Assessment of fire blight introduction in the wild apple forests of Kazakhstan

Elina R. Maltseva[1,2,3], Galiya A. Zharmukhamedova[1,3], Zhulduzay K. Jumanova[1,3], *Dinara A. Naizabayeva[1,2], Zhanna A. Berdygulova[1,2], Karina A. Dmitriyeva[1], Sagi S. Soltanbekov[4], Assel M. Argynbayeva[5], Yuriy A. Skiba[1,2,3], Natalya P. Malakhova[1], Fabio Rezzonico[6], Theo H.M. Smits[6]

[1] *Institute of Molecular Biology and Biochemistry, Almaty, Kazakhstan*

[2] *Almaty Branch of National Center for Biotechnology, Almaty, Kazakhstan*

[3] *Tethys Scientific Society, Almaty, Kazakhstan*

[4] *Kazakh Fruit and Vegetable Research Institute, Almaty, Kazakhstan*

[5] *Institute of Plant Biology and Biotechnology, Almaty, Kazakhstan*

[6] *Environmental Genomics and Systems Biology Research Group, Institute of Natural Resource Sciences, Zürich University of Applied Sciences (ZHAW), Wädenswil, Switzerland*

* Corresponding author: Dinara A. Naizabayeva. dinara.naizabaeva@gmail.com

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Assessment of the fire blight introduction in the wild apple forests of Kazakhstan

Fire blight disease of apples continues its worldwide spread, having reached Kazakhstan in 2010. It is a great threat to the wild apple forests of *Malus sieversii*. The introduction of fire blight is already showing a considerable impact on cultural apple growing and demands radical conservation efforts of the wild apple forests. A number of studies have been conducted to examine the presence of fire blight distribution within apples in agricultural areas, however, there have been no large-scale monitoring of wild apple tree populations. Here we present the results of three years of monitoring wild apples in three protected areas of Kazakhstan, looking for the presence of fire blight (*Erwinia amylovora)*. A visual inspection showed no signs of fire blight on the trees of *M. sieversii* in three consecutive years. These findings were confirmed by lateral flow immunochromatography, and conventional and real-time PCR tests of the asymptomatic samples. The findings of this study will be used to produce recommendations for state authorities to prevent fire blight in wild apple forests of Kazakhstan.

Keywords: *Malus sieversii*, wild apple forests, fire blight, *Erwinia amylovora*, conservation, specially protected natural territories

Introduction

The wild apple forests of Kazakhstan are of global importance both as a natural heritage and as a source of valuable gene diversity (Dzhangaliev, 2007, 2010; Radionov, 2013). It has been established that the domesticated apple in Kazakhstan originated from these trees (Cornille et al., 2012; Harris et al., 2002; Velasco et al., 2010). *Malus sieversii*, native to Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, and China, is listed as 'vulnerable' on the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN) (https://www.iucnredlist.org/species/32363/9693009), with Kazakhstan owning 17,800 ha of the distribution area (Radionov, 2013).

Introduction of the fire blight pathogen in the distribution area of the wild apple poses a certain threat for *M. sieversii* being affected by the disease (Djaimurzina et al., 2014; Doolotkeldieva et al., 2021; Feurtey et al., 2020; Umiraliyeva et al., 2021). The studies of *M. sieversii* responding to this pathogen show a range of reactions with observed increased resistance in comparison with other cultured varieties (Harshman et al., 2017; Luby et al., 2002). At the same time, Harshman et al. noticed a unique resistance response of *M. sieversii* to the infection characterised by low incidences of infection in the beginning, but showing increased severity of the disease once successfully initiated (Harshman et al., 2017).

In cases where the wild apples are infected with the fire blight, it is hard to implement the recommended measures (i.e cutting the trees, treatment with chemicals) as the trees are located in protected natural territories (SPNTs) of the Republic of Kazakhstan where a special protection regime is being applied.

Most of the *M. sieversii* distribution area is located within the SPNTs of the Republic of Kazakhstan that correspond to the IUCN category Ia (strict nature reserves) and II (national parks), providing the highest protection regime with no activities allowed in the core zone except for scientific research and monitoring in accordance with the Law on Specially Protected Natural Territories (https://adilet.zan.kz/rus/docs/Z060000175_/z060175.htm). Some of the territories are also internationally recognized as UNESCO World Heritage sites and UNESCO biosphere reserves in the frames of the Man and the Biosphere Programme. If a pathogen such as fire blight is found within the territory of the SPNT, the countermeasures must be reviewed and approved by the corresponding national authorities, and in case of UNESCO World Heritage site a special procedure for the approval from the Secretariat of the World Heritage Convention should be followed.

Another factor adding to the severity of the problem is that the symptoms of fire blight can often go unnoticed given that rangers are not informed of the disease, nor are they trained in symptoms recognition, and the territories of wild apple distribution are vast and some of them are barely visited.

