Phytopathol. Mediterr. (2008) 47, 204–213

RESEARCH PAPERS

A real-time PCR quantitative detection assay for *Pseudomonas savastanoi* pv. *nerii* in *Nerium oleander*

PATRIZIA BELLA[1,2], GRAZIA LICCIARDELLO[1,2], MATILDE TESSITORI[1] and VITTORIA CATARA[1]

[1]Dipartimento di Scienze e Tecnologie Fitosanitarie, Università degli Studi di Catania, Via Santa Sofia 100, 95123 Catania, Italy
[2]Parco Scientifico e Tecnologico della Sicilia, Z.I. Blocco Palma 1, 95131 Catania, Italy

Summary. A real-time PCR assay based on TaqMan chemistry was developed for the detection of *Pseudomonas savastanoi* pathovars that cause bacterial knot disease on different plant species. Primers and probe sequences were based on the *iaaL* gene coding for (indole-3-acetyl)-L-lysine synthetase and previously used in conventional PCR tests. Assay specificity was tested with an extended range of strains of *P. savastanoi* from eight hosts, with 13 other *Pseudomonas* spp., and with other microorganisms naturally occurring on or in oleander plants. A pure culture cell suspension was quantified over a seven log concentration range (10^8 to 10^2 cfu ml^{-1}). Different protocols were developed for the detection and quantification of *P. savastanoi* pv. *nerii* from symptomatic and asymptomatic oleander plants. A 24-h bacterial enrichment step either on PVF-1 or OKA-M broth improved the sensitivity of the assay, making it suitable to screen planting material for latent infections.

Key words: oleander knot, *iaaL* gene, TaqMan chemistry, bacterial enrichment, diagnosis.

Introduction

Pseudomonas savastanoi includes three pathovars, pv. *glycinea*, pv. *phaseolicola* and pv. *savastanoi sensu* Gardan *et al.* (1992). The latter includes strains that cause bacterial knot disease in a number of plant species. In "Names of Plant Pathogenic Bacteria" (http://www.isppweb.org/names_bacterial_revised.asp), four pathovars of *P. savastanoi* which induce knots and cankers on different hosts are listed: pv. *savastanoi* (olive and other *Oleaceae*), pv. *nerii* (oleander), pv. *fraxin*i (ash) and pv. *retacarpa* (Spanish broom). Nevertheless, according to Gardan *et al.* (1999), based on DNA-DNA hybridization studies, *P. savastanoi* is included in genomospecies 2 with a number of *P. syringae* pathovars, and other related *Pseudomonas* species.

Corresponding author: P. Bella
Fax: +39 095 7147287
E-mail: patrizia.bella@unict.it

Knot development is incited by the production of the phytohormone indoleacetic acid (IAA) and cytokinins produced by the bacterium (Surico and Iacobellis, 1992). The Hrp/hrc genes of *P. savastanoi* have a role in the pathogenicity and multiplication of the pathogen within the host (Sisto *et al.*, 2004).

Pseudomonas savastanoi pv. *nerii* is the causal agent of oleander knot. It not only causes knots on stems, twigs and leaves but also deforms inflorescences and seedpods, reduces blooming and kills pistils (Smith, 1928). The severity of the disease is mainly due to the invasion of the laticifers by the pathogen which causes extensive secondary knotting (Wilson and Magie, 1964). The inoculum carried endophytically or epiphytically in propagation materials, such as cuttings and plantlets, represents a threat for the spread of the pathogen (Wilson and Magie, 1964; Azad and Cooksey, 1995). It would thus be helpful to have a sensitive method to detect this bacterium early in source plants and

cuttings, and to produce disease-free propagation material for certification programs.

Oleander knot disease is diagnosed mainly by visual inspection of the host plants, as in other diseases also caused by *P. savastanoi* pathovars. Bacteria can be isolated from the knots and asymptomatic tissues on general media (nutrient agar sucrose, NAS, King's B medium, KB) and on semiselective media, ANS-S (Varvaro, 1983) and PVF-1 (Surico and Lavermicocca, 1989). A semiselective medium, oleander knot agar (OKA), was specifically developed to isolate *P. savastanoi* pv. *nerii* from oleander plants and to monitor the systemic movement of the pathogen (Azad and Cooksey, 1995). Bacterial isolation is more difficult when performed on old, cracked or lignified knots because of the high presence of saprophytic bacteria and a low viable population of the pathogen (Surico and Lavermicocca, 1989; Azad and Cooksey, 1995). Furthermore, bacterial concentration in asymptomatic samples is very low, and detection is further reduced on semi-selective media where the recovery of *P. savastanoi* pathovars varies, and some strains do not grow at all (Azad and Cooksey, 1995; Surico and Marchi, 2003).

Pure cultures of *P. savastanoi* are identified by biochemical and serological techniques or by fatty acid methyl ester profiling (Casano *et al*., 1987; Janse, 1991; Young and Triggs, 1994). The *iaaL* gene coding for (indole-3-acetyl)-L-lysine synthetase involved in the conversion of IAA to IAA-lysine has proved useful as a target to identify and detect the bacterium by conventional PCR, enriched PCR and nested PCR (Penyalver *et al.,* 2000; Bertolini *et al.,* 2003). The primers that were developed amplified the target sequence in strains isolated from different host plants, but a protocol for plant material was developed to detect the bacterium in olive (Penyalver *et al*., 2000).

