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Original scientific paper

SPECIFICITY AND SENSITIVITY OF THREE PCR-BASED METHODS FOR DETECTION OF *Erwinia amylovora* IN PURE CULTURE AND PLANT MATERIAL

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Three PCR methods, referred in this study as „conventional“, „nested“ and „chromosomal“ PCR and suggested for routine detection of *Erwinia amylovora* in pure culture and plant material, were evaluated according to their specificity and sensitivity. Specificity of PCR methods was analyzed by using 42 strains of *E. amylovora*, originating from different locations and plant species, with diverse PFGE profiles, representing distant populations of the pathogen. Sensitivity of PCR protocols in pure culture was studied by using nine different concentrations of *E. amylovora* in sterile ultrapure water as a template in PCR reactions. In order to study inhibitory effect of plant DNA and other inhibitors on sensitivity of the three PCR methods bacterial dilutions were mixed with plant macerate of pear, apple and quince prior to the PCR reaction. In specificity assays, tested PCR protocols were able to detect all *E. amylovora* strains regardless of the host of the strain, its origin or PFGE group, indicating primer specificity. On the other hand, sensitivity among tested methods varied, depending on bacterial

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concentration and selected plant material used in the PCR. When working with pure cultures nested PCR showed the greatest sensitivity by detecting 1.9 bacterial cells per PCR reaction, followed by detection limit of 9.5 cells per PCR reaction with conventional PCR and 1.9×10^5 cells/PCR reaction with chromosomal PCR. In spiked samples plant inhibitors either did not affect or they decreased the sensitivity of the PCR reaction, depending on the protocol and/or type of plant macerate. In our experiments, inhibitors from pear and quince macerates did not affect sensitivity of nested PCR, while apple macerate reduced its sensitivity by a factor of 10. Conventional PCR protocol was able to detect 95 cells/PCR reaction in pear and apple macerate, but only 9.5×10^3 cells/PCR in quince macerate. Greatest decrease in sensitivity of the PCR method was observed in spiked samples with chromosomal PCR since bacterial DNA was not detected in each of the spiked samples. Our research shows that all three PCR protocols are specific for detection of *E. amylovora*, but nested PCR proved to be most sensitive when working with pure cultures and plant material.

Keywords: molecular detection, conventional PCR, nested PCR, quantification, fire blight

INTRODUCTION

Fire blight, caused by a Gram-negative bacterium *Erwinia amylovora* (Burrill) Winslow *et al*., is one of the most destructive diseases of pear, apple, quince and many other rosaceous plants (BONN and VAN DER ZWET, 2000). Infections may kill flowers, fruitlets, leaves and shoots, while cankers on branches and trunks may cause partial destruction or complete plant death (THOMSON, 2000). Fire blight was first observed in North America more than 230 years ago (BONN and VAN DER ZWET, 2000). Since then, it has spread worldwide and the number of countries in which *E. amylovora* has been detected has tripled in the past 30 years, from 15 in 1977, to 46 in 2004 (VAN DER ZWET, 2006) and over 50 countries in 2013 (BALAŽ *et al*., 2013). In Serbia, symptoms of fire blight were first observed in 1989 on pear and quince in the western part of the country (ARSENIJEVIĆ *et al*., 1991). Due to inadequate diagnostic tools and inefficient control measures fire blight has spread throughout the country over the last two decades. Contaminated propagating material is the most common way of dissemination of fire blight. Therefore, detection of bacteria in seedlings and young plants in early stage, when symptoms on plants are not evident, is crucial for preventing the spread of fire blight.

Accurate detection of *E. amylovora* in plant material is essential for setting early and proper diagnosis of fire blight. If typical symptoms, such as shepherd's crook or bacterial ooze are evident, presumptive diagnosis of fire blight can be relatively simple. However, symptoms are not always specific and can be confused with those caused by other biotic or abiotic factors (THOMSON, 2000). In addition, low concentration of bacteria can occur in symptomless plants. Latent infections of budwood or plantings are recognized as important mean of fire blight dissemination to non-contaminated areas (BONN and VAN DER ZWET, 2000). Thus, highly sensitive and reliable protocols are required for pathogen detection in both symptomatic and asymptomatic plant material.

