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EXPERIMENTAL ARTICLES

Detection of *Erwinia amylovora* by Novel Chromosomal Polymerase Chain Reaction Primers

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Abstract—A new sensitive and specific method for the detection of *Erwinia amylovora* was developed. The method is based on the detection of a chromosomal DNA sequence specific for this bacterial species and enables detection of *E. amylovora* pathogenic strains, including recent isolates that lack plasmid pEA29 and thus cannot be detected by the previously popular PCR methods based on the detection of this plasmid. A species-specific random amplified polymorphic DNA (RAPD) marker was identified, cloned, and sequenced, and sequence characterized amplified region (SCAR) primers for specific PCR were developed. The *E. amylovora* specific sequence, 1269 bp long, was amplified in polymerase chain reaction and detected with electrophoresis in agarose gel stained with ethidium bromide. Amplification with other bacterial species did not produce any PCR product detectable by electrophoresis. Matching of the *E. amylovora* specific sequence to chromosomal DNA was confirmed by computer analysis of the *E. amylovora* genome. A consistent sensitivity limit of the method was 3 CFU/reaction, and in some cases it was possible to detect 0.6 CFU/reaction. Due to its high sensitivity and specificity, our method of *E. amylovora* detection is currently the most reliable, taking into account that the reliability of PCR methods based on plasmid pEA29 has been compromised by the isolation of pathogenic *E. amylovora* strains that lack this plasmid.

Key words: PCR, molecular test, bacterium, chromosome, pEA29, *Erwinia amylovora*.

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INTRODUCTION

There are several methods for the detection of the gram-negative bacterium *Erwinia amylovora*. *E. amylovora* can be detected by its growth on semi-selective medium [1, 2]. However, the detection on semi-selective medium takes several days, and saprophytic organisms can cause problems by their excessive growth. Serological tests can also be applied in the detection. Their disadvantage is that they often lack of sensitivity and specificity [3]. Monoclonal antibodies are overly selective with respect to specific strains and cannot recognize all *E. amylovora* strains [4].

Molecular methods have been widely applied for the detection of this bacterium. DNA hybridization tests are time consuming. PCR-based methods are the most attractive because of their high specificity and sensitivity, and they are rapid and reliable. This kind of test can be performed in less then one working day (usually, in less than 5 hours).

Most PCR methods for *E. amylovora* detection are based on the detection of a 29 kb plasmid (pEA29) contained in *E. amylovora* cells in more than one copy, which increases sensitivity of these methods. This advantage of pEA29 based methods was one of the main reasons to neglect the development of chromosome based methods. Because of the fact that virulent strains lacking the plasmid were unknown, it was believed for a long time that methods based on pEA29 are highly reliable [5]. These methods were considered the best for the detection of *E. amylovora* because of their high specificity and sensitivity; however, very recently an indigenous virulent strain lacking plasmid pEA29 was isolated [6]. This strain contains a previously unknown plasmid of about 70 kb, involved in virulence and having no sequence similarity with pEA29. It is obvious that methods based on pEA29 cannot detect strains lacking pEA29, and their application in this case would give false negative results. Thus, reliable detection of *E. amylovora* is not possible with PCR methods based on the detection of pEA29. That problem can be avoided by the use of PCR methods based on chromosomal DNA. There are four chromosome based methods developed so far, but all of them have low sensitivity or specificity (or both) [7–10].

A novel chromosome based method was developed in this research. During development of the test, we identified a species-specific (*E. amylovora*-specific) ran-

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dom amplified polymorphic DNA (RAPD) marker [11, 12] by analyzing RAPD products of *E. amylovora* and some other plant pathogens by agarose gel electrophoresis. A band characteristic for *E. amylovora* was cut and DNA was isolated from the gel. We cloned and sequenced that DNA and designed sequence characterized amplified region (SCAR) primers [13], which we used in PCR to amplify a 1269 bp sequence for the detection of *E. amylovora*. Using these primers and appropriate reaction conditions that we experimentally determined, a large amount of PCR product was obtained at a high number of cycles, enabling high sensitivity of this method. Our method is specific and it can detect *E. amylovora* strains without plasmid pEA29.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. Bacteria were grown in liquid LB medium to the midlogarithmic growth phase (culture density of 1.22×10^8 CFU/ml as determined by plating aliquots of serial tenfold dilutions onto solid LB medium) and pelleted by centrifugation of 1.5 ml of culture for 10 min at 13000 *g*. Supernatant was removed, and the pellet was resuspended in 300 μl of double distilled H_2O. Cells were lysed by heating for 15 min at 99°C in a dry heating block. This lysate (or its dilutions) was used as a DNA source and was added to PCR mixtures in necessary amounts. All bacterial strains used in this work are listed in the table. NCPPB strains are from National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, UK. All other strains in the table were obtained from Department for Environmental and Plant Protection, Faculty of Agriculture, Trg Dositeja Obradovica 8, Novi Sad, 21000 Serbia (except *Pseudomonas fluorescens* strain F1, kindly provided by Jozsef Nemeth, Plant Health and Soil Conservation Station County Baranya, Bacteriological Laboratory, Pecs, Hungary, and *Xanthomonas campestis* pv. *phaseoli* strains XB 90373 and XB 9047, kindly provided by Ivan Kyryakov, Institute for Wheat and Sunflower "Dobrinja" near General Toshevo, Bulgaria).

