**Czech University of Life Sciences**

**Faculty of Agrobiology, Food and Natural Resources**

**Department of Plant Protection**

# Viruses of *Allium* plants transmissible by eriophyoid mite *Aceria tulipae*: mode of transmission, natural host range and occurrence in the CZECH REPUBLIC

Doctoral thesis

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## Statement of authorship

I hereby declare that I have written my dissertation thesis entitled "Viruses of *Allium* plants transmissible by eriophyoid mite *Aceria tulipae*: mode of transmission, natural host range and occurrence in the Czech Republic" independently and by my own. All literature sources used in this thesis are properly cited according to requirements of the Faculty of Agrobiology, Food and Natural Resources, CULS Prague, and are listed in the chapter References and *vice versa*.

Moreover, it is also to be declared that the research work presented here is original and has not been submitted to other institutions for any degree or diploma.

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## ABSTRACT

*Allexivirus* is a genus of the family *Alphaflexiviridae*. Since this genus was first described in 1970 by Razvjakina, *Allexivirus* species were reported all around the world. Although this genus primarily infects the family Amaryllidaceae, it is also able to infect hosts from Fabaceae, Rosaceae and Orchidaceae. To date, thirteen species have been assigned to the genus. Eight species have been reported to be restricted to *Allium* species: shallot virus X (ShVX), garlic virus A (GarV-A), garlic virus B (GarV-B), garlic virus C (GarV-C), garlic virus D (GarV-D), garlic virus E (GarV-E), garlic virus X (GarV-X), and garlic-mite filamentous virus (GarMbFV) and five described from non-*Allium* hosts: blackberry virus E (BVE), vanilla latent virus (VLV), alfalfa virus S (AVS), Arachis pintoi virus (ApV), and Severe yellow mosaic virus (SSYMV). The *Allium*-infecting allexiviruses have been discovered all over the world including the Czech Republic. These allexiviruses often occur in mixed infection with viruses from different families including *Potyviridae* (genus *Potyvirus*), *Betaflexiviridae* (genus *Carlavirus*) and *Tospoviridae* (genus *Orthotospovirus*). The goal of the study was to investigate the distribution of allexiviruses, their occurrence in mixed infection, and the host range of these viruses in the Czech Republic. Another goal of the study was to determine the transmission characteristics of the member of the genus *Allexivirus*.

The study was carried out during three growing seasons (2016-2018) in four different regions of the Czech Republic (Prague, Průhonice, Brno and Olomouc). During vegetation period, 65 vegetable, wild and ornamental *Allium* species cultivated or naturally occurring were collected from six habitat categories (botanical gardens, private gardens, cultivated fields, ornamentals from markets, cultivated plants of wild origin and wild origin) and tested for the presence of fifteen viruses. Most of the collected samples were infected by at least one or more virus(es). The infection rate of *Allium* samples differed depending on the habitat origin and phylogenetic relatedness of the hosts. Viruses were most frequently detected on cultivated species and low infection rates and virus free samples were detected on species of wild origin.

*Allium*-infecting allexiviruses have been reported to be transmitted by eriophyoid mites, *Aceria tulipae*. However, little is known about their transmission characteristics. The transmission characteristics of members of the genus *Allexivirus* to leek (*Allium porrum* L.) by its eriophyid mite vector, *Aceria tulipae* (Keifer), were studied. Prior to conducting transmission tests, colonies of nonviruliferous *A. tulipae* were established on healthy leek seedlings. None of the mites that originated

from eggs deposited on infected plants transmitted allexiviruses, indicating that the viruses are not transmitted transovarially. Acquisition access period, inoculation access period, and retention period tests, suggest a semi-persistent mode of transmission of *Allexivirus* members by *A. tulipae*.

The data from this study provide a useful basis and reveal several practical applications of future research. The understanding of the biology of allexiviruses, their occurrence in mixed infection, their spread, transmission, and host range is an important and essential key research to be able to choose appropriate method of protection of plants against these viruses.

**Keywords:** *Allium*, *Allexivirus*, host range, mixed infection, *Aceria tulipae*, transmission.

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## List of abbreviations

| | |
|---|---|
| aa | = Amino Acid |
| AlkB | = oxidative demethylase domain (alpha-ketoglutarate-dependent hydroxylase) |
| CP | = Coat Protein |
| CRP | = Cysteine Rich Protein |
| dNTP | = Nucleoside Triphosphate |
| DTT | = Dithiothreitol |
| Exp | = Experiment |
| Gn and Gc | = glycoproteins |
| H | = Helicase |
| HC-Pro | = Helper Component Proteinase |
| ICTV | = International Committee on Taxonomy of Viruses |
| ISEM+DECO | = Immunosorbent electron microscopy and decoration |
| Kb | = Kilobases |
| KDa | = Kilodaltons |
| M | = Methyltransferase |
| $m_7G$ | = 7-methylguanosine cap |
| mil. t | = million tons |
| MS medium | = Murashige and Skoog medium |
| N | = Nucleocapsid protein |
| NABP | = Nucleic Acid Binding Protein |
| NJ | = Neighbour Joining |
| No | = Number |

| | |
|---|---|
| NSm | = Non-STructural protein M |
| NSs | = Non-Structural protein S |
| nt | = Nucleotides |
| NTP | = Nucleoside Triphosphate |
| ORF | = Open Reading Frame |
| pCA | = Principal Component Analysis |
| pCCA | = Phylogenetic Canonical Correlation Analysis |
| poly(A) | = polyadenylation |
| R | = RNA-dependent RNA polymerase |
| RH | = Relative Humidity |
| RNA | = Ribonucleic acid |
| rRNA | = ribosomal RNA |
| t | = tons |
| Ta | = Annealing Temperature |
| TGB | = Triple Gene Block |
| UTR | = Untranslated Region |

# 1 GENERAL INTRODUCTION

*Allium* is one of the largest monocotyledonous plant genera with 950 species (Friesen *et al*., 2006), including species of great economic importance. The genus includes widely used vegetable crops such as garlic (*Allium sativum* L.), onion (*A. cepa* L.), shallot (*A. cepa* var. *ascalonicum*), leek (A. *porrum*), and several wild and ornamental species. For decades, pathogens have been a major constraint on *Allium* production, reducing yields, and affecting the quality and sustainability of global agriculture (Conci *et al*., 2003). With climate change, the spread of pathogens is predicted to increase (Pautasso *et al*., 2012). Common vegetative propagation, poor sexual reproduction, especially in garlic, and the limited resistance sources in onion are the main barriers to the conventional breeding of these major *Allium* crops, making it hard to develop disease and vector-resistant varieties (Khandagale *et al*., 2020).

Viruses are among the most economically important agricultural pathogens worldwide, affecting yield and quality of crops. The following viruses have been reported to infect cultivated *Allium* crops, mainly garlic, onion, shallot, and leek: onion yellow dwarf virus (OYDV); leek yellow stripe virus (LYSV); shallot yellow stripe virus (SYSV) (genus *Potyvirus*; family *Potyviridae*); garlic common latent virus (GCLV); shallot latent virus (SLV) (genus *Carlavirus*; family *Betaflexiviridae*); garlic virus A (GarV-A); garlic virus B (GarV-B); garlic virus C (GarV-C); garlic virus D (GarV-D); garlic virus E (GarV-E); garlic virus X (GarV-X); garlic-mite filamentous virus (GarMbFV); and shallot virus X (ShVX) (genus *Allexivirus*; family *Alphaflexiviridae*); iris yellow spot virus (IYSV) and impatiens necrotic spot virus (INSV) (genus *Orthotospovirus*; *Tospoviridae*) (Van Dijk, 1993; Dovas *et al*., 2001; Chen *et al*., 2001; Conci *et al*., 2003; Klukáčková *et al*., 2007; Mahmoud *et al*., 2008; Ward *et al*., 2009; Cramer *et al*., 2011; Chodorska *et al*., 2014; Winiarczyk *et al*., 2014; Godena *et al*., 2020).

These viruses frequently occur in mixed infected *Allium* crops, and they have been referred to as a 'garlic viral complex' (Van Dijk, 1993). The multiple infections by more than one virus species on the same host plant are expected to occur in natural vegetation (Cooper and Jones, 2006). Therefore, it is useful to know how frequently specific viruses occur together, and whether such co-existence might cause competitive or facilitative interactions among viruses. In natural environments, viruses can rapidly adjust to changes in host genotypes after new host encounters by adapting evolutionary strategies, which can result in epidemics (Lefeuvre *et al*., 2019). Therefore, it is more likely that the

distribution patterns of plant viruses in natural vegetation are different from those found in cultivated fields. Studies on plant viruses are concentrated mainly on viruses that cause crop diseases, but viral infection of wild and ornamental *Allium* species both in natural and agricultural ecosystems is only occasionally tested (Van Dijk, 1993; Dovas *et al.*, 2001; Ward *et al.*, 2009; Cramer *et al.*, 2011; Gawande *et al.*, 2014; Bampi *et al.*, 2015; Scrace *et al.*, 2015; Paduch-Cichal and Bereda, 2017). Although viruses tend not to damage wild plants (Cooper and Jones, 2006), the abundance of *Allium* viruses in nature and their ability to move between hosts should be further explored for disease resistance (Khandagale *et al.*, 2020).

*Allexivirus*, a genus that includes eight species primarily infect alliums, is considered as a threat due to the occurrence of its members in mixed infection. To date, these allexiviruses have been shown to be transmitted by their major vector, the eriophyoid mite *Aceria tulipae* (Van Dijk *et al.*, 1991). Previous records about the virus vector *A. tulipae* provided limited information regarding the transmission characteristics of allexiviruses. Studies on the mite's capabilities as a virus vector are demanding because of the difficulties in rearing healthy mite colonies and manipulating individual mites. One study suggested that viruses are only acquired by the first and the second instar nymphs and mites can transmit allexiviruses up to eight days after they acquired the virus (Ahmed and Benigno 1985). However, further details of the transmission mechanism of allexiviruses have not been described yet.

During the study, several questions have been addressed including:

(i) What are the potential *Allium* hosts of plant viruses?
(ii) How often do coinfections by different viruses occur in *Allium* hosts?
(iii) What is the incidence of viruses in *Allium* plants from different habitats (e.g., cultivated fields, botanical gardens, wild habitat)?
(iv) What is the mode of transmission of *Allium*-infecting allexiviruses?

At present, considerable progress has been made in characterizing the genome and expression of allexi-, poty-, carla- and orthotospoviruses. Determining their host range, distribution in the country, epidemiology, and the mode of transmission of allexiviruses is important to be able to apply appropriate control methods.

# 2 LITERATURE REVIEW

## 2.1 Biology of *Allexivirus* members

### 2.1.1 Taxonomic position

The genus *Allexivirus* has been assigned to the family *Alphaflexiviridae* of the order *Tymovirales* (Kreuze *et al*., 2020). The genus currently comprises thirteen recognized species by International Committee on Taxonomy of Viruses (ICTV) distinguished mainly by their coat protein (CP) and replicase coding regions. According to the species demarcation criteria, members of the genus *Allexivirus* share less than 72 % nucleotide sequence identity (or 80% amino acid sequence identity) between their CP and replicase and react differently with antisera (King *et al*., 2012).

Eight viruses have been detected primarily in *Allium* species, including shallot virus X (ShVX), garlic virus A (GarV-A), garlic virus B (GarV-B), garlic virus C (GarV-C), garlic virus D (GarV-D), garlic virus E (GarV-E), garlic virus X (GarV-X) and garlic mite-borne filamentous virus (GarMbFV) (Adam *et al*., 2004). Five additional viruses were assigned to the genus based on the genome organization and the level of sequence identity between their replicase and/or CP sequences; these viruses are blackberry virus E (BVE), vanilla latent virus (VLV), alfalfa virus S (AVS), Arachis pintoi virus (ApV), and severe yellow mosaic virus (SSYMV) (Sabanadzovic *et al*., 2011; Gutiérrez Sánchez *et al*., 2016; Grisoni *et al*., 2017; Nemchinov *et al*., 2017; Alves *et al*., 2020).

*Allium*-infecting allexiviruses were initially found infecting garlic and onion crops (Razvjazkina 1970). Later, these viruses were described more thoroughly by Van Dijk *et al*. (1991). They were first referred to as onion mite-borne latent virus (OMbLV), shallot mite-borne latent virus (SMbLV) and garlic strain of onion mite-borne latent virus (OMbLV-G) (Van Dijk *et al*., 1991). Later, Kanyuka *et al*. (1992) and Vishnichenko *et al*. (1993) reported the presence of a virus on shallot and named it shallot virus X (ShVX). ShVX was later considered as synonymous to OMbLV and SMbLV (Van Dijk and Van der Vlugt, 1994). Allexiviruses were first assigned to the genus *Rymovirus* (family *Potyviridae*) based on their transmission by mites and the morphology of the virus particles (Barg *et al*., 1994). Later, they were classified to the genus "*Allexivirus*" within the family *Alphaflexiviridae*, for which ShVX is the type member (Pringle, 1999).

Few species were also shown to be related to the genus but remain unassigned until an assessment by the ICTV members. Based on sequence analysis, the unassigned viruses share high degree of nucleotide (nt) and amino acid (aa) sequence similarity with the existing members of *Allexivirus*. The unassigned viruses comprised three species listed in ICTV and includes: SMbLV that was identified as ShVX isolate (GeneBank accession EU835196.1), Allexivirus DS-2013/CZE isolate (JX682826.1), and Cassia mild mosaic virus (CaMMV) (Beserra *et al.*, 2011). The CaMMV isolate found in *Senna macranthera* was at first tentatively identified and given the name cassia mild mosaic virus based on the particle morphology and host range. However, since there was no available information about the nucleotide sequence of this virus, the authors referred to the newly identified allexivirus as Senna virus X (SVX) (Beserra *et al.*, 2011). In addition to the unassigned species, new viruses have been deposited in GenBank as tentative *Allexivirus* species and include: papaya virus A (PaVA; MN418120.1) (Read *et al.*, 2020), garlic virus F (GarV-F; MN059330.1), garlic virus H (GarV-H; MN059332.1), garlic virus G (GarV-G; MN059331.1), garlic virus I (GarV-I; MN059334.1), and garlic yellow virus (GYV; MN059396.1).

### 2.1.2 Genome organization and virion properties

Members of the *Allexivirus* genus are single-stranded positive-sense RNA viruses with a genome size of approximately 9 kb in length (Chen *et al.*, 2004). Virions of allexiviruses are flexuous and filamentous, approximately 800 nm in length and 12 nm in diameter (Fig. 1). Allexiviruses induce the formation of granular inclusion bodies and small bundles of flexible particles in the cytoplasm of the invaded cells of infected plants (Kang *et al.*, 2007). The RNA of *Allexivirus* species is capped at the 5' untranslated region (UTR) terminus with a 7-methylguanosine cap ($m_7G$) and has a polyadenylated tail at the 3' UTR terminus.

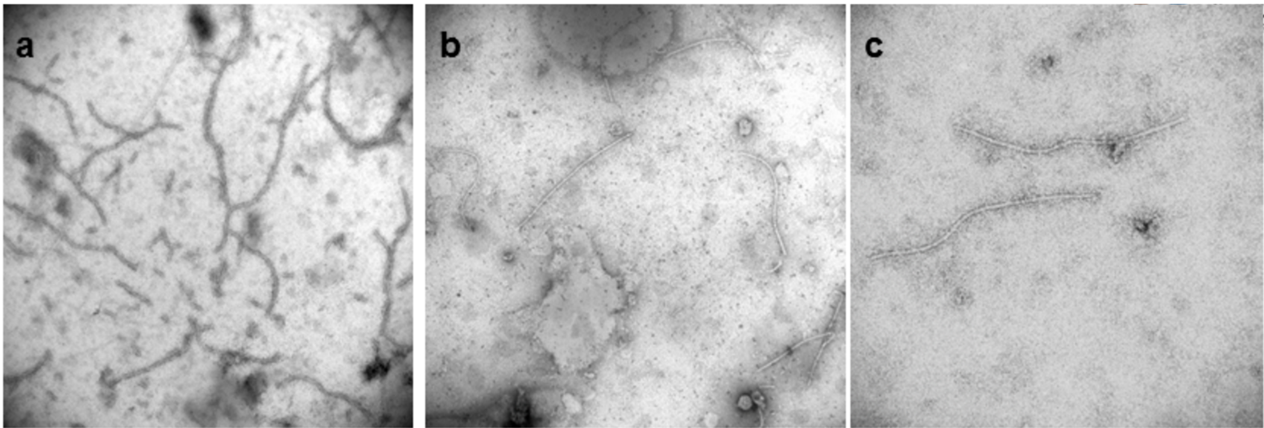


**Figure 1** Transmission electron microscopy showing particles of *Allexivirus* from garlic plants. **a:** Dip preparation (negative staining with 1 % uranyl acetate), **b:** Immunosorbent electron microscopy and decoration (ISEM+DECO) of GarV-B, **c:** ISEM+DECO of GarV-D. (© Julius-Kühn-Institut, Braunschweig).

The number of open reading frames (ORFs) varies among species (Table 1; Fig. 2). The genome of seven *Allium*-infecting allexiviruses (GarV-A, -B, -C, -D, -E, -X, ShVX) contains seven ORFs, whereas AVS, VLV, ApV and SSYMV contains six ORFs, and BVE contains five ORFs (Table 1; Fig. 2). There is no complete genome sequence of GarMbFV, but it is assumed that it is likely to have six ORFs due to its close relationship to GarV-A. The first ORF in all *Allexivirus* species is the largest gene and encodes putative replicase protein with three conserved motifs: methyltransferase (M), NTPase/helicase (H) and RNA-dependent RNA polymerase (R) (Song *et al*., 1998). The replicase protein of ShVX and AVS contains an oxidative demethylase domain (AlkB) located between the methyltransferase and helicase domains that is found in other members of the *Alphaflexiviridae* family (Van den Born *et al*., 2008; Nemchinov *et al*., 2017).

