Eur J Plant Pathol (2007) 118:11–22
DOI 10.1007/s10658-006-9078-4

FULL RESEARCH PAPER

Real-time Scorpion-PCR detection and quantification of *Erwinia amylovora* on pear leaves and flowers

Palmira De Bellis · Leonardo Schena · Corrado Cariddi

Received: 24 July 2006 / Accepted: 26 October 2006 / Published online: 29 March 2007

Abstract A specific primer couple (E3–E4) amplifying a single DNA fragment of 111 bp from plasmid pEA29 was designed to identify, detect and quantify *Erwinia amylovora* by real-time Scorpion-PCR. Specificity of primers and probe was assessed both by means of BLAST analyses and by using genomic DNA from a large number of *E. amylovora* isolates and other bacteria. In Scorpion-PCR, the limit of detection was of 1 pg of total DNA and a high correlation ($r = 0.999$) was achieved between target DNA quantity and cycle threshold (Ct). Combining two sequential amplifications with conventional reported primers (PEANT1–PEANT2) and Scorpion primers (E3 Scorpion-E4) the detection limit was of 1 fg (nested Scorpion-PCR). Using serial dilution of the bacterial suspensions the limit of detection was 3.2×10^4 CFU ml^{-1} in Scorpion-PCR and 2.8×10^2 CFU ml^{-1} in nested Scorpion-PCR. Real-time PCR combined with effective procedures for DNA extraction enabled the detection and the quantification of the epiphytic population of *E. amylovora* in the washings of flowers and leaves of artificially inoculated pear. A significant correlation ($r = 0.92$) was achieved between pathogen CFU on semi-selective media and the corresponding target DNA concentration evaluated by real-time PCR.

Keywords Fire Blight · Molecular detection · Nested PCR · Plasmid pEA29 · Scorpion-PCR (duplex format)

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Introduction

Fire Blight, caused by *Erwinia amylovora*, is the most destructive bacterial disease of pear (*Pyrus communis*), apple (*Malus domestica*), and several other rosaceous plants (Vanneste, 2000). The disease is indigenous in North America from where it has spread world-wide (Vanneste, 1995). The pathogen is now present in 43 countries (van der Zwet, 2002), but it has not been recorded either in South America, or in most African and Asian countries (with the exception of some Mediterranean countries), and only once in

Australia (Bonn & van der Zwet, 2000). In Europe *E. amylovora* is considered a quarantine organism and is included in European Plant Protection Organization (EPPO) A_2 list (http://www.eppo.org/).

The pathogen can survive as an epiphyte and as an endophyte (Thomson, 2000) and can be systemically distributed in plants (Momol et al., 1998). The bacterium transferred from overwintering cankers by crawling insects and splashing rain, infects host plants primarily through flowers or wounds of succulent tissues and is spread from flower to flower by rain and pollinating insects such as bees (Thomson, 2000). Long-distance dispersal of the pathogen mainly occurs with infected propagating material; however, other means such as contaminated shipping containers, aerosol and migrating birds are also possible (Thomson, 2000).

In recent years awareness about the importance of the monitoring of *E. amylovora* populations on flowers for the management of the disease has increased the interest for reliable and sensitive detection methods suitable for qualitative and/or quantitative analyses. Such methods are also essential to evaluate the hygiene of propagating materials and to prevent the dispersal of the pathogen over long distances and its introduction into countries still free of the disease (Llop, Bonaterra, Penalver, & Lopez, 2000).

Molecular detection methods based on the polymerase chain reaction have been developed for a number of phytopathogenic bacteria including *E. amylovora* (Bereswill, Bugert, Bruchmuller, & Geider, 1995; Bereswill, Pahl, Bellerman, Zeller, & Geider, 1992, McManus & Jones, 1995; Taylor, Guilford, Clark, Hale, & Forster, 2001). These techniques have contributed to the alleviation of some of the issues associated with the identification and diagnosis of the pathogen; however, technical limitations related to post-amplification procedures (gel electrophoresis and ethidium bromide staining) still limit their large-scale application. Furthermore, conventional PCR is unreliable for quantitative analysis (Ginzinger, 2002). Real-time PCR combines the sensitivity of conventional PCR with the generation of a specific fluorescent signal, which can be measured at an early stage of the reaction when the rate of amplification is still in its linear phase, enabling the quantification of the initial amount of target DNA (quantitative analyses).

Real-time PCR chemistries utilised to detect and study phytopathogenic microrganisms can be grouped into amplicon sequence non-specific (SYBR Green) and sequence-specific methods (TaqMan, Molecular Beacons, Scorpion-PCR, etc.) (Schaad et al., 2003; Schena, Nigro, Ippolito, & Gallitelli, 2004). Recently real-time PCR assays based on SYBR Green I and TaqMan chemistries have been developed to detect and quantify *E. amylovora* from apple leaves and flowers (Salm & Geider, 2004). Specific primers and probes were developed on DNA by Salm and Geider (2004) from the common *E. amylovora* plasmid (pEA29) previously utilised in a colony-hybridization procedure (Falkenstein, Bellemann, Walter, Zeller, &Geider, 1988). Plasmid pEA29 is the most utilised target (Bereswill et al., 1992; Llop et al., 2000; McManus & Jones, 1995) because it is non-transmissible and seems to be contained in all naturally occurring strains of *E. amylovora* (McGhee & Jones, 2000). PCR primers based on chromosomal DNA are also available, but they are less sensitive than those involving pEA29 (Bereswill et al., 1995).

