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Molecular Assessment of Apple Varietal Diversity in Uzbekistan For Genetic Resistance to Fire Blight

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Abstract: Apple fire blight (*Erwinia amylovora*) is the most common diseases of the apple in almost all apple-growing regions. Being a part of Central Asian region, Uzbekistan has a long apple breeding history. Therefore, a large amount of apple varietal diversity is concentrated in this country, which has not yet been evaluated at the molecular genetic level. In the last decade, new commercial varieties have been introduced from different regions of the world, as a result, in recent years there has been a significant increase in diseases that were rarely seen before. Current study is the first aimed to conduct a molecular screening of the local apple varietal diversity to identify distribution of genetic resistance markers to apple fire blight in Uzbekistan.

Molecular genetic analysis of 109 of local apple cultivars growing in three regions of Uzbekistan have been analyzed for resistance to fire blight by using three specific DNA markers. Screening for host genetic resistance to fire blight revealed that 50,45% of Uzbek apple varieties have moderate to high resistance to *E. amylovora* on genetic level, and 83,48% of varieties have at least one fire blight resistance loci. Revealed genetic diversity indicates great potential of Uzbek varietal apple germplasm for breeding programs. Further studies involving more apple varieties and genetic markers associated with genes or QTLs for valuable agronomic traits including disease resistance need to be done in Central Asia.

Key words: fire blight, molecular marker, resistance

Introduction

The subfamily *Maloideae* which accounts for apples includes 22-25 genera and about 600 species, growing mainly in the temperate zone of the northern hemisphere. The genus Malus, belonging to this subfamily, is the most important in terms of the commercially valuable fruits. This genus has a wide geographical distribution. The range of the genus is within Europe, Asia and North America. Due to the genetic variability in Central Asia this region is generally considered the center of origin for apples (Janick et al., 1996). Therefore, apples of this region may

contain valuable genetic backgrounds, which can be used to breed more environmentally sustainable cultivars.

Many different types of fungi and some varieties of bacteria can cause serious apple diseases. Among them, the most common disease is bacterial blight (*Erwinia amylovora*) (Vincent et al., 2010).

Disease control measures are practically ineffective. In some regions, treatment with chemical agents up to 20-25 times during the vegetation period. The use of a large number of fungicides, in turn, affects the environment and human health, causing an increase in the price of apple fruits.

As current food production systems are key drivers of climate change and environmental degradation, there is an urgent need to reduce the dependency on chemicals and excess fertilization, to increase organic farming, and to fight against the progressive loss of biodiversity. (Höfer et al., 2021). The best way to prevent this is to create apple varieties that are genetically resistant to these diseases (Podwyszyńska eat al., 2021).

In early studies it was shown that wild apple species including *M. robusta, M. sublobata, M. atrosanguinea, M. prunifolia*, and *M. fusca*, are carriers of genetic resistance to various diseases (Aldwinckle et al., 1979).

The goal of many apple breeding programs is to combine high fruit quality traits with disease resistance (Brown and Maloney, 2003). Molecular markers are widely used in marker assisted selection (MAS) programs by apple breeders to genotype greenhouse seedlings and select desirable genotypes for wide planting in the field, thereby cutting down on cost and land requirements (Brown and Maloney, 2003). To that end, the release of an apple reference genome (Velasco et al., 2010) has greatly aided the efforts of MAS in apple breeding.

Fire blight is another devastating disease of apple and plant species in the Rosaceae family caused by bacterial pathogen *Erwinia amylovora* (Burrill 1882) (Winslow et al., 1920). Yearly losses due to fire blight can be substantial in many countries worldwide. Control of fire blight is difficult due to spread of strains with resistance to antibiotics and currently there are no available synthetic compounds with systemic properties that directly affect the pathogen (Aćimović et al., 2015). Like many resistance traits, resistance to *E. amylovora* is quantitative in nature and occurs in both wild and cultivated plants (Brisset et al., 2002; Dondini et al., 2004; Durel et al., 2004).

To identify genetic resistance loci there are several SCAR and SSR markers have been developed and successfully applied in breeding programs and varietal characterization (Khan et al., 2007).

Being a part of Central Asian region, Uzbekistan has a long apple breeding history. Therefore, a large amount of apple varietal diversity which may contain valuable genetic backgrounds is concentrated in this region, and can be used to breed more environmentally sustainable cultivars.