Real-time PCR has recently been employed to detect and quantify phytopathogens (Schaad and Friederick, 2002; Schena *et al.,* 2004). This technique combines the sensitivity of conventional PCR with specific real-time fluorescent signals throughout the reaction, allowing faster and less labour-intensive quantification of the target than is possible with conventional PCR as it eliminates the need for post-PCR processing. An increasing number of real-time PCR assays have been developed for phytopathogenic bacteria in the plants themselves (Schaad and Friederick, 2002; Schaad *et al.,* 2003; Mumford *et al.,* 2006). This technique also accurately and reliably quantifies target DNA in various environmental samples to study epidemiology and host-pathogen interactions as well as inoculum threshold levels as has been described for different phytopathogens (Schaad and Friederick, 2002; Schena *et al*., 2004).

In this paper we describe a sensitive, quantitative real-time PCR assay targeted at the *iaaL* gene which is a suitable target for all *P. savastanoi* gall-forming strains (Penyalver *et al*., 2000; Bella, 2002). Since the primers described by Penyalver amplified a fragment of 454 bp in length that was too long for real-time PCR, new primers and a TaqMan probe were designed. Different sample preparation protocols to detect *P. savastanoi* pv. *nerii* in oleander plants were examined.

Materials and methods

Bacterial and fungal strains and culture conditions

Fifty strains of *P. savastanoi* isolated from oleander, olive, privet, jasmine, *Phillyrea*, myrtle, ash, and Spanish broom, from different countries, and 13 strains of other *Pseudomonads* and some phytopathogenic fungi isolated from oleander were used to test the specificity of the primers (Table 1). Fluorescent bacterial strains were routinely grown on King's B medium (KB) and other bacteria on nutrient agar plus 1% D-glucose (NDA), and the fungal isolates were grown on potato dextrose agar (PDA).

For DNA extraction, bacteria were grown overnight at 28°C in KB broth (fluorescent pseudomonads) or nutrient broth NB (other *Pseudomonas* spp.). To extract DNA, fungi were grown in 30 ml of potato dextrose broth (Oxoid Ltd, Basingstoke Hampshire, UK) at 24°C for 5 days, then the total DNA was extracted from 60 mg of fresh mycelium washed twice in sterile distilled water. Bacterial enrichment and selective plating were carried out on PVF-1 (Surico and Lavermicocca, 1989) and a modified Oleander Knot Agar formulation (OKA-M), in which SDS was added at 0.04% and four antibiotics (bacitracin, ampicillin, novobiocin and cycloheximide) were omitted (Azad and Cooksey, 1995).

DNA isolation

DNA was isolated from pure cultures of bacterial strains and from various tissue extracts (healthy, symptomatic and asymptomatic oleander stems),

Table 1. Bacterial and fungi isolates used in this study.

Pathogen	No. of strains	Strain designation[a]	Host	Geographical origin	Real-time PCR
P. savastanoi pv.:					
pv. *fraxini*	2	CFBP5965	Ash	France	+
		CFBP2094	Ash	Algeria	+
pv. *glycinea*	1	CFBP2214	*Glycinea max*	New Zealand	-
pv. *nerii*	11	PVCT49; PVCT51; PVCT83; PVCT87.1.6;PVCT89.1.1; PVCT90.1.1; PVCT90.1.2;PVCT91.1.1; PVCT92.1.1A; PVCT99.2.3;PVCT100.1.3;	Oleander	Italy	+
	1	CFBP6022	Oleander	Spain	+
	5	NCPPB3334; CFBP1839; CFBP2087; CFBP5967; CFBP6005	Oleander	France	+
	1	CFBP5051	Oleander	Ex Yugoslavia	+
	2	CFBP2083; CFBP2088	Oleander	Algeria	+
	1	CFBP6008	Oleander	Greece	+
pv. *phaseolicola*	1	1449B	*Lablab purpureus*	Ethiopia	-
pv. *retacarpa*	3	CFBP5510 CFBP5511; CFBP5512	Spanish broom	Spain	+
pv. *savastanoi*	10	PVCT3; PVCT10; PVCT13; PVCT18; PVCT26; PVCT40; PVCT55; PVCT63; PVCT97; PVCT101	Olive	Italy	+
	1	CFBP1670	Olive	Ex Yugoslavia	+
	1	CFBP2073	Olive	France	+
	1	CFBP6013	Olive	Syria	+
	1	CFBP5050	Olive	Portugal	+
	3	CFBP5081; CFBP5082;CFBP5083	Privet	Italy	+
	2	CFBP1751 T12.4	Jasmine	Greece	+
	3	Ph1; T51.1; T51.2	*Phillyrea* sp.	Algeria	+
	2	U58.1; U58.2	Mirtle	Syria	+
Other Pseudomonads					
P. fluorescens	1	CFBP2102	Pre-filter tanks	UK	-
P. cichorii	1	CFBP2101	Endive	Germany	-
P. corrugata	1	CFBP5454	Tomato	Italy	-
P. marginalis pv. *marginalis*	1	CFBP1387	Chicory	USA	-
P. mediterranea	1	CFBP5447	Tomato	Italy	-
P. putida	1	CFBP2066	Soil	USA	-
P. syringae pv. *syringae*	2	PVCT10.2, PVCT26	Lemon, pear	Italy	-
P. syringae pv. *pisi*	1	CFBP2704	Pea	USA	-
P. syringae pv. *tomato*	1	PVCT28.3.1	Tomato	Italy	-
P. viridiflava	1	PVCT26.1.1	Red-leaved chicory	Italy	-
Other oleander pathogens					
Colletotrichum sp.	1	PVCT CO1.1	Oleander	Italy	-
Phoma exigua var. *heteromorpha*	1	PVCT PeH 1.1	Oleander	Italy	-
Septoria oleandrina	1	PVCT PO 1	Oleander	Italy	-
Unidentified bacteria	12		Oleander	Italy	-

