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A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction

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Abstract

A simple and rapid method for extracting DNA from plants based on the use of an extraction buffer and precipitation with isopropanol was assayed to see its usefulness in detecting pathogenic bacteria in plant material. The method was compared with a phenol–chloroform standard procedure obtaining higher sensitivity levels of detection. The protocol developed was efficient for detecting a Gram-positive bacterium, *Clavibacter michiganensis* subsp. *sepedonicus* and several Gram-negative pathogenic bacteria (*Ralstonia solanacearum, Erwinia amylovora, Xanthomonas axonopodis* pv. *citri*) with a sensitivity of 10^2–10^3 cfu/ml in spiked samples. It was also efficient to specifically identify such bacteria in naturally infected plant material. This procedure is proposed as a routine tool for detection of plant pathogenic bacteria, as well as in environmental microbiology and biotechnology studies.

Keywords: DNA extraction; Detection; Plant pathogenic bacteria; *Clavibacter michiganensis* subsp. *sepedonicus*; *Erwinia amylovora*; *Ralstonia solanacearum*; *Xanthomonas axonopodis* pv. *citri*

1. Introduction

Current assays to identify pathogenic bacteria in plant tissue by means of plating on selective or common media, immunoassays, host inoculation, etc., are valuable but labour-intensive and in some cases not sensitive enough. The need for rapid, reliable, sensitive and specific methods for detection and identification of plant pathogenic bacteria is especially worthy in the case of those responsible of important losses and when plant material is object of trading towards countries or areas for which a sanitary passport or a certificate is needed. The application of molecular techniques, such as PCR, can resolve most of the difficulties the other methods present when a rapid and accurate analysis is needed to allow or not the entrance of plant material in a country or in a protected zone to prevent the spread of diseases not present. However, when applied to infected plants, there are some drawbacks affecting the advantages of this technique. Direct amplification is not always successful because of the presence of phenolic compounds that inhibit the PCR reaction and other components that bind to the DNA after cell lysis (Sarkar et al., 1990; John, 1992; Henson and French, 1993; Pich and Schubert, 1993). The application of DNA extraction procedures can resolve these problems, but the protocols normally employed

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Table 1
Bacterial strains employed and sensitivity obtained with pure cultures and artificially inoculated plant material employing the PCR with the extraction method proposed

Bacterial species	Strain/collection[a]	Origin	Sensitivity pure cultures	Spiked samples
R. solanacearum	2779 PD	Netherlands	10^2 cfu/ml	10^2 cfu/ml
E. amylovora	PMV 6089[b]	France	10^2 cfu/ml	10^2 cfu/ml
C. michiganensis subsp.	2137 NCPPB	Denmark	10^3 cfu/ml	ND[c]
sepedonicus	2140 NCPPB	Denmark	10^3 cfu/ml	ND[c]
X. axonopodis pv. *citri*	1814 CNBP	Réunion (France)	10^2 cfu/ml	10^2 cfu/ml
	2911 CNBP	Pakistan	10^2 cfu/ml	10^2 cfu/ml

[a] PD. Plantenziektenkundige Dienst, Wageningen, The Netherlands. NCPPB. National Collection of Plant Pathogenic Bacteria, York, UK; CNBP. Collection Nationale de Bactéries Phytopathogènes, Angers, France.
[b] Avirulent mutant of strain 1430 CNBP kindly provided by J. Laurent (INA, Paris) and J.P. Paulin (INRA, Angers).
[c] ND: Not determined.

involve the use of numerous and complex reagents (phenol–chloroform, CTAB, digestion with proteinase enzymes, etc.) (Klotz and Zimm, 1972; Maniatis et al., 1982; Schneider et al., 1993). The high risk of contamination related to the steps necessary for preparing the samples and the use of toxic compounds such as phenol and chloroform, make these procedures difficult for processing a high number of samples and to implement them in a routine laboratory. The PCR is used for detection of several plant quarentine bacteria since the appearance of this technique, and numerous sets of primers have been designed (Bereswill et al., 1992; Seal et al., 1992, 1993; Hartung et al., 1993; Henson and French, 1993; Schneider et al., 1993; Bereswill et al., 1995; Li and De Boer, 1995; Hartung et al., 1996) but scarce protocols have been set up for detection routinely, that means, with few steps and simple handling, without using toxic compounds. Also, there are few comparative studies comparing the results of the detection by PCR against the conventional methods in use in plant pathology. A protocol to overcome those problems was set up and is described in this paper, and the results obtained with the standard procedures and PCR are compared.

