

Causative Pathogen of Fire Blight of Fruit Trees in Georgia

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Abstract. Fire blight is a devastating disease of fruit trees that first appeared in Georgia in 2016. Above 40 samples – fruit trees twigs, buds, flowers and fruitlets, suspected on the fire blight disease were collected in the Mtskheta-Mtianeti and Shida Kartli Regions, eastern Georgia in Summer of 2020. Based on visual observation and immunological test (Ea AgriStrip), 20 plant samples were selected for further study. PCR analysis was performed to detect pathogen in these samples with primers pair A: 5'-CGG TTT TTA ACG CTG GG-3' and B: 5'-GGG CAA ATA CTC GGA TT-3' and 2XPCR BIOTAG Mix using PCR BIOSYSTEMS. The pathogen *Erwinia amylovora* was detected in 6 samples: N10 and N11 apple samples (Ksovrisi); N12 apple sample (Kekhijsvari); N14 apple sample (Karaleti); N16- apple sample (Tirdznisi); N27 pear sample (Osiauri). King's B, NSA and CCT media were used for the isolation of the pathogen from PCR-confirmed diseased samples serial dilutions. For the identification of *Erwinia amylovora*, PCR was performed in 25 µl reaction mixture with primers pair G1-F: 5'-CCT GCA TAA ATC ACC GCT GACAGC TCA ATG 3' and G2-R: 5GCT ACC ACT GAT CGC TCG AATCAA ATC GGC3'. The presence of *E. amylovora* was confirmed in the following isolates, ## 1012, 1022, 1023, 1131, 1133, 1212, 1412, 1612, 1742, and 2763. Study of the physiological-biochemical properties of the *E. amylovora* Georgian isolates revealed a little difference in the sugars utilization and the esculin hydrolysis abilities.

Keywords: Fire blight of fruit trees, *Erwinia amylovora*, PCR

Phytopathogenic bacteria affect a wide range of crops worldwide. *Erwinia amylovora* is a pathogen, causative of a fire blight disease, distinguished by its harmfulness. It damages various cultural and wild plants of the fruit trees, in particular 180 cultural and wild plants belonging to the *Rosaceae* family (Pulawska and Sobiczewski P. 2012). Apple, pear and quince are the most susceptible to the disease (OEPP/EPPO Bulletin, 2013).

The fire blight disease causes enormous economic losses around the world and poses threat of eradication to entire fruit tree garden. In 1971-1972, during the fire blight initial wave, 18 000 plants of thornapple and pears were destructed in Germany, and 10km thornapple hedges and about 175 000 bushes – in Holland (Van der Zwet and Keil 1979).

Erwinia amylovora is included in the European and Mediterranean Plant Protection organization (EPPO) A2 list of quarantine organisms (EPPO 2007). It is also registered in the list of quarantine organisms of Australia (AQIS 1998) and South Africa. Since 2010 *E.amylovora* is included in list of quarantine organisms in Georgia.

The fire blight disease was first observed in New York State in 1780, detected in New Zealand in 1910, in UK in 1958 and then spread progressively accross Europe and Asia (Van der Zwet, 1996). The fire blight disease occurs now in almost 60 countries (<https://gd.eppo.int/taxon/ERWIAM/distribution>). Its spread in the temperate zones of the world

appears to be unavoidable (Paulin 1997).

While long range spread is mainly due to trade of plant material the local source of dispersal is transmission of bacteria through milky exudates (ooze) from the damaged tissues of the plant by rain, wind, insects, birds and agricultural practices (infected tools, vehicles, fruits, etc.) (Beer and Norelli, 1977; Pusey 2002). The most infections take place through the flowers. High air humidity and temperature promote its proliferation and distribution (Billing, 2000).

