Final Report of NKFIH-OTKA funded project K127951

Investigation of plant pathogens causing decline in fruit tree plantations by molecular biology methods

The project has been started in September 2018 and lasted till the end of November 2023. We asked and granted 15 moths' extension, needed because of the difficulties caused by both the COVID pandemic and unification of our research institute to the MATE. This time extension helped us to complete most parts of the planned work.

As a result, we published **5 papers in English** (5 Q1, 1 Q2, 1 Q3 and 1 Q4) and **2 papers in Hungarian**. We had **4 oral presentations at international**, and **13 at national conferences**, presented **7 posters at international** and **4 at national conferences**.
The project supported 1 DSc, 1 PhD, 1 postgraduate, 4 MSc and 2 BSc thesises.

Some parts of the project served as a base of the DSc thesis of Eva Varallyay.

Várallyay Éva: Növényeket fertőző vírusok gazdanövényre gyakorolt hatásának vizsgálata, diagnosztikája és felhasználása a molekuláris biológiában (2021, DSc)

The main results and achievements are described below in chapters which number reflects numbers of the original research proposal.

1. Metagenomics survey of declining fruit trees at phytoplasma infected apricot and apple orchards.

During the first year of the project, we had sampled an apricot orchard at Somogy and an apple orchard at Nyírség where fruit tree decline became visible. 3 symptomatic and 3 asymptomatic tree was sampled. DNA and RNA from the trees were extracted. DNA was analysed using a new method LOOP sequencing (barcoding every single DNA in the sample and its targeted sequencing at Illumina MySeq). This method can be useful to describe the presence of bacteria and fungi at a species level. Bioinformatic analysis of the sequenced 16SrRNA gene (in case of bacteria) and the complete 18SrRNAITS region (in case of fungi) has been done. During sequencing the 16SrRNA gene of bacteria in a plant host, the chloroplast rRNA gene also serves as a template, why a large number of plant chloroplast 16S rRNA gene hits were obtained. In the identification of eukaryotic fungi, reads of the ITS genome of the plant host were also generated for similar reasons. For plant microbiome identification, reads of only microbial and fungal origin were considered, yielding 9-1872 bacterial and 335-16096 fungal molecular reads per library. Annotation resulted 2-66 bacterial and 7-26 fungal species per library. respectively. In the bacterial microbiome, *C.phytoplasma prunorum* and *C.phytoplasma mali* molecules were identified in symptomatic samples of apricot and apple and were not found in the microbiome of symptom-free individuals. Interestingly, molecules annotated as *C.phytoplasma mali* were also found in apricot samples and *C.phytoplasma prunorum* in the apple samples, but only in negligible numbers. The number of phytoplasma molecules identified in each symptomatic sample varied widely from 1 to 208 what is proportional to the concentration of the pathogen in the sample. However, since the sample represents only 3 leaves of the tree, this concentration is not representative of the phytoplasma concentration of the whole tree, the proportion of which may vary significantly in the trees studied.
In the analysis of the list of bacterial and fungal species obtained, no correlation was found between the presence of bacteria and fungi and the decline or symptoms on the tree. According to the literature, phytoplasma infection can modify the composition of endophytic bacterial

communities, therefore, the data analysis was performed to examine the proportion of single bacterial classes present in the dying, symptomatic and healthy individuals. By examining the distribution ratios of bacterial classes, we concluded that the proportion of species belonging to the Gammaproteobacteria class is higher in infected trees and the proportion of species belonging to the Alphaproteobacteria class is higher in healthy trees.
HTS of fungal ITS region identified several plant pathogenic fungal species, but none of them can be directly linked to the tree decline. *Valsa mali* is the causal agent of the disease known as 'valsa canker disease' has been detected in both apricot and apple samples, in both dying and healthy individuals. *Rhizophagus irregularis*, a root-colonizing, non-pathogenic fungal species, was present in all the apricot libraries. The presence of *Epicoccum nigrum* was also detected in both infected and healthy apple samples during loop sequencing. Interestingly enough this species is known and is used as a biocontrol agent against phytoplasma. *Monilia fructigena* and *Podosophera leucotricha*, causative agent of apple powdery mildew and *Alternalia alternata*, the causal agent of storage black rot, was also detected in apple libraries, but both in the declining and healthy individuals.
In parallel with the LOOP sequencing, we tested the presence of phytoplasmas by nested PCR and LAMP technique. Using these techniques, we could only detect the phytoplasmas (ESFY and AP) in the symptomatic trees.
Viromes of the investigated trees were also determined using small RNA HTS detecting several viral species including AHVd, CCGaV, ALV-1 in apple and HSVd in apricot whose presence has not yet been described in the country. The presence of the viruses seemed random and did not correlate with the tree decline.
Unfortunately, we could not finish the manuscript of this study till the end of the project. We still need some statistical analysis to present the metagenome data. We plan to finish the manuscript and publish it next year.

