

First Occurrence of Fire Blight on Apricot (*Prunus armeniaca*) in Hungary

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Abstract

During July 2012, a severe unusual disease symptom was observed on young shoots on apricot (*Prunus armeniaca* 10/13 hybrid) in the city of Pomáz, near Budapest. The naturally infected shoots showed typical symptoms of fire blight including terminal shoots with brown to black necrotic lesions. Symptoms were the same as fire blight symptoms reported from other hosts and locations. The first occurrence of fire blight on an apricot tree in Europe was recorded in Czech Republic in 2011. Samples of the leaves and shoots with symptoms were macerated and spread on King's medium B. After 24 hours of incubation at 26 °C, bacteria morphologically similar to *E. amylovora* were detected. Isolate induced hypersensitive reaction on tobacco (*Nicotiana tabacum* L. 'White Burley') leaves. Biochemical test was also used for identification, and the result of API 20E kit (Biomérieux, Marcy l'Etoile, France), demonstrate that the bacterium belongs to Enterobacteriaceae family. A pathogenicity tests were positive on young apricot shoots and immature fruits. For molecular identification of the pathogen the 16S rDNA region was amplified from isolate Ea-ApricotPo1 with a general bacterial primer pair (63f forward and 1389r reverse). The PCR products were cloned into a pGEM T-Easy plasmid vector (Promega, Madison, WI USA) and were transformed into *Escherichia coli* DH5α cells. A recombinant plasmid (2A2.5) was sequenced by M13 forward and reverse primers. The sequence was deposited in GenBank (Accession No. HF546214) and showed 99-100% sequence homology with a number of *E. amylovora* isolates, including type strain FN666575 with 100% similarity. On the basis of the symptoms, colony morphology, biochemical tests, and 16S rDNA sequence homology, the pathogen was identified as *E. amylovora*. This is a first record of a natural outbreak of fire blight on apricot in Hungary.

Keywords: apricot, *Erwinia amylovora*, fire blight, stone fruits

Introduction

The serious disease of apple, numerous ornamental and wild plants (van der Zwet and Keil, 1979), called "fire blight" is caused by the bacteria *Erwinia amylovora* (Burrill) Winslow *et al.* (1920). According to the recommendations of the EPPO, the European and Mediterranean Plant Protection Organization, it is throughout Europe – and therefore in Hungary as well – a harmful quarantine pest. *Erwinia amylovora* is a gram-negative bacterium. This necrotic disease is sporadic but it can be very destructive for some very susceptible genotypes of Maloideae. *E. amylovora* can infect leaves, shoots, rootstocks and fruits as well. Symptoms first appear on young succulent shoots, which turn black and may show droplets of ooze.

The disease endemic in the USA and known for about 200 years had been introduced to Europe (England) and the basin of the Mediterranean (Egypt) in the middle of the 1950-s, and has been spread now to almost all of the countries of the region. In the last 25 years this pathogen became widespread all over the world. The illness was detected first in Hungary in summer 1995 close to Nyárlőrinc in a 5-6 year old 43,5 acre size apple plantation (Hevesi, 1996). Since its appearance the damage caused by the bacteria can be serious in years provided the weather is favorable for the bacteria.

Within the *Rosaceae* family 200 species belonging to 40 genres can be regarded as its host plant (Steiner and Zeller, 1996). The most important host plants from view of commerce are the species from the *Cotoneaster*, *Crataegus*, *Cydonia*, *Malus*, *Pyrus*, *Photinia*, *Pyracantha* and *Sorbus* genus. The hawthorn myrtle (*Stranvaesia davidiana*) and loquat (*Eriobotrya japonica*) are also host plants, though they are not as important in our country. The disease has also been detected in the USA on thornless blackberries (*Rubus rusticanus* var. *inermis*) (Evans, 1996) and raspberry (Schnabel and Jones, 2001), but these are not infectious on apples and pears. Reports have been issued about the natural infection of the Japanese plums (Mohan and Thomson, 1996). Publication have been issued about the drying of spouts of plums and apricots in the USA, (Mohan, 2007), and the natural infection of European plums in Germany and Hungary (Vanneste *et al.*, 2002; Végh *et al.*, 2012) and apricots in the Czech Republic (Korba and Sillerova, 2010).

