European stone fruit yellows (ESFY) and its vector (*Cacopsylla pruni*, Scopoli) presence in Borsod-Abaúj-Zemplén County

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Abstract

European stone fruit yellows is one of the most serious diseases of peaches, cherries, sour cherries and in addition to apricots. The pathogen causing the disease now known as '*Ca*. Phytoplasma prunorum'. The disease was diagnosed first time in 1992 in Hungary. During the spread of the disease it has reached Borsod-Abaúj-Zemplén County as well. Since the release, it became generally widespread and seriously threatens the growing of susceptible plants, especially of apricot. In 2009 and 2010 in Borsod-Abaúj-Zemplén County more stone plantation was carried out tests to determine the extent of the infestation. The majority of stocks evaluated severe infection have been identified that were confirmed by laboratory tests. During the tests, samples from 28 different stone fruit plant (apricots, peaches, cherries, sour cherries, wild plum / Prunus cerasifera /) were analyzed, of which 13 proved phytoplasma infected in the laboratory tests. From the time of the tests were carried out in 2009, the rate of destruction due to ESFY in the apricot trees of the region of Boldogkőváralja amounted to 70-80% to the year 2014. In 2014 the samples from a three years old apricot tree, derived from the settlement also gave positive results in the PCR test, to which varieties was 'Gönci kajszi'. Previously samples from such a young apricot tree has not been detected phyatoplasma infection. Vector of the ESFY, the plum psylla (Cacopsylla pruni) was observed during the examination of possible swarming routes, and 41 individuals were collected to search phytoplasma infection in 2016 in region of Boldogkőváralja. The '*Ca*. Phytoplasma prunorum' can be said to be widespread in the stone fruit orchards of Borsod-Abaúj-Zemplén County, and the disease means serious hazards to the stone fruits, especially the cultivation of apricots.

Keywords: European stone fruit yellows, peach, sourceherry, cherry, apricot, *Prunus ceasifera*, plum psylla, ESFY, *'Ca.* Phytoplasma prunorum'

Összefoglalás

A csonthéjasok európai sárgasága az őszibarack, a cseresznye, és a meggy mellett a kajszinak is az egyik legsúlyosabb betegsége. A betegséget okozó kórokozó jelenleg *'Ca*. Phytoplasma prunorum' néven ismert. A betegséget először 1992-ben diagnosztizálták Magyarországon. Terjedése során a betegség elérte Borsod–Abaúj-Zemplén megyét is. Megjelenése óta általánosan elterjedté vált és komoly mértékben veszélyezteti a fogékony növények termesztést, különösen a kajsziét. 2009-ben és 2010-ben Borsod–Abaúj-Zemplén megyében több csonthéjas ültetvényben végeztek vizsgálatokat, a fertőzöttség mértékének megállapítására. A vizsgált állományok többségénél súlyos mértékű fertőzést állapítottak meg, amelyet laboratóriumi vizsgálatokkal is igazoltak. A vizsgálatok során 28 különböző csonthéjas növényről (kajszi, őszibarack, meggy, cseresznye, vadszilva /*Prunus cerasifera*/) származó mintát vizsgáltak, amelyből 13 fitoplazma fertőzöttnek bizonyult a laboratórium vizsgálatok során. A 2009-ben elvégzett vizsgálatok idejétől Boldogkőváralja térségében a kajszi fák ESFY miatti pusztulásának aránya 2014-re elérte a 70-80%-ot. 2014-ben a településről származó 3 éves Gönci kajszi fajtájú fáról származó minták is pozitív eredményt adtak a PCR vizsgálat során, korábban ilyen fiatal kajszi fáról származó mintából még nem mutattak ki fitoplazma fertőzöttséget. 2016-ban Boldogkőváralja térségében az ESFY vektorának, a szilva levélbolhának (*Cacopsylla pruni*, Scopoli) vizsgálata során megfigyeltük a lehetséges rajzási útvonalakat és 41 egyedet gyűjtöttünk fitoplazma fertőzöttséget keresve bennük. Borsod-Abaúj-Zemplén megyében, a csonthéjas ültetvényekben a '*Ca*. Phytoplasma prunorum' általánosan elterjedtnek mondható és komoly veszélyeket jelent a csonthéjasok, különösen a kajszi termesztésére.