2. Materials and methods

2.1. Pure cultures and inoculated plant material

Assays with pure cultures and inoculated plant material were made. Several series of experiments were conducted using bacterial cultures and different samples of plant material, inoculated with the following bacteria: *Ralstonia solanacearum* (Rs), *Erwinia amylovora* (Ea), *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) and *Xanthomonas axonopodis* pv. *citri* (Xac). All are quarantine organisms for the EU countries. The strains and the plant material employed in the different assays performed are listed in Tables 1 and 2. The plant material was inoculated following several procedures, trying to mimic naturally infected material. For Rs and Cms,

Table 2
PCR results obtained from different plant material. Comparison between analysis by PCR with DNA extraction and without DNA extraction

Bacterium	Host/plant material	N. positive samples/N. samples analysed	
		PCR without extraction	PCR with extraction
E. amylovora	*Crataegus*/*Cotoneaster* Buds, leaves, twigs	8/15	15/15
C. michiganensis subsp. *sepedonicus*	Potato/heel ends	35/49	49/49
X. axonopodis pv. *citri*	Orange, lemon/fruits	4/10	10/10

two potato extracts consisting of 200 homogenised heel ends prepared according to the official European protocols (OJEC, 1993, 1997) were mixed up with known amounts of bacterial suspensions giving a final concentration of 10^6 to 10^1 cfu/ml in the extracts. For Ea, the samples consisted of ten pear twigs spiked with suspensions of 10^6 to 10^1 cfu/ml of the bacterium. With Xac the inoculation was made cutting leaves with a sterile scalpel and putting on the wounds 20 μl of a bacterial suspension of 10^8 cfu/ml, meanwhile orange fruits were inoculated by spiking the surface of the peel and soaking the oranges in bacterial suspensions from 10^7 to 10^3 cfu/ml, giving a final concentration into the orange peel of 10^5 to 10^1 cfu/ml/cm^2. The final bacterial concentration on each experiment was measured by plating several dilutions on general medium (YPGA) (Lelliot and Stead, 1987). Serial bacterial dilutions and the spiked samples were employed in direct PCR analysis and following the extraction protocol described below. The assays were repeated at least twice.

2.2. Analysis with naturally infected plant material

The need for a DNA extraction step to detect plant pathogenic bacteria in naturally infected samples employing the PCR technique was studied. In a comparison assay with plant extracts obtained by means of the official protocols established we performed direct amplification of the extracts and PCR after extraction of the DNA. The extracts obtained from different plant material infected with each of the bacterium studied and healthy material as negative controls were subjected to amplification with specific primers, as described in Section 2.4. The results are shown in Table 2.

The efficiency of the protocol developed was also demonstrated using plant material naturally infected with the different bacteria, comparing the results obtained by PCR using this DNA extraction protocol with other techniques appropriate for detecting every species. The techniques selected were isolation and serological detection (IF, ELISA), which are the ones recommended. For Rs the samples assayed were 138 potato tubers and extracts and they were also analysed by IF using two antisera (IACR, Rothamsted, Harpenden, UK and SANOFI, France), and plating on common and selective media (Lelliot and Stead, 1987; Englebrecht, 1994). For Ea the plant material consisted of buds, shoots, leaves and flowers of apple (78), pear (10), *Crataegus* (34), *Pyracantha* (14) and *Cotoneaster* (8). PCR results were compared with plating on selective CCT medium (Ishimaru and Klos, 1984) and King's B medium (King et al., 1954) and ELISA with specific monoclonal antibodies and a previous enrichment step in the same selective medium (Gorris et al., 1996). For Cms the samples were 51 potato tubers and tuber extracts obtained as described in Section 2.3. The samples were assayed by plating on YPGA medium (Lelliot and Stead, 1987) and indirect immunofluorescence (IF) (OJEC, 1993) with specific monoclonal antibodies (Agdia, Elkhart, IN, USA). For Xac the material analysed was leaves and fruits of 20 plants suspected of having infections caused by this bacterium. PCR was compared against plating on YPGA (Lelliot and Stead, 1987). Healthy samples were included in all the experiments.

2.3. Sample preparation

Plant material was prepared employing official protocols established by OJEC (1993, 1997) and EPPO (Anonymous, 1990a,b). With potato tubers, for Rs and Cms the protocol was based on maceration. This procedure involves removal of heel-end cores from samples of 200 tubers, homogenisation until complete maceration in a phosphate buffer 50 mM, pH 7.2 (PB), filtration and centrifugation of the homogenate at 10 000 *g* 10 min. The resuspended pellet in the same buffer was employed for each type of analysis.