Conventional methods of detection and identification of *E. amylovora* are used for routine diagnosis. However, isolation of the bacterium and its detection on semi-selective medium takes several days and saprophytic organisms can cause problems by their excessive growth (PALACIO-BIELSA *et al*., 2009). Also, confirmation of the pathogen identity by other techniques is required

(LELLIOTT and STEAD, 1987; SCHAAD *et al*., 2001). Serological techniques are not sensitive enough, except the enrichment-enzyme-linked immunosorbent assay (ELISA) method (GORRIS *et al*., 1996). However, ELISA test requires 3 days to complete and the sensitivity could be affected by other bacteria present in the sample. Furthermore, the actual population of epiphytic and endophytic *E. amylovora* in symptomless plant material could be well below the detection levels of these techniques (LLOP *et al*., 2000). Molecular-based methods have also been widely used for the detection of *E. amylovora*, among which Polymerase Chain Reaction (PCR) is most attractive due to its specificity, low detection limits and robustness (PALACIO-BIELSA *et al*., 2009).

The aim of this work was to study specificity and sensitivity of three PCR methods: Nested PCR in a single tube, conventional PCR and PCR detecting chromosomal DNA. All three PCR methods are recommended by EPPO diagnostic protocol (ANONYMOUS, 2013) for routine detection of *E. amylovora* in pure culture and in plant material. Primers in Nested PCR and in the conventional PCR target the pEA29 plasmid, until recently thought to be ubiquitous for *E. amylovora* strains. Recent discovery of indigenous virulent *E. amylovora* strains lacking pEA29 has put these protocols into question (LLOP *et al*., 2006). For that reason evaluation of primers that target the chromosomal DNA was included in the study.

Since PCR is an enzymatic reaction it is prone to inhibiting substances. These substances, so called PCR inhibitors, may be present in the sample or introduced during sampling process. They can affect the sensitivity of the PCR assay or even lead to false-negative results. Regarding fire blight host-derived compounds copper products have been reported as PCR inhibitors (MINSAVAGE *et al*., 1994; MCMANUS and JONES, 1995; MAES *et al*., 1996). Therefore, we examined the influence of plant DNA and other inhibitors present in plant material by spiking pear, apple and quince macerates with *E. amylovora* cells prior to performing PCR reactions.

MATERIALS AND METHODS

Bacterial strains, growth conditions and specificity of PCR reactions

Strains of *E. amylovora* and their origin used in the specificity assay are listed in Table 1. The strains were isolated from different host plants, at different localities and in different years in Serbia and had various PFGE patterns in their genome, representing distant populations of the pathogen. The strains were previously identified based on biochemical properties, immature pear fruit assay, Biolog^{TM} MicroPlate System and Fatty acid analysis (IVANOVIĆ *et al*., 2012). Type strain of *E. amylovora* NCPPB 595 was used as a positive control.

Genomic and chromosomal DNA templates were prepared from pure bacterial cultures grown on King's medium B at 27ºC for 24h. Bacterial suspensions (approx. 10^8 CFU mL$^-$1) were heated at 95°C for 10 min. Lysates were incubated on ice for 5 min and centrifuged 5 min at 8.000 rpm. Supernatants were used directly for PCR amplifications.

PCR sensitivity for E. amylovora detection in pure culture

Bacterial cells of strain KFB 148, grown for 24 h on KB at 27°C, were suspended in sterile ultrapure water to achieve an optical density of $A_{600} \approx 0.3$ (cca. 10^8 CFU mL$^-$1). From this concentrated suspension serial tenfold dilutions, ranging from 10^8 to 1 CFU mL$^-$1 were made. Furthermore, to determine the exact concentration of each suspension, 50µL of each tenfold dilution was spread in triplicate onto KB plates and CFU's of each dilution after 36-48 h was counted. Bacterial DNA from each of the serial dilutions was extracted by heating at 95°C for 10

min, followed by incubation on ice for 5 min and centrifugation for 5 min at 8.000 rpm. Supernatants were used as a template in the sensitivity PCR assays. Number of detected bacteria in each PCR reaction is calculated by using determined concentrations of each dilution and volume of template DNA used in the PCR reaction mix (1 µL for nested PCR, 5 µL for the conventional and 10 µL for the chromosomal PCR). All PCR assays were performed in duplicate.