Kulcsszavak: csonthéjasok európai sárgasága, őszibarack, meggy, cseresznye, kajszi, vadszilva, szilvalevélbolha, ESFY, '*Ca*. Phytoplasma prunorum'

Introduction

The phytoplasma diseases cause increasing damage to orchards around the world. Particularly, the European stone fruit yellows (ESFY) causing increasing concern in our country. The disease has had many names as mycoplasma-like organisms (MLOs) (Mergenthaler, 2004). Since the first appearance in the country, the disease stepped forward to one of the most serious disease of the apricots, cherries, peaches, and sour cherries. In Hungary, the pathogen was detected first time by laboratory tests from apricots in 1992 (Süle, 1999). The disease during its spreading

reached Borsod-Abaúj-Zemplén County, thus greatly endandering the apricot cultivation, because the most fertile terroir of the country (Gönci terroir) is in this county, which accounts for 40% of the national apricot yield. The pathogen causing yield loss, deterioration, reduction in the life of the bearing trees or tree death. Progress is rapid, appropiet protection against it is not yet known, so the producers can only base the protection on prevention. The prevention is limited to the infection-free planting material use, and the defense against the vector (Viczián et al., 1998). In 2009 and 2010, more comprehensive studies have been conducted in the county stone plantations, in order to shed light on the extent of their infection. In doing so 28 different samples from stone plant (apricot, peach, sour cherry, cherry, wild plum) were examined, which came from different towns. 13 from them proved to be infected according to laboratory tests (Tarcali et al., 2010). From samples which was from a 3 years old apricot, which varieties was 'Gönci Kajszi', infection was detected. Previously, from samples which came from younger than 3 year-old trees, this failed. In 2016 we studied the swarming routes of the vector, the plum psylla (*Cacopsylla pruni*, Scopoli), of the pathogen, and also investigated their phytoplasma infection in the areas of Boldogkőváralja.

Material and method

1.

The terrain tests in 2009 and 2010 were carried out from August onwards in several stone fruit orchards in the county, in order to visually diagnose the disease and judging the extent of the infestation. (Figure 1)

Figure 1. Examined areas

In the examined plantation the infection ratio (I%) and infection index (Ii) (according to a classification system, which is contained in Table 1) were determined based on visible symptoms in which the trees were qualified in a 1-5 grading scale number.

Table 1. Classification scale to determine the infection index (Ii)

Degree	Symptoms
I	Healthy tree
II	Initial symptoms on 1 branch
III	Initial symptopms on several branches
IV	One or more dead branches
V	Dead or felled tree

The first stage was the aseptic tree, the second stage was the tree with initial symptoms on one branch of the tree, the third one was the tree with initial symptoms on more branches of the tree, the fourth one was the tree with one or more dead branches of the tree, and the fifth stage was the tree which was dead or had been cut. To determine the extent of the infestation, we selected sample area, which contained 100 pieces of tree or fruit trees in a row in each plantations, where the trees were grade 1-5 grading scale numbers were qualified. Plant samples were collected from the supposedly infected trees, based on the visible symptoms (living leaves, pieces of branches, which were about as thick as a pencil and pieces of roots). The collected samples were placed in bags with identification and stored and transported in cooler.

In laboratory tests the PCR method was used to detect infection. The DNA extraction was performed based on method of Doyle and Doyle (1990) using CTAB extractin buffer (2.5% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris (pH 8.0), 1% PVP 500 ml distilled water which was added 0.2% mercaptoethanol before to use). The processed crop samples of about 0.5 g in a sterile mortar with addition of CTAB buffer was homogenized, and then follow the steps provided we extracted the DNA from the samples. The DNA pellet was dissolved in sterile distilled water 50-100µl or modified TE solution (10 mM Tris, 0.1 M EDTA). The DNA was stored at -20 ° C.

To determining the infection with phytoplasma we worked with universal primers designed to phytoplasmas (fP1/rP7, fU5/rU3, Kirkpatric et al, 1994). To determine the phytoplasma group-specific preimers (fO1/rO1, Kirkpatric et al, 1994 and ECA1/ECA2, Jarausch et al, 1998) were chosen. The patterns in DNA amplified by polymerase chain reaction (PCR).

To testing the samples from Bükkaranyos nested PCR was used during which to the specific PCR we used about 1 µl from the product which was amplified with the primers P1/P7 as template. The reaction mixture in both cases contained 1,4mM 10x buffer, 200µM dNTPs, 0,2-2,0 µ forward and reverse primers, 0,7U Taq polymerase enzyme, 1µl DNA and distilled water. The success of the reaction was checked by running the resulting product in a 1% agarose gel.

