

EVALUATION OF METHODS FOR *ERWINIA AMYLOVORA* DETECTION

Monika KAŁUŻNA, Joanna PUŁAWSKA*, Artur MIKICIŃSKI

Research Institute of Horticulture

Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

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ABSTRACT

Phytosanitary measures to control diseases require sensitive and reliable detection methods. In case of *Erwinia amylovora*, the causal agent of fire blight, there are several methods developed. We tested six of them: (1) a conventional method; (2) standard PCR; (3) BIO-PCR; (4) Real-time PCR with SYBR Green; (5) Real-time PCR with TaqMan probe; and (6) Loop Mediated Isothermal Amplification (LAMP) in terms of their specificity, sensitivity and repeatability. Of the methods tested, only Real-time PCR with SYBR Green amplified non-specific fragments, which could be however identified by melting curve analysis. Real-time PCRs (both with SYBR Green and TaqMan probe) and LAMP were most sensitive and allowed to detect *E. amylovora* even in the samples where the concentration of cells was lower than 2×10^1 cfu/reaction. The highest repeatability was obtained using conventional method and Real-time PCRs. Of the methods used, only the LAMP technique was very insensitive to plant impurities.

Key words: asymptomatic plant material, fire blight, LAMP, PCR, Real-time PCR

INTRODUCTION

Fire blight, caused by *Erwinia amylovora*, is one of the most destructive diseases of apples and pears in Poland. Besides parasitic relationship, the pathogen can survive in plants as endophyte or as an epiphyte on plant surfaces. Early and quick detection of *E. amylovora* plays an important role in disease prediction, elimination of potential fire blight foci, as well as in testing of trees before planting and planning control treatment schedules. The most common methods of fire blight diagnostics, like isolation of bacteria on selective or semi-selective media, pathogenicity tests and serological assays are time consuming and/or not always specific and sensitive enough. DNA-based methods, particularly PCR, offer the possibility to overcome these constraints and allow fast detection of *E. amylovora* with high specificity and sensitivity. The aim of our study was to evaluate and compare six methods (a conventional and five molecular) of *E. amylovora* detection in terms of their specificity, sensitivity and repeatability.

MATERIALS AND METHODS

Bacterial strains and DNA isolation

In the years 1982-2011, strains of *Erwinia amylovora* were isolated on SNA medium (Nutrient Agar (Difco) with 5% saccharose) from symptomatic apple and pear tree tissues originating from different geographical regions of Poland. After isolation, they were purified, tested for their pathogenicity on immature pear fruitlets (Sobiczewski & Millikan 1985) and deposited in the collection of the Laboratory of Bacteriology at the Research Institute of Horticulture. As a reference, a well-described and sequenced strain B62 (ATCC 49946) (Sebahia et al. 2010) was used. Bacterial DNA from pure culture or from plant tissue was isolated using the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland). One μ l of DNA was used in all techniques based on DNA amplification.

Methods of *E. amylovora* detection

Six methods of *E. amylovora* detection were tested:

*Corresponding author:
e-mail: Joanna.Pulawska@inhort.pl

1. Conventional method

Bacteria were plated on microbiological media – SNA and King's medium B (King et al. 1954). After 48 h of incubation at 26 °C, bacteria were identified based on colony morphology on these two media.

2. Standard PCR with primers complementary to chromosomal DNA (Maes et al. 1996)

Bacterial DNA (1 µl) was amplified by PCR with primers EaF (5'-GCGCAGTAAAGGGTGACAGCCCCGTACACAAAAGGCACT-3') and EaR (5'-CCCTAGCCGAAACAGTGCTCTACCCCCGG-3'). Amplification was carried out in a Biometra T3000 Thermocycler (Biometra GmbH, Gottingen, Germany) with reaction conditions described by Maes et al. (1996). PCR products were separated on a 1.5% agarose gel in TBE buffer, which was stained with ethidium bromide (0.5 mg·l⁻¹) and visualized under a UV transilluminator.

