HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

Survey of the Potyvirus infection of bulbous and tuberous ornamental plants

Thesis of Ph.D. dissertation

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The applicant met the requirement of the Ph.D. regulations of the Hungarian University of Agriculture and Life Sciences and the thesis is accepted for the defense process.

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1 INTRODUCTION

Flower bulbs are herbaceous perennial plants, and are valuable as cut flowers and potted plants. They are utilized in parks and public plantings or in flower tubs on patios as part of the landscape architecture.

The vast majority of flower bulbs are propagated vegetatively, grown under open field conditions in monoculture for several years, which are all favorably increases their pathogen load, most importantly their viral load.

These plants are transported during their dormancy period, so the mosaic symptoms – which are indicative of viral infection – are not present. Symptoms appear only in vegetation, sometimes only for a short period of time. During this period vectors may carry the virus to other agricultural crops or weeds. Sometimes a virus does not cause any symptoms on their original host, but can cause serious losses on its new host.

Member species of the Genus *Potyvirus* cause significant losses in all branches of agriculture. In the last few years more and more candidate species have been described by scientists all over the world, thanks to the availability of Next Generation Sequencing techniques. In my thesis I would like to assess the potyviral load of Hungarian grown bulbous ornamental plants. My main goals are:

1. Assessment of potyvirus infection of tulips in private and community gardens and parks in Hungary. Identification of the causative agents of tulip breaking with serological and molecular methods. Distribution and cultivar preference of the viruses. Phylogenic and taxonomic relations of the pathogens, recombination detection of the isolates. Observation of the symptom development on infected plants. Inoculation of Darwin-hybrid ‘van Eijk’ bulbs with single and multiple viruses, observation of the symptoms.
2. Assessment of the viral load of calla plants used for cut flower production. Serological and molecular identification of the pathogen. Symptom observation through a growing cycle. Phylogenic relation of the Hungarian isolate to other isolates.
3. Assessment of the viral load of forced potted daffodils Serological and molecular identification of the pathogen. Symptom observation through a growing cycle. Phylogenic relation of the Hungarian isolates to other isolates.

4. Assessment of the viral load of a grape hyacinth producing nursery. Serological and molecular identification of the pathogen. Symptom observation through a growing cycle. Phylogenic relation of the Hungarian isolate to other isolates.
5. Assessment of the viral load of miscellaneous flower bulbs. Serological and molecular identification of the pathogen. Symptom observation through a growing cycle. Phylogenic relation of the Hungarian isolates to other isolates.

2 MATERIALS AND METHODS

Sample collection was carried out in 2014 in the Netherlands, and in 2017-2018 in Hungary. *Sternbergia lutea* (648), *Tulipa* Fringed 'Lambada' (704) and *Tulipa* Fringed 'Crystal Beauty' (705) samples were collected in the Buda Arboretum. Samples are 1-5 g of symptomatic leaf tissue. Samples were transported to the lab on ice of frozen (-20 °C), then stored at -70 °C until further processing.

All samples were first ELISA tested. Positive samples were then RT-PCR tested, simultaneously laboratory test plants were sap inoculated, and Koch's postulates were carried out where virus free hosts were available.

Infection of laboratory host plants and Koch's postulates were carried out by the methods described by HORVÁTH and GÁBORJÁNYI (1999). Transmission electron microscopy was carried out by PÁL VÁGI according to the method of HORVÁTH and GÁBORJÁNYI (1999). Recombination detection was carried out with RDP v4.97 (MARTIN *et al.*, 2015, 2017).

2.1 Serological assay

The potyvirus group specific ELISA kit – based on MAb PTY1 detection antibody (JORDAN and HAMMOND, 1991) – was supplied by Agdia. Tests were carried out on each sample in duplicates according to the manufacturer's instructions. The monoclonal detection antibody was raised in mouse against potyvirus coat protein, the enzyme conjugate was a polyclonal antibody to mouse IgG and was raised in rabbit. Negative control was prepared from *Chenopodium amaranticolor* seedlings grown in a vector free greenhouse. The final dilution of both the detection antibody and the enzyme conjugate were 1:100 (v:v). Plates were read at 405 nm wavelength on a Labsystems Multiskan MS ELISA reader after 15, 30, and 60 min incubation. A sample was considered positive if the absorbance of the sample was at least three times greater than that of the negative control.

