al., 1993; Hu et al., 1995; Fredslund et al., 2001) in other contexts.

Table 7
Protocols proposed for the detection of *R. solanacearum* in different sample types

Methodology	Plant samples			Seeds samples		Water samples			Soil samples		
	Protocol 1	Protocol 2	Protocol 3	Protocol 1	Protocol 2	Protocol 1	Protocol 2	Protocol 3	Protocol 1	Protocol 2	Protocol 3
Preparation method	Grinding and maceration	Grinding and maceration	Grinding and maceration	maceration	maceration	Filtration and centrifugation			Suspension, grinding and Nycodenz gradient	Suspension, grinding and centrifugation	suspension, grinding and centrifugation
Additive to maceration or lysis buffer	PVPP									PVPP	PVPP
DNA isolation	boiling	DNA capture	QIAamp® kit	DNA capture	QIAamp® kit	boiling	DNA capture	QIAamp® kit	boiling	DNA capture	QIAamp® kit
Additive to PCR mixture	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA	BSA or P_{32}	BSA or P_{32}	BSA or P_{32}
Reliability[a]	++	++	+++	+++	+++	ND[b]	ND	ND	+++	++	+++
Cost[c]	+	++	+++	++	+++	+	++	+++	++++	++	+++

[a] The number of + symbols indicates the reliability of each protocol. For example, in the case of plant samples, protocol 3 enables detection of the bacterium in all kinds of samples whereas protocols 1 and 2 are less effective with geranium rosat samples.

[b] Not determined because we tried only one kind of water.

[c] The number of + symbols indicates the relative cost of each protocol. For example, in the case of plant samples, protocol 3 is more expensive than protocols 1 and 2.

Apart from detection of *R. solanacearum* in soil samples, PCR-based methods are useful alternative for bacterial wilt diagnosis. A combination of several independent methods is the most reliable procedure for diagnosis (Elphinstone et al., 1996, 1998) since results may vary according to the method. Indeed, we show that semi-selective medium and PCR-based methods are complementary for the effective detection of *R. solanacearum* in plant extracts, each procedure having its own advantages and drawbacks. Both methods display a similar sensitivity, allowing the detection of at least 10^3 cfu/ml, but the former is cheaper and allows the enumeration of viable cells whereas the latter is faster, might be more specific and allows the detection of viable but nonculturable *R. solanacearum* cells (Grey and Steck, 2001). Some of our results, particularly from tobacco, indicated that plant extracts could inhibit the growth of *R. solanacearum* following plating out onto solid culture medium. We also observed the inhibition of PCR amplification, particularly with extracts from geranium rosat samples. However, for these samples, this inhibition was successfully eliminated when the QIAamp® kit was used.

In conclusion, using an appropriate procedure, PCR amplification can be considered to be a powerful alternative method for the diagnosis of bacterial wilt since it showed the maximum sensitivity for the reliable detection of *R. solanacearum* populations in their natural habitats where the commonly used detection tools are often inefficient.

Acknowledgements

This work was supported in part by a grant from the Ministère de l'Enseignement Supérieur et de la Recherche.

We are grateful to Delphine Quantin, Coralie Cesbron, Cécile Guinefoleau and Henri Retali for their technical assistance. We thank André Trigalet and Nigel Grimsley for the critical reading of the manuscript.

References

Audy, P., Braat, C.E., Saindon, G., Huang, H.C., Laroche, A., 1996. A rapid and sensitive PCR-based assay for concurrent detection of bacteria causing common and halo blights in bean seed. Phytopathology 86, 361–366.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1991. Current Protocols in Molecular Biology. Greene Publishing Associates-Wiley Interscience, New York.

Bakken, L.R., Lindahl, V., 1995. Recovery of bacterial cells from soil. In: Trevors, J.T., van Elsas, J.D. (Eds.), Nucleic Acids in the Environment, Methods and Applications. Springer, Berlin, pp. 9–27.

Deng, G., Yu, M., Smith, H.S., 1993. An improved method of competitive PCR for quantitation of gene copy number. Nucleic Acids Res. 21, 4848–4849.

Elphinstone, J.G., Hennessy, J., Wilson, J.K., Stead, D.E., 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. Bull. OEPP/EPPO Bull. 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. In: Prior, P., Allen, C., Elphinstone, J. (Eds.), Bacterial Wilt Disease: Molecular and Ecological Aspects. INRA Editions, Paris, pp. 133–139.

Engelbrecht, M.C., 1994. Modification of a semi-selective medium for the isolation and quantification of *Pseudomonas solanacearum*. Bact. Wilt Newsl. 10, 3–5.