3. BIO-PCR

PCR with primers complementary to chromosomal DNA (Maes et al. 1996) was preceded by overnight incubation of a homogenate of the tested sample on SNA medium. Bacterial growth was washed from the medium with 3 ml of 0.01 M PBS buffer (0.27% Na₂HPO₄; 0.04% NaH₂PO₄; 0.8% NaCl) and 1 ml of suspension was used for isolation of DNA using the Genomic Mini kit according to manufacturer instructions (A&A Biotechnology, Gdynia).

4. Real-time PCR with SYBR Green

Real-time PCR was conducted with primers complementary to plasmid pEA29 and the amplification product was detected with SYBR Green dye (Fast Start Universal SYBR Green Master, Roche Diagnostics GmbH/Applied Science, Mannheim, Germany). One µl of bacterial DNA was used for PCR with primers P29TF 5'-CACTGATGGTGCCGTG-3' and P29TR 5'-CGCCAGGATAGTCGCA TA-3' (Salm & Geider 2004). Amplification was carried out on Rotor Gene 6000 (Corbett Life Science) with the protocol and reaction conditions as described by Salm and Geider (2004). In order to verify the specificity of amplification in Real-time PCR with SYBR Green, the melting curve analysis of the obtained products was performed at the end of the reaction. After preconditioning of PCR products at 60 °C for 90 s, progressive denaturation of products was carried out at a temperature rising

from 60 to 90 °C with 1 °C increment. As the temperature increases, PCR products dissociate (SYBR Green is released) and their melting temperature depends on the length and the composition of the sequence. As a result, it is possible to observe a decrease in fluorescence for dissociating products. The peaks corresponding to the melting temperature of DNA products can be identified on the plot of -dF/dT (negative first derivative of fluorescence versus temperature). At this step, by comparison with the results of positive control, the specificity of obtained products is being determined.

5. Real-time PCR with TaqMan probe

The primers and the TaqMan probe were complementary for plasmid pEA29: primers P29TF and P29TR as above and P29TM (TaqMan probe) 5'-TACCTCCGCAGCCGTCATGG-3' (Salm & Geider 2004). The TaqMan probe was labelled at the 5' end with 6-carboxyfluorescein (FAM) and at the 3' end with 6-carboxy-tetramethyl-rhodamine (TAMRA). The protocol and reaction conditions were as described by Salm and Geider (2004) but Platinum Taq DNA Polymerase (Invitrogen) was used. The reaction was conducted on Rotor Gene 6000 (Corbett Life Science).

6. Loop Mediated Isothermal Amplification (LAMP)

LAMP is a one-step amplification reaction that amplifies a target DNA sequence under isothermal conditions. LAMP employs a *Bst* or *Bsm* DNA polymerase with strand-displacement activity, along with two inner primers (FIP, BIP) and outer primers (F3, B3) which recognize six separate regions within a target DNA (Notomi et al. 2000). The high number of DNA regions, which has to be simultaneously recognized by the primers to obtain amplification products, provides high specificity of detection. Detection of *E. amylovora* using LAMP was carried out with primers complementary to *amsH* gene located on chromosomal DNA (Moradi et al. 2012). One µl of bacterial DNA was added to the amplification mixture of which the content was as originally described by Moradi et al. (2012) but *Bsm* polymerase (Thermo Scientific) was used. Incubation was performed at 60 °C as optimal for *Bsm* polymerase. LAMP products were separated on 1.5% agarose gels in TBE buffer, which were stained with

Table 1. Origin and characteristics of *E. amylovora* strains used in detection sensitivity tests

Strain	Origin	Host plant	Year of isolation*	Virulence
E2	Poland/Oświęcim	<i>Crataegus</i> sp.	2000	low
692	Poland/Nowy Dwór	<i>Sorbus</i> sp.	1997	moderate
659	Poland/Toruń	<i>Malus</i> sp.	1986	moderate
A90	USA	<i>Malus pumila</i>	nd	high
B62	USA	<i>Malus pumila</i>	nd	high

* No data

ethidium bromide (0.5 mg·l⁻¹) and visualized under a UV transilluminator.