The difficulty of fire blight control is conditioned by the lack of registered specific preparations effecting directly on pathogen. Spraying of the antibiotic streptomycin and the use of copper-containing preparations are the most effective control procedures, but not allowed in most countries, including Georgia, due to the fear of the emergence of resistances (Mc Ghee and Sundin, 2011). Among the current complex range of the disease control is the removal of the very first symptoms as soon as they appear, which reduces drastically subsequent infection (Paulin, 1997).

In 2016, the employees of the Diagnostics Department of Plant Harmful Organisms, Laboratory of the Ministry of Agriculture identified *Erwinia amylovora* in apple seedlings from Mtskheta district, village Ksovrisi (Kartli). In 2018 *E. amylovora* was confirmed in symptomatic fruit trees samples in Shida Kartli, Kvemo Kartli and Imereti regions in Georgia by Real time PCR (Gaganidze et al., 2018). The disease distribution among the regions of Georgia and genetic characterization of some *E.amylovora* isolates was also reported (Gaganidze et al., 2021).

The spread of the disease in eastern and western Georgia, posing a great challenge for the country's economy since pomiculture is one of the oldest and important fields of its agriculture

The goal of the paper is to isolate and study of the fire blight causative pathogen from affected fruit trees in eastern Georgia.

Materials and methods

Sample collection

Collection of samples has been conducted in 2020 in the orchards of different regions of Eastern Georgia. Mtskheta-Mtianeti region: Mtskheta district, villages Ksovrisi and Mukhrani; Shida Kartli region: Gori district, villages – Karaleti, Pkhvenisi and Tidznisi; Kareli district, village Kekhijvari, Khashuri district villages Osiauri and Gomi. Twigs, buds, flowers and fruitlets (with necrosis and exudates) from the plants, suspected on the fire blight disease were collected in the orchards of the above presented locations, where they were found. For the initial detection of the *E.amylovora* in the collected material, immunological fast test was conducted (AgriStrip Complete kit “BIoreba”). Positive and suspected samples were transferred to the laboratory as soon as possible.

DNA extraction protocol

DNA isolation has been carried out from collected plant samples. One gram of the tissue from the above stated samples were taken from the region between the diseased and healthy tissue and homogenized in 50 ml 1XPBS buffer. The macerate was centrifuged at 10,000g for 5 min and the pellet was resuspended in 500 µl of Tris-HCl buffer, pH 7.5, containing 24.2 g NaCl, 14.6 g EDTA, 9.3 g SDS, 5 g PVP10 per liter. After 1h incubation at room temperature, the suspension was centrifuged at 4,000g for 5 min. DNA was precipitated by adding of equal volume of isopropanol and centrifuged at 10,000g for 15 min. The pallet was washed with 70% ethanol twice and then suspended in 100 µl TE (Tris-EDTA buffer, Merck).

Detection of E. amylovora in plants

Detection of *E.amylovora* was carried out by conventional PCR based on amplification of targeted sequence of the plasmid pEA29, which plays important role in plant-pathogen interaction. Primer pair: A (5'-CGG TTT TTA ACG CTG GG-3') and B (5'-GGG CAA ATA CTC GGA TT-

3)' were applied in PCR reaction (Bereswill et al., 1992). Predicted PCR product is 900-1100 bp.

DNA extracted from the symptomatic plant twigs, buds, flowers and fruitlets was used as a matrix in PCR analysis. DNA isolation was performed by a method modified by us using the kit (GC proba, Agrodiagnostika). 50 ml of 1XPBS buffer (1:50) was added to 1 gram of plant tissue (sampled from the boundary of damaged and healthy tissue) and incubated on a shaker at 25 ° C for 1 hour; the macerate was centrifuged at 14,000 g for 5 minutes. The resulting precipitate was dissolved in a 50 µl 1XPBS buffer, added 170 µl sorbent containing lysis buffer of the kit and incubated at 55 ° C for 20 minutes. The elution buffer of the kit was used for DNA elution.