These results were only presented as a talk at MBK Napok, (2019) and online at the FIBOK2020 conference and as a MSc thesis of Vajda Zsófia (2020) and as a base of the postgraduate thesis of Galovics Attila (2023).

Nagyné, Galbács Zsuzsanna és Várallyay Éva (2020) Investigation causative agents of tree decline in apricot and apple orchards in Hungary (Fapusztulások okainak kivizsgálása magyarországi alma és kajszi ültetvényekben), FIBOK online, 2020 november 5.
Nagyné Galbács Zsuzsanna, Almash Jahan, Varga Tünde, Baráth Dániel, Jaksa-Czotter Nikoletta, Várallyay Éva (2020) Új vírusok és viroid detektálása almaültetvények virológiai vizsgálata során. MBK Napok online, 2020 november 26.
Vajda Zsófia: Fitoplazma fertőzések vizsgálata molekuláris biológiai módszerekkel (2020, BME, biomérnök MSc)
Galovics Attila: Csonthéjasok európai sárgaság fitoplazmájának (ca. Phytoplasma prunorum) és vektorának, a szilvalevélbolhának (Cacopsylla pruni) vizsgálata magyarországi kajsziültetvényeken (2023, MATE, Növényvédelmi szakmérnök)

2. Survey of viral pathogens at different stock collection of apricot, cherry, plum and apple variety collections.

The main part of the project was to survey and characterize viromes of different fruit tree orchards.
For this study we use small RNA HTS, what method was optimised by our group mainly during the research what was carried out within the frame of this project.

The optimised and modified protocol was published in the Viral Metagenomics Book, of Humana Press acknowledging this project.

Jaksa-Czotter, N., Nagyné Galbács, Z., Jahan, A., Demián, E., Várallyay, É. (2024). Viromes of Plants Determined by High-Throughput Sequencing of Virus-Derived siRNAs. In: Pantaleo, V., Miozzi, L. (eds) Viral Metagenomics. Methods in Molecular Biology, vol 2732. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-3515-5_13, Q3

We surveyed apricot, peach, apple, cherry and sour cherry orchards at different part of the country and determined their viromes using small RNA HTS. As a result, we found not only viruses known to be present in the country and infecting these species, but 8 viruses and 2 viroids which presence in Hungary, or not even on the investigated plant had been described:
Nectarine stem pitting associated virus (NSPaV), peach luteovirus (PaLV) and peach latent mosaic viroid (PLMVd) in peach, Citrus concave gum associated virus (CCGaV), apple luteovirus (ALV-1), apple rubbery wood associated virus (ARWaV) and apple hammerhead viroid (AHVd) in apple, cherry virus A (CVA), Prunus virus F (PrVF) in cherry and sour cherry, Prunus virus I (PrVI) in Clematis.

In the research proposal we showed our unpublished results indicating that we have found CVA and LChV1 in apricot.
That results were published during the review procedure of the proposal (Baráth, et al. Small RNA NGS Revealed the Presence of Cherry Virus A and Little Cherry Virus 1 on Apricots in Hungary. *Viruses* 2018, *10*, 318, https://doi.org/10.3390/v10060318), why it is not acknowledging the project and is not referred in this final report.

Initial data about the comparison of small RNA HTS and biotest for virus diagnostics indicated that some of the previously unknown viruses cannot be detected by biotest. This finding in its early form was presented as a talk at the Final Meeting of COST-DIVAS Action: HTS Technologies for the study and diagnostic of plant viruses, Liège.

Daniel Barath, David Czako, Rozalia Zsovakne-Hangyal, Tunde Varga, Aliz Furton, Mohammad Omran, Klara Nyerges and Eva Varallyay (2018) Comparison of biotest and smallRNA NGS as a virus diagnostics method on fruit trees. Final Meeting of COST-DIVAS Action: HTS Technologies for the study and diagnostic of plant viruses, Liège (Belgium) 26th to 30th November 2018.

