*Plant Pathology* (2009) **58**, 872–881 Doi: 10.1111/j.1365-3059.2009.02083.x

# Improved fireblight diagnostics using quantitative real-time PCR detection of *Erwinia amylovora* chromosomal DNA

M. Pirc[a]*, M. Ravnikar[a], J. Tomlinson[b] and T. Dreo[a]

[a]*National Institute of Biology, Department of Biotechnology and Systems Biology, Vecna pot 111, SI-1000 Ljubljana, Slovenia; and* [b]*Plant Health Group, The Food and Environment Research Agency, York, YO41 1LZ, UK*

Specific and sensitive TaqMan real-time PCR assays were developed targeting chromosomal DNA of *Erwinia amylovora* (*ams*C gene and ITS region). These assays increased the reliability of detection of *E. amylovora* strains, regardless of their plasmid profile, and have the ability to differentiate between *Erwinia* spp. strains from Hokkaido, *Erwinia pyrifoliae* and *Erwinia* spp. isolated from necrotic pear blossoms in Spain. The assays were used for testing the efficiency of three different extraction methods to remove plant-based PCR inhibitors. Combined with an automated DNA-extraction method based on magnetic beads (QuickPick$^{TM}$), the real-time PCR assays reliably detected at least $10^3$ cells $mL^{-1}$ (*c.* four cells per reaction) of the pathogen from blighted woody plant material. In testing of symptomless samples, absolute quantification of *E. amylovora* before and after enrichment in liquid media provided proof of *E. amylovora* viability and its ability to multiply, including in cases when subsequent isolation in pure culture was unsuccessful.

*Keywords:* chromosomal DNA, *Erwinia amylovora*, molecular diagnostics and detection, TaqMan real-time PCR

## Introduction

*Erwinia amylovora* causes fireblight, a devastating disease that continues to spread to new geographical areas (van der Zwet, 2006). Pome fruit trees are among the most economically important hosts, but natural infections occur also on other plants, including *Rubus* spp. (Starr *et al.*, 1951; Evans, 1996) and *Prunus salicina* (Mohan & Thomson, 1996). In addition to biological and chemical control, the detection, removal and eradication of sources of inoculum is needed to limit the spread of the disease. Detection of *E. amylovora* in samples exhibiting symptoms is usually straightforward as bacteria are present in large numbers and grow well on artificial media. Reliable diagnosis, however, can be difficult when bacteria are hindered in their growth on artificial media, or the population size is low because of latent infections. Detection of latent or symptomless infection is especially problematic, but important since infected budwood or trees are important means of its spread to pathogen-free areas (Bonn & van der Zwet, 2000).

Real-time PCR assays have been designed for detection of various plant pathogens, with benefits such as rapidity, automation, reduction in post-PCR handling, high sensitivity, and the possibility of quantification (e.g. Weller *et al.*, 2000; Dreo *et al.*, 2007). Several PCR and real-time PCR methods are available for detection of *E. amylovora* targeting the pEA29 plasmid (Bereswill *et al.*, 1992; McManus & Jones, 1995; Llop *et al.*, 2000; Salm & Geider, 2004; DeBellis *et al.*, 2007). Recently, strains lacking the pEA29 plasmid were found in nature (Llop *et al.*, 2006). Whilst the biological significance of strains lacking pEA29, or any plasmid, is not yet resolved, methods based on detection of chromosomal DNA may be the first choice as screening tests. Existing conventional PCR methods targeting chromosomal DNA (Bereswill *et al.*, 1995; Guilford *et al.*, 1996; Maes *et al.*, 1996) do detect strains lacking pEA29, but are not reliable enough for testing symptomless samples because of amplification of other targets and lower sensitivity (Bereswill *et al.*, 1995; Maes *et al.*, 1996; Llop *et al.*, 2000; Roselló *et al.*, 2002).

The objective of this study was to increase the reliability of *E. amylovora* diagnosis by designing a fast, reliable and sensitive real-time PCR detection system targeting chromosomal DNA. The *ams* region and the spacer region between the 16S and 23S rRNA genes (ITS) were chosen as targets for amplicon design. The *ams* region of the *E. amylovora* chromosome is involved in synthesis of the capsular polysaccharide amylovoran, which seems to be unique to *E. amylovora* (Bereswill *et al.*, 1995; Bugert & Geider, 1995) and is strongly associated with its multiplication in plants and its pathogenicity (Steinberger & Beer, 1988; Menggad & Laurent, 1998). 16S–23S ribosomal intergenic spacer regions (ITS) are extremely variable in size and sequence, even within closely related taxonomic groups (Gurtler & Stanisich, 1996) and have

*E-mail: manca.pirc@nib.si

*Published online 21 May 2009*

been proven useful in detection of many bacterial species (Li & DeBoer, 1995; Pastrik *et al*., 2002).

The developed assays were used to evaluate methods of DNA extraction from plants exhibiting symptoms and to follow enrichment of *E. amylovora* from symptomless samples in liquid media, non-selective King's B and semiselective CCT media (Gorris *et al*., 1996). Using real-time PCR *E. amylovora* cells were quantified before and after enrichment, and the results compared with the rate of success of isolating *E. amylovora* in pure culture.

## Materials and methods

### Bacterial strains and plant material

Bacterial strains (Table 1) were grown on King's B medium (King *et al*., 1954) at 25°C. Suspensions of bacteria were prepared in sterile Millipore Milli Q-UF water. Plant material was collected by the Phytosanitary Inspectorate in the frame of fireblight monitoring coordinated by the Phytosanitary Administration of the Republic of Slovenia from 2003 to 2007. Extracts of plant material with and without symptoms were prepared and analysed according to EPPO protocols (EPPO, 1992, 2004). Symptomless samples of different hosts were collected in autumn. Typically, one sample consisted of 100 twigs of 1- to 2-year wood, from which 30 twigs were sampled. Smaller pieces were cut from sampled twigs (four per twig) and covered with sufficient volume (approximately 80 mL) of sterile 10 mM phosphate buffered saline (PBS; pH 7·2) with 0·1% Tween 20. The weight of pieces of wood depended on the host plant, e.g. ≈ 70 g for apple and ≈ 35 g for cotoneaster plant material. Samples were incubated with shaking (100 r.p.m.) for 90 min at room temperature. Supernatants were centrifuged for 10 min at 1500 ***g***, transferred to a new tube and centrifuged for 20 min at 7000 ***g***. Pellets were suspended in 2 mL sterile phosphate buffer. Immunofluorescence was used to test these crude extracts directly, and real-time PCR (Salm & Geider, 2004) was used to test crude extracts enriched in liquid media (CCT and King's B media). Selective plating on CCT media (Ishimaru & Klos, 1984) was used as an additional test.

