Emerging Plant Viruses in Urban Green: Detection of the Virome in Birch (*Betula sp.*)

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Abstract

Data from next generation sequencing indicate the complexity of the birch virome in the urban landscape of Berlin. It is well known that plant viruses are widespread and contribute to the decline of birch trees. A mixed infection by Cherry leaf roll virus (CLRV), Apple mosaic virus (ApMV) and two newly discovered viruses from the genus Badna- and Carlavirus were investigated in southern Berlin (Steglitz-Zehlendorf) in 2015 and 2016. To gain a more detailed view on epidemiology of this viral complex in birch, the study was enlarged in 2017 including eight districts all over Berlin. Birch trees with symptoms like defoliation and degeneration were selected for determination of viral pathogens by molecular biological methods. Within the complex occurring symptoms in birch trees, new types of symptoms have been identified. Different combinations of plant viruses in single and mixed infection were detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR.) CLRV and Badnavirus combinations have shown to be distinct and widely distributed. Heterogeneity is also known from the symptomatology of virus containing birch leaves. As the correlation of symptoms and viral infection is not shown yet for the mixed infections, it is unknown if the complexity of the virome is the cause of the variability of symptoms. Epidemiology and pathogenicity of the newly discovered viruses as well as species specificity, life cycle, mode of transmission, host plant range and phylogeny are totally unknown and have to be investigated within the next years.

Keywords: Epidemiology; Molecular diagnostics; Symptomatology; Birch virome

Introduction

Urban trees have an immense impact on the health of our population and have to be protected and preserved as such. Over recent decades, research has shown that urban trees are integral to environmental quality of cities and towns around the world [1,2]. They provide many ecosystem services such as energy conservation, carbon storage, reduced storm water runoff, improved air quality, enhanced human health and well-being [3,4]. Within the scope of biotic factors, viral plant diseases have attracted attention as contributing factor to the decline of trees in urban green areas. Plant viruses are widespread, alter plant predisposition and interact with biotic and abiotic impacts [5]. Since trees provide ecosystem services, decline in tree branches and other parts would adversely affect the morphological structure. This contributes to instability of the tree and can have negative impact on road safety. The loss of branches corresponds to a loss of biomass, reduces oxygen production and changes the CO_2 balance influencing the quality of air in the cities.

Birch as a pioneer deciduous tree with fundamental ecological importance is one of the species affected by viruses in temperate and boreal urban green. Since 1990, there has been observed a decline in birch population in Berlin which can be attributed to abiotic factors such as extremely high temperature creating drought problems, weakening trees and predisposing them to viral pathogens which take advantage of their reduced vigor [6]. The decline in birch tree is associated with a heterogenic viral symptomatology summarized as "birch leaf roll disease" (BLRD). The infection often spreads over the whole leaf blade, having begun at one focal point [5]. Young growing leaves infected during early development are often deformed or involute. Leaf areas that are yellow or slightly faded are considered chlorotic and develop around the primary site of infection. Areas with brown or dead tissue are considered necrotic [5]. Virus associated symptoms have been observed in domestic birch species in Berlin (*Betula pendula*, *Betula pubescens* and hybrids) [7-9]. Most of the long term observed trees exhibit virus-suspected leaf symptoms like mottling, chlorotic ringspots and line patterns [10]. The diversity of symptoms in *Betula spp.* leads to the assumption of a mixed infection of unknown viral origin.