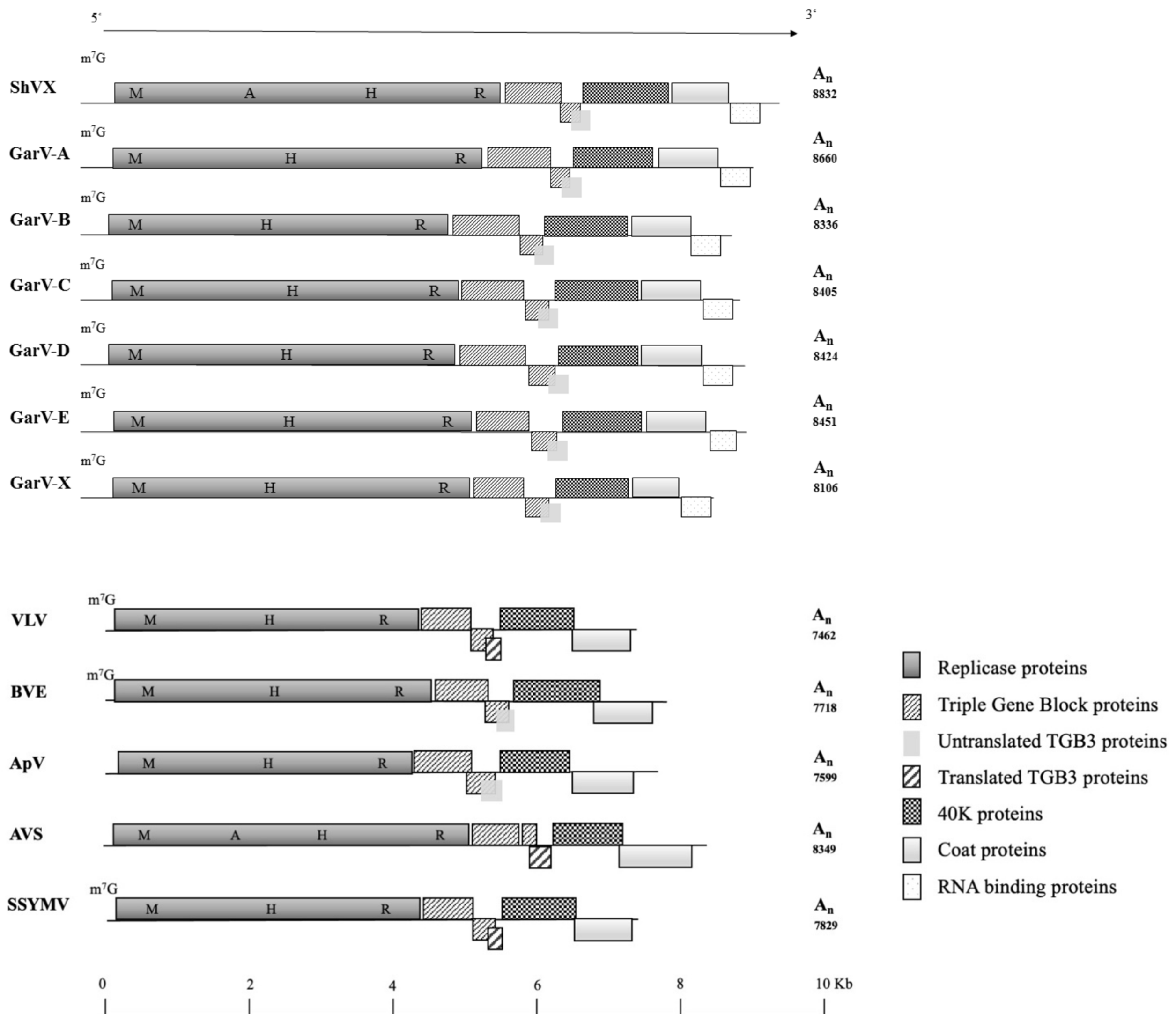


**Figure 2** Diagram showing genome organization of allexiviruses. Blocks represent predicted open reading frames (ORFs). The methyltransferase (M), AlkB (A), RNA helicase (H), and RNA-dependent RNA polymerase (R) domains of the replicase are shown. Untranslated TGB proteins are TGB3 with a non-AUG initiator codon.

Allexiviruses also contain triple gene blocks (TGBs); TGB1 and TGB2 have helicase and viral movement domains, respectively. The two TGBs were shown to be required for viral cell-to-cell movement through plasmodesmata and systemic transport via the phloem (Lezzhov *et al*., 2015). Another TGB-like protein, namely TGB3 was found in all the *Allium*-infecting allexiviruses and BVE and ApV but lacks the initiation codon. TGB3 synthesis requires a leaky ribosome scanning initiated by a TGB3 CUG initiator codon, rather than internal ribosome entry (Lezzhov *et al*., 2015). Such mechanism is commonly used by RNA viruses to translate functionally multicistronic messages (Firth

and Brierley, 2012). On the other hand, TGB3 of the allexiviruses (VLV, AVS, and SSYMV) and the unclassified virus (PaVA) had the initiation AUG start codon (Grisoni *et al*., 2017; Nemchinov *et al*., 2017; Read *et al*., 2020; Alves *et al*., 2020). Presumably, the TGB3 may have an accessory function alongside the TGB1 and TGB2 in the cell-to-cell movement of the virus (Morozov and Solovyev, 2003).

Another large region, downstream of the TGB, encoding a protein of approximately 42 KDa, was found in all allexiviruses and showed no homology to any known protein of other genera (Adam *et al*., 2004). This 42 KDa protein is rich in a serine and threonine-rich protein. It has been shown to be involved in virion assembly and acts as a co-factor to facilitate the interaction of the capsid protein with genomic RNA during assembly (Vishnichenko *et al*., 2002). Another protein, containing a nucleic-acid-binding domain (NABP), has been found only in ShVX, GarV-A, -B, -C, -D, -E, -X, and GarMbFV. This ORF contains a small cysteine-rich protein (CRP), a basic arginine-rich domain and a zinc-finger motif at the 3'-terminal regions (Song *et al*., 1998; Kanyuka *et al*., 1992). Although, CRPs of many plant viruses were shown to act as RNA silencing suppressors (Senshu *et al*., 2011; Fujita *et al*., 2018), CRP of ShVX does not act similarly (Arkhipov *et al*., 2013). Presumably, the *Allexivirus* CPR is necessary for the regulation of viral RNA replication, together with their function as pathogenicity determinants during *Allexivirus* evolution and the control the interaction of the virus with the plant host (Lukhovitskaya *et al*., 2014, Yoshida *et al*., 2018).

Finally, there is the coat protein, which shares the conserved structural core and evolutionary origin of some families of filamentous plant viruses (Martelli *et al*., 2007). Various nucleotide insertions between CP and CRP genes have been observed in GarV-B, GarV-C and GarV-X. These insertions are complementary to garlic 18 S ribosomal RNA (rRNA) and appear to be involved in the termination-reinitiation translation mechanism (Yoshida *et al*., 2018). It is possible that GarV-B, -C, and -X utilize such mechanism to regulate the expression level of viral protein to enable adaptations to specific hosts and vectors. Viral proteins are often multifunctional, and each function is essential for viral survival and can be dependent on viral species, host, and vector. Considerable further research is needed to clarify the entire process of infection by allexiviruses. How these viruses act to favor viral infection and to avoid host defense mechanisms and the functional features of the viral genomes are essential questions that necessitate further research to fully elucidate the expression of allexiviruses and their interactions with their hosts.

**Table 1** Genome size, number and molecular weight of proteins encoded by the genes of each *Allexivirus*.

| Species | Size (nt) | Nb of ORF | Molecular weight (KDa) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | R | TGB1 | TGB2 | TGB3 | 42K p | CP | NABP |
| **GarV-A** | 8660 | 7 | 183 | 28 | 11 | 7* | 39 | 28 | 15 |
| **GarV-B** | 8327 | 7 | 168 | 27 | 12 | 7* | 39 | 27 | 14 |
| **GarV-C** | 8405 | 7 | 175 | 27 | 11 | 7* | 41 | 28 | 15 |
| **GarV-D** | 8424 | 7 | 177 | 26 | 11 | 7* | 40 | 27 | 15 |
| **GarV-E** | 8451 | 7 | 176 | 27 | 11 | 7* | 40 | 35 | 15 |
| **GarV-X** | 8458 | 7 | 174 | 26 | 12 | 7* | 32 | 36 | 15 |
| **ShVX** | 8890 | 7 | 195 | 26 | 11 | 7* | 42 | 28 | 15 |
| **BVE** | 7718 | 5 | 169 | 27 | 12 | 11* | 40 | 25 | -** |
| **AVS** | 8349 | 6 | 188 | 26 | 11 | 10 | 38 | 32 | -** |
| **ApV** | 7599 | 6 | 158 | 26 | 12 | 10 | 41 | 26 | -** |
| **VLV** | 7462 | 6 | 161 | 26 | 11 | 8 | 41 | 25 | -** |
| **SSYMV** | 7829 | 6 | 164 | 26 | 11 | 9 | 37 | 28 | -** |

*Untranslated TGB-like gene that lacks the initiator AUG codon and partially overlapping with the TGB2 genes.
**No sequence was identified.

### 2.1.3 Phylogenic relationships among members

Given the global distribution of allexiviruses, insights from a phylogenetic analysis provides a better understanding of the origin and the relatedness among members of the genus. Phylogenetic tree was constructed from nucleotides sequences using the neighbour-joining (NJ) method with a bootstrap value of 1000 using MEGA X (http://www.megasoftware.net/mega.php) (Kumar *et al.*, 2018; Stecher *et al.*, 2020) (Fig. 3). Sequence alignments were performed with MUSCLE (Edgar, 2004) and the tree was constructed from selected allexiviruses and closely related unassigned members sequences available in NCBI (https://www.ncbi.nlm.nih.gov/). Only partial sequences of GarMbFV were available in GenBank, thus, it was only included in the analysis of the CP. Sequences of isolates used in this study, originating from different parts of the world, and GarMbFV CP sequences included in the CP phylogenetic analysis, were retrieved from GenBank. The viruses (PaVA, GarV-F, -H, -G, -I) have been included in the analysis. However, GYV appears to lack the 42 KDa protein and is more related to members of the genus *Carlavirus* (> 76% nt and aa identity of the CP to *Garlic latent virus*),

thus, GYV was not considered in the analysis. Potato virus M (PVM; genus *Carlavirus*, Family *Betaflexiviridae*) was used as an outgroup. Sequence similarity and identity analyses were performed in BioEdit (Hall, 1999).

Whole genome phylogenetic analysis divided the *Allexivirus* into the *Allium* and the non-*Allium*-infecting allexiviruses (Fig. 3A). Two clades were observed within the *Allium*-infecting allexiviruses, separating ShVX and GarV-A from the remaining *Allium*-infecting allexiviruses. SSYMV and the unassigned PaVA, together with the non-*Allium* allexiviruses (BVE, VLV, ApV, AVS) formed a monphyletic group of distant accessions. Similarly, the CP-based phylogenetic analysis showed the same two clades of the *Allium*-infecting allexivirus group and the non-*Allium* group (Fig. 3B). Within the *Allium*-infecting allexivirus clade, two groups were observed: the first group comprised GarV-X, -B, -I, -H, -F, -G, and -C with ShVX isolates. Second group included GarV-D and -E with -A and GarMbFV isolates. The second clade included the four non-*Allium* allexiviruses (AVS, BVE, VLV, ApV, and SSYMV), while PaVA formed a very distant monophyletic group. Although the unassigned species (PaVA) lack the NABP found in the *Allium*-infecting allexiviruses (Alves *et al*., 2020; Read *et al*., 2020), based on the phylogenetic trees, PaVA group with allexiviruses and can be considered as new species of the genus *Allexivirus*.

On the other hand, sequence identity analysis shows that SMbLV sequence shared 90,5-97,1 % nt sequence identity (97-98 % aa sequence identity) to ShVX isolates (GeneBank: MH389253.1, MH389252.1, KY012791.1). Similarly, DS-2013/CZE isolate (GeneBank: JX682826.1) shared 84,2 % nt sequence identity (95,0 % aa sequence identity) to GarV-D isolates (GeneBank: JX682863.1, AJ551490.1). Therefore, we conclude that SMbLV is an isolate of ShVX, and
DS-2013/CZE isolate is an isolate of GarV-D.

Identity pairwise comparisons of the CP gene showed that GarV-I, GarV-F and GarV-G share high degree of sequence similarity (99,1-99,6 % nt sequence identity (97,9-98,92 % aa sequence identity)), while GarV-H shares 73 %, 72,6 %, and 72,5 % nt sequence identity (66,1 %, 65,7 %, and 65,9 % aa sequence identity) with GarV-I, -G, and -F, respectively. The sequence analysis of the CP gene showed that GarV-I shares 85,4-99,5 % nt sequence identity (72,7-98,9 % aa sequence identity) to GarV-B isolates, however the BLASTN analysis of the replicase gene of GarV-I identified the highest identity values with GarV-D isolates (92,1-95,3 % nt sequence identity (96,1-99,7 % aa

sequence identity)). On the other hand, CP sequence analysis also showed that GarV-F shares 86,1-99,8 % nt sequence identity (75,6-98,91 % aa sequence identity) to GarV-B, GarV-G shares high similarity to GarV-B isolates sharing 87,2-99,6 nt sequence identity (75,7-98,9 % aa sequence identity), while GarV-H shared 68,6-72,6 nt sequence identity (83,6-87,7 % aa sequence identity) to GarV-B. These values are higher than the demarcation criteria of *Allexivirus* genus, suggesting that the isolates GarV-F, -G, and -H are different isolates of GarV-B. Since information about these new accessions is limited, additional information is required to give a proper taxonomic assignation.

The CP and replicase genes have been used to classify species within the genus. Although this criterion has been widely used, there is growing evidence of high similarities existing between some of *Allexivirus* species (Celli *et al*., 2018; Geering and McTaggart, 2019). For example, when available GarMbFV CP sequences were compared with GarV-A CP sequences, the identity values for CP among the isolates were 79,5-81,1 % nt sequences identity (76,6-81,8 % aa sequence identity), all values above the taxonomic classification criterion. Based on the phylogenetic analysis, both species formed a well-supported monophyletic cluster (Fig. 3B), with the exception of GarV-A Tunisian isolate (GenBank: MN995837.1) which formed a cluster with GarMbFV isolates (Fig. 3B), and shared 91,5-93,6 % nt sequence identity (97,1-98,5 % aa sequence identity) with GarMbFV isolates. The data analysis therefore suggests that the two species are very similar and may be different isolates of the same species. The high similarity observed between the species has been clearly demonstrated by Geering and McTaggart (2019), that clarified the taxonomic position of GarMbFV and GarV-A and concluded that the two species are conspecific. Additionally, since the replicase region used in taxonomic classification is absent, the risk of errors in classification can occur especially in cases when only partial sequences have been determined. Presumably, the isolation of GarMbFV complete genome sequence is required to give a bigger picture if the specie should be unclassified from the genus *Allexivirus*.

During the CP and replicase gene analysis, another high similarity was observed between GarV-B and GarV-X isolates. GarV-B and GarV-X isolates shared 75.4-78.1 % nt sequences identity (84.4-89.6 % aa sequence identity) between the CP sequences and 73.4-74.1 % nt sequences identity (81.6-82.9 % aa sequence identity) between the replicase sequences, both values are higher than demarcation criteria of *Allexivirus* genus (King *et al*., 2012). Although, the different accessions of both isolates are well separated in the phylogenetic analysis, GarV-B and GarV-X have enough homology to be considered as different strains of the same species. The data analysis confirms previously

described results by Celli *et al*. (2018), that also indicated possible recombination events within the complete genome of *Allium*-infecting allexiviruses. To sum up, based on the data analysis, SMbLV is an isolate of ShVX, DS-2013/CZE is an isolate of GarV-D, the new viruses GarV-F, -G, -H, and -I are isolates of GarV-B, while GarMbFV and GarV-A are conspecific, and GarV-B and GarV-X are also conspecific. In addition to the high similarity mentioned above, it has been shown that the different *Allium*-infecting species are serologically related (Lu *et al*., 2008), with considerable homology among the species. A weak serological reaction was observed between the different species using antisera produced by CP genes over-expressed in bacteria of the GarV-A, B, C, D, E and -X (Lu *et al*., 2008).

The genetic diversity of *Allexivirus* populations have been reported worldwide when comparing the CP and the replicase sequences, indicating population differentiation (Chen *et al*., 2001; Melo-Filho *et al*., 2004; Mohammed *et al*., 2013). The data indicates that the phylogenetic clustering among *Allexivirus* isolates was clearly independent of geographical area and the host plant (garlic, shallot, blackberry, vanilla, alfalfa, and forage peanut) (Fig. 3B), which means that the overall variability is due in a large part to the evolutionary forces such as the occurrence in mixed infection and the interactions between different genotypes of the same species (Melo-Filho *et al*., 2004; Mohammed *et al*., 2013). More analysis of the population genetic of allexiviruses is needed to allow a more precise understanding of their evolution and the genomic diversity within virus populations.

