ISSN 1392-3196
Zemdirbyste-Agriculture, vol. 95, No. 3 (2008), p. 19–26
UDK 634.1:632.3

A THREE-YEAR STUDY OF FIREBLIGHT IN LITHUANIA

Laima BARANAUSKAITĖ, Vaida JOGAITĖ, Lina JANKUVIENĖ

Phytosanitary Research Laboratory
State Plant Protection Service of Lithuania
Sukilėlių g. 9A, Vilnius, Lithuania
E-mail: vaatla@vaat.lt

Abstract

Lithuania has a protected zone status in respect of a quarantine bacterium *Erwinia amylovora* that causes devastating infections of fireblight. Seeking to detect, identify and determine the distribution of bacterium, monitoring of fireblight was performed in Lithuania. For the first time fireblight was observed on hawthorn with symptoms during surveys in 2005.

During the next two years despite quarantine measures fireblight was established in eight regions of the country. 183 symptomatic and 9 symptomless fireblight infections were detected using different diagnostic methods – immunofluorescence (IF), plating, bioassay, nutritional and enzymatic tests and nested polymerase chain reaction (PCR).

Key words: monitoring, *Erwinia amylovora*, symptomatic and latent (symptomless) infections.

Introduction

Fireblight – the causal agent *E. amylovora* (Burr.) Winslow et al. – has become one of the destructive bacterial diseases of fruit trees of family *Rosaceae* in Lithuania.

In our country, the disease has been monitored since 1998 /Baranauskaite, 2004/. Economically most important hosts of bacterium are apple and pear trees in our country. The area of apple plantations covers 18,616 ha, 14,856 ha of them – fruit bearing area, pear plantations make up 1,221 ha and 946 ha, respectively. Ornamentals such as hawthorn, cotoneaster and mountain ash are very popular in home gardens design and are not less important hosts of pathogen.

Lithuania is characterized by maritime climate in the west changing into continental in the east with high possibility of spring frosts after warm and dewy days during blossom. Despite the opinion that the northern half of Europe (above 48th parallel) has fewer problems with the disease than the southern half /Van der Zwet, 1996/, due to climate changes the bacterium attacks very aggressively gardens and nurseries in Lithuania. The development of fireblight symptoms follows the seasonal growth development of the host plant /Van der Zwet, Beer, 1995/.

In the first year, three distinct kinds of infections described by Steiner & Lightner (1996) were observed: bloom blight, canker blight and trauma blight. The next year spring was very cold and no blossom blight of fruit tree was found, but bright orange shoot tips demonstrated canker blight.

Fireblight has passed the borderline of our northern neighbour Latvia. In 2007 the National Plant Protection Service of Latvia announced the first finding of *Erwinia amylovora* in Latvia /First report, 2007/. This outbreak was detected near the northernmost focus of fireblight in Lithuania.

E. amylovora causes rapid necrosis of the phloem its hosts /Janse, 2005/. It can survive as an epiphyte /Thomson, 2000/ or endophyte /Vanneste, Eden-Green, 2000/ and cause latent (symptomless) infections whose detection is not often successful in artificially inoculated hibernating shoots /Crepel et al., 1996/ or symptomless apple scion tissues /Momol et al., 1996/. Besides enrichment isolation, enriched bioassay and IF analysis method of molecular biology – nested-PCR based on amplified fragment analysis of plasmid pEA29 was used. This 29-kb plasmid is involved in bacterial metabolism of thiamine /Laurent et al., 1989/ and according to other studies, all *E. amylovora* strains with very few exceptions possess it. There is no known homology of plasmid pEA29 to the DNA of other plant pathogenic bacteria in nature.

The aim of our study was to review the occurrence of *E. amylovora* in Lithuania by using different diagnostic methods for pathogen detection in our routine surveys.

Materials and Methods

Monitoring involves periodical surveys of the orchards, nurseries, home gardens, host plants growing individually or in small groups, as well as the surrounding zone with the radius of 250 m at least twice a year: the end of May–July and the end of August–beginning of October.