The material for Xac and Ea was processed following a washing protocol. For Ea, it consisted of placing ten twigs cut in four pieces each or placing a similar number of buds, shoots, leaves or flowers into a flask containing PB buffer plus 0.5 M NaCl (PBS) sufficient to cover the samples, and shake them in a rotary shaker at room temperature, filter the supernatant and centrifuge for 20 min at 10 000 *g*. The pellet was suspended in PBS and the suspension was used for the analysis.

For Xac, the orange peel or leaves were cut in pieces and put into a flask, following the same protocol as for Ea. In addition to these analysis,

samples of individual potato tubers, leaves, stems, buds or shoots of rosaceous plants and orange leaves and fruits, infected with the different plant pathogenic bacteria, were also analysed taking aliquots from comminuted samples for further extraction with our protocol.

2.4. Extraction procedure

The protocol is as follows: 500 μl of the samples, prepared following the methods described above, were placed into an eppendorf and centrifuged at 10 000 *g* for 10 min. The pellet was resuspended in 500 μl extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP), vortexed and left for 1 h at room temperature with continuous shaking. Then it was centrifuged at 5000 *g* for 5 min, 450 μl of the supernatant were taken and 450 μl isopropanol added, mixed gently and left for 1 h at room temperature. The mixture was centrifuged at 13 000 *g* for 10 min, the supernatant discarded and the pellet dried under vacuum. Finally it was resuspended in 100 μl water.

Amplification reactions were performed in 50 μl volume consisting of 5 μl DNA extraction suspension and the PCR mix corresponding to the bacterium to be amplified. The common concentrations were: 200 μM dNTPs (Promega) and 2 U of Taq (Gibco, BRL). For Rs the primers obtained from Seal et al. (1993) that give an amplification band of 288 bp (OLI-1-Y2) were employed. Another set designed by the same author (Seal et al., 1992) which gives a band of 148 bp (PsI–PsH) was used for confirming the results. The reaction mix contains 5 mM of each primer and 1.5 mM $MgCl_2$. Cycling and temperature conditions were as described by the authors. For Ea the primers employed were designed by Bereswill et al. (1992), giving an amplification band of 900 bp. The reaction mix contains 50 μM of each primer and 3 mM $MgCl_2$. Cycling and amplification temperatures were those proposed by the authors. For Cms the primers designed by Schneider et al. (1993) that give an amplification band of 258 bp were used. The reaction mix included 6 μM of each primer, 1.5 mM $MgCl_2$, 2.5% (v/v) formamida. Temperatures were as described by the authors, using 40 cycles of amplification. For Xac the primers used were designed by Hartung et al. (1993) from a plasmid giving a specific band of 222 bp with pathotype A exclusively. The reaction mix include 50 μM of primers and 3 mM $MgCl_2$. The cycling conditions were also the authors' proposal. The apparatus employed in all cases were a Perkin-Elmer 9600 and a TECHNE PHC3. The specificity of all the primers employed was previously checked against a collection of 50–150 representative strains of the corresponding bacteria, other pathogens of the same host and saprophytic bacterial flora (data not shown). The results showed no false positive bands with the non-target bacteria, concluding that the primers used were specific. When a confirmation of the PCR positive results could not be obtained by plating or serological techniques, a second set of primers was used for each bacterium (Bereswill et al., 1995; Li and De Boer, 1995; Hartung et al., 1996).

2.5. Sensitivity comparison of extraction methods

In order to compare the features of our proposed protocol against the conventional and widely used phenol–chloroform DNA extraction (Klotz and Zimm, 1972), several series of assays were performed. Serial dilutions of Ea and Rs were prepared and mixed with extracts obtained from different plant material (buds and leaves of pear for Ea and potato heel ends for Rs) obtaining samples with bacterial concentrations ranging from 10^5 to 10^0 cfu/ml and 10^7 to 10^2 cfu/ml respectively, confirming these concentrations by plating. With 1 ml of each sample we performed, in parallel, both protocols (this paper and (Klotz and Zimm, 1972)). The assays were repeated twice for Rs and four times for Ea. The primers employed were as described above for each bacterium (Bereswill et al., 1992; Seal et al., 1993). With Cms we compared both extraction methods using a suspension of pure cultures (10^6 cfu/ml) and tuber extracts artificially inoculated with the same bacterial concentration.