### Real-time PCR primers, assay design and setup

Two regions of chromosomal DNA were selected as targets for the design of primers and TaqMan probes: (i) the *ams*C gene, internal to the target sequence of the primers designed by Bereswill *et al*. (1995), and (ii) the 16S-23S rRNA intergenic spacer region (ITS). The ITS assay targets a 139-bp sequence present on some rRNA operons (McGhee *et al*., 2002). In the original study this sequence was present only in *Erwinia amylovora* strains from fruit trees and not in the isolate from *Rubus* spp. (McGhee *et al*., 2002). Publicly available sequences from GenBank® and sequence data produced by the *E. amylovora* Sequencing Group at the Sanger Institute, which can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/era/, were aligned using VECTOR NTI software (Invitrogen) to select suitable regions for assay design. The primers and probes (hereafter referred to as the Ams and ITS assays) were designed using PRIMER EXPRESS© software version 2·0 from Applied Biosystems (Table 2).

Real-time PCR reactions were performed on an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) using universal cycling conditions [2 min at 50°C (AmpErase® UNG activation), 10 min at 95°C (AmpliTaq Gold activation), followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, with 9600 emulation]. Reaction volumes of 10 μL contained, in final concentrations: 900 nM primers (Eurofins MWG Operon), 200 nM probe (Eurofins MWG Operon), 1 × TaqMan® Universal PCR Master Mix (Applied Biosystems) and 2 μL sample (DNA extract, bacterial suspension or enriched plant extract). Each sample was tested with the Ams and ITS assays, and the results compared to those obtained using a previously developed real-time PCR assay targeting the pEA29 plasmid (Salm & Geider, 2004), adapted to universal cycling conditions and use of universal master mix (Pirc *et al*., 2008). SDS 2·2·2 software (Applied Biosystems) was used for fluorescence acquisition and calculation of threshold cycles (Ct). The baseline was set automatically, and the fluorescence threshold was set manually to intersect with the linear part of the amplification curves of all real-time PCR assays, resulting in the final Ct value for each well.

### Specificity of real-time PCR assays

The specificity of all three real-time PCR assays was checked *in silico* and by amplification of: (i) 235 strains of *E. amylovora* of different geographic origins and characteristics (levan production, plasmid content, etc.); (ii) 27 isolates of other bacteria pathogenic to rosaceous plants and other selected pathogenic bacteria; (iii) seven strains of selected biocontrol agents; (iv) 24 bacterial isolates from necrotic apple, pear and quince samples which tested negatively for *E. amylovora*; and (v) plant DNA extracted from 14 samples of different hosts with blight symptoms in which *E. amylovora* was not detected using standard methods (Table 1).

### Performance characteristics of real-time PCR assays

In order to determine the limit of detection of each real-time PCR assay, serial dilutions of *E. amylovora* in water ranging from $1{\cdot}8 \times 10^8$ to 18 cells $mL^{-1}$ were prepared by counting cells using an immunofluorescence test. Diluted bacterial suspensions were tested directly by real-time PCR. The slope ($k$) of the linear regression line between logarithmic values of cell numbers (x-axis) and Ct values (y-axis) was used to calculate the amplification efficiency, $E = (10^{[-1/k]}) - 1$, where a value of one corresponds to 100% amplification efficiency (Pfaffl, 2001).

### Evaluation and comparison of DNA-extraction methods

In a preliminary experiment, DNA extracts from tissue with symptoms, from different plant hosts, showed different

Table 1 Bacterial strains and extracted DNA used in this study, their origin and results of real-time PCRs using TaqMan chemistry

| Bacterial species and strain[a] | | Host plant | Origin, year of isolation | Real-time PCR result | | |
|---|---|---|---|---|---|---|
| | | | | plasmid | ITS | Ams |
| *Erwinia amylovora* | | | | | | |
| 219 strains | | *Malus domestica, Pyrus communis, Cydonia oblonga, Cotoneaster* sp., *Photinia* sp., *Crateagus* sp. | Slovenia, 2007 | + | + | + |
| NCPPB 311 | | *Pyrus communis* | Canada, 1952 | + | + | + |
| NCPPB 2791 | | *Pyrus communis* | USA, 1975 | + | + | + |
| NCPPB 683[T] | | *Pyrus communis* | UK, 1959 | + | + | + |
| NCPPB 4306 | | *Pyrus communis* | Egypt, 1982 | + | + | + |
| NCPPB 775 | | *Crataegus* sp. | UK, 1959 | + | + | + |
| CFBP 1367 | | *Crataegus* sp. | France, 1972 | + | + | + |
| LMG2020 – no levan synthesis | | *Pyrus communis* | Netherlands, 1981 | + | + | + |
| ICMP 13 293[b] | | *Cotoneaster* sp. | Australia, 1997 | + | + | + |
| MB1[b] | | *Prunus domestica* | Germany, 2000 | + | + | + |
| MB2[b] | | *Rosa rugosa* | Germany, 2000 | + | + | + |
| Pear 8[b] | | | New Zealand, 1992 | + | + | + |
| RGC 53 mucoid[b] | | | New Zealand, 1991–92 | + | + | + |
| RGC 53 non-mucoid[b] | | | New Zealand, 1991–92 | + | + | + |
| IVIA 1596 without pEA29 | | *Pyrus communis* | Spain, 1996 | – | + | + |
| IVIA 1614-2 without pEA29 | | *Crataegus* sp. | Spain, 1996 | – | + | + |
| NCPPB 1859 | | *Rubus idaeus* | USA, 1966 | + | – | + |
| *Erwinia* strains isolated | IVIA 2055-2 | *Pyrus communis* | Spain, 1999 | – | – | – |
| from necrotic pear | IVIA 2056-4 | *Pyrus communis* | Spain, 1999 | – | – | – |
| blossom[c] | IVIA 2057-5 | *Pyrus communis* | Spain, 1999 | – | – | – |
| | IVIA 3978 | *Pyrus communis* | Spain, 2000 | – | – | – |
| *Erwinia pyrifoliae* | | | | | | |
| CFBP 4172[T] | | *Pyrus pyrifolia* | South Korea, 1996 | – | – | – |
| *Erwinia* strains from | 223b | *Pyrus communis* (Barlett) | Hokkaido, 1996 | – | + | – |
| Hokkaido, Japan[b] | J.41 | *Pyrus ussuriensis* (Mishirazu) | Hokkaido, 1995 | – | + | – |
| | J.71 | *Pyrus ussuriensis* (Mishirazu) | Hokkaido, 1995 | – | + | – |
| *Erwinia billingiae* | | | | | | |
| DSMZ 17 872[T] | | *Pyrus communis* | UK, 1959 | – | – | – |
| *Erwinia tasmaniensis* | | | | | | |
| DSMZ 17 950[T] | | *Malus domestica* | Tasmania, 1999 | – | – | – |
| *Erwinia* sp. | | | | | | |
| ICMP 10 125[b] | | *Pyrus pyrifolia* | Australia | – | – | – |
| *Pectobacterium atrosepticum* | | | | | | |
| Kr 20 | | *Solanum tuberosum* | Slovenia | – | – | – |
| *Dickeya chrysanthemi* | | | | | | |
| NCPPB 402[T] | | *Chrysanthemum morifolium* | USA, 1956 | – | – | – |
| *Pseudomonas* spp. | | | | | | |
| Five strains (HR[d] positive) | | *Pyrus communis, Cydonia oblonga, Cotoneaster* sp., *Pyracantha* sp. | Slovenia, 2003 | – | – | – |
| Five strains (HR[d] negative) | | *Malus domestica, Sorbus* sp., *Chaenomeles* sp., *Crateagus* sp. | Slovenia, 2003 | – | – | – |
| *Pseudomonas syringae* pv. *syringae* | | | | | | |
| NCPPB 281[T] | | *Syringa vulgaris* | UK, 1950 | – | – | – |
| NCPPB 2684 | | *Phaseolus vulgaris* | New Zealand, 1973 | – | – | – |
| *Pseudomonas syringae* pv. *morsprunorum* | | | | | | |
| CFBP 2351 | | *Prunus domestica* | USA, 1977 | – | – | – |
| *Pseudomonas syringae* pv. *papulans* | | | | | | |
| CFBP 1754 | | *Malus sylvestris* | Canada, 1973 | – | – | – |
| *Pantoea agglomerans* (DNA from biocontrol agent strains)[b] | | | | | | |
| Eh252, Eh318, C9-1, P5d, A9 c, P5a, P10-c | | | | – | – | – |
| DNA extracted from plant tissue | | | | | | |
| 14 samples | | *Malus domestica, Pyrus communis, Cydonia oblonga, Cotoneaster* sp., *Crateagus* sp., *Pyracantha* sp., *Sorbus* sp., *Chaenomeles* sp. | Slovenia, 2003–05 | – | – | – |
| Strains isolated from necrotic tissue[e] | | | | – | – | – |