RAPD, molecular cloning and PCR. RAPD was performed with bacterial sample numbers 1, 2, 12, 14, 21, 29, and 31 (table). The volume of the reaction mixture was 16 μl. The reaction mixture contained: dNTPs (440 μM each); 2.5 μM primer T1 (5'-GCAGGGTTTGAATCCGAGAC-3'); 2.5 mM $MgCl_2$; 2 units of *Taq* DNA polymerase (Fermentas); 1× PCR buffer (75 mM Tris–HCl, pH 8.6; 17 mM $(NH_4)_2SO_4$; 0.1% Tween 20); and DNA (6.4 μl of 100-fold diluted cell lysate, corresponding to about 4×10^4 CFU). The reaction conditions were as follows: initial denaturation for 3 min at 94°C; 4 min at 94°C, 5 min at 36°C, 5 min at 72°C (2 cycles); 20 s at 94°C, 10 s at 61°C, 30 s at 72°C (35 cycles); and the final extension step for 3 min at 72°C. These reaction conditions are valid for thermal cyclers with the tube control mode, where the specified temperatures are maintained inside the test tube according to an appropriate algorithm, but not for thermal cyclers in which the specified temperatures are maintained in the heating block. The tube control mode is employed in Eppendorf thermal cyclers, and as well as in those of many other manufacturers (GeneAmp PCR System 2000, Perkin Elmer; UNOII, Biometra; Omni-E, Hybrid; etc.). The PCR product was analyzed in 1.5% agarose electrophoretic gel stained with 0.5 μg/ml ethidium bromide. A band specific for *E. amylovora* was cut and DNA was isolated from the gel as described in [14] and amplified by hot-start PCR to obtain an amount necessary for molecular cloning. The aim of the hot start was to decrease the amount of nonspecific PCR products.

The reaction mixture for hot-start PCR was divided into two parts (mixture 1 and mixture 2) to prevent premature execution of reaction and attain hot start. The reaction could begin only after mixing of mixtures 1 and 2. Mixture 1 was placed on the bottom of a PCR test tube and covered with a drop of wax (melting point, 45°C) warmed up to 95°C. Mixture 2 was placed on hardened wax and covered with a drop of PCR oil. DNA sample, isolated from the gel, was added to mixture 2 by introducing it under oil with a pipette. The test tube was placed into a thermal cycler preheated to 94°C and execution of the PCR program was started. After warming of the test tube, melted wax moved up into the upper layer with PCR oil (after mixing with the PCR oil, wax was in the liquid state even at room temperature). Simultaneously, mixtures 1 and 2 mixed and PCR started.

The volume of mixture 1 was 10 μl. It contained 10 nmol of each dNTP, 41 pmol of primer T1 and double distilled H_2O to 10 μl. The volume of mixture 2 was 20 μl. It contained 5 μl of 10× PCR buffer (750 mM Tris-HCl, pH 8.6; 170 mM $(NH_4)_2SO_4$; 25 mM $MgCl_2$), 2 units of *Taq* polymerase (Fermentas) and double distilled H_2O to 20 μl. DNA was isolated from the gel, precipitated, redissolved, and added with a pipette to mixture 2 under PCR oil. The total volume of the PCR reaction mixture was 50 μl. After the test tubes were placed into the thermal cycler preheated to 94°C, the following program was carried out: initial denaturation for 3 min at 94°C; 10 s at 94°C, 10 s at 61°C, 30 s at 72°C (35 cycles); and final extension step for 3 min at 72°C (the thermal cycler was operated in the tube control mode).

Molecular cloning was performed by inserting the PCR product into vector pGEM-T. The procedure was carried out according to the manual of the vector manufacturer (Technical Manual: pGEM-T and pGEM-T Easy Vector Systems; Promega, USA).

Sequencing of the cloned fragment was carried out with an automated sequencer (ABI PRISM 3100-Avant) using the ABI PRISM BigDye Terminator version 3.1 Sequencing Reagents Kit according to manufacturer manual of the automated sequencer and manual of the reagents kit (Applied Biosystems, Foster City, USA).

Bacterial samples used in PCR specific for *E. amylovora* (+ and – indicate presence or absence of PCR product) and RAPD (• indicates samples used in RAPD)

No.	Species	Strain	Isolation source	RAPD	PCR
1	*Ralstonia solanacearum*	NCPPB 325	*Lycopersicon esculentum*, USA	•	–
2	*Clavibacter michiganensis* subsp. *sepedonicus*	NCPPB 2137	*Solanum tuberosum*	•	–
3	*Pantoea stewartii* subsp. *stewartii*	NCPPB 2295	USA		–
4	*Xanthomonas fragariae*	NCPPB 2473	*Fragaria vesca*, Italy		–
5	*Xanthomonas arboricola* pv. *pruni*	NCPPB 3156	*Prunus persica*, Italy		–
6	*Xanthomonas hortorum* pv. *pelargonii*	NCPPB 3330	*Pelargonium zonale*, UK		–
7	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	NCPPB 559	*Phaseolus vulgaris*, USA		–
8	*Pseudomonas syringae* pv. *glycinea*	NCPPB 3318	*Glycine max*, Italy		–
9		B 13/2	*Glycine max*, Serbia		–
10		S 5/2	*Glycine max*, Serbia		–
11	*Erwinia carotovora* subsp. *carotovora*	Tam 1	*Capsicum annuum*, Serbia		–
12		Tam 8	*Capsicum annuum*, Serbia	•	–
13		Tam 9	*Capsicum annuum*, Serbia		–
14	*Pseudomonas syringe* pv. *phaseolicola*	Gajd 19	*Phaseolus vulgaris*, Serbia	•	–
15		Gajd 20	*Phaseolus vulgaris*, Serbia		–
16		RS 21	*Phaseolus vulgaris*, Serbia		–
17		RS 22	*Phaseolus vulgaris*, Serbia		–
18	*Xanthomonas campestris* pv. *pelargonii*	X 25	*Pelargonium zonale*, Serbia		–
19		X 48	*Pelargonium zonal*e, Serbia		–
20		X 49	*Pelargonium zonale*, Serbia		–
21	*Xanthomonas campestris* pv. *phaseoli*	XB 90373	Bulgaria	•	–
22		XB 9047	Bulgaria		–
23		Mol 1	*Phaseolus vulgaris*, Serbia		–
24		Mol 2	*Phaseolus vulgaris*, Serbia		–
25		Mol 3	*Phaseolus vulgaris*, Serbia		–
26		Mol 4	*Phaseolus vulgaris*, Serbia		–
27	*Xantomonas campestris* pv. *vesicatoria*	Aleva 5	*Capsicum annuum*, Serbia		–
28		Aleva 7	*Capsicum annuum*, Serbia		–
29	*Pseudomonas fluorescens*	F 1	Hungary	•	–
30	*Escherichia coli* (DH5α)		Lab. colection		–
31	*Erwinia amylovora*	NCPPB 595	*Pyrus communis*, USA	•	+
32		1/1	*Malus domestica*, Serbia		+
33		1/2	*Malus domestica*, Serbia		+
34		2/1	*Malus domestica*, Serbia		+
35		2/2	*Malus domestica*, Serbia		+
36		3/1	*Malus domestica*, Serbia		+
37		3/2	*Malus domestica*, Serbia		+
38		4/1	*Malus domestica*, Serbia		+
39		4/2	*Malus domestica*, Serbia		+
40		5/1	*Malus domestica*, Serbia		+
41		5/2	*Malus domestica*, Serbia		+
42		6/1	*Malus domestica*, Serbia		+
43		6/2	*Malus domestica*, Serbia		+
44		7/1	*Malus domestica*, Serbia		+