3. Results and discussion

The method described here is based on modified protocols from Edward et al. (1991) and Pich et al. (1993) both focusing on extraction of plant DNA. Our objective was to take advantage of the simplicity

of the modified protocols to detect bacteria infecting plants, extracting the DNA and amplifying that DNA by PCR, thus resolving the problems of inhibition, increasing the sensitivity of detection and avoiding the problem of presence of foreign bacteria (saprophytic microflora). Finally, our major goal was to set up a protocol with few handling and with no toxic compounds, for use in routine analysis.

3.1. Pure cultures and inoculated samples

The protocol proposed was first assayed in several experiments with artificially inoculated material to test the sensitivity threshold. As shown in Table 1, for every bacterium and plant material assayed, the sensitivity was similar in all cases, reaching a level of 10^2 –10^3 cfu/ml corresponding to 1–10 cells/reaction. These levels of detection are more sensitive (ten times higher) than the results described by other authors (Hartung et al., 1993; Bereswill et al., 1995; Hartung et al., 1996). For Rs and Ea the PCR technique was able to detect all contaminated samples. For Cms, meanwhile with other preparative methods (lysis by heating, alkaline lysis (Birboim and Dolly, 1979)) the positive samples were not always detected (data not shown), with the described protocol they were always amplified. For Xac the results obtained were positive even in samples without external symptoms and in oranges from which it was not possible to isolate the bacterium by plating. The negative samples and controls did not give any amplification signal in any case.

3.2. Naturally infected samples

The results obtained in the PCR comparison assay with and without DNA extraction show the necessity of this step to get a higher degree of sensitivity (Table 2). This is specially important when analysing samples with low population of pathogenic bacteria and/or with samples that normally do not present symptoms, are in bad conditions or suffer great dilutions through the preparation process (i.e. with Rs and Cms with the 200 heel end tuber extracts). The sensitivity level of the PCR detection with the DNA extraction was always higher than with the PCR without DNA extraction.

As shown in Table 3, using naturally infected samples the PCR performed with DNA extracted with our method gave very consistent results, without false positives. The positive samples could be confirmed, sometimes by plating or using serological techniques or with a second set of primers. Comparing the results obtained with each of the bacterium

Table 3
Comparison of detection of several bacteria by different techniques[a] in plant material healthy and naturally infected

Bacterium	Host	N. positive samples/N. samples analysed		
		Isolation	IF or ELISA	PCR
R. solanacearum	Potato	44/125	101/125	94/125
	Tomato	12/13	12/13	13/13
	Total[b]	56/138	113/138	107/138[c]
E. amylovora	Apple	37/78	33/78	47/78
	Crataegus	27/34	25/34	31/34
	Cotoneaster	3/8	3/8	7/8
	Pear	4/10	4/10	10/10
	Pyracantha	3/14	3/14	13/14
	Total	74/154	68/154	108/154[d]
C. michiganensis subsp. *sepedonicus*	Potato	14/51	22/51	31/51[e]

[a] Media and antibodies employed described in Section 2.
[b] The number of positive samples by IF is higher than by PCR due to cross-reactions of the antibodies employed. By PCR no false positives were obtained.
[c] Healthy samples analysed: 31.
[d] Healthy samples analysed: 46.
[e] Healthy samples analysed: 20.

studied, the number of positive samples detected by PCR with the DNA extraction method was higher than with the other routine techniques in use. Healthy samples were always negative by PCR.

In the potato samples analysed for Rs the isolation was much less efficient than IF or PCR. The comparison of the positive samples detected by IF and PCR showed that seven potato and one tomato samples were IF positive but PCR negative. Taking into account that the sensitivity of the IF with the antibodies employed is lower than the PCR with our protocol, these results could be due to cross reacting bacteria, giving false positive results or to inhibition of the PCR, leading to false negatives. It is necessary to point out that no universal monoclonal antibodies for Rs were available, and that several bacteria showing cross reactions with the antisera used were isolated from potato in some samples (Caruso et al., 1997). In addition, these positive IF results could not be confirmed with isolation nor selective enrichment followed by PCR and isolation. Thus, we may conclude that most probably the higher number of positives by IF could be attributed to cross reacting organisms. Moreover, when specific monoclonal antibodies were used in the comparative analysis for Ea and Cms, this higher specificity led to all the positive serological (IF or ELISA) results were always confirmed by other methods.