[a]NCPPB, National Collection of Plant Pathogenic Bacteria, FERA, York, UK; CFBP, Collection Francaise des Bacteries Phytopathogenes, Institut National de la Recherche Agronomique, Beaucouzé Cedex, France; BCCM/LMG Belgian Co-Ordinated Collections Of Micro-Organisms, Laboratorium voor Microbiologie, Universiteit Gent (UGent), IVIA, Instituto Valenciano de Investigaciones Agrarias, Spain; GSPB, Göttinger Collection of Phytopathogenic Bacteria, Göttingen, Germany; ICMP, International Collection of Microorganisms from Plants, New Zealand.
[b]Supplied by Joel L. Vanneste, HortResearch Ruakura Research Centre (Hamilton, New Zealand).
[c]Roselló *et al.*, 2006.
[d]HR, hypersensitive reaction on tobacco plants.
[e]Twenty-four bacterial strains isolated from necrotic apple, pear and quince samples which tested negative for *E. amylovora* by conventional methods.
[T]Type strain.

Table 2 Primers and TaqMan probes designed for real–time PCR detection of *Erwinia amylovora*

| Assay | Name | Sequence (5′–3′) | Amplicon length |
|---|---|---|---|
| Ams (*ams*C gene) | Ams116F | TCC CAC ATA CTG TGA ATC ATC CA | 74 bp |
| | Ams189R | GGG TAT TTG CGC TAA TTT TAT TCG | |
| | Ams141T | FAM-CCA GAA TCT GGC CCG CGT ATA CCG-TAMRA | |
| ITS (16S–23S rRNA ITS) | ITS15F | TGA GTA ATG AGC GAG CTA AGT GAA G | 79 bp |
| | ITS93R | CGC AAT GCT CAT GGA CTC AA | |
| | ITS43T | FAM-AGG CGT CAG CGC GCA GCA AC-TAMRA | |

levels of inhibition of real-time PCR. Based on these results crude extracts prepared from different host plants were selected and pooled together to provide enough starting material for comparison of DNA isolation methods. The crude extracts were spiked with *E. amylovora* to final concentrations from $1{\cdot}8 \times 10^5$ to $1{\cdot}8 \times 10^2$ cells $mL^{-1}$. DNA was extracted from 100-$\mu$L aliquots of spiked crude extracts using (i) the silica-column-based DNeasy Plant Mini Kit (Qiagen); (ii) the magnetic-bead-based QuickPick$^{TM}$ SML Plant DNA Kit (Bio-Nobile) with the KingFisher$^{R}$ mL system (Thermo Labsystem); and (iii) a simple extraction method (Llop *et al*., 1999). The DNeasy Plant Mini Kit was used according to the manufacturer's protocol for purification of total DNA from plant tissue, with final DNA elution into $2 \times 50$ $\mu$L AE buffer. The protocol for extraction using the QuickPick$^{TM}$ SML Plant DNA Kit was as follows: 100 $\mu$L of sample were mixed with 400 $\mu$L lysis buffer and 25 $\mu$L proteinase K, incubated for 30 min at 65°C and centrifuged at 6000 ***g*** for 1 min. Next, 300 $\mu$L lysate were transferred to tube 1 of a KingFisher$^{R}$ mL tube strip. Strips contained 20 $\mu$L MagaZorb$^{TM}$ magnetic particles and 500 $\mu$L binding buffer (tube 1), 800 $\mu$L wash buffer (tubes 2 and 3), 100 $\mu$L elution buffer (tube 4) and 100 $\mu$L water (tube 5). Instrument programme in KingFisher$^{R}$ mL was used with minor modifications: binding time in well A, $3 \times 1$ min release plus 2 min binding; wash in well B, 15 s; wash in well C, 15 s; elution in well D, 10 min. The simple extraction procedure was performed according to the protocol given by Llop *et al*. (1999), except that 100-$\mu$L aliquots of uncentrifuged crude extract were used. DNA extracts were analysed by real-time PCR three times, with at least three replicates. Amplification efficiencies of the real-time PCR assays were compared using DNA extracts from samples containing $1{\cdot}8 \times 10^5$ *E. amylovora* cells $mL^{-1}$ and 10-fold dilutions of these samples.