Table. Contd.

No.	Species	Strain	Isolation source	RAPD	PCR
45		7/2	*Malus domestica*, Serbia		+
46		Kot 1	*Cotoneaster horisontalis*, Serbia		+
47		Kot 2	*Cotoneaster horisontalis*, Serbia		+
48		Kot 3	*Cotoneaster horisontalis*, Serbia		+
49		Kot 4	*Cotoneaster horisontalis*, Serbia		+
50		Musm 1	*Mespilus germanica*, Serbia		+
51		Musm 2	*Mespilus germanica*, Serbia		+
52		Musm 3	*Mespilus germanica*, Serbia		+
53		Kr.B.O. 1	*Pyrus communis*, Serbia		+
54		Kr.B.O. 2	*Pyrus communis*, Serbia		+
55		Kr.Lipol 1	*Pyrus communis*, Serbia		+
56		Kr.Lipol 2	*Pyrus communis*, Serbia		+
57		Jab.Odz.1	*Malus domestica*, Serbia		+
58		Jab.Odz 2	*Malus domestica*, Serbia		+
59		Jab.Cv.Fak. 5	*Malus domestica*, Serbia		+
60		Jab.Cv.Fak. 6	*Malus domestica*, Serbia		+
61		Jab.Gr.B.O. 1	*Malus domestica*, Serbia		+
62		Jab.Gr.B.O. 2	*Malus domestica*, Serbia		+
63		J-K	*Malus domestica*, Serbia		+
64		But 3/1	*Pyrus communis*, Serbia		+
65		But 3/2	*Pyrus communis*, Serbia		+
66		Kot 2/1	*Cotoneaster horisontalis*, Serbia		+
67		Kot1/1	*Cotoneaster horisontalis*, Serbia		+
68		Kot1/2	*Cotoneaster horisontalis*, Serbia		+
69		Kot1/4	*Cotoneaster horisontalis*, Serbia		+
70		8/1	*Chaenomeles japonica*, Serbia		+
71		8/2	*Chaenomeles japonica*, Serbia		+
72		8/3	*Chaenomeles japonica*, Serbia		+
73		8/4	*Chaenomeles japonica*, Serbia		+
74		L-D	*Cydonia oblonga*, Serbia		+

Primers FER1-F and FER1-R were designed on the basis of the obtained sequence. They were suitable for amplification of a 1269 bp long sequence in an *E. amylovora* specific PCR assay. Hot start PCR was used. The total volume of the reaction mixture (mixture 1, mixture 2 and DNA sample) was 25 µl. The volume of mixture 1 was 5 µl. It contained: 5 nmol of each dNTP; 10 pmol of primer FER1-F (5'-AGCAGCAATTAATGGCAAGTATAGTCA-3'); 10 pmol of primer FER1-R (5'-AATTTAATCAGGTCACCTCTGTTCAAC-3'); and double distilled H_2O to 5 µl. The volume of mixture 2 was 10 µl. It contained: 5 µl of 5× PCR buffer (335 mM Tris–HCl, pH 8.3; 85 mM $(NH_4)_2SO_4$; 7.5 mM $MgCl_2$; 0.5% Tween 20; 0.6 mg/ml BSA; 40% glycerol; 2 mM cresol red); 0.5 µl of *Taq* polymerase (5 units/µl; Fermentas); and double distilled H_2O to 10 µl. Mixture 1 (5 µl) was placed on the bottom of test tube. It was covered with a drop of wax (melting temperature 45°C) warmed to 95°C. Mixture 2 (10 µl) was placed on hardened wax, covered with a drop of PCR oil, and DNA sample (10 µl of an appropriate dilution of cell lysate) was added under oil to mixture 2 with a pipette. Test tubes were placed into a thermal cycler preheated to 94°C. After placing of the test tubes, the following