Prominent sequences inferred from NGS datasets from 2015 indicated the existence of new Badna- and Carlaviruses in birch [11]. With the aid of NGS generated sequences, molecular biological methods (RT-PCR) for identification of the viral agents were developed for a routine screening of trees. In 2015 and 2016 the viral status of 142 declining birch trees were investigated in the southern Berlin. Trees were investigated by molecular-biological methods (RT-PCR) testing for Cherry leaf roll virus (CLRV), Arabis mosaic virus (ArMV) and Apple mosaic virus (ApMV) classically known to infect birch. Moreover, the existence of the two new uncharacterized virus species of the genera Badna- and Carlavirus have been established in birch trees [8,9]. The major research goal of the next years is the characterization of these unknown or new discovered viruses, which is necessary to provide information about the pathogenicity and management recommendations. The classical request to characterize new discovered viruses is already given in Koch's postulate. Identification of a single

infected trees and isolation of the particular virus and their transmission into a healthy host is the basic requisite to evaluate symptomatology and determine pathogenicity. Elementary requirement is a screening of a large birch population as conducted within this study. Screening of birch trees can provide additional information about the incidence of viral combinations and their influences in the BLRD.

Experimental

Symptomatology

Visual inspection of leaf samples was followed by a second step including sampling and laboratory analysis. Viral symptoms in birch are mostly distinguished from other leaf structures by mosaic-like leaf patterns, chlorotic ring spots, lines and mottling of light and dark green, yellow and white color as well as deformation. A selection of prominent viral associated symptoms collected in May and June 2017 in Berlin with an average leaf size of 3-4 inches long is shown in Figure 1.

Sampling

With aid from the Berlin parks departments and the urban green cadaster, 72 declining virus-suspected birch trees were chosen from eight districts in Berlin. For each declining birch tree, symptomatic leaves were carefully selected based on the observed symptoms at different locations in all eight districts. Symptomatic leaves were taken using a pair of secateurs and after photographic documentation of symptomatology, frozen and stored at -80°C for further research. RNA was extracted from all symptomatic leaf samples according to Boom et al. [12]. Diagnostic of plant viruses was done by using molecular-biological methods (RT-PCR) as published before to test for CLRV, ApMV, Badnaviruses and Carlaviruses, which were previously associated with declining birch trees [9]. Using Bioedit, Primer sequences were designed in conserved regions at the 5' and 3' ends of assembled plant viruses scaffolds in order to complete the genome sequences and confirm genome circularity [13,14].

NGS was applied to the leaf sample Figure 1A with new discovered ring spots. Following the protocol of Adams et al. [15] 1-2 µg purified cDNA was (Figure 1A) sent to BaseClear (Netherlands) for RNA-Seq analysis with the Illumina HiSeq2500 system. This resulted in 100 bp-long paired end sequence reads corresponding to 50-100 MB data/sample. All NGS data processing and analysis were performed using a bioinformatical workstation applying BlastX in Biolinux for filtering plant viral sequences.

Results and Discussion

Symptomatology

Large heterogeneity was found during 2017 rating of birch trees in Berlin. Sixteen different symptoms could be described as shown in Table 1. In some cases different prevalent symptoms overlap making it difficult to interpret the leaf pattern and sort them in categories. Symptoms at different developmental stages were observed during rating and this made interpretation of leaf patterns difficult. For instance necrosis is the final stage of chlorosis. The definition of symptomatology was still a matter of personal interpretation, therefore it is highly recommended to use a computer based tracking system for categorization of symptoms. Newly discovered leaf symptoms in birch are described as follows: ringspots (A) and oak leaf pattern (B) (Figure1).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Results obtained by RT-PCR for 72 symptomatic leaf samples are given in Figure 2. Out of the four tested plant viruses, Badnavirus recorded with 55.6% (40) as the most prominent virus in the investigated trees followed by CLRV with 26.4% (19), ApMV with 22.2% (16) and Carlavirus 8.3% (6). Infection by two different viral species was recorded for Badnavirus/CLRV 12.5% (9), Badnavirus/ApMV 11.1% (8), CLRV/ApMV 2.8% (2), Badnavirus/Carlavirus 1.4% (1), and CLRV/Carlavirus 1.4% (1). The following combinations of viruses were found in two single trees: Badnavirus/Carlavirus/CLRV and Badnavirus/CLRV/ApMV. In 19.4% (14) of trees, no specific detection of the four investigated viruses was recorded. Correlation of complex symptomatology with molecular biological diagnostics (RT-PCR) was however not successful.