Specificity and sensitivity of tested methods

Specificity of each method was evaluated with 60 strains of *E. amylovora* or their DNA, one *Pseudomonas syringae* strain 110 and two *Xanthomonas arboricola* strains: X507 and L31.

To estimate the sensitivity of *E. amylovora* detection, five strains: B62, A90, 692, 659 and E2, isolated from different plant species, in different years and different geographical regions and of different virulence were selected (Table 1). The suspensions of these strains (2×10^8 cfu·ml⁻¹) were prepared. Then the suspensions were serially diluted 10 times and each dilution was used in the conventional method and in BIO-PCR. From 100 µl of each dilution, bacterial DNA was isolated and afterwards used in methods based on DNA amplification.

For assessment of the sensitivity of *E. amylovora* detection in plant material, 2-3 cm long apple shoot fragments (1.2 ± 0.2 g) were surface sterilized with 70% ethanol, cut into small fragments, and placed in a glass tube with 3 ml of 0.01 M PBS buffer with addition of 100 µl *E. amylovora* suspension of different concentrations (10⁵, 10⁴, 10³, 10², 10¹ and 10⁰ cfu·ml⁻¹). The concentration of bacterial cells in each suspension was estimated by plating 100 µl of suspensions of 10¹–10⁴ cfu·ml⁻¹ (each in three repetitions) on SNA medium and counting colonies grown after overnight incubation at 26 °C. After addition of bacterial suspension to plant material, samples were incubated on a rotary shaker for 1 h at room temperature. One hundred µl of obtained washings was plated on SNA medium to estimate the sensitivity of conventional method and BIO-PCR. Additionally, 1 ml of washings was centrifuged, the pellet was re-suspended in 100 µl of TE

buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and DNA was isolated as described above. The obtained DNA was used in tests on sensitivity of DNA-based detection methods.

Repeatability of tested methods

E. amylovora suspensions of five strains: B62, A90, 692, 659 and E2 (2×10^5 , 2×10^4 , 2×10^3 , 2×10^2 and 2×10^1 cfu·ml⁻¹ – the number of bacterial cells was counted as described above), were used for testing repeatability of the conventional method and BIO-PCR. For estimation of repeatability of all tested DNA-based detection methods, DNA of *E. amylovora* isolated from the prepared suspensions as described above was applied. Each bacterial suspension, and each of its DNA, was tested five times.

Susceptibility of DNA-based detection methods to contamination by plant impurities

Fragments of apple tree tissue (leaves and shoot) were macerated and shaken in 2 ml of 0.01 PBS buffer for 30 min. The plant washings as well as their three 10-fold dilutions were mixed (1:1 v/v) with 1 µl of *E. amylovora* DNA isolated from bacterial suspensions containing 2×10^5 , 2×10^4 , 2×10^3 , 2×10^2 and 2×10^1 cfu·ml⁻¹. The mixture was applied for tests with all DNA-based detection methods. Each combination of plant impurities – DNA was tested two times.

Detection of *E. amylovora* in asymptomatic material

For detection of *E. amylovora* in asymptomatic plant material, eight samples were collected from five orchards localized in central Poland in June 2013. Each sample consisted of 100 twigs, approximately 10 cm long, collected from 100 newly planted apple trees. In the laboratory, the twigs of each sample were disinfected, cut into small pieces

and incubated in 500 ml of sterile 0.01 M PBS buffer for 30 min on the rotary shaker at room temperature. Afterwards, the washings were centrifuged and the pellet was re-suspended in 2 ml of 0.01 M PBS buffer. One hundred μl of the suspension was plated on King's medium B, SNA and CCT medium (Ishimaru & Klos 1984). The remaining suspension was centrifuged, supernatant removed and the pellet used for DNA isolation with AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, Biosciences, USA). After overnight incubation on applied media, bacteria were washed off the plates with 2 ml of PBS buffer and centrifuged. The pellets obtained were subjected to DNA isolation using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, Biosciences, USA). The isolated DNA was used for *E. amylovora* detection with application of standard PCR, Real-time PCRs and LAMP as described above. In parallel, we checked if fire blight occurred during summer in the orchards from which the samples were collected.