2.2 Molecular identification

Total nucleic acid extraction was carried out by the method of WHITE and KAPER (1989). Extracted nucleic acids were stored at -70 °C until further processing.

First strand cDNA was synthesized at 10 µl end volume. Synthesis started by measuring out 2 µl total nucleic acid, 2 µl of sterile nuclease free H_2O, and 1 µl 100 mM poly T_2 reverse primer (5'-CGGGGATCCTCGAGAAGCTTTTTTTTTTTTTTTT-3') (SALAMON and PALKOVICS, 2005). The mixture was incubated at 65 °C for 5 minutes, then set on ice for 2 minutes. The protocol continued by measuring out 1 µl 10 mM dNTPs, 1 µl nuclease free H_2O, 2 µl 5× RT buffer (250 mM Tris-HCl (pH 8.3, 25 °C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT) (Thermo Scientific), 0,5 µl RiboLock RNase Inhibitor (Thermo Scientific), 0,5 µl RevertAid Reverse Transcriptase (Thermo Scientific), then continued with incubation at 42 °C for 1 hour, then set on ice for 2 minutes to stop the synthesis. cDNS was stored at -20 °C until further processing.

PCR reactions were carried out at 50 µl end volumes. The mixture contained: 2 µl cDNA, 5 µl 10× Taq buffer + KCl (100 mM Tris-HCl (pH 8.8, 25 °C), 500 mM KCl, 0,8% (v/v) Nonidet P40) (Thermo Scientific), 3 µl 25 mM $MgCl_2$, 2 µl 10 mM dNTPs, 1 µl poty7941 forward primer (5'-GGAATTCCCGCGGNAAYAAYAGYGGNCARCC-3') (SALAMON and PALKOVICS, 2005), 1 µl poly T_2 reverse primer, 1 µl *Taq* DNA Polymerase (recombinant) (Thermo Scientific), and 35 µl sterile nuclease free H_2O.

The PCR program started with 3 minutes of initial denaturation at 94 °C, the cycle started with 30 seconds of denaturation at 94 °C, followed by 30 seconds at 50 °C for anellation and ended with 2 minutes at 72 °C for elongation. The cycle was repeated 40 times, then followed by 10 minutes of 72 °C final elongation and cooling down to 4 °C.

PCR products were visualized on 1% TBE agarose gels containing ethidium-bromide.

Specific PCR products were excised from the gel and cleaned with High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions.

For the molecular identification I used the O strain of Potato virus Y (BASKY and ALMÁSI, 2005) as positive control, which was maintained on cecei type Hungarian wax pepper (*Capsicum annuum* 'Cecei').

2.2.1 Hybridization assay

Dot-blot hybridization of daffodil samples were carried out by KATALIN NEMES at the Plant Protection Institute, Centre for Agricultural Research. 100 ng (5 µl) of total nucleic acid was mixed with 5 µl denaturing buffer (5 µl formamid, 1 µl 200 mM HEPES, 1 µl 10 mM EDTA, 1,64 µl 35% formaldehyde, 0,36 µl H_2O, 1 µl 10× FDE stain), and incubated for 5 minutes at 65 °C then set on ice. Denatured samples were dripped directly onto Whatman Nytran N blotting membrane,

air dried, then cross-linked to the membrane with UV light. The hybridization was carried out as Northern hybridization (SAMBROOK and RUSSELL, 2001). For probe and positive control we used purified PCR products of NLSYV and HiMV. Probes were synthesized by using Digoxigenin-11-dUTP with Random Primed DNA Labelling Kit (Roche) according to the manufacturer's instructions. After the hybridization of the probe the membrane was washed then prepared for detection. Detection was carried out with 150 mU/ml Anti-DIG-AP antibodies – Anti-Digoxigenin IgG was raised in sheep and conjugated with alkaline-phosphatase – 100 mM malic acid, 150 mM NaCl (pH 7.5). Absorbance was measured with iBright CL 1000 at 405 nm after15 minutes of incubation after adding 1 mg/ml p-Nitrophenyl Phosphate (PNPP) solution.

2.2.2 Ligation, transformation, sequencing

Ligations were carried out at 10 µl end volume. The mixture contained: 3 µl purified PCR product, 1 µl 10× T4 DNA Ligase buffer (300 mM Tris-HCL (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP) (Promega), 0,2 µl pGEM®-T Easy vector (Promega), 1 µl T4 DNA Ligase (Promega), 4,8 µl nuclease free sterile H_2O. Ligations were carried out at 4 °C overnight.