The higher sensitivity of the PCR detection could be due to the amplification of living and dead cells (Josephson et al., 1993) or viable but non-culturable bacteria (Manahan and Steck, 1997), showing the advantages of applying this extraction method before performing the PCR analysis. With symptomless samples but with latent infections this technique is specially useful, because of its high sensitivity and rapid results obtained.

3.3. Sensitivity comparison: phenol–chloroform versus the proposed protocol

Comparing the protocol developed against a standard phenol–chloroform method (Klotz and Zimm, 1972) the detection by PCR for Rs and Ea with our method reached a sensitivity ten times higher than with the phenol–chloroform extraction. These results were obtained in all cases we repeated the assay (Fig. 1). With Cms, a Gram-positive bacterium, the

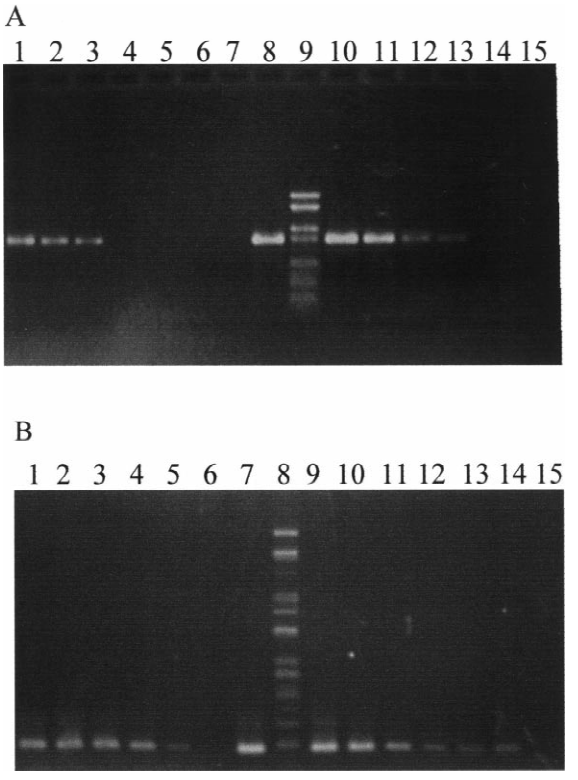

Fig. 1. Sensitivity comparison of extraction methods by PCR. Phenol–chloroform versus the proposed protocol. Same volume of sample extracted with both methods. (A) *E. amylovora* in pear buds. Lanes 1 to 6: serial dilutions (10^5–10^0 cfu/ml) extracted with the phenol–chloroform method. Lanes 10 to 15: serial dilutions (10^5–10^0 cfu/ml) extracted with the proposed protocol. Lane 7: negative control. Lane 8: positive control. Lane 9: DNA Marker VI (Boehringer–Mannheim). (B) *R. solanacearum* in potato extracts. Lanes 1 to 6: serial dilutions (10^7–10^2 cfu/ml) extracted with the phenol–chloroform method. Lanes 9 to 14: serial dilutions (10^7–10^2 cfu/ml) extracted with the proposed protocol. Lane 7: positive control. Lane 15: negative control. Lane 8: DNA Marker VI (Boheringer–Mannheim). Primers employed: for Ea, primers A–B (Bereswill et al., 1992) that give a band of 900 bp. For Rs, primers PsI–PsH (Seal et al., 1992), giving a band of 148 bp.

PCR results were similar, showing that the quality of the DNA extracted is equally good eliminating the use of toxic compounds (Fig. 2).

The analysis of different plant material has shown the effectiveness of this procedure in rapid and accurate detection of plant pathogenic bacteria (Fig. 3). The protocol proposed is being applied routinely for analysing a large number of samples avoiding the

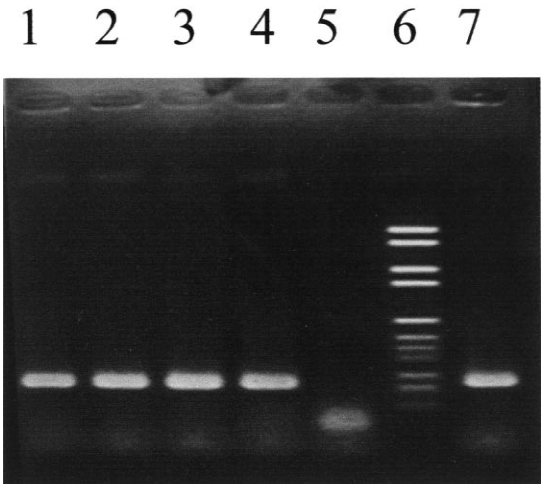

Fig. 2. Amplification of extracted DNA from *C. michiganensis* subsp. *sepedonicus* with the proposed procedure and with the standard phenol–chloroform method on pure cultures and potato tuber extracts. Lanes 1, 2: amplification bands obtained with the proposed method on pure culture and tuber extract respectively. Lanes 3, 4: bands obtained with the same samples with the phenol–chloroform method. Lane 5: negative control. Lane 6: DNA molecular marker VI (Boehringer–Mannheim). Lane 7: positive control. Primers employed: Cms6–Cms7 (Schneider et al., 1993) giving a band of 258 bp.