Table 2. Used sequences and programs on laboratory examination

Name of primer	**Sequences (5'->3')**	**Position (bp)**	**Programme**
P1	AAGAGTTTGATCCTGGCTCAGGATT	6-28	94°C-5min; 94°C-1min; 55°C-1min; 72°C-2min (35 ciklus), 72°C-10min
P7	TTCTCGGCTACTTCCTGC	1818-1836	
fU5	CGCAATGGAGGAAACT	370-387	95°C-3min; 95°C-1min; 55°C-1min; 72°C-1min (35 ciklus); 72°C-5min
rU3	TTCAGCTACTCTTTGTAACA	1230-1250	
ECA1	AATAATCAAGAACAAGAAGT		95°C-1min; 95°C-30sec; 55°C-30sec; 72°C-30sec (35 ciklus); 72°C-3min
ECA2	GTTTATAAAAATTAATGACTC		
fO1	CGGAAACTTTTAGTTTCAGT	61-81	94°C-3min; 94°C-1min; 55°C-1min, 72°C-1min (35 ciklus); 72°C-7min
rO1	AAGTGCCCAACTAAATGAT	1115-1135	

Since inhibitory substances produced by the stone fruits often influence the outcome of a PCR, despite the negative results do not exclude the presence of phytoplasma each semaple. For this reason, several cases of repeated DNA extraction and verification. Tree apricots and peaches phytoplasma DNA extraction section 880 nucleotide base pair sequenece is determined, and compared to sequences in the international database (www.ncbi.nlm.nih.gov) the phytoplasma identified our target. To this DNA fragment amplified by fU5/rU3 universal primer then the product was purified from the unwanted byproducts by using QIAquick PCR Purification KIT (QIAGEN), according to the manufacturer's insturcions. Determination of sequences of patterns was in the Sequence Laboratories Göttingen in Göttingen laboratory of GmBH.

2.

In 2014, during the tests, we collected samples from trees which have shown symptoms of infection, so that the suspected presence of the pathogen was confirmed by laboratory tests. Samples from trees belonging to different age groups and different varieties were collected. The trees from which the samples were collected was 3, 5, and 17-18 years old, the cultivars of apricot were Magyar Kajszi, Ceglédi Óriás and Gönci Kajszi. As samples living petioles, pieces of branches, and pieces of roots were collected to bags with identification and were stored and transported in cooler.

Table 3. Data of analyzed samples

Laboratory code	Date of collection	Date of DNA extraction	PCR, Nested PCR	Plant (apricot)	Measured quantity	Comment
T1	2014.09.21.	2014.09.25.	2014.09.29.	3 éves floém	0,77 g	Gönci Kajszi
T2	2014.09.21.	2014.09.25.	2014.09.29.	3 éves levélnyél	0,70 g	Gönci Kajszi
T3	2014.09.21.	2014.09.25.	2014.09.29.	3 éves gyökérfloém	0,57 g	nagyon barnul Gönci Kajszi
T4	2014.09.21.	2014.09.25.	2014.09.29.	5 éves floém	1,04 g	Magyar Kajszi
T5	2014.09.21.	2014.09.25.	2014.09.29.	5 éves levélnyél	0,61 g	Magyar Kajszi
T6	2014.09.21.	2014.09.25.	2014.09.29.	5 éves gyökérfloém	1,03 g	közepesen barnul Magyar Kajszi
T7	2014.09.21.	2014.09.25.	2014.09.29.	17-18 éves floém	1,03g	Ceglédi Óriás
T8	2014.09.21.	2014.09.25.	2014.09.29.	17-18 éves levélnyél	0,65 g	Ceglédi Óriás
T9	2014.09.21.	2014.09.25.	2014.09.29.	17-18 éves gyökérfloém	0,81 g	egyáltalán nem barnul, rózsaszínes Ceglédi Óriás

The DNA extraction was performed according to the Delladoyle Method (Ahrens-Seemúller, 1992) using CTAB extraction buffer (12,5g CTAB, 140ml of 5M NaCl, 20ml 0.5M EDTA, 50 ml 1M Tris, 5 g PVP-40 500 ml of distilled water before use to which 1ml / 500ml mercaptoethanol was added, the pH was adjusted to 8.0). About 0,5g of processed crop material was homogenized in ice-cooled friction mortars with 7ml 1x Dellaporta solution (1:1 ration Dellaporta solution and distilled water, and added ascorbic acid 0,636mg/120ml on pH 7,6 immediately prior to the use of it) and with quartz sand. Then DNA extraction was made from the samples followed the steps provided. The DNA pellet was dissolved in 25 ml of sterile distilled water and stored at -20 ° C. To determine the infection with phytoplasma universal primers (Eof/Eor, Mergenthaler) were used. Primers used in the second stage was ECA/ECA (Jarausch et al., 1998).