### Enrichment of *E. amylovora* in liquid media

The ability of liquid CCT and King's B media to support the growth of *E. amylovora* in the presence of other organisms and plant material was determined by real-time PCR quantification of *E. amylovora* before and after enrichment. Thirty-three crude sample extracts of symptomless tissue that had previously tested negative for *E. amylovora* were used. Enrichments were prepared by mixing 500 $\mu$L crude sample extract spiked with a low concentration of *E. amylovora* ($10^3$ cells $mL^{-1}$) with the same volume of either CCT or King's B liquid medium. Mixtures were immediately sampled for real-time PCR, enriched (incubated for 72 h at 25°C as advised by EPPO (2004) and again sampled for real-time PCR. Pre- and post-enrichment samples were analysed in real-time PCR directly with no DNA extraction (Pirc *et al*., 2008).

### Isolation of *E. amylovora* in pure culture from samples enriched in liquid media

Isolation in pure culture was attempted by plating 50 $\mu$L of enriched samples and their further 10-fold dilutions on plates of CCT medium. Plates were incubated at 25°C, and observed after 72 and 96 h. *Erwinia amylovora*-like colonies were selected, transferred to King's B medium and identified by an agglutination test. Results were compared between immediate plating of enriched extracts and plating after short-term freezing at −20°C with addition of glycerol to a final concentration of 10%.

## Results

### Specificity of real-time PCR assays

All three real-time PCR assays were found to be specific for detection of *E. amylovora* and results were in accordance with the characteristics of the target regions. No non-specific amplifications were observed with other *Erwinia* spp., *Pseudomonas* spp. present on host plants, selected biocontrol strains, bacteria isolated from host-plant tissues, or host-plant DNA (Table 1).

As expected, all *E. amylovora* strains gave positive signals when amplified with real-time PCR targeting the *ams*C sequence, regardless of their plasmid profile, thus broadening the detection range compared to plasmid real-time PCR (Table 1). The ITS assay detects strains with the 139-bp sequence on rRNA operons that seems to be lacking from *Rubus* strains as reported previously (McGhee *et al*., 2002); therefore the negative result obtained with the *E. amylovora* strain from *Rubus* was expected (Table 1). All other *E. amylovora* strains from different hosts (> 200 strains) tested positive, indicating that this sequence is widely present. Whilst no non-specific amplifications were observed, the ITS assay gave a clear positive signal with *Erwinia* spp. strains from Japan (Hokkaido), possibly reflecting their intermediate taxonomic status, as already described (Won-Sik *et al*., 2001; Maxson-Stein *et al*., 2003). Real-time PCR based

Table 3 Performance characteristics of real-time PCRs with water suspensions of *Erwinia amylovora*

| | Dynamic range[a] (cells mL$^{-1}$) | | Linear regression[b] | | | LOD[c] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Assay | from | to | Slope ($k$) | $R^2$ | $E$ | cells mL$^{-1}$ | pos./all | average Ct | cv |
| Ams | $1{\cdot}8 \times 10^3$ | $1{\cdot}8 \times 10^8$ | −3·30 | 0·99 | 1·009 | $10^3$ | 4/4 | 34·2 | 1·2% |
| ITS | $1{\cdot}8 \times 10^3$ | $1{\cdot}8 \times 10^8$ | −3·22 | 0·99 | 1·046 | $10^3$ | 4/4 | 33·6 | 1·2% |
| Plasmid | $1{\cdot}8 \times 10^3$ | $1{\cdot}8 \times 10^8$ | −3·40 | 0·99 | 0·970 | $10^3$ | 4/4 | 34·9 | 2·0% |

[a]The range of concentrations for which Ct values were in linear relationship with logarithms of concentrations (determined by exploring slope values across sections of Ct values × log number of the cells).
[b]Linear regression of all positive samples in a plot of Ct values against logarithmic number of *E. amylovora* cells: $k$ = slope of the linear regression line; $R^2$ = average square regression coefficient; $E$ = efficiency of amplification.
[c]LOD = limit of detection, for the purpose of this study defined as concentration at which more than half the parallel reactions were positive; pos./all = ratio between number of positive and all reactions; cv = coefficient of variation of Ct values.

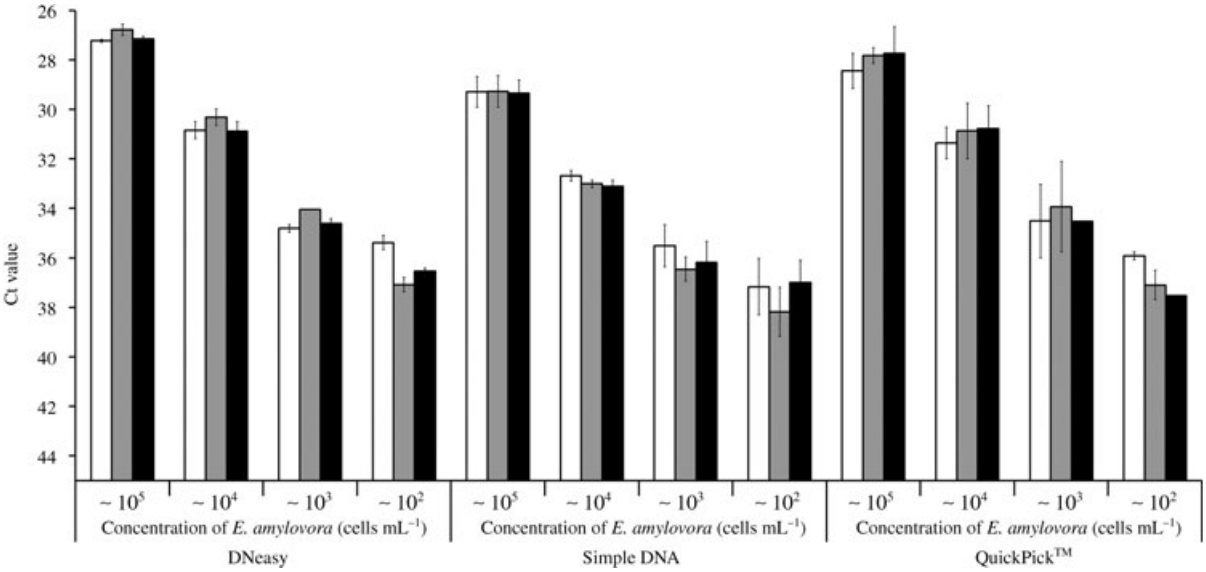


Figure 1 Influence of *Erwinia amylovora* concentrations and DNA-extraction method on real-time PCR results in analysis of samples with symptoms. Ams (white), ITS (grey) and plasmid (black) real-time PCR systems were used. Three DNA-extraction methods were compared: DNeasy Plant Mini Kit (DNeasy), simple DNA protocol (simple DNA) and QuickPick™ SML Plant DNA Kit (QuickPick™). Error bars indicate standard deviation.

on Salm & Geider (2004) was adapted to ABI 7900 universal cycling conditions (Pirc *et al.*, 2008) as described for the other assays; transfer to a higher annealing temperature than that used in the original reference did not have any adverse effects on its specificity (Pirc *et al.*, 2008).