Table 1. E. amylovora strains used in PCR specificity assays

Strain[a]	Host	Origin	Year of isolation	PFGE group[b]
KFB 146	Apple	Sombor	1998	Pt7
KFB 147	Apple	Bačka Palanka	2000	Pt7
KFB 148	Apple	Čačak	2005	Pt2
KFB 149	Apple	Topola	2005	Pt2
KFB 150	Apple	Topola	2005	Pt2
KFB 151	Apple	Topola	2005	Pt2
KFB 152	Apple	Topola	2005	Pt2
KFB 153	Pear	Šid	1998	Pt8
KFB 154	Pear	Šid	1998	Pt2
KFB 155	Pear	Kraljevo	2005	Pt2
KFB 156	Pear	Kraljevo	2005	Pt2
KFB 158	Pear	Mladenovac	2005	Pt9
KFB 159	Pear	Mladenova	2005	Pt9
KFB 160	Pear	Begaljica	2005	Pt9
KFB 161	Pear	Begaljica	2005	Pt9
KFB 162	Pear	Topola	2005	Pt2
KFB 163	Pear	Topola	2005	Pt2
KFB 164	Pear	Topola	2005	Pt2
KFB 165	Pear	Topola	2005	Pt2
KFB 166	Quince	Bečej	1998	Pt8
KFB 167	Quince	Topola	2005	Pt6
KFB 168	Quince	Arilje	2005	Pt2
KFB 169	Quince	Niš	2005	Pt2
KFB 170	Quince	Niš	2005	Pt2
KFB 172	*Sorbus* sp.	Niš	2005	Pt2
KFB 173	*Sorbus* sp.	Niš	2005	Pt2
KFB 174	*Sorbus* sp.	Niš	2005	Pt2
KFB 175	*Sorbus* sp.	Niš	2005	Pt2
KFB 176	Japanese pear	Čačak	2005	Pt2
KFB 177	Japanese pear	Čačak	2005	Pt2
KFB 178	Japanese pear	Čačak	2005	Pt2
KFB 179	Japanese quince	Bačka Palanka	2000	Pt2
KFB 180	Japanese quince	Bačka Palanka	2005	Pt8
KFB 181	*Cotoneaster* sp.	Futog	2000	Pt3
KFB 182	*Cotoneaster* sp.	Futog	2000	Pt3

Table 1. E. amylovora strains used in PCR specificity assays cont.

Strain[a]	Host	Origin	Year of isolation	PFGE group[b]
KFB 183	Medlar	Šid	2001	Pt2
KFB 184	Medlar	Niš	2005	Pt2
KFB 185	Medlar	Niš	2005	Pt2
KFB 186	Apple	Bela Crkva	2003	Pt9
KFB 187	Apple	Bela Crkva	2003	Pt9
KFB 188	Apple	Župa Nikšićka	2003	Pt2
NCPPB 595	Pear	United Kingdom	1958.	Pt1

a E. amylovora strains conserved in the following collections: KFB, Collection of Phytopathogenic Bacteria, University of Belgrade, Faculty of Agriculture, Serbia; NCPPB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, United Kingdom; b PFGE groups differentiated by IVANOVIĆ *et al*. (2012) and for strain NCPPB 595 by DONAT *et al*. (2007).

PCR sensitivity for E. amylovora detection in plant macerates

To study the influence of plant DNA and other inhibitors on sensitivity of three tested PCR methods, spiked pear, apple and quince macerates were mixed with serial dilutions of strain KFB 148. Asymptomatic shoots of pear, apple and quince were tested for presence of the pathogen by isolation on KB and NAS medium. Only shoots that were negative for the presence of the bacterium were used for preparing the macerates. Small pieces of healthy pear, apple or quince shoots containing 1g of buds, twigs and leaves were crushed in sterile distilled water. Serial dilutions of *E. amylovora* strain KFB 148, ranging from 10^8 to 1 CFU mL^-1, were made. As described before, dilutions were plated in triplicate on KB to determine the exact concentration of each dilution. The dilutions were mixed with the above-mentioned plant macerates to give a final concentration ranging from 1.9×10^7 to 1.9 CFU mL^-1. With these samples, a DNA extraction protocol of LLOP *et al*. (1999) was applied prior to the PCR. Reactions were tested in two separate experiments.

PCR reaction mixtures and conditions

Conventional PCR target the pEA29 sequences and was carried out with A/B primer pair according to BERESWILL *et al*. (1992) (Table 2). Reaction mixture of the final volume of 45 µL contained: water (PCR grade) 30.1 µL, PCR buffer 10× 5 µL, $MgCl_2$ 25 mM 6 µL, dNTPs 10mM 0.5 µL, primer A 10 pmol μL^-1 0,5 µL, primer B 10 pmol μL^-1 0.5 µL, Taq polymerase 5 U μL^-1 0.4 µL and 5 µL of DNA sample. The reaction conditions were: a denaturation step of 93ºC for 5 min followed by 40 cycles at 93ºC for 30 s, 52ºC for 30 s, and 72ºC for 1 m 15 s and a final step at 72ºC for 10 min. The amplicon size for this protocol is 900 bp.