RESULTS

Specificity and sensitivity of tested methods

All tested methods, except Real-time PCR with SYBR Green, allowed for specific detection of all tested *E. amylovora* strains. In case of Real-time PCR with SYBR Green, non-specific products were obtained in reactions with DNA of *Pseudomonas syringae* and *Xanthomonas arboricola*. To differentiate specific and non-specific products, it was necessary to apply the product melting curve analysis. In case of samples with lower concentration of *E. amylovora* ($< 10^2$ cfu per reaction), the fluorescence signal for true *E. amylovora* appeared even later than in case of DNA of *Pseudomonas* and *Xanthomonas* strains, applied in a concentration of $10 \text{ ng} \cdot \mu\text{l}^{-1}$. Real-time PCR with TaqMan probe was specific in our hands, with the samples we used, and detected only DNA of *E. amylovora*.

The sensitivity of detection of *E. amylovora* in pure bacterial culture differed depending on the methods tested. Both conventional method and standard PCR had the sensitivity threshold of 2×10^2 cfu per reaction (Fig. 1). Application of preincubation of a sample on microbiological medium (BIO-PCR) increased the sensitivity 100 times (2×10^0 cfu per reaction). Out of the DNA-based methods, the most sensitive detection was obtained with Real-time PCR (both with SYBR Green and TaqMan probe) and LAMP (Figs 2 & 3). These techniques allowed for detection of *E. amylovora* even when the concentration of bacterial cells was lower than 2×10^1 cfu per reaction.

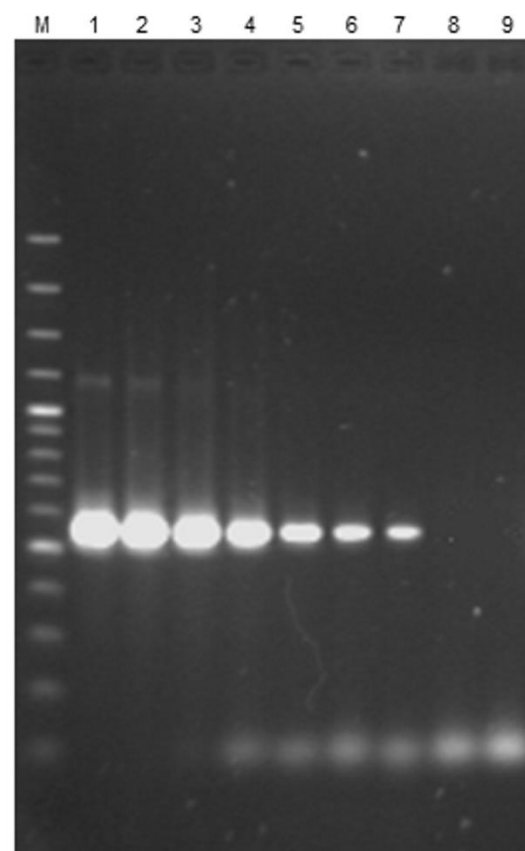


Fig. 1. Sensitivity of detection of *E. amylovora* in pure bacterial culture using standard PCR with primers EaF/EaR: M – marker 100 bp Ladder (Thermo Scientific Fermentas), 1 – 2×10^8 , 2 – 2×10^7 , 3 – 2×10^6 , 4 – 2×10^5 , 5 – 2×10^4 , 6 – 2×10^3 , 7 – 2×10^2 , 8 – 2×10^1 and 9 – 2×10^0 cfu per reaction