Sampling and screening tests of symptomatic samples. Symptomatic samples (typical 'shepherd's crook' in the shoots, shrived turned brown to black flowers, leaves, oily or water–soaked then sinked or cracked bark with droplets of bacterial ooze, reddish-brown streaks in subcortical tissues) from naturally infected plants were prepared according to diagnostic protocol /OEPP/EPPO, 2004/. Immunofluorescence (IF), plating on King's B medium and sucrose nutrient agar (SNA) or Levan medium, CCT medium /Lelliot, Stead, 1987/, PCR were performed as screening tests for samples with symptoms following the protocol. In order to escape false positive *E. amylovora* detection caused by cross reaction, *E. amylovora* positive samples or samples with atypical *E. amylovora* bacteria (atypical cell size, wall structure etc.) we tested by PCR. In the cases when the concentration of *E. amylovora* in the extract of sample was very high ($\geq 10^8$ cells/ml) and cells were with typical morphology PCR analysis was not performed.

DNA isolation from symptomatic samples. DNA for PCR analysis was extracted from *E. amylovora* bacteria cultivated on SNA or CCT agar media: one colony of bacteria was transferred to 500 µl of sterile distillate water. Suspension was heated at 95^{o} C for 13 min and centrifuged at 10 000 x g for 2 min. Five microliters of supernatant were used for the first PCR with external primers and 1 µl of 1000 times diluted PCR product (approximately 1 ng DNA) was used for the second PCR with internal primers.

PCR analysis and digestion of PCR products. Nested-PCR was carried out by using two pairs of primers based on *E. amylovora* plasmid pEA29: the external primers: A: 5'-CGG TTT TTA ACG CTG GG-3'/ B: 5'GGG CAA ATA CTC GGA TT-3' /Bereswill et al., 1992/ and internal primers: AJ 75: 5'-CGT ATT CAC GGC TTC GCA GAT-3'/ AJ 76: 5'-ACC CGC CAG GAT AGT CGC ATA-3 /McManus, Jones, 1995/.

The 25 µl PCR mixture contained 1x Taq buffer with 500 mM KCl (Fermentas, Lithuania), 1.5 mM $MgCl_2$ (Fermentas, Lithuania), 10 mM 2-mercaptoethanol (1:10) (Sigma, USA), 0.16 mg/ml BSA (Fermentas, Lithuania), 5% DMSO (Sigma, USA), 1% Tween 20 (Sigma, USA), 0.2 mM dNTPmix (Fermentas, Lithuania), 0.5 µM forward and reverse primers (The Biopolymer Factory, Germany), 1U Taq DNA polymerase (Fermentas, Lithuania).

Positive (DNA extracted from *E. amylovora* pure culture) and negative (molecular grade water) controls were included in all PCRs.

The DNA amplification was performed in Mastercycler personal (Eppendorf, Germany) in the following conditions: initial DNA denaturation at 93° C for 2 min., followed by 30 cycles of 93° C for 1 min, 52° C for 1 min, 72° C for 1 min. After the final reaction cycle the PCR mixture was incubated at 72° C for 5 min. 10 µl of the PCR products were mixed with 2µl 6x Loading Dye Solution (Fermentas, Lithuania) and analyzed in 1 x TBE buffered 1.5% agarose gel for 1 h at 80 V (6 V/cm), stained with ethidium bromide (0.5 µg/ml) and visualized under UV light. PCR products were diluted 1000 times and 1 µl of solution (approximately 1 ng DNA) was used for the second PCR with internal primers under the same conditions.

Restriction patterns of PCR products obtained with internal primers were examined with restriction endonucleases *Bgl II* and *Taq I* (Fermentas, Lithuania) by incubation of samples for 2.30 hours at 37° C and 65° C respectively.

Sampling and screening tests of symptomless samples. Samples from symptomless plant material were collected according to phytosanitary procedure /OEPP/EPPO, 1992/ for analysis of plants growing in nurseries. We used this method for sampling from orchards, hedges etc. The sample composed of 100 (if possible) twigs about 10 cm in length from 100 different plants, but not less than 30 twigs per sample. From each sample, randomly we took 30 twigs, cut into four pieces (120 stem pieces), put into plastic bag with sterile PBS and 0.1% Tween 20 /OEPP/EPPO, 2004/ and shaked at room temperature for 1.5 h. After centrifugation for 20 min. at 10 000 x g the pellet was suspended in 4.5 ml sterile PBS. According to EPPO diagnostic protocol, as screening tests were used IF and nested-PCR analysis. In order to multiply the initial population of *E. amylovora* in the sample extract an enrichment step was included in our study. Two liquid media: non-selective King's B and semi-selective CCT were used in parallel. One millilite of suspended pellet of the sample was poured into each of the two tubes prepared in advance with four milliliters of each sterile medium. The tubes were incubated without shaking at 25° C for 72 h. Enriched extract from both tubes was performed for nested-PCR test and isolation by plating on CCT medium. 50 µl of each enriched extract and of the 1/10 up to 1/10000 dilutions prepared in sterile distilled water was spread onto CCT medium. Plates were incubated at 27° C for 72–96 h.