### Performance characteristics of real-time PCR assays

All real-time PCR assays were highly efficient and showed high sensitivity, detecting less than four cells per reaction (corresponding to $1{\cdot}8 \times 10^3$ cells mL$^{-1}$) when suspensions of *E. amylovora* in water were added directly to reactions. Ct values were below 35 at the detection limit for all assays (Table 3). Inter-run reproducibility was high for all three assays, with coefficients of variation of Ct values below 3% for all concentrations within the dynamic range.

### Comparison of DNA-extraction methods from blighted plant material

Preliminary study showed some variability in the level of inhibition between DNA extracts. However, whilst the highest inhibition was observed with two pyracantha and hawthorn samples, other samples of these host plants showed only moderate levels of inhibition. Three DNA isolation methods were tested on a mixture of *E. amylovora*-negative samples showing high levels of inhibition (pyracantha, quince and rowan) spiked with *E. amylovora* to final concentrations of $1{\cdot}8 \times 10^5$ to $1{\cdot}8 \times 10^2$ cells mL$^{-1}$. At higher concentrations, DNA-extraction method influenced Ct values and amplification efficiency, with the QuickPick™ SML Plant DNA Kit and DNeasy Plant Mini Kit extractions performing best (Fig. 1). With all three real-time PCR assays, samples with $1{\cdot}8 \times 10^3$ or more

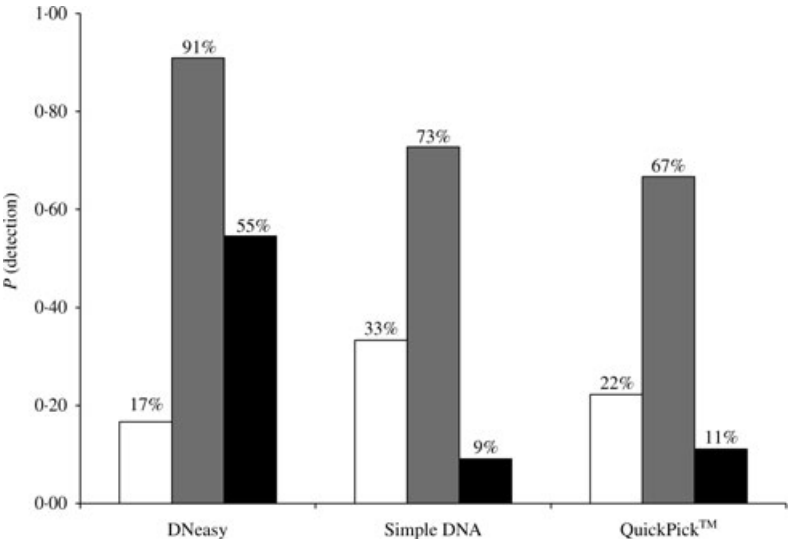


Figure 2 Probability of detecting $10^2$ *Erwinia amylovora* cells $mL^{-1}$ in DNA extracts isolated from spiked samples of plant tissue with symptoms (ratio of positive against all reactions) with Ams (white), ITS (grey) and plasmid (black) real-time PCR according to the method of DNA extraction [DNeasy Plant Mini Kit (DNeasy), simple DNA protocol (simple DNA) or QuickPick™ SML Plant DNA Kit (QuickPick™)]. At $10^3$ *E. amylovora* cells $mL^{-1}$ all methods successfully detected the pathogen.

*E. amylovora* cells per mL were positive, irrespective of the choice of DNA-extraction procedure. At a concentration of less than one cell per reaction (samples with $10^2$ cells $mL^{-1}$), the probability of detection with the Ams and plasmid real-time PCR assays was low and probably affected most by inherent limitations of the real-time PCR system. In contrast, these samples were reliably detected using the ITS assay, with probability of detection ranging from 67 to 91% depending on DNA-extraction method (Fig. 2).

Compared to amplification of *E. amylovora* cells from water suspensions, only slightly lower amplification efficiencies were observed when DNA was isolated from spiked plant samples with the QuickPick™ SML Plant DNA Kit or the DNeasy Plant Mini Kit; the simple DNA extraction was not as efficient in removal of inhibitors, and amplification efficiencies were lower (amplification efficiencies of Ams, ITS and plasmid real-time PCR assays were 0·96, 0·93 and 0·70, respectively with the DNeasy Plant Mini Kit; 0·73, 0·74 and 0·74, respectively with the simple extraction procedure; and 0·98, 0·96 and 0·83, respectively with the QuickPick™ SML Plant DNA Kit).

The probability of detection of low concentrations of *E. amylovora* in tissue with symptoms was determined in crude sample extracts from eight different plant hosts (Table 4). The crude sample extracts (previously tested as negative using standard methods) were spiked with *E. amylovora* to final concentrations from $1{\cdot}8 \times 10^4$ to $1{\cdot}8 \times 10^2$ cells $mL^{-1}$, and DNA was extracted from these spiked samples using the QuickPick™ SML Plant DNA Kit because this method was the most succesful in removing plant inhibitors in previous assays. It was observed that host species did influence the sensitivity of detection (Table 4). However, with the QuickPick™ SML Plant DNA Kit, *E. amylovora* was always detected when present in concentrations at or above $10^3$ cells $mL^{-1}$.

