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Effect of Abiotic Factors on Herbicide Tolerance in *Solanum Nigrum* Populations

Abiotikus tényezők hatása a fekete csucsor (Solanum nigrum) herbicid toleranciájára

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Abstract: Abiotic factors such as light, temperature, relative humidity, soil moisture and carbon dioxide have an effect on herbicide efficacy and performance. As weeds continue to evolve with changes in climate, it is important to understand the impact on herbicide effectiveness in management strategies. In this paper, we describe changes in the sensitivity of black nightshade (*Solanum nigrum*) population to glyphosate under different growing conditions and demonstrate that resistance mechanism may be climate dependent. Young *Solanum nigrum* plants were treated with normal and double dose rates of glyphosate. Observations were recorded 5, 14, 21 and 28 days consecutively after treatment with glyphosate. Some of the *Solanum nigrum* populations exhibited some level of tolerance to glyphosate in the first round of the experiment for both normal and double dose under ambient conditions. However, a repeat experiment using the same samples and treatment in a controlled environment (growing chamber) where growing conditions was set did not yield the same observations made under ambient conditions. Further investigations and experiments are recommended and may provide more explanation to the differences in results obtained for initial tolerance in *Solanum nigrum* samples which could be attributed to the differences in growing conditions.

***Keywords**: Solanum nigrum; abiotic factors; herbicide resistance; climate; glyphosate*

Összefoglalás: Az abiotikus tényezők - mint a fény, a hőmérséklet, a talajnedvesség, légköri széndioxid koncentráció - jelentősen befolyásolják a gyomirtó szerek hatékonyságát. Mivel a gyomállomány összetételére az éghajlati tényezők jelentős hatást gyakorolnak, fontos megérteni a herbicidek hatékonyságára gyakorolt hatást is, a sikeres kezelési stratégiák kidolgozása érdekében. Ebben a munkában a fekete csucsor (*Solanum nigrum*) glifozáttal szembeni reakcióját vizsgáltuk annak érdekében, hogy bizonyítsuk, a herbicidekkel szembeni reakciót a klimatikus tényezők is befolyásolhatják. Ezért fiatal fekete csucsor növények glifozáttal szembeni reakcióját vizsgáltuk dózis-hatás kísérletekben szabadföldön, és kontrollált körülmények között klímakamrában. Az eredmények eltérőek voltak, ami azt valószínűsíti, hogy az abiotikus tényezők jelentősen hatnak a vizsgált gyomfaj/biotípus herbicid érzékenységére, de ennek egzakt bizonyítására további vizsgálatok szükségesek.

***Kulcsszavak**: Solanum nigrum; abiotikus faktorok; herbicid ellenálló képesség; klíma; glifozát*

1. Introduction

Climate is a critical factor in food production and climate change remains one of the defining global issues in recent times. However, irrespective of how the climate patterns change, weeds will continue to be present due to their ability to adapt to various environmental conditions (Bajwa et al., 2021; Mahaut et al., 2020). Agricultural weeds are of particular concern since they have severe impact on global food security and as such, it is important to highlight the effects of abiotic conditions on weed management (Matzrafi et al., 2016). Weeds are highly adaptive and able to withstand several stressors related to climate change such as increase in temperatures, increased carbon dioxide (CO2) levels, drought and waterlogging (Hicks et al., 2018). The effect of weeds on crop production can be very severe if not properly managed (Chen et al., 2020; Kathiresan and Gualbert, 2016). Herbicides have been used in many management strategies across the globe, this is because herbicides provide rapid action, efficient and cost effective mechanism for controlling weeds. However, the increasing numbers of recorded cases of herbicide resistant weeds worldwide is quite alarming and calls for more research focused on resistance development (Travlos et al., 2020). Climate conditions such as temperature, atmospheric CO2 and precipitation are likely to affect weed biology. These factors affect the penetration of herbicide into the plant and indirectly influence the movement of herbicide within the plant. (Ziska et al., 2019). Herbicide performance can also be affected by abiotic, physiological, and biological factors thus, limiting weed control effectiveness (Torra et al., 2021). Study by Ge et al. (2011) reported resistance of horseweed (Conyza canadensis) to glyphosate in response to temperature. The particular increase in herbicide resistance cases especially metabolism based non-target site (NTS) resistance and multiple resistance calls for in-depth study into environmental effects on herbicide performance for improved weed control. Research has shown that a significant proportion of reported cases of NTS herbicide resistance is related to acetyl-CoA carboxylase (ACCase) inhibitors used to control grass weeds in cereal crops and to 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor, glyphosate, most intensively used herbicide to control weeds globally (Délye 2013; Matzrafi et al., 2016).

In Hungary, herbicides are used in many weed management strategies and there have been several reported cases of herbicide resistant weeds. Common ragweed (*Ambrosia artemisiifolia*), johnsongrass (*Sorghum halapense*), Italian cocklebur (*Xanthium italicum*) and green amaranth (*Amaranthus powellii*) have been found resistant to a number of acetolactate synthase (ALS) inhibitors in Hungary (Szabó et al., 2018; Kazinczi-Torma, 2016). Glyphosate-resistant *Conyza canadensis* have been reported (Kutasy et al., 2021; Heap, 2023). Although there are currently no recorded cases of herbicide resistant *S. nigrum* in Hungary, the weed is common occuring in areas with high nitrogenous soils and farmlands in Hungary where herbicides are used to management strategies (Kazinczi et al., 2002). Since forecast for the future continue to predict changes in climate patterns globally, the phenomenon of metabolic resistance to herbicides will dramatically increase in agricultural systems. This highlights the need for more research in the area of herbicide resistance development. In this paper, changes in the sensitivity of *Solanum nigrum* population to glyphosate under different growing conditions is studied and described.

2. Materials and Methods

Matured seeds of *Solanum nigrum* were collected from vineyards in Balatonboglár and Cserszegtomaj where the main method of weed control was with the use of herbicides. Seed samples were taken from individual plants suspected to be resistant after treatments with

glyphosate. Seeds were also collected from wastelands at Kaposvár. The collected seeds were cleaned and cold stratified to break dormancy. After stratification, the seeds were sown in three different trays and allowed to grow under ambient conditions. At 3-4 leaf stage, dose-response experiment was conducted and observations recorded. In the subsequent experiment, a dose-response experiment was performed under controlled conditions where the planted seeds were allowed to grow in a controlled environment with day temperature of 28°C and night temperature 20°C. For the experiment, treatments applied were, control (no herbicide), normal dose (1440 g glyphosate/ha in 250 l/ha of water) and double dose (2880 g glyphosate/ha in 250 l/ha of water). Each sample had four (4) replications per treatment as shown in Table 1. Observations were taken 5, 14, 21 and 28 days after treatment (DAT) with glyphosate.

Table 1: Experiment layout for glyphosate dose-response experiment with *Solanum nigrum*

	Tray 1	**Tray 2**	**Tray 3**
	Each sample replicated 4 times per tray		
Place of collection	No Treatment (Control)	Treatment with normal dosage	Treatment with double dosage
Balatonboglár	10 samples	10 samples	10 samples
Kaposvar	1 samples	1 samples	1 samples
Cserszegtomaj	9 samples	9 samples	9 samples
Total	80 samples	80 samples	80 samples

3. Results

It was observed during the experiment that the plants exhibited varied responses under ambient conditions and in the growth chamber. Initial symptoms of yellowing and wilting were observed 14 DAT and proceeded till death of plant by the 28 day for most of the plants. However, its was observed that some plants from seeds collected from Balatonboglár survived for both normal and double dose rates under ambient conditions with slight yellowing 28 DAT as observed in Figure 1. However, the same samples grown under controlled environment were susceptible to the effects of glyphosate after treatment with normal and double doses.

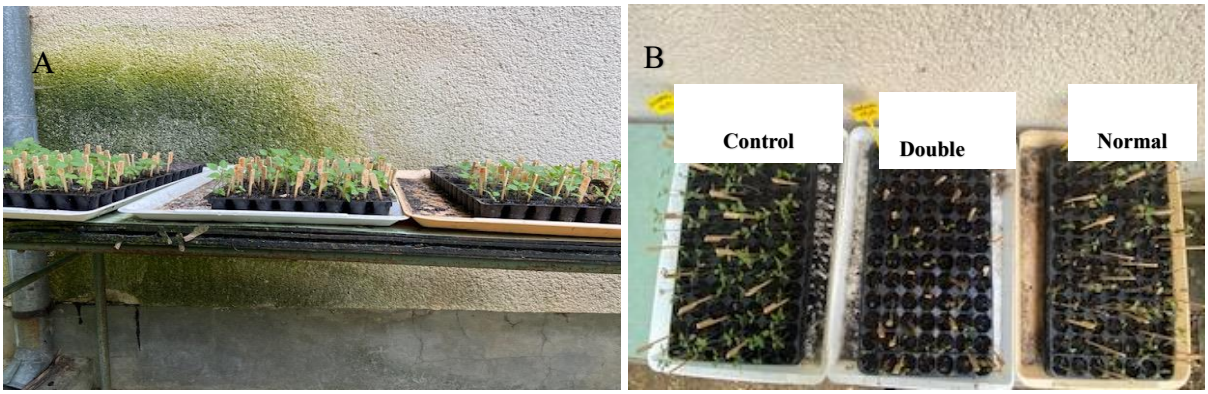

Figure 1. *(A) S. nigrum plants before treatment under ambient conditions. (B) S. nigrum plants 28 DAT. left: control with no treatment; middle: sprayed with double dose rate of glyphosate; right: sprayed with normal dose rate of glyphosate under ambient conditions. Photo: Rita Ofosu*

4. Discussion

The outcome of this experiment could be because of the differences in the environmental conditions under which the plants were grown. This result is comparable to previous work done by Vila-Aiub et al. (2013) where the efficacy of glyphosate on *S. halapense* was reported to have increased with high temperature. In an earlier work done by Kudsk (2017), abiotic factors such as temperature, light intensity and humidity can affect herbicide performance. The ambient temperature recorded for the period when the experiment was conducted was higher than the temperature in the controlled climate chamber with day temperature of 28°C and night temperature 20 °C which supports the finding that resistance mechanism is climate dependent, and that air temperature affects the growth of plants and the ability of the herbicide to produce targeted results. Also, previous studies have shown that increased temperature can affect levels of resistance to glyphosate (Ge et al., 2011). Thus the outcome of this experiment could suggest that the decrease in sensitivity of the *S. nigrum* population as observed under ambient growing conditions may be due to increased herbicide metabolism. Another reason could also be that the herbicide did not get contact with the weeds during the treatment application hence, the weeds survived. The time of application, precipitation, wind direction, humidity and temperature are important and should be considered when applying herbicides to achieve optimum results (Vila-Aiub et al., 2013). Further studies using the same weed populations are recommended and may provide in-depth explanation for the ineffectiveness of glyphosate on weed populations present at the sites of collection, the type of mechanisms used in decreasing the sensitivity of the population to glyphosate dose on the growing field and impact of environmental conditions on herbicide tolerance.

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Assessment of the Auchenorrhyncha Fauna of Winter Oilseed Rape Stands in Connection with the Disease Caused by Aster Yellows Phytoplasma

Őszi káposztarepce állományok kabócafaunájának felmérése az őszirózsa sárgulás fitoplazma okozta betegséggel összefüggésben

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Abstract: The highly polyphagous aster yellows phytoplasma can infect winter oilseed rape as well. In Hungary, the first detection of the aster yellows phytoplasma in oilseed rape was reported in 2020. Leafhoppers are mentioned as the primary vectors of the pathogen, but only a few experiments have proven their transmission ability in Europe. In recent years, we aimed to survey the Auchenorrhyncha fauna of winter oilseed rape. Yellow sticky traps were primarily used to monitor insect populations, but sweep netting was also carried out. *Zyginidia* and *Empoasca* genera were the most abundant, but specimens of potentially aster yellows phytoplasma vectors such as *Macrosteles* and *Psammotettix* genera were also caught.

***Keywords**: winter oilseed rape; leafhoppers; aster yellows phytoplasma*

Összefoglalás: A rendkívül polifág őszirózsa sárgulás fitoplazma képes az őszi káposztarepcét is megbetegíteni. Hazánkban először Zala vármegyéből származó repcenövényekből mutatták ki 2020-ban. A kórokozó terjesztésében a kabócák szerepét szokták kiemelni, habár Európában még kevés az ezt bizonyító tanulmány. Az utóbbi években végzett rovargyűjtéseink célja az őszi káposztarepce állományok kabócafaunájának felmérése volt. Sárga ragacslapos csapdázás mellett, kiegészítő módszerként, fűhálót is alkalmaztunk. Nagyobb egyedszámban a *Zyginidia* és *Empoasca* genus egyedeit fogtuk, de találtunk irodalmi adatok alapján potenciális őszirózsa sárgulás fitoplazma vektorként számon tartott *Macrosteles* és *Psammotettix* nembe tartozó kabócákat is.

***Kulcsszavak**: őszi káposztarepce; kabócák; őszirózsa sárgulás fitoplazma*

1. Introduction

A new plant disease of oilseed rape was first discovered in Poland and the Czech Republic around the year 2000. The symptoms (floral virescence, phyllody, reduced leaves, malformation of siliques) visible in May and June indicated phytoplasma infection. Ten years later, after a more severe infection spreading large areas in Poland, Zwolińska and co-workers (2011), using molecular methods, confirmed the presence of aster yellows phytoplasma (AYP) in the diseased rape plants. Plants showing symptoms of possible phytoplasma infection could be found sporadically in smaller patches in Hungarian oilseed rape fields in the last two decades. In the

spring of 2020, oilseed rape plants with characteristic phytoplasma symptoms could be observed in more significant numbers in several fields in Zala County. Varga and co-workers (2020) detected aster yellows phytoplasma in the diseased plants. Several Auchenorrhyncha species can transmit AYP. In North America, 24 AYP vector leafhopper species are known (Stillson and Szendrey, 2020), but our knowledge about insect vectors is limited in Europe. The European species of the genus Macrosteles are the most examined. AYP was detected in several leafhopper species, but successful transmission experiments with oilseed rape are unknown. This study aimed to investigate the Auchenorrhyncha fauna of oilseed rape and to identify potential AYP vectors.

2. Materials and Methods

Leafhoppers were collected for four years (2020-2023) from oilseed rape fields near the town of Zalaegerszeg. As a primary method, 10x16 cm yellow sticky traps of Csalomon® were used. Trapping started with the emergence of oilseed rape plants and ended at the beginning of the flowering. Two yellow sticky traps (yellow on both sides) were used in every field and changed bi-weekly. Sweep netting was an auxiliary method carried out (always with 100 net strokes) when the developmental stage of the crop made it possible. The collected insects were stored in a refrigerator (-20 °C) till identification. Insects were identified using Ossiannilsson's (1978-1983) work and other sources (Holzinger et al., 2003; Biedermann and Niedringhaus, 2004) with more recent keys of Central European species by morphological analysis with stereo microscopy.

3. Results

Based on our data from four years, it can be stated that with the selected methods, true hoppers could be collected chiefly in the autumn (September, October) from oilseed rape. In March and April, when the frosty days of winter ended, no leafhoppers could be collected except a few individuals. Later (May, June), insect sampling became almost impossible when the plants grew taller and the stand became dense. Examining the results of the sampling period of 2020 near Pózva, Auchenorrhyncha specimens belonging to seven different species or genera (identification to species level can be pretty tricky in case of individuals captured by sticky traps) were captured by yellow sticky traps (Table 1). Most of the collected individuals belonged to the genera *Empoasca* and *Macrosteles*. Fewer specimens from fewer species could be collected with sweep netting from the same field during the same sampling period (Table 2). Auchenorrhyncha species captured with sweep netting were identical to those captured with yellow sticky traps, with only one exception. There was a several years old lucerne field right next to the oilseed rape field. When rape was sampled, sweep netting was carried out in lucerne too. Summarised sampling results in lucerne are shown in the last column of Table 2. Comparing the data of the last two columns in Table 2, it can be seen that the species captured in oilseed rape could also be found in lucerne, except for one species. Populations of the common species living in lucerne were present in higher abundance.

Table 1. Number of sampled Auchenorrhyncha specimens on yellow sticky traps in oilseed rape (2020, Pózva)

Species	17.09-28.09	28.09-12.10	12.10-19.10	19.10-03.11	Σ
Macrosteles spp.	8	17	0	2	27
Psammotettix spp.	0	0	0	6	6
Ph. spumarius	3	3	0	2	8
Eupteryx spp.	0	0	0	8	8
Empoasca spp.	0	5	10	32	47
Cixius spp.	2	0	0	1	3
N. fenestratus	0	0	0	1	1

Table 2. Number of Auchenorrhyncha specimens sampled with sweep netting in oilseed rape and (shown in the last column) in neighbouring lucerne field (2020, Pózva)

Species	23.09	30.09	14.10	21.10	03.11	Σ rape	Σ lucerne
Macrosteles spp.	2	1	0	1	0	4	7
Psammotettix spp.	0	1	1	0	2	4	5
Ph. spumarius	1	1	0	0	0	2	8
Eupteryx spp.	0	1	0	0	0	1	0
L. striatella	1	0	0	0	0	1	7

Yellow sticky traps placed in an oilseed rape field near Gősfa in 2021 captured 156 Auchenorrhyncha individuals, which belonged to eight different taxa (Table 3). Like the results of Pózva (Table 1), *Empoasca* specimens were caught in high numbers, but nearly four times as many individuals of *Zyginidia* were captured. True hoppers were collected from another field near Vasboldogasszony in the autumn of 2022 with similar results to Table 3, considering the dominant species (data not shown). Similar results were achieved by another sampling in 2023 near Gősfa (the three most common genera: *Zyginidia, Eupteryx and Empoasca*).

Table 3. Number of sampled Auchenorrhyncha specimens on yellow sticky traps in oilseed rape (2021-2022, Gősfa)

Species	08.09 -24.09	24.09 -08.10	08.10 -25.10	25.10 -09.11	15.02 -28.02	28.02 -16.03	16.03 -28.03	Σ
Macrosteles spp.	5	3	1	0	0	0	0	9
Psammotettix spp.	1	1	2	0	0	0	1	5
Zyginidia spp.	31	55	11	1	0	0	0	98
Empoasca spp.	1	5	15	2	1	0	0	24
Eupteryx spp.	1	5	1	0	0	0	0	7
Arboridia spp.	0	0	1	0	0	0	0	1
Typhlocibinae	4	0	1	0	0	0	0	5
Cicadellidae	0	0	3	4	0	0	0	7

4. Discussion

Based on our multi-year sampling, it can be stated that in oilseed rape, few Auchenorrhyncha species can be found in relatively small numbers compared to the fauna of certain orchards. According to Nickel (2003), there is not any European leafhopper feeding on *Brassica* plants exclusively, so primarily polyphagous or grass specialist species can be found in oilseed rape that migrate into the field from the surrounding, less disturbed vegetation. Our survey also supports this idea because the same species in higher numbers were captured in the lucerne field situated next to the oilseed rape field. (It is worth mentioning here that lucerne had a more diverse leafhopper fauna because five additional species were present besides those captured in both fields.) We know that some of the genera collected during this survey may carry the AYP. Among *Macrosteles* species, *M. laevis* (Zwolińska et al., 2016), *M. sexnotatus* (Schneller et al., 2016) and *M. quadripunctulatus* (Bosco et al., 2007) are reported in the literature as species that can acquire the phytoplasma. In Turkish and Serbian carrot fields, *Psammotettix* species are the most probable vectors of AYP (Drobnjanković et al., 2011; Randa Zelyüt et al., 2022). Italian authors (Galetto et al., 2011) proved that *Empoasca decipiens* can be an experimental vector of AYP. Among the Auchenorrhyncha species caught in the oilseed rape fields of Zala County, some can be potential vectors of AYP, so the information on their possible role in a future phytoplasma epidemic is essential in developing effective control methods.

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Investigation of Viromes of Solanaceous Weeds

Solanaceae családba tartozó gyomok viromjának vizsgálata

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Abstract: Within the scope of our research study, we delve into a comprehensive investigation of virome of solanaceous weeds at the edge of crop fields and natural habitats. For this pilot study leaves of symptomatic *Datura stramonium* and *Solanum nigrum* were collected at two different locations near Keszthely in 2022. High-throughput sequencing (HTS) of small RNAs and RNAs were conducted and the sequenced reads were analysed using bioinformatic methods. Out of the identified viruses we confirmed the presence of the cucumber mosaic virus (CMV), encompassing RNA1, RNA2, and RNA3 components, as well as broad bean wilt virus 1 (BBWV1) RNA2 using RT-PCR. HTS resulted infection with several other viruses which presence are currently under validation. This pilot study contributes to our understanding of the viral diversity within *Solanaceae* plants, shedding light on the role of these plants as virus reservoirs. The implications of these findings may help to develop strategies for virus management of both endemic and invasive plant populations.

Keywords: *Virus diagnostics; Solanaceae; endemic plants; invasive weeds; cucumber mosaic virus; broad bean wilt virus; PCR; RT-PCR; HTS; virus reservoir*

Összefoglalás: Kutatásunk során a szántóföldek szélén és természetes élőhelyeken található Solanaceae családba tartozó két gyomnövény faj viromját vizsgáltuk. Kísérleteinkhez 2022-ben Keszthely közelében két különböző helyszínen gyűjtöttünk levélmintát vírusfertőzés tüneteit mutató Datura stramonium és Solanum nigrum növényekről. Kis RNS-ek és RNS-ek nagy áteresztőképességű szekvenálását (HTS) végeztük el, és a szekvenált olvasatokat bioinformatikai módszerekkel elemeztük. Az azonosított vírusok közül RT-PCR segítségével igazoltuk az uborkamozaikvírus háromosztatú genomjának mindhárom RNS-ét, valamint a széles babhervadás vírus 1 (BBWV1) RNS2 jelenlétét. A HTS a minták több más vírussal való fertőzését is mutatta, amelyek jelenlétének igazolása jelenleg folyamatban van. Eredményeink hozzájárulnak a Solanaceae növényeket fertőző vírusok változatosságának azonosításához és megértéséhez, és rávilágítanak e növények vírusrezervoárként betöltött szerepére, így segíthetik az endemikus és invazív növénypopulációk vírusfertőzöttségének megelőzését szolgáló stratégiák kidolgozását.