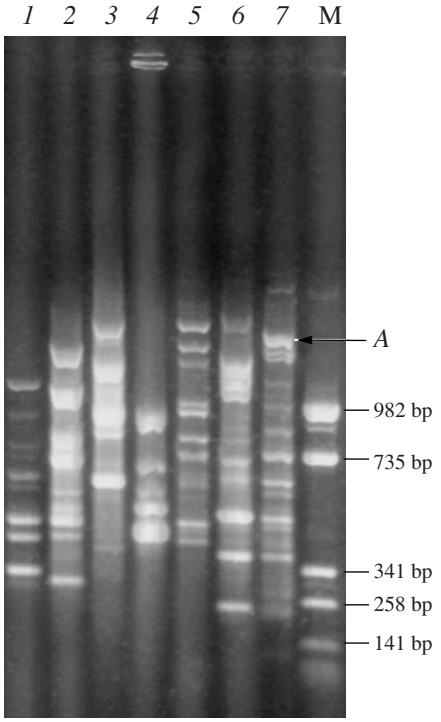

Fig. 1. RAPD products of *E. amylovora* and some other plant pathogens analyzed by agarose gel electrophoresis. The band characteristic for *E. amylovora*, indicated by an arrow and letter A, was cut, and DNA was isolated from the gel. Lanes: (*1*) *Ralstonia solanacearum* (NCPPB 325); (*2*) *Erwinia carotovora* subsp. *carotovora* (Tam 8); (*3*) *Pseudomonas syringae* pv. *phaseolicola* (Gajd 19); (*4*) *Xanthomonas campestris* pv. *phaseoli* (XB90373); (*5*) *Clavibacter michiganensis* subsp. *sepedonicus* (NCPPB 2137); (*6*) *Pseudomonas fluorescens* (F 1); (*7*) *Erwinia amylovora* (NCPPB 595); (M) marker DNA.

PCR program was run: initial denaturation for 3 min at 94°C; 10 s at 94°C, 10 s at 62°C, 30 s at 72°C (41 cycles); and the final extension step for 3 min at 72°C. These conditions are valid for the tube control mode of thermal cycler. PCR product was analyzed by electrophoresis in 1.5% agarose gel stained with 0.5 μg/ml of ethidium bromide. The product was placed into the gel directly from the test tube (there was no need for gel loading buffer).

PCR specificity tests. These tests were performed to check whether the specific 1269-bp PCR product can be obtained in the reaction with *E. amylovora* DNA only and not with DNA of any other species. In specificity tests, dilutions of bacterial DNAs corresponding to 6×10^6 CFU/ml, or 6×10^4 CFU/reaction, were used as potential templates. Specificity tests were performed with all bacterial strains listed in table.

PCR sensitivity tests. Sensitivity of the PCR was assayed by preparing *E. amylovora* strain NCPPB 595 cell lysate serial tenfold dilutions corresponding to CFU concentrations of 6.1×10^7 to 6.1×10^2 CFU/ml and dilutions corresponding to 3×10^2, 1.5×10^2, and 61 CFU/ml. Ten microliters of these DNA dilutions were included in each PCR.

Another sensitivity assay was performed in the presence of plant (apple) extract obtained as described in [15] to test the influence of plant DNA and inhibitors on the reaction sensitivity. The assay was carried out with dilutions obtained by mixing 5 μl of plant extract with 5 μl of cell lysate serial tenfold dilutions corresponding to CFU concentrations of 12.2×10^7 to 12.2×10^2 CFU/ml and dilutions corresponding to 6×10^2, 3×10^2, and 122 CFU/ml (after mixing with the plant extract, the resulting dilutions were the same as in the test without plant extract). Ten microliters of the obtained mixture was added to each PCR.

The sequence amplified in the *E. amylovora* specific PCR was compared with genome sequences of other plant pathogens and some other pathogenic bacteria available online at the internet site of Wellcome Trust Sanger Institute, Cambridge, UK (http://www.sanger.ac.uk/Projects/Microbes) using blast software available at the same site. The sequence was compared to genomes of the plant pathogenic bacteria *Clavibacter michiganensis* subsp. *sepedonicus*, *Erwinia amylovora* (strain Ea273), *Erwinia carotovora* subsp. *atroseptica* (strain SCRI1043, ATCC BAA–672), and *Streptomyces scabies* (strain 87.22), and to genomes of nonplant pathogenic bacteria of the family Enterobacteriaceae (the family to which *E. amylovora* belongs): *Escherichia coli* (strains: 042, E2348/69, H10407, K1), *Shigella dysenteriae* (strain M131649), *Shigella sonnei* (strain: 53G), *Salmonella bongori* (strain 12419, ATCC 43975), *Salmonella enterica* (strain PT4, NCTC 13349), *Salmonella paratyphi* A, *Salmonella typhimurium* (strain DT104, NCTC 13348), *Yersinia pestis* (strain 8081, NCTC 13174), and *Yersinia pestis* (strain C092).

Sequences produced by other PCR-based methods for *E. amylovora* detection [7–10] were compared to the genome of *E. amylovora* strain Ea273.

RESULTS

RAPD, molecular cloning and PCR with *E. amylovora*-targeted primers. RAPD products were analyzed by electrophoresis in agarose gel (Fig. 1). A band specific for *E. amylovora* (pointed out by the arrow and letter A) was cut and isolated from the gel. The size of this DNA band was about 1300 bp. It was cloned in plasmid pGEM-T and sequenced. The primary structure of the DNA was analyzed, and two PCR primers specific for *E. amylovora* were designed (Fig. 2). Both primers, FER1-F and FER1-R, were 27 nucleotides long. Length of sequence to be amplified in PCR with these primers was 1269 bp. The annealing temperature of PCR was determined experimentally (62°C at the tube control mode).