Table 4 Comparison of three different real-time PCR assays for detection of *Erwinia amylovora* in spiked samples of different hosts. DNA was extracted using the QuickPick™ SML Plant DNA Kit

| | | Real-time PCR assays | | |
|---|---|---|---|---|
| Host | Concentration of *E. amylovora* (cells $mL^{-1}$) | Ams | ITS | Plasmid |
| *Malus domestica* | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | – | + | – |
| *Pyrus communis* | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | + | + | – |
| *Cotoneaster* sp. | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | – | + | + |
| *Cydonia oblonga* | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | – | + | + |
| *Mespilus germanica* | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | – | + | – |
| *Sorbus domestica* | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | – | – | – |
| *Crataegus* sp. | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | – | + | – |
| *Pyracantha* sp. | $10^4$ | + | + | + |
| | $10^3$ | + | + | + |
| | $10^2$ | – | + | + |

## Enrichment of *E. amylovora* from symptomless samples

Thirty-three crude sample extracts from symptomless tissue were spiked with $10^3$ *E. amylovora* cells $mL^{-1}$. *Erwinia amylovora* cells were quantified by real-time PCR before and after enrichment by comparison of sample Ct

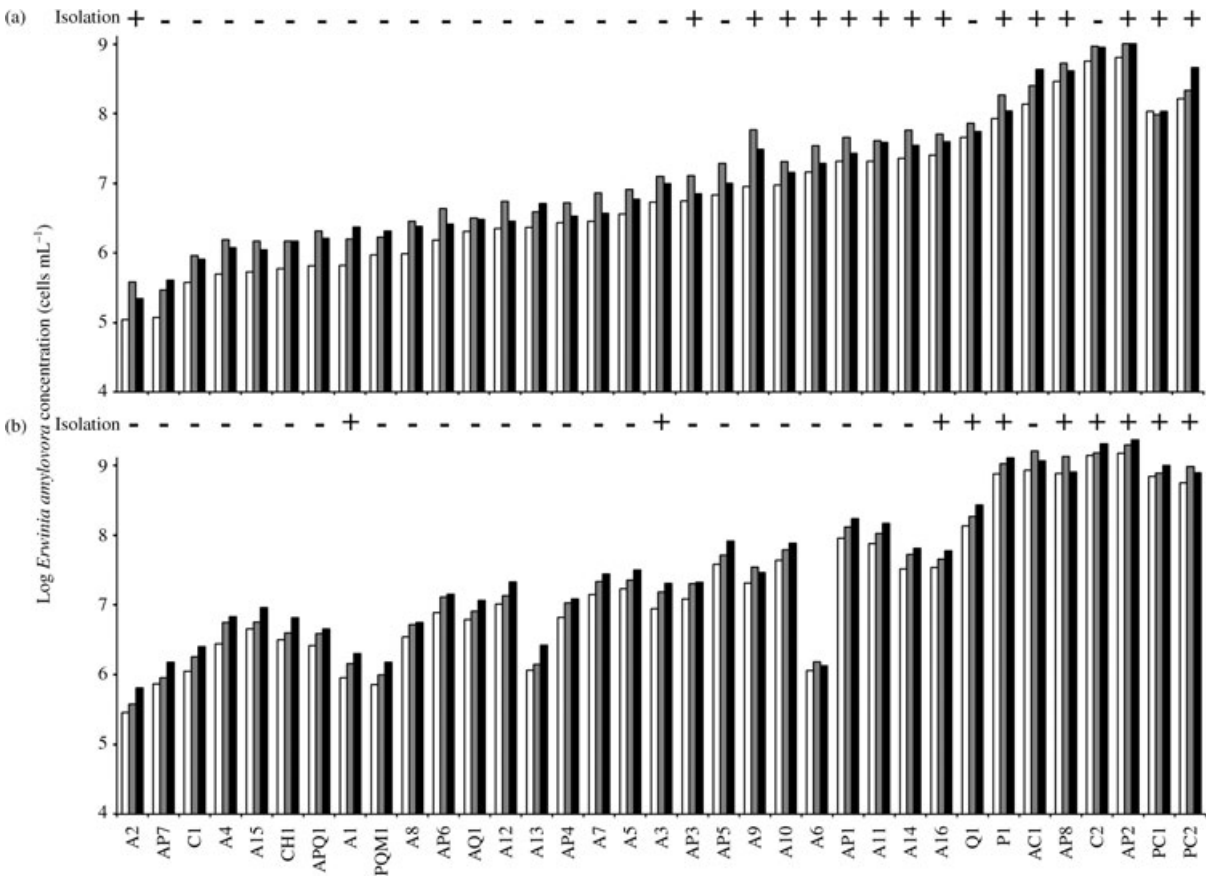


Figure 3 Concentration of *Erwinia amylovora* after enrichment of spiked symptomless samples in liquid media as determined with Ams (white), ITS (grey) and plasmid (black) real-time PCR systems: (a) in King's B medium and (b) in CCT medium. Results of isolation of *E. amylovora* in pure culture from enriched samples are marked above concentration bars. Sample labels designate host plant (A = apple, P = pear, C = cotoneaster, Q = quince, H = hawthorn, M = medlar) and positive control (PC, *E. amylovora* without plant extract).

values with a standard curve of *E. amylovora* in known concentrations. Ct values obtained prior to enrichment corresponded to the concentrations added ($\approx 10^3$ cells $mL^{-1}$). Post-enrichment real-time PCR showed that *E. amylovora* amplified in all samples tested and in both media, King's B and CCT (Fig. 3a,b). Concentrations after enrichment were variable, ranging from $10^5$ to $10^9$ cells $mL^{-1}$, the highest concentrations being comparable to the levels obtained when *E. amylovora* was enriched in the absence of plant tissue. With few exceptions, enrichment of *E. amylovora* in King's B medium correlated well with enrichment in CCT, showing that the use of semiselective medium did not provide a significant advantage over enrichment of *E. amylovora* in liquid medium. There was no correlation observed between species of host plant and enrichment of *E. amylovora* (Fig. 3).

## Isolation from enriched samples

Isolation of *E. amylovora* in pure culture was attempted for all 33 enriched samples (altogether 66 enrichments in King's B and CCT media) using CCT media plates. The success of isolation in pure culture roughly correlated with concentrations of *E. amylovora* (Fig. 3). In general, concentrations of at least $10^7$ *E. amylovora* cells $mL^{-1}$ in enriched extracts were required. Isolation from frozen enrichments in King's B was successful in 39% of samples. Success of isolation from frozen CCT enrichments was lower, with only 24% of enriched samples enabling isolation of *E. amylovora* in pure culture, despite high concentrations determined by real-time PCR. When selected enriched samples (31) were analysed with immediate plating and after short-term storage at −20°C, it was observed that freezing of CCT enrichments reduced the probability of *E. amylovora* isolation (successful isolation was possible from 13 (42%) and seven (23%) of non-frozen and frozen samples, respectively). No such effect was observed for King's B enrichments. As the same effect was observed in enrichment of *E. amylovora* alone, without any plant tissues present (data not shown), it was assumed that it was probably the result of the effect of CCT media on the ability of *E. amylovora* to grow. In most cases, use of both enrichment media was necessary as *E. amylovora* could be enriched and isolated only from one of the media used (from four samples enriched in CCT and from further nine enriched in King's B).