Kulcsszavak*: vírusdiagnosztika; Solanaceae; endemikus növények; invazív gyomok; uborka mozaik virus; széles babsorvadás virus; PCR; RT-PCR; HTS; virus rezervoár*

1. Introduction

The Solanaceae family represents a cohesive group of dicotyledonous plants, encompassing a range of extensively cultivated crops. Within this family, various species have significant roles, whether as vital food sources, suppliers of bioactive compounds, or even as decorative ornamental plants (Gebhardt, 2016). Members of this botanical family, including the potato (Solanum tuberosum), tomato, pepper and tobacco, thrive in regions across the globe with temperate or tropical climates (Hančinský et al., 2020). These plants have become staples in households worldwide, gracing kitchens, gardens, and fields, and contributing to global agriculture and culinary traditions (Olmstead et al., 2008). In addition to the commonly cultivated crops, the Solanaceae family is also home to various medicinal plants known for their alkaloid production. Notable examples include deadly nightshade (Atropa belladonna), black henbane (Hyoscyamus niger), and jimson weed (Datura stramonium) (Hančinský et al., 2020). Over the last century, plants from the Solanaceae family have played a crucial role in genetic research, contributing significantly to advancements in our understanding of plant genetics (Gebhardt, 2016). Cultivated species from the Solanaceae family are frequently found thriving alongside their wild counterparts in the same ecosystems. In many cases, wild Solanaceae species are considered common weeds within these shared habitats. In diverse agricultural settings, solanaceous plants are subject to various infectious pathogens, including viruses (Hančinský et al., 2020). Plant viruses are responsible for widespread epidemics in significant crops, posing a significant challenge to global food security. Consequently, virologists have traditionally prioritized their research efforts on economically vital crops, sometimes overlooking nearby weeds and wild plant species (Wren et al., 2006). In the realm of virus research, exploring the role of weeds as potential reservoirs has become pivotal. Scientists delve into the intricate dynamics between weeds and viruses, unravelling how these plants may serve as silent carriers. Understanding weed-associated viruses is crucial for devising effective strategies to mitigate agricultural threats and protect global crops. Weeds can act as reservoirs for both viruses and the insects that transmit them. In such circumstances, the spread of viruses can be highly pronounced. (Duffus, 1971). In our previous study we have tested the presence of viruses in solanaceous weeds using serological methods (Takács et al, 2001; 2006). In this current work we carried investigated viromes of solanaceous weeds using high-throughput sequencing based metagenomic methods.

2. Materials and Methods

In the year 2022, a study was conducted in Keszthely, Hungary, focusing on solanaceous plants displaying symptomatic features. This investigation encompassed two distinct fields, denoted as Field I (*Datura stramonium, Solanum nigrum*) and Field II (*Solanum nigrum*).

Field I: comprised *Datura stramonium*, germinated in early August, and *Solanum nigrum*, germinated from seeds in May. These plants coexisted alongside horticultural crops, including pepper, eggplant, and tomato. A comprehensive sampling strategy was employed, where five samples were collected from each plant. Specifically, leaves were collected from *Datura stramonium*, while both leaves and flowery shoots were gathered from *Solanum nigrum*.

Field II: was exclusively dedicated to *Solanum nigrum*, which had been germinated in May. This field had a previous history of wheat cultivation and potato farming, resulting in diverse dicot species covering the terrain. Similar to Field I, a systematic approach was adopted, involving the collection of five samples per plant from *Solanum nigrum*, encompassing both leaves and flowery shoots.

From the collected plant material total nucleic acid was isolated. To streamline the investigation, a strategic approach to sample pooling was employed. Three distinct pools were created: one for Field I *Datura stramonium*, one for Field I *Solanum nigrum*, and one for Field II *Solanum nigrum*. Each pool combined the RNA extracts from the respective plants within its category, resulting in a comprehensive representation of the viral content within each field. The three pools were mixed to prepare a pool containing extracts originating from both fields and plants. The pooled RNA samples were subsequently subjected to the construction of small RNA libraries, and RNA sequencing (RNA-seq). High throughput sequencing on Illumina platform was ordered as a service. Sequenced reads were analysed using the Qiagen CLC Genomic Workbench. Reads were trimmed and used for contig assembly.

Virus diagnostics were performed by conducting a BLAST search of the assembled contigs, referencing all known plant-hosted viruses in the NCBI database. The results were ordered based on their lowest E-value. We also mapped reads (both redundant and non-redundant) to the reference genomes of the HTS identified viruses. Consensus sequences were generated and the coverage of viral genomes by sRNA or RNA reads were determined. To validate the results of HTS and directly confirm the presence of cucumber mosaic virus (CMV) and broad bean wilt virus 1 (BBWV1), RT-PCR was performed. After cDNA synthesis, we conducted an actin test to assess cDNA quality. The resultant cDNA served as a template for RT-PCR using virus-specific primers to confirm the presence of CMV (RNA 1, RNA 2, and RNA 3) and BBWV1 RNA 2.

3. Results and Discussion

As a result of the HTS we got 16,5 small RNA and 21,6 million RNA reads, Form these reads 132523 (RNAseq) and 3177 (sRNAseq) contigs were built, respectively (Table 1.).

Table 1. Statistics of the HTS results

Library code	Sequenced reads	Trimmed reads all (containing redundants)	Non-redundant reads	Number of contigs
SOL_KES_10	16253182	15972388	15766344	132523
255_KSOL_S17	21955551	21573415	3178957	3177

Annotation of the contigs using reference genomes of known plant infecting viruses during the BLAST analyses revealed the presence of several different viruses: CMV, BBWV1, tomato vein clearing virus (TVCV), tomato yellow virus (TVY), Chenopodium quinoa mitovirus (CHQM), pepper vein clearing virus (PVCV), potato leafroll virus (PLRV) and Solanum symptomless mottle virus (SSMV).

Table 2. Summary of the results of the bioinformatics analysis of sRNAseq and RNAseq

HTS method	Feature	CMV			BBWV1		TVCV	TYV	CHQM	PVCV	PLRV	SSM
		RNA1	RNA 2	RNA 3	RNA 1	RNA 2						
sRNAseq	Number of contigs	62	54	31	16	5	25	0	0	0	0	3
	Coverage of the genome	99%	99%	100%	67%	64%	80%	43%	48%	63%	40%	55%
RNAseq	Number of contigs	1	1	2	1	1	19	5	7	5	4	22
	Coverage of the genome	99%	99%	99%	98%	97%	89%	90%	92%	90%	90%	88%

While RNAseq detected TVY, CHQM, PVCV, and PLRV in the sample we did not find contigs built up from sRNA reads specific to them. However, we observed extensive coverage of these viruses, even where no contigs were detected. This observation suggests that these viruses may be present in the samples, but they did not induce that strong silencing signal. Further studies are needed to fully understand the possible consequences of this finding and to determine the presence and possible function of these viruses.

The result of the bioinformatic analysis tells us how many different viruses could be detected in the sequenced pools, but does not contain information about the infection is the different species and individuals. Validation of the CMV and BBWV1 was done by RT-PCR (Figure 1) not only in the sequenced mixed pool, but also in the pools corresponding to the fields and plant species. Once the pool containing the virus was identified through RT-PCR, in-depth investigations were conducted on individual plants within that field (Figure 1.)

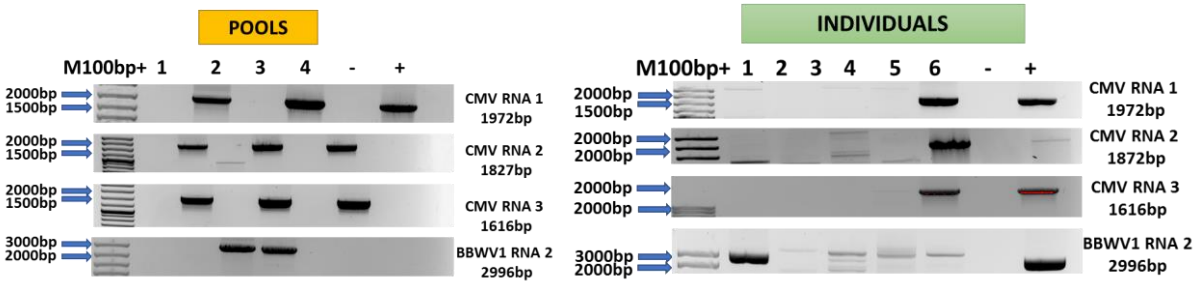

Figure 1. *Result of the RT-PCR analysis for testing the presence of CMV, and BBWV1 in Field I and Field II. M100bp+ is Thermo Scientific GenRuler 100bp+, 1 stand for pool Field I Datura stramonium, 2 stands for pool Field I Solanum nigrum, 3 stands for pool of Field II Solanum nigrum, while 4 is the pool of 1+2+3. - is MQ used as a negative control, while + was the positive control*

This methodology efficiently detected viruses in the selected fields, allowing us to focus on relevant subsets for a comprehensive analysis.

Notably, we observed infections with CMV and BBWV1, which were further confirmed by RT-PCR analysis. Both three RNAs of CMV could be detected in *S. nigrum* growing at field I. Testing the individuals growing at that field showed that only one plant (5) was infected with this virus. RNA2 of BBWV1 was detected in *S. nigrum* growing at field II, where one plant (1) was tested positive for its presence.

RT-PCR validation for the presence of the other viruses is currently ongoing.

4. Conclusions

During our work sRNA and RNA HTS showed their ability to reveal virus infection in the sampled weeds even when a pool of 15 plant extracts were sequenced. To reveal how frequent the coinfection of the plant with the presenting viruses we need further validation of the viruses in the individuals. The coexistence of multiple viruses in this small pilot survey highlights the complexity of the viral community within solanaceous plants and emphasizes the need for comprehensive monitoring and management strategies in agricultural settings. It is essential to continue studying the interplay between these viruses and their impact on crop health, as well as to develop targeted approaches to mitigate the potential negative effects on agricultural productivity if needed.

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Phytophthora Infestans Induced Gene Expressional Changes in Different Potato Cultivars

Phytophthora infestans által kiváltott génexpressziós változások különböző burgonya fajtákban

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Abstract: The oomycete fungus *Phytophthora infestans* is the most damaging pathogen of potatoes. Sources of resistance are identified in different wild potato species, and some of these genes have already been isolated. During the six decade long resistance breeding program in Keszthely, race-specific *P. infestans* resistance genes were used in breeding. One of these cultivars, White Lady (WL), was used in this study together with the susceptible Sárvári borostyán (Sb) and Kastia (K) for transcriptomic analysis. Purpose of the present experiment was to analyse the *Phytophthora* inoculation induced stress response in the three varieties. Transcriptomes were reconstructed from samples collected 18, 24, 48 and 72 hours after inoculation. The results clearly revealed, that in the resistant WL significantly more genes, in some cases three-five times more genes are upregulated, than in the two susceptible cultivars. Similarly, significantly more genes are downregulated in the WL, than in Sb or K. It is concluded that the response to *P. infestans* inoculation is more comprehensive in the resistant cultivar, and possibly this reorganisation of tens of thousands of functioning genes leads to a successful resistance response. This process is suggested to be triggered by the race-specific late blight resistance genes in White Lady.

Keywords: *Phytophthora infestans; late blight; potatoes; resistance; transcriptomes*

Összefoglalás: A burgonyavészt előidéző Phytophthora infestans a burgonya legveszélyesebb kórokozója. Ez idáig számos rezisztenciagént azonosítottak különféle vad burgonyafajokban, és néhányat ezek közül izoláltak is. A több, mint hat évtizedre visszatekintő keszthelyi rezisztencianemesítési program során rassz-specifikus P. infestans rezisztenciagéneket építettek be számos fajtába. Ezek egyikét, a White Lady-t (WL) használtuk a jelen tanulmány transzkriptomikai vizsgálataiban két másik fajtával a fogékony Sárvári borostyánnal (Sb) és a Kastiával (K) együtt. A jelen vizsgálatok célja a három fajtában a Phytophthora fertőzésre adott stressz válasz molekuláris genetikai jellemzése volt. A transzkriptomokat a fertőzés után 18, 24, 48 és 72 órával gyűjtött levélmintákból készítettük. Az eredmények világosan mutatják, hogy a rezisztens WL-ben szignifikánsan több gén, egyes esetekben 3-5-ször annyi gén expressziója növekedett, mint a fogékony fajtákban. Hasonlóképpen, szignifikánsan több gén expressziós szintje csökkent a WL-ben mint a fogékony fajtákban. Ezek az eredmények arra utalnak, hogy a rezisztens fajtában az egész genetikai apparátusra kiterjedő változások mennek végbe,

melyben gének tízezreinek működése módosul. Feltételezhetően ezen átfogó génexpressziós változásokat a White Lady-ben jelenlévő rassz-specifikus rezisztenciagének indukálják, és egyben ez a feltétele a sikeres rezisztencia válasznak.

Kulcsszavak: *Phytophthora infestans; burgonyavész; burgonya; rezisztencia; transzkriptom*

1. Introduction

The late blight disease caused by the oomycete fungus Phytophthora infestans is still considered the most damaging disease in potato (Solanum tuberosum) production (Gao et al., 2013). The yield loss caused by P. infestans is estimated to be about 16% globally (Sanju et al., 2015) and the annual cost of crop damage and chemical control is typically around 5.6 billion euros globally (Haverkort et al. 2009). Hence, genetics-based plant protection would be desirable for crop safety, as well as for lowering production costs, and not at least for the mitigation of agrochemical pressure on the environment. In 32 Solanum species 70 P. infestans resistance genes have been identified so far, and some of them, like the R1, R2, R3a, R3b, and R8, which derived from the hexaploid Solanum demissum (Ballvora et al. 2002; Huang et al. 2005; Li et al. 2011; Lokossou et al. 2009; Vossen et al. 2016), and the Rpi-blb1, Rpi-blb2, Rpi-blb3, Rpi-abpt, and Rpi-bt1, which originate from the diploid S. bulbocastanum (Ea et al. 2005; Vossen et al. 2003; Lokossou et al. 2009; Oosumi et al. 2009) have already been cloned. The R genes convey race-specific resistance to P. infestans, while the Rpi genes are considered broad spectrum resistance genes against late blight. The pyramiding of different resistance genes could be the solution to procreate durable late blight resistance in new potato varieties. Marker assisted selection based on isolated P. infestans resistance genes would facilitate such a breeding effort. The complex resistant potato cultivar White Lady (WL), that was bred at the Potato Research Centre (now MKSzN) at Keszthely, Hungary, contains most of the R1-R11 genes, and by molecular methods previously the R2, R3a and R3b genes have already been revealed from this cultivar.

To explore the genetic background of late blight resistance, as well as to understand the difference between a resistance and susceptibility response to *P. infestans* inoculation, in the present study we analysed the gene expression changes induced by *P. infestans* in three different potato cultivars. The cultivar Kastia (K) was previously found by Gergely (2004) to have outstanding good horizontal resistance to *P. infestans*, although the origin of its resistance was unclear. A susceptible cultivar, Sárvári borostyán (Sb) was used also in the experiments, as well as the cultivar WL for the identification of not yet isolated R-genes. In the present publication we focus on the *P. infestans* induced gene expressional changes, by identifying the up and down regulated transcripts in different time points after inoculation. The expressional differences are compared between the cultivars, and annotation of the significantly upregulated transcripts of the resistant cultivar WL was performed.

2. Materials and Methods

2.1. Plant material

Plants were grown from minitubers in 3 L pots filled with peat. The growing conditions in the phytotron were: 50% relative humidity, 16:8 hours day:night period, with the start of the lighting period the temperature increased gradually in two hours from 20ºC to 25ºC, and at the

end it decreased also in two hours from 25ºC to 20ºC. From each cultivar five plants were grown for inoculation and one for control.

2.2 *P. infestans* inoculations and samplings

The Polish *Phytophthora* isolate, MP-1548 was used. The culture was grown on tuber slices of the cultivar Hópehely. From the harvested mycelium sporangia were released in distilled water, and its concentration was adjusted to 15 000 sporangium/mL. One drop of this solution was applied to the abaxial surface of a leaflet on vigorously growing plants before flowering. The control plants obtained just a drop of clean distilled water.

For molecular analysis the neighbouring leaflet of the inoculated one on the same leaf was collected, and was put immediately on dry ice, and until RNA extraction the leaf samples were stored at -80ºC. Samples were collected at 18, 24, 48 and 72 hours after inoculation from the inoculated and control plants.

2.3. RNA-sequencing and transcriptome construction

RNA was extracted by Direct-zol RNA Miniprep kit (Zymo Research, USA). For poly-A enrichment the Poly(A) RNA Selection kit (Lexogen, Austria) was used. The sequencing libraries were prepared using the NEXTFLEX Rapid Directional RNA_Seq kit 2.0 (Perkin Elmer, USA), and transcriptome sequencing was done on a NextSeq 500 (Illumina, USA) platform, using a High Output 150 sequencing kit.

Using the SOAPdenovo-Trans (Xie et al. 2014), a *de novo* transcriptome assembly was done from White Lady RNA-seq data, and this *de novo* transcriptome was used as the mapping index. Quantification of transcripts in White Lady, Sárvári borostyán and Kastia samples was done with the "index" and "quant" commands of the Salmon program (Patro et al. 2017). Statistically significant differentially expressed genes (DEGs) were identified with the DESeq2 (Love et al. 2014) Bioconductor package in the R environment.

3. Results

The inoculation proved the expected resistance of WL, as well as the expected susceptibility of Sb to *P. infestans.* Surprisingly, the cultivar Kastia showed symptoms of infection, and similarly to the cultivar Sárvári borostyán, the late blight disease developed on the five tested Kastia plants, while the control plants remained healthy.

Transcriptomes of the control plants and of the four test time points were constructed for each cultivar. In the resistant cultivar WL with 8% ($\pm$0.3%) more transcripts were identified, than in the two susceptible cultivars. The total number of transcripts was the following: White Lady - 111 228 (100.0%), Sárvári borostyán - 102 654 (92.3%), Kastia - 101 919 (91.7%).

Besides the number of expressed genes, strong differences in the number of genes with inoculation induced expressional changes in the different time points was observed. As revealed in Figure 1., three to five times more genes were upregulated in WL than in the sensitive cultivars. In WL the number of upregulated transcripts continuously increased during the sampling period, while in both sensitive cultivars decrease in the number of upregulated genes could be observed at 24 hpi and 48 hpi, and an increase at 72 hpi. For the sensitive cultivars exactly the same tendency could be observed also for the downregulated genes. Further, the number of downregulated genes was in all cases characteristically higher in the sensitive cultivars, but in the resistant WL the tendency was the opposite, i.e.: there were in all time point much more up than downregulated genes. The number of downregulated genes was always higher, in WL than in SB and K, and in some cases this was 2-3 times more.

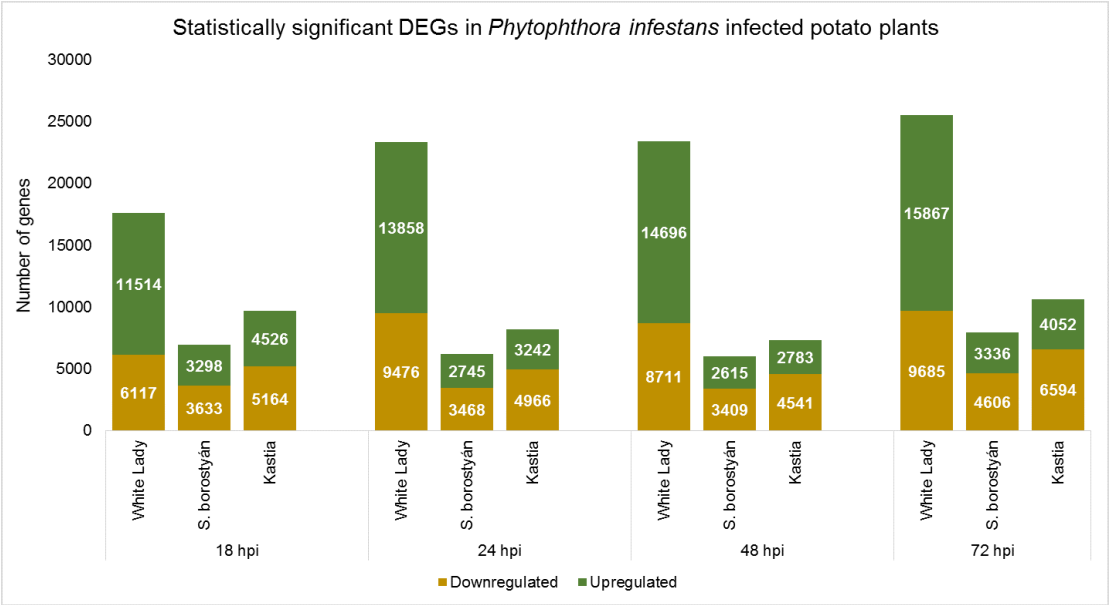

Figure 1. *The number of statistically significant differentially expressed genes (DEGs) of the three analysed cultivars in the four tested time points*

However, the transcripts revealed in Figure 1., are those which showed a two-times up- or down regulation, in each sampling time point a characteristically higher number of transcripts were found to be significantly up- or downregulated, but with a magnitude smaller than twice. The number of these transcripts are listed in Table 1.

Table 1. The number of differentially expressed transcripts in the three potato cultivars in different post inoculation time points

	Cultivars	LFC >1 and padj <0.05	LFC >0 and padj <0.05	LFC <0 and padj <0.05	LFC <-1 and padj <0.05
All time points	WL	6 735	8 315	4 272	2 014
	Sb	976	1 640	2 222	1 248
	Kastia	897	1 902	2 546	1 059
18 hpi	WL	11 514	14 368	9 191	6 117
	Sb	3 298	4 388	4 878	3 633
	Kastia	4 526	7 232	7 732	5 164
24 hpi	WL	13 858	16 614	12 429	9 476
	Sb	2 745	3 871	4 600	3 468
	Kastia	3 242	6 095	7 403	4 966
48 hpi	WL	14 696	17 462	11 866	8 711
	Sb	2 615	3 728	4 593	3 409
	Kastia	2 783	5 219	6 941	4 541
72 hpi	WL	15 867	18 519	12 839	9 685
	Sb	3 336	4 417	5 683	4 606
	Kastia	4 052	7 241	9 039	6 594

Notes: LFC: log to fold change; padj: adjusted P-value. LFC>1 and LFC<-1 are the more than two-times up- or downregulated transcripts, respectively. LFC>0 and LFC<0 are the less than two-times up- or downregulated transcripts, respectively. padj<0.05 indicates the 5% probability. hpi – hours post inoculation. WL – White Lady, Sb – Sárvári borostyán

4. Discussion

Instead of the expected high horizontal resistance of Kastia, that was previously described in unheated foil tent provocation experiments (Gergely, 2004), under the adjusted conditions of the phytotron this cultivar was found to be sensitive to *P. infestans*. Besides the differing test conditions of the two experiments, the *Phytophthora* isolate was also different, that may give an explanation for the observed alteration in stress response.

During the testing period more and more genes have been activated in WL and a high number of genes were downregulated, obviously a phenomenon closely linked to the successful resistance response. In contrast much less genes were affected by the *P. infestans* inoculation in the sensitive Sárvári borostyán and Kastia cultivars. This difference indicates that the late blight resistance genes of WL, similarly to other *P. infestans* resistance genes with proved function, are possibly transcription initiation factors, which after recognition of the pathogen trigger a cascade of genes expression changes to avoid infection.

For the identification of those genes and gene families which are mostly affected by the *P. infestans* inoculation we are using now a GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) annotation approach. Further, with the DNA motif-based identification of resistance gene like sequences we are identifying the possible late blight resistance gene candidates in the cultivar White Lady.

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In the Search of Viral Suppressors of *Prunus* Infecting Viruses

Prunus fajokat fertőző vírusok géncsendesítést gátló fehérjéinek keresése

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Abstract: Perennial woody plants can be infected by a wide range of viruses and viroids. During infection, the plant's defence system, the RNA interference (RNAi), the highly effective and specific defence reaction of the host, is induced. Throughout their evolution, viruses have developed strategies to block this defence process in various ways. One of these strategies involves the encoding of proteins that function as viral suppressors of RNA interference (VSR). In our study, we examined the potential RNAi silencing suppressor activity of P21 proteins of little cherry virus-1 (LChV-1) from different host plants (cherry and apricot) and of P4 of peach-associated luteovirus (PaLV). Our results revealed that the P21 protein of LChV-1apricot strain displayed systemic VSR activity, whereas the P4 protein of PaLV showed only weak local suppressor activity.