There we presented early data about apple, peach, sour cherry and sweet cherry results, which research were continued. The biotest sRNA data were or is still planned to be published according to the plant species.

Below I will summarize our results according to the investigated plants species:

2.1. Peach

Peach trees can be infected with viruses and viroids. As we do not have efficient plant protection methods against these pathogens, the prevention of infection is crucial. Fruit trees are maintained by vegetative propagation. Planting material such as certified mother trees and rootstocks should be free from viruses and viroids, and this status has to be regularly checked to prevent infections. We surveyed certified peach trees for the presence of viruses and viroids using small RNA high-throughput sequencing (HTS), an unbiased virus diagnostic method. The results of the bioinformatic analysis of HTS were validated by other molecular methods

including RT-PCR, Northern blot hybridization and loop-mediated isothermal amplification (LAMP). We found the presence of plum pox virus and peach latent mosaic viroid (PLMVd) in the vector-free isolator houses, whose presence should be regularly tested. Moreover, we detected frequent infection with recently described viruses such as nectarine stem pitting-associated virus and peach-associated luteovirus (PaLV). During the survey, PLMVd and PaLV were detected for the first time in Hungary. The analysis of the presenting virus variants and possible sources of infection suggests that the source of the viral infection could be the infected propagating material. Our study emphasizes the importance of using sensitive and trustworthy diagnostic techniques to be able to detect viral infections and successfully prevent their spread by propagation material.
We found mixed infections on several occasions. During our surveys, the decline in additional trees occurred. Although in some cases it coincided with the presence of mixed viral infections, there were cases where no virus or PLMVd were detected in the declined tree while their causative agent could not be identified. The distribution of virus-infected trees did not follow any pattern, and due to their high frequency, it is very difficult to identify the origin of their infection. We showed that the PPV infection of the Springcrest cultivar could originate from its *in vitro* source.
We could not correlate the presence of viruses to any specific symptoms and not even to the decline in trees. This does not mean that infection did not play any role in the decline, but it is also possible that other factors, i.e., non-viral pathogens or abiotic effects alter or expedite their synergistic effect.
Connecting the presence of the pathogen to any specific symptoms requires the molecular characterization of the viruses described by HTS, which needs special attention in the future

These results were presented as a talk at a national conference (Növényvédelmi Napok) as a poster at an international conference (LIFE) and published in Hungarian (Növényvédelem) and in English in MDPI Plants and served as a base of the MSc thesis of Mohammed Omran and PhD thesis of Daniel Barath.

Baráth Dániel, Jaksa-Czotter Nikoletta, Varga Tünde, Bükki Alexandra, Balássy Júlia, Oláh Beatrix, Szabó Luca, Kirilla Zoltán, Balássy Júlia, Preininger Éva Várallyay Éva (2019) Kajszi és őszibarack ültetvény vírusdiagnosztikai vizsgálata kis RNS-ek újgenerációs szekvenálásával, 65. Növényvédelmi Napok, Budapest, 2019 február 19-20

Dániel Baráth, Nikoletta Jaksa-Czotter, Tünde Varga, Luca Szabó, Zoltán Kirilla, Éva Preininger, Éva Várallyay (2019) Virome of Hungarian peach plantations identified by small RNA NGS, LIFE Nemzetközi Élettudományi Konferencia, Eger, 2019 március 29-31.

Baráth Dániel, Jaksa-Czotter Nikoletta, Varga Tünde, Várallyay Éva (2020) Őszibarackfák vírusdiagnosztikai vizsgálata kis RNS-ek szekvenálásával Növényvédelem 2020, 81:11, 523-532.