Nested PCR in a single tube was carried out with two pairs of primers, external AJ75/AJ76 and internal primers PEANT1/PEANT2, both based on sequences from pEA29 (Table 2). According to LLOP *et al*. (2000) reaction mixture of the final volume of 49 µL contained: water (PCR grade) 31.76 µL, PCR buffer 10× 5 µL, $MgCl_2$ 25 mM 6 µL, dNTPs 10mM 1µL, primer AJ75 0.1 pmol μL^-1/µL 0.32 µL, primer AJ76 0.1 pmol μL^-1 0.32 µL, primer PEANT1 10 pmol μL^-1 1 µL, primer PEANT2 10 pmol μL^-1 1 µL, Taq polymerase 5 U μL^-1 0.6 µL and 1 µL of DNA sample. The reaction conditions were: 94°C for 4 min followed by 25 cycles at 94°C for 60 s and 72°C for 90 s. This first round PCR is followed in the same thermocycler by a second denaturation step at 94°C for 4 min and 40 cycles at 94°C for 60 s,

56°C for 60 s, and 72°C for 60 s, and a final step at 72°C for 10 min. The amplicon size for these primers and conditions is 391 bp.

Chromosomal sequences were amplified with the primer par FER1-F/FER1-R developed by OBRADOVIĆ *et al*. (2007) (Table 2). In order to avoid „hot start" of the reaction the original protocol was modified in our study and carried out without separation of reaction mixtures at the beginning of the reaction. The modified method saves time and labor minimizing the possibility of cross contamination of samples. The altered PCR reaction was performed in a 25 µL reaction mixture and contained: 8 µL water (PCR grade), PCR buffer 10× 2.5 µL, $MgCl_2$ 25 mM 1.5 µL, dNTPs 10 mM 0.5 µL, primer FER1-F 10 pmol $\mu L^{-}1$ 1 µL, primer FER1-R 10 pmol $\mu L^{-}1$ 1 µL, Taq polymerase 5U $\mu L^{-}1$ 0.5 µL and 10µL of template DNA. The following program was carried out: initial denaturation for 3 min at 94°C, followed by 41 cycles at 94°C for 10 s, 62°C for 10 s and 72°C for 30s, and final extension step for 3 min at 72°C. The amplicon size for this method is 1269 bp.

In all experiments PCR products were separated by 1.5% (w/v) agarose gel electrophoresis in 0.5×TAE buffer for 30 min at 100 V, stained with ethidium bromide for 20 min, and visualized under UV light.

Table 2. PCR protocols used in the study for evaluation of specificity and sensitivity in detection of E. amylovora

PCR protocol	Target DNA	Primers	Size of amplicon (bp)	Reference
Conventional	Plasmid (pEA29)	A/B	900	BERESWILL *et al*., 1992
Nested	Plasmid (pEA29)	AJ75/AJ76 (External) PEANT1/PEANT2 (Internal)	391	LLOP *et al*., 2000
Conventional	Chromosomal	FER1-F/FER1-R	1269	OBRADOVIĆ *et al*., 2007*

* modified protocol as referred in the text.

RESULTS AND DISCUSSION

Specificity of selected PCR protocols

The specificity of three PCR procedures was tested by using pure cultures of 42 *E. amylovora* strains with different PFGE patterns. Regardless of their PFGE pattern type, host, origin or year of isolation, all three PCR methods resulted in amplicons of the expected size. A single amplification band of 391 bp, 900 bp and 1269 bp was observed with AJ75/76-PEANT1/2, A/B and FER1-F/FER1-R pair of primers, respectively. Unspecific banding was not observed with any of the analyzed protocols and all 42 strains of *E. amylovora* examined produced a single amplification band. LECOMTE *et al*. (1997) reported variations in amplicon size between 900 and 1100 bp when A/B pair of primers were employed, but in our study all strains produced a single, 900 bp band. The variation in band size is due to the number of 8 bp repeats sequences within the amplified DNA fragment (JONES and GEIDER, 2001).