***Keywords**: Velarivirus; Luteovirus; LChV-1; PaLV; viral suppressor; agroinfiltration*

Összefoglalás: Az évelő fásszárú növényeket sokféle vírus és viroid fertőzi. A fertőzés során a növény védekezőrendszere, az RNS interferencia indukálódik, mely igen hatékony és specifikus. A vírusok evolúciójuk során olyan stratégiákat fejlesztettek ki, melyekkel ezt a védekező folyamatot különböző módon képesek blokkolni. Ilyenek, a legtöbb növényt fertőző vírus által kódolt, virális RNS csendesítést gátló, szupresszor fehérjék (VSR.). Kutatásunk célja VSR fehérjék keresése és jellemzése a cseresznye aprógyümölcsűség vírus 1 (little cherry virus-1 - LChV-1) és az őszibarack-asszociált luteovírus (peach-associated luteovirus - PaLV) különböző gazdanövényeket fertőző variánsaiban. Eredményeink alapján megállapítható, hogy a LChV-1 P21 fehérje kajsziból izolált variánsa szisztemikus VSR aktivitást mutatott, míg a PaLV P4 fehérjéje gyengén és csak lokálisan volt képes gátolni az RNS interferenciát.

***Kulcsszavak**: Velarivirus; Luteovirus; LChV-1; PaLV; virális szuppresszor; agroinfiltrálás*

1. Introduction

During viral infection, the plant's defence system, the RNA interference, is induced. It is a conserved, sequence-specific eukaryotic gene regulation mechanism (Covey et al., 1997). During evolution, viruses have evolved different VSR proteins that can block this defence reaction (Csorba et al., 2015). VSRs can inhibit the production or activity of siRNAs in the infected tissue (local silencing), and/or their spread to systemic leaves (systemic silencing) (Burgyán and Havelda, 2011).

LChV-1 is a member of the *Velarivirus* genus in *Closteroviridae* family of plant viruses. It has a single-stranded positive-sense RNA genome of 16–17 kb and includes eight open reading

frames (ORFs). Together with little cherry virus 2 and X-disease phytoplasma, it is associated with the little cherry disease. LChV-1 can infect sweet and sour cherry and other *Prunus* species such as almond, peach, plum and apricot (Baráth et al, 2018). VSR activity of ORF7 of a Greek sweet cherry strain encoding P21 was tested positive previously (Katsiani et al., 2017).

PaLV is a member of the genus *Luteovirus,* formerly belonging to the family *Luteoviridae*, and has recently been re-assigned to the family *Tombusviridae* (Miller & Lozier, 2022). The virus was detected in peach, flat peach and nectarine and described from Hungary (Barath et al., 2018). The PaLV genome is single-stranded positive-sense RNA of 5-6 kb and includes six ORFs (Khalili et al., 2023). Based on previous studies, the P4 protein of barley yellow dwarf virus-PAV (BYDV-PAV), a member of the same genus to which PaLV belongs, acts as a VSR (Fusaro et al., 2017).

The aim of this study was to investigate the possible local and systemic silencing suppressor activity of P21 protein of LChV-1 (apricot and sour sweet cherry strain) and P4 protein of PaLV.

2. Materials and Methods

The transient gene expression system is a well-established method for characterizing VSRs (Voinnet & Baulcombe, 1997). The potential VSR coding regions of LChV-1 and PaLV isolates were cloned into a BinHA binary plasmid using In-fusion method. The resulting recombinant BinHA constructs were transformed into the *Agrobacterium tumefaciens* (strain C58C1) by triparental mating and then infiltrated into 3-week-old *Nicotiana benthamiana* leaves together with a GFP expressing construct in 0.4 :0.6 ratio (Hamilton, 2002). The GFP fluorescence signal of the infiltrated leaves (local silencing, 3,5 days post inoculation (dpi)) and whole plants (systemic silencing, 20 dpi) were examined visually under UV light. The level of GFP and tested protein expression was determined by Western blotting while the level of GFP mRNA expression was determined by real-time PCR.

3. Results and Discussion

3.1. Assaying LChV-1 encoded P21 for silencing suppressor activity

The ORF7 of LChV-1 has a possibility to encode a shorter and a longer protein. In the sweet cherry strain investigated before only a short P21 protein is translated and found to act as a VSR (Katsiani et al., 2017). In the reference genome of LChV-1, an ORF7 encodes a P21 protein that is 181 amino acids long. In contrast, our isolates, specifically the sweet cherry strain 'Alex' and apricot strain 'Magyar kajszi,' exhibit a longer version of P21, with an additional 50 amino acids, alongside the shorter variant. Moreover, the short P21 version in the apricot strain is 6 amino acids longer than its counterpart in the cherry variant.

In this research, we amplified both a short (S) and a long (L) form of P21 from sweet cherry (*Prunus avium* 'Alex') (Ch) and apricot (*Prunus armeniaca* 'Magyar kajszi') (Ap) infected by LChV-1 strains to compare their possible VSR in both local and systemic silencing suppression assays. We used the well-characterized VSR, P19 from Tomato bushy stunt virus (TBSV) as a positive control, and BinHA empty plasmid for negative control.

The LChV1-P21 variants showed a weak, transient local VSR activity that could be detected only visually (Figure 1A) through the mild increase in GFP fluorescence when compared to the control empty vector. However, these differences were not detected in molecular analysis (Figure 1B, C).

This contradicts with a previous report that P21 from LChV-1 has local VSR activity (Katsiani et al., 2017), but could be explained by differences of the P21 proteins originating other LChV-1 isolates (Figure 1.).

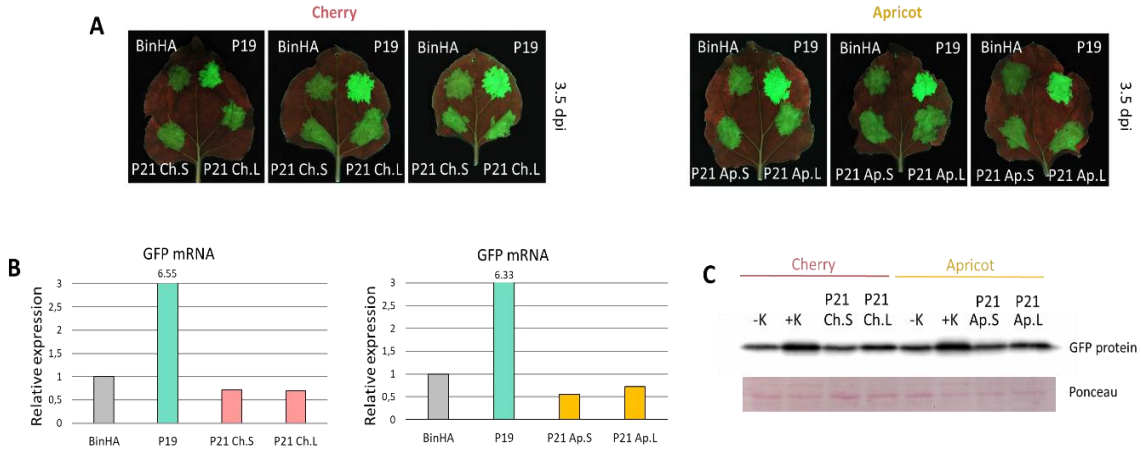

Figure 1. *(A) Local silencing suppression assays of LChV-1 short and long P21 protein of cherry and apricot isolates. (B) Quantitative real-time PCR for GFP mRNA expression measurement. (**C**) Western blot analysis of GFP protein*

Some VSRs, such as the apple chlorotic leaf spot virus (ACLSV) P50, suppress systemic RNA silencing without interfering with local silencing (Yaegashi et al., 2008). To find out whether P21 of LChV-1 can inhibit systemic RNA silencing, we tested its activity in a systemic silencing suppression assay (Figure 2).

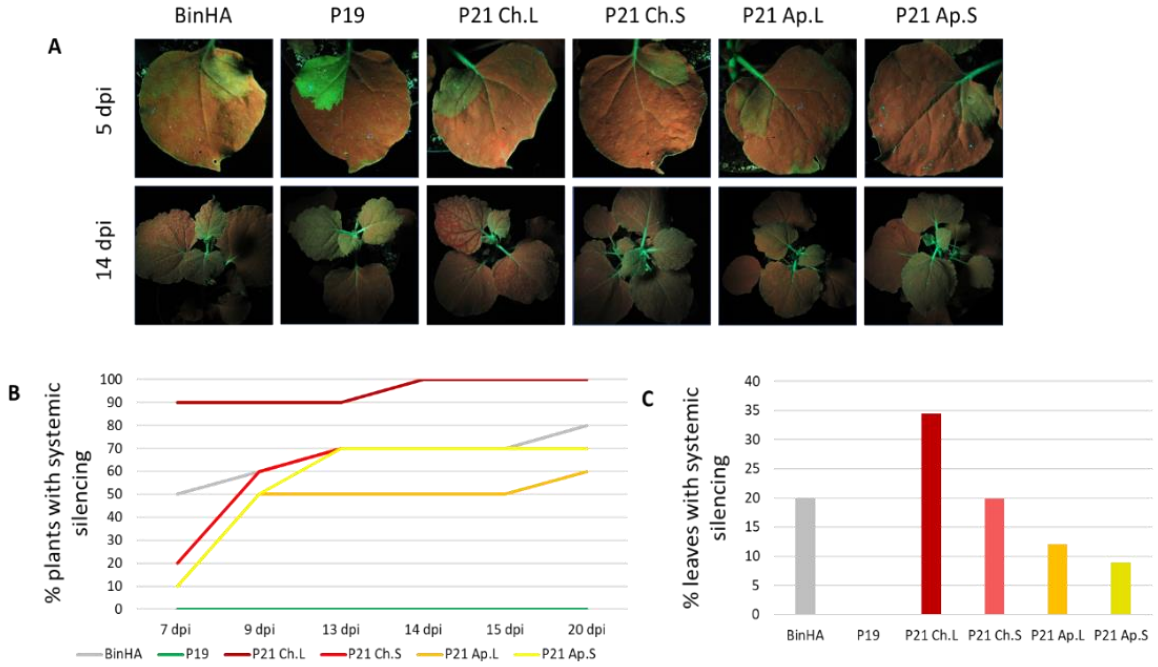

Figure 2. *(A) Systemic silencing suppression assays of LChV-1 short and long P21 protein of cherry and apricot isolates.(B) Percentage of plants with systemic silencing. (C) Percentage of leaves with systemic silencing*

Plants (n=10) and leaves were scored for the appearance of systemic silencing daily till 20dpi. The spread of the silencing signal was detected through the emission of red light resulting from chlorophyll fluorescence. Absence or minimal presence of red veins under UV light indicates a suppressor effect of the protein. In contrast to the negative result in the local silencing assay P21 apricot variants, both short and long proteins, showed mild suppression activity in the systemic silencing assay delaying the movement of the silencing signal. This activity was detected not only in decrease of the number of the plants showing the silencing signal (Figure 2B), but also in decrease in the number of the leaves with red veins (Figure 2C).

3.2 Assaying PaLV encoded P4 for silencing suppressor activity

Based on previous studies on BYDV-PAV (Fusaro et al., 2017), a close relative to PaLV, we investigated VSR activity of two variants of P4 proteins, differing in 11 amino acid, encoded by different strains of PaLV identified in 'Aranycsillag3' (Acs3) and 'Elvira' (Elv) cultivars.

In the local silencing suppression assay (Figure 3), both P4 'Acs3' and 'Elv' proteins showed a slightly stronger GFP fluorescent signal than the negative control (empty vector BinHA) (Figure 3A). The increase in the GFP signal could be detected, both by RT-qPCR and western blot analysis (Figure 3B, C).

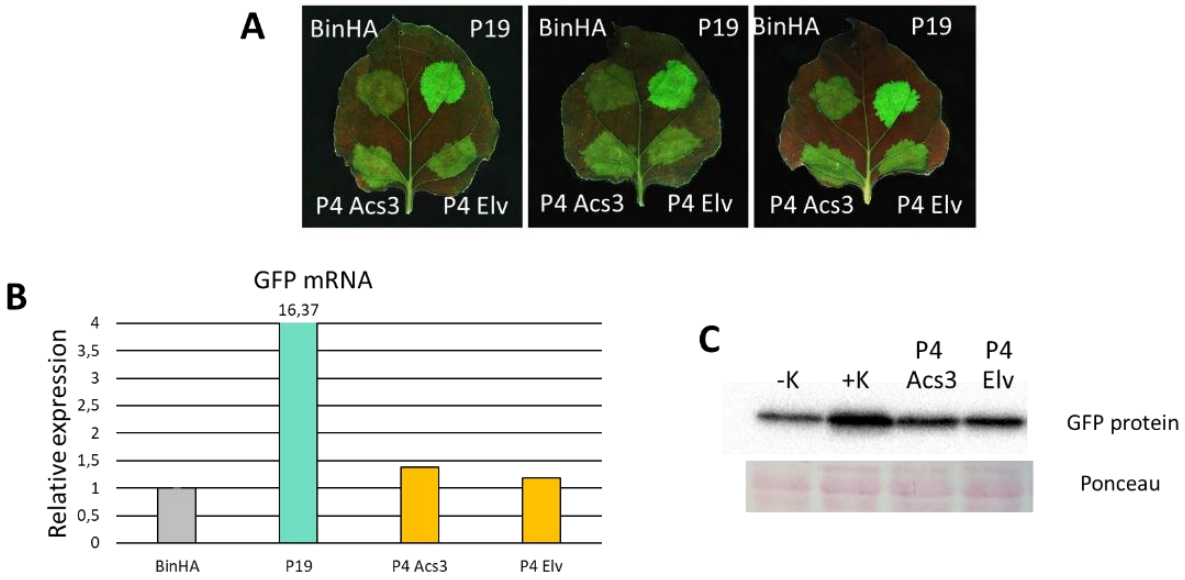

***Figure 3**. (A) Local silencing suppression assays of PaLV P4 isolates Acs3 and Elv. (B) Quantitative real-time PCR for GFP mRNA expression measurement. (C) Western blot analysis of GFP protein*

In the systemic silencing suppressor activity assays, P4 'Acs3' and 'Elv' variants failed to show VSR activity compared to the controls in the percentage of plants showing systemic silencing (data not shown).

4. Conclusions

In our study, we aimed to identify proteins with potential viral suppressor activity in two different *Prunus* infecting viruses. Based on our results, the P21 of LChV-1 isolate from apricot displayed mild systemic VSR activity, in both case of short and long variants. However, local silencing activity was not observed in either P21 variants. In the future, we plan to repeat the study using another GFP variant as silencing inducer, suited for detecting weak RNA silencing

suppressors (Mann & Dietzgen, 2017). P4 of luteoviruses can act as a viral suppressor, what was found also in case of PaLV: P4 protein showed only weak local but no systemic silencing activity. PaLV is usually present in asymptomatic trees which are frequently infected with other viruses. Its weak potential VSR activity can explain its latent presence. Its effect on the host and its role in co-infection with other viruses in terms of synergetic effects, is still unclear and needs further investigation in the future.

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First Description of Apple Rubbery Wood Virus 2 in Hungary by Small RNA High Throughput Sequencing

Az alma fapuhulás vírus 2 hazai jelenlétének kimutatása kis RNS-ek nagy-áteresztőképességű szekvenálásával

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Abstract: Apple (*Malus domestica*) is one of the most widely produced and economically important fruits in temperate regions worldwide. Recently novel negative-stranded RNA viruses have been identified in fruit trees, including pome fruit trees. Apple rubbery wood virus 2 (ARWV2) is one of them belonging to family Phenuiviridae in the order Bunyavirales. ARWV2 possess negative-sense single-stranded tripartite RNA genome small (S), medium (M) and large (L) segments. Apple rubbery wood infectious disease is characterized by unusual flexibility of twigs and smaller branches due to lack of rigidity caused by decreased lignification of xylem vessels and fibers in the symptomatic trees. In our work we detected and validated the presence ARWV2 first time in Hungary in asymptomatic apple trees via small RNA high-throughput sequencing (HTS), and detected its presence in different apple, pear and quince trees by a reverse-transcription PCR (RT-PCR) based survey.

***Keywords**: apple rubbery wood virus 2; small RNA; HTS; RT-PCR*

Összefoglalás: Az alma (*Malus domestica*) a világ mérsékelt égövi régióinak egyik legszélesebb körben termesztett és gazdaságilag legfontosabb gyümölcse. A közelmúltban új, negatív szálú RNS-vírusokat azonosítottak gyümölcsfákban, köztük almatermésűekben. Az apple rubbery wood virus 2 (ARWV2) az egyik ilyen vírus, amely a Bunyavirales rendbe a Phenuiviridae családba tartozik. Az ARWV2, egyszálú, negatív olvasatú háromosztatú RNS genommal rendelkezik kis (S), közepes (M) és nagy (L) szegmensekkel. Az alma fapuhulás fertőző betegségét a gallyak és kisebb ágak merevségének hiánya, szokatlan rugalmassága jellemezi, ami a xilém elemek csökkent lignifikációja miatt következik be. Munkánk során Magyarországon először mutattuk ki kis RNS nagy áteresztőképességű szekvenálással (HTS) a ARWV2 jelenlétét tünetmentes almafában és reverz transzkripciós PCR (RT-PCR)-n alapuló virológiai felmérésben, különböző alma, körte- és birsfák vizsgálatakor.

***Kulcsszavak**: alma fapuhulás vírus 2 (ARWV2); kis RNS; HTS; RT-PCR*

1. Introduction

Apple is an economically important fruit crop cultivated in different parts of world on large hectares of land. Apple trees are propagated vegetatively through grafting, cuttings or layering, which facilitates frequent transmission and accumulation of viruses. Apple rubbery wood virus

2 (ARWV-2)(Rott et al., 2018) is a member of the genus Rubodvirus and family Phenuiviridae. (Kuhn et al., 2020). It was first identified in apples in an association with apple rubbery wood disease (ARWD), which is characterized by unusual flexibility of stems and branches, reduced growth, shortened internodes, and increased cold sensitivity. The flexibility (rubber-like feature) of the branches in susceptible apple varieties is caused by the decreased lignification of xylem vessels and fibers in the infected symptomatic trees. ARWD was first reported in 1935 in England on apple later was found on quince and pear fruit trees. Apple rubbery wood virus 1 (ARWV-1) and ARWV-2 were discovered in apple trees together showing rubbery wood disease symptoms in Germany and USA (Jakovljevic et al., 2017; Rott et al., 2018). ARWV-2 has a tripartite negative-sense single-stranded RNA genome. The complementary RNA (cRNA) of each genomic RNA contains one open reading frame (ORF). RNAs1–3 (also named: large (L), medium (M), and small (S)) encode an RNA-dependent RNA polymerase (RdRp), a movement protein (MP), and a nucleocapsid protein (NP), respectively. Some isolates of ARWV2 have two distinct M and S RNA segments, referred as ARWV2 Ma, Mb, Sa, and Sb (Wang et al., 2022). HTS technologies are potential universal diagnostic methods which can be used to detect and identify any known or unknown pathogens present in the investigated sample (Hou et al., 2020). In our previous work we used HTS to survey the presence of pathogens in apple trees (Várallyay et al., 2022). In that work we focused on the description of citrus concave gum-associated virus, apple luteovirus 1 and apple hammerhead viroid. Detailed bioinformatic analysis of that samples indicated the presence of ARWV2 in Freedom cultivar, which was analyzed in details and validated in this current study. The introduction should briefly place the study in a broad context and highlight why it is important.

2. Materials and Methods

Apple leaf samples were collected from production orchards and germplasm collections from different geographical locations of Hungary. These samples were collected from four different branches of the trees and were used for RNA extraction by the CTAB method. For sRNA HTS, the RNA pools representing individual trees or a mixture of ten different trees were prepared by mixing equal amounts of RNA. The sRNA sequencing libraries were prepared from the purified small RNAs using a TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, CA, USA) and our in-house modified protocol (Czotter et al., 2018). These small RNA libraries were sequenced using a single index on a HiScanSQ by UD-Genomed (Debrecen, Hungary) (50-bp, single-end sequencing). The sequenced reads were analysed using Qiagen CLC Genomic workbench. The reads were trimmed, both redundant and non-redundant list of sequences were prepared. This later one was used for contig building. Virus diagnostics were done by BLAST search of assembled contigs using all plant hosted viruses in the NCBI. The result list was ordered according to their lowest E-value. Both redundant and non-redundant reads were mapped to the ARWV2 reference genome. Based on this analysis the consensus sequences were prepared and the coverage of the viral genome by small RNA reads were calculated. Validation of the bioinformatic analysis to test the presence of ARWV2 was done using RT-PCR. RNA extracts were reverse-transcribed by a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) using random primers according to the manufacturer's instructions. The quality of the cDNA was tested by amplifying a part of the *Malus domestica* actin gene. RT-PCR was carried out using cDNA as template and virus specific diagnostic primers for different segments of ARWV2. The PCR products were evaluated by gel electrophoresis. PCR products intended for Sanger sequencing were purified from the agarose

gel using a GeneJET Gel Extraction Kit (Thermo Fisher). The products were cloned into the CloneJET vector (Thermo Fisher) and Sanger-sequenced.

3. Results and Discussion

To investigate the virome of apple orchards and apple germplasm collections small RNA HTS was applied as an unbiased diagnostic method. Bioinformatics analysis of different libraries indicated the presence of ARWV2 originated contig in one library, which was prepared from a Freedom cultivar grown at Újfehertó originating from a production orchard. Coverage of the ARWV2 viral genome reached the threshold level in further samples, but although in most of them we could validate the presence of the virus using diagnostic primers designed to amplify part of the S segment, only in case of Freedom we could amplify part of the M and L segments. As ARWV2 was detected at different geographical locations we designed an RT-PCR survey to obtain further insight of its distribution and presence. Beside apple trees we sampled pear and quince at collected in different years. Out of total 77 samples15 tested positive for ARWV2 by RT-PCR using the ARWV2 310F & 824R diagnostic primers amplifying partial S segment (Table 1).

Table 1. Summary of ARWV2 positive samples by RT-PCR for S segment

Sample Origin	Position (row, tree)	Variety	ARWV2_S segment
Érd_Elvira _new certified stock2020	B VII. 41/9	Granny Smith* (apple)	+
Érd_Elvira _2019	8_13	Ozark gold (apple)	+
	8_14	Ozark gold (apple)	+
	16_3	Jonica (apple)	+
Olcsvaapáti_2020	18_4	Reglindis(E1) (apple)	+
Érd_Elvira _old certified stock_2019	1_5;1_7	Nyári fontos (apple)	+
	6_3;6_4	Akane (apple)	+
	10_2;10_3	Jonatán M41 (apple)	+
Érd_Elvira _new certified stock2021	40_28	Golden Del. Reinders (apple)	+
	37_35	Bereczki (quince)	+
	38_1	Packhams Triumph (pear)	+
	38_46	Tongre (pear)	+
	39_2	Vilmos (pear)	+
	41_1	Piros vilmos (pear)	+
		BA-29 (Rootstock)	+

While samples collected in 2019 and 2020 were confide to apples, in 2021 pears, quince and BA-29 rootstock trees were also sampled. Out of the 15 samples one apple, one quince, four pears and the rootstock were also tested positive (Figure 1).