In few cases bioassay – prick-inoculation of immature pear fruits – was successfully performed as enrichment. Fruits were washed, sterilized for 30 min. with 70% alcohol, repeatedly washed with sterile distilled water. The halves of fruits were inoculated with suspended pellet and kept at 25° C at about 80% relative humidity. After 5–7 days probably contaminated extract was taken from prick and tested by nested-PCR and plating as described above.

The interpretation of IF reading (calculation of cells) was done according to the Commission Directive (2006).

DNA isolation from symptomless samples. DNA of *E. amylovora* was extracted as described by Pastrik & Rainey (1999) after an enrichment step in CCT or King's B medium, which was removed before DNA extraction: 1 ml of culture suspension was centrifuged at 15 000 x g for 5 min. The pellet was resuspended in 500 µl of PBS buffer /OEPP/EPPO, 2004/.

PCR conditions for DNA amplification were the same as mentioned above.

Identification and pathogenicity. The identification of pure cultures of bacterial isolates was carried out according to the nutritional and enzymatic tests described by EPPO diagnostic protocol.

The pathogenicity of all isolates was tested by tobacco (*Nicotiana tabacum* 'Samsun') hypersensitivity reaction (HR). Collapse of the infiltrated tissue after 24 h was recorded as positive reaction.

Confirmation. Every identified *E. amylovora* strain was confirmed by IF and double pathogenicity on immature fruits of pears and pear shoots or pear *Pyrus communis* 'Piguva Davidov' seedlings. Aquatic bacterial suspension of 10^8 cfu/ml was used for inoculation by syringe. Inoculated fruits and shoots were kept in wet chamber. Production of typical bacterial ooze verified pure culture as *E. amylovora*.

Control tests (positive and negative) were used in parallel with all tests performed for detection and identification of *E. amylovora* in the samples in our study. As control material we used *Erwinia amylovora* reference strains PD 437 and NCPPB 683. For IF tests we used polyclonal antibodies, validated in ring tests from Loewe Biochemica GmbH.

Results and Discussion

Due to the absence of spring frosts and excellent environmental (climatic) conditions for multiplication of bacteria the primary infection of symptomatic fireblight was detected in 2005 on the hedge of a 30-year old hawthorn *Crataegus monogyna* Jacq. The first detection and identification of causative agent of fireblight *Erwinia amylovora* was done in the north of the country (Šiauliai region) at the beginning of June. Gram negative, facultative anaerobes, motile, urease and oxidase negative, levan producing, gelatine hydrolysing, reducing substances from sucrose, acid producing from glucose, fructose, galactose bacteria were identified as *E. amylovora.* The identification of a representative isolate LT 61 was confirmed by the Central Science Laboratory in York, UK. After two weeks a few plants of hawthorn and cotoneaster were found infected by *E. amylovora* in the nursery near the contaminated hedge. The same year nine new outbreaks were confirmed near these two foci. The same month a big outbreak site (overlapped survey zones within the radius of 1.000 m from focus and buffer zones within 50 km^2) was found in the northwest of the country on apple trees in an old industrial orchard. The last detection of fireblight was in the middle of October. In total 107 samples were positive in regard to *E. amylovora*. In a few cases it was suspicious that no symptoms were observed, especially on apple tree shoots. After the repeated detailed examination specific orange tips of twigs and small cankers were observed. In

2005 17 outbreak sites of fireblight were recorded in Šiauliai, Klaipėda, Telšiai, Vilnius, Panevėžys, Kaunas and Tauragė regions.