Although *E. amylovora* is still considered a homogenous species, recent genomic studies have shown that different PFGE patterns (JOCK *et al*., 2013) or different plasmid content (LLOP *et*

al., 2006; ISMAIL *et al*., 2014) within pathogen population may occur. For diagnostic laboratories it is of great importance to have reliable protocols which can detect all strains within one species. BERESWILL *et al*. (1992) were the first to propose a procedure for specific detection of *E. amylovora* by PCR. This method is based on the amplification of a fragment of the plasmid pEA29, which was considered to be common to all *E. amylovora* strains (FALKENSTEIN *et al*., 1989; LAURENT *et al*. 1989; SALM and GEIDER, 2004). Another widely accepted PCR-based technique for detection of *E. amylovora* is nested PCR in a single closed tube, also amplifying a DNA fragment in the pEA29 (LLOP *et al*., 2000). The discovery of a virulent strain of *E. amylovora* lacking the 29 kb plasmid in Spain (LLOP *et al*., 2006) and later in Iran, Egypt and Germany (MOHAMMADI *et al*., 2009) has brought concerns and confusion in the specificity of PCR methods targeting the pEA29 (PIRC *et al*., 2009). These strains contain a previously unknown plasmid of about 70kb, involved in virulence and having no sequence similarity with pEA29. A chromosome-based method developed by OBRADOVIĆ *et al*. (2007) may overcome the possible disadvantage of plasmid-based detection methods. Since all 41 strains originating from Serbia were detected with both pEA29-derived primers it can be concluded that all *E. amylovora* strains from our country carry the common pEA29 plasmid. This finding was supported by ISMAIL *et al*. (2014) who confirmed no other plasmid than the pEA29 in the *E. amylovora* population from Serbia.

Sensitivity of selected PCR protocols for E. amylovora detection in pure culture

In sensitivity test different levels of detection limits were observed among three PCR methods (Table 3). When using pure cultures of *E. amylovora* suspended in sterile ultrapure water as a template, the greatest sensitivity was achieved with nested PCR in a single tube. With this method *E. amylovora* cells could be detected at concentrations as low as 1.9×10^3 CFU mL^-1 (Figure 1b, line 6). Considering that only 1 μL of template DNA was used in nested PCR reaction this sensitivity corresponds to a detection limit of 1.9 bacteria per PCR reaction (Table 3).

Table 3. Sensitivity of three PCR-based methods for detection of E. amylovora in water suspension and spiked samples of three most common plant hosts

Sample [a]	Lowest positive dilution (bacterial cells/PCR reaction) detected by PCR procedure		
	BERESWILL *et al*., 1992	LLOP *et al*., 2000	OBRADOVIĆ *et al*., 2007
KFB 148 + ultrapure water	9.5	1.9	1.9×10^5
KFB 148 + pear extract [b]	95	1.9	/
KFB 148 + apple extract	95	19	/
KFB 148 + quince extract	9.5×10^3	1.9	/

[a] Serial dilutions of *E. amylovora* strain KFB 148 in ultrapure water and in different plant extracts; [b] Samples of plant extracts were analyzed following DNA extraction of LLOP *et al*. (1999); / *E. amylovora* DNA not detected in the sample.

Conventional PCR using A/B primers also showed high sensitivity when working with suspensions derived from pure cultures. Detection limit was also 1.9×10^{3} CFU $mL^{-}1$ (Figure 1a, line 6), but since higher volume of template DNA (5 µL) was used in the PCR reaction, the sensitivity corresponds to 9.5 bacteria per PCR reaction. The modified chromosomal PCR was not sensitive as previous two methods in detecting *E. amylovora* in pure culture. The lowest detected concentration was only 1.9×10^{7} CFU $mL^{-}1$ (Figure 1c, line 2). Taking into account that 10µL of DNA template was used in the reaction this corresponds to 1.9×10^{5} cells/PCR reaction. The same results were obtained when the analysis was repeated.