PCR mixture in a total volume of 25 µl contained 12.5 µl XPCR BIO Tag Mix, PCR BIOSYSTEMS, Primers A and B at final concentration 0.1 µM each and 2 µl DNA. Positive controls contained DNA of *E.amylovora* reference strains NCPPB #683 (National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, York, UK). Negative control contained PCR grade water instead of DNA. PCR reaction was conducted in thermocycler TECHNE, TC 3000. Cycling conditions were: initial denaturation at 93°C for 5 min, followed by 40 cycles of 93°C for 30 s, 52°C for 30 s and 72°C for 1 min 15 s, with a final elongation step at 72°C for 10 min.

Isolation and purification of *E. amylovora*

Plant samples, in which the fire blight causative bacteria *E. amylovora* were detected were washed with water and surface disinfected by soaking in 70% ethanol for 3 min, rinsed three times in sterile distilled water and gently dried with sterilized tissue paper. Small pieces of plant tissues were macerated in 1ml of sterile phosphate buffer saline (PBS) during 30 min. After 30 min incubation at 25°C 10⁻¹, 10⁻² and 10⁻³ dilutions were prepared. One drop (30 µl) of macerate were and streaked onto three different media in Petri plates King's B (King et al., 1954), NSA (Nutrient Sucrose Agar) (Billing et al., 1961) and CCT (Crystal violet-cycloheximide-tergitol) agar medium (Ishimaru & Klos, 1984) as is recommended for maximum recovery of *E.amylovora*. The plates were incubated at 25°C for 48–72 h. After development of the bacteria colonies suspicious ones were purified on none selective King's B medium. For long-term storage cultures in 30% glycerol were placed in low-temperature freezer at -80°C.

Molecular identification of *E. amylovora* isolates

Molecular identification of *Erwinia amylovora* was conducted by PCR based on amplification of sequence near termini of the chromosomal pEA71 insert region with a synthesis of a 187 bp PCR product as expected from the nucleotide sequence (Taylor et al., 2001). With this aim the specific primer pair: G1-F: 5'-CCT GCA TAA ATC ACC GCT GAC AGC TCA ATG-3' and G2-R: 5'GCT ACC ACT GAT CGC TCG AAT CAA ATC GGC-3' was used to amplify DNA.

DNA was extracted from the isolates by the boiling. The 24-hour culture grown on solid medium was transferred to a 100 µl TE buffer (Tris-EDTA, pH 7.4) with a sterile loop, incubated at 100 ° C for 10 minutes, then cooled at 4 ° C for 10 minutes and centrifuged 2 min at 3000 rpm. A supernatant containing DNA was used for the PCR reaction.

PCR mixture in a total volume of 25 µl contained 12.5 µl XPCR BIO Tag Mix, PCRBIOSYSTEMS, Primers G1 and G2 at final concentration 0.2 µM each and 2 µl DNA extracted from the isolates. Positive controls contained DNA of *E.amylovora* NCPPB #683, Negative control contained PCR grade water instead of DNA.

PCR reaction was conducted in thermocycler TECHNE, TC 3000. Cycling conditions were: initial denaturation at 95°C for 3 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final extension step at 72°C for 5 min and cooling at 8°C.

Electrophoresis of PCR products was conducted on 1% agarose gel in TAE buffer during 40 min at 80V. Staining was carried out with ethidium bromide and viewed by UV Transilluminator (Sambrook et al., 2000).

Phenotypic identification of *E. amylovora* isolates

For morphological identification of the suspected isolates pure cultures King's B (King et al.,

1954), CCT agar and NSA were used. Observation of colony morphology, such as size and shape was carried out by stereoscopic microscope (Leica M50). Bacterial cells were studied by light microscope (Leica DM 1000).

For phenotypic identification following biochemical tests were performed: Gram Test, oxidase, nitrate reduction, H₂S formation, citrate utilization, esculene hydrolysis, gelatin liquefaction, growth ability at 39⁰C, fluorescent pigment production in King's B (under UV), sugars utilization ability and acid formation from glucose, sucrose, lactose, maltose, galactose, xylose, mannitol, sorbitol and levan formation from sucrose according to the methods of Jones & Geider (2001).