In the present study, we used plasmid pEA29 to develop a new real-time PCR method based on the duplex format of the Scorpion-PCR (Solinas et al., 2001). Furthermore, to increase sensitivity of the detection, we developed a nested approach in which a first amplification round with the conventional primers PEANT1–PEANT2 reported by Llop et al. (2000) is combined with a second round real-time PCR amplification. This approach, named nested-Scorpion-PCR, increases sensitivity of the reactions without losing some of the most important advantages of real-time PCR (Ippolito, Schena, Nigro, Soleti Ligorio, & Yaseen, 2004; Schena & Ippolito, 2003).

Materials and methods

Isolates

Isolates of *E. amylovora* and other bacterial species are listed in Tables 1 and 2, respectively.

Most of the isolates were from the collection of the Department of Plant Protection and Applied Microbiology (DPPMA) of the University of Bari (Italy) and were collected from several Apulian localities (southern Italy) and Albania from 1990 to 2002. Additional isolates were obtained from other research groups or purchased from international culture collections. Isolates were stored at –80°C in an aqueous solution containing 20% glycerol. To extract DNA, isolates were transferred to 5% Sucrose Nutrient Agar (SNA) and grown overnight at 27°C in Luria-Bertani (LB).

Primer selection and specificity

Five primer pairs were designed from *E. amylovora* plasmid pEa29 (accession number AF264948) using the 'Steve Rozen, Helen J. Skaletsky (1998) Primer3 software' and arranged in different combinations for DNA amplifications (Table 3). All primer pairs amplified short DNA fragments (96–114 bp) suitable for the development of a detection method based on real-time PCR.

A primer pair (E3–E4) was selected in preliminary tests with *E. amylovora* DNA for its ability to amplify a single bright PCR band of 111 bp and for the absence of primer dimers. Primers E3–E4 were flanked by the reported primers PEANT1–PEANT2 utilised to detect *E. amylovora* in asymptomatic plant material (Llop et al., 2000). This primer localization was convenient for the development of a nested-PCR providing a first amplification round with primers PEANT1–PEANT2 and a second amplification round with primers E3–E4 (Fig. 1).

Specificity of selected primers was preliminarily assessed by means of Basic Local Alignment Search Tool (BLAST) to exclude the presence of similar sequences in other microrganisms among available GenBank databases. Moreover, primer specificity was assessed using genomic DNA from 63 different isolates of *E. amylovora* (Table 1) and 68 isolates of other bacteria (28 species) (Table 2). For this purpose total DNA was extracted using the AquaPure Genomic DNA Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Conventional PCR reactions were performed in a total volume of 25 µl containing 50 ng of genomic DNA, 2.5 µl of reaction buffer 10× (Promega), 1.5 mM of $MgCl_2$, 20 µM each dNTPs, 0.5 µM each primer and 1 unit of *Taq* polymerase (Taq DNA polymerase, Promega Corporation, WI, USA). The PCR reaction was incubated in a programmable thermal cycler (iCycler Thermal Cycler, Bio-Rad) starting with 5 min denaturation at 95°C, followed by 35 cycles at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. A negative control

Table 1 *Erwinia amylovora* isolates utilised in the present study

Host plant	Number of isolates	Source[a]	Geographic origin	Year of isolation
Pyrus communis	40	DPPMA	Apulia, southern Italy	1990–2002
Crataegus monogyna	1	DPPMA	Apulia, southern Italy	1995
Crataegus azarolus	1	DPPMA	Apulia, southern Italy	1995
Cydònia oblònga	1	DPPMA	Apulia, southern Italy	1995
Malus domestica	1	DPPMA	Apulia, southern Italy	2001
Cydònia oblònga	1	DPPMA	Apulia, southern Italy	2001
Pyrus communis	9	DPPMA	Albania	1995
Pyrus communis	4	ISF	Emilia-Romagna, northern Italy	2001–2002
Pyrus communis	1	IPVBO	Emilia-Romagna, northern Italy	2001
Cydònia oblònga	1	ISF	Emilia-Romagna, northern Italy	2001
Pyrus pyrifolia	1	ISPaVe	Campania, southern Italy	1996
Pyrus communis	1	CUCPPBEa 547	Apulia, southern Italy	1990
Pyrus communis	1	NCPPB 595	United Kingdom	1958

[a] DPPMA: Department of Plant Protection and Applied Microbiology, University of Bari, Italy; ISF: Fruit Tree Research Institute, Rome, Italy; IPVBO: Department of Agroenvironmental Sciences and Technologies, University of Bologna, Italy; ISPaVe: Plant Pathology Research Institute, Rome, Italy; CUCPPB: Cornell University Collection Plant Pathogenic Bacteria, Ithaca, USA; NCPPB: National Collection of Plant Pathogenic Bacteria, Harpenden, UK