[a] PVCT, Istituto di Patologia Vegetale, Dipartimento di Scienze e Tecnologie Fitosanitarie, Catania, Italy; CFBP, Collection Française des Bacteries Phytopathogènes, INRA, Beaucouzé Cedex, France; NCPPB, National Collection of Plant Pathogenic Bacteria, York, UK.

with two commercial kits according to the manufacturer's instructions: Puregene ® genomic DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) and Generation Capture Column kit (Gentra Systems). DNA was extracted from fungi as previously described using the Puregene ® genomic DNA isolation kit (Licciardello *et al.,* 2006). DNA concentration was measured using a biophotometer (Eppendorf Srl, Milan, Italy).

TaqMan probe design and PCR

The primers and TaqMan probe were designed with the Primer Express v2 software (Applied Biosystems, Monza, Italy) using sequence information from the DNA target sequence of the IAA-lysine synthetase (*iaaL*) gene used for the PCR detection of *P. savastanoi* pv. *savastanoi* by Penyalver *et al.* (2000). Of the potential primer pairs identified, the primers IAAL-F (TTCCGCAGCAACGGCTTTAT) and IAAL-R1 (CCGGCCATCATGGTAATCGT) were selected, as well as the TaqMan probe IAAL-P (5'-FAM-TGCCCGGTGATCGCTGCGC-TAMRA-3'). They amplified a 101-bp fragment. The specificity of the primer pairs designed for real-time quantitative PCR was first tested by conventional PCR. Standard PCR was performed in a 25 μl reaction containing:1× PCR buffer (Invitrogen Srl, Milan, Italy), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 400 nM of each primer and 1.25 U Taq DNA recombinant polymerase (Invitrogen). Amplification was carried out in a GeneAmp PCR thermal cycler system 9700 (PE Applied Biosystems, Forster City, CA, USA) programmed for one cycle of 10 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, with a final extension step of 5 min at 72°C. Amplified products were resolved by electrophoresis at 5.7 volt cm^{-1} in 2% agarose gel in 0.5× Tris-acetate EDTA buffer and stained with ethidium bromide (Sambrook *et al.,* 1989).

The real-time PCR assay was performed using OmniMix HS lyophilised beads (Cepheid, Sunnyvale, CA, USA) in a final volume of 50 μl (for two reactions). We added 400 nM primers, 200 nM fluorogenic probe and 1 μl genomic DNA. The negative control reaction contained the same mixture with 1 μl sterile water replacing the DNA template. All PCR reactions were performed in 25 μl tubes (Cepheid) in a Smart Cycler II System (Transportable Device, TD Configuration; Cepheid). The thermal cycling conditions for *P. savastanoi* pv. *nerii* DNA template amplification were: an initial denaturation at 95°C for 120 s, followed by 40 cycles at 95°C for 10 s, 60°C for 15 s and 72°C for 15 s, with heating ramp rates (max) of 10°C s^{-1} and cooling ramp rates (max) of 2.5°C s^{-1}. For each sample, detection was by online monitoring to identify the exact Ct value where the log-linear phase could be distinguished from the background. To confirm that only the PCR product was amplified, all reactions were analysed by 2% agarose gel electrophoresis.

Sensitivity of the real-time PCR assay

The sensitivity of real-time PCR and the standard curve for absolute quantification were assessed by analysing *P. savastanoi* pv. *nerii* strain PVCT 89.1.1. One-ml aliquots of a pure culture grown in KB medium (10^8 cfu ml^{-1}) were washed twice with 1 ml of 0.1 M Tris-HCL. The pellet was resuspended in 100 μl of 0.1 M Tris-HCL and heated for 5 min at 95°C. The cell lysate was serially diluted in sterile distilled water and 1 μl aliquots were subjected to conventional PCR and real-time PCR amplification. Standard curves were obtained by plotting the Ct values versus the logarithm of the concentration of each 10-fold-dilution series. Ct values for each standard reaction were calculated for three PCR replications, performed in three separate runs.

Plant samples

Spiked samples were used to optimise detection of *P. savastanoi* pv. *nerii* from oleander by real-time PCR. Plant extracts were prepared from stems collected from uninfected nursery-grown oleander plants. Stem portions of about 1 g were cut with a razor blade and homogenized in 20 ml of either PVF-1 or OKA-M using a Polytron PTA 20 TS. Then 30 ml of the corresponding medium was added, making a final volume of 50 ml. Known amounts of a fresh overnight culture of *P. savastanoi* pv. *nerii* PVCT89.1.1 from 1 to 10^6 cfu ml^{-1} final concentration were added to the plant extracts. One millilitre of spiked plant samples from each bacterial concentration was added to 50 ml of the semi-selective medium, either PVF-1 or OKA-M, for bacterial enrichment and incubated at 28°C at 180 rpm for 24 h. Spiked samples were subjected to DNA extraction before and after enrichment using the extraction kits.