Fredslund, L., Ekclund, F., Jacobsen, C.S., Johsen, K., 2001. Development and application of a Most-Probable Number-PCR assay to quantify flagellate populations in soil samples. Appl. Environ. Microbiol. 67, 1613–1618.

Graham, J., Lloyd, A.B., 1978. An improved indicator plant method for the detection of *Pseudomonas solanacearum* race 3 in soil. Plant Dis. Rep. 62, 35–37.

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., 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29, 65–87.

Hu, X., Lai, F.-M., Reddy, S.N., Ishimaru, C.A., 1995. Quantitative detection of *Clavibacter michiganensis* subsp. *sepedonicus* by competitive polymerase chain reaction. Phytopathology 85, 1468–1473.

Ito, S., Ushijima, Y., Fujii, T., Tanaka, S., Kameya-Iwaki, M., Yoshiwara, S., Kishi, F., 1998. Detection of viable cells of *Ralstonia solanacearum* in soil using a semiselective medium and a PCR technique. J. Phytopathol. 146, 379–384.

Jacobsen, C.S., 1995. Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridization and PCR amplification assay. Appl. Environ. Microbiol. 61, 3347–3352.

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

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

Kelman, A., 1954. The relationship of pathogenicity in *Pseudomo-*

nas solanacearum to colony appearance on a tetrazolium medium. Phytopathology 44, 693–695.

Kreader, C.A., 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62, 1102–1106.

Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K., Jackson, P.J., 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol. 64, 2463–2472.

Leite Jr., R.P., Jones, J.B., Somodi, G.C., Minsavage, G.V., Stall, R.E. 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed by DNA amplification. Plant Dis. 79, 917–922.

McCarter, S.M., Dukes, P.D., Jaworski, C.A., 1969. Vertical distribution of *Pseudomonas solanacearum* in several soils. Phytopathology 59, 1675–1677.

McGregor, D.P., Forster, S., Steven, J., Adair, J., Leary, S.E.C., Leslie, D.L., Harris, W.J., Titball, R.W., 1996. Simultaneous detection of micro-organisms in soil suspension based on PCR amplification of bacterial 16S rRNA. BioTechniques 21, 463–471.

Minsavage, G.V., Thompson, C.M., Hopkins, D.L., Leite, R.M.V.B.C., Stall, R.E., 1994. Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. Phytopathology 84, 456–461.

Nesmith, W.C., Jenkins, S.F., 1979. A selective medium for the isolation and quantification of *Pseudomonas solanacearum* from soil. Phytopathology 69, 182–185.

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.

Porteous, L.A., Seidler, R.J., Watrud, L.S., 1997. An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. Mol. Ecol. 6, 787–791.

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

Poussier, S., Vandewalle, P., Luisetti, J., 1999. Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-Restriction Fragment Length Polymorphism analysis of the *hrp* gene region. Appl. Environ. Microbiol. 65, 2184–2194.

Robinson-Smith, A., Jones, P., Elphinstone, J.G., Forde, S.M.D., 1995. Production of antibodies to *Pseudomonas solanacearum*, the causative agent of bacterial wilt. Food Agric. Immunol. 7, 67–79.

Seal, S.E., Jackson, L.A., Young, J.P.W., Daniels, M.J., 1993. Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and the Blood Disease Bacterium by partial 16S rRNA sequencing: construction of oligonucleotides primers for sensitive detection by polymerase chain reaction. J. Gen. Microbiol. 139, 1587–1594.

Sokhal, R.R., Rohlf, F.J., 1969. Biometry: The Principles and Practice of Statistics in Biological Research. Freeman, San Francisco.

Stead, D.E., Elphinstone, J.G., Pemberton, A.W., 1996. Potato brown rot in Europe. Brighton Crop Protection Conference—Pests and Diseases. BCPC, Farnham, pp. 1145–1152.

Steffan, R.J., Goksoyr, J., Bej, A.K., Atlas, R.M., 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. 54, 2908–2915.

Tebbe, C.C., Vahjen, W., 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl. Environ. Microbiol. 59, 2657–2665.

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

Volossiouk, T., Robb, E.J., Nazar, R.N., 1995. Direct DNA extraction for PCR-mediated assays of soil organisms. Appl. Environ. Microbiol. 61, 3972–3976.

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.

Widjojoatmodjo, M.N., Fluit, A.C., Torensma, R., Verdonk, G.P.H.T., Verhoef, J., 1992. The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. J. Clin. Microbiol. 30, 3195–3199.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, 3741–3751.

Young, C.C., Burghoff, R.L., Keim, L.G., Minak-Bernero, V., Lute, J.R., Hinton, S.M., 1993. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Appl. Environ. Microbiol. 59, 1972–1974.