Table 2 Bacterial species utilised in the present study

Species	Strains[a]
Agrobacterium radiobacter	NCPPB 2407
Agrobacterium tumefaciens bv. 1	NCPPB 396; DPPMA 1Agr
Agrobacterium tumefaciens bv. 2	NCPPB 1651; DPPMA 14Agr
Agrobacterium vitis	NCPPB 3554; DPPMA 2Agr
Clavibacter michiganensis subsp. *michiganensis*	NCPPB 1064; DPPMA 3Cmm, 4Cmm
Clavibacter michiganensis subsp. *sepedonicus*	NCPPB 2140
Erwinia carotovora subsp. *carotovora*	NCPPB 312; DB-PPS 3389; DPPMA 6Ec, 60Ec, 63Ec
Erwinia carotovora subsp. *atroseptica*	DB-PPS Eca; DPPMA 1Ec, 62Ec
Erwinia chrysantemi	DB-PPS Echr
Erwinia herbicola	NCPPB 666
Erwinia persicinus	NCPPB 3774
Erwinia sp.	DPPMA 31Ba
Pseudomonas spp. (saprophytes)	DPPMA 1Psap, 2Psap
Pseudomonas cichorii	NCPPB 943; DPPMA 1Pci, 4Pci
Pseudomonas corrugata	NCPPB 2445; DPPMA 1Psc, 33Psc
Pseudomonas fluorescens	NCPPB 1964; DPPMA 3Psf, 5Psf
Pseudomonas marginalis pv. *marginalis*	NCPPB 667; DPPMA 61Psm
Pseudomonas putida	NCTC 10936
Pseudomonas syringae pv. *morsprunorum*	NCPPB 2995
Pseudomonas savastanoi pv. *savastanoi*	NCPPB 639; DPPMA 1Psav
Pseudomonas syringae pv. *syringae*	NCPPB 281; DPPMA 1Pss, 2Pss, 3Pss, 4Pss, 5Pss, 6Pss, 11Pss, 16Pss, 21Pss
Pseudomonas syringae pv. *tomato*	NCPPB 1106; DPPMA 20Pst, 33Pst
Pseudomonas viridiflava	NCPPB 635; DPPMA 1Psv, 2Psv
Rhodococcus fascians	DB-PPS PD1236
Serratia fonticola	NCTC 12147
Serratia proteomaculans	NCPPB 245
Xanthomonas arboricola pv. *juglandis*	NCPPB 411; DPPMA 1Xan, 9Xan
Xanthomonas vesicatoria	NCPPB 422; DPPMA 18Xan, 20Xan
Xanthomonas campestris pv. *campestris*	NCPPB 528; DPPMA 12Xan, 14Xan

[a] NCPPB: National Collection of Plant Pathogenic Bacteria, Harpenden, UK; DPPMA: Department of Plant Protection and Applied Microbiology, University of Bari, Italy; DB-PPS: Department of Bacteriology, Plant Protection Service, The Netherlands; NCTC: National Collection of Type Cultures, London, UK

Table 3 Set of primers utilised to amplify specific regions of the of plasmid pEA29 from *E. amylovora* and length of the amplified fragments

Forward primer (5′–3′)	Reverse primer (5′–3′)	Fragment size (bp)
PEANT1 TATCCCTAAAAACCTCAGTG[a]	PEANT2 GCAACCTTGTGCCCTTTA[a]	383
E1f CACGTAAAGGGCACAAGGTT	E2 TTCCGATGATGTCTCTGCAA	110
E3 AAGACATCCGGCTTCTGAAA	E4 GGATTACGGGATGACAAGA	111
E5 GGGGTAATTACGGCAACAAA	E1r AACCTTGTGCCCTTTACGTG	114
E6 AAGGTTGCGATGGTCGATAC	E2 TTCCGATGATGTCTCTGCAA	96
E7 TCGGATTGCTCATAGCAGAA	E8 TCGCACTGAGGTTTTTAGGG	102

[a] Primers reported by Llop et al. (2000)

(no template DNA present in PCR reaction) was included in every experiment. Amplicons were analysed by electrophoresis in 2% agarose gels in Tris–borate–EDTA (TBE) buffer and visualized by staining with ethidium bromide.

Real-time Scorpion-PCR

Primer E3 was modified according to Solinas et al. (2001) to obtain a Scorpion primer (duplex format) and utilised in conjunction with primer

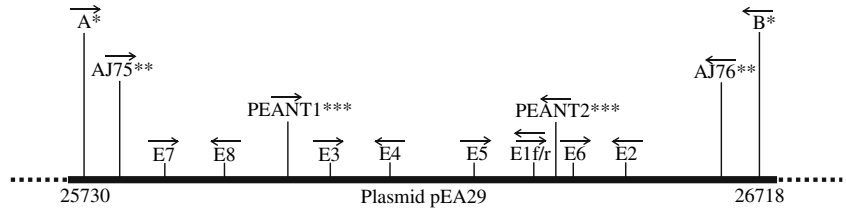

Fig. 1 Schematic representation of a fragment of plasmid pEA29 of *E. amylovora* with specific primers reported by Bereswill et al. (1992) (*), McManus and Jones (1995) (**), Llop et al. (2000) (***) or developed during this study (E). Represented fragment is between position 25730 (primer B) and 26718 (primer A) according to the complete nucleotide sequence of plasmid pEA29 reported by McGhee and Jones (2000) (accession number AF264948). Arrows on primers indicate orientation

E4 for real-time identification and detection of *E. amylovora* (Fig. 2). The Scorpion primer was designed by Eurogentec ltd (Belgium). All reactions were conducted using a spectrofluorometric Bio-Rad thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA) in sealed tubes in a 96-well microtiter plate (Bio-Rad).