Specificity of the test. The specificity was tested by performing PCR with DNA samples from all bacterial

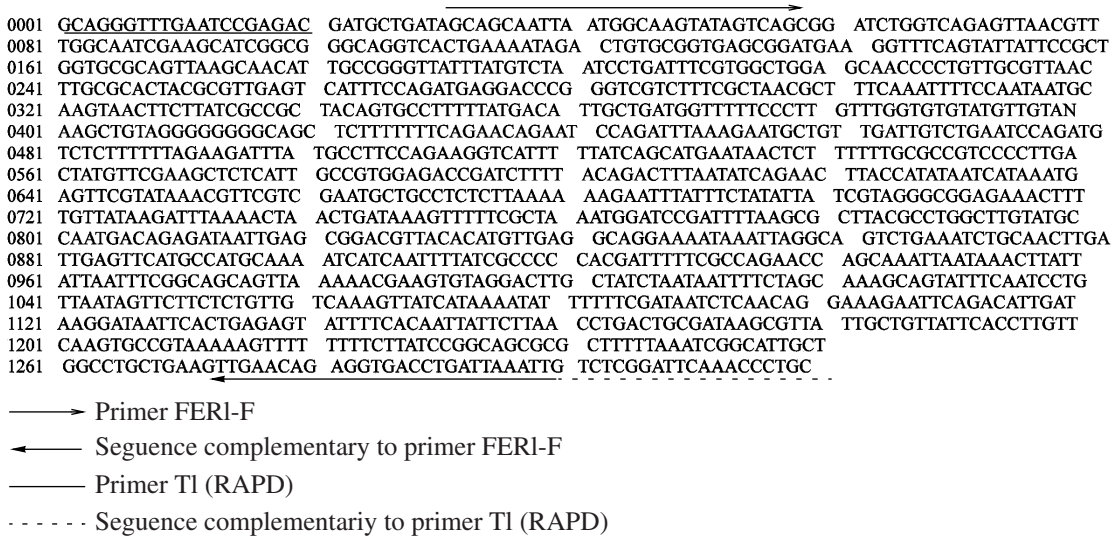

Fig. 2. Nucleotide sequence of the DNA isolated from the RAPD band specific for *E. amylovora*.

strains listed in table. All bacterial species were plant pathogens except *E. coli*, chosen because it belongs to the family Enterobacteriaceae, like *E. amylovora*, and is closely related to it. Negative PCR with the closely related *E. coli* additionally supports the high specificity of the method. Figure 3 shows the electrophoretic gel of PCR products of some species representatives. There was no bacterial species, except *E. amylovora*, that gave any specific or nonspecific PCR product. The *E. amylovora* sample produced the specific 1269 bp product. PCR products of 43 *E. amylovora* isolates were analyzed in agarose electrophoretic gel, and some are shown in Fig. 4. All these samples produced the specific PCR product. Application of PCR with hot start was important. Without hot start, an additional nonspecific band can appear in PCR with *E. amylovora* sample, about 600 bp long, which is easy to differentiate from the specific 1269 bp band (Fig. 5). In spite of that, application of hot start is important, because every nonspecific band can decrease sensitivity of PCR. In hot start PCR of *E. amylovora* samples, there was no nonspecific band even if 46 cycles of PCR were performed, and there were a few after 51 cycles. Even after 51 cycles, the size of nonspecific bands was very different from that of the specific band. Anyhow, 41 cycles were chosen for this assay because this cycle number always gave very stable PCR product, without any nonspecific band.

The sequence amplified in *E. amylovora* PCR was compared in silico with genomes of other plant pathogens and of some non plant pathogen bacteria. No significant sequence similarity was found with the 1269 bp long sequence specific for *E. amylovora*. The nucleotide sequences of primers FER1-F and FER1-R were different enough from the most similar sequences occurring in those genomes; thus, amplification of any sequence is unlikely to occur, especially of a sequence about

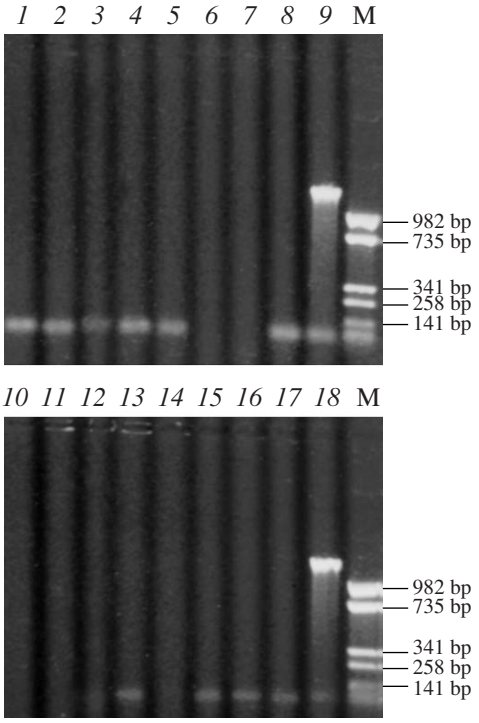

Fig. 3. Specificity of the polymerase chain reaction. Lanes: (*1*) *Pseudomonas syringae* pv. *glycinea* (NCPPB 3318); (*2*) *Pantoea stewartii* subsp. *stewartii* (NCPPB 2295); (*3*) *Xanthomonas campestris* pv. *vesicatoria* (Aleva 5); (*4*) *Xanthomonas campestris* pv. *phaseoli* (Mol 1); (*5*) *Pseudomonas fluorescens* (F1); (*6*) *Pseudomonas syringe* pv. *phaseolicola* (RS 21); (*7*) *Erwinia carotovora* subsp. *carotovora* (Tam 8); (*8*) negative control (H_2O); (*9*) *Erwinia amilovora* (NCPPB 595); (*M*) marker DNA; (*10*) *Escherichia coli (DH5)*; (*11*) *Clavibacter michiganensis* subsp. *sepedonicus* (NCPPB 2137); (*12*) *Ralstonia solanacearum* (NCPPB 325); (*13*) *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (NCPPB 559); (*14*) *Xanthomonas hortorum* pv. *pelagonii* (NCPPB 3330); (*15*) *Xanthomonas arboricola* pv. *prunii* (NCPPB 3156); (*16*) *Xanthomonas fragariae* (NCPPB 2473); (*17*) *Xanthomonas campestris* pv. *pelargonii* (X 25); (*18*) *Erwinia amylovora* (NCPPB 595); (M) marker DNA.