To evaluate the detection of *P. savastanoi* in naturally infected plants, we selected three sample types: 1. stem segments including knots; 2. adjacent

portions without symptoms; and 3. segments from asymptomatic stems. The 1–2 cm long segments were surface sterilized in 10% sodium hypochlorite, homogenised in PVF-1 like the spiked samples, and incubated at 28°C at 180 rpm for 24 h in the same medium as that used for bacterial enrichment. Aliquots of plant extracts were spread on PVF-1 with the Eddy Jet Spiral Plater (LabScientific Inc., NJ, USA) for bacterial isolation, or subjected to DNA extraction with the Puregene® genomic DNA isolation kit for conventional and real-time PCR analysis.

Plant extracts from knots were also prepared according to Azad and Cooksey (1995). Briefly, knots were excised and macerated in sterile distilled water at 4°C for 30 min. Aliquots were spread on PVF-1 or used directly for PCR or real-time PCR.

To assess whether real-time PCR could be further exploited to detect *P. savastanoi* pathovars in other hosts, we prick-inoculated the following combinations of bacterial strains and hosts: PVCT31-olive, CFBP5081-privet, CFBP1751-jasmine, T51.1-*Phillyrea,* U58.1-myrtle and CFBP2094-ash.

Results

Primers and probe specificity testing

The specificity of the primers (IAAL-F, IAAL-R1) and probe (IAAL-P) was evaluated using the bacteria and fungi listed in Table 1. Conventional PCRs performed with primers IAAL-F and IAAL-R1, preliminarily tested on a number of *P. savastanoi* strains agents of galls, produced a single DNA amplicon of 101 bp (data not shown) whereas no amplicon was obtained with any of the other *Pseudomonas* species. The strains were analysed by real-time PCR. All fifty *P. savastanoi* strains isolated from 8 different hosts, produced threshold cycle values (Cts) between 12 and 19, with DNA concentrations between 50 and 180 ng DNA μl^{-1}, indicating amplification. No fluorescence was detected for various *Pseudomonas* species belonging to DNA-RNA homology group I or other microorganisms naturally occurring on or in oleander tissues or oleander DNA, indicating that amplification had not occurred. Real-time PCR amplicons were further visualised by gel electrophoresis, displaying the expected band of 101 bp (data not shown).

Sensitivity and linear range of quantification

To estimate the sensitivity of the method, a ten-fold dilution series of cell lysates from *P. savastanoi* pv. *nerii* strain PVCT89.1.1, covering seven logs ranging from 10^8 to 10^2 cfu ml^{-1} was measured in triplicate by real-time quantitative PCR (Fig.1a). Amounts as small as 10^2 cfu ml^{-1} could be detected. The correlation coefficient (r^2) 0.998 demonstrated the linearity of the quantification over a range of seven logs (from 10^8 to 10^2 cells ml^{-1}) (Fig.1b). The standard deviation for each point on the standard curve increased with decreasing initial target concentration. The slope of the standard curve was –3.43, which was equivalent to a PCR efficiency of 95.4%.

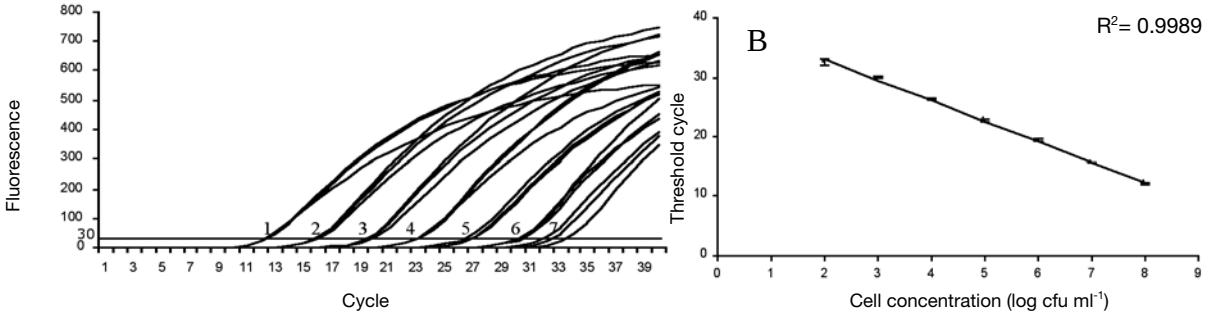

Fig. 1. Calibration curve for the absolute quantification of *P. savastanoi* pv. *nerii* PVCT89.1.1 cell lysate targeting the *iaaL* gene. (A) Amplification plot generated from the change in fluorescence vs. the threshold cycle number of the 10-fold dilution series (1–7 from 10^8 to 10^2 cells). (B) Standard curve were generated from threshold cycle number (Ct value) of dilution of *P. savastanoi* pv. *nerii* PVCT89.1.1 cell lysate plotted vs. the log of the number of cells in the lysis reaction, illustrating a 7 log linearity range. Error bars represent the standard deviation from three replicates for each dilution obtained in three independent runs. R^2 = correlation coefficient. Data of both graphs from Smart Cycler software 2.0 were exported as Microsoft Excel data to build black-and-white graphs.