Real-time PCR reactions were performed in a total volume of 25 µl containing 50 ng of genomic DNA, 2.5 µl of reaction buffer 10× (Promega), 2.5 mM $MgCl_2$, 40 µM each dNTPs, 0.5 µM each primer, 2.5 µM quencher oligonucleotide and 1.5 unit of *Taq* polymerase (Promega). In preliminary tests, amplifications consisted of an initial denaturing step at 95°C for 2 min followed by 40 cycles, each consisting 5 s of denaturation at 95°C and 10 s of annealing-extension at 55°C. Fluorescence was monitored for real-time data collection at 55°C. Relative normalised fluorescence (ΔRn) and cycle thresholds (Ct), i.e., the PCR cycles at which fluorescence exceeded the threshold fluorescence intensity, were automatically generated by the iCycler iQ™ associate software (Real-Time Detection System Software, version 3.0).

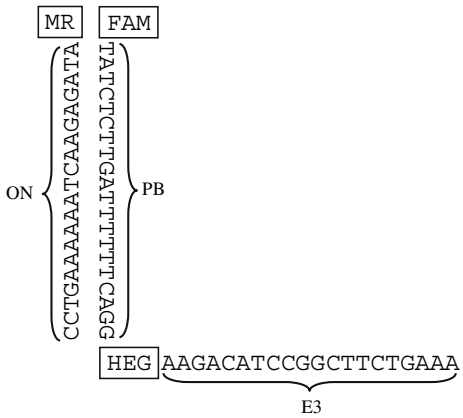

Fig. 2 Schematic representation of E3 Scorpion probe ('duplex format'). E3 Scorpion is composed of a primer element (E3), a non-amplifiable monomer (HEG, hexethylene glycol), a probe element (PB) and a fluorophore (FAM, 6-carboxy-fluorescein). The probe element is annealed to a complementary oligonucleotide (ON) bearing a quencher (MR, methyl red monomer) at the 3′-end

Melting curves of real-time PCR products were evaluated from 30°C to 95°C to confirm the amplification of a single PCR band and assess the optimal annealing-extension temperature. The following cycling conditions were utilised: initial denaturation for 5 min at 95°C, cooling to 30°C and melting from 30°C to 95°C with a 0.2°C transition rate every 7 s.

Specificity of real-time PCR with primers E3 Scorpion-E4 was assessed using the same isolates as described for conventional PCR. Compared to preliminary tests, real-time PCR amplification conditions were slightly modified and provided an annealing-extension temperature of 49°C, since this temperature was found to be optimal in melting curve analysis experiments (Fig. 3).

Sensitivity of the reactions

To assess sensitivity of the reactions, total DNA extracted from a pure culture of *E. amylovora* was serially diluted 10-fold to obtain final DNA concentrations ranging from 10 ng μl^{-1} to 100 ng μl^{-1}. One microliter of each DNA dilution was amplified using primers E3 Scorpion-E4

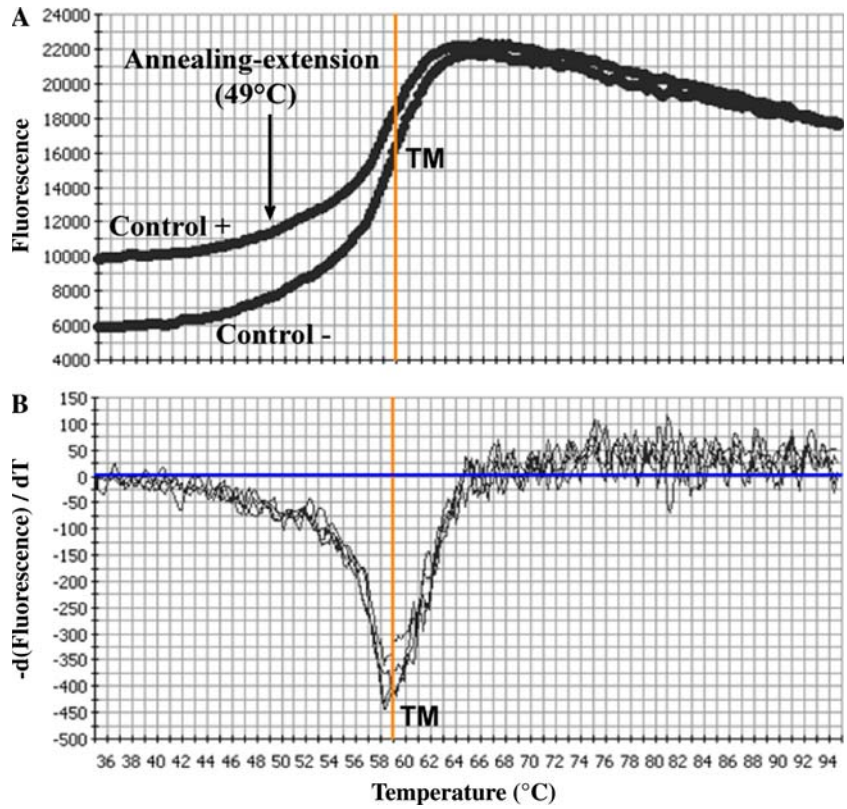

Fig. 3 Fluorescence melting curves drafted as linear (**A**) or as first negative derivative (**B**) of primers E3 Scorpion-E4. In (A) the two curves represents a negative sample in which target DNA was replaced with water (control –) and a positive sample containing target DNA amplified during PCR (control +). 49°C was considered the highest possible temperature for Δ fluorescence between positive and negative samples (**A**); this temperature was selected for the annealing-extension phase in all PCR reactions

(Scorpion-PCR) or combining a first round amplification with primers PEANT1–PEANT2 and a second round amplification with E3 Scorpion-E4 (nested-Scorpion-PCR). In the latter case, 1 µl of amplified product from the first round was utilised as target in the second round amplification. Water was used as a negative control to replace template DNA in PCR reactions. Each reaction was repeated twice. Conventional and real-time PCR amplifications were conducted as previously described in a total volume of 25 µl. Standard curves and corresponding correlation coefficients (r) were automatically generated by the iCycler iQ^{TM} associate software by interpolating Ct values against the logarithm of the initial DNA concentrations.

