

Detection of *Pseudomonas savastanoi* pv. *savastanoi* in Olive Plants by Enrichment and PCR

RAMÓN PENYALVER, AMPARO GARCÍA, AMPARO FERRER, EDSON BERTOLINI,
AND MARÍA M. LÓPEZ*

Departamento de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias,
46113 Moncada, Valencia, Spain

Received 30 November 1999/Accepted 21 March 2000

The sequence of the gene *iaaL* of *Pseudomonas savastanoi* EW2009 was used to design primers for PCR amplification. The *iaaL*-derived primers directed the amplification of a 454-bp fragment from genomic DNA isolated from 70 strains of *P. savastanoi*, whereas genomic DNA from 93 non-*P. savastanoi* isolates did not yield this amplified product. A previous bacterial enrichment in the semiselective liquid medium PVF-1 improved the PCR sensitivity level, allowing detection of 10 to 100 CFU/ml of plant extract. *P. savastanoi* was detected by the developed enrichment-PCR method in knots from different varieties of inoculated and naturally infected olive trees. Moreover, *P. savastanoi* was detected in symptomless stem tissues from naturally infected olive plants. This enrichment-PCR method is more sensitive and less cumbersome than the conventional isolation methods for detection of *P. savastanoi*.

Pseudomonas savastanoi and its pathovars *savastanoi*, *fraxini*, and *nerii* incite a disease of olive (*Olea europaea* L.), ash (*Fraxinus excelsior* L.), other *Oleaceae* plants and oleander (*Nerium oleander* L.) that is characterized by tumorous outgrowths (4, 19). This development of knots is dependent on bacterial production of the phytohormone indoleacetic acid (IAA) and cytokinins (3, 7, 16, 18). *P. savastanoi* can conjugate IAA with lysine to form 3-indoleacetyl- ϵ -L-lysine (IAA-lysine) (6). The two enzymes involved in IAA biosynthesis are tryptophan monooxygenase, which converts tryptophan to indoleacetamide, and indoleacetamide hydrolase, which catalyzes the conversion of indoleacetamide to IAA (12). The enzyme involved in the conversion of IAA to IAA-lysine is (indole-3-acetyl)-L-lysine synthetase (5). The genes for tryptophan monooxygenase (*iaaM*), indoleacetamide hydrolase (*iaaH*), and IAA-lysine synthetase (*iaaL*) reside on the 52-kb plasmid pIAA1 in the oleander *P. savastanoi* strain EW2009, and they have been sequenced (3, 5, 14, 20). Sequence analysis revealed that *iaaM* and *iaaH* have significant similarity with homologous genes of other plant-associated bacteria (13, 20). In contrast, to date, no nucleotide homologies have been found with the *iaaL* gene.

Detection of *P. savastanoi* is currently based on bacterial isolation followed by pathogenicity tests and biochemical or serological techniques (2, 8, 17, 21). These conventional methods are time-consuming and expensive, requiring bacterial isolation. We used the published sequence of *iaaL* (14) to design specific primers for amplification of this gene. We report here the development of a new sensitive and specific detection method for *P. savastanoi* based on amplification of *iaaL* after a bacterial enrichment. The developed enrichment-PCR assay can be applied to specifically detect low levels of *P. savastanoi* in inoculated and naturally infected plants.

Specificity of the PCR assay. Seventy strains of *P. savastanoi* isolated from olive, oleander, ash, and jasmine (*Jasminum officinalis* L.) plants from different countries, 23 outgroup strains,