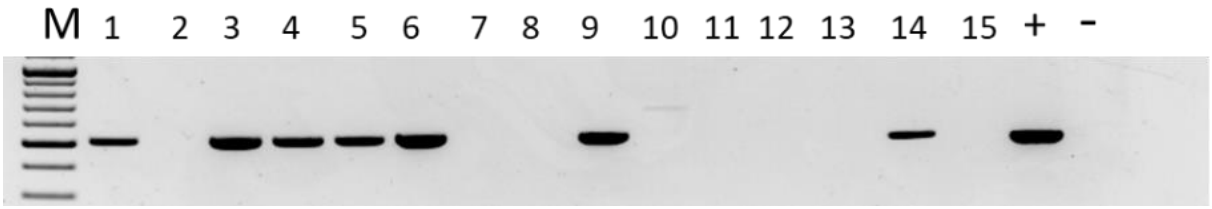

Figure 1. *RT-PCR analysis for testing the presence of ARWV2_Sa segment positive samples from location Érd_Elvira _KTÜ_2021(M-Molecular maker 100 base pair plus, 1- Golden Del. Reinders (apple), 3- Bereczki (quince), 4- Packhams Triumph (pear), 5-Tongre (pear), 6- Vilmos (pear), 9- Piros vilmos (pear), 14- BA-29 (Rootstock) and + positive control (Freedom) and -MQ H2O*

The phylogenetic tree indicates that the samples originated from Hungary clustered together, irrespectively to the host (Figure 2).

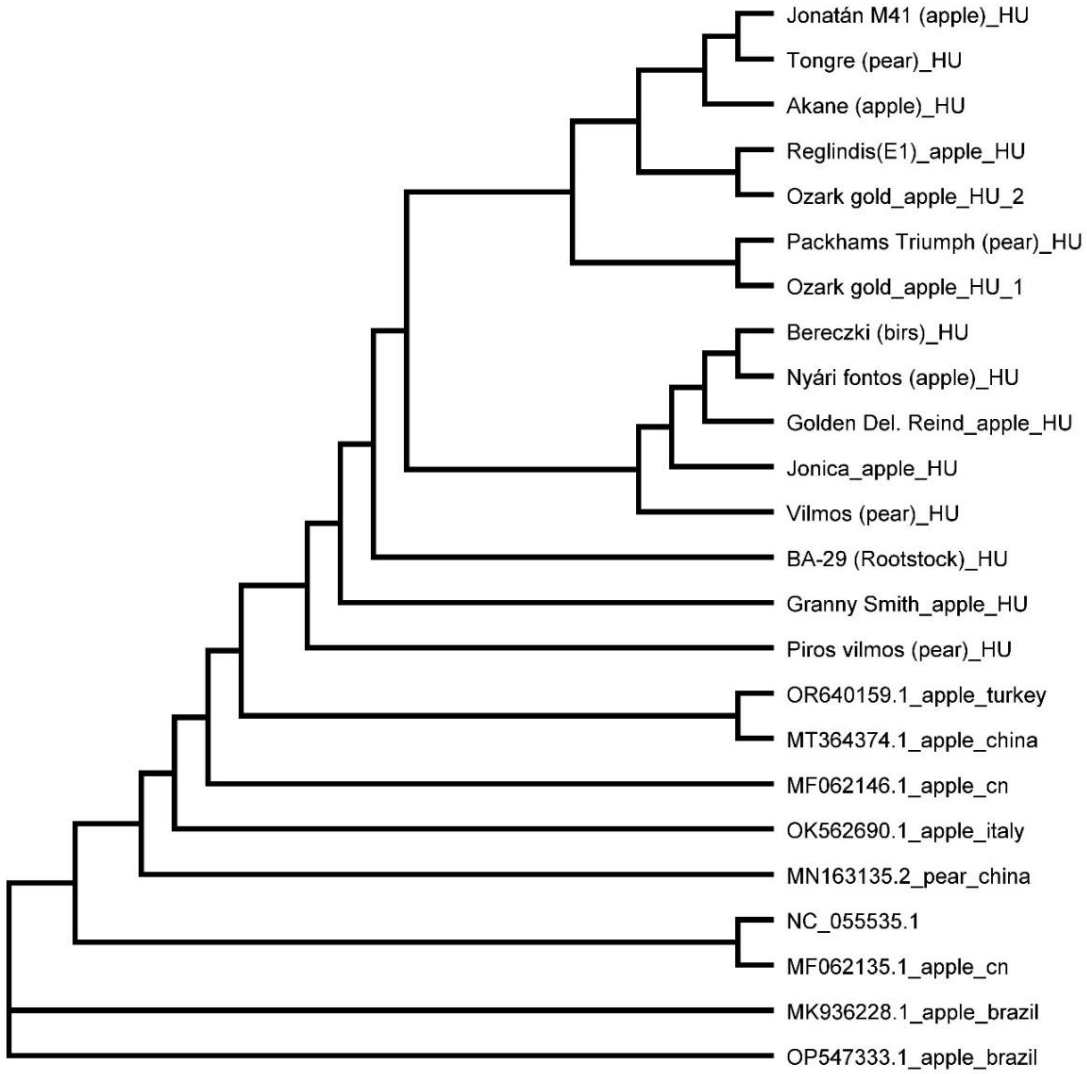

Figure 2. *Phylogenetic tree of all the Hungarian positive samples and including all the available sequences in NCBI for ARWV2 Sa segments (Geneious prime software was used to make the tree using substitution model-TN93 and 1000 bootstrap value) (HU-Hungary, CN-Canada and NC_055535.1-NCBI Id of reference genome of ARWV2_S segment*

4. Discussion

Our study provides new data and knowledge in the following areas:
1. ARWV2 was first described in Hungary using HTS and presence of all three genomic segments were validated in case of Freedom cultivar.
2. The presence of ARWV2 showed its further presence in several different apple cultivars originating from production orchards and germplasm collections from different geographical locations of Hungary and also in pear and quince.
3. Insight into the variability of ARWV2.

During our survey we detected the presence of ARWV2 in asymptomatic trees, usually infected with other viruses. To reveal the importance and possible connection between ARWV2 infection and ARWD further research is currently ongoing focusing on the changes in the miRNA regulation of the lignification process possibly induced by the ARWV2 infection.

Acknowledgements

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Abiotic Stress Impacts on Soybean (*Glycine Max L. Merr.*) Seed Viability

Abiotikus stresszhatások a szója (Glycine max L. Merr.) csírázóképességére

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Abstract: Abiotic stress factors may have adverse impact on the growth and development of crop plants. In a crop physiology experiment at the Hungarian University of Agriculture and Life Sciences, Gödöllő, Hungary soybean seeds were exposed to various levels of temperature, salinity and water supply. Viability and initial growth were evaluated. The results obtained suggest that viability and early development of soybean plants depend on the optimum level of abiotic external factors like temperature, salinity and water availability.

Keywords*: abiotic stress; viability; soybean; waterlogging; salinity; temperature*

Összefoglalás: Az abiotikus stresszhatások eltérő módon befolyásolják a növények növekedését és fejlődését. Gödöllőn, a Magyar Agrár- és Élettudományi Egyetemen végzett kísérletben a hőmérséklet, a sókoncentráció és a vízellátottság szója magvak életképességére gyakorolt hatását vizsgálták. A kísérletben elemezték a csírázóképesség és a kezdeti fejlődést. A kapott eredmények szerint a szója magvak csírázását és a kezdeti fejlődését nagymértékben meghatározzák a vizsgált abiotikus tényezők; a hőmérséklet, a sókoncentráció, illetve a vízellátottság mértéke.

Kulcsszavak*: abiotikus stressz; csírázóképesség; szója; belvíz; szikesedés; hőmérséklet*

1. Introduction

Live organisms are exposed to various impacts that may influence the regular physiological processes in various ways. Selye (1974; 1983) proposed four variations of stress. On one hand he determined good stress (eustress) and bad stress (distress). On the other is over-stress (hyperstress) and understress (hypostress). He advocates balancing these: the ultimate goal would be to balance hyperstress and hypostress perfectly and have as much eustress as possible.

Viability of plant seeds and the development of germinated plants require certain environmental conditions. The most fundamental factors of these processes are the optimal presence of moisture, temperature, and the availability of oxygen. Any of them whenever missing or the presence of that is out of the optimum range may induce stress impacts on the germination and sprouting processes.

It is essential to study the effect of abiotic stress in seed germination and have an idea about the germination rate of seeds and the tolerant and sensitive species under abiotic stress to increase the efficiency and production of crops. In the present study, our main aims were as

follows: studying the effect of abiotic stress – temperature, salinity and waterlogging – on soybean seed germination, and trying to determine the optimal amount, range or threshold level of these factors for soybean seed germination.

2. Materials and Methods

The present trial was conducted in the laboratory of crop production MATE in Gödöllő, Hungary (Huynh Anh Kiet, 2022). The objective of the trial was studying the effect of abiotic stresses – among them temperature, salinity and waterlogging – on soybean seed germination. We used the following materials: transparent Petri dishes; micro pipette; filter paper; distilled water; soybean seeds; climate chamber; precision scale. Soybean seeds of Martina variety used for the experimentation were as follows: the methods and the description of the trial were by general laboratory standards. Samples of 9 seeds were tested for viability under these experimental conditions in three replications, run in a Memmer-type climatic chamber.

During the experiment, soybean seeds germination was tested under three abiotic factors. The treatments were set in a randomized complete block design (RCBD), having 3 repetitions. For each factor of abiotic stress, we set 3 treatments with 3 replications.

Temperature treatment. We carried out a study on the effect of three levels of temperature (T20; T25; and T30) on the germination of seeds as follows: 20°C, 25°C and 30°C respectively.

Salinity treatment. Two levels of NaCl concentrations on seeds germination (1000 ppm) and (1500 ppm) compared to control seeds (0 ppm) as follows: S0: control seeds. S1: seeds tested to germinate under 1000 ppm of salt. S2: seeds tested to germinate under 1500 ppm of salt.

Water treatment. In this trial, we tried to test three amounts of water in each seed of soybean using Petri dishes. The treatments were as follows: W0: seeds soaked in 6 ml of water, W1: seeds soaked in 9 ml of water, W2: seeds soaked in 12 ml of water.

3. Results and Discussion

3.1. Effect of different levels of temperature on soybean seed germination

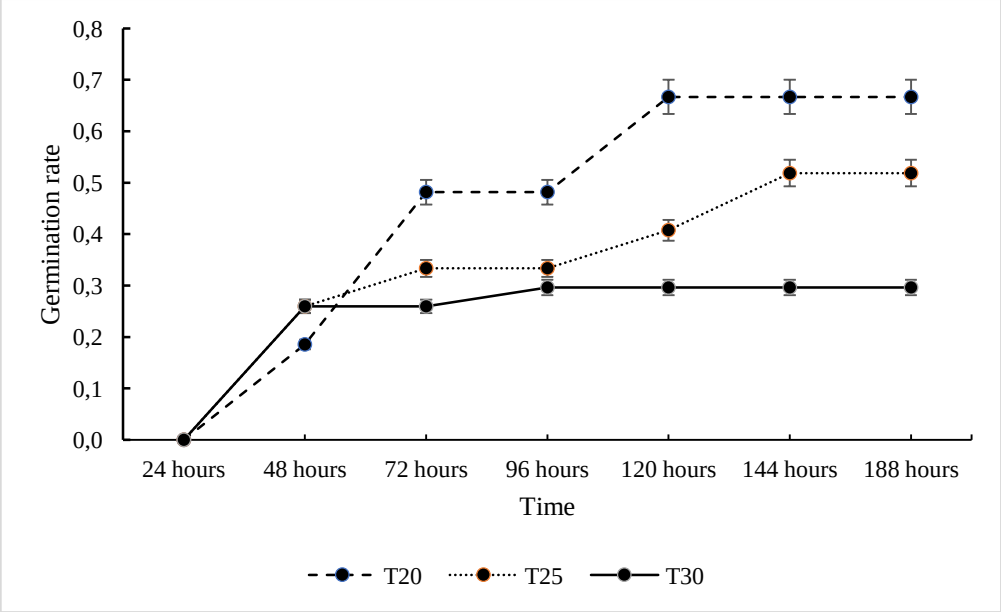

Figure 1. *Germination response of soybean seeds to temperature levels. Each point represents a mean ± SEM (n=3)*

Figure 1 presents the effects of three levels of temperature on the germination of soybean seeds during seven days. Germinated seeds varied between 1 to 6 per petri dish. The temperature of 20ºC resulted in the highest percentage of germination in all of the measured levels, while the temperature of 30ºC produced the lowest percentage of germination. The germination rate of soybean seeds decreased with increasing temperature. Total percentage of germinated seeds was 67%; 52%; and 30% at temperature 20ºC; 25ºC; and 30ºC, respectively.

A one-way ANOVA between treatments subjected to heat stress was conducted to compare the effect of different temperature levels (20°C; 25°C; 30°C) on the germination of soybean seeds. There was a significant difference effect ($p<0,05$) of different temperature levels on germinated seeds, radicle length, and plumule length of germinated seeds, presented in Table 1.

Table 1. Summary of one-way ANOVA showing a degree of freedom (Df), F, and probability for each analysis under temperature stress conditions. Significant P-values are highlighted in bold

Parameters	Df	F	P
Germinated Seeds	80	3,984	0,023
Radicle length	80	4,926	0,010
Plumule length	80	3,594	0,032

There was no significant difference between temperatures of 20°C and 25°C; 25°C and 30°C in total germinated seeds treated. The germination ratio of soybean seeds was affected by temperature. We observed a non-significant difference in seed germination between 20°C and 25°C of about -0,148 at p=0,265 and between 25°C and 30°C of about -0,222 at p=0,096 between 20°C and 25°C for germinated seeds (Table1). However, we found a significant difference in germinated seeds ratio between 20°C - and 30°C of around -0,370 at p=0,006. The ratio of germinated seeds decreased as the temperature increased.

Based on these findings, our results agreed with that of Hatfield and Egli (1974) and Gilman et al. (1973) at 20ºC the time required for a given percentage of soybean seed germination was twice of that at 30ºC. Wuebker, Mullen, and Koehler (2001) explain that at higher germination temperatures, the damaging effects of impact damage were more significant than at lower temperatures. However, our findings disagree with Szczerba et al., (2021). In their experiment, four types of soybean seed demonstrated a high ability to germinate at 25°C.

3.2. Effect of different salt levels on soybean seeds germination

Figure 2 demonstrates the effect of salt concentrations on the germination of soybean seeds. The highest percentage of germination was recorded at 0 ppm and the lowest at 1500 ppm for three salt concentrations applied. The total proportion of germinated seed was about 70%; 44%; 33%, respectively in the levels of salt 0 ppm; 1000 ppm; and 1500 ppm.

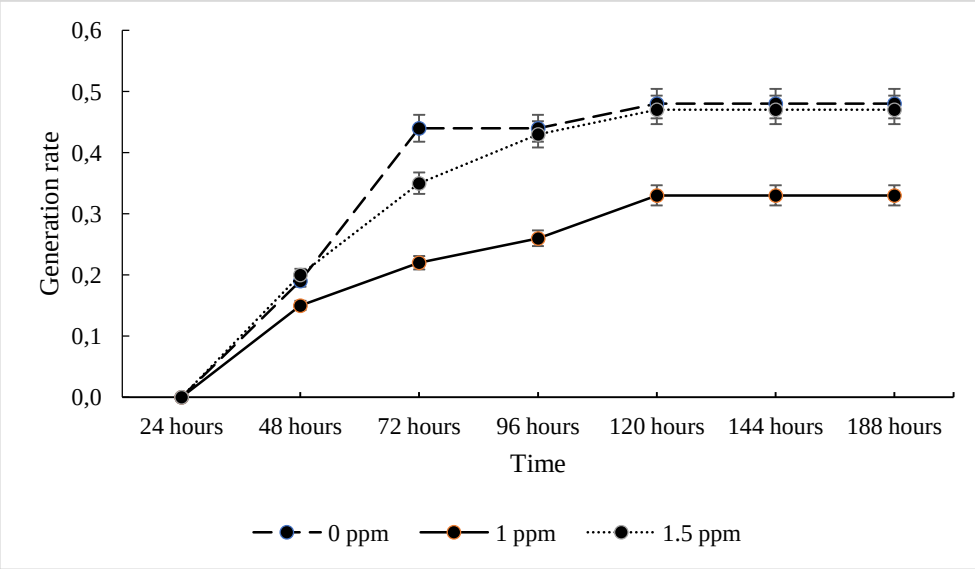

Figure 2. *Germination response of soybean seeds to salt level (x1000 ppm). Each point represents a mean ± SEM (n=3)*

A one-way ANOVA between salt stress treatments was conducted to compare the effect of different salt levels 0 ppm; 1000 ppm; and 1500 ppm on soybean seeds germination. There was a significant difference percentage of germinated seeds. The results are shown in Table 2.

Table 2. Summary of one-way Anova showing a degree of freedom (Df), F, and probability for each analysis under salt stress conditions. Significant P-values are highlighted in bold

Parameters	Df	F	P
Germinated Seeds	80	4,158	0,019
Radicle length	80	6,902	0,002
Plumule length	80	5,508	0,006

The results suggest that the percentage of soybean seed germination was reduced with increasing salt concentration. This decrease can be due to the increase in external osmotic pressure, which affects the rate of water absorption by seeds, and limits their germination (Ashraf, 2012). Our results are similar to Shu et al., (2017), the germination rate of the soybean seeds under salt stress conditions was about two to three-folds less than normal during the germination processes. Other results also supported that the delayed-germination phenotype was caused by the NaCl treatment. (Xu et al., 2011), the concentration of 100 mmol/L NaCl affected the ability of soybean seed germination. On the other hand, moderate salt stress intensity just delayed germination time and had no significant effect on the final germination percentage.

3.3. Effect of different water levels on soybean seeds germination

A water-stress experiment was carried out with three levels of water supply. Our study indicates that soybean seeds germination ratios are highest at two levels, namely 6 ml and 9 ml of water, with seeds germination percentages of 40.74 and 22.22 for 12 ml of water, respectively. After a few days at the 12 ml water treatment, however, waterlogged soybean seeds were blocked in further germination and development (Figure 3).

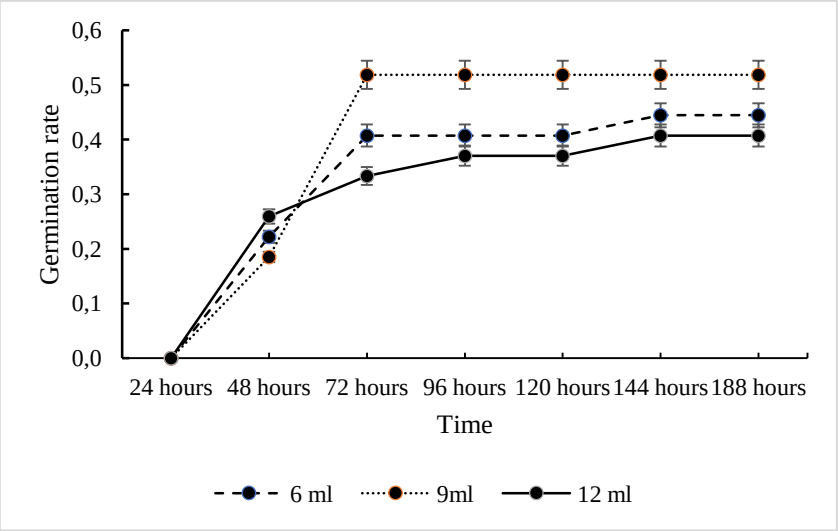

Figure 3. *Germination response of soybean seeds to water level. Each point represents a mean ± SEM (n=3)*

One-way ANOVA between treatments was conducted to compare the effect of levels of water 6 ml water; 9 ml; and 12 ml on soybean seed germination (Table 3). There was a significant difference effect of levels of water on the radicle length and plumule length of seeds germinated, as Table 2 and 3 show.

Table 3. Summary of one-way Anova showing a degree of freedom (Df), F, and probability for each analysis under water stress conditions. Significant P-values are highlighted in bold

Parameters	Df	F	P
Germinated Seeds	80	0,338	0,714
Radicle length	80	3,495	0,035
Plumule length	80	2,226	0,115

The germination ratio of soybean seeds was not significantly different between levels of water. On the other hand, the waterlogging condition damaged the soybean seedling 7 days after sowing at a water level of 12 ml. Similar results were obtained in a pea crop seed long-term waterlogging experiment (Zaman et al., 2019), where the lack of oxygen, caused membrane degradation and leakage of cellular contents, resulting in seed mortality and/or germination failure. Carrera et al (2021) reported that drought stress of soybean plants resulted in future yield losses. In our trial the best germination performance was recorded in the 9 ml treatments however the magnitude was not significant. The results suggest that water logging may deteriorate soybean germination activities and reduce the number of germinated seeds.

4. Conclusions

Summarising the trial, the results obtained suggest, that abiotic stress conditions caused changes of various magnitude in the viability and initial growth and development of soybean seeds and sprouts. Temperature proved to be a strong environmental factor influencing the performance of the seed. The higher is the temperature the poorer are the viability and growth values. Salinity was also an influencing factor for these parameters. Control and low level of NaCl concentrations had no or less impact on the seed development, however high salt concentrations in general have blocked germination and the growth of initial organs. Waterlogging conditions have obstructed germination after the second day, while normal and abundant but no flood water

supply applications contributed to higher germination. The stress processes were detected and identified, however more detailed physiological observations are needed in the future to specify external and internal stresses of a certain crop, since that is the only way to improve stress tolerance in field crops.

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In Vitro Technics for Determination of the Eye Irritant Properies of the Agrochemicals

In vitro technika az agrokemikáliák szemirritációs tulajdonságának meghatározására

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Abstract: Until now the most popular method to classify substances' eye irritation potential is the OECD 405 test guideline. The basis of this is the Draize-test, which is one of the most criticized in vivo methods, because of the injuries of the test animals and subjective nature of the test in recording the results. Nowadays, several alternative tests are available which can be partly or totally replaced the in vivo eye irritation testing depending on the circumstances. The Isolated Chicken Eye Test (ICET) is part of these alternative methods. Four different agrochemicals (Biscaya, Dezormon, Kyleo, Pulsar 40 SL) were examined with this in vitro method. In ICET the eye irritation potential of test items were predicted based on the combination of three endpoints: corneal swelling, corneal opacity and fluorescein retention. The basis of determination of each endpoint was the differences between values of the base line measurement and values of any observation time points after the post-treatment rinse. Three agrochemicals showed different severity irritation potential and one agrochemical did not show any effect on the treated corneas. Comparing these in vitro results with the available in vivo data of the tested agrochemicals, results are found to be corresponding to each other.