On the next morning 100 µl *Escherichia coli* DH5α (SAMBROOK and RUSSELL, 2001) competent cells were put on ice to thaw for 10 minutes. The total volume of the ligate were mixed with the competent cells and were incubated on ice for 20 more minutes, then incubated on 42 °C for 1 minute, then were set on ice for 5 minutes. Then 400 µl of LURIA-BERTANI (LB) broth (ATLAS, 2010) were added and incubated with shaking for 1 hour at 37 °C. 150 µl the bacterium suspension was spread on ampicillin (100 mg/l) LB plates (1000 ml LB broth + 20 g/l agar) supplemented with 10 µl IPTG (100 mM/ml) and 40 µl X-Gal (20 mg/ml). Colonies were grown at 37 °C overnight, then incubated at 4 °C for 1 hour before blue-white screening. A white colony was suspended in 3-5 ml ampicillin (100 mg/l) LB broth and incubated overnight with shaking at 37 °C. Plasmids were extracted with GeneJET Plasmid miniprep kit (Thermo Scientific). Sequencing of the inserts were carried out by service providers using the method of SANGER (SANGER and COULSON, 1975; WALKER and LORSCH, 2013). For sequencing I used M13 primers in the beginning: M13-for (5'-GTAAAACGACGGCCAGT-3'), M13-rev (5'-GGAAACAGCTATGACCATG-3'); but later I designed new primers which were 75-77 bases closer to the Multiple Cloning Site (MCS) of the vector sqGMTZ-for (5'-GGGCGAATTGGGCCCGACG-3') and sqGMTZ-rev (5'-CCAACGCGTTGGGAGCTCTCCC-3'). Using these new primers the mapping of chromatograms to reference sequences were better.

2.2.3 Sequence analysis

Chromatograms were first analyzed with megaBLAST (MORGULIS *et al.*, 2008) available from National Center for Biotechnology Information, U.S. National Library of Medicine (NCBI)

GenBank in order to identify to Genus and species level (ADAMS *et al*., 2005; WYLIE *et al*., 2017). For each virus species present in the database a reference sequence was chosen. If there were no sequences available in the database with 100% query cover and more than 76% identity chromatograms were loaded into CLC Sequence Viewer v8.0. Alignments were made with less accurate option, then the consensus alignment for each isolate were saved and this became the reference. Each chromatogram was then loaded into Unipro Ugene v1.30 (OKONECHNIKOV *et al*., 2012) and were mapped to the references with trimming quality threshold set to 0, and mapping minimum similarity set to 50%. Each mapped read was then manually corrected according to peaks, then saved as GenBank flat file format. Sequence annotation were carried out in the same program after removing the adapter regions of the primers (poty 7941, 5'-GGAATTCCCG-3'; poly T_2, 5'-CGGGGATCCTCGAGAAGC-3'), then they were uploaded to GenBank.

2.2.4 Phylogenic analysis

For phylogenetic analysis I used MEGA X program (KUMAR *et al*., 2018).

First ClustalW alignments were built (LARKIN *et al*., 2007; THOMPSON *et al*., 1994) with the most identical isolates present in the database. Weigh matrix was set to ClustalW v1.6.

Then I asked the program to find the best fit DNA substitution model, with the lowest corrected Akaike Information Criterion (AICc) (NEI and KUMAR, 2000). Maximum Likelihood (ML) trees (FELSENSTEIN, 1981; GUINDON and GASCUEL, 2003) were built with this best fit model. Reliability of the trees were tested with the Bootstrap method (FELSENSTEIN, 1985) with 1000 replicates. *Tobacco etch virus* (RefSeq: NC_001555) were used as outgroup in each case, trees were rooted to this sequence.

3 RESULTS AND DISCUSSION

Based on my serological assays all tested samples turned out to be positive for potyvirus group specific ELISA. During the 6 week observation period no symptoms occurred on the laboratory test plants.