Dániel Barát, Nikoletta Jaksa-Czotter, Tünde Varga, Éva Varallyay: Viromes of Hungarian Peach Trees Identified by High-Throughput Sequencing of Small RNAs. MDPI Plants 2022, 11, 1591. https://doi.org/10.3390/plants11121591 IF: 4,658 (Q1)

Mohammad Omran: Molecular Detection of Peach-associated Luteovirus (PaLV) in Peach Trees. (2019, SZIE Mezőgazdasági biotechnológus MSc)

Baráth Dániel: Kajszi és őszibarack fajták vírusdiagnosztikai vizsgálata új generációs szekvenálással (2021, december 8, PhD, MATE, BTDI)

2.2. Apple

Our initial results on apple were shown at an international conference as a talk. As our Czech colleagues had similar finding, we decided that we will collaborate and publish our research together. Our finding below served as a base of a joint publication:

Grafting cultivars onto rootstocks is a widely used practice by the apple industry predominantly aimed at faster fruit bearing. Using high-throughput sequencing, we revealed the presence of recently described viral agents, namely apple hammerhead viroid (AHVd), apple luteovirus 1 (ALV-1), and citrus concave gum-associated virus (CCGaV), in germplasm collections and production orchards in the Czech Republic and Hungary. The HTS results were validated with RT-(q)PCR, and Northern blotting techniques. To obtain further insight about the presence of these agents, RT-PCR based surveys were carried out and showed their widespread presence alone or in mixed infections. The pathogens were present both in production areas and in feral samples. In addition, rootstock-to-scion transmission of ALV-1 and CCGaV was confirmed using commercial rootstock materials. Phylogenetic relationships based on partial sequences of distinct variants were also investigated. Furthermore, the rosy apple aphid was found to be ALV-1-positive, suggesting that it might be a potential vector of the virus.
Our study provides new data and a substantial extension of knowledge in the following areas:

- Occurrence and high incidence of AHVd, ALV-1, and CCGaV in germplasm, mother stock, propagation materials, orchards and feral trees in CZ and HU.
- Insight into the variability of these pathogens by HTS and Sanger sequencing.
- First data on the possible spread of ALV-1 and CCGaV by rootstock, grafting, and possible vectors and hence a need for testing of apple propagation material for ALV-1 and CCGaV to limit their spread into new orchards.

We believe that our extensive data on AHVd, ALV-1, and CCGaV in apple, the principal fruit crop in Europe, will be of interest to a broad community of apple breeders, nurseries, apple growers and phytosanitary administrations.

Further analysis of the small RNA libraries revealed the presence of apple rubbery wood associated virus 2 (ARWaV2). In our regular survey in 2021 we also surveyed pear and quince trees and found a viru ARWaV2 in them too. We are working on the characterization of the different strains of the virus, trying to amplify parts of both five segments of the viral genome.

These results were shown and reported as talks at national conferences (MBK Napok, Növényvédelmi Napok, Fiatal RNS kutatók Fóruma) and at an international conference (ICVF) as a poster at international conferences (Advances in Plant Virology, FIBOK2022) and published in the Növényvédelem and in MDPI Viruses. and was published as the MSC thesis of Fürtön Alíz. Some of the research (hazelnut, ARWaV2) is still continuing and together with the published data serve as a base of the PhD thesis of Almash Jahan (planning to finish in 2025).

Almash Jahan, Baráth Dániel, Majercsik Nándor, Varga Tünde, Jaksa-Czotter Nikoletta és Várallyay Éva: Small RNA HTS revealed the presence of CCGaV and AHVd in apple trees in Hungary, MBK Napok, 2019 november 28-29

Almash Jahan, Baráth Dániel, Nagyné Galbács Zsuzsa, Varga Tünde, Jaksa-Czotter Nikoletta és Várallyay Éva (2020): Magyarországon eddig nem detektált vírusok jelenlétének kimutatása almaültetvényeken, 66. Növényvédelmi Tudományos Napok, Budapest, 2020 február 18-19

Almash Jahan, Daniel Barath, Nandor Majercsik, Tünde Varga, Nikoletta Jaksa-Czotter, Éva Várallyay (2019): Small RNA HTS revealed the presence of CCGaV and AHVd in apple trees in Hungary, Advances in Plant Virology, Rome, 2019 október 29-31.

Jahan Almash, Zsuzsanna Nagyne Galbacs and Varallyay Eva: Analysis of molecular variability of apple hammerhead viroid found in different apple cultivars, FIBOK 2022 április 11-12, Gödöllő, Hungary, ISBN 978-963-269-999-8

Almash Jahan, Nagyné Galbács Zsuzsanna, Várallyay Éva: Uncovering phytosanitary status of apple germplasm and orchards using using HTS as diagnostic method. Fiatal RNS Kutatók Fóruma, Gödöllő, 2023 június 29.