and 70 saprophytic isolates from olive plants were used to test for the specificity of the primers designed (Table 1). All strains were routinely grown in King's medium B (9). The bacterial DNA preparation method was based on the protocol described by Llop et al. (10). The primers designed for *iaaL* amplification were as follows: primer IAALF, 5'-GGCACCAGCGGCAAC ATCAA-3'; primer IAALR, 5'-CGCCCTCGGAACTGCCAT AC-3'. PCR reactions were performed by combining the following reagents in a reaction mix: 10 \times *Taq* buffer (GIBCO-BRL), 1.5 mM MgCl₂, 5% formamide, 0.2 mM concentrations of each deoxynucleoside triphosphate (Pharmacia LKB), 0.6 μ M concentrations of each primer, and 1.5 U of *Taq* DNA polymerase (GIBCO-BRL) per reaction. Then, 5 μ l of the DNA extraction from bacterial cultures (or plant samples) was added. Samples were amplified through 1 cycle of 94°C (5 min), followed by 35 cycles of 94°C (30 s), 62°C (30 s), and 72°C (30 s) and then 1 cycle of 72°C for 5 min in a 9600 Perkin-Elmer thermocycler. Negative controls with uninfected olive plants were included in every DNA extraction series. Furthermore, to help detect carryover contamination, duplicate samples and two sets of two negative controls each with sterile purified water were routinely included in every reaction. One set was loaded just after reaction mix preparation, and the other one was added just after loading the samples. Amplification was also conducted in a separate laboratory. Amplified products (5 μ l) were separated by electrophoresis (100 V) on a 1.5% agarose gel or digested with the restriction enzyme *Hae*III (GIBCO-BRL) at 37°C for 2 h and separated by electrophoresis (100 V) on a 2% agarose gel. Electrophoresis gels were stained with ethidium bromide.

A 454-bp product was obtained from all *P. savastanoi* strains, showing that *iaaL* sequences were present in strains from different countries and isolated from four hosts (Table 1). Two fragments of 279 and 175 bp were generated from all amplified fragments after *Hae*III digestion, a result in agreement with previously published sequence data (14). Genomic DNA from outgroup species did not produce any discrete bands upon amplification. Similarly, we found no amplification of the 454-bp fragment with genomic DNA from isolates of olive microbiota (Table 1). Only the DNA from one saprophytic isolate identified by biochemical tests as *Pseudomonas putida*

* Corresponding author. Mailing address: Departamento de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias, Apartado Oficial, 46113 Moncada, Valencia, Spain. Phone: 34-96-1391000. Fax: 34-96-1390240. E-mail: mlopez@master.ivia.es.

TABLE 1. Bacterial strains used

Strain(s) ^a (no. of isolates)	Origin	Host	Amplification with <i>iaaL</i> primers	Source
<i>Pseudomonas savastanoi</i>				
1613-1, 1624-a, 1613-4, 1624-b, 1628, 1629, 1637-b, 1637-c, 1649, 1651-a, 1651-b, 1651-c, 1657-2, 1657-8, 1663, 1664-1, 1664-6, 1668, 1669, 1670, 78/55, 1862-3	Spain	Olive	+	This study
CFBP 1670, NCPPB 1479	Yugoslavia	Olive	+	
NCPPB 2780, NCPPB 3335, CFBP 2073	France	Olive	+	
NCPPB 2327, NCPPB 1506	Italy	Olive	+	
PBa206, PBa223, PBa221, PBa230, PBa225, PVFi1, PVFi2, ITM101, ITM317	Italy	Olive	+	N. Iacobellis
NCPPB 1342, NCPPB 1344	United States	Olive	+	
ITM301, ITM302	United States	Olive	+	N. Iacobellis
CFBP 2074	Algeria	Olive	+	
CFBP 71	Tunisia	Olive	+	
NCPPB 64	Portugal	Olive	+	
BPIC 264, BPIC346	Greece	Olive	+	P. G. Psallidas
1610, 1611, 910-1, 910-4, 1889-2, 1889-3, 1889-4, 1889-5, 1889-6, 1889-7	Spain	Oleander	+	This study
ITM519, ITM601, PBa204		Oleander	+	N. Iacobellis
NCPPB 640	Yugoslavia	Oleander	+	
CFBP 2088	Algeria	Oleander	+	
BPIC 344, BPIC 345	Greece	Oleander	+	P. G. Psallidas
NCPPB 1464	United Kingdom	Ash	+	
F ₂ , F ₃		Ash	+	N. Iacobellis
NCPPB 3474	France	Ash	+	
BPIC 464, BPIC 465	Greece	Jasmine	+	P. G. Psallidas
Outgroup species ^b (23)			—	IVIA ^c
Microbiota from olive plants				
Fluorescent pseudomonads (9)	Spain	Olive	—	This study
<i>Pseudomonas putida</i> (1)	Spain	Olive	+ ^d	This study
<i>Pantoea agglomerans</i> (10)	Spain	Olive	—	This study
Unidentified isolates (52)	Spain	Olive	—	This study

^a CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, York, England; BPIC, Benaki Phytopathological Institute Collections, Athens, Greece.