From the 40 tested samples I was able to molecularly identify 7 known and 1 unknown potyvirus, with 42 isolates altogether. Each sample was infected with one species of potyvirus, but occasionally I was able to detect different variants of the same virus species, which confirms that viruses are quasi-species (DOMINGO *et al*., 2008):

- tulips: lily mottle virus (13), Rembrandt tulip-braking virus (4), tulip breaking virus (16)
- daffodils: Hippeastrum mosaic virus (2), Narcissus late season yellows virus (2)
- calla: konjac mosaic virus (1)

- grape hyacinth: Muscari mosaic virus (1), unknown potyvirus, what we named Muscari chlorotic mottle virus (2)
- autumn daffodil: Narcissus late season yellows virus (1).

In brackets I indicate the number of strains isolated from a given virus.

3.1 Tulip (*Tulipa*)

In regard of the symptoms my results are significantly in accordance with referenced works (DE BEST *et al*., 2000; BLOEMBOLLENKEURINGSDIENST (BKD), 2010, 2019; NÉMETHY, 1990, 1994a, 1994b). In the case of ReTBV the cultivar 'Absalon' showed hair thin, 1-2 mm long purplish linear streaks on the underside of the leaf, while 'Insulinde' cultivar showed wide purple bands on both sides of the leaf. The same purplish linear patterns have been observed on the flower stalk during bloom. There have been no correlation between the infectious agent and the developed symptoms. One cultivar showed different symptoms when infected with the same virus species and different virus species caused identical symptoms (Figures 2-4).

Electron microscopic assays confirmed the findings of the ELISA tests. Sample 36/3 (Fig. 12) and A1 (Fig. 13) showed approximately 780 nm long and 14 nm in diameter flexuous filamentous particles which are characteristic of potyviruses.

According to the molecular assays I was able to identify 3 virus species (TBV, ReTBV and LMoV), two of them (TBV, LMoV) are widespread in Hungary (Fig. 1). In the population studies I found that both TBV and LMoV are present in equal rates in a single tulip population, and there is no host cultivar preference. The distribution of the species is random in the country and their occurrence is approximately equal.

The fulfillment of the Koch's postulates are in contrast to the cited literature (DE BEST *et al*., 2000; KRABBENDAM and BAARDSE, 1966). The Darwin-hybrid cultivar 'van Eijk' is susceptible to TBV, but symptoms – especially flower breaking – are mild (Fig. 15A). In the case of ReTBV transfection was also successful, but symptoms were also mild (Fig. 15C). Based on my results I conclude that Darwin-hybrid tulips are also susceptible to ReTBV not only the members of Rembrandt cultivar group. I was also successful in transfecting this cultivar with three different virus at the same time proving the possibility of complex infection (Fig. 15F).

Phylogenetic analysis of the species revealed both on the phylogenetic tree (Fig. 16) and on the phylogenetic network (Fig. 17) that all three species separated from each other, which is supported by high Bootstrap values. It was visible all three species can be traced back to a common ancestor (Fig. 16). The nucleotide sequence identity in every species is also above 76% on the complete coat protein gene coding region (Table 6), meaning Rembrandt tulip-breaking virus can be elevated to species level.

In the case of *Lily mottle virus* my results support the results of RIVAS *et al.* (2016). I was also able to identify two subgroups (I. and II.) in LMoV, the phylogenetic position of the isolates are in accordance with earlier reports.

Recombination detection revealed two recombinant isolates in LMoV (JN127341, AJ310203), from which JN127341 strain showed two distinct recombination events. In the case of TBV three recombination events were detected (X63630, KF442403, KT923168). In both viruses there were at least one isolate which had at least one recombinant parent (Table 9). Each recombination event were supported by at least 3 different method, and each method was individually significant (Table 10).

3.2 Daffodil (*Narcissus*)

I was able to identify two viruses in daffodils (HiMV and NLSYV). Daffodil was described as a new host of *Hippeastrum mosaic virus*. Symptoms were in accordance with earlier reports (HANKS and CHASTAGNER, 2018), but they were not virus species specific, as other daffodil infecting viruses (*Narcissus mosaic virus*, *Narcissus latent virus*) may also cause these symptoms. Correct identification of the infectious agent based solely on symptoms is not possible.

Hybridization assays for NLSYV were positive for samples 660 and 661, for HiMV only sample 644 were positive, confirming the results of ELISA tests and molecular identification.

Phylogenetic analysis of the viruses showed both had a common ancestor and are separate species as they group into separate clades (Fig. 18). The results are supported by high Bootstrap values.