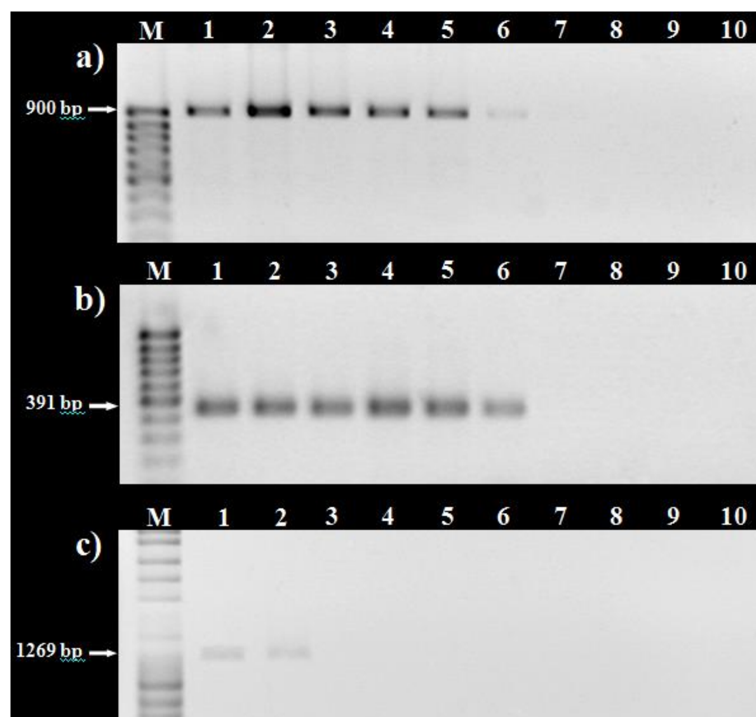

Figure 1. Sensitivity comparison of three PCR protocols in detection of *E. amylovora* in pure culture with: a) A/B; b) AJ75/76-PEANT1/2; c) FER1-F/FER1-R pair of primers. Lines M, marker (a and b-Low range DNA Ladder; c-Mix DNA Ladder, MBI Fermentas, Lithuania); lines 1 to 9, serial dilutions of strain KFB 148 ($1,9\times10^{8}$-1,9 CFU $mL^{-}1$); 10, negative control.

Although the protocol of OBRADOVIĆ *et al*. (2007) in our study revealed good specificity by detecting all PFGE groups from Serbia, it did not meet the expectations in the sensitivity test. In the original protocol of OBRADOVIĆ *et al*. (2007) the authors report the sensitivity level of 3 CFU $mL^{-}1$ in pure culture. The altered protocol proved to be more user friendly, saves time and labor, but in our study it could only detect bacteria at concentration of 1.9×10^{7} CFU $mL^{-}1$ or higher. Furthermore, with this protocol we could not detect any bacteria in spiked pear, apple or quince macerate. While the modification minimizes the possibility of cross contamination of

samples, it greatly affects the sensitivity of the protocol. Further analyses are needed to look into the mechanism of the reaction and find the reasons of poor sensitivity.

Sensitivity of selected PCR protocols for E. amylovora detection in plant macerates

We also compared sensitivity of conventional PCR, nested PCR in a single tube and chromosomal PCR for detection of *E. amylovora* cells in presence of plant material. Macerates of three most common hosts of fire blight pathogen: pear, apple and quince were used in the test. Inhibitors from pear macerate did not influence sensitivity of nested PCR reaction keeping the same level of detected bacteria, 1.9×10^3 CFU $mL^{-}1$ (Figure 2b, line 5). Sensitivity level of conventional PCR with A/B primers was reduced by a factor of 10 compared to pure culture, to 1.9×10^4 CFU $mL^{-}1$ (Figure 2a, line 4). On the other hand, sensitivity of chromosomal PCR greatly decreased and no bands were visible in pear macerate spiked with *E. amylovora* cells (Figure 2c). Furthermore, no bacteria were detected in apple and quince macerate when this modified protocol was applied.

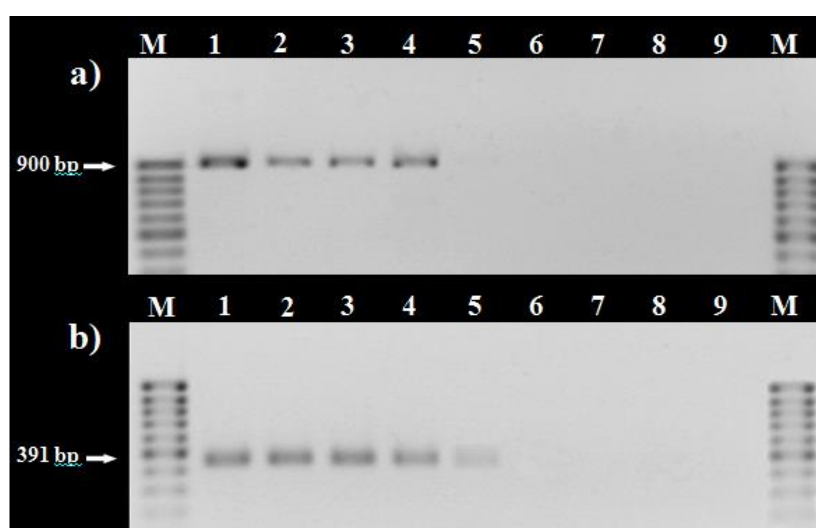

Figure 2. Sensitivity comparison of three PCR protocols in detection of *E. amylovora* in pear macerate with: a) A/B; b) AJ75/76-PEANT1/2. Lines M, marker Low range DNA Ladder, MBI Fermentas, Lithuania); lines 1 to 9, serial dilutions of pear extract spiked with strain KFB 148 (1.9×10^7-1.9 CFU $mL^{-}1$); 9, negative control.