Winter 2006 was very cold with a lot of snow, spring was quite cold and late, the beginning of summer was warm but dry. There were no blossom blight that year, but canker blight was detected for the first time in the middle of May on cotoneaster. Fireblight was observed on hawthorn, pear at the end of spring – beginning of summer. But the main spread of fireblight occurred in the second half of summer or even in the autumn when warm and humid weather had set in. During 2006 ten outbreak sites were identified, seven of which were identified repeatedly. The outbreaks were confirmed in Šiauliai, Klaipėda, Telšiai, Marijampolė, Kaunas and Tauragė regions.

Despite very strict measures – trees and shrubs were eradicated and burned – new outbreak sites were detected in 2007, fireblight for the first time was identified in a fruit tree nursery. Climatic conditions were very favourable for the spread of bacteria into new territories in the north of the country. All types of infection were found with the exception of rootstock blight that year. Eleven outbreak sites were detected in five regions (Šiauliai, Telšiai, Panevėžys, Kaunas and Marijampolė) of the country.

The pathogen was spread in eight regions (from ten) of the country and covered 37 outbreak sites in a three-year period (Table).

Table. Summary of investigation of *Erwinia amylovora* during three years' monitoring

Year	Number of outbreak sites	Number of regions	Samples investigated by IF, plating, nutritional, HR, enzymatic assays		Samples investigated by PCR assays	
			Number of investigated samples	Number of positive samples	Number of investigated samples	Number of positive samples
2005	17	7	379	107	–	–
2006	10	6	280	22	13	5
2007	11	5	334	54	94	52
In total			993	183	107	57

During monitoring bacteria were isolated from host plants belonging to six genera of *Rosaceae* family (Figure 1): *Crataegus* L., *Malus* Mill., *Pyrus* L., *Cotoneaster* Ehrh., *Sorbus* L., *Chaenomeles* Lindl.

Hawthorns were determined as very influential sources of fireblight inoculums in Lithuania.

During the three years' monitoring 993 samples were tested, 269 of which were expected to be positive by visual examination. Only 183 samples were established as positive according to *E. amylovora* after the detailed testing. The biggest part of samples – 724 were supposed asymptomatic, 9 of which after IF, nested-PCR, enriched bioassay, plating on semi-selective CCT medium were detected to be contaminated with quarantine bacterium. Isolates from asymptomatic two hawthorn, two pear and five apple samples were identified and pure cultures as *E. amylovora* were confirmed. In two cases after positive PCR and enriched bioassay isolation of bacterium was not successful.

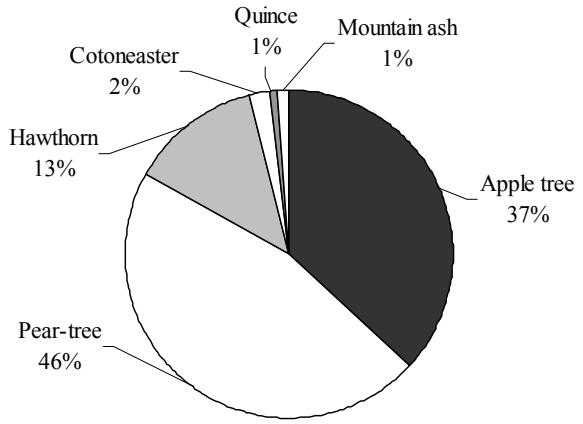

Figure 1. The range of infected host plants during the period 2005–2007

Detection of *E. amylovora* with PCR method. PCR analysis for monitoring of *E. amylovora* in Lithuania has been applied since 2006. During the two years 107 DNA samples were tested with PCR method, 57 samples of which were found to be infected with *E. amylovora*.

DNA samples, which possessed *E. amylovora* specific 900–1100 bp PCR product produced with external primers A and B or/and 750 bp length PCR product amplified with internal AJ 75 and AJ 76 primers were estimated as *E. amylovora* positive samples (Figure 2).