In the last decade, an increase in the spread of fire blight has been observed in Uzbekistan (Khasanov B. and Guzalova A., 2021; Umarov Z., 2021).

In this regard, there is an acute demand of conducting a comprehensive assessment of the genetic diversity of local apple varieties in Uzbekistan to identify resistant genotypes. To date, local varietal diversity has not yet been characterized on molecular genetic level for disease resistance.

Thus, the aim of this study was to screen and genotype 109 local apple cultivars growing in three regions of Uzbekistan for the presence of resistance genes to *E. amylovora* using

polymorphic DNA markers. Genotypes of varietal diversity with different loci combinations and varying degrees of disease resistance were identified. The data obtained will help breeders and farmers to determine a further strategy for the selection and cultivation of varieties depending on the pathogenic background in different regions of the country.

Material and methods

Plant material

Leaf samples were collected from domestic apple cultivars from various specialized family farms (where apple trees have historically grown for several generations) located across two geographical regions of Uzbekistan. The plant material of the present study included 109 local apple genotypes from North-West (Karakalpakstan and Xorazm province) and South (Surxondaryo province) regions of Uzbekistan. Each variety was coded depending on geographic origin, where the first letter corresponds to region (**Supplementary Tab 1**). For each plant, a phenotypic description and geographic location according to GPS were also recorded. Leaf samples were placed into individual Ziplock bags containing silica gel and, after collection, were transported to the laboratory for subsequent DNA isolation and molecular-genetic analysis.

Molecular analysis

Genomic DNA from each cultivar was extracted using the PureLink® Plant Total DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's guide.

After isolation, the genomic DNA were measured on BioSpec-nano (Shimadzu Corporation, Kyoto, Japan) spectrophotometer for quantity and quality. Finally, all DNA samples were diluted to 20-30ng/µl, visualized on 0,8% agarose gel, and stored at -20°C for further analysis.

Genomic DNA samples were analyzed by PCR using molecular markers for scab, powdery mildew, and fire blight resistance genes.

PCR amplifications of the specific fragments were performed using 20 ng of genomic DNA in 10µl volumes containing 10 mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM MgCl2, 100µM of each dNTP, 200nM of each primer and 0.6U Taq polymerase. PCR amplifications were performed on Veriti™ Thermal Cycler (Thermo Fisher Scientific) using an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 40 s at 94 °C, 1 min at annealing temperatures based on the primers used for each marker (**Table 2**), 1 min at 72 °C, and a final extension at 72 °C for 10 min. The amplification PCR products were visualized in electrophoresis. The bands on the gels were transformed in binary data by being scored as 1 and 0 for the presence and absence, respectively, of alleles for each cultivar per marker. The sizes of the fragments corresponding to the allelic sizes were accurately scored based on a molecular weight DNA ladder of 100 bp (NEB).

Statistical analysis of genetic markers was performed in GenAIEx 6.5 (Peakall and Smouse 2006, 2012). A Jaccard similarity co-efficient test was employed in the data matrix and a cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). A principal component analysis (PCA) was performed with a bootstrap value of 1000 replicates using the Clustvis tool (Metsalu et al., 2015).

Table 2.

List of the molecular markers for the fire blight resistance genes along with their primer sequences, annealing temperature and amplicon sizes.

№	Marker name	Primer pairs	Ta* (°C)	PCR band** (b.p.)	Reference
Fire blight					
1	AE10-375	F 5'-CTGAAGCGCACGTTCTCC-3' R 5'-CTGAAGCGCATCATTTCTGATAG-3'	60	**375**/-	Khan et al., 2007
2	CH-F7-Fb1	F 5'-AGCCAGATCACATGTTTTCATC-3' F 5'-ACAACGGCCACCAGTTTATC-3'	60	**210**/ 174	
3	GE-8019	F 5'-TTGAGACCGATTTTCGTGTG-3' R 5'-TCTCTCCCAGAGCTTCATTG-3'	60	**397**/-	

Results

The genetic screening successfully generated genotypes for all studied 109 local apple cultivars from three regions of Uzbekistan for the presence of several resistance loci to *E. amylovora* using polymorphic DNA markers.