Table 4. Primers used for the detection and identification of phytoplasma

Name of primer	Target DNA	Sequences (5'-3')	Postition (bp)
Eof	16S rDNS	CCA AC TTA ATA ATA GCA ATA GGA A	
Eor	16S rDNS	TGA TTT ATG TTT TCA ACT TTT CCA	471
ECA1	16s rDNS	AAT AAT CAA GAA CAA GAA GT	
ECA2	16S rDNS	GTT TAT AAA AAT TAA TGA CTC	237

The reaction mixture in each case consisted of the following components: 2x distilled water: 2,9 µl, DreamTaq Green PCR Master Mix (2x) (Scientific Bio): 7,5 µl, forward primer (10pmol): 1,8µl, reverse primer (10pmol):1,8µl, DNA solution: 1µl. In the first PCR the DNS was the template, and in the second PCR the first PCR product was used as template. The parameters of bith PCR reaction were the same. The phytoplasma DNA fragments were amplified by 35 cycles, which duration were 30 sec. in the cases under the following parameters: 95°C-1min, 95°C-30sec; 55°C-30sec; 72°C-30sec; 72°C-3min. The success of the reaction was checked by the running of the resulting products in 1% agarose gel.

3.

During the 2016 tests, plum psylla's possible swarming routes were determined with the help of satellite map, and we considered the host plants and the possible place of overwintering according to literature.

Figure 2. Possible routes swarming of Cacopsylla pruni in the region of Boldogkőváralja

Psyllas were captured with jar glasses which contained 70% pharmacy alcohol. The phytoplasma infection of the captured psyllas were tested in laboratory with the method of Nested PCR. The DNA extraction was carried out on the basis of the modified Doyle and Doyle (1990) (Marzachi et al, 1998). The samples were homogenized with the addition of 2% CTAB buffer, and then the DNA was extracted in accordance with specified steps. The resulting DNA pellet was dissolved in TE buffer and till the test were stored in -20°C. The phytoplasma infection was tested by nested PCR. The success of the reaction was checked by running the resulting products in 1% agarose gel.

In order to test the correlation of phytoplasma infection in the area and the vectors, samples were collected from apricot trees on which previously plum psyllas sucking and showed visible signs of infection as a result of phytoplasma infection. Samples (petioles, pieces of roots and pieces of branches, and also bark) collected from two apricot tree which were previously designed during the time of plum psyllas catches. Samples were stored and transported in labeled bags in cooler. DNA extraction was performed also according the Delladoyle Method (Ahrens-Seemüller, 1992). The phytoplasma infection was tested by nested PCR. The success of the reaction was checked by running the resulting products in 1% agarose gel.

Results

1.

For the cherry orchards based on the studies the infection was 36-62% in Bekecs in 2009, and it was 59% in Bükkaranyos in 2010. The infection was confirmed by laboratory tests as well. We detected 30% infection in cherry orchards of Bekecs in 2009. In the case of peach the infection was 70% in Bekecs in 2009. The wild plum samples from Bükkaranyos proved to be infected according to laboratory tests, though it did not show visual symptoms of the disease. In the apricot orchards the infection was 55-70% in Bekecs in 2009, the infection was 78-84% in Bükkaranyos, 59% in Rátka, 37% in Göncruszka, 8% in Vizsoly, 77% in Boldogkőváralja, and 10% in Abaújkér in 2010.

Table 5. Phytoplasma infection data on the examined fruit platations in Borsod-Abaúj-Zemplén County in 2009

Settlement	Time of field examination	Tree kind	Age of trees (year)	Number of trees	Degree of infection					Ii	I%
					I	II	III	IV	V		
Bekecs	2009.10.02.	kajszi	4	100	98	1	1	-	-	1,03	2
Bekecs	2009.10.02.	kajszi	8-9	100	45	4	6	5	40	2,91	55
Bekecs	2009.10.02.	kajszi	~8	100	15	7	7	6	65	3,99	85
Bekecs	2009.10.02.	kajszi	12-13	100	30	6	4	35	25	3,21	70
Bekecs	2009.10.02.	őszibarack	~8	100	79	7	2	2	10	1,57	21
Bekecs	2009.10.02.	cseresznye	~10	100	70	9	4	6	11	1,79	30
Bekecs	2009.10.02.	meggy	8-9	100	38	14	10	8	30	2,78	62
Bekecs	2009.10.02.	meggy	7	100	91	3	1	1	4	1,24	9
Bekecs	2009.10.02.	meggy	~30	100	64	6	9	13	8	1,95	36