## Discussion

Using real-time PCR assays targeting chromosomal DNA to detect *E. amylovora* has potential advantages over previously described methods. With the two newly designed real-time PCR assays described here, it is possible to detect *E. amylovora* strains regardless of the plasmid profile and to differentiate among related bacteria, and the sensitivity of the method allows the testing of samples containing low concentrations of *E. amylovora*.

All real-time PCR assays gave a positive signal with more than 200 strains of *E. amylovora* isolated from different hosts, whilst no signal with plant tissues was observed (Table 1). Both of the real-time PCR assays targeting chromosomal DNA (Ams and ITS), enabled detection of *E. amylovora* strains lacking the pEA29 plasmid. Although strains lacking pEA29 seem to be in a minority in nature, their potential importance was recognized very early in molecular-biology-based detection (Bereswill *et al*., 1995). Whilst their biological significance is not yet resolved, further reports on their presence in various geographical areas (Llop *et al*., 2008) certainly have implications for detection methods targeting this plasmid. Using a combination of real-time PCR assays targeting different sequences enabled the detection of *E. amylovora* strains regardless of their plasmid profile, with the ability to differentiate among *Erwinia* spp. strains from Hokkaido, *E. pirifoliae* and *Erwinia* spp. from necrotized pear blossoms in Spain (Table 1), providing more reliable testing and additional information on the present strains. No signal was detected with other *Erwinia* spp., *Pseudomonas* spp. present on host plants, or other bacteria isolated from host plant tissues (Table 1).

With all real-time PCRs, $10^3$ *E. amylovora* cells $mL^{-1}$ (less than four cells per reaction) were reliably detected in water suspension or DNA extracts of tissue with symptoms, showing that sensitivity of all the assays is close or equal to the theoretical sensitivity of the method. Lower concentrations of *E. amylovora* could be detected more reliably with the ITS assay than the Ams or plasmid assays (Fig. 2). The 139-bp target sequence of the ITS assay is present in higher copy numbers than the *ams* region (McGhee *et al*., 2002), providing higher sensitivity. A comparison between nested PCR (Llop *et al*., 2000), which is highly sensitive and part of a recommended protocol of EPPO (2004), and the real-time PCR assays showed a higher probability of detecting a positive sample using real-time PCR systems. Nested PCR was comparable to real-time PCR in some cases, but showed high variability in sensitivity and failed to detect some samples, even at higher concentrations (data not shown).

Real-time PCR was applied to blighted plant material in combination with different DNA-extraction methods, obtaining the same level of detection as in analysis of bacterial suspensions [limit of detection (LOD) $= 1{\cdot}8 \times 10^3$ cells $mL^{-1}$]. Samples with symptoms could be tested directly without DNA isolation provided the extracts were analysed diluted 1:100 in water. However, since the inhibition seemed to depend not only on plant host species, but also on the particular sample, DNA isolation is needed to assure reliable detection of lower concentrations of target bacteria. In this study, the magnetic-bead- and silica-column-based methods were most successful in removing inhibitors, leading to increased amplification efficiencies in real-time PCR compared to a simple extraction procedure of plant tissues. Together with real-time PCR both DNA-extraction methods are easily amenable to high-throughput analysis. The probability of *E. amylovora* detection was highest with the ITS assay, irrespective of the DNA-extraction method used (Fig. 2).

In testing of field samples, *E. amylovora* could be reliably detected using real-time PCR, even when concentrations were below the detection limit of performed serological methods (data not shown).

In the European diagnostic protocol (EPPO, 2004), enrichment of *E. amylovora* in liquid CCT and King's B media is suggested prior to further analysis for selected material with symptoms (i.e. advanced necrosis, or samples treated with copper or antibiotics), and for latent testing, because of the low numbers of bacteria in these samples and possible viable-but-nonculturable (VBNC) state (EPPO, 2004; Ordax *et al*., 2006). Although the diagnostics protocol for *E. amylovora* has been validated in a ring test among 10 laboratories (López *et al*., 2006), limited data are available on the practical usefulness of this method. In the present study, real-time PCR was used to quantify *E. amylovora* in spiked symptomless twig samples before and after enrichment. From initial concentrations at LOD level ($\approx 10^3$ cells $mL^{-1}$) *E. amylovora* cells multiplied in all 33 symptomless samples to a variable degree, with final concentrations ranging from $10^5$ to $10^9$ cells $mL^{-1}$ (Fig. 3). Isolation of *E. amylovora* in pure culture was attempted by plating enriched extracts on CCT media. Despite using five 10-fold dilutions for plating to avoid problems with overgrowth by other bacteria [an increase from the three 10-fold dilutions suggested by EPPO (2004)], target bacteria could be isolated in pure culture mainly from samples with at least $10^7$ *E. amylovora* cells $mL^{-1}$ enriched extract. Short-term freezing of enriched CCT extracts seems to have an adverse effect on *E. amylovora* growth on media, further lowering the sensitivity of the method.

Whilst giving information on the possible presence of *E. amylovora*, the methodology of latent testing thus seems to fail in definitively proving the presence of the target bacterium in the form of pure culture, even when bacteria are present in large numbers and able to grow in liquid media. Knowledge of the physiological state of *E. amylovora* cells associated with latent infections is limited, but it can safely be assumed that their amplification rates, especially if resuscitation from VBNC state is needed, will lag behind the rates observed for the reference strain adapted to artificial media. In such cases, quantification of *E. amylovora* cells before and after enrichment indicates not only the presence of the pathogen, but also bacterial viability and ability to multiply, and having a test targeting several different targets in

*E. amylovora* DNA minimizes possibilities of cross-reactions and increases the reliability of diagnosis in the absence of conclusive evidence.

In this study it was shown that the real-time PCR assays tested – the newly developed chromosomal Ams and ITS assays and the assay targeting pEA29 (Salm & Geider, 2004) – are suitable for sensitive detection of *E. amylovora*. For quantification purposes, use of the Ams assay is encouraged since the number of targets is expected to be constant, as opposed to known variability in copy numbers of ITS regions. In cases when reliable detection of a low number of *E. amylovora* is desired, use of a combination of these tests is advised to minimize possibilities of cross-reactions and false-negative results. In general, these tests have applications in the diagnosis of difficult samples where isolation of *E. amylovora* in pure culture on media is unsuccessful, when quantification of bacteria is desired, or in high-throughput screening.

## Acknowledgements

This work was partially financially supported by the Phytosanitary Administration of Slovenia, the Slovenian Research Agency and the Slovenian Technology Agency (TIA-MISIS). We thank our partners from COST864 and EUPHRESCO ERWINDECT projects for valuable discussions and Dr Joel L. Vanneste from HortResearch Ruakura Research Centre (Hamilton, New Zealand), Dr Maria M. López and Dr Pablo Llop from Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain) for providing bacterial strains.