Dominant SCAR markers AE10-375 and GE-8019 associated with resistance to *E. amylovora* produced single bands of 375 bp and 397 bp in 11.0% (n=12) and 79.81% (n=87) of samples respectively. Of them, 70,64% (n=77) of samples had AE10-375 loci only, 1,82% (n=2) of samples had GE-8019 only, 9,17% (n=10) amplified both loci, and in 18,34% (n=20) of samples both markers were absent (Tab. 1). Codominant STS marker CH-F7Fb1 produced two loci 174 bp and 210 bp, the last one is conferring resistance to disease (Khan et al., 2007). Out of 109 analyzed varieties the CH-F7Fb1 loci was present in 83,48% (n=91) of samples. Genotypes and alleles distribution for CH-F7Fb1 in varietal population deviated from Hardy-Weinberg equilibrium (X^2=7,99; p=0,0047) with the following frequencies: 174/174 – 48,69%, 174/210 – 42,17%, and 210/210 – 9,13%; allele 174bp – 69,78%, and allele 210bp – 30,22% (Tab 3).

Tab.3.

Allele and genotype frequencies of fire blight resistance markers in apple varieties from geographic regions of Uzbekistan

Marker	CH-F7Fb1					GE-8019	AE10-375
amplicon size, bp	210	174	210/210	174/174	210/174	397	375
Surkhandaryo (n=50)	0.4186	0.5814	0.1752	0.338	0.4867	0.16	0.92
Karakalpak (n=33)	0.4231	0.5769	0.179	0.3328	0.4882	0.12	0.55
Xorazm (n=26)	0.2054	0.7955	0.0418	0.6327	0.3254	0.00	0.88
Total in population (n=109)	0.3022	0.6978	0.0913	0.4869	0.4217	0,11	0,79
	X2=7,99; p=0,0047						

Thus, from all studied varieties only 8,25% (n=9) did not have any of studied fire blight resistance markers, and 8,25% (n=9) were homozygous by susceptible CH-F7Fb1 allele (174bp)

only. Phylogenetic analysis distributed all varieties to two main clusters with several subclusters: the first cluster presented by high (n=21+2), moderate (n=16) and low (n=2) fire blight resistant genotypes, whereas the second were mainly presented by low (n=34) and susceptible (n=18) genotypes followed by moderate (n=16) resistant genotypes (Fig 1.). In the first cluster, only two varieties "Sariq olma" (yellow apple) and "Besh barmoq" (Five fingers) had all fire blight resistance loci. Due to high homogeneity within subclades, there were no specific clustering of apple varieties by geographic regions. Overall, pairwise Nei genetic identity based on three fire blight resistance markers between populations was very high, with the lowest identity of 96,7% between Xorazm and Surkhandaryo varieties, and the highest identity of 99,7% between Surkhandaryo and Karakalpak varieties (Tab 4). Analysis of molecular variance (AMOVA) also showed very small differentiation among populations (Fst=0.001), and among individuals (Fst=0.036), and very high genetic differentiation within individuals (Fst=0.376) (Tab.5). Dominant fire blight marker GE-8019 was distributed among varieties in Surkhandaryo and Karakalpak regions (16% and 12% respectively), and was not present in Xorazm region (Tab. 3) Dominant fire blight marker AE10-375 was mostly present among varieties in Surkhandaryo (92%) and Xorazm (88%) regions. Fire blight resistance codominant marker allele CH-F7Fb1 210 bp was predominantly present among varieties in Karakalpak (42,31%) and Surkhandaryo region (41,86%) (Tab 3). Analysis of the genotype combinations, revealed presence of the AE10-375 loci alone or AE10-375 + CH-F7Fb1 210 in highly resistant varieties, and AE10-375 + CH-F7Fb1 210/174 in moderately resistant varieties (Tab 1.).

Thus, 50,45% of Uzbek apple varieties have moderate to high resistance to *E. amylovora* on genetic level, and 83,48% of varieties have at least one fire blight resistance loci.