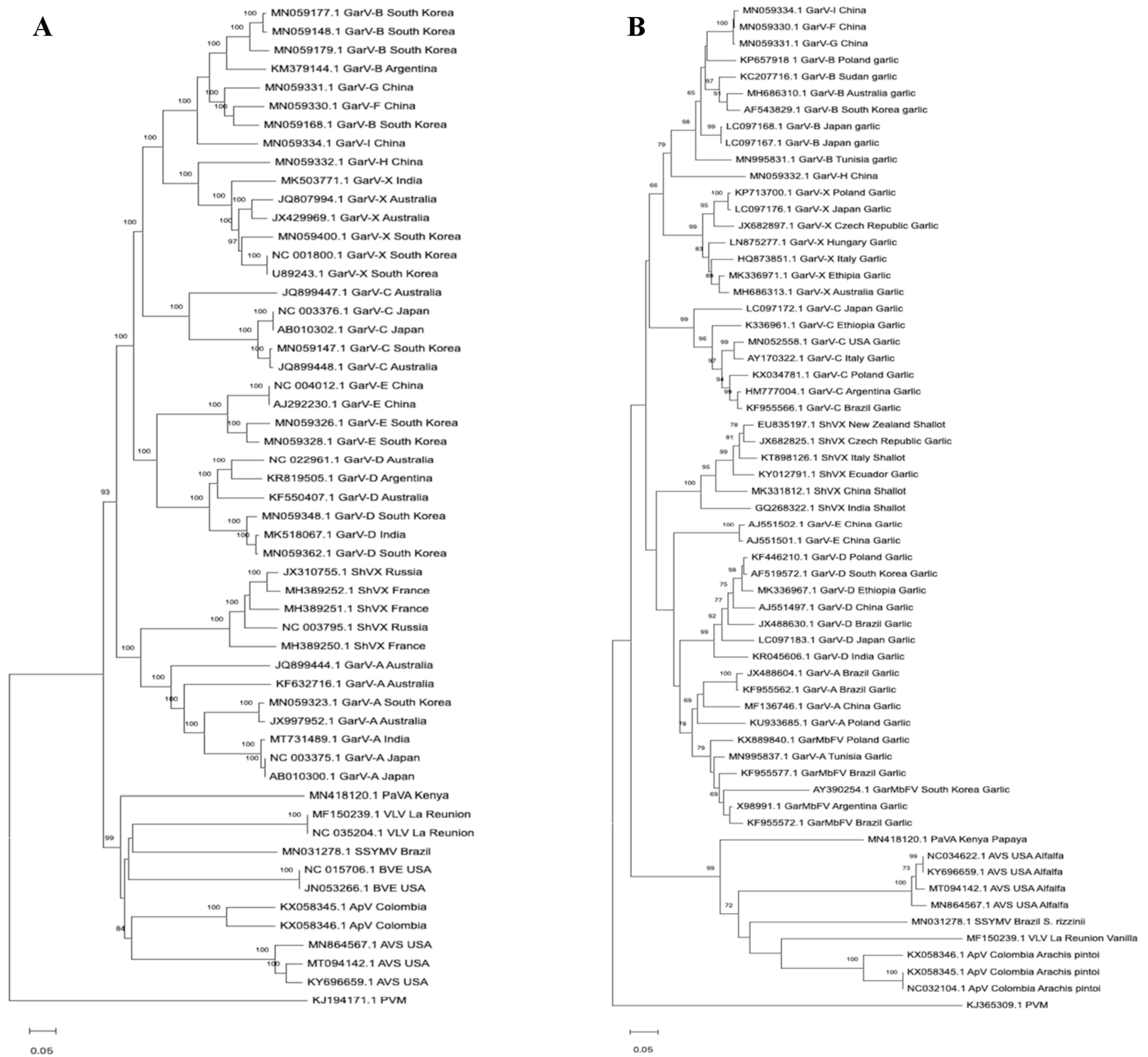


**Figure 3** Phylogenetic analysis among *Allexivirus* species and isolates (ShVX, GarV-A, -B, -C, -D, -E, -X, -F, -G, -H, -I, GarMbFV, AVS, BVE, ApV, VLV, and unclassified PaVA and SSYMV) based on the alignment of some nucleotide sequences. PVM was used as the outgroup. **(A)** Based on the complete genome sequences. **(B)** Based on the coat protein gene sequences. The neighbour-joining method was used for the construction of the tree and the reliability of the branches was inferred from a bootstrap analysis of 1000 replicates. The country of origin and the accession numbers of the selected *Allexivirus* sequences retrieved from the GenBank are shown next to their acronyms.

## 2.2 Allexiviruses pathology and management

### 2.2.1 Host range

The first allexiviruses described were the ones infecting *Allium* crops, primarily garlic (*A. sativum*) and onion (*A. cepa L.*) (Van Dijk *et al*., 1991). The natural host range of allexiviruses has been previously reported to be restricted to several cultivated, ornamental, and wild *Allium* species (Table 2). *Allium*-infecting allexiviruses (GarV-A, -B, -C, -D, -E, -X, ShVX, and GarMbFV), have been shown to only infect monocotyledon plants in the order Asparagales (Van dijk *et al*., 1991; Fidan *et al*., 2013). The only exception to date is the detection of GarV-D on *Drimia maritima* (L.) (Family *Asparagaceae*), which is the first time any of the *Allium*-infecting allexiviruses were reported to naturally infect a non-Alliaceae plant species (Fidan *et al*., 2013). The presence of *Allium*-infecting allexiviruses in *D. maritima* is particularly surprising as it is not an *Allium* plant, suggesting that either the virus is expanding its host range, as a consequence perhaps of its increased prevalence in the field, or that it has always been present in other host families.

Members of the genus ApV, AVS, BVE and VLV naturally infect dicotyledonous plants in the families Rosaceae, Fabaceae, Orchidaceae and Caricaceae (Table 2). The unclassified allexiviruses, PaVA was detected on *Carica papaya* L. (Read *et al*., 2020), SSYMV on *Senna rizzinii* (syn. *Cassia chrysocarpa* var. *psilocarpa* Benth.) and CaMMV *S. macranthera* (Beserra *et al*., 2011; Alves *et al*., 2020).

Most members of the genus *Allexivirus* are transmissible from natural to experimental hosts by mechanical inoculation. *Allium*-infecting allexiviruses can be transmitted to several diagnostic experimental hosts, including *Chenopodium quinoa*, *C. murale*, *C. amaranthicolor, G. globosa, Nicotiana occidentalis* and *Atriplex hortensis* (Van Dijk *et al*., 1991; Yamashita *et al*., 1996). VLV is mechanically transmitted to their natural hosts vanilla plants (*Vanilla planifolia*) (Grisoni *et al*., 2017). The SSYMV is mechanically transmitted to *S. rizzinii*, *S. occidentalis, C. amaranticolor*, *G. globosa* and CaMMV is mechanically transmitted to *G. globosa* and *Phaseolus vulgaris* (Beserra *et al*., 2011; Alves *et al*., 2020).

**Table 2** Natural hosts and experimental host range when mechanically inoculated with one of allexiviruses.

| Species | Natural hosts | References | Experimental hosts |
|---|---|---|---|
| **GarV-A** | *A.sativum, A.ampeloprasum, A. victorialis var. Platyphyllum, A, senescens, A. ascalonicum, A.chinense* | Yamashita *et al*., 1996; Ward *et al*., 2009; Park *et al*., 2011 | *Chenopodium murale*, *Gomphrena globosa*, *C. amaranticolor*, *C. quinoa, Atriplex hortensis, C. foliosum, C. opulifolium*, *A.cepa*, *A. ampeloprasum, Nicotiana benthamiana* (Van Dijk *et al*., 1991, Yamashita *et al*., 1996; Melo-Filho *et al*., 2004; Cafrune *et al*., 2006a; Dąbrowska *et al*., 2020) |
| **GarV-B** | *A.sativum, A.chinense, A. caeruleum, A sphaerocephalum* | Ward *et al*., 2009; Bampi *et al*., 2015; Paduch-Cichal and Bereda, 2017 | |
| **GarV-C** | *A.sativum, A.cepa L., A.ampeloprasum, A. caeruleum, A sphaerocephalum* | shahraeen *et al*., 2008; Ward *et al*., 2009; Bampi *et al*., 2015 | |
| **GarV-D** | *A. sativum, A.cepa L., Drimia maritima, A. ascalonicum, A. fistulosum, A. caeruleum, A sphaerocephalum* | Ward *et al*., 2009; Fidan *et al*., 2013; Bampi *et al*., 2015; Paduch-Cichal and Bereda, 2017 | |
| **GarV-E** | *A.sativum, A. caeruleum, A sphaerocephalum* | Chen and Chen, 2002; Bampi *et al*., 2015 | |
| **GarV-X** | *A.sativum* | Song *et al*., 1998 | |
| **GarMbFV** | *A.sativum, A.cepa L.* | Dovas *et al*., 2001 | |
| **ShVX** | *A.sativum, A. cepa L. var. Aggregatum, A.cepa L., A. ascalonicum, A. caeruleum, A.vineale, A. sphaerocephalum* | Ward *et al*., 2009; Hamed *et al*., 2012; Bampi *et al*., 2015; Taglienti *et al*., 2017; Paduch-Cichal and Bereda, 2017 | |
| **BVE** | *Rubus L.* | Sabanadzovic *et al*., 2011 | |
| **VLV** | *Vanilla planifolia, V. pompona, V. humblotii* | Grisoni *et al*., 2017 | *V. planifolia* |
| **ApV** | *Arachis pintoi* | Gutiérrez Sánchez *et al*., 2016 | |
| **AVS** | *Medicago sativa* | Nemchinov *et al*., 2017 | |
| **SSYMV** | *Senna rizzini* | Alves *et al*., 2020 | *S. rizzinii*, *S. occidentalis* |

### 2.2.2 Economic impact

The *Allexivirus* genus comprises species with important economic impacts, despite causing only mild symptoms (mild mosaic, yellow stripes, and stunted growth) in natural infection (Kang *et al*., 2007). Allexiviruses have been reported to cause yield loss and deterioration in the quality of several *Allium* crops (Cafrune *et al*., 2006b). The single infection with either GarV-C or GarV-A resulted in a decrease in bulb weight and diameter (losses of approximately 15 % and 5 %, respectively) (Cafrune *et al*., 2006a; Perotto *et al*., 2010). Single infection with GarV-D caused a reduction of 12.3 % of garlic bulb weight and 6.7 % of bulb calibre (Celli *et al*., 2016). Although infection of garlic crops by one allexiviruses could result in disease, yield losses were considerably more severe when allexiviruses occurred in mixed infections, especially in the presence of viruses from the genera *Potyvirus* (Family *Potyviridae*) and *Carlavirus* (Family *Betaflexiviridae*) (Conci *et al*., 2003).

On the other hand, little research has been done on the non-*Allium* allexiviruses since their discovery, suggesting that they have low impact on their host crop. Although, BVE and ApV cause mild symptoms such as chlorosis and vein yellowing in their host, it is possible that these symptoms are exhibited due to their occurrence in mixed infection (Sabanadzovic *et al*., 2011; Martin and Tzanetakis, 2015; Gutiérrez Sánchez *et al*., 2016).

### 2.2.3 Transmission mode

*Allium*-infecting allexiviruses are transmitted by their major vector, the eriophyid mite *Aceria tulipae* Keifer (Van Dijk *et al*., 1991). *A. tulipae*, also known as the garlic mite, the dry bulb mite, tulip mite or wheat curl mite (Lange and Mann, 1960) was first found in tulip bulbs by Keifer in 1938. Later, it was found in garlic and onion bulbs in 1952 (Keifer, 1952). Razvjazkina (1970) described filamentous shaped virus particles (Fig. 4) that were isolated from mite-infested garlic and onion plants with mosaic symptoms (Fig. 5).

The host range of *A. tulipae* includes plant species in the families Liliaceae, Amaryllidaceae, Melanthiaceae, and Asparagaceae (Kiedrowicz *et al*., 2017). The pest is considered one of the most important eriophyid plagues of bulbs attacking bulbous crops such as onion, garlic, and tulip (Conijn *et al*. 1996). *A. tulipae* is widespread around the world, with infestations recorded from 33 countries to date, representing all continents except Antarctica (Perring, 1996; Navia *et al*., 2010). Infested plant

parts are affected in multiple ways due to the activity of eriophyoid mites on the surface of bulbs and leaves. The feeding of eriophyoid mites on epidermal cells induces damage and deterioration of epidermal tissue and promotes virus transmission (Stenger *et al*., 2016). Consequently, bulb drying or decay, leaf twisting, curling, and discoloration of *Allium* crops are often observed (Lange, 1955; ChannaBasavanna, 1966; Debnath and Karmakar 2013). It was reported that high infestations of garlic cloves can result in a 32 % loss in bulb crops (Budai *et al*., 1997; Debnath and Karmakar 2013).

High population densities of the dry bulb mite have been reported on the bulb segment and middle sections of leaves (Sapáková *et al*., 2012; Beltran 2020). Once established on bulbs or leaves, mites can develop high population density. Courtin *et al*. (1999) reported that the mite develops from egg to egg within eight days under ideal environmental conditions (25 °C, 80 % relative humidity) and in this time develops four stages: eggs, larva, nymph, and adults, what is typical for eriophyid (Manson and Oldfield 1996). Moreover, this pest may easily spread by crawling to neighboring plants, through insects, airflow, and irrigation water (Debnath and Karmakar 2013; Kiedrowicz *et al*., 2017).

*Allexivirus* transmission probably occurs during storage of bulbs, where the mite *A. tulipae* can proliferate to enormous amounts, thus viruses can be spread very effectively, but also the mite may feed on growing leaves and flowers. According to Van Dijk and Van Vlugt (1994), *A. tulipae* was found infesting shallot, rakkyo and garlic bulbs, and their number increased during storage of bulbs causing more damage and one single clove can contain thousands of mites (Sapáková *et al*., 2012).

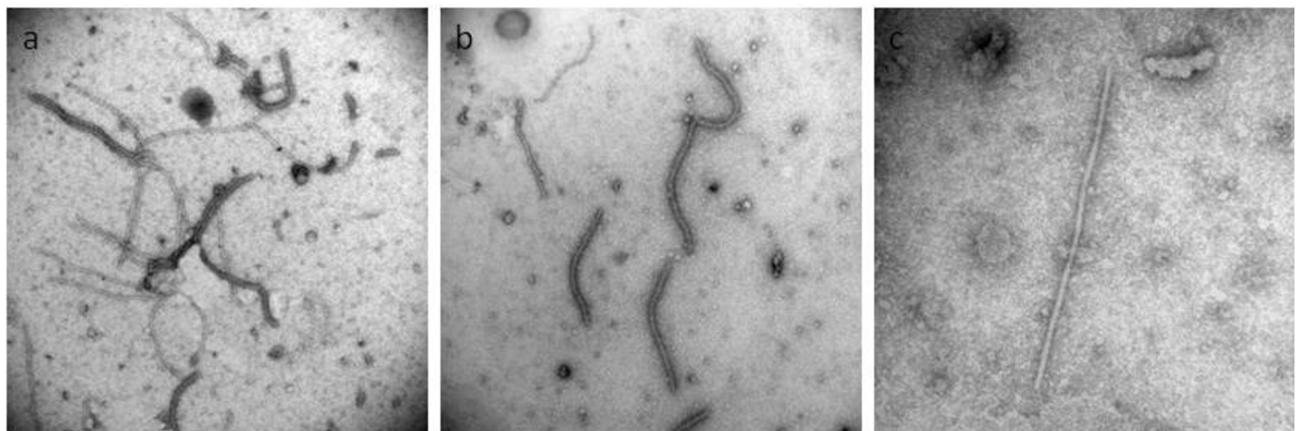


**Figure 4** Transmission electron microscopy showing particles of *Allexivirus* extracted from mites; **a:** ISEM+DECO GarV-B (42000X), **b:** ISEM+DECO GarV-D (42000X), **c:** Dip preparation from mites (110000x). (© JKI)

Garlic virus B, -C, -D and -X have been shown to be successfully transmitted to leek plants (*A. ampeloprasum*) by *A. tulipae* (Dąbrowska *et al*., 2020). To date, details about the acquisition time, persistence of the virus in the vector and effectiveness of the transmission have not been clarified. It was only reported that garlic mite-borne mosaic viruses are only acquired by first- and second-instar nymphs (Yamashita *et al*., 1996). On the other hand, several studies on eriophyid mites have described their transmission of plant viruses as semi-persistent (Gispert *et al*., 1998; Kulkarni *et al*., 2002), thus, it is possible that the transmission manner of *Allium*-infecting allexiviruses is similar to other eriophyid mites. Moreover, Ahmed and Benigno, 1985 published a detailed study of a mite-borne virus and the different developmental stages of mites in virus transmission in garlic. According to these experiments, allexiviruses can be transmissible by nymphs and adults of *A. tulipae* and the efficiency of transmission increased with the increase of the acquisition-feeding period on infected plants (Yamashita *et al*., 1996; Ahmed and Benigno, 1985). However, Ahmed and Benigno, 1985 concluded that once mites acquired the virus, they could transmit it up to 8 days, then transmission of the virus will be declined with the increase of days. Almost all previous authors proved only generally transmissibility of allexiviruses by mites without giving further important details about the acquisition time, persistence of the virus in a vector, and effectiveness of the transmission.

Dissemination of infected propagation materials and subsequent transmission by grafting and vectors are largely responsible for the worldwide deterioration of the sanitary status of many crops associated with many virus species. In vegetatively propagated hosts, virus dissemination takes place primarily through the distribution of infected propagative materials such as bulbs, offshoots, propagating stocks, and nursery stocks and this is a pathway for allexiviruses (Mandal *et al*., 2017). Such means of propagation are usually used by farmers to grow vanilla and blackberry, which can lead to the dissemination VLV and BVE. It is suspected that BVE can propagate through root cuttings given the fact that blackberry yellow vein-associated virus (BYVaV, genus *Crinivirus*), a virus found in mixed infection with BVE in blackberry, is propagated through root cuttings and spread in nursery stock (Susaimuthu *et al*., 2007).

Alfalfa and forage peanut are considered to be a natural reservoir of viruses and important in disease spread (Van Leur and Kumari, 2011), which can play a role in the introduction of ApV and AVS in legumes. Although alfalfa and forage peanuts are more frequently propagated from seeds, seed transmission of allexiviruses is not reported. The vector of some *Allexivirus* members has not been

determined to date, whether *A. tulipae* is a potential vector of BVE, ApV, AVS, VLV, SSYMV and the unassigned virus (PaVA) is an essential question that has not been answered.