## References

Bereswill S, Pahl A, Bellemann P, Zeller W, Geider K, 1992. Sensitive and species-specific detection of *Erwinia amylovora* by PCR-analysis. *Applied and Environmental Microbiology* **58**, 3522–6.

Bereswill S, Bugert P, Bruchmüller I, Geider K, 1995. Identification of the fire blight pathogen, *Erwinia amylovora*, by PCR assays with chromosomal DNA. *Applied and Environmental Microbiology* **61**, 2636–42.

Bonn WG, van der Zwet T, 2000. Distribution and economic importance of fire blight. In: Vanneste JL, ed. *Fire Blight. The Disease and its Causative Agent, Erwinia amylovora*. Wallingford, UK: CABI Publishing, 37–53.

Bugert P, Geider K, 1995. Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. *Molecular Microbiology* **15**, 917–33.

De Bellis P, Schena L, Cariddi C, 2007. Real-time Scorpion-PCR detection and quantification of *Erwinia amylovora* on pear leaves and flowers. *European Journal of Plant Pathology* **118**, 11–22.

Dreo T, Gruden K, Manceau C, Janse JD, Ravnikar M, 2007. Development of a real-time PCR-based method for detection of *Xylophilus ampelinus*. *Plant Pathology* **56**, 9–16.

EPPO (European Plant Protection Organization), 1992. EPPO Standards PM 3/40 *Erwinia amylovora*. Sampling and test methods. *EPPO Bulletin* **22**, 225–31.

EPPO (European Plant Protection Organization), 2004. Diagnostic protocols for regulated pests PM 7/20, *Erwinia amylovora*. *EPPO Bulletin* **34**, 159–71.

Evans IR, 1996. Fire blight of raspberries in Alberta. *Acta Horticulturae* **411**, 69–72.

Gorris MT, Cambra M, Llop P *et al*., 1996. A sensitive and specific detection of *Erwinia amylovora* based on the ELISA-DASI enrichment method with monoclonal antibodies. *Acta Horticulturae* **411**, 41–5.

Guilford PJ, Taylor RK, Clark RG, Hale CN, Forster RLS, 1996. PCR based techniques for the detection of *Erwinia amylovora*. *Acta Horticulturae* **411**, 53–6.

Gurtler V, Stanisich VA, 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* **142**, 3–16.

Ishimaru ES, Klos EJ, 1984. New medium for detection of *Erwinia amylovora* and its use in epidemiological studies. *Phytopathology* **74**, 1342–5.

King EO, Ward MK, Raney DE, 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* **44**, 301–7.

Li X, DeBoer SH, 1995. Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus*. *Phytopathology* **85**, 837–42.

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

Llop P, Bonaterra A, Peñalver J, López MM, 2000. Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. *Applied and Environmental Microbiology* **66**, 2071–8.

Llop P, Donat V, Rodriguez M *et al*. 2006. An indigenous virulent strain of *Erwinia amylovora* lacking the ubiquitous plasmid pEA29. *Phytopathology* **96**, 900–7.

Llop P, López MM, Cabrefiga J, Ruz L, Montesinos E, 2008. Study of the virulence in wild-type strains of *Erwinia amylovora* devoid of the plasmid pEA29. *Acta Horticulturae* **793**, 145–8.

López MM, Llop P, Gorris MT *et al*. 2006. European protocol for diagnosis of *Erwinia amylovora*. *Acta Horticulturae* **704**, 99–106.

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 Pathology* **45**, 1139–49.

Maxson-Stein K, McGhee GC, Smith JJ, Jones AL, Sundin GW, 2003. Genetic analysis of a pathogenic *Erwinia* sp. isolated from pear in Japan. *Phytopathology* **93**, 1393–9.

McGhee GC, Schnabel EL, Maxson-Stein K *et al*. 2002. Relatedness of chromosomal and plasmid DNAs of *Erwinia pyrifoliae* and *Erwinia amylovora*. *Applied and Environmental Microbiology* **68**, 6182–92.

McManus PS, Jones AL, 1995. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse blot hybridizations. *Phytopathology* **85**, 618–23.

Menggad M, Laurent J, 1998. Mutations in *ams* genes of *Erwinia amylovora* affect the interaction with host plants. *European Journal of Plant Pathology* **104**, 313–22.

Mohan SK, Thomson SV, 1996. An outbreak of fire blight in plums. *Acta Horticulturae* **411**, 73–6.

Ordax M, Marco-Noales E, López MM, Biosca EG, 2006. Survival strategy of *Erwinia amylovora* against copper: induction of the viable-but-nonculturable state. *Applied and Environmental Microbiology* **72**, 3482–8.

Pastrik KH, Elphinstone JG, Pukall R, 2002. Sequence analysis and detection of *Ralstonia solanacearum* by multiplex PCR amplification of 16S–23S ribosomal intergenic spacer region with internal positive control. *European Journal of Plant Pathology* **108**, 831–42.

Pfaffl MW, 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, 2002–7.

Pirc M, Dreo T, Ravnikar M, 2008. Real-time PCR for testing of symptomless fire blight samples. *Acta Horticulturae* **793**, 533–8.

Roselló M, Garcia Vidal S, Tarin A *et al*. 2002. Characterization of an *Erwinia* sp. isolated from necrotic pear blossoms in Valencia, Spain. *Acta Horticulturae* **590**, 139–42.

Roselló M, Peñalver J, Llop P, *et al*. 2006. Identification of an *Erwinia* sp. different from *Erwinia amylovora* and responsible for necrosis on pear blossoms. *Canadian Journal of Plant Pathology* **28**, 30–41.

Salm H, Geider K, 2004. Real-time PCR for detection and quantification of *Erwinia amylovora*, the causal agent of fireblight. *Plant Pathology* **53**, 602–10.

Starr MP, Cardona C, Folsom D, 1951. Bacterial fire blight of raspberry. *Phytopathology* **41**, 915–9.

Steinberger EM, Beer SV, 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. *Molecular Plant-Microbe Interactions* **1**, 135–44.

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE, 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied and Environmental Microbiology* **66**, 2853–8.

Won-Sik K, Jock S, Paulin JP, Rhim SL, Geider K, 2001. Molecular detection and differentiation of *Erwinia pyrifoliae* and host range analysis of the Asian pear pathogen. *Plant Disease* **85**, 1183–8.

van der Zwet T, 2006. Present worldwide distribution of fire blight and closely related diseases. *Acta Horticulturae* **704**, 35.