Erwinia amylovora (Burrill) (Winslow et al., 1920) is the bacterium causing fire blight, the devastating pome disease that threatens plants of the *Rosaceae* family throughout the world. First detected in North America, fire blight has further spread to New Zealand, Europe, North Africa, the Middle East, Russia, and most recently to Central Asia, the Caucasus and South Korea (Djaimurzina et al., 2014; Gaganidze et al., 2021; Kurz et al., 2021; Myung et al., 2016). The first record of fireblight in Kazakhstan was registered in 2008 (Drenova et al., 2012).

The introduction of disease into Kazakhstan was mainly due to an increased import of planting material in the frames of state program increasing apple plantations in the country. The relative proximity of the wild apple forests to the cultural varieties of apple that might be infected with fire blight should be taken into account, especially since the pathogen spreads with rain, wind, and insects. The goal of this study was therefore to reveal whether the pathogen *E. amylovora* is already present in some of the wild apple forests of Kazakhstan.

Materials and Methods

The objects of study were wild trees of *M. sieversii*, located within the SPNT of the Republic of Kazakhstan: the Ile-Alatau State National Park, Zhongar-Alatau State National Park, and Aksu-Zhabagly Nature Reserve.

Phytopathological assessments of trees for susceptibility to fire blight was carried out on two diagonals of the wild tree forest or on separately standing trees. The crowns, trunks, branches and flowers of the trees were carefully examined for the presence of fire blight-compatible symptoms. At least 10-20 trees were inspected in each wild apple forest, depending on the area of their growth. In the case of tree stands or separate trees all of them were inspected. The prevalence of the disease and the degree of its development was determined by a special formula (see below).

The susceptibility was measured on a 6-point scale, modified by us taking into account the peculiarities of fire blight: 0 – healthy tree; 0,1 – barely visible signs of illness; 1 – the initial stage of disease manifestation (single wilting and blackening of the flowers, twisting and browning of shoots and leaves are noticeable); 2 – flowers, shoots and leaves are affected more than 10%; 3 – damage to the bark of branches, trunks, fruits (bacterial exudate is released on the affected areas); 4 – more than 75% of the crown is burned, trees look as after a fire; 5 – tree dead from disease.

The percent of distribution (frequency of occurrence) of the disease was calculated using the following formula:

$$P = \frac{H \cdot 100}{N}, \qquad (1)$$

where P referred to the prevalence or frequency of disease, %; H – number of infected trees; N – number of studied trees.

The degree of disease development is calculated by the following formula:

$$R = \frac{\Sigma (a \cdot b) \cdot 100}{NK}, \quad (2)$$

where R referred to the level of disease development, %; Σ – the sum of multiplication of a and b; a – the number of trees with same disease lesions; b – lesion score corresponding to this symptom; N – total number of trees; and K – the highest score of the lesion scale.

Samples (flowers, shoots, fruitlets and stem segments) were taken from the trees and processed according to (Anonymous, 2022). All asymptomatic samples were enriched on liquid King's media prior to plating and serological/molecular tests. Enriched cultures were plated on King's solid media and levan media in accordance with the same international guidelines (Anonymous, 2022).

Serological tests were conducted with the Ea AgriStrip (Bioreba, Switzerland) according to the manufacturer's instructions. Nucleic acids were extracted with EasyPure Bacteria Genomic DNA Kit (TransGen Biotech, China) according to the manufacturer's instructions. Molecular tests were carried out with the protocols of Stöger et al. (Stöger et al., 2006) for conventional polymerase chain reaction (PCR) and the protocol of Pirc et al. (Pirc et al., 2009) for real-time PCR according to the protocol provided in (Anonymous, 2022) and run on QuantStudio 5 (Applied Biosystems, USA), Bio-Rad (Bio-Rad, USA) and Eppendorf Mastercycler X50s (Eppendorf, Germany) thermal cyclers. The products of conventional PCR were analised in 1% agarose gel in tris-acetate buffer.

Results

The apple forests, stands and separate trees were examined in 2019, 2020 and 2021 by visual observation in three main clusters of their distribution area in Kazakhstan: in Aksu-Zhabagly State Nature Reserve in the southern part of their distribution area, in Ile-Alatau State National Park in southeast, and in Zhongar-Alatau State National Park in the eastern reach of their distribution. The location of the sampled sites is provided in the map below (Figure 1).

[*figure 1 here*]

Figure 1. The geographic location of the phytopathologic inspection and sample collection sites in 2019-2021

Visually, symptoms of fire blight on the trees of the wild apple trees were not detected. The number of locations for phytopathological assessment and sample collection with the results of visual observations are presented in Table 1.