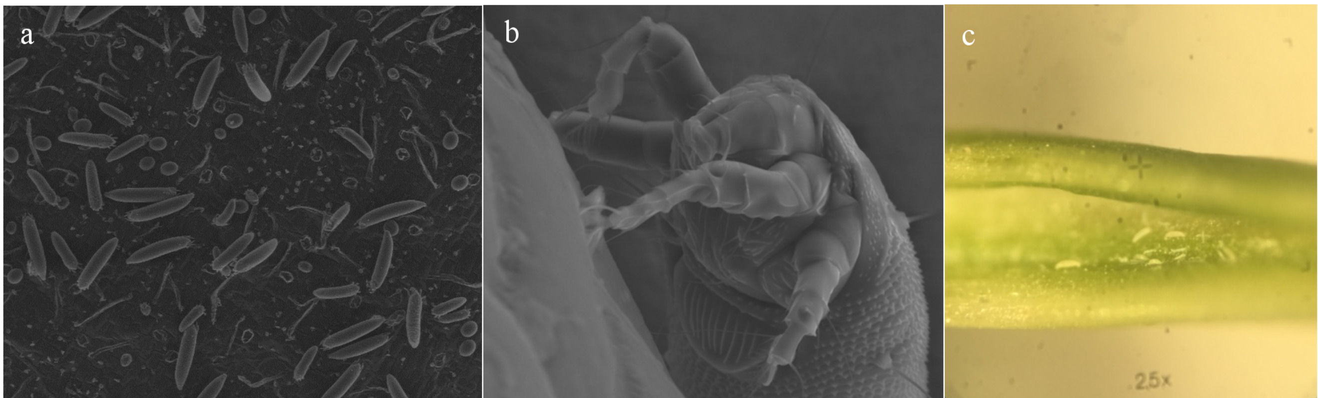


**Figure 5** Scanning electron and light microscopy of garlic bulb infested with *Aceria tulipae*: **a:** different stages of mites including eggs, larvae, nymphs and adults, **b:** mite female adult, **c:** garlic leaf infested with mites. (© JKI)

### 2.2.4 Geographical distribution

To date, *Allium*-infecting allexiviruses have been recorded in all continents, except Antarctica in *Allium* producing regions (Table 3), whereas ApV, AVS, BVE and VLV have not been reported outside of the countries in which they were first described (Sabanadzovic *et al*., 2011; Gutiérrez Sánchez *et al*., 2016; Grisoni *et al*., 2017; Nemchinov *et al*., 2017). However, it is most likely that allexiviruses are more broadly distributed than currently known, especially where their host plants are cultivated.

**Table 3** Geographical distribution of members of *Allexivirus* genus

| Location | Detected viruses | References |
|---|---|---|
| China | GarV-A, -D, -E, -X, ShVX, GarV-B | Chen *et al*., 2001, 2004 |
| Japan | GarV-A, -B, -C, -D, GarMbFV | Sumi *et al*., 1993; Yamashita *et al*., 1996 |
| South Korea | GarV-A, -B, -C, -D, -E, -X | Kang *et al*., 2007; Lee *et al*., 2007 |
| India | GarV-A, -C, -D, -X, GarMbFV, ShVX | Mandal *et al*., 2017 |
| Iran | GarV-A, -B, -C, -D, ShVX | Shahraeen *et al*., 2008 |
| Russia | ShVX | Kanyuka *et al*., 1992 |
| Turkey | GarV-B, -D, -X, GarMbFV | Fidan, 2010; Fidan *et al*., 2013 |
| Italy | GarV-B, -D, -X, ShVX | Taglienti *et al*., 2017 |
| Poland | GarV-A, -B, -D, -X, GarMbFV, ShVX | Bereda *et al*., 2017 |
| France | ShVX | Marais *et al*., 2019 |
| Greece | GarV-C, -D, GarMbFV | Dovas *et al*., 2001 |
| Czech Republic | GarV-A, -B, -C, -D, -E, -X, GarMbFV, ShVX | Klukáčková *et al*., 2007 |
| Slovenia | GarV-A, -B, -C, -D, -E, -X, GarMbFV, ShVX | Mavrič and Ravnikar, 2005 |
| Spain | GarV-B, -D, -X | Tabanelli *et al*., 2004 |
| United Kingdom | ShVX | Ryabov *et al*., 1996 |
| Netherlands | OMBLV, SMbLV | Van Dijk *et al*., 1991 |
| Argentina | GarV-A, -B, -C | Cafrune *et al*., 2006a |
| Mexico | GarV-D | Rocha and Esmeralda, 2019 |
| Brazil | GarV-A, -B, -D, -X, GarMbFV, SSYMV | Oliveira *et al*., 2014 |
| Colombia | ApV | Gutiérrez Sánchez *et al*., 2016 |
| Ecuador | ShVX | Granda *et al*., 2017 |
| USA | GarV-B, -C,-D, -E, ShVX, AVS, BVE | Gieck *et al*., 2009; Sabanadzovic *et al*., 2011; Wijayasekara *et al*., 2019 |
| Sudan | GarV-A, -B, -X, ShVX | Mohammed *et al*., 2013; Hamed *et al*., 2012 |
| La Reunion | VLV | Grisoni *et al*., 2017 |
| Ethiopia | GarV-B, -C, -D, -X | Jemal *et al*., 2015; Abraham *et al*., 2019 |
| DR Congo | GarV-D | Majumder *et al*., 2019 |
| New Zealand | GarV-A, -B, -D, ShVX | Perez-Egusquiza et al., 2008; Ward *et al*., 2009 |
| Australia | GarV-A, -B, -C, -D, -E, -X | Wylie *et al*., 2014; Nurulita *et al*., 2020 |

*Allium*-infecting allexiviruses have been widely studied and have been detected worldwide (Table 3), infecting primarily garlic, onion and other vegetable, wild and ornamental alliums. Several surveys identified allexiviruses on garlic from China, Spain, Argentina, and other countries that import garlic bulbs (Melo-Filho *et al.*, 2004; Wylie *et al.*, 2014; Bereda *et al.*, 2017). The exchange of plant materials, mainly vegetatively propagated species, could be a reason for the widespread introduction of new virus species, as well as new isolates of previously characterized viruses in new geographical areas.

## 2.3 Control and management

Allexiviruses present distinct challenges for the control and management of their spread and emergence in several crops. They are not seedborne, but these viruses have been introduced in different countries via infected plant material. Control management such as diagnosis, sanitation, sanitary certification, plant resistance and vector management are all key factors for the appropriate control of these diseases. Furthermore, diverse approaches, such as conventional breeding, transgenic approaches, and gene silencing strategies, have been used to control RNA viruses (Chaudhary, 2018); hence, it is important to study the biology of viruses to be able to apply these measures and limit the spread of viruses.

According to several studies, the use of virus-free propagative planting material is one of the most effective method that helps controlling viruses and increasing yield (Torres *et al.*, 2000; Salomon, 2002). In-vitro tissue culture techniques like micro-propagation, meristem culture, thermotherapy, chemotherapy, cryotherapy, and somatic embryogenesis have been used for the multiplication of virus-free propagules (Conci and Nome, 1991; Nagakubo *et al.*, 1997; Torres *et al.*, 2000; Senula *et al.*, 2000; Ayabe and Sumi, 2001; Ramirez–Malagon *et al.*, 2006; Manjunathagowda *et al.*, 2017). The embryonic stem cells are the most suitable tissue for initiation of virus-free culture. Although these methods have been successfully used for the elimination of carla- and potyviruses, it is hard to produce 100 % elimination of allexiviruses (Yamashita *et al.*, 1996). Elimination efficiencies of viruses by meristem tip culture of garlic can depend on the type of the virus, it can vary from 54 % to 100 % (Verbeek *et al.*, 1995) and the rate of virus-free plants was improved when combined with meristem tip culture (Conci and Nome, 1991).

Kamenetsky *et al*. (2015) studied the distribution of allexiviruses in different parts of garlic plants and reported that for the production of virus-free garlic seed, the flowers, inflorescence, leaves and basal plate are ideal since the amount of RNA was observed to be low in those parts comparing to cloves and roots, which had the highest amount of allexiviruses particles. More virus-free plants are generated from inflorescence meristem, bulbils, and roots than the apical meristem (Xu *et al*., 2001). A reduction of level of GarMbFV were also obtained in plantlets grown from immature bulbils (Ebi *et al*., 2000). While there are no reports about *Allexivirus* free plants, there are traditional and cultural means of reducing the effect of virus, in addition to the above-mentioned practices, including fumigation of infested bulbs after harvest and before storage and cultural practices that help avoid infestation and remove infected materials.

The current knowledge offers little prospect of control of allexiviruses of its geographical spread or of its emergence in new crops. The re-infection of viruses in fields is a major obstacle for utilization of meristem-tip derived seed bulbs and the number of infected plants gradually increased within next growing seasons. The reinfection occurred by vector transmission from nearby infected crops, such as garlic and onion plants (Taglienti *et al*., 2017). Therefore, continuous inspection for vector and strict pest control management are essential throughout. For mite-borne allexiviruses, the use of propergite and dicofol followed by wettable sulfur treatments in the field were significantly successful against *A. tulipae* (Debnath and Bala, 2017), which help reducing the spread of allexiviruses and limit their damage to alliums. Fumigation and the use of sulfur after harvest and before storage is an efficient treatment for reducing mite infestation in stored garlic bulbs (Estrada-Venegas *et al*., 2015). Traditional methods, such as exposing garlic cloves before storage to hot water treatment at temperatures of 35 °C and 40 °C for 15 and 20 minutes, respectively, are effective to reduce mite infestation (Kamali *et al*., 2013; Hammad *et al*., 2017).

To date, there is no report of *in vitro* sanitation and control managements for BVE, ApV, AVS, VLV, SSYMV and the newly unassigned viruses. However, the use of virus-free planting material can be an effective tool for the control of these viruses, in addition to the development of transgenic berry crops and legumes, which has been already reported to limit damaging viruses belonging to different families (Hill *et al*., 1991; Divakaran *et al*., 2008; Martin and Tzanetakis, 2015). Therefore, the use of resistance and transgenic varieties may be a promoting strategy for the control of these allexiviruses. Although limited detection has been reported, more biological research is needed in the mode of transmission of the non-allium allexiviruses.

## 2.4 Other viruses infecting *Allium* species

### 2.4.1 Potyviruses

The family *Potyviridae* (genus *Potyvirus*) is considered as one of the most important taxonomic groups affecting a wide range of monocotyledons/dicotyledons species causing mild to severe disease symptoms. Members of the genus *Potyvirus* are usually the most abundant and cause most of the damage induced. Among the potyviruses affecting *Allium* spp., onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV) have been detected worldwide (Conci *et al*., 1992; Van Dijk, 1993). These viruses cause damage to garlic and onions crops, reducing garlic bulb weight between 24 and 60 % for OYDV and between 17 and 54 % for LYSV (Canavelli *et al*., 1998; Lot *et al*., 1998). In the Czech Republic, both potyviruses OYDV and LYSV were detected in mixed infected shallot plants with an incidence of 53.2 and 59.9 %, respectively (Smékalová *et al*., 2017) and in garlic plants with an incidence of 75.4 % by OYDV and 31.2 % by LYSV (Klukáčková *et al*., 2007). Moreover, two other potyviruses, shallot yellow stripe virus (SYSV) and turnip mosaic virus (TuMV), have been reported but they are less frequent and cause less damage (Van Dijk, 1993; Gera *et al*., 1997; Van der Vlugt *et al*., 1999). SYSV and TuMV incidence in *Allium* crops is lower, and they have been reported only in certain geographic regions (Gera *et al*., 1997; Van der Vlugt *et al*., 1999).

The members of *Potyvirus* have filamentous flexuous particles of 680- 900 nm size, and 11-13 nm in diameter (Fig. 6). The genome of potyviruses is single stranded positive-sense RNA, with approximately 10 Kb in size. The genome contains one ORF which is translated as a large polyprotein (340-368 kDa), that is cleaved into ten functional proteins, (protein) P1, helper component, P3, cylindrical inclusion, viral genomic protein, nuclear inclusion protein A, nuclear inclusion protein B, capsid protein (CP), and two small putative proteins known as 6 K1 and 6 K2 (Riechmann *et al*., 1992). Potyviruses are transmitted by aphids in a non-circulative, nonpersistent and stylet borne manner using a helper Component Proteinase (HC-Pro), which facilitates binding of virus particles to the maxillary stylet of aphids (Blanc *et al*., 1998).

OYDV is one of the important pathogens, causing severe economic losses in *Allium* spp. seeds and bulb production. In onion, the virus causes distortion of flower stems, reduction in the number of flowers and seeds, and bulbs deterioration during storage (Paludan, 1980), whereas, in garlic, OYDV causes stunting of the plants, yellow striping, and chlorotic striping (Van Dijk, 1993). OYDV is

transmitted by over 50 aphid species in a nonpersistent manner with *Myzus ascalonicus*, *M. persicae*, and *Rhopalosiphum maidis* being the most important virus vectors (Van Dijk, 1993).

LYSV is one of the most frequent and important viruses in leek and garlic crops worldwide (Diekmann, 1997). LYSV symptoms depend on virus strain and host genetic background (Van Dijk, 1993). In leek, it causes chlorotic stripes in leaves, and yellowing (Diekmann, 1997). Symptoms are more severe when leeks are coinfected with shallot latent virus (SLV, genus *Carlavirus*) (Paludan, 1980). In garlic, LYSV causes mild streaking and up to severe yellow streaking and reduction of bulb size. LYSV is transmitted experimentally by *Myzus persicae*, *Aphis fabae*, *A. gossypii*, and several other aphid species (Lunello *et al*., 2002).

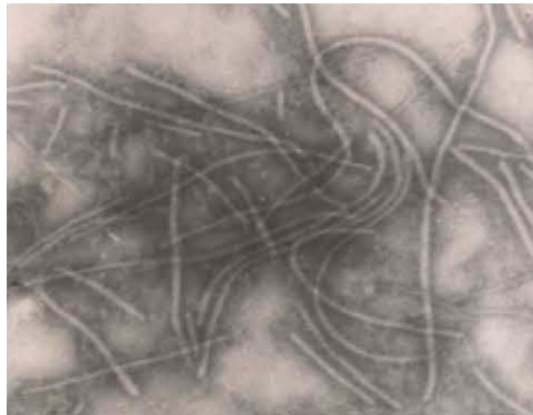

**Figure 6** Electron microscopy of purified filamentous and flexuous OYDV virions particles (Mahmoud *et al*., 2008).

### 2.4.2 Carlaviruses

*Carlavirus* genus (Family *Betaflexiviridae*) have a wide host range within members of the genus *Allium*. They cause latent infections, and limited crops losses (Perotto *et al*., 2010). However, they can cause significant yield losses when the plants are coinfected with potyviruses due to synergistic effects (Celli *et al*., 2016). Among the carlaviruses affecting *Allium* spp.: shallot latent virus (SLV) and garlic common latent virus (GCLV). These viruses are transmitted in a nonpersistent manner by aphids and the transmission is less efficient than potyviruses (Van Dijk, 1993). The two viruses have been detected on garlic and leek worldwide. In the Czech Republic, SLV and GCLV have

been reported to have high incidence of 81.1, and 99.6 %, respectively on garlic and leek (Klukáčková *el al*., 2007).

Members of *Carlavirus* genus have filamentous and flexuous particles of 470-1000 nm size, and 12-13 nm in diameter (Fig. 7). The genome is single stranded positive-sense RNA, with approximately 5.8-9 kb in size (Adam *et al*., 2004). The genomic RNAs have a 3'-poly(A) tract and a 5'-cap. They contain six ORFs including Replicase, TGBs (TGB1, 2, 3), CP, and binding protein.

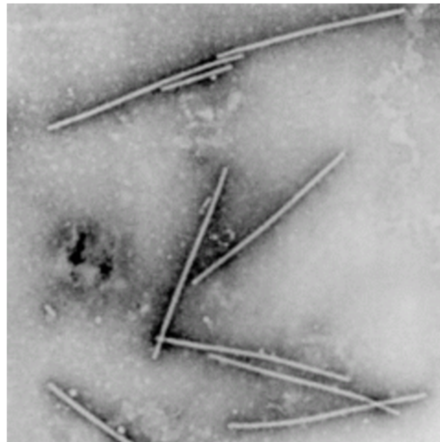

**Figure** 7 Electron microscopy of purified *Carlavirus* particles (Foster *et al*., 2002).

SLV was the first carlavirus detected in *Allium* species (Bos *et al*., 1978). SLV host range is restricted to members of the genus *Allium*, such as onion, leek, garlic, shallot, *A. scorodoprasum*, *A. cepa*, *A. ampeloprasum*, *A. chinense*, and *A. fistulosum* (Van Dijk, 1993). SLV is symptomless and it acts synergistically with potyviruses. In leek, mild chlorotic streaking appears in singly infected plants and severe chlorotic or white streaking and even plant death in plants coinfected with LYSV (Paludan, 1980). SLV is transmitted in the nonpersistent manner by *Aulacorthum solani*, *M. ascalonicus*, *M. persicae*, *Neutoxoptera formosana*, and *A. gossypii* (Sako *et al*., 1990).

GCLV is restricted to *Allium* species, and it infects more than 50 species (Van Dijk, 1993). GCLV is latent in garlic, leek, and onion, but it acts synergistically with potyviruses. SLV and GCLV are widely distributed all over the world where *Allium* species are cultivated, however, GCLV incidence is usually higher than SLV (Barg *et al*., 1994).

### 2.4.3 Orthotospoviruses

Orthotospoviruses are plant-infecting members of the family *Tospoviridae*. These viruses have a broad host range infecting more than 1090 plant species over 90 families (Parrella *et al*., 2003) that include several important vegetables, legumes, ornamental crops, and weeds. They are transmitted by polyphagous thrips in a circulative-propagative manner (Mumford *et al*., 1996). Among the orthotospoviruses affecting *Allium* spp.: Iris yellow spot virus (IYSV) and Impatiens necrotic spot virus (INSV) were reported.

The genome of orthotospoviruses is tripartite of negative or ambisense polarity single strand RNA (King *et al*., 2012). Orthospoviruses have enveloped isometric virions of 80- 120 nm (Fig. 8) containing three segments of single stranded RNA [small (S), medium (M) and large (L) RNA] coated with nucleocapsid protein (N) (Mumford *et al*., 1996). The L RNA is of negative polarity and, after transcription, is translated into the RNA-dependent RNA polymerase. Segment M is ambisense and encodes the precursor of two glycoproteins (Gn and Gc), and non-structural protein M (NSm) involved in cell-to-cell movement in plants (Feng *et al*., 2006). Segment S is ambisense and encodes the nucleocapsid (N) protein and non-structural protein S (NSs), which is a suppressor of gene silencing (Garcia-Ruiz *et al*., 2018). Segments M and S contain two non-overlapping open reading frames in opposite polarities that separated by an intergenic region (253–620 nt long) that is highly rich in A and U stretches, and folds into a stable hairpin structure (Hedil *et al*., 2014).