Keywords*: eye irritation; in vitro; corneal swelling; corneal opacity; fluorescein retention*

Összefoglalás: A Draize-féle primer szemirritációs tesztet nagyon sok kritika éri az eredmények szubjektív értékelése, de legfőképp a vizsgálatok során felhasznált állatok szenvedése miatt. A napjainkban elérhető alternatív módszerek a körülményektől függően részben vagy akár teljes mértékben képesek kiváltatni az in vivo tesztet. Ezen eljárások közé tartozik az izolált csirkeszem vizsgálatán alapuló szemirritációs vizsgálati módszer (ICET). Ezen vizsgálati módszer elvégzése során a kezelést követően a szaruhártya-duzzadás, -homály és fluoreszein megtartás mértékét az alap értékhez viszonyítva határoztuk meg, és az így kapott végpontok kombinációiból következtettünk a vizsgálat agrokemikáliák (Biscaya, Dezormon, Kyleo, Pulsar 40 SL) szemirritációs potenciáljára. A vizsgált agrokemikáliák közül három eltérő mértékben, de szemirritáló tulajdonságúnak mutatkozott, míg egy vizsgálat anyag esetében nem állapítottunk meg szemirritációs potenciált az alkalmazott in vitro módszer alapján. A rendelkezésre álló in vivo eredményekkel összehasonlítva azt tapasztaltuk, hogy az összes vizsgált anyag esetében a saját in vitro és az in vivo eredmények megegyeztek.

***Kulcsszavak**: szemirritáció; in vitro; szaruhártya-homály; szaruhártya-duzzadás; fluoreszein megtartás*

1. Introduction

In the Isolated Chicken Eye Test (ICET) the agrochemicals are applied in a single dose onto the cornea of isolated chicken eyes. The purpose of this test is to classify the agrochemical as ocular corrosive and/or severe irritant (UN GHS Category 1) or to identify the test item as a chemical that does not require classification for eye irritation (UN GHS No Category) or serious eye damage under the UN GHS classification system.

The ICET does not fully replace the in vivo Draize-test (OECD 405), because it cannot be identified of the agrochemicals, that should be classified as slight or moderate eye irritant (UN GHS No Category 2). The reason of lack of this ability, that the necessary physiological properties of the chicken eyes can be hold on only few hours after the chickens slauthered, but day or weeks are necessary for the examination of the reversibility of the observed effects (Buda et al., 2013). However, this method is used as part of a tiered testing strategy for regulatory purposes (Budai et al., 2004; Tavaszi and Budai, 2006; Tavaszi et al., 2008).

Four different agrochemicals (Biscaya, Dezormon, Kyleo, Pulsar 40 SL) were tested with this method. Our goal was to determinate how the results, which got this in vitro test method, can be collerate with the available in vivo test results and based on that how effectively use this in vitro test system for the determination of the eye irritation properties of the agrochemicals in the future.

2. Materials and Methods

The Isolated Chicken Eye Tests were performed based on the OECD 438 (2023) guideline.

Chicken heads collection and transport: Breed of chicken was ROSS 308. The heads were transported at the earliest convenience for use approximately within 2 hours from collection.

Selection and preparation of eyes for the test: Corneal integrity was checked by fluorescein solution. Eyes that had high baseline fluorescein staining or corneal opacity score or any additional signs of damage after enucleation was rejected. A minimum of seven eyes were used for each test (three treated eyes, three positive control eyes and one negative control eye).

The base line assessments: Baseline values were required to evaluate any potential agrochemicals related effects after treatment.

Treatment: Biscaya (Bayer Hungária Ltd., Hungary), Dezormon (Nufarm Hungária Ltd., Hungary), Kyleo (Nufarm Hungária Ltd., Hungary) and Pulsar 40 SL (BASF Hungária Ltd., Hungary) were applied onto the centre of the cornea (standard amount was 30µL) such that the entire surface of the cornea was covered.

Test item removal: After an exposure period of 10 seconds from the end of the application, the cornea surface was rinsed thoroughly with ~20 mL isotonic saline at ambient temperature.

Observation: The cornea thickness and opacity of all eyes (control and test eyes) were evaluated pre-treatment and at approximately 30, 75, 120, 180 and 240 minutes after the post-treatment rinse. The fluorescein retention was measured on two occasions, baseline (t=0) and 30 minutes after the post-treatment rinse.

Evaluation: The endpoints evaluated were corneal opacity, swelling, fluorescein retention, and morphological effects (e.g., pitting or loosening of the epithelium).

3. Results

The purpose of ICETs was to evaluate the potential ocular corrosivity and irritancy of the agrochemicals Biscaya, Dezormon, Kyleo and Pulsar 40 SL by their ability to induce toxicity in enucleated chicken eyes:

The overall ICE classes of the Pulsar 40 SL treated corneas were thrice I (based on corneal swelling of 3 % within 240 minutes, based on the corneal opacity score of 0.5 and based on the fluorescein retention of 0.3) in the experiment.

The overall ICE classes of the Biscaya treated corneas were thrice II (based on corneal swelling of 8 % within 240 minutes, based on the corneal opacity score of 1.3 and based on the fluorescein retention of 1.3) in the experiment.

The overall ICE classes of the Kyleo treated corneas were thrice III (based on corneal swelling of 19 % within 240 minutes, based on the corneal opacity score of 1.7 and based on the fluorescein retention of 2.0) in the experiment.

The overall ICE classes of the Dezormon treated corneas were thrice IV (based on corneal swelling of 48 % within 240 minutes, based on the corneal opacity score of 3.7 and based on the fluorescein retention of 3.0) in the experiment.

The positive control was classified as corrosive/severely irritating, UN GHS Classification: Category 1 in each experiment. The negative control had no significant effects on the chicken eye in these tests and was categorized as UN GHS Non-Classified in each experiment. So, the positive and negative controls showed the expected results in each experiment and confirmed the validity, sensibility and suitability of the tests.

4. Discussion

The Pulsar 40 SL did not show eye irritation property in this in vitro eye irritation test, which result is harmonizing with the available in vivo result (see Table 1).

The agrochemicals Biscaya and Kyleo have been categorized as ‘no prediction can be made’. This means, that they did not cause serious eye damage, but they exact eye irritation properties cannot be determined with this method, because the lack of the observation of the reversibility processes prevents the identification of the agrochemicals, which should be classified as slight or moderate eye irritant (UN GHS No Category 2). However, these results are perfectly harmonized with the available in vivo results (see Table 1).

The Dezormon showed serious eye damage. This means it can be classified as Category 1, which is the same as the available in vivo result (see Table 1).

Table 1. The in vitro irritation categories of the tested agrochemicals obtained in in vitro tests and the in vivo eye irritation categories on the safety data sheets

Agrochemicals	In vitro GHS[1] Classification based on the ICET results	In vivo GHS[1] Classification based on the SDS[2]
Pulsar 40 SL	No Category	No Category
Biscaya	No prediction can be made	Category 2
Kyleo	No prediction can be made	Category 2
Dezormon	Category 1	Category 1

[1]*GHS: Globally Harmonized System*

[2]*SDS: Safety Data Sheet*

According to the results above, the available in vivo date are fully supported by the in vitro isolated chicken eyes tests.

Based on the correlation between the in vivo and in vitro results, it can be established in accordance with the opinion of other authors (Adriaens et al., 2014; Budai et al., 2021), that The Isolated Chicken Eye Test method is applicable for, that partly or totally replace the in vivo test method with its weakness of the lack of the observation of reversibility processes.

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Model Study to Investigate the Toxic Interaction between Spirotetramat and Myclobutanil on Pheasant Embryos in the Early Phase of Development

A spirotetramat és a miklobutanil közötti toxikus kölcsönhatás vizsgálata fácánembriókon, a fejlődés korai szakaszában

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Abstract: The toxic effects of the Movento insecticide (100 g/l (9.29 m/m%) spirotetramat) applied alone or in combination with Systhane 20 EW fungicide (200 g/l myclobutanil (19.4 m/m%) were studied on pheasant embryos, in the early phase of embryonic development. The test materials were injected in 0.1 ml volume into the eggs' air chamber on the first incubation day. Subsequently, on the third day of incubation, permanent preparations were made using the embryos to study the early developmental stage. Embryos fixed on microscope slides and stained with osmium tetroxide solution were studied by light microscopy. The embryonic mortality and the occurrence of developmental anomalies were analysed statistically by the Fisher test. Based on the experiment's results, the embryonic mortality of pheasant embryos increased in every treated groups (individual or combined administration of the chemicals) compared to the control. The differences in the individually treated groups could not be statistically proven. The simultaneous application of Movento and Systhane 20 EW significantly increased the mortality of pheasant embryos compared to the control. Both test substances were embryotoxic in pheasants, and an additive toxic interaction was revealed between Movento and Systhane 20 EW.

Keywords: *spirotetramat; myclobutanil; interaction, embryonic mortality, pheasant embryo*

Összefoglalás: Munkánk során a Movento inszekticid (100 g/l (9,29 m/m%) spirotetramat) és a Systhane 20 EW gombaölő szer (200 g/l miklobutanil (19,4 m/m%) toxikus hatásait vizsgáltuk, a növényvédő szereket önmagukban és kombinációban alkalmazva fácánembriókon, az embrionális fejlődés korai szakaszában. A vizsgálati anyagokat 0,1 ml térfogatban a tojások légkamrájába fecskendeztük az inkubáció első napján. Ezt követően az inkubáció harmadik napján az embriókból tartós preparátumokat készítettünk a korai fejlődési szakasz tanulmányozására. A tárgylemezeken rögzített és ozmium-tetroxid oldattal megfestett embriókat fénymikroszkóp alatt vizsgáltuk. Az embriómortalitás és a fejlődési rendellenességek statisztikailag Fisher-féle egzakt teszttel elemeztük. A kísérlet eredményei alapján a fácánembriók embriómortalitása minden kezelt csoportban (az egyedileg és az

anyagok kombinációjával kezelve) nőtt a kontrollhoz képest, a különbségek az egyedileg kezelt csoportokban statisztikailag nem igazolhatók. A Movento és Systhane 20 EW egyidejű alkalmazása szignifikánsan növelte a fácánembriók mortalitását a kontrollhoz képest. Mindkét vizsgálati anyag embriótoxikus volt fácánban, közöttük additív toxikus kölcsönhatás igazolódott.

***Kulcsszavak**: spirotetramat; miklobutanil; interakció, embriómortalitás; fácánembrió*

1. Introduction

The increase in the Earth's population places an ever-greater task on agriculture, as the food needs of the rapidly increasing population must be produced in an ever-smaller agricultural area, and the highest possible amount of crops in proper quality must be harvested from the available areas.

Integrated plant protection, which includes mechanical and chemical agrotechnical solutions, dramatically contributes to production safety. The use of pesticides is essential in the control of harmful organisms present today, and for the time being, no alternative in the future could replace chemical control to a greater extent than at present.

The solution for harmonising plant protection product use and the interests of environmental protection and safe food production is the reasonable, specific use of pesticides with the lowest environmental impact.

Pesticides and various chemical substances released into the environment pose a danger to plants, animals, and humans through the food chain. Sprayed agents can have a negative effect not only on adult wild birds, causing direct (immediate death) or indirect (reproductive biology) issues, but also on the embryo already developing in the egg (Nagy, 1984; Faragó, 1997; Szabó et al., 2003). Cunningham and Woodworth-Saigo (1995) investigated the problem of the decrease in shell thickness of bird eggs, which they attributed to calcium metabolism disturbances caused by chemicals. Wild bird species are exposed to the effects of agricultural chemicals, especially during reproduction and rearing. Since their reproduction period coincides with the time of pesticide spraying, extra attention should be paid to the ecotoxicological examination of the harmful effects of pesticides on living organisms. The possibility of direct exposure to wild bird eggs is increased by, among others, the non-prescribed application of the preparations, the inappropriate spraying technique, and the dangers arising from drifting (Keserű et al., 2004; Várnagy et al., 2003; Szabó et al., 2020).

2. Materials and Methods

Our experiment was carried out in 2021. The 0.75% water suspension of Movento (Bayer Hungária Ltd., Hungary) insecticide with 100 g/l (9.29 m/m%) spirotetramat active ingredient and the 0.225% fungicide Systhane 20 EW (Dow AgroSciences Hungary Ltd., Hungary) with 200 g/l myclobutanil (19.4 m/m%) active ingredient of oil emulsion was used, which corresponded to practical spray concentration. The embryotoxic effect of their single and combined administration was investigated in the early embryonic stages of the pheasant as a test organism.

Forty fertile pheasant eggs derived from the main colony of the Hubertus Hunting Association (Abádszalók, Hungary) were used in the experiment. The eggs were incubated after transportation and rested for 24 hours. The eggs spent 23–24 days within a Ragus-type incubator (Vienna, Austria). During the incubation, the appropriate temperature (37.5–37.8°C), air humidity (48–65%) and the daily rotation of eggs were provided (Bogenfürst, 2004).

The treatment of eggs (n=10/group) was performed on the first day of incubation. In the individual treatments, suspensions and emulsions made from test chemicals in 0.1–0.1 ml end volume were used, while in the case of combined treatments, 0.2 ml of the test materials were injected into the air chambers of eggs (Clegg, 1964; Lutz, 1974).

A hole was punched in the calcic eggshell, and the shell membrane above the air space, and then the proper quantity of the suspensions and emulsion of the test materials was injected into the air chamber (Clegg, 1964; Várnagy et al., 1996). After the injection, the hole was sealed with paraffin, and the eggs were returned to the incubator. In the control group, avian physiological saline solution (0.75 w/v%) was injected into the air space of the eggs in the manner described above. The incubation was started immediately after the treatments.

To study the early development phase, permanent preparations were made from 10 embryos per group on the third day of incubation. Above the air chamber, the calcic eggshell and the shell membrane were removed. The germinal disk was cut around and, with a filter paper placed on it, was put into avian physiological saline (0.75 w/v %) at 38ºC temperature. After blotting up the saline solution, the embryo placed on a slide was stained with 0.1% osmium tetroxide solution and fixated, then mounted with DPX histological adhesive and covered with a coverslip. The permanent preparations were examined by light microscopy (Sinkovitsné and Benkő, 1993).

The number of embryonic deaths and developmental abnormalities of embryos were recorded.

In the case of the biometric processing of embryonic mortality and malformations, an exact test, according to Fisher, was used.

3. Results

Three days after treatments, no dead embryo was found in the control group.

As a result of the treatment with Movento, the rate of embryonic mortality was 10.0%, but the difference was insignificant compared to the control group.

The single administration of Systhane 20 EW increased the embryonic mortality up to 30.0%. This change was also not significant as compared to the control group.

The combined administration of the insecticide and the fungicide resulted in a 40.0% embryonic mortality. According to the statistical evaluation, the change was statistically significant as compared to the control group ($p<0.05$) (Table 1).

Table 1. Embryonic mortality from teratogenicity test of Movento insecticide and Systhane 20 EW fungicide in pheasant embryos after single and combined administration

Treatment	Death No. / No. of fertile eggs	Rate of embryonic mortality (%)
Control	0/10	0.00
Movento	1/10	10.00
Systhane 20 EW	3/10	30.00
Movento + Systhane 20 EW	4/10[a]	40.00

[a]*Significant difference as compared to the control group ([a]$p<0.05$)*

During the light-microscopic evaluation of permanent preparations, only a single developmental anomaly (10.0%) was found in the control group (Table 2-3).

Two embryos treated with Movento insecticide showed developmental anomaly (22.22%). This rate was not significantly different from the control group's (Table 2). The developmental

anomaly was diagnosed as a retarded development of the embryo and its vascular system (Table 3).

Two embryos (28.57%) showed abnormal development as a result of the treatment with Systhane 20 EW fungicide alone. This change was insignificant compared to the control group (Table 2). The developmental anomaly was identified as a retarded development of the embryo and its vascular system (Table 3).

Due to the combined treatment, the rate of developmental anomalies was 16.67%. The change was insignificant compared to the control group and the groups treated with either insecticide or fungicide alone (Table 2). The type of developmental anomaly was also a retarded development of the embryo and its vascular system (Table 3).

Table 2. Developmental anomalies from teratogenicity test of Movento insecticide and Systhane 20 EW fungicide in pheasant embryos after single and combined administration

Treatment	No. of embryos showing developmental anomalies / No. of alive embryos	Rate of developmental anomalies (%)
Control	1/10	10.00
Movento	2/9	22.22
Systhane 20 EW	2/7	28.57
Movento + Systhane 20 EW	1/6	16.67

Table 3. Types of developmental anomalies diagnosed in the teratogenicity test of Movento insecticide and Systhane 20 EW fungicide in pheasant embryos after single and combined administration

Treatment	Types of developmental anomalies (incidences of developmental anomalies)
Control	Less developed body (1)
Movento	Poorly developed vasculature and body (2)
Systhane 20 EW	Poorly developed vasculature and body (2)
Movento + Systhane 20 EW	Poorly developed vasculature and body, undeveloped embryo (1)

4. Discussion

Based on the experiment's results, it can be established that the embryonic mortality found in the groups treated with the insecticide or the fungicide alone was not significantly different from that seen in the control group.

At the same time, it can be stated that combined treatment with the pesticides enhanced the embryotoxicity since the rate of embryonic mortality found in the group receiving the combined treatment was significantly higher than that obtained in the control group.

As a result of the treatments, developmental delay appeared in the form of the retarded development of the vascular system and the body. Movento and Systhane 20 EW applied together proved to be embryotoxic for the pheasant embryo in the initial stage of its development. Based on the incidence of malformations, a teratogenic effect cannot be confirmed because the detected developmental disorders can later be compensated (Juhász et al., 2005).

These results are in harmony with the results of previous studies in which eggs were treated with various pesticides at different periods of incubation, and signs of embryotoxicity were detected at necropsy, but teratogenicity was not proven (Budai et al., 2002; Varga et al., 1999).

The interaction avian teratology test results also confirmed the pheasant embryo's increased sensitivity to the toxic effects of pesticides applied together, which may exceed the consequences of individual exposure. Furthermore, in parallel with the opinion of other authors, it can be stated that the interaction avian teratology studies indicate with appropriate sensitivity the unique toxic effects that are modified as a result of the joint exposure of different xenobiotics (Varga et al., 1999; Juhász et al., 2006).

There are differences in sensitivity between different species of wild birds to various chemicals (Kertész, 2001). The larger pore volume and specific surface area of some bird eggs can increase the exposure. Compared to the species of the *Phasianidae* family, including the pheasant, the species belonging to the *Anatidae* family (e.g. mallard duck) are more sensitive to spirotetramat (Maus, 2008).

The scientific and literary sources are convincing that the bird embryo can be used well and efficiently in first-line embryotoxicity, as it reacts with great sensitivity to the damaging effects of various physical and chemical agents affecting the body. The morphological and functional changes in the embryogenesis of birds show similarities with the embryonic development of mammals from many points of view, providing an opportunity for extrapolations (Pan and Fouts, 1978; Hill and Hoffman, 1984; Major et al., 2022).

5. Conclusions

Based on our experimental data, an additive effect was observed at the embryonic mortality due to the simultaneous injection treatment of Movento and Systhane 20 EW.

Besides the injection treatment method, it would be advisable to perform complete examinations with immersing treatments to model expositional circumstances during the plant protection practice.

In addition, the experiment could be supplemented with pathological processing, during which it is also possible to evaluate and examine histological samples from the blood and different organs (e.g., the liver). Moreover, the examination could also be supplemented with skeletal staining, revealing possible developmental abnormalities in the skeletal system. Also, using groups with a more significant number of elements would be recommended during the test replication.

The experiments that model the practical use more efficiently can provide a more accurate picture of the effects of chemical stresses on the environment. All information obtained can help to protect nature and wildlife in the future.

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Dose-Dependent Embryotoxicity and Teratogenicity Study of Prochloraz on Chicken Embryos

A prokloráz hatóanyagú Faxer fungicid dózisfüggő embriótoxicitási és teratogenitási vizsgálata házityúk-embriókon

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Abstract: The aim of the present study was to determine the dose-dependent embryotoxic and possible teratogenic effects of Faxer fungicide (450 g/l prochloraz) on the development of chicken embryos. The pesticide was applied at a practical spray concentration (3.33 µl/ml), and at twofold (6.66 µl/ml) and fivefold (16.65 µl/ml) doses. Emulsions of the test material were injected in 0.1 ml volume into the air chamber of the chicken eggs before starting the incubation. The chicken embryos were examined on day 19 by the followings: rate of embryo mortality, body weight, prevalence and type of developmental anomalies by macroscopic examination. The body weight of the live embryos was evaluated statistically by one-way ANOVA, the embryo mortality and the developmental anomalies were analysed by Fisher's exact test. Based on the results, we found that the embryotoxic effect increased with higher concentrations. In the fivefold dose (16.65 µl/ml), the average body weight of the chicken embryos was significantly lower ($p<0.05$) than that of the control group. The highest concentration of prochloraz-containing fungicide significantly increased ($p<0.001$) the mortality of chicken embryos compared to the control. Treatment induced growth retardation and embryonic minor structural anomalies were sporadic. No teratogenic effect was confirmed even in the fivefold dose.

Keywords*: prochloraz; chicken embryo; dose-dependent embryotoxicity; teratogenicity; injection*

Összefoglalás: Vizsgálatunkban a Faxer (450 g/l prokloráz) gombaölő permetezőszer házityúk-embrió tesztszervezetre gyakorolt embriókárosító hatását tanulmányoztuk. A növényvédő szer engedélyokiratában rögzített legmagasabb koncentráció (3,33 µl/ml) mellett, a túldozírozás által kiváltott méreghatás megítélése céljából a gyakorlati permetlé-töménység kétszeres (6,66 µl/ml) és ötszörös (16,65 µl/ml) dózisa is beállításra került. A keltetés megkezdése előtt a fungicid három koncentrációjából készült emulziókat 0,1 ml végtérfogatban a tyúktojások légkamrájába injektáltuk. Az inkubáció 19. napján a tojások feltárását követően rögzítettük az embriómortalitás alakulását, valamint a fejlődési rendellenességek előfordulási gyakoriságát és típusát, illetve lemértük és feljegyeztük a testtömegüket. Az embriómortalitási adatok és a morfológiai elváltozások biometriai értékelését Fisher-féle egzakt teszttel, míg a testtömeg

adatok statisztikai vizsgálatát egytényezős varianciaanalízissel hajtottuk végre. A prokloráz hatóanyagú gombaölő szer gyakorlati permetlé-töménységben (3,33 µl/ml), továbbá annak kétszeres dózisában (6,66 µl/ml) fokozta ugyan az embrióelhalást és a malformációk előfordulási gyakoriságát, valamint csökkentek az élő házityúk-embriók testtömegei a kontroll csoporthoz képest, de statisztikailag igazolható eltérés a vizsgált paraméterekben nem volt megállapítható. Az eredmények alapján feltételezhető a dózisfüggő embriótoxikus hatás, mivel az ötszörös dózisban (16,65 µl/ml) a fungicid szignifikáns mértékű embriómortalitás-emelkedést ($p<0,001$) és embrionális testtömegcsökkenést ($p<0,05$) indukált a kontrollhoz viszonyítva. Teratogén hatás nem volt igazolható, mert fejlődési rendellenesség - a legmagasabb dózis esetében is - csak sporadikusan jelentkezett, végtagdeformitás, nyitott mellkas és hasüreg formájában.