^b *Agrobacterium tumefaciens* (1 strain); *Clavibacter michiganensis* subsp. *michiganensis* (1 strain); *Erwinia amylovora* (1 strain); *Pectobacterium carotovorum* (2 strains); *Pectobacterium chrysanthemi* (1 strain); *Pseudomonas chlororaphis* (1 strain); *Pseudomonas marginalis* (1 strain); *Pseudomonas syringae* pv. *syringae* (12 strains); *Rhodococcus facians* (1 strain) *Serratia fonticola* (1 strain); *Xanthomonas hortorum* pv. *pelargonii* (1 strain).

^c IVIA, bacterial collection from the Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain.

^d DNA from this isolate yielded a ca. 440-bp fragment. After *Hae*III digestion, the amplified DNA yielded 310- and 130-bp fragments that were perfectly distinguishable from those of the *iaaL* gene of *P. savastanoi*.

gave a smaller product of ca. 440 bp upon amplification. However, it was perfectly distinguishable from that of *P. savastanoi* because after *Hae*III digestion we did not find the corresponding pattern of *P. savastanoi iaaL* (Table 1). Formamide (5%) was required in the amplification cocktail to eliminate nonspecific bands.

Detection of *P. savastanoi* in plants. Olive plant samples (1 g of stem tissue from uninfected greenhouse-grown plants comminuted in 50 ml of sterile distilled water) were subsequently amended with known amounts of *P. savastanoi* 1628 from ca. 1 to 10⁷ CFU/ml of the plant extract. Then, 0.5 ml of amended plant samples from each bacterial concentration was amplified after performing the DNA extraction described above or was added to 5 ml of the nonselective King's medium B (9) and the semiselective PVF-1 medium (17) for bacterial enrichments. Samples were incubated for 3 days at 26°C, and bacterial DNA extracts using 500 µl of each sample were then subjected to PCR amplification as described above. Three replicates of the experiment were performed. The detection limit was 10² to 10³ CFU/ml of plant extract (Fig. 1A). A previous bacterial enrichment using King's medium B slightly improved the detection level, allowing detection of ca. 10² CFU/ml of plant ex-

tract. However, preenrichment in PVF-1 improved the detection level, allowing detection of ca. 10 to 100 CFU/ml of plant extract (Fig. 1B).

We then evaluated the detection of *P. savastanoi* in knots from inoculated and naturally infected plants. The stems of 1-year-old olive plants were wounded and subsequently inoculated with 10 µl of a bacterial suspension (ca. 10⁹ CFU/ml) of strain 1628 to develop knots. They were analyzed 8 months after inoculation. In addition, knots from olive, oleander, and Spanish broom (*Retama sphaerocarpa* [Boiss] L.) from naturally infected plants were also collected. From inoculated and naturally infected samples, 500 µl of comminuted knot tissues in sterile water (1:50 [wt/vol]) were subjected to the protocol described above for amended plant samples, and subsequently the amplified product was digested with *Hae*III. Conventional bacterial isolations were performed from each sample before or after bacterial enrichments by plating serial dilutions on King's B (9) and PVF-1 (17) media. The identity of the putative *P. savastanoi* colonies was confirmed by biochemical tests (21) and by the developed PCR assay. In all of the knot samples analyzed from inoculated plants, we found the expected PCR product of 454 bp and also the corresponding restriction