Results

Above 40 samples – twigs, buds, flowers and fruitlets of the plants suspected on the fire blight disease were collected in the Mtskheta-Mtianeti and Shida Kartli Regions, Eastern Georgia in Spring of 2020. Based on visual observation and immunological test (Ea AgriStrip), 20 plant specimens were selected for further study.

Fig.1 shows diseased plant samples collected in Mtskheta-Mtianeti and Shida Kartli regions in which the pathogen *E.amylovora* was detected by the PCR (Bereswill et al., 1992) (Fig.2).

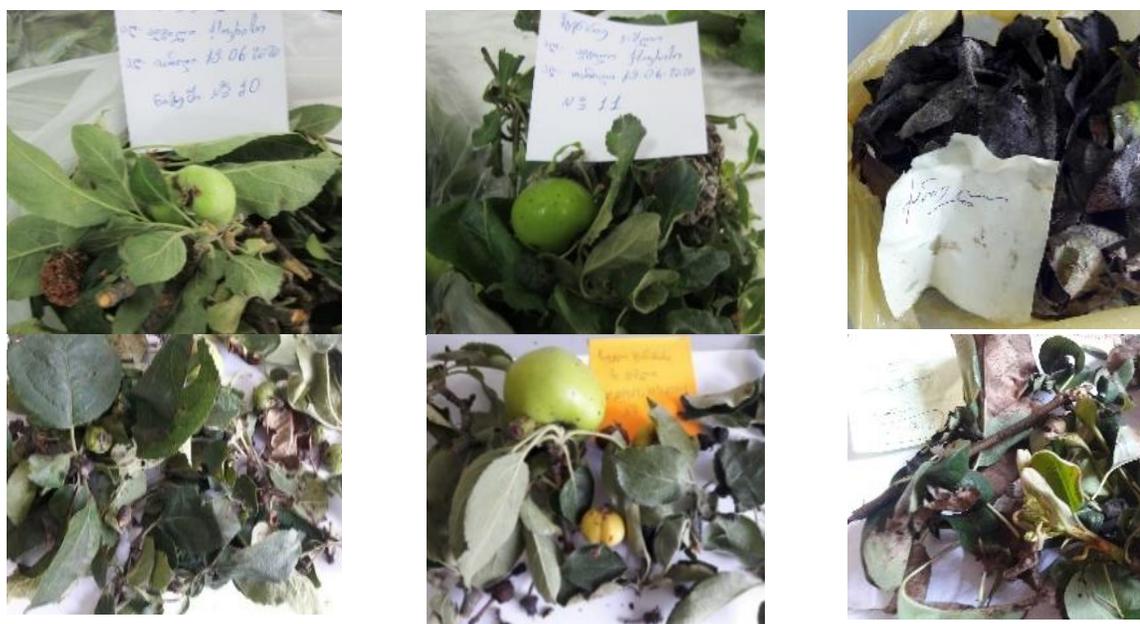


Fig.1. The fire blight diseased plant samples collected in Mtskheta-Mtianeti and Shida Kartli regions in 2020.

1-2 Apple (Ksovrisi), 3 –apple (Kekhijsvari), 4 – apple (Karaleti), 5 – apple (Tirdznisi), 6 – pear (Osiauri).

Results of PCR-based detection of *E.amylovora* in symptomatic plant samples is represented on Fig.2.