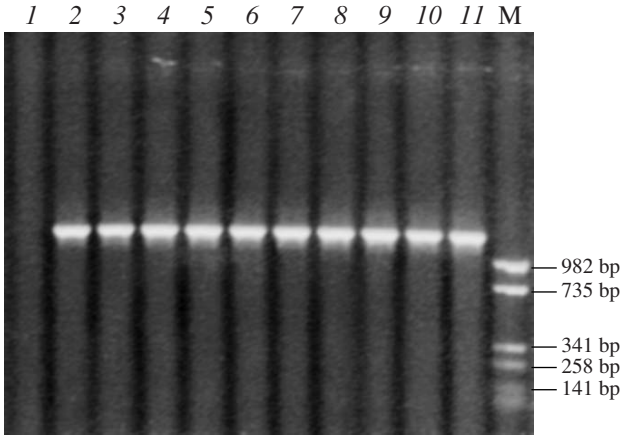

Fig. 4. PCR amplification of various *E. amylovora* isolates with primers FER1-F and FER1-R. Lanes: (*1*) negative control (H_2O); (*2*) Kot 2; (*3*) Kr. Lipol. 1; (*4*) Musm 1; (*5*) J-K; Kr. B. O. 2; (*6*) But 3/1; (*7*) Jab. Odz. 2; (*8*) L-D; (*9*) 1/2; (*10*) 8/3; (*11*) NCPPB 595; (M) marker DNA.

1300 bp long. The 1269 bp PCR sequence specific for *E. amylovora* (derived from strain NCPPB 595) was compared with the genome of *E. amylovora* strain Ea273. We found that the *E. amylovora* specific sequence was present in the genome of strain Ea273 (there were distinctions in only 5 out of 1269 bp), confirming the chromosomal origin of this sequence. The sequence was present in one copy in the genome of *E. amylovora*. The sequences corresponding to primers FER1-F and FER1-R were at the ends of the *E. amylovora* specific sequence present in the genome of Ea273, and their sequence similarity with the primers was 100%.

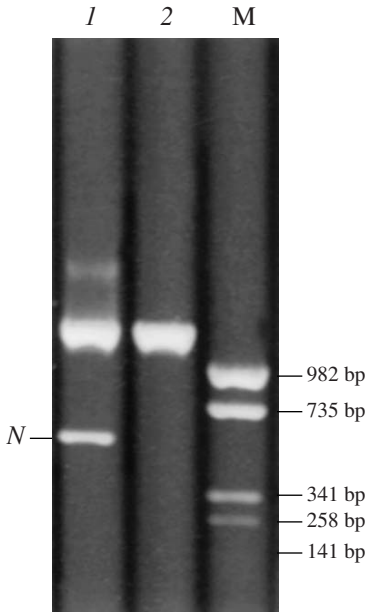

Fig. 5. Application of hot start eliminates a nonspecific band, indicated by letter *N*, in PCR specific for *E. amylovora*. Lanes: (*1*) *E. amylovora* (PCR without hot start); (*2*) *E. amylovora* (PCR with hot start); (M) marker DNA.

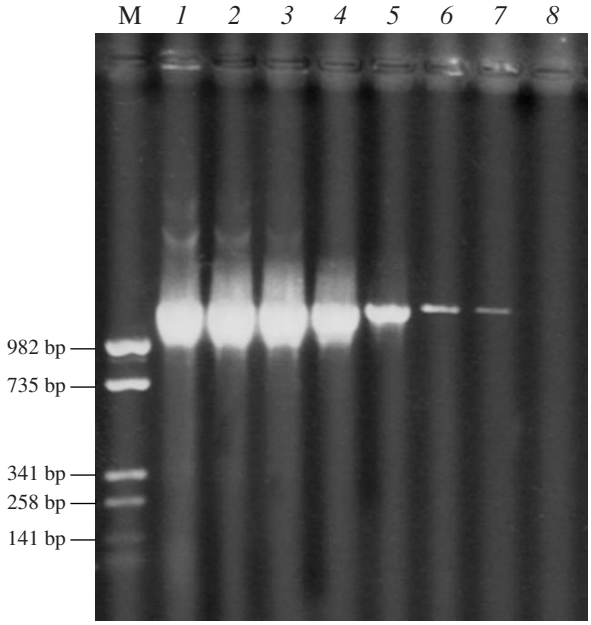

Fig. 6. Sensitivity of *E. amylovora* detection by polymerase chain reaction. All concentrations are expressed as CFU/reaction. Lanes: (M) marker DNA; (*1*) 6.1×10^5; (*2*) 6.1×10^4; (*3*) 6.1×10^3; (*4*) 6.1×10^2; (*5*) 6.1×10; (*6*) 6.1; (*7*) 0.61; (*8*) negative control (H_2O).

Sensitivity of the PCR. Sensitivity was tested in PCRs with aliquots from dilutions of *E. amylovora* cell lysates. The amounts of DNA added corresponded to 6.1×10^5, 6.1×10^4, 6.1×10^3, 6.1×10^2, 6.1, 3, 1.5, and 0.61 CFU/reaction.