During July 2012, a severe unusual disease symptom was observed on young shoots on apricot (*Prunus armeniaca* 10/13 hybrid) in the city of Pomáz, near Budapest. The naturally infected shoots showed typical symptoms of fire blight including terminal shoots with brown to black necrotic lesions. Symptoms were the same as fire blight symptoms reported from other hosts and locations.

Materials and methods

Isolation and propagation

The infected stems were first disinfected with ethanol. Samples then were taken under sterile conditions from the border of the dead and healthy plant parts. Next the plant tissues were homogenized with distilled water and transferred to King-B agar medium (King *et al.*, 1954). The Petri-dishes were incubated at 26 °C for 1-2 days. The separate colonies were then inoculated into sterile medium. Clean cultures were made and incubated at 26 °C. The plates were then stored at 4 °C. The bacterium was typified by colony types (Mazzucchi, 1977). The bacterium was evaluated after 24-48 hours, under microscope. The colony types were distinguished by consistence, shape, surface, margin and color (Puskás, 1986; Klement *et al.*, 1990).

Classical bacteriological tests

Identification of Gram-features

24 hours after inoculation, 1-2 colonies from the clean, fresh medium are placed on sterile slides. Three percent potassium hydroxide were added and then homogenized. The bacteria is Gram-negative in case the potassium hydroxide dissolves the cell wall (the mixture is stinky), while the bacteria is Gram-positive if the cell wall is left intact (the mixture is watery) (Suslow *et al.*, 1982).

Hypersensitive reaction

The bacterium suspension (5×10^7 cells/ml) was injected into tobacco leaves (*Nicotiana tabacum* L. cv. *xanthi*). After 24-48 hours hypersensitive reaction (tissue necrosis) was monitored (Klement, 1963).

Pathogenicity test

Pathogenicity tests (Koch, 1976) were carried out in 4 repetitions on each isolates. The surfaces of the plants used in the pathogenicity test were disinfected with ethanol.

The fresh, young apricot shoots were 20-25 cm long when inoculated. The bacterium suspension (5×10^7 cells/ml) was injected with a syringe into the base of the second fully matured leaf from the top of the shoot. In order to assure optimal conditions for the infection to spread, the shoots were then kept in the laboratory, in 80-90% relative humidity at 25-27 °C. The untreated control was stung with a sterile syringe with distilled water. The control was kept under the same conditions as the treated ones only separated.

Contamination of the fruits (apricot) was carried out under sterile conditions. The fruits were stung 3 places with bacterium suspension (5×10^7 cells/ml). The control was again treated with distilled water. The treated fruits were incubated in 80-90% relative humidity at 25-27 °C.

Evaluation of the pathogenicity tests were done after 5 days on the fruits and 18 days on the shoots. The results were given after the analysis of the typical symptoms of

Erwinia amylovora (van der Zwet and Keil, 1979): brownish, blackish colorization and the deformation of the shoot and the water-soaked tissue lesions on the fruits.

Biochemical characteristics

Newly isolated *Erwinia amylovora* isolates was studied with API 20E (Biomérieux, Marcy l'Étoile, France) strips. The instruction of the manufacturer (Biomérieux, Marcy l'Étoile, France) was followed during API 20E test. In case of each isolate, 5×10^7 cells/ml bacterium suspension was used for the sample places containing special media of the kits. Test was incubated on 36 °C and evaluated after 24-48 hours. The evaluation of the test is based on color change. The evaluation of the API 20E test was done with the help of positive and negative test strips provided by the manufacturer.

Molecular bacteriological tests

Amongst molecular bacteriology tests, for the identification of bacteria and for taxonomy studies, determination of the sequence encoding 16S rRNA is the most common method (Choi *et al.*, 1996; Clarridge, 2004). Since detecting the presence of *Erwinia amylovora* on apricot in Hungary has high significance, it was especially important to identify the isolate by determining the sequence of 16S rDNA.

DNA was recovered 24 hours after the inoculation of bacteria on King-B media. For 16S rDNA sequence determination universal primers (63f: 5'-CAGGCCTAACACATGCAAGTC-3', 1389r: 5'-ACGGGCGGTGTGTACAAG-3') were used. In the reaction was carried out in a Perkin-Elmer 9700 thermocycler (Applied Biosystems) with an initial denaturation step at 94 °C for 3 min followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, elongation at 72 °C for 1.5 min, and a final elongation at 72 °C for 10 min. Amplified products were cloned into pGEM-T Easy (Promega) and sequenced by using M13 reverse and M13 forward primers and the ABI PRISM BigDye Terminator Cycle Sequencing kit on ABI PRISM 310 instrument (Applied Biosystem). Sequence analysis was performed using CLC SEQUENCE Viewer 6.5.1 program package. The recombinant plasmid sequence was determined and then matched to homologue sequences found in the international databases (NCBI Genebank-National Center for Biotechnology Information).