Table 6. Phytoplasma infection data on the examined fruit plantations in Borsod-Abaúj-Zemplén County in 2010

Settlement	Time of field examination	Tree kind	Age of trees (Year)	Number of trees	Degree of infection					Ii	I%
					I	II	III	IV	V		
Bükkaranyos	2010.09.07.	kajszi	13	70	11	12	2	10	35	3,66	84
Bükkaranyos	2010.09.07	kajszi	13	78	17	6	3	11	41	3,68	78
Bükkaranyos	2010.09.07	meggy	7	104	43	7	12	12	30	2,78	59
Rátka	2010.09.07	kajszi	21	100	41	10	9	11	28	2,72	59
Göncruszka	2010.09.07	kajszi	4	54	34	4	4	3	9	2,06	37
Vizsoly	2010.09.07	kajszi	~12	50	46	1	2	1	-	1,16	8
Boldogkőváralja	2010.09.07	kajszi	~25	100	23	24	12	21	26	3,21	77
Abaújkér	2010.09.07	kajszi	~15	50	45	3	1	1	-	1,16	10

Table 7. Rates of examined DNA-isolated samples of different fruit trees and the results of phytoplasma detection

Fruit tree species	Number of examined amples	Number of positive saamples	Identified phytoplasma
apricot (*Prunus armeniaca*)	16	8	ESFY
peach (*Prunus persica*)	4	1	ESFY
sour cherry (*Prunus cerasus*)	7	3	ESFY
wild plum (*Prunus cerasifera*)	1	1	ESFY

2.

During the 2014 tests, 5-year-old ‘Magyar Kajszi’ , 17-18-year-old ‘Ceglédi Óriás’ and 3-year-old ‘Gönci Kajszi’ were tested in laboratory to detect phytoplasma infection. During the test, the ESFY can be detected surest from the root floe samples. It can be said that although from the T7 lab code floe samle failed to detect the ESFY, it can be successfully demonstrated from the other two samples (petioles, and root floe) which was from the same 17-18 years old tree. (Figure 3).

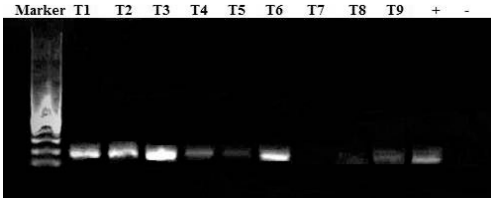

Figure 3. PCR results of apricot samples

From all the thre tree that gave the samples was proved that the deterioration started to the disease of ESFY (European stone fruit yellows) caused by the pathogen of *Ca*. Phytoplasma prunorum. The phytoplasma was the safest deteected from the samples which were from the three years old tree of the three tree. This was followed in the row by the samlpes from the 5 years old tree, especially the root floe sample, and then the samples from the 17-18 years old tree. The reasons of this sequence may be age caracteristics. From the root flo in all three cases we were able to detect the pathogen more sure. The phytoplasma ESFY so far only four-year-old trees, or from older ones have been shown, by contrast one of our tree was 3 years old, and the PCR reaction was the strongest in the samples from it.

This confirmed that the symptoms that can be actually recoreded on the trees caused by the pathogen Ca. Phytoplasma prunorum (Figure 4).

Figure 4. Apricot trees showing the symptoms of phytoplasma infection

3.

The first place we catch psyllas on 09/04/2016, which was a neglected orchard lane, and from the host plants plum, apricot and wild plum were also presented, 8 females and 5 males were caught. Females deposited eggs in that time from what a photo was made which is shown on Figure 5.

Figure 5. Egg depositing Cacopsylla pruni

The second catch place on 16/04/2016 which was an apricot orchard 9 females were caught, of which two has darker coloured wings than the typical. It would be worthwhile to look at the darker wing color can be connected to one biotype. Males could not caught in this area. The darker colored wing individuals are shown in Figure 6.

Figure 6. Darker winged individuals

The third catch place, 6 females and 1 male were caught in a band blackthorn on 04/16/2016.

The fourth catch place, which was an apricot orcahard located right next to a pine forest, 6 females and 5 males were captured on 16/04/6016. Among the males was an interesting body shape individual whose shape resembled to the bodyshape of a female, it was swollen abdomen, but it had a male genitalia organ. The microscopic photo from that male shown in Figure 7.