IYSV is the major constraint for the production of onion and garlic in some countries (Pappu *et al*., 2009). It was first identified in the USA in 1989, later the virus was reported in other countries (Hall *et al*., 1993; Gent *et al*., 2006). The symptoms of IYSV include chlorotic spindle or diamond shaped lesions on the leaves, and necrotic patches on leaves and scapes (Gent *et al*., 2006). These symptoms are more visible on older leaves and as the disease progresses (Gawande *et al*., 2014). The natural hosts of IYSV include onion, garlic, and chives. On experimental hosts, the virus induces chlorotic local lesions in *C. amaranticolor* and necrotic lesions in *Datura stramonium, D. metel* and *D. alba*, whereas systemic chlorosis, curling and twisting in *Nicotiana rustica* (Ravi *et al*., 2006). The major vector of IYSV is *Thrips tabaci* but also can be transmitted by *Frankliniella fusca* with lower efficiency (Srinivasan *et al*., 2012).

For the *Tospovirus* INSV, it was formerly called *Tomato spotted wilt virus*-Impatiens strains, isolated from *Impatiens* spp. in 1989 (Lebas *et al*., 2008). Later, the virus was considered to be distinct to TSWV due to the different symptoms induced on host plants and INSV has a serologically distinct nucleocapsid protein, N protein with different size and no homology of the S and M RNAs (Vaira *et al*., 1993). INSV is an economically important pathogen in a broad host range of ornamental plants (Shahraeen *et al*., 2002). INSV has been reported to infect more than 300 plant species, including some weed species (Mertelík *et al*., 2000). The nature of symptoms depends on the virulence of virus strain, host plant, and environmental conditions and include necrosis, chlorosis, ring patterns, mottling, stunting, and death of the plant (Lebes *et al*., 2008). The most effective vector of INSV is the Western flower thrips, *F. occidentalis* (Ullman *et al*., 1998). INSV spreads rapidly if the vector is present, resulting in high level of infection. INSV is also transmitted by *F. fusca* and *F. intonsa* but less efficiently.

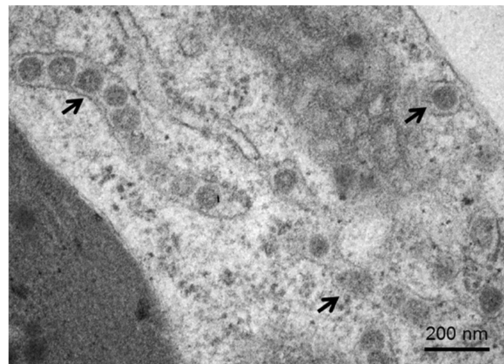


**Figure 8** Vesicles (arrows) containing tospovirus particles observed by transmission electron microscopy of symptomatic onion leaf tissue (Montero-Astúa *et al*., 2017).

# 3 HYPOTHESES

- Wide range of *Allium* species (cultivated, wild, and ornamental), growing in different habitat in the Czech Republic is infected by viruses. These viruses often occur in multiple infections in naturally infected *Allium* plants and belong to different genera including *Allexivirus*, *Carlavirus*, *Potyvirus,* and *Orthotospovirus*. The infection rate of *Allium* samples depends on the origin and phylogenetic of the hosts.

- Allexiviruses are transmitted to *Allium* species by eriophyoid mites *Aceria tulipae*. *Aceria tulipae* egg stages are virus free. Mode of mite transmission and its persistence influence transmission characteristics of the virus.

# 4 AIMS OF THESIS

The aims of the PhD thesis are:

- To investigate the incidence of allexiviruses, in addition to viruses belonging to the genera *Potyvirus*, *Carlavirus*, and *Orthotospovirus* occurrence in six habitat categories in the Czech Republic.

- To identify potential *Allium* hosts of the above-mentioned viruses and to analyze how often do coinfections by different viruses occur in *Allium* host and what virus species participate in such coinfections.

- To study the transmission characteristics of allexiviruses, including acquisition, retention, and inoculation period by their vector Eriophyid mite, *Aceria tulipae*.

# 5 MATERIALS AND METHODS

## 5.1 Experiment 1: Distribution and host range of fifteen viruses in the Czech Republic

### 5.1.1 Plant material and sampling

The survey of viruses infecting *Allium* species was carried out during three growing seasons (2016-2018) in four different regions of the Czech Republic. *Allium* species were sampled without symptom assessment from the following regions: Prague city, Průhonice near Prague, Brno city, and Olomouc city (Fig. 9). Samples were taken during the vegetation period (May–August) from six habitat categories: botanical gardens; private gardens; cultivated fields; a collection of wild species originally sampled in wild and cultivated for a short period (2 years) at Palacký University Olomouc (UPOL) experimental garden; wild origin; and some ornamental species were bought from farmer's market (Table 4). In total, 883 samples from 65 *Allium* species were collected from different localities within the abovementioned regions and habitats. *Allium* species collected during the survey belonged to the different subgenera (*Allium, Amerallium, Anguinum, Butomissa, Cepa,Cyathophora, Melanocrommyum, Nectaroscordum, Polyprason, Porphyroprason, Reticulatobulbosa, Rhizirideum*) (Table 12).

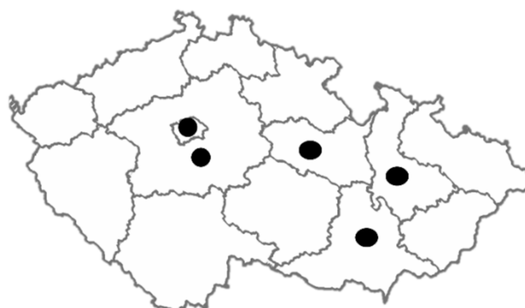

**Figure 9** Map of the Czech Republic showing the areas surveyed for *Allium* viruses.

Sampled *Allium* plants were determined at the (sub)species level by Dr. Krahulec, CSc and Dr. Duchoslav, put separately into labeled bags, and stored at 5 °C for further serological assays. Sample size differed depending on the abundance of each plant species in each study site. A single fully developed young leaf was collected from each plant. For species growing in clumps, several leaves

from the same clump were sampled and tested together. Garlic (*A. sativum*) plants from cultivated fields were collected as reference material since garlic is usually heavily infected (Klukáčková *et al.*, 2007). Leek (*A. porrum*) and onion (*A. cepa*) plants from different fields were also sampled. All sampled plants were analyzed first by ELISA for the presence of eleven viruses, and later tested by RT-PCR to confirm virus presence. Since commercial antibodies are not available for the detection of four allexiviruses (GarV-D, -E, -X, and GarMbFV), all samples were tested using RT-PCR for the detection of the four allexiviruses.

**Table 4** Sources and geographical distribution of the species collected during the survey.

| Habitat categories | *Allium* species (Nb of samples collected) | Site |
|---|---|---|
| University botanical gardens | *A. altaicum* (1), *A. anisopodium* (2), *A. atropurpureum* (5), *A. bidentatum* (1), *A. caesium* (5), *A. callimischon* (1), *A. cernuum* (1), *A. cristophii* (5), *A. cyathophorum* (1), *A. flavum* (3), *A. fistulosum* (5), *A. haematochiton* (3), *A. hybridum* (8), *A. insubricum* (2), *A. karataviense* (5), *A. macrostemon* (2), *A. moly* (5), *A. mongolicum* (1), *A. platycaule* (1), *A. runyonii* (2), *A. scabriscapum* (5), *A. schubertii* (7), *A. sphaerocephalon* (1), *A. thunbergii* (6), *A. zebdanense* (1) | Prague |
| | *A. angulosum* (3), *A. cernuum* (4), *A. carinatum* (16), *A. cyathophorum* (9), *A. ericetorum* (2), *A. flavum* (6), *A. giganteum* (2), *A. karataviense* (1), *A. ledebourianum* (4), *A. longicuspis* (1), *A. macrostemon* (4), *A. macrorrhizum* (2), *A. moly* (7), *A. nutans* (6), *A. oreophilum* (5), *A. oleraceum* (8), *A. rotundum* (4), *A. sativum* (10), *A. stipitatum* (3), *A. schoenoprasum* (3), *A. scorodoprasum* (2), *A. senescens* subsp. *montanum* (11), *A. tuberosum* (5), *A. thunbergii* (2), *A. ursinum* (5), *A. victorialis* (6) | Brno |
| Private gardens | *A. altaicum* (1), *A. angulosum* (4), *A. bucharicum* (1), *A. bulgaricum* (5), *A. carinatum* (3), *A. cepa* (8), *A. flavum* (1), *A. fistulosum* (3), *A. ledebourianum* (2), *A. moly* (1), *A. porrum* (28), *A. sativum* (25), *A. schoenoprasum* (2), *A. scorodoprasum* (1), *A. senescens* subsp. *montanum* (6), *A. vineale* (1), | Prague |
| | *A. cernuum* (5), *A. cristophii* (1), *A. flavum* (2), *A. fuscum* (1), *A. hollandicum* (4), *A. sphaerocephalon* (1), *A. nutans* (1), *A. oleraceum* (6), *A. paradoxum* (5), *A. rotundum* (1), *A. schoenoprasum* (4), *A. scorodoprasum* (5), *A. senescens* subsp. *montanum* (4), *A. ursinum* (5), *A. victorialis* (1), *A.* × *proliferum* (3), | Průhonice |
| | *A. cyathophorum* (1), *A. flavum* (9), *A. microdictyon* (1), *A. narcissiflorum* (3), *A. neapolitanum* (1), *A. nigrum* (2), *A. nutans* (2), *A. oleraceum* (5), *A. przewalskianum* (1), *A. pskemense* (2), *A. ramosum* (3), *A. roseum* (1), *A. suaveolens* (2), *A. stipitatum* (2), *A. tuberosum* (6), | Brno |
| Wild origin | *A. sphaerocephalon* (3), *A. rotundum* (1), *A. scorodoprasum* (3), *A. senescens* subsp. *montanum* (1), *A. strictum* (2), *A. vineale* (7) | Říp Hill |
| | *A. paradoxum* (23), *A. oleraceum* (17), *A. vineale* (20) | Průhonice park |
| | *A. oleraceum* (5), *A. senescens* subsp. *montanum* (9), *A. strictum* (3), *A. vineale* (1) | Prague |

| Collection of species | *A. angulosum* (1), *A. flavum* (2), *A. oleraceum* (20), *A. porrum* (3), *A. rotundum* (4), *A. sativum* (3), *A. senescens* subsp. *montanum* (7), *A. sphaerocephalon* (3), *A. scorodoprasum* (10), *A. strictum* (1), *A. vineale* (11) | UPOL |
|---|---|---|
| Bought ornamentals | *A. caeruleum* (10), *A. cernuum* (4), *A. cristophii* (1), *A. fistulosum* (1), *A. hollandicum* (3), *A. porrum* (15), *A. sativum* (5), *A. sphaerocephalon* (40), *A. stipitatum* (3), *A. tuberosum* (10), *A. senescens* subsp. *montanum* (10) | Farmer's market |
| Cultivated fields | *A. cepa* (18), *A. porrum* (83), *A. sativum* (81) | CZU |

### 5.1.2 Virus detection by ELISA and RT-PCR

The detection of eleven viruses was first carried out using commercial antibodies (Leibniz Institute DSMZ, Germany and LOEWE Biochemica, Germany) in accordance with the manufacturer's instructions. Double antibody sandwich ELISA (DAS-ELISA) was performed for the detection of GarV-A, GarV-B, GarV-C, SHVX, GCLV, OYDV, LYSV, and SLV and Triple antibody sandwich ELISA (TAS-ELISA) for the detection of IYSV, INSV, and SYSV. Leaves were homogenized using mortar and pestle in extraction buffer in a ratio 1:10. Positive and negative controls supplied by the respective manufacturer were used to check the reliability of the reaction. Optical density of ELISA plates was measured at 405 nm using Sunrise reader (Tecan).

For further virus detection and to confirm ELISA tests, total RNA was extracted from each sample using the modified silica capture method described by Malinowski (1997). A final RNA precipitate was resuspended in 50 µl deionized water. The concentration of RNA was determined spectrophotometrically. Two-step RT-PCR was done using specific primers (Table 13) for the detection of different virus species. Reverse transcription was performed using approximately 750 ng of total RNA in a 50 µl mixture containing 1×First-Strand Buffer (Invitrogen™, Life Technologies, Gaithersburg, MD, USA), 0.5 µg random hexamers (Roche Diagnostics, Mannheim, Germany), 0.5 mM dNTP, 4 mM DTT (Invitrogen™, Life Technologies) and 140 U Mu-MLV Reverse Transcriptase (Invitrogen™, Life Technologies) for 55 min at 42 °C, with a final incubation at 70 °C for 10 min. PCR was carried out in the PTC 200 thermal cycler (BIORAD) and the cycling parameters were as follows: Initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at different temperatures according to the primers used (Table 13), extension at 72 °C for 45 s, and a final extension at 72 °C for 7 min. The PCR-amplified fragments were visualized after electrophoresis on ethidium bromide-stained 1.5 % agarose gels. Positive

controls from the manufacturers of ELISA kits were used in most cases, while garlic infected by GarMbFV, and GarV-X was kindly provided by Dr. M. Bereda (Poland).

### 5.1.3 Statistical analysis

Associations between viruses were compared by Fisher's exact test (two-sided) and the chi-square test. Results were considered significant if $p<0.05$ using IBM SPSS Statistics software. Partial correspondence analysis (pCA; Legendre and Legendre, 1998) was used to find the main gradients in the distribution of viruses on their hosts and similarities among *Allium* taxa and virus species incidence. The number of occurrences of each virus was used as the species data, *Allium* species were considered as samples, and the number of analyzed samples per *Allium* species was considered as a covariate in the analysis. Only those *Allium* species infected with at least one virus (57 species in total) were analyzed.

Partial canonical correspondence analysis (pCCA; Legendre and Legendre, 1998) was used to test for potential effects of *Allium* phylogeny and origin of samples (habitats) on the virus incidence and composition within the dataset. Each studied *Allium* species was classified into the respective subgenera, representing simplified information about phylogenetic relationships between taxa (Friesen *et al.*, 2006). Samples were classified according to their origin into 6 habitat categories (botanical gardens; private gardens; field cultivation; ornamentals from markets; cultivated plants of wild origin; wild origin). We used two separate pCCAs with either subgenus identity or sample origin (habitats) as an explanatory variable and the remaining variable as a block in the analysis to disentangle the effect of both variables on the virus species composition in *Allium* samples. Additionally, the number of analyzed samples per *Allium* species was considered as a covariate in all analyses to reduce the effect of unequal sampling size per *Allium* species and locality. Forward selection of explanatory variables was implemented to obtain a subset of available explanatory variables with significant effect; each of the levels of the explanatory variables was treated as a separate predictor in the analyses. Multivariate analyses were performed in Canoco 5 (ter Braak and Šmilauer, 2012).

## 5.2 Experiment 2: Transmission characteristics of allexiviruses by the eriophyid mite, Aceria *tulipae* (Keifer) (Acari: Eriophyidae)

### 5.2.1 Plant material

Leek plants (*Allium porrum* L.) were established from seeds, grown, and maintained in growth chambers at 25 °C on a 16 hr (hours) photoperiod, with a constant 80 % relative humidity (RH). Colony plants were regularly tested for allexiviruses and checked for possible contamination by mites. None of these plants was found infected with allexiviruses during the study.

All experimental plants were covered individually with mite-proof polyamide cages with 40µm in diameter mesh and maintained in separate growth chambers operating under the above-described temperature and light conditions (Fig. 10). Garlic bulbs bought from a farmer's market were screened for the presence of mites and tested for allexiviruses. Several cloves with high mite infestations (> 1000 mites per clove) were placed in paper bags and kept in a dark storage room (25 °C, 80 % RH) for further experiments. To obtain mite-free garlic plants, garlic cloves were put into hot water at temperatures of 40 °C for 20 minutes, to eliminate mite infestation. Each garlic clove was then planted and caged. Infected garlic plants were put into a separate growth chamber (25 °C, 80 % RH); later garlic leaves were checked for mite presence, tested for allexiviruses, and used for further experiments.

**Figure 10** Mite-proof polyamide cages used during mite transmission experiments.

### 5.2.2 Mite rearing

Mites used during experiments were obtained from infested garlic cloves bought from a farmer's market and stored in paper bags, as described above (Fig. 11). In all transmission experiments, mites were handled using a human eyelash affixed to a six cm-long wooden stick under a stereomicroscope. Adult mites were carefully taken to avoid their injury and transferred to young leek leaves under an adjacent stereomicroscope. Leek plants were immediately covered with mite-proof polyamide cages and carefully moved to growth chambers (25 °C, 80 % RH).

To obtain nonviruliferous *A. tulipae*, eggs were collected from garlic cloves and transferred to allexiviruses-free and mite-free leek leaves as confirmed by reverse transcription polymerase chain reaction (RT-PCR). For initial culture of *A. tulipae,* 5×5 mm pieces were cut out from leek leaves and were placed into Plexiglas® plates containing an artificial culture medium. The *in vitro* MS medium was prepared according to the method described by Murashige and Skoog (1962) and modified by Karpicka-Ignatowska *et al.* (2020). Plexiglas plates were covered with white paper to reduce the intensity of light that might damage the eggs and placed in a growth chamber (25 °C, 80 % RH). After seven days, eggs were hatched and nymph and/or adults *A. tulipae* stages were observed. Mites were then carefully transferred onto three-week-old leek plants, caged, and placed into a growth chamber (25 °C, 80 % RH). The same experiment was repeated a few times to obtain enough mite colonies. To ensure that mite colonies originating from eggs, were nonviruliferous, plants were assayed for viruses 21 days post-inoculation (pi) with mites and again 42 days pi. To test the transmission of allexiviruses from mixed infections, viruliferous mites obtained from *Allexivirus*-infected garlic were used in most experiments.