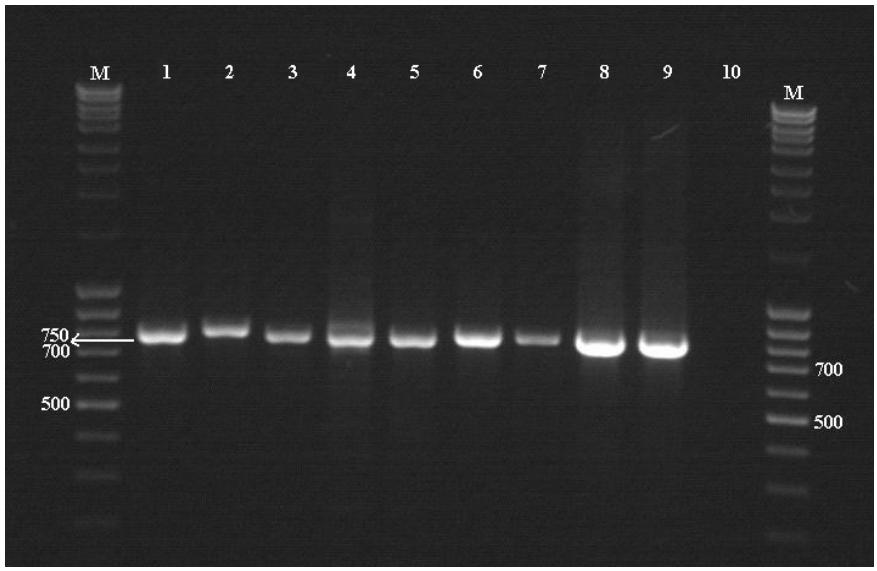

Figure 2. Gel electrophoresis of nested PCR of E. amylovora, extracted from different outbreak sites in Lithuania: 1 – Kaunas, 2 – Marijampolė, 3 – Telšiai, 4 – Panevėžys, 5 – Šiauliai, 6 – Tauragė, 7 – Klaipėda, 8 – Alytus, 9 – positive control of E. amylovora, 10 – negative control (molecular grade water), M – DNA size markers

In our study, a few DNA samples produced slightly longer than 750 bp PCR products. Nevertheless, DNA restriction analysis of PCR products with *Taq I* and *Bgl II* revealed the same number and similar size of restricted fragments in all samples supporting the identity of PCR products. This can be explained by the variability in the number of 8 bp repeats in the amplified sequence of pEA29 described by other authors /Lecomte et al., 1997; Schnabel, Jones, 1998/. Analysis of the 8 bp repeats in fragment of plasmid pEA29 revealed high variance in the number of this repeats. Depending on *E. amylovora* strain it can range from 3 to 14.

High concordance of PCR results with *E. amylovora* detection by other methods (IF, platig etc.) was observed in our study. Only in two cases after positive PCR and enriched bioassay, isolation of bacterium was not successful. In our opinion, a possibility of non-specific DNA amplification, low yield of PCR product, when it is not possible to confirm PCR results with RFLP analysis and non-viable bacteria could explain this contradiction.

In order to estimate the influence of enrichment conditions on PCR results two different media were used in this step – semi-selective CCT medium and non-selective King's B medium. Our primary PCR results revealed that DNA amplification was more successful and PCR bands were stronger when DNA was extracted from bacterium culture cultivated in King's B medium, on the other hand, several unspecific amplification bands appeared more common, when bacteria were cultivated in this medium. Besides, we observed variance among PCR results received from bacteria cultivated in semi-selective CCT and non selective King's B media: some *E. amylovora* positive DNA samples extracted from bacteria cultivated in King's B medium showed *E. amylovora* negative assay when they were cultivated in CCT medium. Only in one case *E. amylovora* positive sample extracted from bacteria cultivated in CCT medium was detected as *E. amylovora* negative after bacteria cultivation in King's B medium. Up to now, we have not found any studies about possible influence of bacteria cultivation medium on PCR results, but it is evident, that circumstantial studies are needed in order to reduce the possibility of misleading results.

Conclusions

Our study revealed, that natural latent infection of fireblight could be detected using several enriched methods in unison: nested-PCR, enriched bioassay, enriched plating. Furthermore, increasing the reliability of detection and identification of causal agent of the disease could help to prevent a quick as a flash spread of fireblight in new areas, to decrease the number of outbreak sites in our country.

The fireblight was detected in the eight out of ten regions of the country. Hasty epidemics of the disease in areas away from one another suggest that bacteria spread naturally. But other reasons of fireblight occurrence could be ascertained by a more comprehensive examination of the strains of *Erwinia amylovora*.