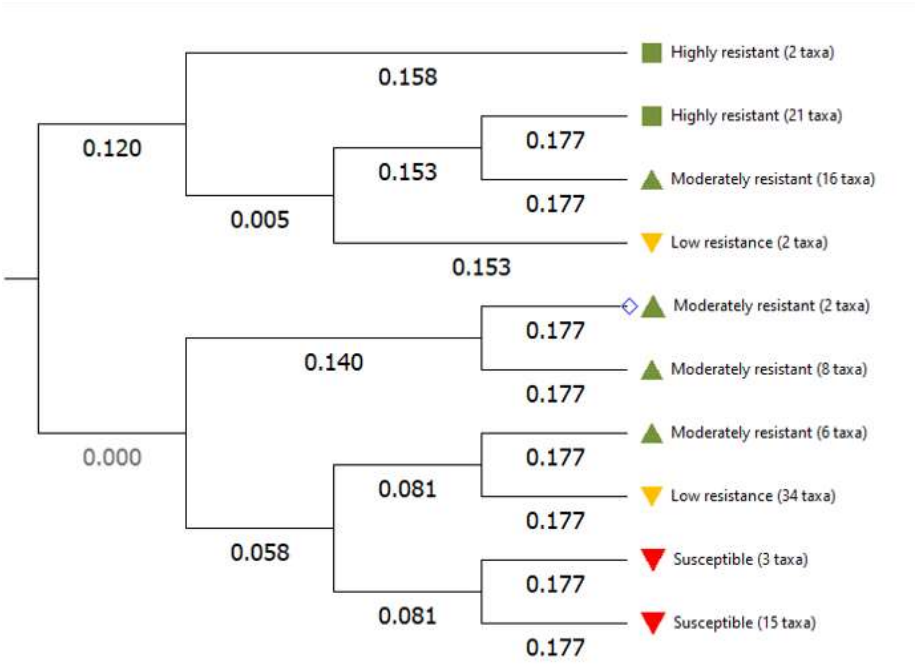

Figure 1. Phylogenetic tree of local apple cultivars based on genotypes for fire blight resistance markers AE10-375, GE-8019, and CH-F7Fb1.

Phylogenetic tree was constructed by UPGMA method using bootstrap (1000 replicates). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site.

Tab.4

Fire blight resistance markers based pairwise population matrix of Nei genetic identity

among apple varieties in Uzbekistan

	Surkhandaryo	Karakalpak	Xorazm
Surkhandaryo	1.000		
Karakalpak	0.997	1.000	
Xorazm	0.967	0.971	1.000

Tab.5.

Summary AMOVA test of Uzbek apple varieties based on fire blight resistance markers AE10-375, GE-8019, and CH-F7Fb.

	df	SS	MS	Est. Var.	%	P-val
Among Pops	2	1.028	0.514	0.001	0%	0.001
Among Indiv	106	47.568	0.449	0.036	9%	
Within Indiv	109	41.000	0.376	0.376	91%	

Discussion

This is the study aimed to screening and analysis of the varietal diversity of local apple varieties presented in Uzbekistan. The data obtained indicate the presence of diverse varieties with genetic resistance to diseases. The combination of markers used are an important tool in determining a variety for cultivation in a particular region, depending on the pathogenic background.

Most local varieties have been cultivated for many years in different regions of the country. We have determined that some local varieties cultivated in different regions have the same names, but may differ in the combination of DNA markers for the degree of disease resistance. This indicates their likely crossbreeding and selection over decades in regions where certain diseases arose.

Phenotypic assessment of varieties for fire blight resistance showed that 25,6% (n=28) of varieties were highly resistant, 16,5% (n=18) moderate resistant, 37,6% (n=41) low resistant, and 20,1% (n=22) susceptible (Tab.1).

Screening for host genetic resistance to fire blight using SCAR AE10-375, GE-8019, and SSR CH-F7-Fb1 markers (Khan et al., 2007) revealed high frequency (79.81%) of dominant marker AE10 and low (11%) frequency of GE-8019 in varietal germplasm, whereas frequency of 210bp allele of codominant CH-F7-Fb1 was 30,2% in total population. Presence of both resistance loci AE10 and CH-F7-Fb1-210 observed in 34.8% of varieties only. Thus, we did not observe co-segregation of resistance loci AE10-375 and CH-F7-Fb1-210, as it was reported previously (Khan et al., 2007). Both loci segregated independently suggesting their recombination due to distant location. The presence of only one of the studied loci in most cases caused moderate or weak resistance. Varieties with combination of AE10-375 and CH-F7-Fb1-210 either GE-8019 and CH-F7-Fb1-210 exhibit moderate to high resistance to fire blight. The greatest phenotypic resistance was observed in two local varieties “Sariq olma” (SS11) and “Besh barmoq” (XK10) these are the only identified varieties with three resistance loci at once. Thus, we suggest that combination of

all three markers should be used to maximize the chance of selecting seedlings that do have increased fire blight resistance.