Detection of *P. savastanoi* pv. *nerii* in oleander spiked samples by PCR and real-time PCR

PCR and real-time PCR assays detected *P. savastanoi* pv. *nerii* PVCT89.1.1 in oleander extracts spiked with known amounts of the bacterium. The sensitivity achieved differed depending on the DNA extraction method, the PCR assay and an enrichment step (Table 2). In tests with conventional PCR the detection limit of *P. savastanoi* pv. *nerii* was 10^4 cfu ml^{-1} with both PVF-1 and OKA-M-prepared samples, but only when the DNA had been extracted with the Puregene® genomic DNA isolation kit (solution kit). After 24 hours of bacterial enrichment in PVF-1, the bacterium was detected in the sample contaminated with 10^1 and 10^2 cfu ml^{-1}, using DNA extracted with the solution kit and the Generation Capture Column kit (column kit) respectively. Similarly, when OKA-M was used as the enrichment medium, the detection limit was 10^1 cfu ml^{-1} for DNA extracted with the solution kit and 10^3 cfu ml^{-1} for column-extracted DNA. In real time PCR assays the presence and amount of target DNA were determined on the basis of Ct values. The Ct values were compared to evaluate the efficacy and sensitivity of the sample preparation methods.

The detection limit by real-time PCR before enrichment of the samples extracted with the solution kit was 10^2 cfu ml^{-1}, and the bacterial concentrations inferred by interpolating Ct values from the standard curve were, in some cases, about one log lower than the cells actually added to the spiked samples. When DNA was extracted with the column kit (10^3 cfu ml^{-1}), sensitivity was lower, but no correlation was found between the concentration of the sample and that inferred by real-time PCR. After enrichment, *P. savastanoi* was detected in spiked samples up to 10^1 cfu ml^{-1} when using DNA extracted with the solution kit, and to 10^2 cfu ml^{-1} when using DNA extracted with the column kit. Considerably lower Cts were obtained compared to the same samples before enrichment, but no quantification in this case was applicable. When comparing the Ct values of the enrichment media, it was generally possible to obtain lower values for samples enriched in PVF-

Table 2. Detection of *Pseudomonas savastanoi* pv. *nerii* PVCT 89.1.1 in oleander tissue extracts spiked with known amounts of bacterial cells by conventional (PCR) and Real-time (ReT) PCR.

Cfu ml^{-1}	PCR method	Without enrichment				With enrichment			
		PVF-1		OKA-M		PVF-1		OKA-M	
		Solutions[a]	Columns[b]	Solutions	Columns	Solutions	Columns	Solutions	Columns
10^5	PCR	+	-	+	-	+	+	+	+
	ReT PCR Ct/ cells ml^{-1}	22.35/ 1.7×10^5	30.87/ 4.6×10^2	24.34/ 3.4×10^4	31.03/ 4.1×10^2	n.t.	n.t.	n.t.	n.t.
10^4	PCR	+	-	+	-	+	+	+	+
	ReT PCR Ct/ cells ml^{-1}	28.72/ 1.9×10^4	32.99/ 1.1×10^2	27.75/ 3.6×10^3	30.59/ 5.5×10^2	n.t.	n.t.	n.t.	n.t.
10^3	PCR	-	-	-	-	+	+	+	+
	ReT PCR Ct/ cells ml^{-1}	30.85/ 4.7×10^2	-	29.71/ 1.0×10^3	33.09/ 1.0×10^2	21.20/ 2.7×10^5	27.72/ 3.7×10^3	23.99/ 4.3×10^4	28.46/ 2.2×10^3
10^2	PCR	-	-	-	-	+	+	+	-
	ReT PCR Ct/ cells ml^{-1}	32.10/ 2.0×10^2	-	30.38/ 6.4×10^2	-	23.42/ 6.2×10^4	30.80/ 4.8×10^2	27.18/ 5.2×10^3	32.32/ 1.7×10^2
10^1	PCR	-	-	-	-	+	-	+	-
	ReT PCR Ct/ cells ml^{-1}	-	-	-	-	27.45/ 4.4×10^3	-	29.58/ 1.0×10^3	-

[a] DNA extraction KIT with solutions (Puregene ® genomic DNA isolation kit [Gentra Systems]).
[b] DNA extraction KIT with columns (Generation Capture Column kit ([Gentra Systems]).
+, PVCT98.1.1 detected by conventional PCR; -, PVCT98.1.1 not detected by conventional PCR.
n.t., not tested.

1 rather than those in OKA-M, with a difference between the two of 2.5–3.5 cycles.