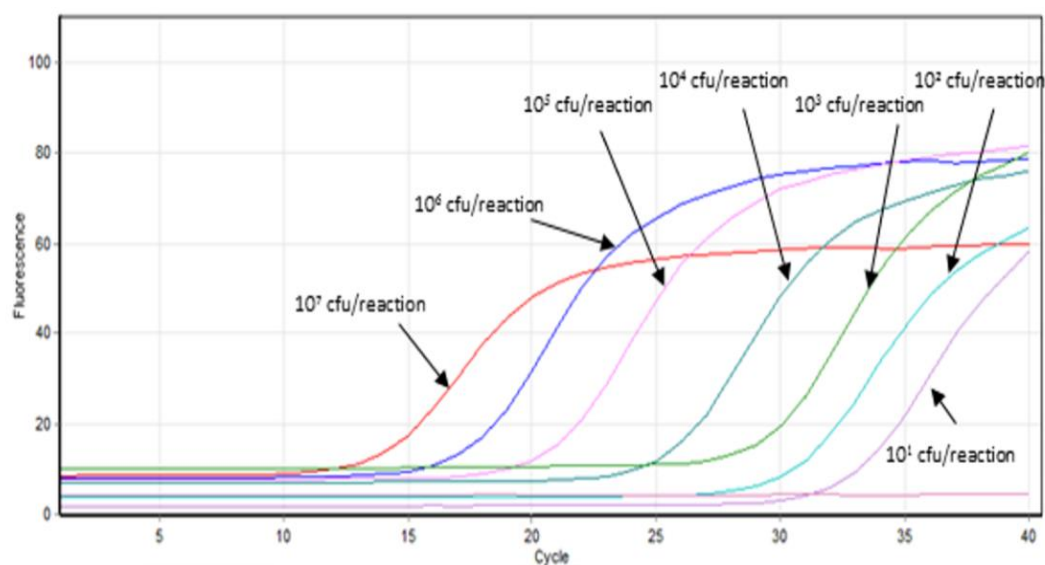


Fig. 2. Real-time PCR with SYBR Green for specific and quantitative amplification of *Erwinia amylovora* DNA. Fluorescence signal in relation to the amount of template

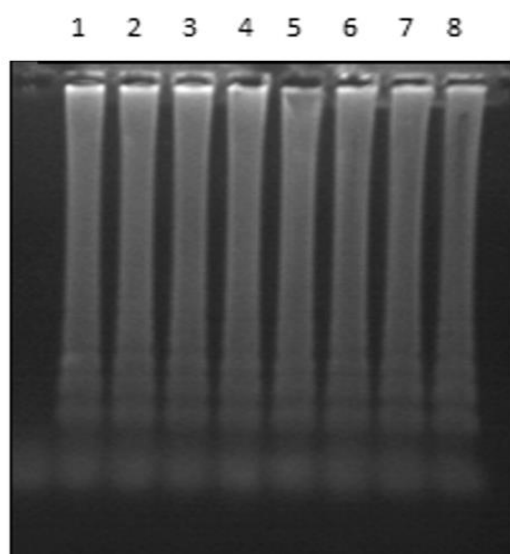


Fig. 3. Sensitivity of detection of *E. amylovora* with LAMP technique. Amplification profile was observed in samples of concentration: 1 – 2×10^7 , 2 – 2×10^6 , 3 – 2×10^5 , 4 – 2×10^4 , 5 – 2×10^3 , 6 – 2×10^2 , 7 – 2×10^1 , 8 – 2×10^0 cfu per reaction

In case of detection of *E. amylovora* in plant material, Real-time PCRs, both with TaqMan and SYBR Green, showed similar sensitivity as in pure bacterial culture ($< 2 \times 10^1$ cfu per reaction) and it was the highest sensitivity out of all the tested methods. The lowest

sensitivity of detection was obtained by the conventional method (10^3 cfu·ml⁻¹). With standard PCR, the detection threshold was 2×10^2 cfu per reaction while BIO-PCR using SNA washings allowed increasing this sensitivity 100 times.

Repeatability of tested methods

All techniques tested differed in terms of repeatability. Classical microbiological method and Real-time PCRs were the most repeatable. Their repeatability was estimated as 100%; this means that the same result was obtained in all repetitions tested. The standard PCR and BIO-PCR had repeatability equal to 80%. The lowest repeatability, 20-60% depending on the bacteria concentration, was observed in LAMP technique.