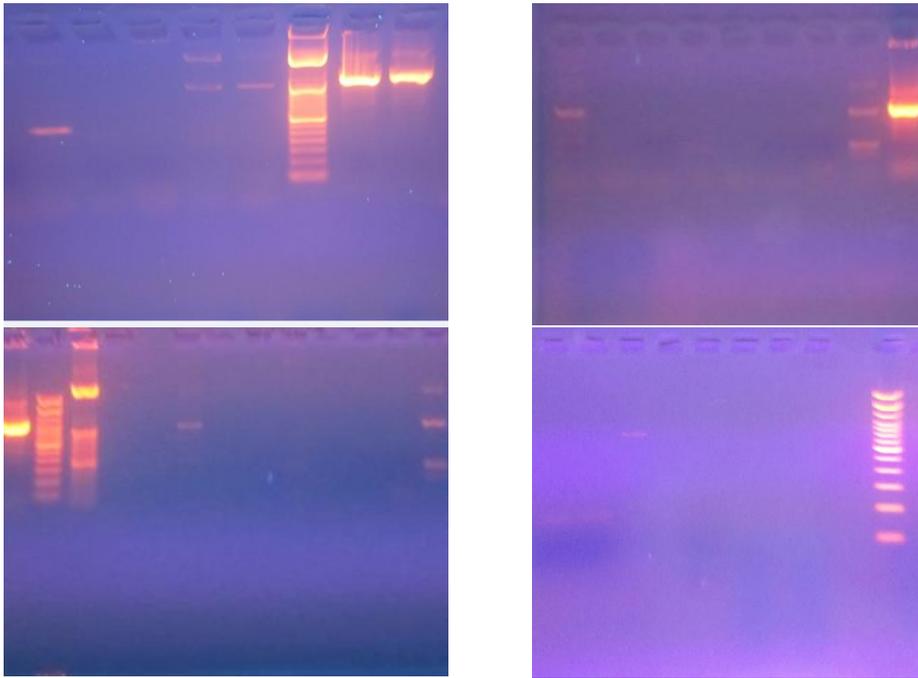


Fig. 2. Detection of fire blight causative pathogen in fire blight diseased plant samples - electrophoregram of PCR fragments on 1.0% agarose gel.

1- Quince (Mukhrani), quince (Ksovrisi), apple (Mukhrani), apple (Ksovrisi, samples #10, 11), DNA ladder, PC NCPPB 683, Ref sample, NC;

2- Apple (Kekhijvari, sample # 12), apple (Tirdznisi), quince (Tirdznisi), apple (Karaleti), quince (Karaleti), apple (Pkhvenisi), NC, apple (Tirdznisi, sample # 16), PC, DNA Ladder 100bp.

3- PC NCPPB 683, DNA 100bp, quince (Pkhvenisi), apple (Pkhvenisi), apple (Karaleti, sample #14 dil.1:10), apple (Tirdznisi dil.1:10), quince (Tirdznisi dil. 1:10), quince (Karaleti dil. 1:10), apple (Pkhvenisi, dil. 1:10, quince (Karaleti), NC, apple (Tirdznisi, sample #16, dil.1:10).

4- NC, pear (Osiauri, sample #27), pear (Osiauri), quince (Osiauri), three samples apple Gomi, DNA ladder 100bp, pear (Gomi), PC NCPPB 683.

Synthesis of PCR amplicons with size of 900 bp indicates on the existence of *E.amylovora* in 5 apple samples #: 10, 11, 12, 14, 16, and one pear sample #27 (Fig.2).

Colonies of some *E. amylovora*, isolated from the fire blight diseased samples, in which the pathogen was confirmed by PCR on different media as shown on Fig.3.



Fig. 3. *E.amylovora* colonies: #1011 and 1133 on King's B medium; #1012 on SNA) medium.

One morphotype colonies were formed on CCT medium after 72 h – pale violet, circular, convex and mucoid, with slower growth than on King's B or SNA. Colonies on King's B appear after 24 h and are creamy white, circular.

The isolates ## 1012, 1022, 1023, 1131, 1133, 1212, 1612, 2763 and 2772, obtained from the 6 pathogen confirmed plant samples from different dilutions were confirmed to be *E.amylovora* by the PCR using primer pair G1 and G2.