Moreover, the study revealed several varieties such as Atlas olma (SS03), Turkish (SS04), Xuboni (SS10), Krasniy jeleznyak (KB01), Shoiy olma (KH16), and Besh barmoq (XK10) which combine marker loci for resistance to fire blight.

Conclusion

This is the first study on the genetic screening for pathogens resistance of apple varieties cultivated in Uzbekistan. It was found that local varieties have diverse degree of resistance, several of them combine genetic loci for resistance to three studied diseases. Thus, using a genetic screening of the apple cultivar collection, several cultivars with high value for resistance breeding and sustainable cultivation were identified. However, more varieties from other regions of the country should be screened in further studies for wider evaluation of host genetic resistance. Furthermore, our work provides a basis for the discovery of fire blight resistant cultivars in historically apple cultivation regions of Central Asia.

Genetic diversity provides the raw material for breeding and plant improvement, allowing breeders to adapt changing environmental conditions as well as satisfy consumers and markets requirements. The information on the resistance/susceptibility of fruit genetic resources towards economically important diseases is important for breeding and for replanting traditional cultivars. In Central Asian countries including Uzbekistan farmers and breeders traditionally selected apple cultivars based on phenotypic characteristics only. Introduction of molecular genetic tools will facilitate and enforce local apple breeding programs.

The markers used in current study showed good discriminatory ability when studying the genetic diversity of varieties for fire blight resistance in local varietal collection. Revealed genetic diversity indicates great potential of Uzbek varietal apple germplasm for breeding programs. Further studies involving more genetic markers associated with genes or QTLs for valuable agronomic traits including disease resistance need to be done in Central Asia.

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Supplementary Table 1

Summary table for fire blight, powdery mildew and scab resistance genotypes among apple varieties growing in Uzbekistan

№	Variety	Marker genotypes for resistance to fire blight (*E.amylovora*)			Resistance Index	№	Variety	Marker genotypes for resistance to fire blight (*E.amylovora*)			Resistance Index
		GE-8019	AE10-375	CH-F7Fb1 (210bp)				GE-8019	AE10-375	CH-F7Fb1 (210bp)	
SS 01	Qirmizak	-	+	-	2	SSh 01	Renet Semerenko	-	-	+	1
SS 02	Renet Semerenko	-	+	-	2	SSh 02	Oq olma	-	-	-	1
SS 03	Atlas olma	-	+	+	4	SSh 03	Besh yulduz	-	-	-	1
SS 04	Turkish	-	+	+	4	SSh 04	Qizil olma	-	-	-	1
SS 05	Atlas kechki	+	+	-	4	SSh 05	Besh yulduz	-	-	-	1
SS 06	Renet Simarenko	-	+	+	3	SSh 06	Renet Semerenko	-	-	-	1
SS 07	Qirmizak	-	+	+	4	SSh 07	Oq olma	-	-	-	1
SS 08	Starkrimson	-	+	+	4	SSh 08	Besh yulduz	-	+	+	4
SS 09	Malus niedzwetzkyana	-	+	+	4	KB 01	Krasniy jeleznyak	-	+	+	4
SS 10	Xuboni	-	+	+	4	KB 02	Jonathan	-	+	+	4
SS 11	Sariq olma	+	+	+	4	KB 03	Yozgi olma	-	-	-	1
SD 01	Mayskiy	-	-	-	1	KB 04	Starkrimson	-	+	-	2
SD 02	Xuboni	-	+	+	4	KB 05	Xazaraspskiy letniy	-	-	-	1