Susceptibility of DNA-based detection methods to contamination by plant impurities

The LAMP technique was insensitive to plant impurities; amplification products were observed in all samples independently from *E. amylovora* DNA or plant extract concentration. Standard PCR was inhibited by concentrated plant extract and the efficiency of DNA amplification in the sample with 10 times diluted plant extract was much lower than that of pure DNA. Real-time PCR with SYBR Green was inhibited only by the most concentrated plant extract while in Real-time

PCR with TaqMan probe fluorescence was observed even in case of highest concentration of plant impurities but the signal was detected 5 cycles later in comparison to pure DNA (Δ ct = 5). Plant extracts diluted 100 and 1000 times had no effect on detection with any of the tested methods.

Detection of *E. amylovora* in asymptomatic material

In asymptomatic samples of young apple shoots, *E. amylovora* was not detected using Real-time with SYBR Green. Although the fluorescent signal was observed for all samples, only for positive control (DNA of *E. amylovora*) the product of expected size (112 bp) was obtained, which was confirmed after melting curve analysis ($T_m = 87$ °C). It could be caused by the presence of high number of other bacteria isolated from samples, which DNA could be also amplified. When the amplification products were analysed on the electrophoresis gel, many DNA fragments of different sizes were observed; they surely were the reason of fluorescence but they had another size than the specific product. Real-time PCR with TaqMan probe gave positive signal for all tested samples, but the number of threshold cycle was higher than 30, which suggests that the amount of bacteria in the samples was rather low – it corresponds to the $10^1 - 10^2$ cfu per reaction according to the sensitivity measured on pure bacterial cultures (Fig. 2). Standard PCR showed positive results only in the samples, which were earlier preincubated on microbiological media. Using LAMP technique, *E. amylovora* was detected in the majority, 19 of 32 tested samples. Monitoring of the orchards from which the asymptomatic samples were collected revealed that fire blight was not observed in any of them in the year we made the survey, although the weather conditions during the season were very favourable for disease development.

DISCUSSION

Out of all tested methods, and with the plant samples we used, only Real-time PCR with SYBR Green showed non-specific results; a positive signal of reaction was observed also for other than *E. amylovora* tested bacteria. Originally, the primers used in this reaction were checked by their authors for specific-

ity (Salm & Geider 2004) but in that test only bacteria of other *Erwinia* species were included. However, based on the BLAST analysis (Altschul et al. 1990) the sequences of primers showed high homology and coverage to other bacteria, also belonging to *Pseudomonas* and *Xanthomonas* genus. This is quite important, because these bacteria, particularly *Pseudomonas* spp., are ubiquitous and are very often isolated from plant material. The disadvantage of both Real-time PCRs is the fact that the primers and probe are complementary to the sequence of the plasmid pEA29. Actually, pEA29 was historically the first target for designing the primers for *E. amylovora* detection (Bereswill et al. 1992) because it was thought that this plasmid is ubiquitous in all *E. amylovora* strains and due to its copy number, primers based on this plasmid will be more sensitive than chromosomal primers. Since then, several strains lacking pEA29 were found (Llop et al. 2006; Mohammadi et al. 2009), which means that application pEA29-based diagnostic system as the only technique can be risky.

Real-time PCRs appeared to be very sensitive and repeatable. Similar sensitivity was shown also by LAMP which has many advantages over PCR: it is reported to be faster, more specific, simpler to learn and interpret, not sensitive to plant origin compounds, doesn't require sophisticated equipment and is more adaptable to on-site implementation for field diagnostics (Gill & Ghaemi 2008; Fang et al. 2010). However, the low repeatability of this technique, often observed in case of samples with low concentration of bacteria (F. Rezzonico, personal communication) can be the limit in reliable detection of pathogens.

Detection of *E. amylovora* in asymptomatic plants, although very important from a phytosanitary point of view, has been shown to be difficult (Anonymous 2013). In our study, although *E. amylovora* was detected in asymptomatic plant material with almost all techniques and methods used, it was not equivalent to the occurrence of fire blight symptoms in the orchards from where samples were collected. On the other hand, it could be connected to regular control treatments with copper preparations, which were done during the season.

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