Detection and quantification of *P. savastanoi* pv. *nerii* in symptomatic and asymptomatic oleander plants

On the basis of the results obtained with spiked samples, naturally infected symptomatic and asymptomatic samples from oleander plants were homogenised and enriched in PVF-1, and then total DNA was extracted with the Puregene® genomic DNA isolation kit and subjected to PCR analysis. Twenty stem portions from each of the three sample types were analysed: 1. stem portions from symptomatic stems with knots, 2. asymptomatic stem portions adjacent to knots, and 3. stem portions from asymptomatic stems of symptomatic plants. *P. savastanoi* pv. *nerii* was detected by PCR and real-time PCR in almost all the stem portions with hyperplasia (either as knots or as secondary excrescences), as well as being isolated on PVF-1 (Table 3). The efficiency of the isolation plating on PVF-1 drastically decreases after enrichment. Ct values from 16.2 to 20.8, corresponding to 2.8×10^6 and 3.7×10^5 cfu ml^{-1}, were observed indicating high concentrations of bacteria in the knots. Of the 20 portions tested before and after enrichment, *P. savastanoi* pv. *nerii* was detected by PCR in 6 asymptomatic portions before enrichment, and in 10 asymptomatic portions after enrichment. The corresponding positive samples were 12 and 18 with real-time PCR. Before enrichment a range of Ct values from 28.87 to 32.10, corresponding to 1.9×10^4 and 1.2×10^2 cfu ml^{-1}, was observed, indicating different concentrations of the bacterium in naturally infected samples. The bacterium was also isolated on PVF-1 from 6 samples at concentrations ranging from 1.1×10^2 to 6.5×10^3 cfu ml^{-1}. Asymptomatic samples were negative by both PCR methods and also by isolation on PVF-1. After enrichment, 4 samples were positive by real-time PCR, of which only two were confirmed positive by conventional PCR and by isolation on PVF-1.

Detection of *P. savastanoi* pathovars in galls of different hosts by real-time PCR

The real-time PCR protocol was used to detect strains of various *P. savastanoi* pathovars artificially inoculated into their principal hosts in which they had produced typical knots. Since those were evident from a visual diagnosis we extracted the DNA using methods that were rapid and easy: either with the Generation Capture Column kit and by comminuting and macerating the knots in sterile distilled water. Knots from olive, ash, privet, jasmine, *Phillyrea* and myrtle gave positive results, irrespective of the extraction method (five knots per species). However, the Ct values from DNA extracted with the column kit were lower than those obtained from knots macerated in sterile distilled water (data not shown).

Discussion

We developed a real-time PCR assay, based on TaqMan chemistry, which detected and helped identify *P. savastanoi* pathovars, the causal agents of knot disease, directly from infected tissue, with a specificity equal to that of other PCR methods, but avoiding post-PCR handling. It enabled sensitive and quantitative detection of *P. savastanoi* pv. *nerii* from oleander tissue extracts and by adding

Table 3. Detection of *Pseudomonas savastanoi* pv. *nerii* from symptomatic and asymptomatic stems of naturally infected oleander plants.

Oleander sample (No.)	*P. savastanoi* pv. *nerii* detection (No. of positive samples)					
	Without enrichment			After enrichment		
	Isolation on PVF-1	Conventional PCR	Real-time PCR	Isolation on PVF-1	Conventional PCR	Real-time PCR
Symptomatic						
With knots (20)	18	18	20	12	20	20
Without knots(20)	6	6	12	10	10	18
Asymptomatic (20)	0	0	0	2	2	4

a bacterial enrichment step the assay was made particularly sensitive.

The *iaaL* gene coding for (indole-3-acetyl)-L-lysine synthetase was the target designated for the PCR primers and probe. This enzyme has been shown in *P. savastanoi* from oleander to further metabolise IAA to an amino acid conjugate, 3-indoleacetyl-ε-L-lysine (IAA-lysine) which influences the IAA pool size and virulence in *P. savastanoi* (Glass and Kosuge, 1988). Apparently olive and privet strains do not do this (Glass and Kosuge, 1986) and no information is available about strains from other hosts. However, the *iaaL* gene proved to be a good diagnostic marker as it was amplified in all seventy strains of *P. savastanoi* isolated from olive, oleander, ash and jasmine tested by Penyalver *et al.* (2000) and also in another forty-three strains from olive, oleander, ash, privet, jasmine, *Phillyrea* and Spanish broom (Bella, 2002).

The primers, IAAL-F and IAAL-R1, developed in this study designed on the *iaaL* gene, reacted with all the *P. savastanoi* strains, both by conventional PCR and with the IAAL-P probe in real-time PCR. The assay was also specific: none of the other microorganisms produced an amplicon. Although the *iaaL* gene has been described in a wide variety of *P. syringae* pathovars (Glickmann *et al.,* 1998), BLAST analysis of the 101 bp target sequence showed no similarity to *P. savastanoi* pv. *phaseolicola* or to pv. *glycinea*. There was however a similarity of 89% with a putative indole acetate-lysine ligase of *P. syringae* pv. *tomato* DC3000, although here three and four nucleotide differences were observed in the forward and reverse primer region respectively. Furthermore, the *P. syringae* pv. *tomato* strain tested gave no amplification product with either PCR or real-time PCR.

Analysis of propagating materials requires a sensitive and reliable assay. The assay developed here, including sample preparation and real-time PCR, enabled reliable detection of *P. savastanoi* pv. *nerii* in oleander samples spiked with 100 cfu ml^{-1}. Combined with bacterial enrichment, it was possible to detect the bacterium even in samples spiked with 10 cells per ml after only a one-day enrichment step. At this level of sensitivity the bacterium was detected even in asymptomatic oleander stems. Comparable results were obtained by Penyalver *et al.* (2000) using conventional PCR both before and after the enrichment of olive plant extracts for three days in PVF1. Only a nested PCR protocol was more sensitive, detecting as little as one cell per ml of plant extract after enrichment (Bertolini *et al.*, 2003).