When *E. amylovora* cells were mixed with apple macerate, sensitivity of nested PCR was reduced by a factor of 10 to 1.9×10^4 CFU $mL^{-}1$ corresponding to 19 cells/PCR reaction (Figure 3b, line 4). Minimal detected concentration of bacterial cells mixed with apple macerate with conventional PCR was 1.9×10^4 CFU $mL^{-}1$ (95 cells/PCR) (Figure 3a, line 4) while chromosomal PCR could not detect the *E. amylovora* cells even at the highest concentration.

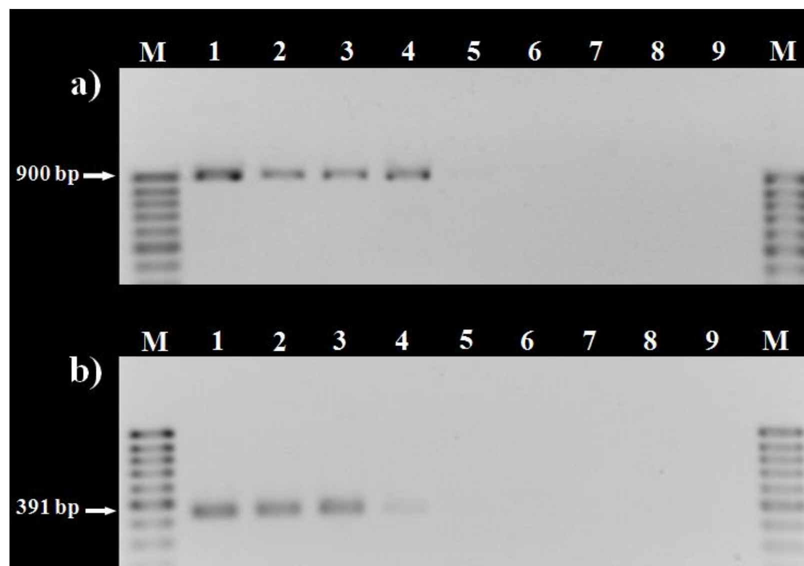

Figure 3. Sensitivity comparison of PCR methods in detection of *E. amylovora* in apple macerate with: a) A/B; and b) AJ75/76-PEANT1/2 pair of primers. Lines M, marker Low range DNA Ladder, MBI Fermentas, Lithuania; lines 1 to 9, serial dilutions of apple extract spiked with strain KFB 148 (1.9×10^7-1.9 CFU mL^-1); 9, negative control.

Figure 4. Sensitivity comparison of protocols in detection of *E. amylovora* in quince macerate with: a) A/B; and b) AJ75/76-PEANT1/2 pair of primers. Lines M, marker Low range DNA Ladder, MBI

Fermentas, Lithuania; lines 1 to 9, serial dilutions of quince extract spiked with strain KFB 148 (1.9×10^7-1.9 CFU mL^{-1}); 9, negative control.

Quince macerate did not influence sensitivity of nested PCR, since the minimal concentration of *E. amylovora* detected was 1.9×10^3 CFU mL^{-1} (Figure 4b, line 5). However, the same macerate reduced the sensitivity of conventional PCR, allowing detection of only 1.9×10^6 CFU mL^{-1} (Figure 4a, line 2), corresponding to 9.5×10^3 cells/reaction. No cells of *E. amylovora* were detected in quince macerate with chromosomal PCR.

Two protocols that target plasmid DNA showed greatest sensitivity in our studies. Plasmid pEA29 is present in the bacterium in more than one copy making its detection easier. With A/B primers we were able to detect 9.5 cells per reaction in pure culture, 95 cells in pear and apple macerate, but only 9.5×10^3 cells per reaction in quince macerate. It was shown earlier that PCR inhibitors, which are very common in fire blight hosts, present a serious drawback for conventional PCR techniques (MCMANUS and JONES, 1995; MAES *et al*., 1996). A low copy number of initial target DNA sequences makes the first amplification cycles critical and PCR inhibitors can result in false negatives, which could have a major impact, especially in quarantine testing. The presence of inhibitors in plant material can be overcome with DNA extraction protocol. However, in quince macerate we were not able to eliminate all inhibitory compounds, suggesting a new technique for extraction of bacterial DNA when working with quince material and A/B primers.