Table 1. Phytopathologic assessment of wild apple forests, stands and separate trees

[*table 1 here*]

A total of 92 asymptomatic samples were taken in 2019, 55 samples were collected in 2020, and 18 samples were taken in 2021. The collected samples were tested with the Bioreba AgriStrip kit onsite in order to exclude storage and transportation bias. The results of the AgriStrip tests were negative for all the tested samples. At the same time, it was decided to process the samples under laboratory conditions after an enrichment step in order to completely exclude the possibility of the presence of pathogens in the material. For that, the asymptomatic samples were processed according to the international guidelines and cultures, enriched in liquid

King’s media, were tested with both conventional PCR that targets sequences located in the plasmid pEA29 (Stöger et al., 2006), and a real-time PCR that targets chromosomal part of the genome (Pirc et al., 2009). The results of both PCR analyses were concordant. The examples of the obtained results are presented in Figure 2.

[*figure 2 here*]

Figure 2. Examples of the results obtained for the biomaterial of *M. sieversii*: an immunochromatographic test - Agristrip (up left), a result of the real-time PCR - the curve belongs to the positive control sample (up right), and a result of conventional PCR where M is a molecular weight marker of 100 bp and K+ is a positive control (down)

The enriched liquid cultures were still plated on solid media (King’s agar and levan media) to search for the colonies with characteristic features of *E. amylovora.* No colonies produced on solid media belonged to this pathogen.

Thus, the absence of *E. amylovora* was proven by a variety of methods – visual inspection, bacteriological analysis (plating of enriched cultures on solid media), lateral flow immunochromatography (AgriStrip tests), and molecular analysis of enriched samples with two separate PCR protocols.

Discussion

The distribution area of wild apples (*M. sieversii*) in Kazakhstan stretches from West Tien Shan mountains in the south to Zhongar Alatau ridges in the east with very small percentages of the population further to the east in the Tarbagatai mountains (Radionov, 2013). According to IUCN Red List, the main factors threatening the wild apple are agricultural expansion and development, genetic erosion and overgrazing, with some sources indicating its habitat decline by over 70% in the last 30 years (https://www.iucnredlist.org/species/32363/9693009#assessment-information). With these factors affecting the conservation of the wild apple, an introduction of a notorious pathogen such as fire blight, which might lead to quick loss of high numbers of the trees, is unacceptable and needs timely prevention. Safeguarding this valuable biodiversity is one of the main goals for a network of protected areas established in the wild apple distribution area. The forests, stands and separate trees of *M. sieversii* are being monitored by rangers in these protected areas, and we relied on their help in finding the locations for our phytopathologic observations.

The phytopathologic observations and laboratory tests of asymptomatic plant material allow us to confirm the absence of *E. amylovora* in the studied zones of wild apple's growth. At the same time, the results of this study alone cannot be regarded as evidence that *M. sieversii* in Kazakhstan are not infected with fire blight, and continuous monitoring studies are needed. The pathogen is present in Kazakhstan (Umiraliyeva et al., 2021) and effective measures for its control and prevention should be taken both in the agricultural lands (e.g. commercial orchards, private households and gardens) and in the lands close to and in the limits of the specially protected natural territories.

Based on this study, a set of recommendations will be created and shared with the corresponding authorities for the prevention of fire blight introduction in the wild apple forests. These recommendations will include regular monitoring of the wild apple forests, stands and separate trees for the symptoms of fire blight, as well as monitoring of the lands within the 10 km buffer zone by the rangers, phytosanitary service inspectors and by the local people themselves. For this, an awareness-raising campaign has already started within the frames of this work, targeting protected areas' rangers and local people. A set of materials describing the symptoms of fire blight, as well as the information on the actions to be taken once these symptoms are registered, was shared at these events.

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Table 1. Phytopathologic assessment of wild apple forests, stands and separate trees

Specially protected natural territory	Number of locations for phytopathological assessment / sampling	Year	Disease index, %	
			P	R
Ile-Alatau State National Park	5	2019	0	0
Aksu-Zhabagly State Nature Reserve	4	2019	0	0
Zhongar-Alatau State National Park	10	2019	0	0
Ile-Alatau State National Park	6	2020	0	0
Aksu-Zhabagly State Nature Reserve	4	2020	0	0
Zhongar-Alatau State National Park	8	2020	0	0
Ile-Alatau State National Park	4	2021	0	0
Zhongar-Alatau State National Park	3	2021	0	0

Figure 1. The geographic location of the phytopathologic inspection and sample collection sites in 2019-2021

Figure 2. Examples of the results obtained for the biomaterial of *M. sieversii*: an immunochromatographic test - Agristrip (*up left*), a result of the real-time PCR - the curve belongs to the positive control sample (*up right*), and a result of conventional PCR where M is a molecular weight marker of 100 bp and K+ is a positive control (*down*)