Results and discussion

Identification of the bacteria with classical methods

Symptoms caused by Erwinia amylovora

Brown or black, typically deformed, shepherd's crook-like shoots were collected from young apricot trees (*Prunus armeniaca* 10/13 hybrid) (Fig. 1). The symptoms implied the presence of *E. amylovora*.

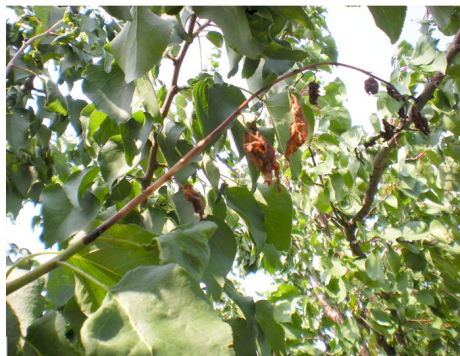


Fig. 1. Symptom caused by the bacterium *Erwinia amylovora* on young apricot shoot

Colony type

The bacterium colonies on King-B media were uniform, milky and cream-colored, smooth surfaced with intact outlines (Fig. 2)



Fig. 2. *Erwinia amylovora* colonies on King-B agar

Gram-test

Since the 3% potassium hydroxide solution dissolved the cellular wall of the bacteria, the isolates collected from the different host plants proved to be Gram-negative.

Hypersensitive reaction

On the leaves of the tobacco plants inoculated with 5×10^7 cell/ml suspension of isolates, quick tissue necrosis formed after 24-48 hours, which is a sign of hypersensitive reaction.

Pathogenicity test

The test plants inoculated with bacterium suspensions showed infection. The apricot fruits and the apricot shoots (*Prunus armeniaca* 10/13 hybrid) showed intensive reaction and formed typical symptoms after 5-18 days. The in vitro test was successful. On the apricot fruits sagged, brown spots formed around the inoculation (Fig. 3). The shoots became brown or black and crook-like. The bacterium was re-isolated from lesions on inoculated shoots, fulfilling Koch's postulates. No lesions were observed on controls.

The biochemical properties of the *Erwinia amylovora* isolate

Result of the API 20E test

The API 20E test is used for the determination of the species belonging to the *Enterobacteriaceae* family. The collected isolates were tested with API 20E kits in order to identify them as *E. amylovora*. The isolates showed positive reaction for β -galactosidase and citrate utilization, acetoin production and also in glucose, mannitol, sorbitol, saccharose, melibiose and arabinose reactions. The results were negative in case of arginin-dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S production, urease, tryptophan deaminase, indole production, gelatinase, inositol, rhamnose, amygdalin tests. The biochemical properties of the tested isolate matched the description of *Erwinia amylovora* given in the API 20E kit.



Fig. 3. Artificial infection with *Erwinia amylovora* on apricot fruits

Molecular test of the 16S rRNA gene of the isolate collected from apricot.

The PCR product of the reaction was about 1300 bp long. The 16S rDNA nucleotide sequence (1323 bp) of the partially sequenced Hungarian Ea-ApricotPo1 isolate were sent to the international databank. Here it can be found under the accession number HF546214.

The sequence of the isolate collected from apricot was matched with the sequences found in the international data base. The sequence of the fragment showed 100% homology with one isolate: number FN666575 (English, host plant: *Malus* sp.). There were other matches with 99% homology with samples from different host plants (*Malus* sp., *Pyrus* sp., *Rubus* sp., *Prunus* sp.).

Conclusions

On the basis of the symptoms, colony type, biochemical tests, pathogenicity and 16S rDNA sequence homology, the pathogen was identified as *E. amylovora*. Natural infection of the pathogen only was reported on apricot in the Czech Republic. In Hungary the pathogen of fire blight was not identified on apricot yet. The occurrence of *Erwinia amylovora* on apricot could cause serious problem to Hungarian fruit growers in the future. It enables to suppose to appearance the pathogen on the other stone fruits as well. This is a first record of a natural outbreak of fire blight on apricot in Hungary.

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