**Figure 11** A single *A. tulipae* taken from infested garlic cloves put into slide containing Berlese buffer (Amrine and Manson, 1996).

### 5.2.3 Transmission tests

Virus-free and mite-free three-week-old leek plants maintained in the growth chamber (25 °C, 80 % RH) were used to determine transmission efficiency, acquisition/inoculation period, and retention of the virus.

#### 5.2.3.1 Acquisition access period

To test the acquisition access period (AAP), nonviruliferous *A. tulipae* were allowed to feed on *Allexivirus*-infected, mite-free garlic leaves for 5 min, 15 min, 30 min, 1hr, 5 hr, 24 hr, and 48 hr. After each acquisition period, a group of 10 mites was transferred onto healthy three-week-old leek seedlings. AAP experiments for each given period were repeated at least two times; In each repetition, five to ten leek plants were used and kept in separate growth chambers. Leek plants were monitored for the absence of mites/symptoms and assayed for allexiviruses three weeks pi. For each experiment, two plants were used as negative control.

#### 5.2.3.2 Inoculation access period

Virus-free and mite-free leek seedlings were also used to determine the inoculation access period (IAP). A group of 10 mites was transferred from infected garlic to individual healthy leek seedlings and allowed to feed for 30min, 1, 5, 24, and 48 hr. Following the respective time, mites were terminated by spraying plants using Substral acaricide (active ingredient acetamiprid used at 0,005 % concentration).

IAP experiments for each given period were repeated at least two times; In each repetition, five to ten leek plants were used and kept in separate growth chambers. Leek plants were monitored for the absence of mites/ symptoms and assayed for allexiviruses three weeks pi. For each experiment, two plants were used as negative control.

#### 5.2.3.3 Virus retention

For virus retention, nonviruliferous mites were fed on *Allexivirus*-infected garlic bulbs for 24 hr. A group of 10 mites were then transferred serially three times to healthy leek seedlings for IAP of 1, 5, and 24 hr. Five plants were used for virus retention in each transfer; plants were caged and assayed for allexiviruses 3 weeks pi.

#### 5.2.3.4 Transmission efficiency

To determine the number of mites required to obtain 100 % virus transmission, adult mites reared on *Allexivirus*-infected garlic cloves were transferred onto healthy three-week-old leek seedlings, either singly or in groups of 5, 10, and 20 mites. Mite-inoculated leek plants were then caged and assayed for allexiviruses 3 weeks pi.

### 5.2.4 Detection of viral infection

All experimental plants were assayed for presences of allexiviruses by looking for typical virus symptoms illustrated by mosaic and chlorotic patterns and leaf deformations and confirmed by RT-PCR. RT-PCR tests were first done using degenerate *Allexivirus* primers (Chen *et al*., 2004). Then positive samples were tested using specific primers for the detection of GarV-A, -B, -C, -D, -E, -X, ShVX and GarMbFV (Table 13). Reverse transcription was performed using approximately 750 ng of total RNA in a 50 µl mixture containing 1×First-Strand Buffer (Invitrogen™, Life Technologies, Gaithersburg, MD, USA), 0.5 µg random hexamers (Roche Diagnostics, Mannheim, Germany), 0.5 mM dNTP, 4 mMDTT (Invitrogen™, Life Technologies) and 140 U Mu-MLV Reverse Transcriptase (Invitrogen™, Life Technologies) for 55 min at 42 °C, with a final incubation at 70 °C for 10 min.

PCR was carried out in the PTC 200 thermal cycler (BIORAD) and the cycling parameters were as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at different temperatures according to primers used (Table 13), extension at 72 °C for 45 s and a final extension at 72 °C for 7 min. The PCR-amplified fragments were visualized after electrophoresis on ethidium bromide-stained 1.5 % agarose gels.

# 6 RESULTS

## 6.1 Experiment 1: Distribution and host range of fifteen viruses in the Czech Republic

### 6.1.1 Virus incidence

To determine which viruses were present in *Allium* species collected from the six habitats, all samples were first analyzed using ELISA and positive samples were then confirmed by RT-PCR, while the presence of the allexiviruses (GarV-D, -E, -X, and GarMbFV) was identified only by RT-PCR. Due to various reasons, some samples were not tested for all viruses. In addition, the thrip-transmitted viruses INSV and IYSV were detected in few samples by ELISA, but their presence was not confirmed by RT-PCR. These samples have not been included in the present data. Therefore, data from all plants are presented as the percentage of the samples tested for each virus by ELISA and confirmed by RT-PCR (Fig. 12).

Infection rates varied greatly among the identified viruses. LYSV was the most common with an incidence of 35.0 % (310 infected samples/ 883 tested samples), followed by ShVX (32.8 %, 290/ 883) and GCLV (27.7 %, 245/ 883) (Fig. 12). Allexiviruses were detected with a recorded infection rate of about 20-26 % (GarV-A, 23.6 %, 209/ 883; GarV-B, 25.7 %, 227/ 883; GarV-C, 21.1 %, 187/ 883). Among mite-transmitted viruses, the most limited spread was recorded for GarV-E and GarMbFV, with an incidence of 2.2 % (19/ 830) and 2.9 % (25/ 830), respectively. Carlavirus SLV and potyvirus OYDV were also commonly found in the tested plants with an overall incidence of 19.9 % (176/ 882) and 17.9 % (158/ 883), respectively (Fig. 12). The potyvirus (SYSV) and the orthotospoviruses (INSV and IYSV) have been recorded on alliums for the first time in the Czech Republic with an overall incidence of 8.9 % (79/ 882), 4.1 % (34/ 817), and 1.2 % (10/ 812), respectively. Negative control samples gave no product.

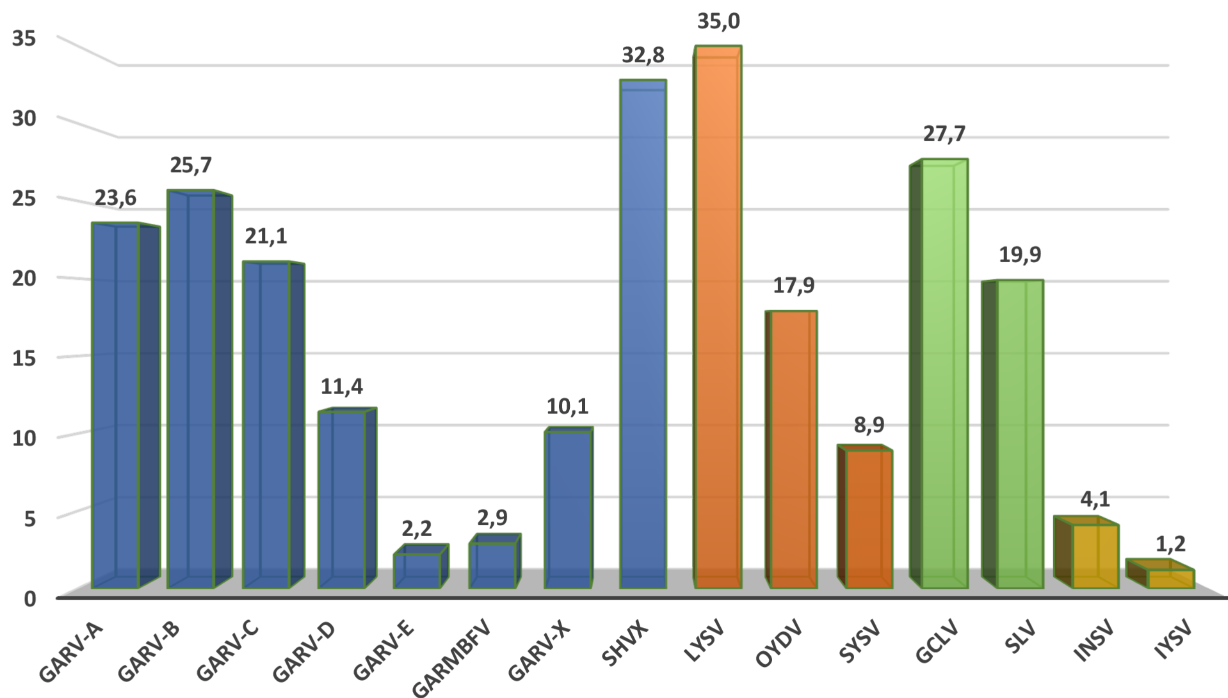


**Figure 12** Incidence of *Allium* viruses in surveyed plants sampled from *Allium*-growing regions.

### 6.1.2 Distribution of viruses among *Allium* species

A total of fifty-seven *Allium* species (57/ 65) were infected with at least one virus. The infection rates differed among samples depending on the habitat origin and host species. The highest infection rate was recorded in species collected from cultivated fields, bought from farmer's market, private and botanical gardens with incidence of 65- 97 %. The lowest incidence was recorded in samples with wild origin and from the species collection at UPOL (Table 5).

**Table 5** Infection rates of *Allium* plants collected in different habitat categories, separately for each sampling sites.

| Habitat categories | Site | Infection rate | Mean of infection |
|---|---|---|---|
| University botanical gardens | Prague | 82 % | 77 % |
| | Brno | 72 % | |
| Private gardens | Prague | 97 % | 84 % |
| | Průhonice | 85 % | |
| | Brno | 70 % | |
| Wild origin | Říp Hill | 29 % | 34 % |
| | Průhonice park | 16 % | |
| | Prague | 0 % | |
| Collection of species | UPOL | 35 % | 35 % |
| Bought ornamentals | Farmer's market | 65 % | 65 % |
| Cultivated fields | CZU | 90 % | 90 % |

Several new virus-host associations were recorded (Table 6). The highest infection rate was registered in garlic (*A. sativum*), onion (*A. cepa*), and leek (*A. porrum*) from cultivated fields with a total incidence of s respectively. In these *Allium* species, SLV and GarV-C were found frequently in addition to ShVX, GCLV, and LYSV (Table 6). However, OYDV was more frequently found in garlic (44.4 %) and onion (34.6 %) in comparison to leek (5.4 %).

High infection rates, mostly exceeding 90 %, were also observed in species collected from botanical gardens, private gardens, and species brought from farmer's markets (e.g., *A. caeruleum*, *A. cernuum*, *A. schoenoprasum*, *A. cristophii*, *A. cyathophorum*, *A. moly, A. sphaerocephalon*, *A. nutans*, and *A. senescens* subsp. *montanum*) (Table 6). LYSV, GCLV, and ShVX were the most detected viruses among these *Allium* species. Besides, a high incidence of GarV-A, -B, -C, and SLV was also recorded in some species (e.g., *A. moly, A. cyathophorum, A. schoenoprasum, A. sphaerocephalon*). GarV-X (74 %) was more commonly found in *A. sphaerocephalon*.

The infection rate was low (5-30 %) in populations of some *Allium* species collected from botanical and private gardens (e.g., *A. carinatum, A. macrostemon*, *A. oleraceum, A. tuberosum, A. paradoxum*, and *A. vineale*). They were infected by only one or two viruses in a single plant. Contrarily, no viral infection was recorded in species with a wild origin and wild species cultivated for

a short period (2 years) in the UPOL collection (e.g., *A. oleraceum, A. tuberosum, A. paradoxum, A. vineale*, and *A. strictum*). No viral infection was detected also in some other cultivated ornamental *Allium* species (e.g., *A. bidentatum, A. callimischon, A. ericetorum, A. fuscum, A. platycaule, A. mongolicum*, and *A. longicuspis*) but more sampling is needed since only a few samples were analyzed per species.

**Table 6** Results of virus screening of *Allium* species collected in the Czech Republic

| ***Allium* species** | **Infected /total** | **GarV-A** | **GarV-B** | **GarV-C** | **GarV-D** | **GarV-E** | **GarMbF** | **GarV-X** | **SHVX** | **LYSV** | **OYDV** | **SYSV** | **GCLV** | **SLV** | **INSV** | **IYSV** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. altaicum* | 1/2 | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - |
| *A. angulosum* | 5/8 | + | - | + | - | - | - | - | + | + | + | - | + | - | - | - |
| *A. anisopodu* | 1/2 | + | + | - | - | - | - | - | + | + | + | - | + | - | - | nt |
| *A. atropurpureum* | 5/5 | - | - | - | + | - | + | + | - | + | - | - | + | - | - | - |
| *A. bidentatum* | 0/1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *A. bucharicum* | 1/1 | + | - | - | - | - | - | - | + | + | - | - | - | - | - | - |
| *A. bulgaricum* | 4/5 | - | - | - | nt | nt | nt | nt | + | - | + | - | + | - | nt | nt |
| *A. caeruleum* | 10/10 | + | + | - | - | - | - | - | + | + | + | nt | + | - | nt | nt |
| *A. caesium* | 5/5 | + | + | + | + | - | + | + | + | + | + | - | + | - | - | - |
| *A. callimischon* | 0/1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | nt |
| *A. carinatum* | 4/19 | + | - | + | nt | nt | nt | nt | + | + | - | - | + | + | - | - |
| *A. cepa* | 24/26 | + | + | + | + | + | + | + | + | + | + | + | + | + | nt | nt |
| *A. cernuum* | 9/14 | + | + | + | - | + | - | + | + | + | + | + | + | + | + | - |
| *A. cristophii* | 7/7 | + | + | + | + | + | - | - | + | + | + | + | + | + | - | - |
| *A. cyathophorum* | 11/11 | - | - | - | nt | nt | nt | nt | + | + | + | + | + | + | - | + |
| *A. ericetorum* | 0/2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | nt |
| *A. fistulosum* | 6/9 | - | + | - | - | - | - | - | + | + | - | - | + | + | - | - |
| *A. flavescens* | 1/2 | - | - | - | nt | nt | nt | nt | - | - | - | - | - | + | - | nt |
| *A. flavum* | 11/23 | + | - | + | + | - | - | + | - | + | - | + | + | - | - | + |
| *A. fuscum* | 0/1 | - | - | - | - | - | - | - | - | - | - | - | - | - | nt | nt |
| *A. giganteum* | 2/2 | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - |
| *A. haematochiton* | 2/3 | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - |
| *A. hollandicum* | 3/7 | - | - | - | - | - | - | - | + | + | - | + | + | + | nt | nt |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. hybridum* | 8/8 | + | + | - | + | - | + | + | + | + | - | - | + | - | - | - |
| *A. insubricum* | 2/2 | - | - | - | - | - | - | - | + | + | - | - | + | - | - | - |
| *A. karataviense* | 5/6 | - | - | - | + | - | + | + | - | + | + | - | - | - | - | - |
| *A. ledebourianum* | 4/6 | + | - | - | nt | nt | nt | nt | + | + | + | - | - | - | - | - |
| *A. longicuspis* | 0/1 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *A. macrorrhizum* | 1/2 | - | - | - | - | - | - | - | - | - | + | - | - | - | - | nt |
| *A. macrostemon* | 1/6 | - | - | - | + | - | - | - | - | - | + | - | - | - | - | - |
| *A. microdictyon* | 1/1 | + | - | + | - | - | - | - | + | + | - | - | - | - | - | - |
| *A. moly* | 13/13 | + | - | - | + | - | - | + | + | + | + | + | + | + | - | - |
| *A. mongolicum* | 0/1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *A. narcissiflorum* | 3/3 | + | + | + | - | - | - | - | + | - | - | - | + | - | - | - |
| *A. neapolitanum* | 1/1 | + | + | + | - | - | - | - | + | + | + | - | + | - | - | - |
| *A. nigrum* | 1/2 | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - |
| *A. nutans* | 9/9 | + | + | + | - | - | - | - | + | + | + | - | + | - | - | - |
| *A. oleraceum* | 8/61 | + | - | + | - | - | - | - | - | - | - | - | + | + | - | - |
| *A. oreophilum* | 5/5 | - | + | + | + | - | - | + | + | - | - | - | + | - | + | - |
| *A. paradoxum* | 2/28 | - | - | - | - | - | - | - | - | + | - | + | - | - | nt | nt |
| *A. platycaule* | 0/1 | - | - | - | - | - | - | - | - | - | - | - | - | - | nt | nt |
| *A. porrum* | 109/129 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *A. przewalskianum* | 1/1 | - | - | - | - | - | - | - | + | + | - | - | - | - | nt | nt |
| *A. pskemense.* | 1/2 | - | - | - | - | - | - | - | - | + | - | - | - | - | nt | nt |
| *A. ramosum* | 3/3 | + | - | + | - | - | - | - | + | + | + | - | + | - | - | - |
| *A. runyonii* | 2/2 | + | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| *A. roseum* | 1/1 | + | - | + | - | - | - | - | - | - | - | - | - | - | nt | nt |
| *A. rotundum* | 5/10 | + | - | + | - | nt | nt | nt | + | + | - | + | + | + | + | + |
| *A. sativum* | 111/124 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *A. scabriscapum* | 3/5 | + | - | - | + | - | - | - | + | + | + | - | + | - | - | - |
| *A. schoenoprasum* | 9/9 | + | + | + | - | - | - | - | + | + | + | + | + | + | - | - |
| *A. schubertii* | 3/7 | + | - | - | + | - | + | + | - | - | + | - | - | - | - | - |
| *A. scorodoprasum* | 19/21 | + | - | - | - | + | nt | + | + | + | + | + | + | + | + | - |
| *A. senescens* | 26/48 | + | + | + | + | - | - | - | + | + | - | + | - | + | + | - |
| *A. siculum* | 48/51 | - | - | - | nt | nt | nt | nt | + | - | + | - | + | - | - | - |
| *A. sphaerocephalon* | 2/8 | - | + | + | + | - | - | + | + | + | + | - | + | - | + | + |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. stipitatum* | 0/6 | + | - | + | - | - | - | - | + | + | - | + | + | + | - | - |
| *A. strictum* | 1/1 | - | - | - | - | - | - | - | - | - | - | - | - | - | nt | nt |
| *A. suaveolens* | 8/8 | + | + | + | - | - | - | - | + | + | + | nt | - | nt | nt | nt |
| *A. thunbergii* | 7/21 | + | - | - | + | - | - | - | - | - | - | - | + | - | nt | nt |
| *A. tuberosum* | 10/10 | + | + | + | - | - | - | - | + | + | - | - | + | - | + | + |
| *A. ursinum* | 3/7 | + | + | + | - | - | - | - | + | + | - | + | + | + | nt | nt |
| *A. victorialis* | 8/40 | - | - | + | - | - | - | - | + | - | - | - | - | - | + | - |
| *A. vineale* | 3/3 | + | - | + | - | - | - | - | - | + | - | - | + | - | - | - |
| *A. x proliferum* | 1/1 | - | + | - | - | - | - | - | + | + | - | - | - | + | nt | nt |
| *A. zebdanense* | 1/1 | + | - | - | - | - | - | - | + | + | + | - | + | + | + | - |

### 6.1.3 Natural occurrence of single and mixed infections

Of the total 883 tested plants, 560 (63.4 %) of them were naturally infected by at least one or more viruses in the same host (Table 7). 109 samples were single infected, accounting for approximately 12.3 % of the total sample. SLV was the most frequent virus in singly infected plants with an incidence of 31.1 % within a single infection (34 single infected/ 109 total single infected), followed by 16.5 % of ShVX (18/ 109) within single infected samples.