80% of investigated birch leaf samples in 2017 confirmed to be infected by plant viruses. The virological examination of diseased and degenerating birches in eight districts in Berlin have shown that single infection by Badnaviruses is widely distributed (27.8%). Even though there were single infections from CLRV, ApMV and Carlavirus respectively, Badnavirus was the most prevalent in all tree samples. In contrast previous study by Landgraf et al. [8] showed CLRV recording about 71% and being more significant than the others. There was no significant difference between data for double infection by CLRV and Badnavirus from 2015/2016 (13.6%) in Steglitz-Zehlendorf [9] and investigated areas in 2017 (12.5%). The combination of CLRV and Badnavirus seems to be common in birch trees and associated with the BLRD.

In contrast to the study in 2015 and 2016, ApMV was detected in single infections as well as in mixed infections with a high percentage (22.2%) by RT-PCR. An additional proof of ApMV in the new discovered symptoms Figure 1A has been achieved by applying NGS. If this ApMV virus strain is birch specific has to be investigated in future. The difference in the detection of ApMV in 2015/2016 and 2017 might be due to regional differences in virus distribution, the investigated area and their specific plant origin or local climatic conditions.
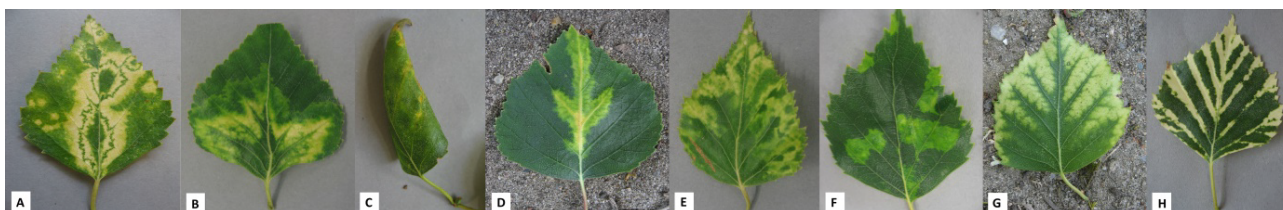
Figure 1: Selection of prominent symptoms observed during tree rating in 2017 in Berlin (Germany). Symptom descriptions, location, result of RT-PCR, sample number are given: (A) Ring spots, Neukölln, ApMV, E56736; (B) Oak leaf pattern, Reinickendorf, Carlavirus, and E54088; (C) Leaf rolling and chlorosis, Köpenick, Badnavirus and Carlavirus, E54047; (D) Vein bending, Pankow, Badnavirus, E54065; (E) Mottle and ring spots, Reinickendorf, ApMV, E54085; (F) Chlorotic spots, Charlottenburg-Wilmersdorf, CLRV, E54030; (G) Vein chlorosis, Mitte, CLRV, E54072; (H) Vein bending, Köpenick, no specific record, E54056.

Symptoms/Year of rating	2015	2016	2017
Intercostal chlorosis	✓	✓	✓
Chlorosis	✓	✓	✓
Mottling	✓	✓	✓
Leaf rolling	✓	✓	✓
Necrosis	✓	✓	✓
Vein bending	✓	✓	✓
Small leaves	✓	✓	✓
Leaf deformation	✓	✓	✓
Chlorotic spot	✓	✓	✓
Mosaic	✓	✓	✓
Line pattern	✓	✓	✓
Ringspot	✓	✓	✓
Variegation	✓	✓	X
Oak leaf pattern	✓	✓	X
Ringspot**	X	X	✓
Oak leaf pattern**	X	X	✓

White color represents specific symptoms, light grey color represent symptoms associated with chlorosis during disease development and dark grey represent different phases of mottling development. ✓Observed symptoms; X No observed symptoms. **New extreme symptoms as shown in Picture A and B (Figure 1)