***Kulcsszavak**: prokloráz; házityúk-embrió; dózisfüggő méreghatás; teratogenitás; injektálás*

1. Introduction

Prochloraz is an imidazole fungicide widely used worldwide in agriculture and horticulture against phytopathogenic microorganisms. Like triazoles, imidazoles are used in veterinary and human medicine to treat fungal infections. Inhibits the enzyme lanosterol 14α-demethylase (CYP51A1), which is involved in the biosynthesis of ergosterol. The end result is damage to the fungal cell membrane and destruction of the fungal cells (Haselman et al., 2018; Heise et al., 2018).

Several toxicological studies have shown that prochloraz is an endocrine disruptor and can damage the reproductive system. Prochloraz has multiple mechanisms of action *in vitro*, including inhibition of the activity of other cytochrome P450 (CYPs) enzymes that catalyse crucial biochemical processes in higher organisms. It may affect aromatase (CYP19A1), a key enzyme in steroidogenesis, and thus interfere with the biosynthesis of estrogens. In addition, as an agonist of the aryl hydrocarbon receptor (AhR), it may also affect the expression of genes encoding cytochrome P450 enzymes, immunity regulation and cell differentiation. Screening tests have shown that prochloraz antagonises the androgen and estrogen receptors, and *in vivo* has been found to be antiandrogen in the Hershberger bioassay (in rats), as evidenced by a reduction in reproductive organs weight, an effect on androgen-regulated gene expression in the prostate and an increase in luteinizing hormone (LH) levels (Vinggaard et al., 2002; Stein et al., 2014).

In developmental biology and reproductive toxicity studies, prochloraz was teratogenic to zebrafish embryos (*Danio rerio*) as well as caused morphological and functional damage to the reproductive organs of adult fish. Furthermore, prochloraz may negatively affect the reproductive capacity of western honey bees (*Apis mellifera*) and thus the probability of population survival (Baumann et al., 2015; Glavinic et al., 2019).

The different agricultural areas offer sources of food, shelter and breeding places to wild birds (common pheasant, for example), therefore the sprayed pesticides can contaminate not only the adults, but the embryos developing in egg, as well. Their toxic effects can result in embryonic lethality and developmental abnormalities (Fejes et al., 2002; Várnagy et al., 2003; Szabó et al., 2020; Juhász et al., 2005). The aim of our study was to examine the dose-dependent embryotoxic and possible teratogenic effects of prochloraz-containing fungicide (Faxer) on the development of chicken embryos (*Gallus gallus domesticus*) after administration by injection technique. The relationship between dose and toxicity of pesticides and other xenobiotics is poorly understood in avian embryo (Szabó et al., 2003; Keserű et al., 2004).

2. Materials and Methods

One hundred and sixty, mixed-use Farm color hen eggs (Goldavis Ltd., Hungary) with good fertility were used in the experiment. They were randomised into four groups (40 eggs/group) based on their size and weight. The eggs were incubated in a Ragus type table incubator (Vienna, Austria), applying adequate temperature (37–38°C), relative humidity (65–70%) and daily rotation of the eggs during the incubation (Szabó et al., 2022). The prochloraz-containing fungicide (Faxer, Belchim Crop Protection Ltd., Hungary) was applied at a practical spray concentration (3.33 µl/ml), and at twofold (6.66 µl/ml) and fivefold (16.65 µl/ml) doses.

The experimental protocol of the study was approved by the local Committee of Animal Welfare at Hungarian University of Agriculture and Life Sciences, Georgikon Campus.

Emulsions of the test material were injected in a volume of 0.1 ml directly into the air chamber of the eggs with a micropipette on day 0 of incubation, and the hole was closed with paraffin after the treatment. Control eggs were treated with avian physiological saline solution (0.75% sodium chloride).

The eggs and the embryos were processed by necropsy on day 19 of incubation, and the following parameters were monitored for evaluation: mortality, body weight, and developmental abnormalities of the embryo (Juhász et al., 2006; Lehel et al., 2021).

Statistical analysis of the body weight of live embryos was performed by one-way analysis of variance (ANOVA). The mortalities and the developmental anomalies of embryos were analysed statistically using Fisher's exact test. Statistical analysis of the data was performed using R statistical software.

3. Results

The mortality and the developmental abnormalities are summarised in Table 1, and the body weight of the live embryos in the groups are shown in Figure 1. The embryonic mortality rate was 2.50%, and no embryos showing developmental anomalies were found in the control group (0.00%). The average body weight of the embryos was 24.30±1.44 g in the control group. Treatment with Faxer fungicide at practical spray concentrations resulted in an increase in embryo mortality rate to 12.50%. Developmental abnormalities were detected in two living embryos (5.71%). The differences were not significant. The treatment did not cause statistically significant change in the body weight (23.93±1.38 g). When prochloraz fungicide was applied at a dose of 6.66 µl/ml, the rate of embryonic mortality was increased to 15.00%, and two embryos shows teratogenic malformation (5.88%). The average body weight of the embryo was 23.86±0.85 g in this group. In the fivefold dose, the average body weight of the chicken embryos was significantly lower (23.51±1.24 g, $p<0.05$) than that of the control group. The highest concentration of prochloraz-containing fungicide significantly increased (30.00%, $p<0.001$) the mortality of the chicken embryos compared to the control, but the incidence of developmental anomalies was sporadic (10.71%). The types of the developmental anomalies were open abdomen and thoracic cavity and leg deformities in all cases.

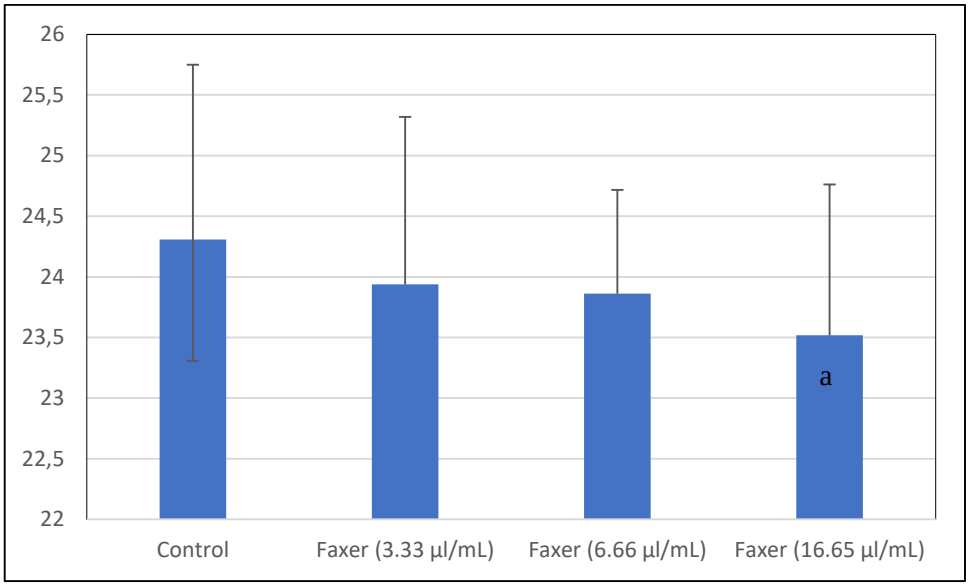

Figure 1. *Body weight of live embryos (average ± SD; g) with increasing concentrations*

[a] *Significant decrease as compared to the control data ($p<0.05$)*

Table 1. Mortality and developmental anomalies of embryos with increasing concentration.

Treatment	No. of embryos showing abnormality/No. of live embryos	No. of deaths/Total eggs	Rate of developmental anomalies (%)	Mortality (%)
Control	0/39	1/40	0.00	2.50
Faxer (3.33 µl/mL)	2/35	5/40	5.71	12.50
Faxer (6.66 µl/mL)	2/34	6/40	5.88	15.00
Faxer (16.65 µl/mL)	3/28	12/40[a]	10.71	30.00

[a] *Significant decrease as compared to the control data ($p<0.001$)*

4. Discussion and Conclusions

The results of our study showed that the embryotoxic effect of the fungicide Faxer, containing prochloraz as active substance applied with increased concentration, was manifested in significant decrease of embryonic body weight, and that of higher embryonic mortality compared to the control group. The embryotoxicity was dose-dependent. However, no teratogenicity was observed at the concentrations tested (3.33 µl/ml; 6.66 µl/ml; 16.65 µl/ml).

Haselman et al. (2018) investigated the effects of chronic prochloraz exposure on *Xenopus laevis* amphibians over several life stages. Treatments were initiated in embryo at concentrations of 0; 6.7; 20; 60 and 180 µg/l. In the pathogenetic study, the hepatotoxic and nephrotoxic lesions detected by the researchers were positively correlated with increasing dose. Furthermore, the study confirmed the antiandrogenic and endocrine disrupting effects of prochloraz with involvement of the hypothalamic-pituitary-gonadal (HPG), thyroid (HPT) and adrenocortical (HPA) axes.

Domingues et al. (2013) studied differences in sensitivity between zebrafish embryos (*Danio rerio*) and adult zebrafish to prochloraz concentrations between 4.6 and 8.5 mg/l after 96 hours of exposure. They conclude that the early life stage is more sensitive to sublethal dose than the adult stage. In their study, prochloraz was found to be teratogenic at medium concentrations. The sensitivity of zebrafish is therefore highly dependent on the developmental stage.

In our study on chicken embryos (*Gallus gallus domesticus*), the dose-dependent embryotoxicity of a prochloraz-containing fungicide induced fatal, significant embryo damage in the fivefold dose (16.65 µl/ml).

Our teratological research supports our thesis that the avian embryo is well suited for developmental biology studies because it is sensitive enough to indicate the damaging effects of different chemical agents (Várnagy et al., 1996; Varga et al., 1999; Budai et al., 2003).

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Investigation of Embryotoxic Interaction between Insecticide Pirimor 50 WG and Fungicide Score 250 EC on Chicken Embryos

A Pirimor 50 WG és a Score 250 EC egyedi és együttes embriótoxikus hatásának vizsgálata házityúk-embriókon

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Abstract: The aim of this study was to investigate the single and combined embryotoxic effects of Pirimor 50 WG insecticide (500 g/kg pirimicarb) and Score 250 EC fungicide (250 g/l difenoconazole) on the development of chicken embryos. The test materials were injected in 0.1 ml volume into the air chamber of the chicken eggs on the first day of the incubation and the embryos were examined on day 18 of the incubation. During the necropsy the followings were recorded: body weight, embryonic mortality and the type of developmental anomalies. Based on the results of the experiment, embryonic mortality increased in all treated groups compared to the control. Developmental anomalies were not recorded in the individually treated groups, while abnormalities occur in only one embryo in the combination treatment group. The average body weight of the embryos in the treated groups was lower than that of the control group, but the difference was only significant as a result of the combined treatment. The combined administration of the test materials increased the embryotoxicity compared to the individual toxicity tests that was presumably manifested as an additive toxic interaction.

Keywords*: interaction; embryotoxicity; chicken embryo; pirimicarb; difenoconazole*

Összefoglalás: Vizsgálatunkban a Pirimor 50 WG rovarölő szer (500 g/kg pirimikarb) és a Score 250 EC gombaölő szer (250 g/l difenokonazol) egyedi és együttes embriótoxikus hatását tanulmányoztuk a csirkeembriók fejlődésére. Az injektálásos kezelést az inkubáció első napján, a kórbonctani vizsgálatot az inkubáció 18. napján végeztük. A boncolás során a testtömeget, az embrionális mortalitást és a fejlődési rendellenességek típusát rögzítettük. A kísérlet eredményei alapján az embrionális mortalitás minden kezelt csoportban nőtt a kontrollhoz képest. Fejlődési rendellenesség nem fordult elő az egyedileg kezelt csoportokban, míg az együttesen kezelt csoportban egy embrió mutatott rendellenességeket. A kezelt csoportokban az embriók átlagos testtömege alacsonyabb volt, mint a kontroll csoporté, de a különbség csak az együttes kezeléssel összehasonlítva volt szignifikáns. A vizsgált készítmények együttes kezelése fokozta az embriótoxicitást, ami feltehetően additív toxikus interakcióként nyilvánult meg.

Kulcsszavak*: interakció, embriótoxicitás, házityúk-embrió, pirimikarb, difenokonazol*

1. Introduction

Currently, agriculture is facing a major challenge, the world's rapidly growing population must be supplied with food, while the size of the cultivated areas is decreasing. Chemical pesticides used in integrated pest management programs are currently the most effective tools in the fight against organisms that damage our cultivated plants. In addition to the benefits, there are many problems associated with the use of pesticides. Some of the problems are: pest resistance to pesticides, toxicity to non-target organisms and general environmental quality. Cultivated land provides source of food, shelter and nesting ground for wild birds. Pesticides sprayed during chemical plant protection procedures can affect not only adult wild birds, but also offspring and embryos developing in eggs (Várnagy et al., 2003; Szabó et al., 2020). In teratological studies, individual treatments are usually used, however, different pesticides can be used simultaneously or consecutively within a short period of time on the cultivated fields in plant protection practice (Várnagy et al., 1996; Varga et al., 1999). The effects of various chemical compounds in the environment (adverse effects, after-effects) must be monitored (Budai et al., 2003; Juhász et al., 2005; Szabó et al. 2020), because the chemical exposure usually occurs in a complex way, so we can count on joint toxic effects (Fejes et. al, 2002; Juhász et al., 2006).

Pirimicarb, as the active ingredient of Pirimor 50 WG insecticide, has a selective action against aphids, and it is used in vegetable, cereal and orchard crops (Cabras et al., 1995). Carbamate insecticides are acetylcholinesterase enzyme inhibitors, similar to organophosphate pesticides (Rosman et al., 2009). Pirimicarb is moderately toxic following acute administration to various animal species (Hoffmann et al., 2008).

Difenoconazole is a broad-spectrum triazole fungicide with action of ergosterol biosynthesis inhibitor that is widely used to prevent and control fungal diseases of vegetables, fruits and cereal crops that act as (Liu et al., 2021). Difenoconazole resulted in slight acute toxicity in laboratory animals during oral, dermal and inhalation treatments, but it can cause liver tumors and increase the incidence of foetal mortality in utero at very high dose (Voiculescu et al., 2022).

The aim of our study was to examine the single and combined embryotoxic effects of a widely used, pirimicarb containing insecticide, Pirimor 50 WG, and a difenoconazole containing fungicide Score 250 EC on the development of chicken embryos. Since the test methods used in ecotoxicology are primarily aimed at the investigation of individual toxic effects, the data on the joint toxic effects of pesticides can be considered as highly important and gap filler, especially in relation to the bird organism.

2. Materials and Methods

For modelling the environmental pesticide load, the concentration of the pesticides used in the experiment corresponds to that usually applied in chemical plant protection. Both during individual and combined treatment, Pirimor 50 WG (500 g/kg pirimicarb) (ADAMA Hungary Ltd., Hungary) was applied in a concentration of 0.04%. The difenoconazole containing fungicide Score 250 EC (250 g/l difenoconazole) (Syngenta Hungary Ltd., Hungary) was administered as a 0.0625% emulsion. For the preparation of emulsions or suspensions as well as in the control treatments, tap water was used.

The experimental protocol of the study was approved by the local Committee of Animal Welfare at Hungarian University of Agriculture and Life Sciences, Georgikon Campus.

One hundred and forty, mixed-use Farm color hen eggs derived from the stock farm of Goldavis Ltd. (Sármellék, Hungary) with good fertility were used in our experiment. The chicken eggs were randomly divided into four homologous groups (35 eggs/group) based on their size and weight. The eggs were incubated in a Ragus type hatcher (Vienna, Austria).

During the incubation, the appropriate temperature (37–38°C), air humidity (55–65%) and the daily rotation of eggs were provided.

On day 0 of incubation 0.1 ml emulsion or suspension of test materials per egg was administered directly into the air space with a micropipette. Before the treatment the egg shell was bored through, then, after the injection, it was sealed with paraffin.

Pathological examination was carried out on day 18 of incubation and the following parameters were recorded: embryonic mortality, body weight, and macroscopic developmental abnormalities of the embryo.

Biometric analysis of the body weight of live embryos was made with one-way analysis of variance. The mortalities and the developmental abnormalities of embryos were analysed statistically using Fisher's exact test. Biometric analysis of the data was performed using R statistical software.

3. Results

The embryonic mortality rate was 8.57%, and no embryos showing developmental anomalies were found in the control group (Table 1, Table 2). The average body weight of the embryos was 21.96±2.39 g in the control group (Figure 1.). As a result of treatment with Pirimor 50 WG, the rate of embryonic mortality was increased to 48.57%. The difference was not significant (Table 1). No embryos showing developmental anomalies were detected in this group (Table 2). Treatment with Pirimor 50 WG did not cause a statistically significant change in the body weight (20.95±1.78 g) (Figure 1). On the effect of Score 250 EC the embryonic mortality increased to 31.43% (Table 1). The changes were not significantly different as compared to the control. There was no any embryo showing developmental anomalies in the group treated with Score 250 EC (Table 2). The single administration of Score 250 EC caused a reduction on the body weight of embryos (21.03±3.28 g) compared to the control (Figure 1.) Due to the combined treatment of Pirimor 50 WG and Score 250 EC, the rate of embryonic mortality was increased to 40% (Table 1), but the incidence of developmental anomalies was sporadic (4.76%) (Table 2). Types of developmental abnormalities were the followings: lack of eye, the shortening of the beak mandible, hernia of brain and malformation of feet. The combined administration of Pirimor 50 WG and Score 250 EC resulted in a significant reduction of the average body weight (19.78±3.11 g) as compared to the control (Figure 1.).

Table 1. The number and rate of dead embryos in the embryotoxicity test of single and joint toxic effect of Pirimor 50 WG and Score 250 EC on chicken embryos

Treated groups	Number of dead embryos/number of fertile eggs (pcs)	Rate of dead embryos (%)
Control	3/35	8.57
Score 250 EC	11/35	31.43
Pirimor 50 WG	17/35	48.57
Score 250 EC + Pirimor 50 WG	14/35	40.00

Table 2. The number and rate of malformed embryos in the embryotoxicity test of single and joint toxic effect of Pirimor 50 WG and Score 250 EC on chicken embryos

Treated groups	Number of malformed embryos/number of alive embryos (pcs)	Rate of malformed embryos (%)
Control	0/32	0.00
Score 250 EC	0/24	0.00
Pirimor 50 WG	0/18	0.00
Score 250 EC + Pirimor 50 WG	1/21	4.76

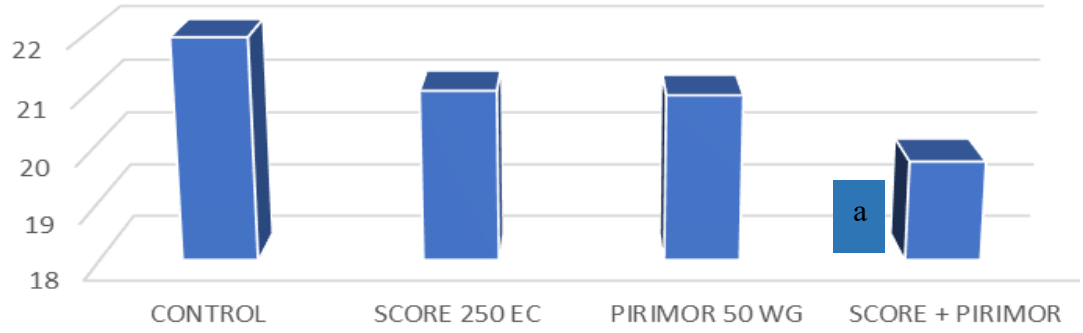

***Figure 1**. Data of embryonic body weights in the embryotoxicity test of single and joint toxic effect of Pirimor 50 WG and Score 250 EC on chicken embryos*

[a] Significant difference compared to the control group ($p<0.05$)

4. Discussion

In our experiment, individual treatments of a 0.04% suspension of Pirimor 50 WG insecticide and a 0.0625% emulsion of Score 250 EC fungicide proved to be embryotoxic, which was manifested in a non-significant decrease in embryonic body weight, and in higher embryonic mortality compared to the control group. The teratogenic effect of individual treatments of pesticides was not justified.

Keserű et al. (2004) studied the adverse effect of some pesticides (BI 58 EC, Flubalex and Dual Gold 960 EC) in chicken embryos after single administration by immersion and injection methods. They found that the body weight of embryos significantly decreased and the embryonic mortality increased markedly due to the single administration of organophosphate insecticides (BI 58 EC) with the same mechanism of action as carbamate insecticides.

Similarly, a study using immersion method of the eggs treated with 0.1% tebuconazole containing fungicide Mystic 250 EW together with single and combined administration of 0.01% lead acetate shown significant reduction in body weight and increased the rate of embryonic mortality in both of applied group (Szemerédy et al., 2018).

The combined administration Pirimor 50 WG insecticide at concentration of 0.04% and Score 250 EC fungicide at concentration of 0.0625% proved to be embryotoxic, which was manifested in a significant decrease in embryonic body weight, and in nonsignificant increase

in embryonic mortality compared to the control group. Since developmental anomalies only occurred sporadically, the teratogenic effect could not be verified.

Szabó et al. (2022) investigated the toxic effect of chlorpyrifos insecticide (Pyrinex 48 EC) and tebuconazole fungicide (Mystic 250 EW) on the development of chicken embryos, when applied them at a concentration corresponds to that usually applied in chemical plant protection. Summarising the findings, it can be established that the combined administration of Pyrinex 48 EC and Mystic 250 EW resulted in enhanced embryotoxicity, which was primarily manifested in an increased embryonic mortality rate and a significant reduction of the body weight.

5. Conclusions

Based on the results of avian teratological studies investigated with Pirimor 50 WG insecticide and Score 250 EC fungicide, the combined toxic effects of both pesticides increased the embryotoxicity, which represented in a significant decrease in body weights of embryos and in an increased embryonic mortality under the circumstances used in our experiment. Joint toxic effects depend on species, age, health status and exposure parameters (dose, duration, frequency) thus it is quite difficult to routinely predict expected effects in laboratory experiments (Thompson, 1996; Lehel et al., 2021).

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Plum-Pox Virus Incidence in Serbian Temerin Municipality

A szilvahimlő-vírus fertőzöttség mértéke a délvidéki Temerinben

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Abstract: During two years of research, incidence of *Plum pox virus* (PPV, sharka disease) on roadside and backyards *Prunus* trees was determined. Altogether 50 samples were analysed by DAS-ELISA technique. All 35 analysed plum trees proved to be infected (35/35), in case of myrobalan plum four samples were infected (11/4), apricot one (3/1) and one collected peach sample was healthy (1/0). These results confirm the widespread presence of *PPV* both on plants with clear symptoms, but also on symptomless *Prunus* species and underline the necessity of growing tolerant or hypersensitive cultivars.

Keywords*: sharka; Plum pox virus; Prunus; tolerant cultivars*

Összefoglalás: Kétéves kutatás során a szilvahimlő virus elterjedését vizsgáltuk a délvidéki Temerin községben. A levélmintákat az útszéli és kiskerti csonthéjasokról gyűjtöttük. Összesen 50 mintát vizsgáltunk DAS ELISA szerológiai módszerrel. Minden megvizsgált szilvaminta fertőzöttnek bizonyult (35/35), a ringló esetében négy minta fertőzött volt (11/4), a sárgabarack esetében a begyűjtött, illetve fertőzött növényminták aránya 3/1, míg az egyetlen őszibarack minta egészségesnek bizonyult. Az eredmények a vírus széleskörű jelenlétét igazolják, ami szükségessé teszi a toleráns fajták termesztésének szükségességét.