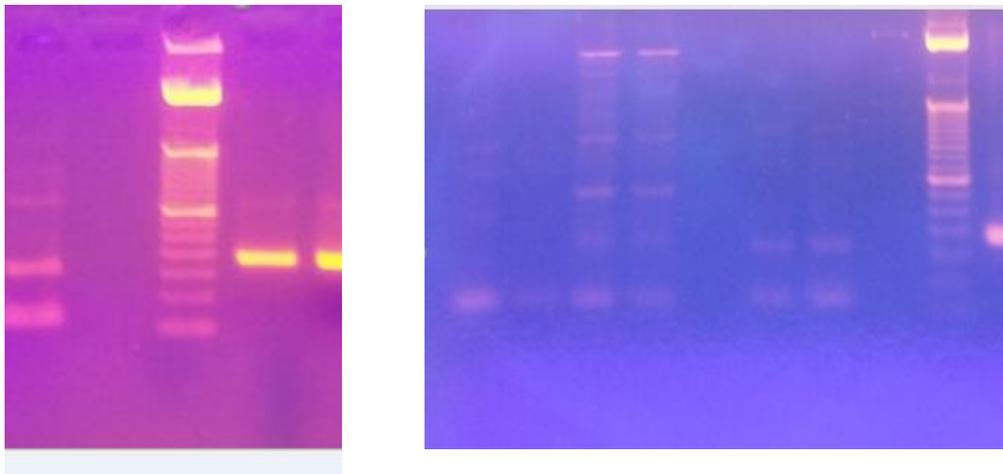


Fig. 4. Representative picture of *E. amylovora* molecular identification by PCR using G1 and G2 primer pair - electrophoregram of PCR fragments on 1.0% agarose gel.

1 – Isolates from sample #11: 1131, NC, 50bp DA ladder, 1133, PC NCPPB 683.

2 - Isolates from sample #27: 2712, 2713, 2733, 2741, 2752, 2763, 2772, NC 50 bp DNA ladder, PC NCPPB 683.

Tests performed for the phenotypic analysis show that all isolates are Gram+ and are characterized by the abilities of the bacterial extracellular polysaccharide – levan formation on NSA medium, gelatin hydrolysis and citrate utilization. The isolates do not develop at 39⁰C, are oxidase negative, are not able to nitrate reduction and H₂S formation.

Study of the physiological-biochemical properties of the *E. amylovora* Georgian isolates revealed a little difference in their sugars utilization ability. All isolates are characterized by acid formation from glucose, sucrose and galactose. Alternatively, none of them utilised lactose, maltose, xylose and mannitol. As about 25% of the isolates utilize sorbitol.

Thus, the above listed properties of the isolates indicate that they are *Erwinia amylovora*.

Discussion

The fire blight disease of fruit trees first appeared in village Ksovrisi, in the Mtskheta-Mtianeti region in 2016 (Gaganidze et al., 2018) and the local farmer relates it to imported apple seedling. Based and referring on this publication EPPO publishes “2019/148 First report of *Erwinia amylovora* in Georgia (EPPO 2019). *E.amylovora* received the status of a limited-distribution quarantine pest in Georgia (Document is under preparation).

Since the first discovery, the fire blight disease is spread in eastern and western Georgia, However, the number of cases is small (Gaganidze et al., 2021). In 2020 out of collected 40 samples in two regions of eastern Georgia – Mtskheta-Mtianeti and Shida Kartli Regions, 6 were confirmed to be diseased by the fire blight based on molecular detection of it's causative *E. amylovora*. In a total of 10 pure isolates were recovered from the disease confirmed samples serial dilutions. The presence of *E. amylovora* has been confirmed by specific PCR (Tailor et al., 2001) in the following isolates, ## 1012, 1022, 1023, 1131, 1133, 1212, 1412, 1612, 1742, and 2763.

Georgian *Erwinia amylovora* isolates according to biochemical and physiological properties are typical representatives of a very homogenous group of the species (Dye 1981; Holt et al. 1994; Paulin 2000). However, there are little differences among the isolates, expressed in the ability to

utilize certain carbohydrates as carbon sources.