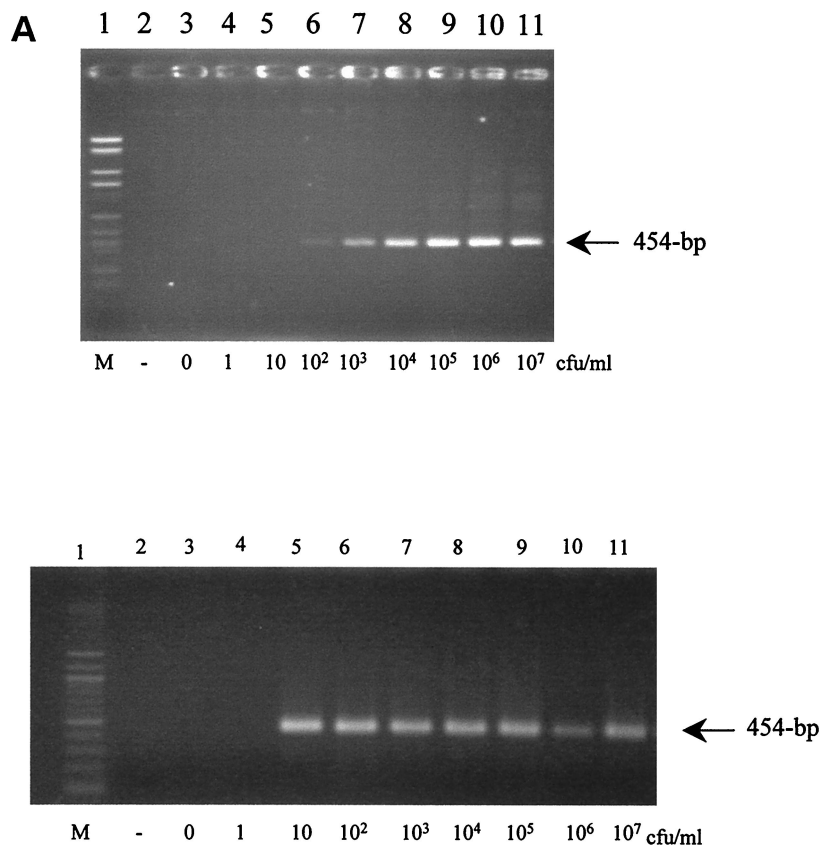


FIG. 1. Sensitivity of the PCR assay in detecting the *iaaL* gene of *P. savastanoi* pv. *savastanoi* in amended plant samples without enrichment (A) or with PVF-1 enrichment (B). PCR products from amended plant samples were inoculated with *P. savastanoi* 1628. Lanes 1, molecular marker (M); lanes 2, PCR-negative control; lanes 3, noninoculated plant material; lanes 4 to 11, PCR-amplified products obtained from amended plant samples inoculated with increased concentrations of bacteria from 1 to 10⁷ CFU/ml of plant extract.

profile (Table 2). We were also able to isolate the inoculated *P. savastanoi* strain (Table 2). We compared the detection efficiency of the developed PCR assay with that of the isolation method in knot samples from naturally infected plants, either with a previous bacterial enrichment step or not. When analyzing 37 knot samples, *P. savastanoi* was detected by PCR in

21 of the 37 samples, without preenrichment (Table 2). One of these amplified products was double strand sequenced, and the obtained sequence was 100% similar to the previous *iaaL* reported sequence. A preenrichment in King's medium B did not improve the detection of *P. savastanoi* by PCR in knot tissues from naturally infected plants because *P. savastanoi* was de-

TABLE 2. Detection of *P. savastanoi* in knots from inoculated and naturally infected plants

Plant group	Host	No. of knot samples analyzed	<i>P. savastanoi</i> detection (no. of positive samples) ^a :					
			Without preenrichment		With preenrichment			
			Bacterial isolation	PCR and restriction analysis	King's B		PVF-1	
				Bacterial isolation	PCR and restriction analysis	Bacterial isolation	PCR and restriction analysis	
Inoculated plants (10 ⁹ CFU/ml)	Olive	15	15	15	ND	ND	ND	ND
Naturally infected plants	Olive	26	5	16 ^b	3	10	3	19
	Oleander	3	0	1	0	0	0	2
	Broom	8	3	4	0	3	0	8
Total no. of analyzed samples ^c		37	8	21	3	13	3	29