Kulcsszavak*: szilvahimlő; Plum pox virus; Prunus; toleráns fajták*

1. Introduction

Plum pox virus is one of the most important member of genus *Potyvirus*. The main host plant is the plum (Barba et al., 2011), but it can infect other grown stone fruit species (apricot, peach, almond, sweet cherry, sour cherry), ornamental plants or wild *Prunus* species grown spontaneously beside roads or parks (myrobalan plum, blackthorn, etc.). According to Jevremović and Paunović (2014) the infection level by sharka disease in some Serbian orchards can reach 80%. Emergence of the virus in Serbia caused economically important losses and reduced the plum production, since the very popular autohtone plum cultivar Požegača is extremely susceptible to virus infection. In 18 century plum was very important export merchandise and contributed to state economy (Demeter, 2016). The anisometric shaped virus is transmitted mechanically and by number of aphid species on non-persistent manner (Levy et al., 2000). Since the transmission time is very short, the infection of healthy trees happens even if the tree was previously treated by insecticides. Another important way of virus spreading is the infected propagation material. Also an important way of virus spreading is grafting, so the

propagation material becomes infected if the rootstock or scion is infected. There are about 10 different virus strains, among them very important are PPV-D (Dideron), PPV-M (Marcus) and PPV-Rec (recombinant) (James et al., 2013). Strains can be differentiated by molecular tools, but from practical standpoint it is important that the strains differ in their pathogenicity toward different stone-fruit species, the symptoms on host plants can also be different and the transmission efficiency by different aphid species (Serçe et al., 2009).

2. Materials and Methods

Leaf sample collection from roadside or backyard *Prunus* species was carried out in 2021 (twenty sample) and 2023 (thirty). During the first year of research the samples were taken from plum (five samples), apricot (three samples), myrobalan plum (11 samples) and peach (1 sample). In the second year of research we were focused on roadside or backyard grown plums, so all thirty samples originated from plum trees. During sample collection the tree`s GPS position was determined and the samples coded. All samples were kept on +4 ºC-on until the serological analysis.

DAS ELISA kit produced by Loewe Biochemica GmbH was used to analyse the samples. After the measurement of 0,25 g of leaf tissue and homogenisation in the extraction puffer the standard DAS ELISA protocol was followed (Clark and Adams, 1977).

After incubation of leaf tissue with specific antibody and conjugated specific antibody the positive samples were detected by BioTek Epoch spectrophotometer. The values were compared to positive and negative controls. All samples with spectrophotometric value above double value of negative controls average were considered as positive.

3. Results and Discussion

On the basis of the results of the serological analysis high *Plum pox virus* incidence can be determined in Temerin locality: during first year of investigation all the analysed plum trees were infected (5/5), in case of apricot one tree (3/1), myrobalan plum four (11/4) were infected, and the only one peach sample was virus-free. During the second year of research all collected plum samples from Temerin roadside or backyard proved to be infected (30/30).

On infected plum trees typical ringspots and mosaic symptoms can be observed (Figure 1 and Figure 2). The symptoms depend from plum cultivar, virus strain and ecological factors too. Fruits from infected trees regularly have less sugar content and other fruit quality parameters are reduced (Kensaku et al., 2020).

Figure 1. *Typical ringspots on plum leaves caused by Plum pox virus*

Figure 2. *Mosaic symptoms of Plum pox virus infected plum leaves*

While in case of infected plum trees the symptoms can be easily discovered in case of myrobalan plum even the symptomless trees proved to be infected. These findings can be very important from the standpoint of virus (sharka) disease control, since in the case of eradication measures it is not enough to destroy the trees with visible symptoms. It is necessary to test all *Prunus* species and destroy all sources of inoculum. Wild or ornamental *Prunus* species often can be the host of virus and serve as virus inoculum source.

This kind of high *Plum pox virus* incidence is not typical just for the Temerin region, but also for regions of the province Vojvodina (Bagi et al., 2021). From practical standpoint it means that the old, autochtone, but very virus susceptible cultivars like Pozegaca can not be grown, but also it means that even in the case of planting virus –tested or virus free planting material, the newly planted orchard after few years became infected, because of broad existence of virus source.

Non-persistent or stylet-borne manner of virus transmission and possibility that different aphid species are capable of virus transmission are facts which makes control measures, (like use of insecticides) uneffective. Basic control measures, like planting a virus tested plant material is still essential, but because of the broad presence of infected *Prunus* species is not a long term solution. There are a number of examples in plant pathology history that eradication measures on some regions, even countries can be effective. In Brasil more than 4 million citrus trees were destroyed to prevent losses from CTV (Citrus tristeza vírus) (Fadel et al., 2018). Similar measures were effective against PPV in Puglia region in Italy (Boscia, pers. com.).

Canada spent 125 million dollars from 2001 for destroying the PPV infected trees and producers compensations (Wang et al., 2006). In Serbia eradication measures were taken in the municipality of Lazarevac.

Without possibility of producer compensation on state or region level after eradication, and in the case of widespread presence of virus infection, the solution can rely just on plant breeding. Since the virus resistance is multigenic (Levy et al., 2000), the traditional breeding programs worked on breeding of tolerant or hypersensitive cultivars (Neumüller et al., 2010). Tolerant stone fruit cultivars secures relatively stable and quality yield even after *Plum pox virus* infection. Hypersensitive cultivars are reacting by necrosis of infected tissue and in this way prevents the spreading of virus to uninfected plant parts. Planting of tolerant cultivars is in some way a coexistence with the virus, since the tolerant, infected trees are also a further source of infection. In some countries by tools of molecular genetics, the genes which are coding the virus coat protein are inserted in the plant genome. In that way transgenic plants became resistant to plant virus (Malinowski et al., 2006).

5. Conclusions

In the Province of Vojvodina every condition for plum pox virus outbreaks exists, since virus and the susceptible hosts are widespread, and the climate is favourable for vector transmission. Among plant protection measures growing of tolerant stone fruit cultivars and planting of virus tested plant material can moderate the economic losses.

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Infection of *'Candidatus* Phytoplasma Vitis' in a Hungarian Vineyard East of the Danube

'Candidatus Phytoplasma vitis' fertőzése egy Dunától keletre fekvő hazai szőlőültetvényben

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Abstract: In Hungary, two phytoplasma species are known to infect grapevines, the *'Candidatus* Phytoplasma vitis' and the *'Ca*. Phytoplasma solani'. The *'Ca.* Phytoplasma vitis' is one of Europe's economically most important grapevine pathogens, as its infection can cause significant yield losses and even the death of infected vines. The *'Ca*. Phytoplasma vitis' was first described in Hungary in 2013, and since then, it has been reported from grapevines and traveller's joys in several transdanubian counties, while east of the Danube, it has only been identified in a few cases in grapevines. In the summer of 2023, leaf yellows and rapid death of vine stocks were observed in Csongrád. After total nucleic acid extraction, nested PCR tests were carried out using universal phytoplasma primers (P1/P7 and fU5/rU3). All tested samples were positive for phytoplasma infection. Based on the results of the *in silico* RFLP analysis using *Tru*9I restriction endonuclease, the infection of '*Ca*. Phytoplasma vitis' were determined, which pathogen has not yet been reported from grapevines in Csongrád-Csanád County.

Keywords*: Vitis; grapevine; phytoplasma; PCR; Flavescence dorée*

Összefoglalás: Hazánkban szőlőn mind ez ideig két fitoplazma faj, a *'Candidatus* Phytoplasma vitis' és a *'Ca*. Phytoplasma solani' jelenlétét azonosították. A *'Ca*. Phytoplasma vitis' egyike a szőlő gazdaságilag legjelentősebb kórokozóinak. Fertőzése következtében a termés mennyisége és minősége jelentősen csökken, a fertőzött tőkék pedig akár néhány éven belül ki is pusztulhatnak. A *'Ca*. Phytoplasma vitis' hazai megjelenéséről elsőként 2013-ban számoltak be Zala vármegyéből, azóta a Dunántúl számos borvidékéről jelezték előfordulását szőlőről és erdei iszalagról, a Dunától keletre azonban szőlőről csupán néhány esetben azonosították. 2023 nyarán, egy csongrádi szőlőültetvényben, a tőkék levélzetének sárgulására és gyors tőkepusztulásra lettek figyelmesek. Össznukleinsav-kivonást követően, univerzális fitoplazma primerpárokat (P1/P7 and fU5/rU3) felhasználva, nested-PCR technikával teszteltük a minták fitoplazma-fertőzöttségét. Minden vizsgált minta fitoplazma fertőzöttnek bizonyult. A *Tru*9I restrikciós endonukleázzal végzett *in silico* RFLP vizsgálat eredményeképpen kapott hasítási mintázat alapján, a *'Ca*. Phytoplasma vitis' fajt azonosítottuk, mely kórokozó előfordulását Csongrád-Csanád vármegyéből, szőlőről eddig még nem jelezték.

Kulcsszavak*: Vitis; szőlő; fitoplazma; PCR; Flavescence dorée*

1. Introduction

Several phytoplasma species worldwide may cause the grapevine yellows (GY) disease. In Hungary, two phytoplasma species have been identified, causing leaf yellows on grapevine. The *'Ca.* Phytoplasma solani' (syn. *Stolbur phytoplasma*), the pathogen of Bois noir (BN) and the *'Ca.* Phytoplasma vitis' (syn. *Grapevine flavescence dorée phytoplasma*), causing Flavescens dorée (FD). The *'Ca*. Phytoplasma vitis', besides the bacterium *Xylella fastidiosa*, is currently considered the most dangerous grapevine pathogen as it can cause significant yield losses (up to 50%) and rapid death of grapevines (Benedek, 2014). *'Ca*. Phytoplasma vitis' is a quarantine pest in Europe. Its vector, the American grapevine leafhopper (*Scaphoideus titanus* Ball) - a pest that only feeds on grapevine species - can spread the pathogen rapidly (Mori et al., 2002).

The *'Ca*. Phytoplasma vitis' was first identified in Hungary in 2013, in Zala County, near the Slovenian border (Dancsházy and Szőnyegi, 2014; Kriston et al., 2013), and since then, it has been detected in many Transdanubian counties and wine districts, both in grapevines and traveller's joys (*Clematis vitalba*). In Hungary, east of the Danube, it was first reported in 2019 from a vineyard in the Hajós-Baja wine district. Since 2019, it has been spreading in vineyards in the east of the Danube as well (Kölber and Lázár, 2023), while previously, the pathogen was only identified from traveller's joys in this part of the country (Dancsházy, 2019; Tóth és Pableczki, 2015). According to Kölber and Lázár (2023), the occurrence of the pathogen in grapevines from the Szekszárd, Villány, Balatonfüred-Csopak, Nagy-Somló, Pannonhalma, Mátra, Eger, Tokaj and Csongrád wine districts is still not reported.

Phytoplasmas are devastating plant pathogens. Protection of our plants against them is complicated and is based on preventing infection. The most essential is producing and using healthy, phytoplasma-free propagating material. Other solutions are controlling the vectors that spread the pathogen and the destruction of infected vines during cultivation. Removing potential host plants in neglected vineyards is also crucial (Benedek, 2014; Ember et al., 2012).

The primary vector of the pathogen, the American grape leafhopper (*Scaphoideus titanus*), was first detected in Hungary in 2006 (Dér et al., 2007), and currently, it is widespread throughout the country (Orosz and Zsolnai, 2010; Szalárdi et al., 2014). The leafhopper feeds exclusively on grapes, primarily on cultivated species (*Vitis vinifera*) in Europe (Mori et al., 2002).

Different symptoms can appear on grapevines after the infection of *'Ca*. Phytoplasma vitis'. The leaves roll downward, forming a triangular shape. Its tissue becomes hard and brittle, and the colour turns yellow or red depending on the variety. The shoots become thin and rubbery. The lignification of the shoots is incomplete. During winter, they blacken and die. The fruit set is reduced. The berries become shrivelled and turn brown, and their sugar content decreases while the acidity increases compared to healthy grapes (OEPP/EPPO, 2007). The symptoms listed above are almost identical in the case of the infection of both phytoplasma species causing grapevine yellows in Hungary. Thus, visual identification is impossible. Molecular diagnostic methods are the only reliable way to determine the infecting phytoplasma species (Dancsházy and Kriston, 2012).

In July 2023, in a small family vineyard in Csongrád, leaf yellowing and rapid death of many vine stockes were noticed. These symptoms could be caused by phytoplasma infection. Therefore, the testing of the infection of grapevine samples was aimed at using the PCR technique.

2. Materials and Methods

In the summer of 2023, leafy grapevine shoots of vines of the 'Chardonnay' variety showing symptoms collected in a 4-year-old plantation in Csongrád (Csongrád wine district, Csongrád-Csanád County) were sent to the laboratory of the Plant Pathology Department of the Hungarian University of Agriculture and Life Sciences. Two-two approximately 20 cm long symptoms showing leafy shoots were collected from four randomly selected vines. The collected plant parts were stored at -70 ºC until use.

Total nucleic acid extraction was performed using a simplified CTAB method (Xu et al., 2004). 0.5 g of plant tissue was used per sample for total nucleic acid extraction. Extracts from the mixture of central veins and petioles and extracts from the phloem tissue of the shoots were prepared (Table 1). The nucleic acid extracts were visualised on a 1% TBE agarose gel containing a fluorescent dye and stored at -70 °C until further use.

Table 1. Grapevine samples

Vines	Collected plant parts	Plant parts used for total nucleic extraction	Samples
Sz1	leafy grapevine shoot	mixture of main vein + petiole	H-FD1
		phloem tissue	H-FD5
Sz2	leafy grapevine shoot	mixture of main vein + petiole	H-FD2
		phloem tissue	H-FD6
Sz3	leafy grapevine shoot	mixture of main vein + petiole	H-FD3
		phloem tissue	H-FD7
Sz4	leafy grapevine shoot	mixture of main vein + petiole	H-FD4

Nested PCR was carried out for the molecular detection. Universal phytoplasma primer pairs, P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) and fU5/rU3 (Lorenz et al., 1995) were used in the first and second PCR rounds for testing phytoplasma infection of all seven grapevine samples. The P1/P7 primer pair amplifies an approx. 1800 bp long DNA fragment that encompasses the 16S rRNA–IGS–5' 23S rRNA region, the fU5/rU3 primers amplify an approx. 880 bp long PCR product. Sterile distilled water was used as a negative control for all PCRs. The following parameters were set for the first PCR round: 94 °C for 5 min, for 35 cycles 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1.5 min, and finally 72 °C for 10 min. The following parameters were set for the second PCR round: 94 °C for 2 min, for 35 cycles 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. The PCR amplicons were visualised on 1% TBE agarose gel staining with fluorescent dye.

The PCR fragments were isolated from the 1% agarose gel with the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions, and two nucleotide sequences were determined (Biomi Kft., Gödöllő). The obtained sequences were aligned and joined using the CLC Sequence Viewer 8.0 program package and compared to the sequences available in the NCBI database using the BLAST program. An *in silico* RFLP analysis was also performed, mapping the cleavage sites of the *Tru*9I restriction endonuclease.

3. Results and Discussion

15-20% of the previously homogeneous, healthy-looking vines finished growing in the second half of the spring of 2023, and rapid grapevine deaths occurred in the vineyard in Csongrád. The leaves on the vines rolled downward and became light in colour (Figure 1). The set bunches dried up. Vines that showed the leaf rolling and yellowing symptoms but did not die developed stunted shoots that broke easily.

Figure 1. *Vines of 'Chardonnay' variety showing symptoms of grapevine yellows in Csongrád. (Photo: Merkely)*

Nested-PCR gave positive results for all seven tested samples prepared from the leafy shoots originating from four vines showing yellowing symptoms. In the cases of all samples, the approx. 880 bp long DNA fragments were amplified. Negative results were obtained in the control water samples (Figure 2).

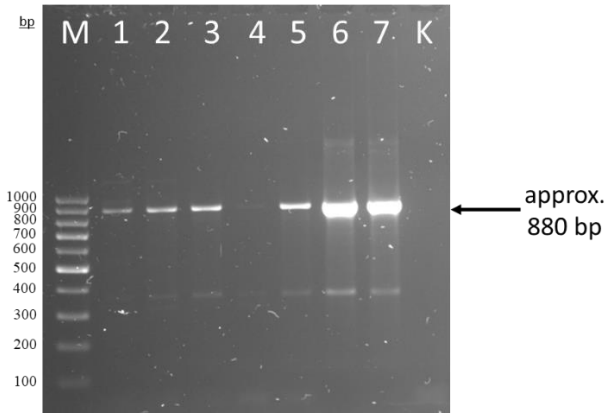

Figure 2. *Nested-PCR analysis of grapevine samples*

Legend: M: DNA marker (ThermoFischer Scientific GeneRuler 100 bp DNA Ladder), K: Negative control, 1: H-FD1, 2: H-FD2, 3: H-FD3, 4: H-FD4, 5: H-FD5, 6: H-FD6, 7: H-FD7 PCR products amplified with primers fU5 and rU3. The approx. 880 bp phytoplasma-specific PCR-fragment (arrow) shows the phytoplasma infection of the sample.

Sequence comparisons were made after determining the nucleotide sequences of two PCR products. When comparing the obtained nucleotide sequences (842 nt) with the nucleotide sequences of the corresponding region of isolates available in the international database, 100% similarity values were obtained with isolates belonging to different phytoplasma species thus both isolates (H-FD1, H-FD6) could be identified as a phytoplasma. H-FD1 and H-FD6 isolates

showed complete identity (100%) with phytoplasma sequences from the database and with each other at the nucleic acid level.

Phytoplasma species can be differentiated by restriction analysis of the PCR-amplified part of the 16S rDNA region. Based on the restriction pattern of this region, phytoplasmas are classified into different groups (Lee et al., 1993, 2000; Schneider et al., 1993; Seemüller et al., 1994), out of which *'Ca*. Phytoplasma vitis' belongs to the 16SrV (Elm yellows, EY; OEPP/EPPO, 2007), while *'Ca*. Phytoplasma solani' to the 16SrXII group (Stolbur, STOL; Quaglino, 2013). RFLP analysis of the PCR product amplified by the fU5/rU3 primer pair results in different cleavage patterns using *Tru*9I restriction endonuclease in the case of phytoplasma species belonging to 16SrV or 16SrXII groups (OEPP/EPPO, 2007). The cleavage sites of *Tru*9I were mapped on the nucleotide sequences of H-FD1 and H-FD6 isolates to determine either *'Ca*. Phytoplasma vitis' or *'Ca*. Phytoplasma solani' caused the leaf yellowing on grapevines in Csongrád. The restriction patterns obtained by the *in silico* RFLP tests were the same as the cleavage pattern typical to phytoplasma species belonging to the 16SrV group according to the data of the diagnostic protocol for the detection of '*Ca.* Phytoplasma vitis' published by the EPPO (OEPP/EPPO, 2007). Based on this result, it was concluded that the samples were infected with *'Ca*. Phytoplasma vitis' belonging to the 16SrV group. To our knowledge, this is the first report of the infection of *'Ca*. Phytoplasma vitis' on grapevines from Csongrád-Csanád County, Hungary.

The pathogen could have entered the vineyard in Csongrád, either with the propagating material used to establish the plantation or via its leafhopper vectors. It spreads over long distances, mainly with infected propagating material, but its vectors, especially the American grape leafhopper, also play a significant role at the local level. Although the size of the population of leafhopper species capable of transmitting the pathogen may change from year to year in a given area (Kutas, 2022), their presence and, thus, the possibility of the infection can not be excluded even though the most effective plant protection treatments, due to individuals flying in from far and potentially carrying the pathogen.

Unfortunately, the infected propagating material is not always recognisable. It is often symptomless, so that the pathogen can be spread unintentionally. Therefore, grapevine propagating material from infected countries must be imported only after careful phytosanitary control, and only certified, pathogen-free grafted plants are purchased from domestic nurseries.

Despite strict phytosanitary regulations, limiting the pathogen's spread is challenging. The Danube, previously referred to as a natural defence line, could not stop the pathogen's spread to wine districts east of the Danube. Viticulturists and home garden grapevine growers are responsible for overcoming the transmission and infection of *'Ca*. Phytoplasma vitis'.

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Naturally Occurring *Aspergillus* Species and their Mycotoxigenic Potential from Hungarian Sorghum (*Sorghum Bicolor* L. Moench) Kernels

Hazai szemes cirok (Sorghum bicolor L. Moench) szemtermésen megjelenő Aspergillus fajok és a mikotoxin-termeléssel összefüggésbe hozható génjeik vizsgálata

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Abstract: This study examined and identified the occurring *Aspergillus* spp. from sorghum (*Sorghum bicolor* L. Moench) kernels. We examined the internal infections of sorghum grains in PPA medium. During our experiments, we also used molecular methods to identify *Aspergillus* species and their genes, which promote aflatoxin production. This study identified two different *Aspergillus* species: *A. flavus* and *A. oryzae*. During identifying the genes, we detected that some of our A. flavus species could be aflatoxin-producing isolates.

Keywords*: aflatoxin; sorghum; aflatoxigenic genes; molecular identification; toxigenic fungi*

Összefoglalás: A szemes cirok (Sorghum bicolor L. Moench) termesztése az utóbbi két év aszályos és kiszámíthatatlan csapadékeloszlású termesztési évei miatt a kukorica alternatívájaként hazánkban is egyre jobban a termesztők érdeklődésének a középpontjába került. A cirok növényvédelméről azonban kevés az elérhető információ, a szemtermésen előforduló egészséget károsító penészgombákról pedig még korlátozottabbak az ismeretek, ami azért problémás, mert a szemtermés takarmány alapanyagként és élelmiszerként egyaránt hasznosul. A gabonanövények szemterméseinek mikotoxinokkal való szennyezettsége az utóbbi termesztési év tapasztalatai alapján nagy problémát okoz a gyakorlatban; az előforduló gombák nagy része polifág, ráadásul szaprofitaként is előfordul a talajban, rezisztenciával pedig nem rendelkeznek a köztermesztésben lévő fajták. A mikotoxin termelő penészgombák közül az Aspergillus fajok szubtrópusi eredetű kórokozók, azonban a fent említett hazai időjárásbeli változások közül is elsősorban az emelkedő hőségnapok számának növekedése ezen kórokozók előfordulásának is kedvez. Mivel magyarországi termőhelyről származó információkban a szakirodalom sem bővelkedik, ezért választottuk kísérletünk alapjának a hazai területekről származó szemes cirok mintákat. Kísérleteink során 30 szemes cirok minta Aspergillus fajok által okozott belső fertőzöttségét vizsgáltuk laboratóriumi körülmények között. Vizsgálataink során a belső fertőzöttségi vizsgálatokhoz PPA (Pentachloronitrobenzene Peptone Agar) táptalajt alkalmaztunk. A tiszta tenyészetek létrehozása után az izolált Aspergillus fajokat molekuláris genetikai úton azonosítottuk a kalmodulin (CaM) génre specifikus primer párral (cmd5/cmd6; CL1/CL2A). A kísérleteink során a Gallo és mtsai. (2012) és Degola és mtsai. (2007) által leírt aflatoxin termelésben szerepet játszó géneket azonosítottuk, amely alapján

megállapítottuk, hogy az általunk azonosított A. flavus izolátumok némelyike lehetséges aflatoxin termelő.