It is difficult to determine whether this disease was spread in Georgia by one or several imported seedling at the same time and whether it is due to further internal distribution. Study of the genetic diversity using modern molecular biology methods is planned to reveal the differences between the isolates. Sequence of the CRISPR regions (Rezzonico et al., 2011) of the four Georgian isolates revealed the presence of two distinct genotypes (Gaganidze et al., 2021). Extension of the genetic analysis to greater number of isolates is very important for epidemiological studies and to identify the pathogen type, source/sources and ways of distribution of the fire blight disease in Georgia.

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საქართველოში ხეხილის ბაქტერიული სიდამწვრის გამომწვევი პათოგენი

ამაშუკელი ნანული, დადანიძე დალი,
აზნარაშვილი მარიამ, ხარაძე შორენა,
სტურუა ნელი, სადუნიშვილი თინათინ
საქართველოს აგრარული უნივერსიტეტი,
სერგი დურმიშიძის ბიოქიმიისა და
ბიოტექნოლოგიის ინსტიტუტი
დავით აღმაშენებლის ხეივანი 240,
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აბსტრაქტი. ბაქტერიული სიდამწვრე არის ხეხილის დამანგრეველი დაავადება, რომელიც საქართველოში 2016 წელს გამოჩნდა. დაავადებისათვის დამახასიათებელი სიმპტომების მქონე ყლორტის, კვირტის, ყვავილისა და ნაყოფის ორმოცზე მეტი ნიმუში იქნა შეგროვებული აღმოსავლეთ საქართველოში, შიდა ქართლისა და მცხეთა-მთიანეთის რეგიონების ზღვებში 2020 წლის ზაფხულში. ვიზუალური დაკვირვებისა და იმუნოლოგიური ტესტის საფუძველზე (*Ea AgriStrip*), კვლევისათვის შეირჩა 20 ნიმუში. პჯრ ჩატარდა ნიმუშებში პათოგენის გამოსავლენად პრაიმერების წყვილით A: 5'-CGG TTT TTA ACG CTG GG-3 და B: 5'-GGG CAA ATA CTC GGA TT-3 და 2XPCR BIOTAG Mix PCR BIOSYSTEMS გამოყენებით. პათოგენი *Erwinia amylovora* გამოვლინდა 6 ნიმუშში: N10 და N11 ვაშლის ნიმუშები (ქსოვრისი); N12 ვაშლის ნიმუში (კეხიჯვარი); N14 ვაშლის ნიმუში (კარაღეთი); N16- ვაშლის ნიმუში (ტირმნისი); N27 მსხლის ნიმუში (ოსიაური). King's B, NSA და CCT არეების გამოყენებით ნიმუშების სერიული განზავებებიდან გამოყოფილ და გასუფთავებულ იქნა პათოგენები. პჯრ რეაქციით პრაიმერებით G1-F: 5'-CCT GCA TAA ATC ACC GCT GACAGC TCA ATG 3' და G2-R: 5GCT ACC ACT GAT CGC TCG AATCAA ATC GGC3 ', დადასტურებულ იქნა რომ იზოლატები ## 1012, 1022, 1023, 1131, 1133, 1212, 1412, 1612, 1742 და 2763 არის *Erwinia amylovora*. *E.amylovora* ქართული იზოლატების ფიზიოლოგიურ-ბიოქიმიური თვისებების შესწავლით გამოვლენილ იქნა მცირე განსხვავება შაქრების შეთვისებისა და ესკულინის ჰიდროლიზის უნარში.

საკვანძო სიტყვები: ხეხილის ბაქტერიული სიდამწვრე, *Erwinia amylovora*, პჯრ

მადლიერება: ნაშრომი დაფინანსებულია საქართველოს შოთა რუსთაველის ეროვნული სამეცნიერო ფონდის (სშრესფ) მიერ, გრანტის ნომერი FR-19-22524.