Total DNA extraction with the solution kit was the most effective method, reliably quantifying *P. savastanoi* pv. *nerii* in spiked samples of oleander. If the aim is to detect the bacteria in suspected samples, where high concentrations of bacteria are supposed, for the purpose of phytosanitary control, the column kit is preferable since sample preparation lasts only 30 minutes, compared with 2.5 hours for the other kit.

Two semi-selective media, PVF-1 and OKA-M, the first developed to isolate *P. savastanoi* from olive plants under field conditions, and the second to detect the epiphytic and systemic populations of *P. savastanoi* from oleander, were evaluated as liquid media for bacterial enrichment. PVF-1, which has been compared to King's B medium for bacterial enrichment, improved PCR detection of *P. savastanoi* in asymptomatic stem tissue from infected olive plants and in knot tissue from naturally infected plants (Penyalver *et al.,* 2000).

In the spiked samples before enrichment, we quantified fewer cells compared to those added. The discrepancy between cells quantified and cells added was lower in PVF-1 than in OKA-M. This may have been due to inhibition by the oleander extracts or by the media employed. Consequently, the quantification of *P. savastanoi* pv. *nerii* in the natural samples may have been underestimated.

Bacterial detection in the spiked oleander samples after enrichment under the experimental conditions was more accurate and sensitive with PVF-1 than with OKA-M, although it is the simplified formulation of OKA proposed by Azad and Cooksey (1995) to reduce the inhibition activity towards *P. savastanoi* pv. *nerii*. Real-time PCR results showed differences of approximately 2.5 to 3.5 Cts between the two media when samples were spiked with the same bacterial concentration, indicating better bacterial growth in PVF-1. By contrast, the effectiveness of isolation plating on PVF-1 decreased after enrichment, probably due to the high concentration of saprophytic bacteria, as already reported by Penyalver *et al.* (2000) and Bertolini *et al.* (2003). *Pantoea agglomerans*, for example, was found frequently associated with *P. savastanoi* pv. *savastanoi* in olive knots (Marchi *et al.*, 2006; Cimmino *et al.*, 2006).

Both PCR and isolation plating may also have been affected by a toxic effect of the medium. It has been shown that both PVF-1 and OKA are toxic to some *P. savastanoi* strains, inhibiting their growth (Surico and Lavermicocca, 1989; Surico and Marchi, 2003). Azad and Cooksey (1995) reported that the recovery of *P. savastanoi* on OKA and PVF-1 varied greatly, depending on the strain, ranging from 5.7 to 93.6% on OKA, and from 11.3 to 83.3%, on PVF-1.

The assay reported here also detected other knot-inducing pathovars of *P. savastanoi* from other hosts, although proper trials are required to optimise the DNA extraction protocol for each of the hosts so as to quantify the pathovars *in planta*. The main advantages of real-time PCR are its speed (40 cycles in thirty minutes), its not requiring any post-PCR handling, and its ability to quantify *P. savastanoi* pv. *nerii* within oleander tissues. The real-time PCR assay quantified the bacterium over a seven log linear range from 10^8 to 10^2 cfu ml^{-1}. Different concentrations of the bacterium were detected both in the knots and in the stems adjacent to the knots. The lowest concentration detected in field samples without enrichment was approximately 100 cfu ml^{-1}; this was close to the detection limit with spiked samples and water cell suspensions.

Since the virulence of *P. savastanoi* pv. *nerii* strains is variable (Bella *et al.*, 2001), and since cultivars vary in their resistance to the bacterium (Bella *et al.*, 2006), real-time PCR could be a valid support to estimate the growth and spread of the pathogen.

Literature cited

Azad H.R. and D.A. Cooksey, 1995. A selective medium for detecting epiphytic and systemic populations of *Pseudomonas savastanoi* from oleander. *Phytopathology* 85, 740–745.

Bella P., 2002. *Caratterizzazione Biochimica, Patogenetica e Genomica di* Pseudomonas savastanoi *pv.* nerii. PhD Thesis, University of Catania, Catania, Italy, pp 127.

Bella P., V. Catara, C. Guarino and G. Cirvilleri, 2006. Evaluation of oleander cultivars for resistance to *Pseudomonas savastanoi* pv. *nerii*. *Journal of Plant Pathology* 88, 273–278.

Bella P., V. Catara, R. Di Benedetto, A. Bertuccio and G. Cirvilleri, 2001. Variability among isolates of *Pseudomonas savastanoi* pv. *nerii* from oleander plants. In: *Proceedings of the 5th Congress of the European Foundation for Plant Pathology*, 18–22 September, 2000, *Taormina-Giardini Naxos*, Italy, 184–187.

Bertolini E., R. Penyalver, A. Garcia, A. Olmos, J.M. Quesada, M. Cambra and M.M. Lòpez, 2003. Highly sensitive detection of *Pseudomonas savastanoi* pv. *savastanoi* in asymptomatic olive plants by nested-PCR in a single closet tube. *Journal of Microbiological Methods* 52, 261–266.

Casano F.J., S.Y. Hung and J.M. Wells, 1987. Differentiation of some pathovars of *Pseudomonas syringae* with monoclonal antibodies. *European and Mediterranean Plant Protection Organization Bulletin* 17, 173–176.