SD 03	Mayskiy	-	+	+	4	KB 06	Xazaraspskiy zimniy	-	-	-	1
SD 04	Xuboni	-	+	-	2	KB 07	Shoyi olma	+	-	-	2
SD 05	Atlas olma	-	+	-	2	KB 08	Besh yulduz	-	+	-	2
SD 06	Mayskiy Qizil	-	+	-	2	KB 09	Golden delicious	-	+	-	2
SD 07	Eshak olma	-	-	-	1	KB 10	Malus niedzwetzkyana	-	+	+	4
SD 08	Qizil kechki olma	-	+	+	4	KB 11	Rozemary white	-	+	+	4
SD 09	Oq olma	-	-	-	2	KB 12	Besh barmoq	-	+	-	2
KH 01	Besh barmoq	-	+	+	3	KB 13	Yanar	-	+	+	4
KH 02	Renet Semerenko	+	-	-	2	KB 14	Qizil olma (Red apple)	-	+	+	4
KH 03	Samarqandskiy ranniy	-	+	+	3	SKO01	Qizil olma (Red apple)	-	+	-	2
KH 04	Starkrimson	-	+	-	2	SKO02	Qizil olma (Red apple)	-	+	-	2
KH 05	Golden delicious	-	+	-	2	SKO03	Qizil olma (Red apple)	-	+	-	2
KH 06	Jonathan	-	+	+	3	SKO04	Qizil olma (Red apple)	-	+	-	2

KH07	Pervenets Samarqanda	-	+	+	3	SKO05	Renet Semerenko	-	+	-	2
KH08	Besh yulduz	-	+	+	3	SKO06	Oq olma	-	+	-	2
KH09	Besh barmoq	-	+	-	2	SKO07	Besh yulduz	-	+	-	2
KH10	Grimes Golden	+	+	-	4	SKO08	Oq olma	-	+	-	2
KH11	Qand olma kishgi	-	+	+	3	SKO09	Oq olma	-	-	-	1
KH12	Mayskiy	-	+	+	3	SKO10	Oq olma	-	+	-	2
KH13	Red delicious	-	+	+	3	SKO11	Besh yulduz	-	+	-	2
KH14	Qizil olma urtagi	-	+	-	2	SKO12	Oq olma	-	+	-	2
KH15	Qizil olma	-	+	+	3	SKb01	Besh yulduz	-	+	+	4
KH16	Shoyi olma	-	+	+	3	Skb02	Atlas	-	+	-	2
KH17	Tosh olma	-	+	-	2	SKb03	Qizil olma	-	-	+	2
KH18	Qand olma	-	+	+	3	SKb04	Oq olma	-	+	-	2
KH19	Krasniy jeleznyak	+	+	-	4	SKb05	Qizil olma	-	-	-	1
XS01	Besh barmoq	-	+	-	2	SKb06	Qizil olma	-	+	-	2
XS02	Grimes Golden	-	+	+	2	SKb07	Oq olma	-	+	-	2

XS03	So’x go’zali	-	+	+	3	SKb08	Renet Semerenko	-	-	-	1
XS04	R.Semerenko	+	+	-	4	SKb09	Qizil olma	-	-	-	1
XS05	Qizil sho’r olma	-	+	-	2	SKb10	Qizil olma	-	+	-	1
XS06	Golden delicious	-	+	+	3	XK01	Karvak qutir olma	-	+	-	1
XS07	Yozgi olma	-	+	+	3	XK02	Avgustovskiy	-	-	-	1
XS08	Krasniy jeleznyak	+	+	-	4	XK03	Ruxi janon	-	+	-	1
XS09	Mayskiy	-	+	-	2	XK04	Qand olma	-	+	-	1
XS10	Qizil olma	+	+	-	4	XK05	Karvak olma	+	+	-	2
XS11	Xazaraspskiy letniy	-	+	+	4	XK06	Qutir qand olma	-	+	+	3
XS12	Qand olma kishgi	-	+	-	2	XK07	Jeleznyak	-	-	-	1
XS13	Qand olma	+	+	-	4	XK08	Muz olma	-	+	-	2
XS14	Muz olma	-	+	-	2	XK09	Renet Semerenko	-	+	+	3
XS15	Xivinskiy beshbaroq	-	+	+	4	XK10	Besh barmoq	+	+	+	4
						XK11	Red delicious	-	+	-	2

The letters in the accession number were assigned depending on its location in the geographical region of Uzbekistan. S -Surkhandarya, K- Karakalpakstan, X – Xorazm. The resistance index was assigned depending on the degree of pathogen infestation; Disease resistance index: 1-susceptible, 2-low resistance, 3-moderate resistance, 4- high resistance