Figure 7. Male with special bodyshape

The ratio of sexes on 04/16/2016 at the second catch place was about the same, therefore that day could be the day of the fight peak ont hat area. It was a relatively high proportion of males rates on first catch place on 09.04.2016. on which the females deposited eggs. During the investigation, a total of 41 individuals were captured, of which 30 were female and 11 male. Two of the captured females were darker wings than the typical, and one of the captured males was abnormal bodyshape. (Figure 8) It would be worth further investigation be carried out to explore that the distortion of bodyshape and infection with phytoplasma linked to eachother.

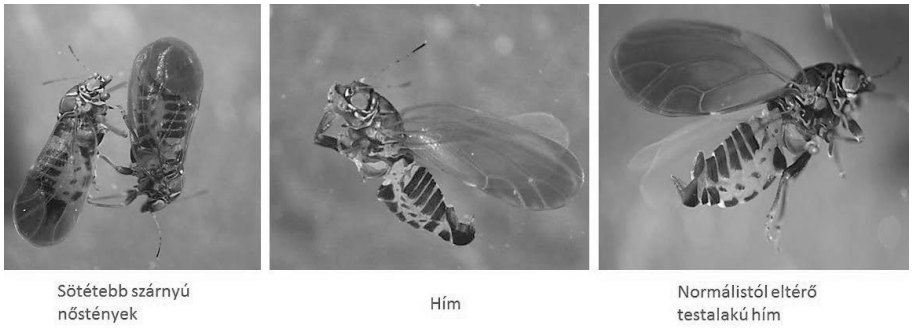

Figure 8. Captured plum psylla individuals

In 2016 studies, from the captured individuals in Boldogkőváralja, there was not any infected with phytoplasma according to the laboratory tests. (Figure 9)

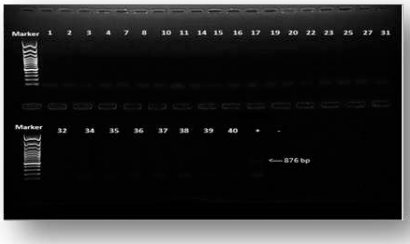

Figure 9. PCR results of plum psyllas

In spite of the fact that the plant samples (bark and pieces of roots), which came from the trees, on which the psyllas sucked at the time of their capture, clearly showed positive results in the Nested PCR test. (Figure 10)

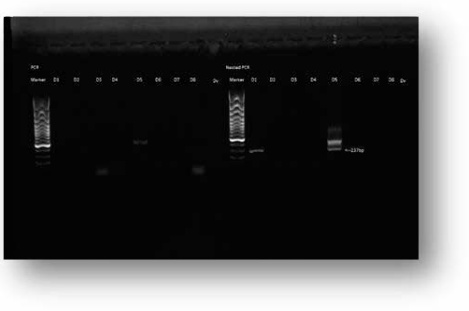

Figure 10. PCR results of apricot samples

During the plum psyllas catching we had chance to observe different behaviors, some of which were associated with the weather. When the weather was sunny, windlees the psyllas always lingered on the young shoots, the lower surfice of the young leaves where the sun was directly shines. In case of windy weather the psyllas retract to the leaves seam. In the case of rainy weather they clung to the brunch with their abdominal side, to the part of the branches which were closer to the ground, that avoid that the rain drops wash them.

Another phenomenon is also observed, which was not dependent on the weather. The phenomenon was observed on both from the selected apricot orchards as catch places. Tha plum psyllas were present in only certain bands within the plantations. The bands retreated in the rows perpendicular. Bands contained the trees which shown the symptoms of phytoplasma infection and also the sapling which were planted to the place of the trees that earlier had wiped out. The bands in the rows of trees infested trees were 1-2 widths in two directions. The plum psyllas were present in these bands in high density. The rest of the plantation is not or only rarely found them.

Discussion

Based on the results, and also supported with laboratory results, stated that the European stone fruit yellows not only present, but very high level can cause damage to stone fruit cultivations in the investigated areas of Borsod-Abaúj-Zemplén County. Extremely high damage can be experienced in peach and apricot orchards. Although the vector is present in the infected orchards and throughout the region, in the individuals which were tested in laboratory, the phytoplasma infection was not proven. However, due to different individuals caught with the typical individuals, further studies should be performed in conjunction with vectors. An important task

of the problem is even more thorough examination of the area, and absolute need to search for an appropriate solution.

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