Cimmino A., A. Andolfi, G. Marchi, G. Surico and A. Evidente, 2006. Phytohormone production by strains of *Pantoea agglomerans* from knots on olive plants caused by *Pseudomonas savastanoi* pv. *savastanoi*. *Phytopathologia Mediterranea* 45, 247–252.

Gardan L., C. David, M. Morel, E. Glickmann, M. Abu-Ghorrah, A. Petit and Y. Dessaux, 1992. Evidence for a correlation between auxin production and host plant species among strains of *Pseudomonas syringae* subsp. *savastanoi*. *Applied and Environmental Microbiology* 58, 1780–1783.

Gardan L., H. Shafik, S. Bedouin, R. Broch, F. Grimont and P.A. Grimont, 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *International Journal of Systematic Bacteriology* 49, 469–78.

Glass N.L. and T. Kosuge, 1986. Cloning of the gene for indoleacetic acid-lysine synthetase from *Pseudomonas syringae* subsp. *savastanoi*. *Journal of Bacteriology* 166, 598–603.

Glass N.L. and T. Kosuge, 1988. Role of indoleacetic acid-lysine synthetase in regulation of indoleacetic acid pool size and virulence of *Pseudomonas syringae* subsp. *savastanoi*. *Journal of Bacteriology* 170, 2367–2373.

Glickmann E., L. Gardan, S. Jacquet, S. Hussain, M. Elasri, A. Petit and Y. Dessaux, 1998. Auxin production is a common feature of most pathovars of *Pseudomonas syringae*. *Molecular Plant-Microbe Interactions* 11, 156–162.

Janse J.D., 1991. Pathovar discrimination within *Pseudomonas syringae* subsp. *savastanoi* using whole-cell fatty acids and pathogenicity as criteria. *Systematic and Applied Microbiology* 13, 79–84.

Licciardello G., F.M. Grasso, P. Bella, G. Cirvilleri, V. Grimaldi and V. Catara, 2006. Identification and detection of *Phoma tracheiphila,* causal agent of citrus Mal secco disease, by real-time polymerase chain reaction. *Plant Disease* 90, 1523–1530.

Marchi G., A. Sisto, A. Cimmino, A. Andolfi, M.G. Cipriani, A. Evidente and G. Surico, 2006. Interaction between *Pseudomonas savastanoi* pv. *savastanoi* and *Pantoea agglomerans* in olive knots. *Plant Pathology* 55, 614–624.

Mumford R., N. Boonham, J. Tomlinson and I. Barker, 2006. Advances in molecular phytodiagnostics – new solutions for old problems. *European Journal of Plant Pathology* 116, 1–19.

Penyalver R., A. Garcia, A. Ferrer, E. Bertolini and M.M. Lopez, 2000. Detection of *Pseudomonas savastanoi* pv. *savastanoi* in olive plants by enrichment and PCR. *Applied and Environmental Microbiology* 66, 2673–2677.

Sambrook J., E.F. Fritsch and T. Maniatis, 1989. *Molecular Cloning: a Laboratory Manual*. 2nd edition, Cold Spring Harbor Laboratory press, Cold Spring Harbor, NY, USA.

Schaad N.W. and R.D. Frederick, 2002. Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology* 24, 250–258.

Schaad N.W., R. D. Frederick, J. Shaw, W.L. Schneider, R. Hickson, M.D. Petrillo and D.G. Luster, 2003. Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annual Review of Phytopathology* 41, 305–324.

Schena L., F. Nigro, A. Ippolito and D. Gallitelli, 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology* 110, 893–908.

Sisto A., M.G. Cipriani and M. Morea, 2004. Knot formation caused by *Pseudomonas syringae* subsp. *savastanoi* on olive plants is *hrp*-dependent. *Phytopathology* 94, 484–489.

Smith C.O., 1928. Oleander bacteriosis in California. *Phytopathology* 18, 503–518.

Surico G. and N.S. Iacobellis, 1992. Phytohormones and olive knot disease. In: *Molecular Signals in Plant-Microbe Communications* (D.P.S. Verma, ed.), CRC Press, Boca Raton, FL, USA, 209–227.

Surico G. and P. Lavermicocca, 1989. A semiselective medium for the isolation of *Pseudomonas syringae* pv. s*avastanoi*. *Phytopathology* 79, 185–190.

Surico G. and G. Marchi, 2003. Olive Knot Disease: new insights into ecology, physiology and epidemiology of *Pseudomonas savastanoi* pv. *savastanoi*. In: Pseudomonas syringae *and related pathogens. Biology and Genetic*. (N.S. Iacobellis, A. Collmer, S.W. Hutcheson, J.W. Mansfield, C.E. Morris, J. Murillo, N.W. Schaad, D.E. Stead, G. Surico, M.S. Ullrich, ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 17–28.

Varvaro L., 1983. Un substrato selettivo per *Pseudomonas syringae* pv. s*avastanoi*. *Phytopathologia Mediterranea* 22, 78–79.

Young J.M. and C.M. Triggs, 1994. Evaluation of determinative tests for pathovars of *Pseudomonas syringae* van Hall 1902. *Journal of Applied Bacteriology* 77, 195–207.

Wilson E.E. and A.R. Magie, 1964. Systemic invasion of the host plant by the tumor-inducing bacterium, *Pseudomonas savastanoi*. *Phytopathology* 54, 576–579.

Accepted for publication: September 23, 2008