To evaluate the detection limit in terms of number of cells detectable in an aqueous suspension, strain CUCPPBEa 547 of *E. amylovora* was grown on Nutrient Agar (NA, Oxoid) for 12 h at 27°C and used to prepare an aqueous suspension containing approximately 10^9cells ml^{-1}. The actual bacterial concentration was evaluated with a densitometer (Densimat, bioMèrieux, France) and diluted to 10-fold serial concentrations ranging from 10^8 to 10^0 cells ml^{-1}. For each concentration 1 ml of cell suspension was centrifuged at 10,000 rpm for 5 min and the pellet resuspended in 40 µl of breaking buffer (2% Triton X–100, 1% SDS, 100 mM NaCl, 10 mM Tris–HCl, pH 8, 1 mM EDTA pH 8). DNA was extracted with 40 µl of phenol/chloroform/isoamyl alcohol (25:24:1) in the presence of 25 mg of acid-washed glass beads (425–600 µm diam). This mixture was blended in a FastPrep FP120 (Qbiogene, France) at 5 m s^{-1} for 40 s and centrifuged at 14,000 rpm for 10 min at 4°C. The upper phase (approximately 40 µl) was mixed with an equal volume of chloroform/isoamyl alcohol (24:1), vortexed for 1 min and centrifuged for 5 min at 14,000 rpm. One microliter of the upper aqueous face was amplified by both Scorpion and nested-Scorpion-PCR as described before.

To evaluate the number of colony-forming units (CFU), 100 µl of bacterial suspension from each bacterial dilution were uniformly distributed on CG medium (Crosse & Goodmann, 1973) and incubated at 27°C for 48–60 h.

Tests on artificially inoculated plants of pear

Qualitative tests with flowers and leaves

A suspension of *E. amylovora* strain CUCPPBEa 547 (10^7cells ml^{-1}) was utilised to inoculate three pear plants maintained in 50 l pots in a greenhouse at 25 ± 2°C (day) and 15 ± 2°C (night). A hand sprayer was used to spray cell suspensions avoiding dripping. After 24 h 10 flowers and 10 leaves were randomly sampled from each tree and washed for 30 min in 20 ml of phosphate buffer (0.05 M, pH 7) on a rotatory shaker maintained at 180 rpm. Flowers and leaves harvested from healthy plants of pear served as negative controls. From each sample, 100 µl of the washings were diluted and plated in triplicate on CG medium. The remaining washing of each sample was filtered through gauze and centrifuged for 10 min at 14,000 rpm. The pellet was resuspended in 100 µl of breaking buffer and processed to extract DNA using the same procedure described previously for water suspensions. Before amplification, DNA was purified using a polyvinylpolypyrrolidone (PVPP) as described by Schena and Ippolito (2003). One microliter of purified DNA was amplified by Scorpion-PCR in a total volume of 25 µl as described previously.

Quantitative tests with leaves

Water suspensions of *E. amylovora* containing 10^4, 10^5, 10^6, 10^7, and 10^8 cells ml^{-1} were utilised to inoculate individual branches of pear separated from each other using plastic sheets. Three different branches were inoculated with each bacterial suspension or with water (negative control). Fifty leaves were collected from each branch and analysed by Scorpion-PCR and by plating on CG medium using the same procedure described previously.

Results

Primer specificity

Bacterial DNA used to evaluate primer specificity had a concentration 50 ng μl^{-1}, a 260/280 nm ratio of between 1.8 and 2.0 and in previous experiments was shown to be amplifiable by Random Amplified Polymorphic DNA (RAPD) (data not shown). All tested primers (E1–E2, E3–E4, E5–E1, E6–E2, E7–E8) amplified unique DNA fragments of the expected size. In particular, primers E3–E4 amplified a single bright PCR band of 111 bp exclusively from *E. amylovora* and did not show any primer dimer (data not shown). BLAST analyses excluded the presence of similar sequences among available GenBank databases for other microrganisms. Primer specificity was confirmed by the amplification of DNA fragments of the expected size (111 bp) from all isolates of *E. amylovora* (Table 1) but not from the other bacteria (Table 2) (data not shown).

Scorpion-PCR specificity and sensitivity

Melting curves of real-time PCR products evaluated from 30 to 95°C confirmed the amplification of a single PCR band and 49°C as the best annealing-extension temperature. The difference between fluorescence of positive samples (presence of amplified DNA) and negative samples (absence of amplified DNA) significantly decreased at higher temperatures (Figure 3).

Specificity tests with primers E3 Scorpion-E4 were carried out using the same DNA samples as for conventional PCR. A significant increase in fluorescence was achieved only with DNA extracted from *E. amylovora*, whereas no increase in fluorescence was observed for other bacterial species (Figure 4). Agarose gel electrophoresis and ethidium bromide staining confirmed the presence of specific PCR bands only for samples with positive increases in fluorescence (data not shown).