^a That is, the number of samples in which *P. savastanoi* was detected. ND, not determined. Bacterial isolations were performed by plating serial dilutions on King's B and PVF-1 media, and after bacterial purifications, isolates were identified as described in Materials and Methods. PCR amplification was done by using the primer pair IAALF-IAALR, and subsequently, the amplified product was digested with the restriction enzyme *Hae*III as described in Materials and Methods.

^b One of these amplified fragments was double strand sequenced, thus verifying *iaaL* detection.

^c Analyzed samples from naturally infected plants.

TABLE 3. Detection of *P. savastanoi* pv. *savastanoi* in asymptomatic stem tissues from infected olive plants

Variety	No. of samples analyzed	<i>P. savastanoi</i> pv. <i>savastanoi</i> detection (no. of positive samples) ^a					
		Without preenrichment		With preenrichment			
		Bacterial isolation	PCR and restriction analysis	King's B		PVF-1	
Bacterial isolation	PCR and restriction analysis			Bacterial isolation	PCR and restriction analysis		
Blanqueta	6	1	0	0	1	0	3
Gordal	3	2	0	0	3	1	2
Manzanilla	6	2	0	3	4	0	5
Picual	20	5	4 ^b	4	6	4	10
Picudo	3	0	0	1	2	1	3
Total no. of samples	38	10	4	8	16	6	23

^a That is, the number of samples in which *P. savastanoi* pv. *savastanoi* was detected. Bacterial isolations were performed by plating serial dilutions on King's B and PVF-1 media, and after bacterial purifications, isolates were identified as described in Materials and Methods. PCR amplification was done by using the primer pair IAALF-IAALR, and subsequently, the amplified product was digested with the restriction enzyme *Hae*III as described in Materials and Methods.

^b One of these amplified fragments was double strand sequenced, thus verifying *iaaL* detection.

tected in only 13 samples. However, a preenrichment in PVF-1 medium improved the detection of *P. savastanoi* by PCR to up to 29 of the 37 samples (Table 2). *P. savastanoi* was isolated by plating in some samples without enrichment (eight samples); however, the detection efficiency of bacterial isolation was not improved by any preenrichment (Table 2).

To evaluate the detection of *P. savastanoi* in asymptomatic olive tissues, stem samples were collected from branches without knots taken from naturally infected plants. A total of 1 to 10 g of stem tissues was processed, and 0.5 ml of the obtained samples was analyzed as described above. Uninfected greenhouse-grown plants were used as negative controls. When analyzing 38 asymptomatic samples, *P. savastanoi* was detected by the developed PCR and subsequent restriction analysis in only 4 of the 38 samples (Table 3). One of these amplified fragments was double strand sequenced showing that it corresponded to the *iaaL* gene. A preenrichment using either King's B (9) or PVF-1 (17) medium greatly improved the detection of *P. savastanoi* by PCR in symptomless stem samples (Table 3). The highest detection level was found with the enrichment-PCR method using PVF-1, which allowed the detection of *P. savastanoi* in 23 of the 38 samples. *P. savastanoi* was isolated by plating in 10 samples without preenrichment; however, the detection level obtained by plating was not improved by any preenrichment in these asymptomatic samples (Table 3).

In this study an enrichment-PCR assay was developed for the detection of *P. savastanoi* in olive plants, and its efficiency was compared by isolation plating. We evaluated the specificity of the PCR assay by testing for amplification of the 454-bp DNA in a collection of *P. savastanoi* strains and other bacteria. Genomic DNA from all of the *P. savastanoi* strains tested was amplified with the *iaaL* primers, and subsequent restriction analysis of the amplified product yielded only the expected fragmentation pattern. It shows that this gene is present among the isolates of *P. savastanoi* from olive, oleander, ash, and jasmine hosts. Genomic DNA from all non-*P. savastanoi* strains tested, including outgroup species and different bacteria associated with olive plants, failed to produce the 454-bp PCR product upon amplification under the conditions described above.