Products of these PCRs were analyzed by agarose electrophoresis, and presence of a 1269 bp band was considered to be positive result (Fig. 6). The highest sensitivity achieved was 0.61 CFU/reaction, but the reproducibility of this result was only 50%. At 0.61 CFU/reaction, the 1269 bp band was very weak and was sometimes detectable only by photographing. At 1.5 CFU/reaction, the PCR product was obtained in about 70% of PCRs. The highest sensitivity that was reliably reproducible was 3 CFU/reaction. The PCR sensitivity was also tested in the presence of plant (apple) extract, and it proved to be the same as in PCRs without plant extract.

DISCUSSION

Four chromosome based PCR methods for the detection of *E. amylovora* have been published so far. They all have certain shortcomings.

Maes et al. developed a method based on amplification of 23S ribosomal DNA (rDNA), the sensitivity of which, according to the authors, was 1.5 CFU/reaction

[8]. The authors assumed that the 565 bp sequence is probably present in several copies per genome. We performed in silico analysis of the entire genome of *E. amylovora* strain Ea273 (sequenced by Wellcome Trust Sanger Institute, Cambridge, UK) to determine how many copies of this 565 bp sequence are present and found seven copies. The authors warned that their assay gave false positive results with *E. coli*, which is not a plant pathogenic bacterium. Rosello et al. applied this method and found lack of specificity amplifying an *Erwinia* sp. (different from *E. amylovora*) isolated from necrotic pear blossoms [16]. So, in spite of pretty high sensitivity, this method is not reliable because of lack of specificity.

Guilford et al. and Taylor at al. developed another PCR method based on amplification of a 187 bp specific sequence for the detection of *E. amylovora* [9, 10]. They supposed that the 187 bp sequence is of chromosomal origin. By computer analysis of *E. amylovora* genome, we confirmed that this 187 bp sequence is of chromosomal origin and found that it is present in one copy. The authors claimed that the sensitivity of this method is 10 CFU/reaction, but other authors reported lower sensitivity of this method (only 7×10^3 CFU/ml of analyzed suspension, or 140 CFU/reaction) [5].

Bereswill et al. developed two PCR methods based on amplification of *E. amylovora ams* genes (1.6 kb) and 16S rDNA genes (1.5 kb) [7]. According to the authors, the sensitivity of the method based on *ams* genes was 500 cells per PCR, and in some cases additional digestion with restriction endonucleases was necessary to distinguish the specific PCR product from background bands. The authors did not specify the sensitivity of the method based on 16S rDNA. rDNA of many gram negative bacteria (*Escherichia coli*, *Erwinia herbicola*, *Erwinia carotovor*a, etc.) have high homology to rDNA of *E. amylovora*. Primers used in this method always give false positive results with those bacteria, and an additional digestion with restriction endonucleases was necessary for proper analysis of the PCR product. Our computer analysis of *E. amylovora* genome shows that ams and 16S rDNA sequences are present in one and seven copies, respectively.

A highly sensitive method (0.0007 CFU/reaction or 7×10^{-1} CFU/ml of the analyzed suspension), based on nested PCR of plasmid pEA29, amplifies a sequence that varies in length from 447 to 391 bp depending on the *E. amylovora* strain [5]. This difference in size may confuse the interpretation of results, especially if background bands are present. Such a high sensitivity was achieved because of application of nested PCR with a high number of cycles, while with the standard PCR procedure based on pEA29 it was possible to detect 7×10^1 CFU/ml [5]. Another pEA29 based method for *E. amylovora* detection and quantification by real-time PCR was developed by Salm et al. [17]; its sensitivity is as low as 50 CFU/reaction. It is the least sensitive PCR method so far in spite of the fact that methods based on plasmid pEA29 can have higher sensitivity in comparison with chromosome based methods, because the plasmid is present in more than one copy in the cell. However, Llop et al. isolated an indigenous virulent strain of *E. amylovora* lacking pEA29 [6]. They were unable to detect that strain with PCR methods based on pEA29, because instead of pEA29 the strain had a previously undescribed plasmid of about 70 kb involved in virulence (with no sequence similarity with pEA29). In other studies [18, 19] several others strains that might lack pEA29 were reported. These facts compromise all PCR methods based on pEA29, because they cannot reliably detect *E. amylovora*.

Thus, all of the earlier developed PCR based methods have serious shortcomings. They lack specificity or sensitivity or both. In the present study, novel chromosomal polymerase chain reaction primers for detection of *E. amylovora* were developed. These primers amplify a 1269 bp sequence, which is present in *E. amylovora* genome in one copy. Nevertheless, this method is highly sensitive. One of the reasons for the high sensitivity is high number of cycles (41), and efficient amplification of a long sequence (1269 bp), which gives a large amount of product. The number of cycles is possible because the PCR product is virtually without background bands (no background bands after 46 cycles and a few after 51 cycles). 41 cycles provide a highly sensitive and stable reaction, without background bands. Application of hot start PCR is very important because it helps to eliminate background bands and thus additionally increases sensitivity. Another factor which increases sensitivity is the tube control mode of thermal cycler, which makes it possible to shorten the duration of each step of PCR cycles and reduces heat inactivation of *Taq* DNA polymerase. BSA is included in the PCR buffer because it prevents the effect of PCR inhibitors [20, 21]. Tween 20 stabilizes *Taq* DNA polymerase and may also suppress the formation of DNA secondary structure, thus increasing product yield [22, 23]. Application of buffer with Tween 20 in our research significantly increased PCR product yield. Glycerol was included because it can also increase product yield [22]. Cresol red and glycerol eliminated the need for post-PCR manipulation; the reaction mixture could be loaded onto the gel without mixing with loading buffer. Elimination of post-PCR manipulation saves labor and time and decreases the possibility of cross contamination of samples with PCR products.

The PCR method described in our work is rapid, sensitive, specific, and reliable. It overcomes the shortcomings present in other existing methods, which lack specificity or sensitivity or both.