Using 10-fold serial dilutions of *E. amylovora* DNA, detection limits were 1 pg for Scorpion-PCR and 1 fg for nested-Scorpion-PCR (Figure 5). Cycle thresholds (Ct) were linearly correlated to the quantity of target DNA and permitted the construction of standard curves after both Scorpion-PCR (Figure 5A) and nested-Scorpion-PCR (Figure 5B). Correlation coefficients (r) were 0.999 and 0.991 after Scorpion-PCR and nested-Scorpion-PCR, respectively.

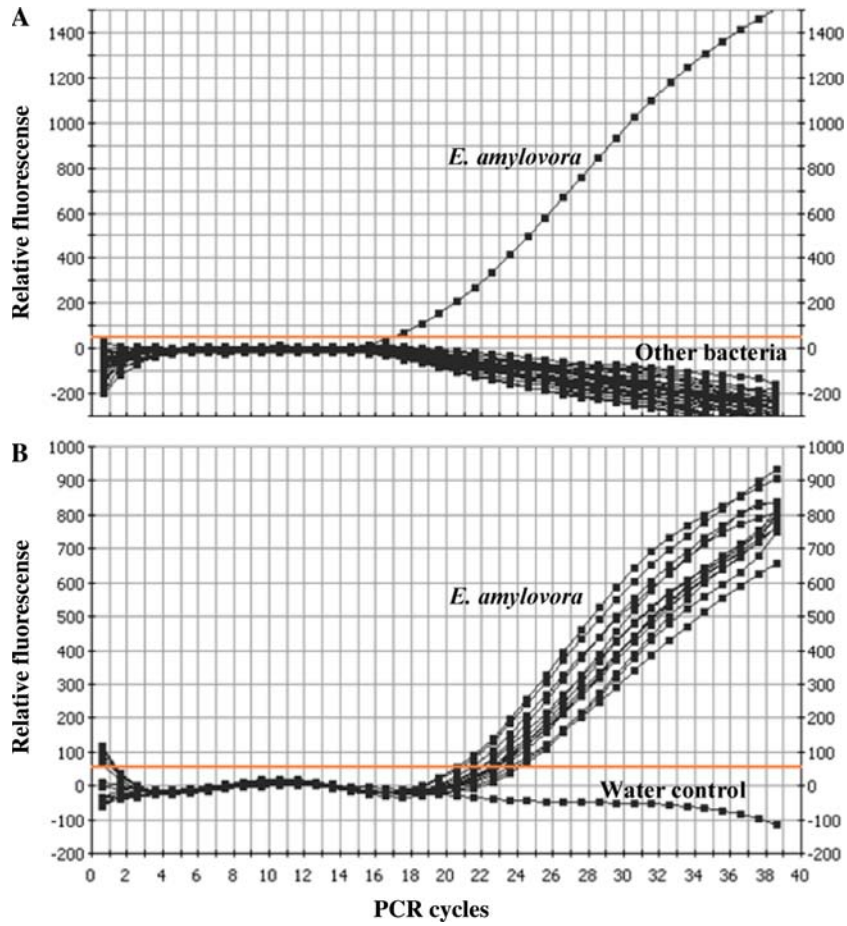

Fig. 4 Results of tests conducted to assess specificity of primers E3 Scorpion-E4. Tests were conducted using DNA from a number of bacterial species and an isolate of *E. amylovora* as positive control (**A**) or a number of *E. amylovora* isolates and water-replacing template DNA as negative control (**B**)

Using serial dilutions of bacterial suspensions, the detection limit was 3.2×10^4 CFU ml^{-1} in Scorpion-PCR and 2.8×10^2 ml^{-1} in nested-Scorpion-PCR. Standard curves showed a high and significant correlation between CFU ml^{-1} and target DNA in Scorpion-PCR ($r = 0.97$) and in nested-Scorpion-PCR ($r = 0.91$) (Figure 6).

Detection of *E. amylovora* from flowers and leaves of artificially inoculated pears

All pear flowers and leaves artificially inoculated with *E. amylovora* produced a positive increase in fluorescence in Scorpion-PCR with an average Ct value of 21.1 (data not shown). Non-inoculated control samples did not produce any increase in fluorescence. The population of *E. amylovora* as assessed on CG medium was of 6×10^7 and 4×10^7 CFU ml^{-1} of washing buffer of leaves and flowers, respectively.

Quantification of *E. amylovora* from pear leaves

Scorpion-PCR enabled the detection of *E. amylovora* on all inoculated leaves whereas the control samples did not produce any increase in fluorescence. A high and significant correlation ($r = 0.92$) was found between CFU leaf^{-1} assessed by plating the washings on CG medium and the quantity of DNA estimated by real-time Scorpion-PCR. The detection limit of Scorpion-PCR was 10^4 CFU leaf^{-1} corresponding to leaves spread with 10^5 cells ml^{-1} (Fig. 7).