Received 2008-06-02
Accepted 2008-08-22

REFERENCES

1. Baranauskaite L. Fireblight monitoring in Lithuania // Bulletin OEPP/EPPO Bulletin. – 2004, vol. 34, p. 335–337

2. Bereswill S., Pahl A., Bellemann P. et al. Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis // Applied and Environmental Microbiology. − 2006, vol. 58, p. 3522–3526

3. Commission Directive 2006/63/CE of 14 July 2006 amending Annexes II to VII to Council Directive 98/57/EC on the control of *Ralstonia solanacearum* (Smith) Yabuuchi et al. // Official Journal of the European Union. – 2006, p. 85

4. Crepel C., Geenen J., Maes M. The latent survival of *Erwinia amylovora* in hibernating shoots // Acta Horticulturae: VII International Workshop on Fire Blight / ed. W. G. Bonn. − St Catherines, Ontario (Canada), 1996, p. 21–26

5. First report of *Erwinia amylovora* in Latvia // EPPO Reporting Service. – Paris, 2007, No. 8, p. 2

6. Janse J. D. Phytobacteriology: principles and practice. – Wallingford (UK), 2005, p. 125–127, 146

7. Laurent J., Barny M. A., Kotoujansky P. et al. Characterization of a ubiquitous plasmid in *Erwinia amylovora* // Molecular Plant-Microbe Interactions. – 1989, vol. 2, p. 160–164

8. Lecomte P., Manceau C., Paulin J. et al. Identification by PCR analysis on plasmid pEA29 of isolates of *Erwinia amylovora* responsible for an outbreak in Central Europe // European Journal of Plant Pathology. – 1997, vol. 103, p. 91–98

9. Lelliot R. A., Stead D. E. Methods for the diagnosis of bacterial diseases of plants // Methods in Plant Pathology. – Oxford (GB), 1987, vol. 2, p. 176–177, 182, 190–191

10. McManus P. S., Jones A. L. Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse blot hybridizations // Phytopathology. – 1995, vol. 85, p. 618–623

11. Momol M. T., Momol E. A., Norelli J. L. et al. Detection of systemic spread of *Erwinia amylovora* through symptomless apple scion tissues into the rootstocks // Acta Horticulturae 411: VII International Workshop on Fire Blight / ed. W. G. Bonn. − St Catherines, Ontario (Canada), 1996, p. 77–78

12. OEPP/EPPO. EPPO Standards PM 3/40 *Erwinia amylovora*. Sampling and test methods // Bulletin OEPP/EPPO Bulletin. – 1992, vol. 22, p. 225–231

13. OEPP/EPPO. Diagnostic protocols for regulated pests PM 7/20(1) *Erwinia amylovora* // Bulletin OEPP/EPPO Bulletin. − 2004, vol. 34, p. 159–171

14. Pastrik K. H., Rainey F. A. Identification and differentiation of *Clavibacter michiganensis* subspecies by polymerase chain reaction-based techniques // Journal of Phytopathology. – 1999, vol. 147, p. 687–693

15. Schnabel E. L., Jones A. L. Instability of a pEA29 marker in *Erwinia amylovora* previously used for strain classification // Plant Disease. – 1998, vol. 82, p. 1334–1336

16. Steiner P. W., Lightner G. W. MARYBLYT 4.3, a predictive program for forecasting fire blight disease in apple and pears. – Maryland (USA), 1996, p. 1–12

17. Thomson S. V. Epidemiology of fireblight // Fire blight, the disease and its causative agent, *Erwinia amylovora* / ed. J. L. Vanneste. – Wallingford (GB), 2000, p. 11–13

18. Van der Zwet T. Present worldwide distribution of fire blight // Acta Horticulturae 411: VII International Workshop on Fire Blight / ed. W. G. Bonn. − St Catherines, Ontario (Canada), 1996, p. 7–8

19. Van der Zwet T., Beer S. V. Fire blight – its nature, prevention and control. A practical guide to integrated disease management // Agriculture Information Bulletin. – Washington, 1995, No. 631, p. 69–82

20. Vanneste J. L., Eden-Green S. Migration of *Erwinia amylovora* in host plant tissues // Fire Blight, the Disease and its Causative Agent, *Erwinia amylovora* / ed. J. L. Vanneste. − Wallingford (GB), 2000, p. 74–81