In this study nested PCR showed to be the most sensitive method in detecting *E. amylovora*, regardless of the tested material. With nested PCR we were able to detect 1.9 cells/PCR reaction in pure culture, pear and quince macerate, and 19 cells/reaction in apple macerate. In nested PCR, sensitivity and specificity of detection are enhanced by performing a second round of PCR with amplified DNA from first-round PCR as the template and primers internal to the first-round primers. The external and internal primer pairs of this method have different annealing temperatures directing amplification of a specific DNA fragment from plasmid pEA29.

CONCLUSIONS

Long-distance as well as local spread of fire blight, caused by movement of *E. amylovora*-contaminated plant material, could be considerably minimized by monitoring such material for the pathogen by using sensitive detection techniques. Numerous PCR-based techniques for the detection and identification of *E. amylovora* have been developed during the last two decades. These protocols must be specific, sensitive and reliable. Three tested PCR protocols and primer pairs in this study showed robustness and good reproducibility. They showed high level of specificity by detecting 42 *E. amylovora* strains with different PFGE patterns. Primers that target the plasmid pEA29 were able to detect low numbers of bacteria in pure culture. However, nested PCR in a single closed tube proved to be more sensitive when working with plant material.

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SPECIFIČNOST I OSETLJIVOST TRI PCR METODE ZA DETEKCIJU *Erwinia amylovora* U ČISTOJ KULTURI I BILJNOM MATERIJALU

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Izvod

Tri PCR metode, označene u radu kao „nested“ „konvencionalni“ i „hromozomalni“ PCR, koje su predložene za rutinsku detekciju *Erwinia amylovora* u čistoj kulturi i biljnom materijalu, ocenjene su na osnovu njihove specifičnosti i osetljivosti. Specifičnost PCR metoda analizirana je korišćenjem četrdeset i dva soja *E. amylovora*, poreklom iz različitih lokacija i biljnih vrsta, sa varijabilnim PFGE profilima, predstavljajući udaljene populacije ovog patogena. Osetljivost PCR protokola u čistoj kulturi proučena je korišćenjem 9 različitih koncentracija *E. amylovora* u sterilnoj ultra čistoj vodi kao uzorak za PCR reakciju. Kako bi se proučio inhibitorni efekat biljne DNK i drugih inhibitora na osetljivost tri PCR metode bakterijska razređenja su pomešana sa biljnim maceratom kruške, jabuke i dunje neposredno pre PCR reakcije. Tokom ispitivanjima specifičnosti bilo je moguće detektovati sve sojeve *E. amylovora* pomoću sve tri PCR metode bez obzira na domaćina iz koga su izolovani, geografsko poreklo ili PFGE grupu, ukazujući na specifičnost prajmera. S druge strane, osetljivost između proučavanih metoda je varirala u zavisnosti od koncentracije bakterija i odabranog biljnog materijala koji je korišćen u PCR reakciji. Prilikom korišćenja čistih kultura nested PCR je pokazao najveću osetljivost detektovanjem 1.9 bakterijskih ćelija po PCR reakciji, zatim konvencionalni PCR sa granicom detekcije od 9.5 ćelija po reakciji i 1.9×10^5 ćelija/reakciji kod hromozomalnog PCR. U veštački inokulisanim uzorcima inhibitori iz biljaka nisu uticali ili su smanjivali osetljivost PCR reakcije, u zavisnosti od protokola i/ili tipa biljnog macerata. U našim eksperimentima inhibitori iz macerata kruške i dunje nisu uticali na osetljivost nested PCR, dok je macerat jabuke umanjio osetljivost metode 10 puta. Pomoću konvencionalnog PCR bilo je moguće detektovati 95 ćelija po jednoj PCR reakciji u maceratu kruške i jabuke, ali samo 9.5×10^3 ćelija po PCR reakciji u maceratu dunje. Najveće smanjenje osetljivosti PCR metode je zabeleženo u detekciji veštački inukulisanih uzoraka pomoću hromozomalnog PCR pri čemu bakterijska DNK nije detektovana ni u jednom od inokulisanih uzoraka. Naša istraživanja pokazuju da su sve tri proučavane metode specifične u detekciji *E. amylovora*, ali da je nested PCR pokazao najveću osetljivost prilikom rada sa čistim kulturama i biljnim materijalom.

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