Almash Jahan, Zsuzsanna Nagyne Galbacs, Eva Várallyay: Unveiling viruses and viroid in apple germplasm and orchards in Hungary using high throughput sequencing. The 25th International Conference on Virus and other graft transmissible diseases of Fruit crops, Wageningen, The Netherlands, 9-13 July 2023

Nagyné Galbács Zsuzsanna, Almash Jahan, Várallyay Éva: Újonnan felfedezett vírusok és viroid jelenlétének felmérése magyarországi almaültetvényeken

Növényvédelem, 83 [N.S.58]:11, 473-483.

Várallyay, E.; Přibylová, J.; Galbacs, Z.N.; Jahan, A.; Varga, T.; Špak, J.; Lenz, O.; Fránová, J.; Sedlák, J.; Koloniuk, I. Detection of Apple Hammerhead Viroid, Apple Luteovirus 1 and Citrus Concave Gum-Associated Virus in Apple Propagation Materials and Orchards in the Czech Republic and Hungary. MDPI Viruses 2022, 14, 2347. https://doi.org/10.3390/v14112347, IF:5,818 (Q1)

Fürtön Alíz: Alma vírusok diagnosztizálási módszereinek összehasonlító elemzése (2019, SZIE Növényorvos MSc)

2.3. Sweet and sour cherry

Leaf samples of different Hungarian cultivars of sour and sweet cherries were collected in 2017, 2019, 2021, 2022 and 2023 at two locations: 1/ at an open field stock nursery in Central - Hungary, Érd, Elvira major and 2/in an isolator house at East-Hungary, Újfehértó (Table samples). Leaf samples from four different branches of the trees were collected to ensure detection of all, possibly unevenly distributed viruses.
The samples for HTS analysis were grouped into seven groups according to the sampling year and location. The first three groups contained samples of sour cherries: 22 cultivars of new breeds and nine Hungarian standard cultivars, collected in 2021. Fourth and fifth group contains sour cherry and sweet cherry cultivars, respectively sampled in 2017 randomly, including trees which were in parallel submitted for official biotest screen. The sixth group contains samples from 32 trees, representing 13 sour cherry cultivars, maintained at an isolator house covered with an insect proof net, sampled in 2017. The 7th group contains sweet cherry trees of Paulus cultivar infected with Prunus necrotic ringspot virus (PNRSV) originating from Hungary, sampled in 2018, and from Germany, sampled in 2019. In total 94 trees were sampled for HTS.
For the detailed survey 106 sweet cherry trees representing 12 cultivars and 25 sour cherry trees representing 5 cultivars grown at an open field germ stock collection was sampled in 2019. From these mother plants grafts were prepared in 2019 using MaxMa14, or *Prunus mahaleb* rootstocks. 47 grafts, representing 17 cultivars were grown in an isolator house covered by an insect proof net and sampled in 2022. Form the new bred and standard variety cultivars (tested as a member of the pool 1_PC_E, 2_PC_E2 and 3_PC_E3) only one representant of the existing 2 or 4 trees were sampled in 2021 and were investigated by HTS. The test was repeated as an RT-PCR based survey in 2023, sampling all of the trees alive from this population.

Detailed study of the surveys revealed the presence of PrVF, a virus which presence has not yet been described in Hungary before. We found widespread distribution of CVA and PrVF, these two non-regulated, recently described viruses in cherry and sour cherry trees. Moreover, at the GenBank, where the trees have to bloom and grow fruits, we detected the spread of PNRSV, a pollen transmitted, but regulated virus. Comparison of biotest with the small RNA HTS data showed that these newly described viruses are overlooked in the biotest.

The manuscript about these findings is almost finished and we want to submit it early 2024 for publication.
The obtained results have been presented as a talk at national conferences (Növényvédelmi Fórum, and MADOSZ), at an international conference (Sloven-Hungarian PhD Student Forum), as a poster at international conferences (LIFE, FIBOK2022, Advances in Plant

Virology and ICVG) served as a base of the BSc thesis of Matyus Nikolett and MSc thesis of David Czako.
Some of the research is still continuing and together with the published data will serve as a base of PhD thesis of Francesco Desiderio (planning to finish in 2024).

Francesco Desiderio, Zsuzsanna Nagyné Galbács and Éva Várallyay: New player in the field: Prunus virus F is present and spread in Hungarian cherry orchards. XXXII. Keszthelyi Növényvédelmi Fórum, 2023. január 18-20.