3.3 Calla (*Zantedeschia*)

Infected plants showed spectacular symptoms compared to healthy ones. Leaves had yellow-green strap shaped mosaic, the white spots almost entirely disappeared. Leaf shape changed from wide spade-like to narrow sagittate with long acuminate tips (Fig. 7C). The length of the spathe were the same as the length of the spadix, or less than half the size, the margins were lacerated, with green and purple striping on the outer surface and they did not opened (Fig. 7B). Plants were stunted in growth, number of leaves were less than normal. As the growing season progressed symptoms become masked, but leaf margins stayed chlorotic. Infected plants grow only one or two inflorescences which were unsalable.

Based on the phylogenetic analysis – on both the nucleic acid and amino acid – of the complete coat protein coding region the plants were infected with KoMV. The closest relative of this isolate was from New Zealand (EU544542) (Figures 19 and 20).

3.4 Grape hyacinth (*Muscari*)

My results of the symptoms differ from the results of Latvian researchers (NAVALINSKIENĖ and SAMUITIENĖ, 2001), as I did not observed necrosis on the plants. At the same time greenish striping and the yellowing of the plants were observed.

Based on my molecular assays I was able to identify two potyviruses from the samples: Muscari mosaic virus – which is a tentative member of the Genus *Potyvirus* in the 9th ICTV report, and Muscari chlorotic mosaic virus, which is new to science.

Phylogenetic analyses showed the two viruses group into separate clades on the tree (Fig. 21) – which is supported by high Bootstrap values – and are well separated from other viruses showing high nucleotide sequence identity on the coat protein.

Based on the pairwise nucleotide identity of viruses showing high nucleotide identity on the CP coding region and the viruses identified by me (Table 8), the serological and phylogenetic assays Muscari mosaic virus and Muscari chlorotic mottle virus can be elevated to species level.

3.5 Autumn daffodil (*Sternbergia*)

Infected autumn daffodils showed vivid symptoms, greenish yellow long stripes were present on the entire length of the foliage, which were stronger in spring (Fig. 11B), masked in autumn. Vitality of the plants decreased, flowering were sparse or absent. Dormancy occurred 10-14 days earlier than healthy plants – around middle of May.

Molecular assays indicated NLSYV infection.

Phylogenetic analyses (Fig. 22) showed highest relationship with isolates from daffodils from Hungary (MK132194) and two other isolates from Japan (LC158450, LC158451).

Sternbergia was described as a new host of NLSYV based on my results.

4 NEW SCIENTIFIC ACHIEVMENTS

1. I reported first that MAb PTY1 antibody based Potyvirus group specific ELISA kits are able to detect the following viruses: *Hippeastrum mosaic virus*, Muscari mosaic virus, Muscari chlorotic mottle virus, Rembrandt tulip-breaking virus.
2. From Hungary I reported first the following viruses: *Hippeastrum mosaic virus*, *Konjac mosaic virus*, Muscari mosaic virus, Muscari chlorotic mottle virus, *Narcissus late season yellows virus*, Rembrandt tulip-breaking virus.
3. I detected first Muscari mosaic virus from Hungary with serological and molecular methods, and fulfilled Koch's postulates.

4. I described a potentially new virus species – Muscari chlorotic mottle virus – with serological and molecular methods, and fulfilled Koch's postulates.
5. I reported first daffodil as a new host of *Hippeastrum mosaic virus*.
6. I reported first autumn daffodil as a new host of *Narcissus late season yellows virus*.
7. I reported first the *Lily mottle virus* infection of tulips in Hungary.
8. I reported, that neither *Lily mottle virus* nor *Tulip breaking virus* has no cultivar or cultivar group preference in tulips. In a tulip population the two species has the same occurrence in Hungary.
9. I detected first intraspecific recombination in *Tulip breaking virus*, and discovered multiple recombination events in a single isolate in *Lily mottle virus*.
10. I supported the emergence of two subgroups in Rembrandt tulip-breaking virus, and provided supportive evidence of the existence of two subgroups within *Lily mottle virus*, in accordance with the findings of (RIVAS *et al*., 2016).

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First report of konjac mosaic virus in Zantedeschia from Hungary

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Identification and Analysis of New Viruses, Virus recombinants and Resistance Breaking Strains in Hungary
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