A bacterial enrichment prior to serological and molecular techniques has been reported to improve the sensitivity of detection of other plant pathogenic bacteria (11, 15). For *P. savastanoi* detection in amended plant samples, knots, and symptomless stem tissues from naturally infected plants, a bac-

terial enrichment prior to PCR analysis in PVF-1 liquid medium (but not in King's medium B) greatly improved the detection level. This shows the higher efficiency of selective enrichment versus bacterial enrichment in common medium. With the developed enrichment-PCR assay it is possible to detect living cells of *P. savastanoi* at low population levels in plant material, thus avoiding the need to obtain bacterial cultures as required for the conventional detection techniques.

In knot samples from inoculated plants the developed PCR assay was as efficient as plating isolation because we recovered the inoculated *P. savastanoi* strain in all of the samples that were also positive by PCR. These samples came from fairly young knots because the analyses were done 8 months after the inoculations, when the knot tissues retain a high population of viable cells as shown by the isolation studies. In contrast, in knot samples from naturally infected plants the developed PCR assay was more efficient than bacterial isolation in detecting *P. savastanoi*. This finding could be due to lower populations of viable bacteria in old cracked knots. For bacterial isolations in these samples, an enrichment step, even using the semiselective PVF-1 medium, decreased the plating efficiency due to the growth of bacteria other than *P. savastanoi* in the plates. This finding suggests that the enrichment allows the multiplication of the *P. savastanoi* living cells; however, the rapid development of the colonies of the microbiota from the olive tissues on the isolation plates (mainly *Pantoea agglomerans* and fluorescent pseudomonads) interfered with the growth of *P. savastanoi* colonies but not with their detection in the enriched culture by PCR. Other authors (1, 17) suggested that fairly young olive knots should be used for the isolation of *P. savastanoi* because the presence of a number of other bacteria and low viable populations of the pathogen in the diseased tissues from old, cracked knots often made isolations very difficult. Our work agrees with these findings and extends it to oleander and broom knots.

The semiselective medium PVF-1 was more effective than King's medium B for *P. savastanoi* enrichment and subsequent PCR detection, probably due to the relative low growth rate of *P. savastanoi* compared to that of some other saprophytic bacteria present in plant samples. This finding also suggests that the enrichment effect was not primarily due to the dilution effect of the potential PCR inhibitors present in plant material, as previously suggested (15).

With the developed enrichment-PCR method we detected *P. savastanoi* in many samples in which we were not able to

isolate the bacterium. We were then concerned about the possibility of false-positives with the PCR technique. To help detect cross-contamination among samples and PCR reagents, multiple controls were used in all steps of the protocol as described in Materials and Methods. Negative controls from every experiment were consistently negative. On the other hand, the designed primers were highly specific, and confirmation that the PCR product is from amplification of the target DNA was always made by restriction analysis and by DNA sequencing in two samples. Besides the conventional isolation methods, no other serological or molecular techniques are available for further confirmation of *P. savastanoi* detection directly in plant samples.

In conclusion, this study indicates that PCR amplification of *P. savastanoi* with the *iaaL*-derived primers and the developed preenrichment assay is a sensitive and specific method for the detection of this phytopathogenic bacterium in plant material. This enrichment-PCR method has advantages over all presently used detection methods for *P. savastanoi* in time, sensitivity, and cost. This assay may be useful for the early detection of low levels of *P. savastanoi* in olive plants during plant material propagation for certification purposes or epidemiological studies.

We thank P. G. Psallidas and N. Iacobellis for kindly providing bacterial strains and J. Piquer for technical support with plant management. We are grateful to A. Olmos for technical advice.

R. Penyalver is a recipient of a contract from the Spanish MEC (Programa de Incorporación de Doctores a Grupos de Investigación en España). This work was supported by grant OLI96-2179 from the CICYT of Spain.

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