Francesco Desiderio, Zsuzsanna Nagyné Galbács, Éva Várallyay: The detection of Prunus virus F and its distribution in Hungarian sour cherry orchards. MADOSZ, Debrecen, 2023, február, Különdíj

Francesco Desiderio, Zsuzsanna Galbács Nagyné, Éva Várallyay
New player in the field: Presence and distribution of PrVF in Hungarian sour and sweet cherry Sloven-Hungarian PhD Student Forum 2023-11-22 Ljubljana, Szlovénia

Francesco Desiderio, Evans Duah Agyemang, Andras Takacs, Eva Varallyay (2021) Prunus virus F is present in Hungarian sour cherries, Hungarian Molecular Life Science Conference, 2021 november 5-7, Eger.

Desiderio Francesco, Galbacs Nagyné Zsuzsanna, Varallyay Éva: New Putative Viruses in Hungarian Sour Cherry Genebank Collection FIBOK 2022 április 11-12, Gödöllő, Hungary, ISBN 978-963-269-999-8

Francesco Desiderio, Zsuzsanna Nagyné Galbács, Éva Várallyay. Prunus virus F is widespread in Hungarian sour cherry orchards. AAB, International Advances in Plant Virology 2022, Ljubljana Slovenia, 2022 october 5-7. P14, 78

Francesco Desiderio, Nagyné Galbács Zsuzsanna, Várallyay Éva: Investigating the presence and distribution of PrVF in Hungarian sour and sweet cherry. The 25th International Conference on Virus and other graft transmissible diseases of Fruit crops, Wageningen, The Netherlands, 9-13 July 2023 (P11)

Mátyus Nikolett: Gyümölcsfákat fertőző vírusok molekuláris jellemzése (2020, ELTE Biológus BSc)

Czakó Dávid Fidél: Meggyültetvények virológiai felmérése (2018, SZIE, Növényorvos MSc)

2.4. Blueberry, hazelnut and strawberry

We prepared and analysed sRNA HTS results of some blueberry samples. According to the bioinformatic analysis we detected indication for the presence of 2-3 different viruses. Although we tried several times, we could not validate their presence in the plants yet. We went back to Zala, sampled the plants again and plan to HTS RNA with ribodepletion hoping that this would reveal a clearer indication of their virological status.
From the work Péli Noémi a BSc student (ELTE Biologist) wrote and defended her BSc diploma work.

Péli Noémi: Bogyós gyümölcsök vírusfertőzöttségének vizsgálata nagy-áteresztőképességű szekvenálással (2021, ELTE Biológus BSc)

During the regular test of hazelnut trees, checking the presence of AMV- a regulated viruses in them, using small RNA HTS we have detected the presence of CCGaV in some of them. This virus has not yet been described from hazelnut before. Although we could validate the presence of one of the viral RNA in them, we had difficulties with the RT-PCR of the other viral RNA. To overcome this problem ribodepleted RNAseq was also carried out for these samples, but the results of the bioinformatic analysis did not show unambiguously the presence of CCGaV. Currently we are trying to amplify part of the viral genome using genus specific primers, and hope that finally we will be able to validate the presence of CCGaV in hazelnut, what would be a new host of the virus.

As a collaboration with my Czech colleague, we characterized and investigated the small RNA profile of strawberry plants which were infected with multiple viruses. In this research the

molecular work was carried out in Ceske Budejovice, while collaborated in the bioinformatic analysis. Results of that study was published in MDPI Plants.

Koloniuk, I.; Matyášová, A.; Brázdová, S.; Veselá, J.; Přibylová, J.; Várallyay, E.; Fránová, J. Analysis of Virus-Derived siRNAs in Strawberry Plants Co-Infected with Multiple Viruses and Their Genotypes. Plants 2023, 12, 2564. https://doi.org/10.3390/plants12132564, Q1, IF:4,5

2.5. Clematis

During our studies we have bumped into a *Clematis* plants showing very strange line pattern symptoms. We have made sRNA HTS from this plant and revealed a presence of a Prunus infecting Ilarvirus (described 2021 March in cherry in Greece – Prunus virus I). WE presented our results at ICVG 2023 in Wageningen as a poster, where our Slovakian colleagues presented their similar finding. We decided to finish and publish our work together. During the finishing analysis we revealed that PrVI is present in a Croation historical sample, originally misdiagnosed as tobacco streak virus Clematis variant, and was deposited to the German viral collection in the 90ties. The summary of this international research is the following:

Clematis vitalba L. is a climbing shrub and a pioneer plant in abandoned orchards or vineyards that are widespread in temperate climate zones. In past years, several viruses infecting the Clematis species have been identified, including different ilarviruses. Prunus virus I (PrVI) is a recently described ilarvirus, which has been shown to infect sweet cherries and peaches in Greece. Moreover, its presence has been detected in ornamental Clematis in Russia. In the present work, we analyzed the virome of wildly growing C. vitalba plants from Hungary, Slovakia and Croatia showing different kinds of symptoms using high-throughput sequencing (HTS) of small RNAs or ribodepleted RNAs. Applying HTS enabled us to identify the presence of PrVI in C. vitalba, and the bioinformatic analyses were further validated with RT-PCR using PrVI-specific primers and Sanger dideoxy sequencing. Nearly full genome sequences of all three viral RNAs of one Hungarian, two Slovak and one Croatian isolate were determined. Their phylogenetic analysis showed high similarity to each other and to other PrVI isolates described from Central Europe. As the sampled plants were co-infected with other viruses, it is not possible to determine a direct correlation between the infection with PrVI and the observed symptoms. Analyses of different Prunus species in stock collection showed infection of several peach and sweet cherry varieties in Hungary. Our results expand the knowledge on the natural host range of PrVI and highlight the necessity to evaluate alternative plant hosts (even non-Prunus) of PrVI and the role of the virus in the etiology of the potential diseases.

This work was presented as a talk at national conferences (Forum For young RNA Investigators, Tavaszi Szél, GBI Napok, Növényvédelmi Fórum), poster at an international conference and published in MDPI Viruses.

Agyemang, Evans Duah, Desiderio, Francesco, Emese Demian, Andras Takacs, Pal Salamon, Eva Varallyay (2021) Searching for the causative agent of a viral-like symptom in Clematis vitalba. Forum For young RNA Investigators (online conference), 2021 március

Agyemang, Evans Duah, Desiderio, Francesco, Emese Demian, Andras Takacs, Pal Salamon, Eva Varallyay (2021) Putative Ilarvirus found in Clematis vitalba showing virus-like symptoms. XXIV. Tavaszi Szél Konferencia, 2021 május 28-30.

Francesco Desiderio, Evans Duah Agyemang, Emese Demian, Andras Takacs, Pal Salamon, Eva Varallyay (2021) Symptoms on Clematis vitalba can be a reason of Prunus virus I infection. GBI Napok, Gödöllő, 2021, December 14-15.

Evans Duah Agyemang, Francesco Desiderio, Emese Demian, Andras Takacs, Pal Salamon, Eva Varallyay: Egy cseresznyét fertőző vírus kimutatása tünetes iszalagon.

Evans Duah Agyemang, Francesco Desiderio, Emese Demian, Andras Takacs, Pal Salamon, Eva Varallyay (2021) Symptom on Clematis vitalba could be a reason for an infection with Prunus virus I. Hungarian Molecular Life Science Conference, 2021 november 5-7, Eger.

Salamon, Evans Duah Agyemang, Francesco Desiderio, Emese Demian, Zsuszsanna Nagyné-Galbács, Andras Takacs, Eva Varallyay: Clematis vitalba is an alternative host plant of Prunus virus I. 25th International Conference on Virus and other graft transmissible diseases of Fruit crops, Wageningen, The Netherlands, 9-13 July 2023 (P18, book of abstract page 59

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2.6. Virus elimination

As our main conclusion from the viromes surveys is that the source of the infection is usually the infected propagation material, it would be very important to optimise methods for virus elimination from the fruit trees. It is usually done by in *vitro system* and heat therapy.
The literature about this possibility has been described as a revies paper and has been accepted for publication in Plant Cell, Tissue and Organ Culture.
In the Fruit Research Institute, a PhD student, Luca Szabo, set a series of experiments, established form virus infected apricot, plum and cherry to test if some chemotherapy agents alone or in combination with heat therapy can be successful to eliminate different kind of viruses. Although the results of the experiments are not fully conclusive, the work was presented as a poster at FIBOK 2022.