Mixed infections, representing 51.1 % of the total sample (451/ 883), were most frequently found among the samples. Double infections were the most common mixed infection with 13.2 % (117/ 883) of the total sample. Fourteen samples were found to be infected with a positive association of ShVX with SLV (11.9 % within double infection, 14/ 117, $p < 0.001$), followed by a positive association of ShVX with LYSV (9.4 % within double infection; 11/ 117, $p = 0.043$). The presence of three and four viruses in one host was detected in 12.0 % (99/ 883) and 10.0 % (93/ 883) of the analyzed plants, respectively. The following viruses showed significantly positive associations within the triple and four virus associations: GarV-A, ShVX, and LYSV (14.1 %, $p < 0.05$); LYSV, GCLV, and SLV (9.1 %, $p < 0.001$), and GarV-A, -B, -C, and -D (18.3 %, $p < 0.001$). The last combination was the most common four-virus association. The occurrence of six, seven, eight, nine, and ten viruses in a single plant was recorded with an overall incidence of 9.4 % (84/ 883) of the total analyzed samples and the combination of viruses was variable.

**Table 7** Natural occurrence of the most frequently detected single and mixed virus infections in *Allium*.

| Single infection and multiple infections | Number of infected plants | Percentage within single or multiple infections (%) |
|---|---|---|
| SLV | 34 | 31.1 |
| ShVX | 18 | 16.5 |
| ShVX + SLV | 14 | 11.9 |
| ShVX + LYSV | 11 | 9.4 |
| GarV-A + ShVX + LYSV | 14 | 14.1 |
| LYSV + GCLV + SLV | 9 | 9.1 |
| GarV-A + B + C + D | 17 | 18.3 |
| LYSV + OYDV + GCLV + SLV | 10 | 10.8 |
| GarV-B + C + D + X + ShVX | 12 | 20.7 |
| GarV-A + C + ShVX +GCLV + LYSV | 8 | 13.8 |

### 6.1.4 Effects of phylogeny and origin of samples on the virus incidence

The results of the multivariate analysis (pCA) of viruses in infected *Allium* taxa showed clustering of LYSV, SLV, GCLV, INSV, GarV-A, and ShVX in the left part of the ordination diagram (Fig. 13a). These viruses frequently occurred in mixed infections in the majority of the analyzed *Allium* species (Fig. 13b, c). The allexiviruses GarV-B, -C, -D, -E, -X, and GarMbFV showed higher dissimilarity both with each other and with other viruses. Their solitary positions within the ordination diagram were related to their increased proportions within mixed infections in several *Allium* species in the right part of the ordination diagram (Fig. 13a, b). This is in line with the significant effects of phylogeny and origin of samples on the virus incidence revealed by pCCA.

*Allium* species of the subgenus *Melanocrommyum* significantly differed in virus species composition from *Allium* taxa of other subgenera (pCCA, forward selection, PseudoF = 4.4, $p = 0.02$; 5.4 % of explained variation after accounting for covariables), having more (GarMbFV, GarV-D, GarV-X) and less (GarV-A, -B, -C) common representation of respective allexiviruses (Fig. 13d). Samples originating from wild were significantly less infected and differed in virus incidence in comparison with samples originating from cultivation, farmer's market, and botanical gardens (pCCA, forward selection, PseudoF = 6.2, $p < 0.001$; 9.0 % of explained variation after accounting for covariables; Fig. 13e).

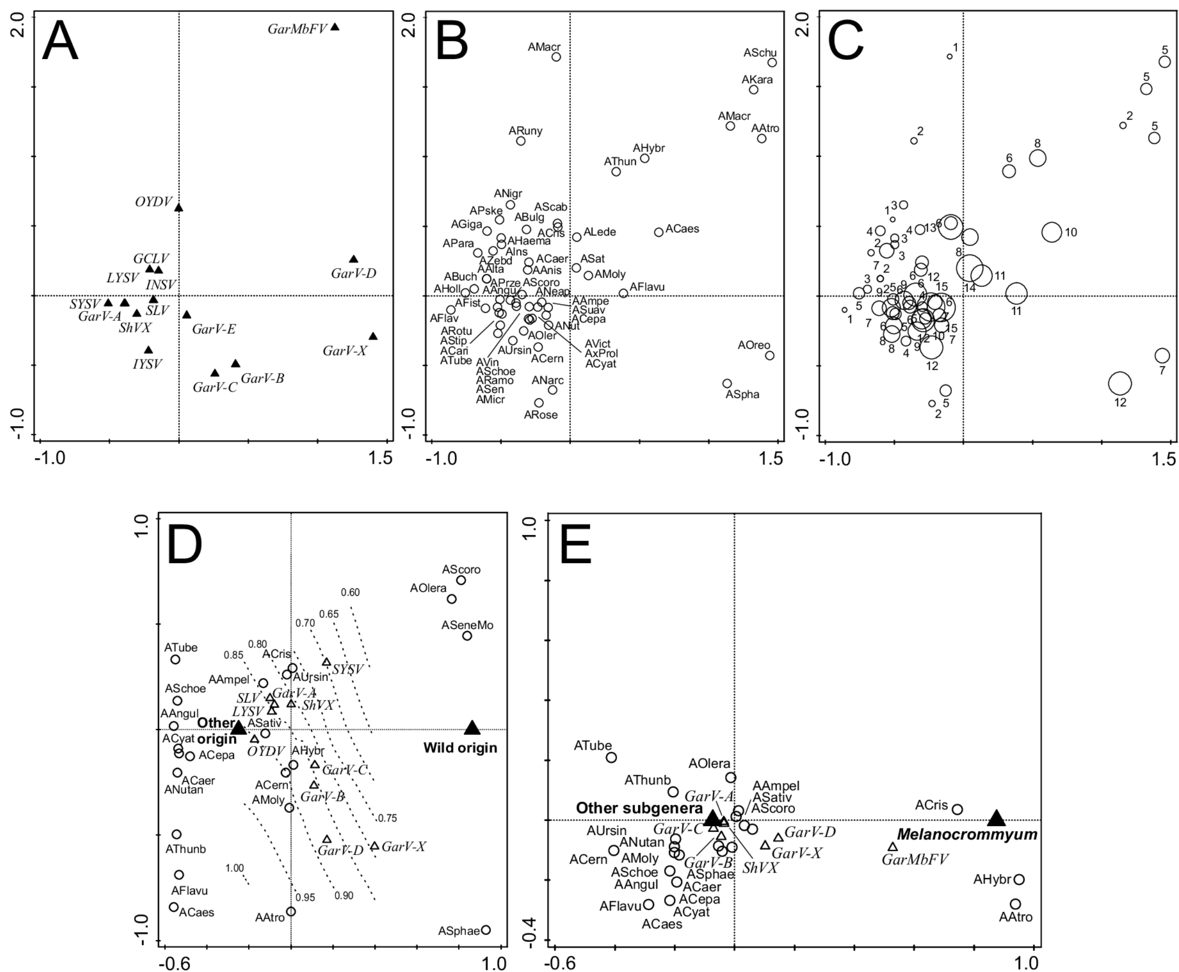


**Figure 13** (**A**) First two axes of the partial correspondence analysis plots (pCA; explained variation: 1st axis - 33.5 %, 2nd axis - 18.6 % after accounting for a covariate) to visualize the viruses characterizing studied *Allium* species. Ordination diagrams of viruses considered as species in the CA. The distance between the symbols representing each virus approximates the dissimilarity between their profiles of values across the *Allium* species as measured by the chi-square distance. Points in proximity correspond to viruses often occurring together. (**B**) Ordination diagram of samples of the same pCA. The distance between the symbols approximates the dissimilarity of their species composition of viruses as measured by the chi-square distance. (**C**) Number of virus species (size of the circle) infecting each of studied *Allium* species. (**D**) First two axes of the partial canonical correspondence analysis plots (pCCA) visualize significant effect of the origin of samples (wild origin vs other types of origin; $p < 0.05$) on the virus species composition in *Allium* species. The distribution of infection rate in the ordination space was visualized by isolines summarizing a fitted loess regression model ($r^2$ = 27.1 %). (**E**) First two axes of the pCCA visualize significant effect of phylogeny of *Allium* species (subgenus *Melanocrommyum* vs other subgenera; $p < 0.05$) on the virus species composition.

## 6.2 Experiment 2: Transmission characteristics of allexiviruses by the eriophyid mite, *Aceria tulipae* (Keifer) (Acari: Eriophyidae)

### 6.2.1 Nonviruliferous *A. tulipae* colonies

Eggs collected from garlic cloves, were carefully transferred to allexiviruses-free and mite-free leek leaves and kept in growth chamber. After few days, nymph or adult mites were transferred to three-week leek seedlings. The mite-inoculated leek plants tested negative for allexiviruses by RT-PCR. Leeks inoculated with *A. tulipae* generated mite colonies within weeks. After two months, high densities of mites were observed and plants showed symptoms triggered by mite feeding and propagation activities such as twisting, curling, and discoloration of leaves (Fig. 14). These nonviruliferous were later used for further transmission experiments.

**Figure 14 (A)** leaf curling caused by *A. tulipae* and mosaic symptoms on leek plants prior to 3 weeks after inoculation with infected mites compared to healthy leek plants on the right side **(B)**; **(C)** colonies of mites observed in the folded leek leaves under stereomicroscope.

### 6.2.2 Acquisition access period

Nonviruliferous mites acquired allexiviruses and transmitted the viruses to two healthy leek plants after 30 minutes (33 % of tested plants) (Table 8). None of the mites provided with a 5- or 15-minute AAP were able to successfully inoculate a healthy leek plant, indicating that a minimum AAP of 30 minutes was required for *A. tulipae* to acquire allexiviruses. When mites were provided with an AAP of 5 and 14 hours, transmission to healthy leek plants increased (40- 91 % of tested plants).

**Table 8** Transmission of allexiviruses to healthy leek seedlings by *A. tulipae* given different AAP and a 24-hour IAP

| | No. of infected plants/ No. of plants (% transmission) | | |
|---|---|---|---|
| **AAP** | **Exp. 1** | **Exp. 2** | **mean (%)** |
| 5min | 0/3 (0) | 0/3 (0) | 0 |
| 15min | 0/5 (0) | 0/5 (0) | 0 |
| 30min | 2/4 (50) | 0/5 (0) | 33 |
| 1hr | 4/5 (80) | 0/5 (0) | 40 |
| 5hr | 3/5 (60) | 1/5 (20) | 40 |
| 24hr | 6/6 (100) | 4/5 (80) | 91 |

### 6.2.3 Inoculation access period

Following different feeding period, mites were terminated by spraying plants with acaricide. After three weeks post inoculation, leeks were tested using RT-PCR for the presence of allexiviruses. The results showed that none of the leek plants exposed to viruliferous *A. tulipae* for an IAP of 30 minutes became infected (Table 9). However, plants exposed to viruliferous *A. tulipae* for more than 1 hr developed curling and mosaic symptoms and these plants tested positive for allexiviruses by RT-PCR using both degenerate and specific primers. The transmission was more efficient and increased by increased inoculation period of 24 and 48 hours, which reached up to 83 % efficiency.

**Table 9** Transmission of allexiviruses to healthy leek seedlings by a group of 10 *A. tulipae* reared on *Allexivirus*-infected garlic and given different IAP

| | No. of infected plants/ No. of plants (% transmission) | | | |
|---|---|---|---|---|
| **IAP** | **Exp. 1** | **Exp. 2** | **Exp. 3** | **mean (%)** |
| 30 min | 0/3 (0) | 0/4 (0) | nt | 0 |
| 1 hr | 2/4 (50) | 1/3 (33) | nt | 43 |
| 5 hr | 2/5 (40) | 3/5 (60) | nt | 50 |
| 24 hr | 4/9 (44) | 4/6 (66) | 9/10 (90) | 68 |
| 48 hr | 5/6 (83) | 5/7 (71) | 8/10 (80) | 78 |

### 6.2.4 Virus Retention

A group of 10 mites reared on *Allexivirus*-infected garlic cloves for 24 hr transmitted allexiviruses to RT-PCR virus-free and mite-free leeks for up to two serial transfers when they were allowed to feed for 5 and 24 hr, (representing 40 % and 80 % of the tested plants, respectively) (Table 10). On the second transfer, only 40 % of the plants were infected and none were infected after the third transfer. When mites were allowed to feed for 1hr, one out of five leek plants were infected; However, none of the plants were infected after the second and third transfer, confirming that continuous feeding on a plant for more than one hour is essential for successful virus transmission.

**Table 10** Retention of allexiviruses by *A. tulipae* after given different IAP and then serially transferred to healthy leek seedlings

| | No. Infected plants/ No. Plants (% transmission) | | |
|---|---|---|---|
| **IAP (hr)** | **Transfer 1** | **Transfer 2** | **Transfer 3** |
| 1 | 1/5 (20) | 0/5 (0) | 0/5 (0) |
| 5 | 2/5 (40) | 1/5 (20) | 0/5 (0) |
| 24 | 4/5 (80) | 1/5 (20) | nt |

### 6.2.5 Transmission efficiency

When a single viruliferous *A. tulipae* was transferred from *Allexivirus*-infected garlic cloves to healthy leek seedlings, only 22 % of the plants were successfully infected. However, virus transmission increased when plants were inoculated with more than 5 mites on the leaf (Table 11). Virus infected leek plants showed in addition to curling symptoms due to mite infestation, increased yellowing, and chlorotic strikes on the leaves.

**Table 11** Transmission efficiency of allexiviruses to healthy leek seedlings by different numbers of *A. tulipae* that were reared on *Allexivirus*-infected garlic bulbs

| | No. of infected plants/ No. of plants (% transmission) | | | |
|---|---|---|---|---|
| **No. of *A. tulipae*/ plant** | **Exp. 1** | **Exp. 2** | **Exp. 3** | **mean (%)** |
| 1 | 4/8 (50) | 1/10 (10) | nt | 22 |
| 5 | 5/9 (55) | 4/10 (40) | nt | 47 |
| 10 | 8/12 (66) | 8/10 (80) | 4/5 (80) | 80 |
| 20 | 10/12 (83) | 9/10 (90) | 5/5 (100) | 89 |

### 6.2.6 Virus detection

In garlic cloves and leaves, allexiviruses (GarV-A, -B, -C, -D, -E, -X, GarMbFV, and ShVX) could be identified (Fig. 15). For each experiment (AAP, IAP, virus retention, and transmission efficiency), leek plants also tested positive for allexiviruses using degenerate primers and specific primers for each species. During this study, all infected and mite-inoculated leek plants were found to be mixed infected with more than one *Allexivirus*. During the acquisition period experiment, out of the inoculated 56 leek plants, a total of 20 plants (35 %) were successfully infected by at least one or two allexiviruses. For the inoculation period, 59 % of the inoculated plants were infected by allexiviruses (43 infected/ 72 total inoculated plants), whereas only 22 % (9 infected/ 40 total) of inoculated plants were infected by allexiviruses during retention period experiments. A total of 58 out of the inoculated 91 leek plants were found infected during the transmission experiments (63 % infected leek plants).

The most frequently detected *Allexivirus* present in mixed infections was ShVX, accounting for 64 % of the total infected leek samples (84 infected with ShVX/ 130 total infected plants) indicating positive detection, followed by GarV-A and GarV-B with 41 % (54/ 130) and 38 % (50/ 130) of the total samples testing positive, respectively. The least detected viruses were GarV-X and GarV- E, with detection occurring in only 7 (5 %) and 13 (10 %) plants of the total tested samples, respectively. GarV-D, GarV-C and GarMbFV were also detected in the inoculated leeks with an infection rate of 28 % (37/ 130), 31 % (41/ 130), and 21 % (28/ 130), respectively (Fig. 15).

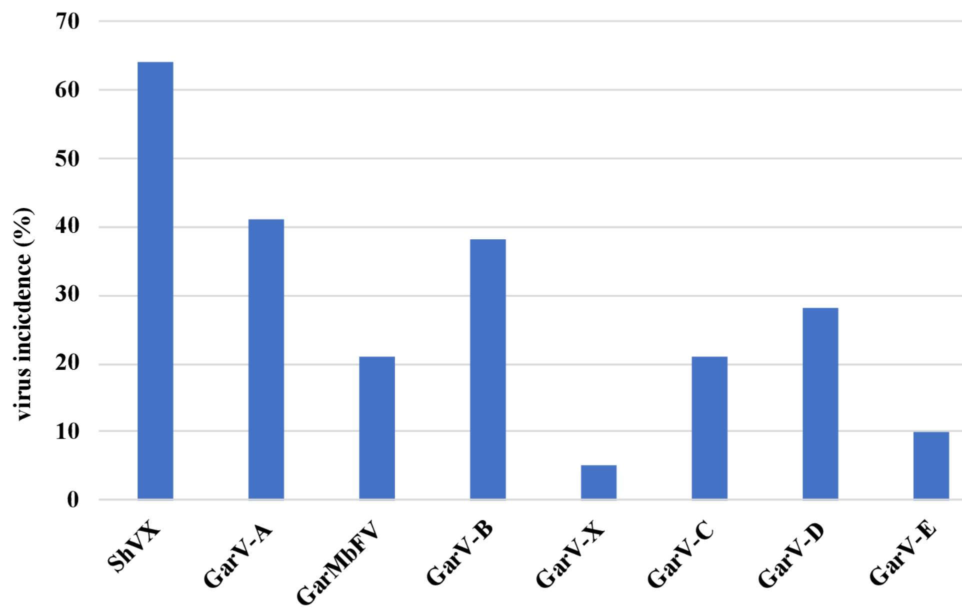


**Figure 15** Incidence of allexiviruses detected from the total of experimental mite-inoculated and infected leek plants.