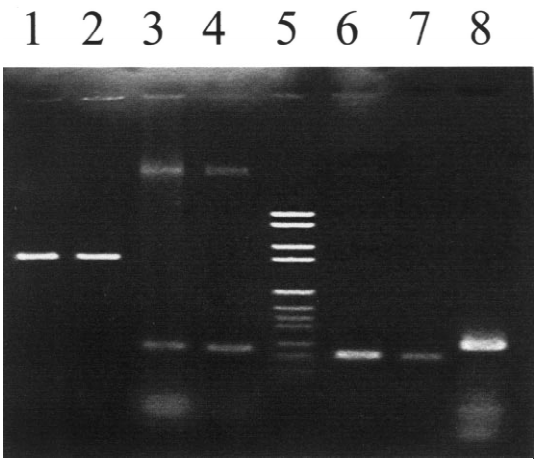

Fig. 3. Different samples infected with quarantine bacteria, amplified after DNA extraction with the proposed protocol in several amplification reactions. Lanes 1, 2: *E. amylovora* amplified from pear buds and pear twigs, respectively, with primers A–B (900 bp). Lanes 3, 4: *C. michiganensis* subsp. *sepedonicus* amplified bands from potato tuber and potato stem with primers Cms6–Cms7 (258 bp). Lane 5: DNA molecular marker VI (Boehringer–Mannheim). Lanes 6, 7: bands obtained with DNA extracted from orange leaves and orange fruits infected with *X. axonopodis* pv. *citri* with primers 2–3 (222 bp) Lane 8: amplification band of *R. solanacearum* from potato tuber with primers Oli1-Y2 (288 bp). Negative controls not included in this picture.

risk of cross contamination due to the small number of steps required and without the use of toxic compounds such as phenol and chloroform. This suggests that this simple and short method can be used for rapid and sensitive detection by PCR of many plant pathogenic bacteria and in other types of studies as in epidemiological surveys. The DNA extracted could be measured, but this method does not discriminate between bacterial and plant DNA. As the PCR technique itself is non-quantitative, we cannot know the infection level of the samples. However, there have been several developments to achieve this quantification employing internal standards that coamplify with the target, leading to the PCR called competitive (Sykes et al., 1992; Deng et al., 1993; Haff, 1994; Niedbalski et al., 1998). Such internal standards detect the presence of inhibitors that can affect the amplification reaction, resulting in a weaker signal than expected for the target DNA present in the sample, and should be obtained for every set of primers employed. In our experiments, the sensitivity assays performed on plant material amended with known amounts of bacterium, have shown a good correlation in different models and hosts, between the intensity of the bands obtained and the amount of target into the plant extract. Therefore, the stronger the signal obtained, we will suspect that there is much bacterium in that sample.

When analysis of bacteria are needed for certification or exportation the possibility of using the OJEC and EPPO recommended protocols to prepare the samples (OJEC, 1993, 1997; Anonymous, 1990a,b) and afterwards perform the DNA extraction procedure proposed allows to have results by PCR in a short time. That gives previous information about the presence or absence of such bacteria in a very fast way allowing, until the final confirmation with the other techniques (IF, plating or plant inoculation), the adoption of measures with the material under analysis. Plant material can also be treated directly with the proposed extraction protocol, taking pieces of each material, crushing them in PB or PBS buffer and the supernatant subjected to the extraction

procedure. The same protocol has been successfully used for detection of some other bacteria in plant material as *Agrobacterium tumefaciens* in stone fruit trees, grapevine and rose (Cubero et al., 1999), *Erwinia chrysanthemi* in potato (Llop et al., unpublished results) and *Pseudomonas savastanoi* pv. *savastanoi* in olives (Penyalver et al., unpublished results). This protocol has been also successful in detecting transformed cells in citrus plants (Cervera et al., personal communication).

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