***Kulcsszavak**: aflatoxin; szemes cirok; aflatoxin termeléssel összefüggésbe hozható gének; molekuláris genetikai vizsgálatok; mikotoxin-termelő gombák*

1. Introduction

Sorghum (Sorghum bicolor L. Moench) is the main staple crop in developing countries, used for both animal and human consumption; in the ranking, it is the fifth most harvested crop worldwide (FAOSTAT 2023). Due to its resistance to drought, efficient use of water, and heat tolerance, sorghum will become an essential plant for human nutrition in the near future; many studies in the literature mention it as the grain of the future (Chhikara et al. 2019; Meena et al. 2022; Safian et al. 2022; Bakari et al., 2023). In addition, regular consumption of sorghum-based products has many health benefits due to its strong antioxidant effect. It can lower cholesterol, have anti-inflammatory and anti-cancer properties, and reduce obesity, type II diabetes, and cardiovascular disease (Xiong et al. 2019). The genus Aspergillus includes a diverse group of species based on morphological, physiological, and phylogenetic traits that have significant implications for biotechnology, food production, indoor environments, and human health. Among them, fungal species belonging to the Aspergillus Flavi section are important from the point of view of agriculture, biotechnology, the food industry, and human and animal health (Frisvad et al. 2018).

Degola et al. (2007) used five primer pairs to specifically amplify the AflD, AflR, AflS, AflO, and AflQ genes associated with aflatoxin production in *Aspergillus* species. The obtained results supported the generally accepted paradigm, according to which the expression of the AflR gene is necessary for the large-scale production of the toxin. The regulatory role of the AflR and AflS genes in regulating the expression of structural genes has already been proven. As expected, the observed structural gene expression follows AflR and AflS transcription. Although AflD, AflO, and AflQ encode enzymes of the aflatoxin biosynthesis pathway, they did not experience the advantages of their use in the laboratory compared to other genes of the aflatoxin cluster. Gallo et al. (2012) also identified the two regulatory genes, AflR and AflS, and the structural genes, AflD, AflM, AflO, AflP, and AflQ. Primer pairs targeting these genes were used in the study of genes related to aflatoxin production. The correlations between the presence of the mentioned genes and the aflatoxin production of the groups were then observed in different groups. They found that only those strains that lacked the presence of three or more of the observed genes were unable to produce aflatoxin. In contrast, all of the aflatoxin-producing isolates showed the complete gene set. Thus, the lack of amplification of aflatoxin biosynthetic genes was consistently associated with the non-aflatoxigenic nature of the strains. Also, in their case, AflO, AflP, and AflQ were amplified genes in a large number of non-aflatoxin isolates.

2. Materials and Methods

2.1. Materials

The *Aspergillus* species were isolated from sorghum kernels, which were collected in several parts of Hungary. During the molecular identification, we identified 21 isolates and two different species. The isolates were: INVT_A001; INVT_A002, INVT_A003, INVT_A004, INVT_A005; INVT_A006, INVT_A007, INVT_A008, INVT_A009, INVT_A010,

INVT_A011, INVT_A012, INVT_A013, INVT_A014, INVT_A015, INVT_A016, INVT_A017, INVT_A018, INVT_A019, INVT_A020, INVT_A021, INVT_A022, INVT_A023. For the molecular identification of the promoting genes of aflatoxin production, these isolates have been used.

2.2. Molecular identifications

For the molecular genetic studies, we isolated the *Aspergillus* cultures into 90-mm-diameter petri dishes on PDA medium. For DNA extraction, we used the ZR Fungal/Bacterial DNA MiniPrep kit, following the manufacturer's instructions. DNA has been taken from fresh fungal mycelia, taking care not to contaminate the process. During the molecular genetic identifications, in the case of belonging to the genera *Aspergillus,* standard polymerase chain reaction (PCR) was used to amplify the CaM (calmodulin) gene region, CL1 (forward primer; 5'-GA(GA)T(AT)CAAGGAGGCCTTCTC-3') and CL2A (reverse primer; 5'-TTTTTGCATCATGAGTTGGAC-3') (O'Donnell et al. 2000). The Pro FlexTM thermocycler (Applied BiosystemsTM Singapore) was used for amplification with the conditions described by O'Donnell et al. 2000. The CaM PCR product was used as a template for DNA sequencing by Macrogen Europe (Macrogen Europe BV, Amsterdam). Sequences were compared using the US National Center for Biotechnology Information (NCBI) database, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. In addition, the sequences were also uploaded and validated in the NCBI Genebank database. During the PCR tests, we examined the presence of seven genes associated with mycotoxin production using the standard PCR method described by Degola et al. (2007) and Gallo et al. (2012). These primers were specific primers for AflR (forward: 5'-AAGCTCCGGGATAGCTGTA-3'). reverse: 5'-AGGCCACTAAACCCGAGTA-3'), AflS (forward: 5'-TGAATCCGTACCCTTTGAGG-3'; reverse: 5'-GGAATGGGATGGAGATGAGA-3'), AflD (forward: 5'-CACTTAGCCATCACGGTCA-3'; reverse: 5'- GAGTTGAGATCCATCCGTG-3'), AflM (forward: 5'-AAGTTAATGGCGGAGACG-3'; reverse: 5'- TCTACCTGCTCATCGGTGA-3'), AflP (forward: 5'-AGCCCCGAAGACCATAAAC-3'; reverse: 5'-CCGAATGTCATGCTCCATC-3'), AflO (forward: 5'- TCGTCCTTCCATCCTCTTG-3'; reverse: 5'-ATGTGAGTAGCATCGGCATTC-3'), AflQ (forward: 5'- TCGTCCTTCCATCCTCTTG-3'; reverse: 5'-ATGTGAGTAGCATCGGCATTC-3') genes, which genes play a role in the aflatoxin production of the fungus.

3. Results

3.1. Molecular identification of *Aspergillus* species

During the species-level identification of the members of the isolated *Aspergillus* genus, 23 pure cultures were created. After extracting the DNA of the 23 pure cultures, the PCR run was performed with the primer pair CL1/CL2A. The PCR products were prepared with the CL1/CL2A primer pair for the amplification of the DNAs. During the molecular genetic tests, 2 *A. oryzae* and 19 *A. flavus* species were identified.

3.2. Molecular identification of aflatoxigenic genes of the identified *Aspergillus* species

Gel electrophoresis was performed on the DNA of the 21 identified *Aspergillus* species with primer pairs of genes linked to aflatoxin production (AflD, AflM, AflO, AflP, AflQ, AflR, and AflS). During the experiment, the last two samples in the gel containing PCR products were the

positive and negative controls. In the case of the positive control, we used DNA samples from the collection of MATE, NVI, and the Department of Integrated Plant Protection. In the case of the negative control, the PCR product did not contain any DNA; molecular water was added to the PCR products instead. Table 1 contains the results obtained during gel electrophoresis for each gene-specific primer.

Table 1. Amplified target sequences of gene specific primers. In the table, positive results where the primer amplified the target sequence were marked with "+" and green, and "-" where the primer did not amplify the target sequence

N°	Code of isolate and DNA	Species	AflD	AflM	AflO	AflP	AflQ	AflR	AflS
1	INVT_A023	*Aspergillus flavus*	-	-	+	+	+	-	-
2	INVT_A022	*Aspergillus flavus*	-	-	-	-	-	-	-
3	INVT_A021	*Aspergillus flavus*	-	-	-	-	-	-	-
4	INVT_A020	*Aspergillus flavus*	+	+	+	+	-	+	+
5	INVT_A019	*Aspergillus flavus*	+	+	+	+	-	+	+
6	INVT_A018	*Aspergillus flavus*	-	-	+	-	-	-	-
7	INVT_A017	*Aspergillus flavus*	-	-	+	+	+	-	-
8	INVT_A016	*Aspergillus flavus*	-	-	+	-	-	-	-
9	INVT_A015	*Aspergillus flavus*	+	+	+	+	+	+	+
10	INVT_A014	*Aspergillus flavus*	+	+	+	+	+	+	-
11	INVT_A013	*Aspergillus flavus*	+	+	+	+	+	+	+
12	INVT_A012	*Aspergillus flavus*	+	+	+	-	+	+	+
13	INVT_A011	*Aspergillus flavus*	+	+	+	-	-	+	+
14	INVT_A010	*Aspergillus oryzae*	-	+	+	+	+	+	+
15	INVT_A009	*Aspergillus flavus*	+	+	+	+	-	+	+
16	INVT_A008	*Aspergillus oryzae*	+	+	-	-	-	+	+
17	INVT_A007	*Aspergillus flavus*	-	+	+	+	-	+	+
18	INVT_A006	*Aspergillus flavus*	+	+	+	-	-	+	+
19	INVT_A005	*Aspergillus flavus*	-	+	+	-	-	+	-
20	INVT_A004	*Aspergillus flavus*	+	+	+	-	-	+	+
21	INVT_A003	*Aspergillus flavus*	+	+	+	+	+	+	+
22	INVT_A002	*Aspergillus flavus*	+	+	+	+	+	+	+
23	INVT_A001	*Aspergillus flavus*	-	+	+	+	+	+	-

For samples INVT_A015 and INVT_A013, all primers were able to amplify the target sequence, which means that we got a positive result for all genes that help mycotoxin production. In the case of the INVT_A022 sample, we did not find the presence of a single gene. Some DNA from *Aspergillus flavus* and *A. oryzae* were put through gel electrophoresis.

The AflD gene was found at 852 base pairs on 12 of the pieces. The presence of the AflM gene was found at 470 base pairs in 15 *A. flavus* and 2 *A. oryzae* DNAs. The presence of the AflO gene was detected in 19 *A. flavus* and 1 *A. oryzae* cases. During gel electrophoresis, the bands appeared at 790 base pairs. In the case of the AflP gene, we found its presence at 870 bp in 12 *A. flavus* and 1 *A. oryzae*. The presence of the gene AflQ, which can be associated with aflatoxin production, was found in the DNA of 9 *A. flavus* and 1 *A. oryzae* of the isolated fungi. The presence of the AflR gene was detected during gel electrophoresis at 1079 bp, which occurred in 15 A. flavus and 2 A. oryzae samples. The presence of the AflS gene was detectable in 12 *A. flavus* and 2 *A. oryzae* cases at 684 base pairs.

4. Discussion

During the isolation of *Aspergillus* species, 23 pure cultures were created, from which 19 *A. flavus* and 2 *A. oryzae* were identified as molecular tools. All of the identified species belong to the section *Flavi*, which is extremely important from the point of view of agriculture, biotechnology, and human and animal health (Frisvad et al. 2018). *A. flavus* is a frequently occurring species of agricultural plant that is often isolated from many parts of the world, and due to their aflatoxin-producing ability, they pose a major threat during food and feed (Palencia et al. 2010; Riba et al. 2010). *A. oryzae* species have also been isolated in many cases, and due to their non-aflatoxin properties, the species is considered safe. Furthermore, the fermentation industry still uses the species for various fermentation processes (Chang and Ehrlich 2010). Species have also been identified in many cases under domestic conditions (Baranyi et al., 2015; Tóth et al., 2012; Dobolyi et al., 2013; Sebők et al., 2016).

In the experiments conducted by Degola et al. (2007) and Gallo et al. (2012), the association of different genes with aflatoxin production was investigated. In our experiments, based on their research, we searched for the presence of the most important genes from the point of view of aflatoxin production. In a 2012 experiment, Gallo and his colleagues discovered that non-aflatoxin isolates were capable of amplifying the AflQ gene in large numbers. The AflQ gene was amplified 10 times in the samples we examined (INVT_A023, INVT_A017, INVT_A015, INVT_A014, INVT_A013, INVT_A012, INVT_A010, INVT_A008, INVT_A002, INVT_A001), which suggests that the 10 species of *Aspergillus* are probably Gallo and others. In his 2012 research, it belongs to the group considered non-aflatoxin. However, since the presence or absence of a single gene does not clearly determine the mycotoxin production capacity, it is also important to evaluate the results of the other tested genes.

In the case of the AflR and AflS genes, the close correlation between the presence of the genes and their ability to produce aflatoxin has been demonstrated (Degola et al., 2007; Gallo et al., 2012). Thus, in the case of our own samples, the presence of these genes would make the production of aflatoxin probable. During our experiments, 14 samples (INVT_A020, INVT_A019, INVT_A015, INVT_A013, INVT_A012, INVT_A010, INVT_A011, INVT_A009, INVT_A008, INVT_A007, INVT_A006, INVT_A004, INVT_A003, INVT_A002) amplified the AflS gene. The AflR gene was detected in 17 samples (INVT_A001, INVT_A002, INVT_A003, INVT_A004, INVT_A005, INVT_A006, INVT_A007, INVT_A008, INVT_A009, INVT_A010, INVT_A011, INVT_A012, INVT_A013, INVT_A 014, INVT_A015, INVT_A019, INVT_A020). Degola et al.'s 2007 experiment indicated aflatoxin production capacity with high certainty in the presence of these genes. Therefore, in the case of the samples, it is likely that an *Aspergillus* species is capable of producing aflatoxin. In addition to all of this, it is important to highlight that the identification of the mentioned genes in the individual samples does not mean a clear aflatoxin production

capacity; it merely makes this capacity probable, since Gallo et al. (2012) also detected aflatoxin production only in the case of the combined presence of the tested genes during their 2012 experiment. During our experiments, we experienced the co-presence of the genes in the cases of samples INVT_A002, INVT_A003, INVT_A010, INVT_A013, INVT_A014, and INVT_A015.

5. Conclusions

Our data are first insights in the identification of *Aspergillus* species in sorghum grains in Hungary. Our results highlighted the appearance of *Aspergillus* species in sorghum grains and their capability to inhibit aflatoxin production. Reliable identification of *Aspergillus* species and defining their capacity for mycotoxin production are important for plant disease management and for food and feed safety. Our data can help in the mycotoxin regulation of cereals grown in small areas. Moreover, it could help sorghum breeders by handing over advanced knowledge on the variability of *Aspergillus* species.

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Colletotricum and *Fusarium* Spp. "Hitch -Hiking" on Imported Mango and Banana Fruits to Hungary

Colletotrichum *és* Fusarium *fajok jelentősége hazánkba importált mangó és banán gyümölcsökön*

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Abstract: Post-harvest losses caused by plant pathogens, both in general and during transport, are a major economic problem. Symptoms of fruit rot, caused by pathogens, are often observed in supermarkets. Importing fruits could be a pathway for pests and pathogens to enter a new region. We aimed to identify the pathogens introduced into Hungary through the fruit trade of bananas and mangoes. We confirmed the presence of *Colletotrichum* and *Fusarium* species in the fruits.

Keywords*: Colletotrichum; Fusarium; ITS*

Összefoglalás: A gyümölcsök nemzetközi kereskedelme megkönnyíti a növényi kórokozók és kártevők globális terjedését is. A boltok polcain gyakran találkozhatunk növénypatogének okozta tüneteket mutató mangó és banán gyümölcsökkel. Kutatásunk során a trópusokról importált mangó és banán gyümölcsökkel érkező *Colletotrichum* és *Fusarium* fajokat és azok jelentőségét vizsgáltuk.

Kulcsszavak*: Colletotrichum; Fusarium; ITS*

1. Introduction

Mangoes (*Mangifera indica* L.) and bananas (*Musa* × *paradisiaca*) are among the most popular fruits and are generally cultivated in tropical, subtropical and other regions and are distributed worldwide. Millions of tons of tropical fruits, including bananas and mangoes, are imported to the EU from more than 130 countries every year, which could become a potential pathway for plant pathogens (Suffert et al., 2018; Freshfel, 2021). Post-harvest losses caused by plant pathogens, both in general and during transport, are a huge economic problem. Fruits showing symptoms of fungal diseases are often observed in grocery stores. The aim of this study was to identify *Colletotrichum* and *Fusarium* species introduced into Hungary with the fruit trade.

2. Materials and Methods

For this study, tropical fruits (bananas and mangoes) showing symptoms of fungal diseases were selected from supermarkets and fruit merchants in Hungary. Isolates were obtained from infected fruit tissues placed into PDA medium. Macroscopic (mycelial colour, shape, edges and pattern of colonies) and microscopic (colour, shape and size of conidia) characteristics of the fungal isolates were recorded. Size and shape of 50 conidia per sample were characterized. Koch's postulates were fulfilled for all isolates. Genomic DNA was extracted from the growing margins of the colonies, using the cetyl-trimethyl-ammonium-bromide (CTAB) method. ITS1 and ITS4 primers were used to amplify the internal transcribed spacer (ITS) region. The partial beta-tubulin (TUB) gene was amplified using Bt1a and Bt1b primers. CAL1 and CAL2 primers were used to amplify a part of the calmodulin (CAL) gene. A part of the beta-tubulin (TUB) gene, the calmodulin (CAL) gene and the ITS region were amplified and sequenced Primers used in this study are listed in Table 1. Sequences were checked and edited according to the chromatogram and compared with the sequences stored in the NCBI database.

Table 1. The list of primers for amplification of internal transcribed spacer, the partial calmodulin gene and the partial beta-tubulin gene

Gene or DNA region	Primer	Primer sequence (5'-3')	References
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC	
calmodulin	CA_CAL1	TGAGTACAAGGAGGCCTT CTCCC	Glass és Donaldson 1995
	CA_CAL2	TTTGCATGAGTTGGACGAACTC	
beta-tubulin	Bt1a	TTCCCCCGTCTCCACTTCTTCATG	Glass és Donaldson 1995
	Bt1b	GACGAGATCGTTCATGTTGAACTC	

3. Results

3.1. Fungal pathogens isolated from banana fruits

Fusarium desaboruense

Symptoms on fruit: Spots on fruits were brown to back, sometimes sunken, 0.3-1 cm in size. Fruit tissues under the spots were mildly rotten.

Morphology and culture characteristics: Colony surface was cottony, white to pale violet. Colony reverse was white to pale violet and becoming violet pigmented over time. Microconidia were 6.7 × 2.6 µm in size, hyalin, ovoid, smooth and thin-walled with 1-2 septate. Macroconidia were 36.9 × 3.5 µm in size, hyalin, slightly curved with 3-4 septate. No chlamydospores were observed.

Molecular identification: BLAST analysis revealed that ITS and TUB gene had 99-100 % identity with the existing sequences of *Fusarium desaboruense.*

Fusarium oxysporum

Symptoms on fruit: Spots on fruits were brown to back, sometimes sunken, 0.3-1 cm in size. Fruit tissues under the spots were mildly rotten.

Morphology and culture characteristics: Colony surface was cottony, white to grey. Colony reverse was orange pigmented. Growth pattern of mycelia was in concentric rings. Microconidia were 2.95 × 7.21 µm in size, hyalin, ovoid, smooth and thin-walled with 0-1 septate. Macroconidia were 40.01 × 2.98 µm in size, hyalin, slightly curved with 2-3 septate. No chlamydospores were observed.

Molecular identification: Based on a BLAST analysis, ITS and TUB gene had 99.79-100% identity with the existing sequences of *Fusarium oxysporum.*

Colletotrichum musae

Symptoms on fruit: Spots on fruits were brown to back, sunken, 0.5-4 cm in size with salmon coloured spore masses and acervuli in the lesion.

Morphology and culture characteristics: Colony was cottony, white on the surface, pale orange to pale pink on the reverse side. The aerial mycelia were white then turned pale grey covered bright pinkish-orange conidial masses in the middle. Conidia were one-celled, 16.2 × 5.9 µm in size, hyaline, cylindrical in shape, with rounded ends.

Molecular identification: BLAST analysis revealed that ITS, TUB and CAL gene had 100% identity with the existing sequences of *Colletotrichum musae.*

3.2. Fungal pathogens isolated from mango fruits

Fusarium oxysporum

Symptoms on fruit: Spots on fruits were brown to back, sometimes sunken, 0.3-1 cm in size. Fruit tissues under the spots were mildly rotten.

Morphology and culture characteristics: Colony surface was cottony, white to grey. Colony reverse was orange pigmented. Growth pattern of mycelia was in concentric rings. Microconidia were 2.60 × 9.01 µm in size, hyalin, ovoid, smooth and thin-walled with 0-1 septate. Macroconidia were 38.7-3.02 µm in size, hyalin, slightly curved with 2-3 septate. No chlamydospores were observed.

Molecular identification: Based on a BLAST analysis, ITS and TUB gene had 99.79-100% identity with the existing sequences of *Fusarium oxysporum.*

Colletotrichum asianum

Symptoms on fruit: Brown to black rot appears at the stem end with orange coloured spore masses and acervuli in the lesion. Spots on fruits were brown to back, sunken, 0.3-1 cm in size sometimes also with spore masses and acervuli in the lesion.

Morphology and culture characteristics: Colony was cottony, white on the surface, pale orange with grey concentric rings and dark grey in the middle on the reverse side. The aerial mycelia were white with bright orange conidial mass in the middle. Morphological characteristics– Conidia were one-celled, 14.8 × 4.3µm in size, hyaline, cylindrical in shape, with rounded ends or with one acute end.

Molecular identification: Based on a BLAST analysis, ITS, TUB and CAL gene had 99.86-100% identity with the existing sequences of *Colletotrichum asianum.*

Colletotrichum fructicola

Symptoms on fruit: The symptoms were identical to those observed in *C. asianum*.

Morphology and culture characteristics: Colony was cottony, white on the surface, pale orange with grey concentric rings and dark grey in the middle on the reverse side. The aerial mycelia was white with bright orange conidial mass in the middle. Conida were one-celled, 14.3 × 4.1 µm in size, hyaline, cylindrical in shape, with rounded ends.

Molecular identification: BLAST analysis revealed that ITS, TUB and CAL gene had 99.79-100% identity with the existing sequences of *Colletotrichum frucicola.*

4. Discussion

The global trade of fruits facilitates the international movement of plant pathogens, with the introduction of new pathogens into a region potentially having economic consequences for local crop production.

In this study we reported the presence of *Colletotrichum asianum, C. fructicola,* on imported mango fruits and *Fusarium desaboruense* (*syn. F. sacchari*) on banana fruits for the first time in Hungary. *C. fructicola* is a polyphagous pathogen, member of the *C. gloeosporioides* complex causing anthracnose, bitter rot and leaf spot diseases of many cultivated plant species, including *Malus domestica, M. pumila, Prunus persica*, which are common fruit crops in Hungary (EFSA, 2021). *Fusarium desaboruense* was described in 2019 as a novel pathogen in the *Fusarium fujikuroi* species complex causing Panama disease in Indonesia (Maryani et al., 2019). Since, a study has shown that *F. desaboruense* should be considered synonymous with *F. sacchari* (Yilmaz et al. 2021). Beside sugarcane, *F. sacchari* infects *Solanaceae* crops, chilli peppers and strawberries (Saseetharan and Zakaria, 2014).

Infected fruit parts can end up in compost bins, providing an opportunity for pathogen establishment. With global warming, climatic factors will become more favourable for many pathogens, therefore more attention should be paid to newly introduced pathogens.

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CONTENT – TARTALOMJEGYZÉK


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