Discussion

The main objective of this study was the development of a new real-time PCR detection method for *E. amylovora* satisfying the need for rapid and

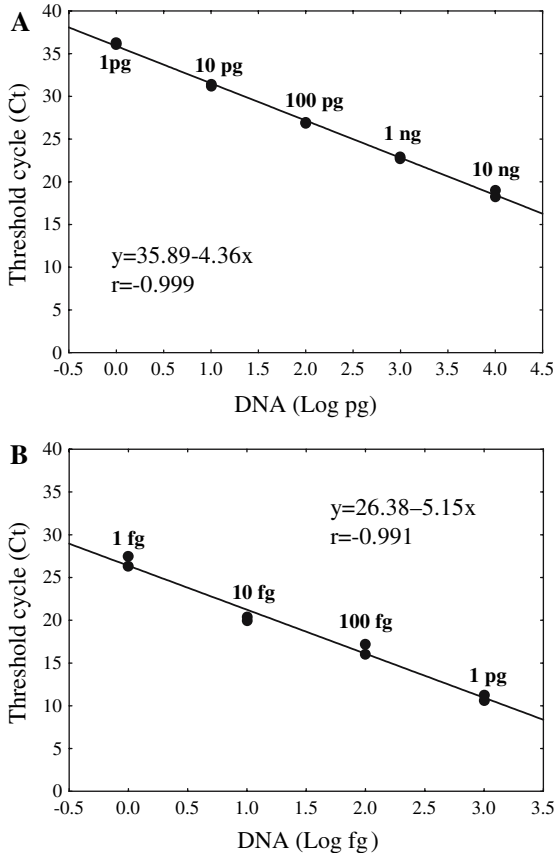

Fig. 5 Sensitivity of Scorpion-PCR (**A**) and nested Scorpion-PCR (**B**) as assessed using 10-fold serial dilutions of *E. amylovora* DNA from 10 ng to 1 pg (**A**) and from 1 pg to 1 fg (**B**). Standard curve and corresponding linear equations were obtained by plotting the threshold cycles against the input DNA target quantity (logarithm scale)

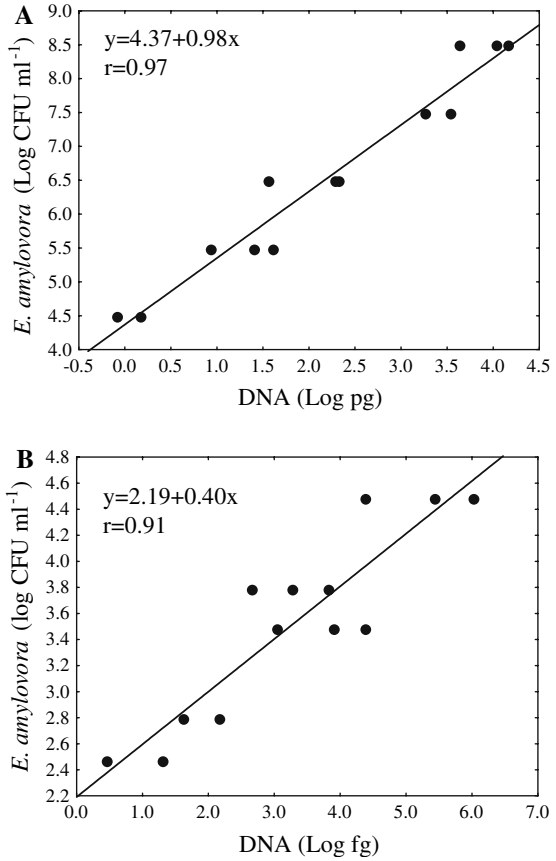

Fig. 6 Standard curves showing the linear correlation existing between *E. amylovora* colony-forming units (CFU) and concentration of *E. amylovora* target DNA after Scorpion (**A**) (from 5×10^8 to 3.2×10^4 CFU ml^{-1}) and nested-Scorpion-PCR (**B**) (from 3.5×10^4 to 2.8×10^2 CFU ml^{-1}). The concentration of target DNA was evaluated by real-time PCR using the specific linear equations developed for Scorpion-PCR and nested-Scorpion-PCR (Fig. 5)

sensitive detection and enabling quantitative and qualitative analyses. A new primer couple amplifying short DNA fragments suitable for real-time PCR (E3–E4) was utilised to develop a real-time PCR detection method based on the 'duplex format' of Scorpion-PCR (Solinas et al., 2001). This method proved to be highly specific as verified by BLAST analysis, the absence of cross-reactivity with DNA of other bacteria and the absence of amplification from non-infected samples of flowers and leaves.

Recently a TaqMan-based detection method has been developed and utilised to amplify *E. amylovora* DNA from field samples and from inoculated leaves and flowers (Salm & Geider, 2004). The method developed in the present study is based on a very recent format of Scorpion-PCR called 'duplex format'. Although in this study there are no comparative experiments, the Scorpion-PCR approach seems to have some advantages compared to the TaqMan approach (Thelwell, Millington, Solinas, Booth, & Brown, 2000). Scorpion-PCR is based on a unimolecular mechanism in which the hybridisation reaction occurs within the same strand. The benefits of a unimolecular rearrangement seem significant, since the reaction is effectively instantaneous and occurs prior to any competing or side reactions such as target amplicon