Luca Krisztina Szabó, Francesco Desiderio, Éva Preininger Éva Várallyay: The effect of in vitro chemotherapy and heat treatment used for virus elimination from stone fruit species. FIBOK 2022 április 11-12, Gödöllő, Hungary, ISBN 978-963-269-999-8

Luca Krisztina Szabó, Francesco Desiderio, Zoltán Kirilla, Attila Hegedűs, Éva Várallyay, Éva Preininger. A mini-review on in vitro methods for virus elimination from Prunus sp. fruit trees. Plant Cell, Tissue and Organ Culture Q1 accepted for publication

3. Investigation of gene expression changes in the small regulatory RNA pattern of phytoplasma infected field trees and in different model systems.

In this part of the research we planned to investigate AY phytoplasma infected *C.roseus* plants, in vitro apple proliferation phytoplasma infected plants and Arabidopsis plants overexpressing Sap11, which usually induced during phytoplasma infection.
From the *in vitro* apple we extracted RNA, prepared small RNA sequencing libraries and sequenced them (3+3 library from infected and non-infected in vitro apple cultures). Bioinformatic analysis indicated that miRNAs induced during stress responses were upregulated in all of them, what made us impossibly to conclude anything correlated with the phytoplasma infection response.
The Sap11 overexpressing A.th plants were segregating even after three years, and we cannot sort out enough plants for further studies.
In a collaboration with the Bologna University micropropagated Catharantus roseus plants infected with ‘Candidatus Phytoplasma asteris’ showed virescence symptoms, witches’ broom symptoms, or became asymptomatic were sampled. Nine plants were grouped into three categories according to these symptoms, which were then employed for investigation. The

phytoplasma concentration, as determined by qPCR, correlated well with the severity of symptoms.
To reveal the changes in the small RNA profiles in these plants, small RNA high-throughput sequencing (HTS) was carried out. The bioinformatics comparison of the micro (mi) RNA and small interfering (si) RNA profiles of the symptomatic and asymptomatic plants showed changes, which could be correlated to some of the observed symptoms.
We have detected phytoplasma derived small RNAs and suspected that they could be a product of the plant self defence system. Our careful analysis however showed that their number correlates with the phytoplasma concentration but are more likely degradation and not DCL products as we originally suspected.
Phytoplasma infection highly interferes with the hormone balance and is associated with developmental changes in plants. MiRNAs and siRNAs can play a role during this altered regulation. The pathogen itself is confined to the phloem in an uneven concentration. This spatially different expression of the pathogen can further change temporally during the infection cycle, thus making it particularly difficult to precisely monitor the molecular mechanisms lying behind the symptom expression. An miR-NA-based hormone regulation can be one of the main components of this complex process. C. roseus is not only the model plant for phytoplasma research, but it is also widely used as a medicinal plant; this is why it is very interesting to know whether the production of its important alkaloids can be affected during phytoplasma infection. The alkaloid content of the phytoplasma-infected plants would be important to further investigate in the future not only to better understand this important process, but also to potentially increase the yield of the beneficial compounds. These results complement previous studies on phytoplasmas and serve as a starting point for small RNA-omic studies in phytoplasma research.

This work was presented on a poster at a national conference (FIBOK2022), asa a talk at an international conference (IPWG) and published in Phytopathogenic Mollicutes and MDPI Genes.

Nagyné Galbács, Zsuzsanna, Yuri Zambon, Nicoletta Contaldo, Várallyay, Éva: Small RNA profiling of aster yellows infected Catharanthus roseus and apple proliferation phytoplasma infected apple plants. FIBOK 2022 április 11-12, Gödöllő, Hungary, ISBN 978-963-269-999-8

Yuri Zambon, Nicoletta Contaldo, Assunta Bertaccini, Eva Varallyay (2019 Small RNA profiling of aster yellows infected Catharanthus roseus plants. 4rd meeting of the International Phytoplasmologist Working Group (IPWG), Valencia, Spain, 2019. September 8-12

Yuri Zambon, Nicoletta Contaldo, Assunta Bertaccini, Eva Varallyay (2019) Small RNA profiling of aster yellows infected Catharanthus roseus plants. Phytopathogenic Mollicutes, Volume-9, Issue-1 (June) Print ISSN: 2249-4669, Online ISSN: 2249-4677, DOI:10.5958/2249-4677.2019.00066.5 Q4

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