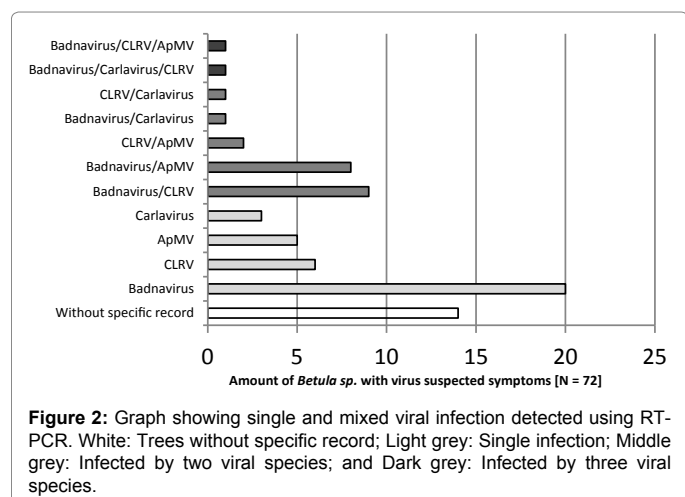**Table 1:** Table of observed symptoms from 2015 to 2017.

Figure 2: Graph showing single and mixed viral infection detected using RT-PCR. White: Trees without specific record; Light grey: Single infection; Middle grey: Infected by two viral species; and Dark grey: Infected by three viral species.

As there were symptomatic leaf samples without specific record of the four investigated viruses, it is obvious that unknown viruses are involved in the symptomatology. The next generation sequencing (NGS) technology provides a powerful tool for plant pathologists in the diagnosis and identification of those unknown viruses and viroids.

New viruses have been identified in NGS data from birch [10]. The potential of new methods in metagenomics offered another interpretation regarding the causal agent of the BLRD revealing a complex etiology engaging various viral pathogenic agents [12]. Successful detection of diverse viruses and viroids by metagenomic approaches has been described in plants since 2009 [15-19].

The advantage of NGS approaches in viral diagnostics is the extraction of new genetic information of hidden viruses and enables scientist to use molecular methods for characterization of species which elude from cultivation, isolation or enrichment. Analysis of NGS data shows the plant virome is nearly unexplored including pathogenic viruses. The most recent report of the International Committee for the Taxonomy of Viruses lists about 2285 virus and viroid species. Most of them are well-characterized viruses, but only a few have been

detected in trees and shrubs. Due to difficulties in isolation of viruses from woody plants the identification of viruses in the forest ecosystem is extremely rare compared to those of viruses in agricultural and horticultural environments. But trees and shrubs can serve as reservoir for pathogenic viruses and as such be a source of infection in agriculture. The utilization of NGS in plant virology will definitely increase the number of viral pathogens significantly as new viruses are being discovered and characterized in different plant host species, including wild and woody plants, as well as in different insect vectors. NGS technologies will also likely transform the inspection and quarantine services required to provide a fast, accurate, and full indexing of viruses in plant samples as it provides the genetic information necessary for routine diagnostics.

Conclusion

Viral infection is non-treatable by any plant protection procedure and the mechanisms of viral interaction within a mixed infection such as the one discovered in birch are unknown. More cultivated areas are affected by viral epidemics than non-cultivated areas. The birch decline seems to be the result of tree management in the nurseries of the past. Diagnosis of viral agents plays a major role in virus-free plant production and disease monitoring in field. The management of viral pathogens in young trees depends strongly on the virus free hygiene in seeds, meristem culture, seedling or grafting. The two requirements for effective control of viruses are the identification of causal pathogen and the determination of the possible mechanism of transmission; this was shown for CLRV by Hamacher and Quadt [20]. Explicit knowledge of virus characteristics, experience in symptomatology and access to reliable and sensitive virus detection are prerequisites for the proper diagnosis of tree viruses and for their effective management. Virus control is based on prevention, and so hygiene strategies have to be applied to the origin of the planting material. Attention has to be focus at the nursery production of planting stock, where critical examination and assessment of vigor can be practised routinely.

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