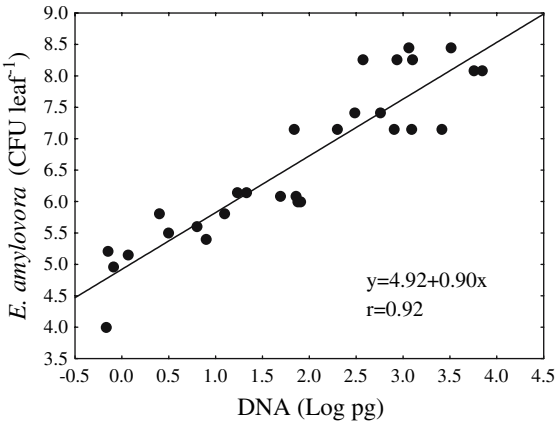

Fig. 7 Standard curves showing the linear correlation existing between the population of *E. amylovora* on pear leaves (CFU leaf^{-1}) and the concentration of target DNA evaluated by Scorpion-PCR using the linear equations reported in Fig. 5

re-annealing or inappropriate target folding. This leads to stronger signals, more reliable probe design, shorter reaction times and better discrimination (Thelwell et al., 2000). Furthermore, the 'duplex format' of the Scorpion-PCR (Solinas et al., 2001) is an improvement on the traditional Scorpion-PCR defined as 'stem-loop format' (Thelwell et al., 2000; Whitcombe, Theaker, Guy, Brown, & Little, 1999). In the latter, quencher and fluorophore remain within the same strand of the DNA and some quenching can occur even in the open form. As opposed to the 'duplex format' the quencher is on a different oligonucleotide and separation between the quencher and fluorophore is greatly increased, thus decreasing the quenching when the probe is bound to the target (Solinas et al., 2001). Disadvantages of Scorpion-PCR compared to TaqMan are the higher cost of probes and the complexity of their design.

Primers E3–E4 were designed from the plasmid pEA29 (McGhee & Jones, 2000) and were flanked by two reported primers (PEANT1–PEANT2) used to detect *E. amylovora* in asymptomatic plant material by conventional PCR (Llop et al., 2000). This primer localization enabled the development of two alternative approaches: (i) Scorpion-PCR, based on a single real-time PCR amplification with primers E3 Scorpion-E4 and (ii) nested-Scorpion-PCR, based on a first amplification round with primer PEANT1–PEANT2 and a second amplification round with the Scorpion primers. Both approaches were shown to be specific and performed well; however, they provided different levels of sensitivity and complexity and therefore their choice should be related to the purpose of the analyses.

Scorpion-PCR gave a detectable amplification product up to 1 pg of template DNA and enabled the detection of 3.2×10^4 CFU ml^{-1}. A similar detection limit was reported for the method of Salm and Geider (2004). Furthermore, Scorpion-PCR was appropriate for quantitative analyses as confirmed by the high correlation achieved between the quantity of DNA and CFU from water suspensions and pear leaves. Although specific quantitative tests were not conducted with flowers, the positive result achieved with qualitative tests suggests that the method is appropriate to quantify *E. amylovora* on both leaves and flowers. The early detection of *E. amylovora* on flowers is very important because they are the main port of entry for the pathogen (Johnson & Stockwell, 1998; Thomson, 2000). Since high levels of populations on flowers are usually required for the development of the disease (Thomson, 1986, 2000), the level of sensitivity of the Scorpion-PCR method developed in the present study (3.2×10^4 CFU ml^{-1} = 10^4 CFU leaf^{-1}) seems to be sufficient to detect and quantify the pathogen on leaves and flowers and to predict the development of the disease.

In nested-Scorpion-PCR the detection limit was of 1 fg of target DNA and enabled the detection of as little as 2.8×10^2 CFU ml^{-1}. To the best of our knowledge, this is the highest level of sensitivity achieved for *E. amylovora* with a molecular-based detection method. A significant correlation was found between quantity of DNA and CFU; however, this correlation was only possible for a restricted range of DNA concentrations (1 pg–1 fg) and correlation coefficients were inferior compared to those of Scorpion-PCR. This result was expected because, as already reported for other microorganisms (Hayden, Rizzo, Tse, & Garbelotto, 2004; Schena, Nigro, & Ippolito, 2002), the requirement for two

amplification rounds reduces the existing correlation between starting quantity of template DNA and Ct values. Furthermore, nested-Scorpion-PCR is more costly, complex and prone to risks of false positives compared to Scorpion-PCR (Schena et al., 2004). On the basis of these considerations, the nested-Scorpion-PCR approach seems to be advisable when very high levels of sensitivity are required, while Scorpion-PCR seems to be more appropriate for quantitative analyses and for routine applications. The nested approach could be utilised practically to prevent the introduction of the bacterium into new areas through contaminated propagating materials.

The method utilised to extract DNA from washings of leaves and flowers is based on physical disruption of cells, phenol–chloroform extraction and removal of co-extracted compounds by the use of PVPP spin column chromatography (Schena & Ippolito, 2003). Protocols based on organic solvents could cause loss of template DNA in the interface and reduce the appropriateness of the method for large-scale assays especially when conducted by regulatory diagnostic laboratories. However, organic solvents ensure high quality DNA compared to direct cell lysis methods and are less expensive compared to commercial kits.

In conclusion, two different approaches based on Scorpion-PCR were developed for the detection and quantification of *E. amylovora* in plant organs. These methods integrate other recently reported approaches (Salm & Geider, 2004) and provide important tools for a series of applications including the development of effective strategies to control and manage the spread of the pathogen as well as to study its biology, ecology and interaction with hosts.

Acknowledgements This work was supported by a grant from the University of Bari: 'Epidemiology and genetics of plant-pathogen microrganisms'. We thank J.D. Janse Jr. at Department of Bacteriology, Plant Protection Service, Wageningen, The Netherlands, C. Bazzi at Department of Agroenvironmental Sciences and Technologies, University of Bologna, Italy, M. Scortichini at Fruit Tree Research Institute, Rome, Italy, and L. Corazza at Plant Pathology Research Institute, Rome, Italy, for kindly supplying bacterial isolates.

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