REFERENCES

1. Miller, T.D. and Schroth, M.N., Monitoring the Epiphytic Population of *Erwinia Amylovora* on Pear with a Se-

lective Medium, *Phytopathology*, 1972, vol. 62, pp. 1175–1182.

2. Ishimaru, C. and Klos, E.J., New Medium for Detecting *Erwinia Amylovora* and Its Use in Epidemiological Studies, *Phytopathology*, 1984, vol. 74, pp. 1342–1345.
3. Roberts, P., Problems Encountered during Immunofluorescence Diagnosis of Fireblight, *Plant pathol*., 1980, vol. 29, pp. 93–97.
4. Lin, C.P., Chen, T.A., Wells, J.M., and van der Zwet, T., Identification and Detection of *Erwinia Amylovora* with Monoclonal Antibodies, *Phytopathology*, 1987, vol. 77, pp. 376–80.
5. Llop, P., Bonaterra, A., Penalver, J., and Lopez, M., Development of a Highly Sensitive Nested-PCR Procedure using a Single Closed Tube for Detection of *Erwinia amylovora* in Asimptomatic Plant Material, *Appl. Environ. Microbiol.,* 2000, vol. 66, pp. 2071–2078.
6. Llop, P., Donat, V., Rodrigez, M., Cabrefiga ,J., Ruz, L., Palomo, J.L., Montesinos, E., and Lopez, M.M., An Indigenous Virulent Strain of *Erwinia amylovora* Lacking the Ubiquitous Plasmid pEA29, *Phytopathology*, 2006, vol. 96, pp. 900–907.
7. Bereswill, S., Bugert, P., Bruchmüller, I., and Geider, K., Identification of the Fire Blight Pathogen, *Erwinia amylovora*, by PCR Assay with Chromosomal DNA, *Appl. Environ. Microbiol*., 1995, vol. 61, pp. 2636–2642.
8. Maes, M., Garbeva, P., and Crepel, C., Identification and Sensitive Endophytic Detection of the Fire Blight Pathogen *Erwinia amylovora* with 23S Ribosomal DNA Sequences and the Polymerase Chain Reaction, *Plant pathol*., 1996, vol. 45, pp. 1139–1149.
9. Guilford, P.J., Taylor, R.K., Clark, R.G., Hale. C.N., and Foster, R.L.S., PCR-Based Techniques for the Detection of *Erwinia amylovora*, *Acta Hortic*., 1996, vol. 411, pp. 53–56.
10. Taylor, R.K., Guilford, P.J., Clark, R.G., Hale, C.N., and Forster, R.L.S., Detection of *Erwinia amylovora* in Plant Material Using Novel Polymerase Chain Reaction (PCR) Primers, *New Zealand J. Crop Horticultural Sci.*, 2001, vol. 29, pp. 35–43.
11. Welsh, J. and McClelland, M., Fingerprinting Genomes using PCR with Arbitrary Primers, *Nucleic Acids Res*., 1990, vol. 18, pp. 7213–7218.
12. Williams, J. G. K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V., DNA Polymorphisms Amplified by Arbitrary Primers are Useful as Genetic Markers. *Nucleic Acids Res*., 1990, vol. 18, pp. 6531–6535.
13. Paran, I. and Michelmore, R.W., Development of Reliable PCR-Based Markers Linked to Downy Mildew Resistance Genes in Lettuce, *Theor. Appl. Genet*., 1993, vol. 85, pp. 985–993.
14. Sambrook. J., Fritsch, E.E., and Maniatis, T., *Molecular cloning: a laboratory manual*, 2nd edition, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.
15. Llop, P., Caruso, P., Cuberto, J., Morente, C., and Lopez, M.M., A Simple Extraction Procedure for Efficient Routine Detection of Pathogenic Bacteria in Plant Material by Polymerase Chain Reaction, *J. Microbiol. Methods*, 1999, vol. 37, pp. 23–31.
16. Rosello, M., Vidal, S.G., Tarin, A., Llop, P., Goris, M.T., Donat, V., Chartier, R., Paulin, J.P., Gardan, L., Penalver., J., and Lopez, M.M., Characterization of an *Erwinia* sp. Isolated from Necrotic Pear Blossoms in Valencia, Spain, *Acta Hortic*., 2002, vol. 590, pp. 139–142.
17. Salm, H. and Geider, K., Real-time PCR for Detection and Quantification of *Erwinia amylovora*, the Causal Agent of Fireblight, *Plant pathol*., 2004, vol. 53, pp. 602–610.
18. Brown, E.W., Janisiewicz, W., and van der Zwet, T., Preliminary Phenotypic and Genetic Differentiation of the Fire Blight Bacterium *Erwinia amylovora*, *Acta Hortic*., 1996, vol. 411, pp. 199–210.
19. Brennan, J.M., Doohan, F.M., Egan, D., Scanlan, H., and Hayes, D., Characterization and Differentiation of Irish *Erwinia amylovora* Isolates, *J. Phytopathol*., 2002, vol. 150, pp. 414–422.
20. Kreader, C.A., Relief of Amplification Inhibition in PCR with Bovine Serum Albumin or T4 Gene 32 Protein. *Appl. Environ, Microbiol*., 1996, vol. 62, pp. 1102–1106.
21. Nagai, M., Yoshida, A., and Sato, N., Additive Effects of Bovine Serum Albumin, Dithiothreitol, and Glycerol on PCR, *Biochem. Mol. Biol. Int*., 1998, vol. 44, pp. 157–163.
22. *PCR Technology: Principles and Applications for DNA Amplification*, Ed. Erlich, H.A. New York: Stockton Press, 1989.
23. *PCR Protocols: A Guide to Methods and Applications*, Eds. Innis, M.A., White, T.J., and Sninsky, J.J. San Diego, California: Academic Press, Inc., 1990.