# 7 DISCUSSION

**Survey of fifteen viruses infecting *Allium* species**

The occurrence of viruses especially in cultivated crops such as garlic, onion, leek, and shallot has been well investigated worldwide (Dovas *et al.*, 2001; Chen *et al.*, 2001; Conci *et al.*, 2003; Klukáčková *et al.*, 2007; Mahmoud *et al.*, 2008; Ward *et al.*, 2009; Cramer *et al.*, 2011; Chodorska *et al.*, 2014; Winiarczyk *et al.*, 2014; Godena *et al.*, 2020) but viral infection of wild and ornamental *Allium* species both in natural and agricultural ecosystems is only occasionally tested (Van Dijk, 1993; Dovas *et al.*, 2001; Ward *et al.*, 2009; Cramer *et al.*, 2011; Winiarczyk *et al.*, 2014; Gawande *et al.*, 2014; Bampi *et al.*, 2015; Scrace *et al.*, 2015; Paduch-Cichal and Bereda, 2017). Some earlier studies investigated viruses in wild plants based on the idea that natural vegetation may have served as a reservoir for the disease (Cooper and Jones, 2006). Although viruses tend not to damage wild plants (Cooper and Jones, 2006), the abundance of *Allium* viruses in nature and their ability to move between hosts should be further explored for disease resistance (Khandagale *et al.*, 2020).

The incidence of fifteen economically important viruses in the range of potential hosts of the genus *Allium,* representing 65 vegetable, wild, or ornamental species have been studied. The survey showed that all tested viruses belonging to the major genera (*Potyvirus*, *Carlavirus*, *Allexivirus*, and *Orthotospovirus*) are widespread throughout the Czech Republic. As expected, garlic, leek, and onion crops were the most heavily infected species (Klukáčková *et al.*, 2004; 2007). Our study confirms earlier reports on the occurrence of twelve viruses including all allexiviruses, potyviruses, and carlaviruses in cultivated garlic and onion crops in the Czech Republic (Klukáčková *et al.*, 2004; 2007). In addition, the potyvirus SYSV, has been reported for the first time in the country. SYSV has been reported to infect shallot (*A. cepa* var. *ascalonicum*), garlic (*A. sativum*), and Welsh onion (*A. fistulosum* L.) (Van Dijk, 1993; Van der Vlugt *et al.*, 1999). In the present study, SYSV was detected in several new host species collected from botanical and private gardens (Table 6). Besides, the orthotospoviruses IYSV and INSV were also recorded for the first time in the Czech Republic on alliums with a relatively low incidence. INSV has been previously detected in the Czech Republic infecting 17 ornamental plants and weed species (*Stellaria media*) (Mertelík *et al.*, 2000), whereas IYSV has been detected on onion and leek in neighboring countries Austria and Hungary (Panel *et al.*, 2012). The presence of INSV and IYSV in alliums is probably due to their adaptation to new crops, the importation of infected bulbs, and the transmission from infected nearby crops and volunteer plants

that contribute to disease spread by acting as reservoirs for the virus and the vector (Cooper and Jones, 2006). Among the species identified as hosts of either carla-, poty-, allexi- or orthotospoviruses, several have not been reported previously.

The survey showed that LYSV, GCLV, ShVX, GarV-A, and GarV-B represent the main threat to vegetable *Allium* species such as *A. sativum*, *A. cepa*, and *A. porrum*, and also to the cultivated wild and ornamental species (e.g., *A. caeruleum*, *A. cernuum*, *A. schoenoprasum*, *A. cristophii*, *A. cyathophorum*, *A. moly, A. sphaerocephalon, A. nutans*, and *A. senescens* subsp. *montanum*). These viruses are economically important due to their wide host range within the *Allium* genus (Dovas *et al*., 2001). Apart from LYSV, which alone can cause severe damage and yield losses of up to 50 % in garlic bulb weight and size (Conci *et al*., 2003), GCLV has been reported to cause latent infections, while ShVX effect on crop yields has not been determined. However, viruses infecting alliums can cause significant damage when the plants are infected by mixtures of viruses, which can lead to even greater yield losses (Van Dijk *et al*., 1991; Van Dijk, 1993; Chen *et al*., 2001; Conci *et al*., 2003). The potyvirus OYDV is considered one of the most important potyviruses affecting garlic and onion crops in many countries in terms of yield loss (Chen *et al*., 2001; Mahmoud *et al*., 2008). However, during our survey, it was encountered at a lower frequency than previously reported (Klukáčková *et al*., 2004, 2007). OYDV was found frequently by us in garlic crops, but less frequently in leek, onion, ornamental, and wild alliums.

Mixed infections of two or more viruses were detected in 51.1 % of the samples with frequent virus combinations presented in Table 7. Mixed infection has been widely studied on vegetable alliums such as garlic and onion but rarely studied on other ornamental and wild species. Two types of virus combinations (LYSV, GCLV, and SLV) and (LYSV, OYDV, GCLV, and SLV) have been recorded on mixed infected garlic plants (Godena *et al*., 2020). Similar virus combinations have been found in several *Allium* samples. In nature, carla- and potyviruses are transmitted by aphids, orthotospoviruses by thrips, and allexiviruses by eriophyid mites (Van Dijk *et al*., 1991). We hypothesized that viruses with common vector species could exhibit a high frequency of co-occurrence. This was supported by our data concerning the mite-transmitted viruses that showed significant positive associations between each other. On the other hand, we found a lack of association between viruses of the same genus in double infection. Thus, virus titer in bulbs may be mainly promoted by vegetative propagation rather than by vector transmission. It is also possible that this co-existence may be the result of a synergistic

effect that enhances multiple infections (Conci *et al*., 2003). Previous reports have indicated that the mixed infection resulted in more severe symptoms than those arising from a single infection (Chen et al. 2001), indicating such possible synergetic relationship. In this study, association analysis revealed that (ShVX + SLV) and (ShVX + LYSV) were more frequently found in multiple infection, suggesting that these viruses might facilitate infection of other virus species.

**Effect of phylogeny and origin of host samples**

The study of the multivariate analysis showed a great variation in virus species incidence among and within *Allium* species. Our search for possible drivers of such variability resulted in two significant explanatory factors: phylogeny and origin of host samples. *Allium* members of the subgenus *Melanocrommyum* differed significantly in virus presence from those of other subgenera, having more GarMbFV, GarV-D, and GarV-X and less GarV-A, -B, and -C frequent incidence of allexiviruses. *Melanocrommyum* subgenus are bulbous plants originating predominantly from Southwestern and Central Asia, representing the dominant subgenus of the second evolutionary line within the genus *Allium* (Friesen *et al*., 2006). However, species of the subgenus *Melanocrommyum* studied by us were introduced into European and North American gardens in the early 19th century, and since then widely cultivated as ornamentals (Gregory *et al*., 1998). Close phylogenetic relationship of the analyzed *Melanocrommyum* species together with their common cultivation and vegetative propagation in a several horticultural companies (Fritsch *et al*., 2006) might be a likely explanation of the observed dissimilarity of virus presence with members of the other subgenera in the other two evolutionary lines of the genus *Allium* studied by us.

In addition to the phylogeny of alliums studied, the origin of the samples was another significant driver of variable virus presence in *Allium* samples. We found that species and/or individuals sampled in the wild were both significantly less infected and differed in virus incidence in comparison with cultivated species collected from farmer markets, botanical, and private gardens. These results are in agreement with previous studies from different regions (Van Dijk *et al*., 1991; Van Dijk 1993; Dovas *et al*., 2001). Virus persistence in cultivated species can be ensured through vegetative transmission via clonal propagation of hosts, a commonly used method of *Allium* production (Chen *et al*., 2001), and dense host populations growing nearby, which facilitate virus transmission via various vectors (Lefeuvre *et al*., 2019).

Wild species are widely known as an extensive collection of viruses and vector sources (Cooper and Jones, 2006). Several wild species (e.g., *A. vineale*, *A rotundum*, *A. oleraceum*) frequently occur near fields with cultivated *Allium* crops (Duchoslav, 2001; Krahulec and Duchoslav, 2010), which might contribute to virus epidemiology (Paduch-Cichal and Bereda, 2017). The study shows that such wild populations are usually virus-free or infected by one or two viruses. Natural plant populations generally vary more in density, genetic diversity, and spatial pattern than crops do (Alexander *et al*., 2014); therefore, it is more likely that they respond to viruses differently from those of cultivated fields. Also, we suspect that populations of wild *Allium* species are probably not easily accessible to vectors; thus, the low virus incidence was recorded. Once wild populations are cultivated (e.g., *A. sphaerocephalon*, *A. scorodoprasum*, and *A. senescens* subsp. *montanum*), they become easily infected. These species are often grown in small groups, which make them more accessible to vectors from nearby infected crops. Therefore, detailed studies comparing virus incidence in wild and cultivated samples of identical species are highly desirable.

The reservoir potential of wild and ornamental host plants and their actual role in the epidemiology of *Allium* viruses have never been verified under field conditions. Several studies on the epidemiology of viruses were reported, but all dealt with disease development in vegetable crops (Chen *et al*., 2001; Conci *et al*., 2003; Chodorska *et al*., 2014). Knowledge of the epidemiology of viruses, host range, vectors, and the ways climate change influences future virus disease epidemics in cultivated plants and natural vegetation is of great importance to both global food security and natural ecosystems (Trebicki, 2020).

**Transmission of allexiviruses by *Aceria tulipae* (Keifer)**

Allexiviruses have been previously reported to be transmitted by their major vector, the eriophyid mite, *Aceria tulipae* (Van Dijk *et al*., 1991). The pest is considered one of the most important pests of bulbous crops (onion, garlic, and tulip), causing bulb drying or decay, leaf twisting, curling and discoloration of *Allium* crops (Debnath and Karmakar 2013). Previous records about the virus vector *A. tulipae* did not address characteristics of allexivirus transmission in greater detail. Studies on mite transmission have been considered as complex because of the difficulties in rearing healthy mite colonies and manipulating individual mites.

During the study allexiviruses were transmitted from mixed infected garlic (*Allium sativum* L.) to leek (*A. porrum* L.) by their major vector, *Aceria tulipae*. A similar study from Poland showed that a mixed infection of Garlic virus B, -C, -D, and -X was successfully transmitted to leek plants by *A. tulipae* (Dąbrowska *et al*., 2020). Our data showed that mixed infection with more than one *Allexivirus* was observed on mite-inoculated leek plants. ShVX, GarV-B, and GarV-A were the most transmitted viruses in the mite-inoculated leaves. These viruses are the most economically important allexiviruses found in the Czech Republic, according to our survey. Although limited data about the concentration of allexiviruses in infected garlic is available, some reports indicated that the concentration of allexiviruses in garlic cloves may depend on the season and that allexiviruses present in low concentration may be undetectable (Conci *et al*., 2002; Cafrune *et al*., 2006a). Therefore, one can speculate that the transmission of some allexiviruses from the mixed infection is probably due to the unequal distribution in the bulb tissue. However, more studies are needed to further understand the distribution and concentration of these viruses in garlic crops.

**The mode of transmission of allexiviruses**

To define the transmission characteristics of *A. tulipae*'s and its potential to transmit allexiviruses in greater detail, we started a series of experiments to investigate the acquisition access period, inoculation access period, virus retention, and transmission efficiency from naturally ixed infected plants. *Aceria tulipae* required a minimum of a 30 min acquisition access period (AAP) and an AAP more than 5 hours to acquire allexiviruses and increase transmission. A minimum of one-hour inoculation access period (IAP) was required for successful transmission. Transmission efficiency of allexiviruses increased with increasing acquisition and inoculation access time, which has also been documented for wheat streak mosaic virus (WSMV, genus *Tritimovirus*) and triticum mosaic virus (TriMV, genus *Tritimovirus*) (Orlob, 1966; Knoell, 2018). Based on the inoculation/acquisition access period, the transmission of allexiviruses may be considered semi-persistent. The semipersistent manner is characterized by an acquisition period of minutes to hours and the virus can be retained for as long as few days but it is lost during molting (Bhat and Rao 2020).

Transmission details for most other eriophyid mite-borne viruses are not well defined due to the difficulties in manipulating such tiny creatures. To date, the best-studied relationship is that of WSMV and its vector *A. tosichella* (previously known as *A. tulipae*). WSMV is transmitted by all stages of its vector, and is retained through the molt, but not through the egg (Orlob, 1966). However,

adults transmit only if they acquire virus during their immature stages; they cannot acquire the virus as adults. *A. tosichella* acquires WSMV in a minimum AAP of 15 min, once acquired, WSMV is transmitted by *A. tosichella* for at least 4 days (Orlob, 1966; Slykhuis, 1955). Based on these findings, it was suggested that the mode of transmission was circulative (Paliwal, 1980). In less detailed studies, fig mosaic virus (FMV, genus *Emaravirus*) was reported to be transmitted in a persistent manner by *A. ficus* with a 6 to 7 hr latent period in the vector, to be retained through the molt, and to be transmitted by viruliferous mites for up to 10 days (Proeseler, 1969). Based on study, the transmission of allexiviruses by *A. tulipae* is best considered to be in a semi-persistent manner. Studies on two other mite-transmitted viruses also indicate a semi-persistent mode of transmission. The transmission of pigeon pea sterility mosaic virus (PPSMV, genus *Emaravirus*) and peach mosaic virus (PMV, genus *Trichovirus*) in a semi-persistent manner by their vector, *Aceria cajani* ChannaBasavannaand and *Eriophyes Insidiosus* Keifer & Wilson, respectively (Gispert *et al*., 1998; Kulkarni *et al*., 2002). Transmission efficiency of allexiviruses via *A. tulipae* decreased as number of mites transferred decreased. Transmission efficiency was greatest with 10 adult mites transferred per plant, similar to results observed for PPSMV (Kulkarni *et al*., 2002). In contrast, relatively high transmission was observed by a single *E. insidiosus* mite compared to when the number of mites was increased (Gispert *et al*., 1998). This can be explained by the fact that different mite species may have different virus transmission efficiencies (Seifers, 2002).

When newly hatched mites from eggs were transferred to healthy leek seedlings, symptoms observed on mite-infested leek plants included twisting and curling of leaves, which are typical symptoms of mite infestation (Debnath and Karmakar 2013). The observed phenotypes of leaf deformations are assumed to protect the free roaming and no gall forming mites from dehydration. Under suitable environmental conditions, mites can proliferate to high numbers (Sapáková *et al*., 2012). In addition, no leek plants developed mosaic symptoms and they tested negative for allexiviruses. Thus, we can conclude that allexiviruses are not transovarially transmitted. Several studies on eriophyid mite-borne viruses reported that viruses are not transmitted through the egg of their vector. WSMV was reported to be transmitted by larvae, nymph, and adults of *Aceria tosichella* Keifer, but not through eggs (Orlob1966; Sánchez-Sánchez *et al*., 2001). In addition, PPSMV and PMV also were not transovarially transmitted by their vectors (Gispert *et al*., 1998 Kulkarni *et al*., 2002).

# 8 CONCLUSION

Since wild and ornamental *Allium* species has become more popular and accessible for common consumers, the outcomes of this study are important for the understanding of virus epidemiology in these species and the assessment of their distribution among these species in the different habitat, which will allow us to prevent their spread in the future. In this study, we presented data about viruses infecting 65 *Allium* species cultivated or naturally occurring in the area of the Czech Republic. The addition of other geographical areas will eventually provide a more complete picture of continental and global trends in *Allium* virus epidemiology. The results of this study suggest that all viruses are widespread throughout the Czech Republic. The potyvirus LYSV, the carlavirus GCLV, and the allexiviruses ShVX, GarV-A, and GarV-B represent the main threat to vegetable, wild, and ornamental *Allium* species. Although, the rest of viruses were detected less frequently, it is important to identify these viruses and limit their spread. The data presented also shows that the viral infection rate was exceptionally high not only in cultivated species (mainly vegetable crops), but also same high level of infection was detected on ornamental species bought from market, private and botanical gardens. The high transmission rate of viruses through vegetative propagation and by arthropod vectors have significantly contributed to their wide dissemination in the Czech Republic. Overall, the transmission of viruses is mainly due to long-term vegetative propagation of *Allium* crops and their adaptation to local cultivars of diverse geographical and climatic regions.

Interestingly, the results showed no viral detection from species collected from natural habitat. As previously concluded, these species are probably not preferable by vectors, which explains the absence of viral infection. However, several wild plants have been used as a source of genetic resistance in breeding programs for cultivated crops. With this in mind, it is important to further study wild species and species from natural habitat for possible source of resistance or tolerance to plant viruses. Due to the importance of alliums in the country and worldwide, and the wide use of vegetable, wild, and ornamental species by consumers, it is necessary to prevent destructive virus spread to limit crop losses and disease outbreaks. This study provides insight in the various viruses and their existence in mixed infected alliums. We also showed the relationship between disease, the habitat origin and phylogenetic relatedness of the hosts, all factors were shown to be important in virus epidemiology. Further investigations in this area are required, and particular attention should be drawn towards alliums grown in natural habitat, ornamental, and wild species.

Our study also provides an insight in the transmission characteristics of allexiviruses by their vector eriophyid mite *Aceria tulipae*. The results show that allexiviruses are transmitted in a semi-persistent manner from infected garlic to healthy leeks. The knowledge of the retention and transmission features of semi-persistent viruses in their vector can help design strategies to prevent virus spread and interfere with virus transmission by vectors including host genetic resistance to virus and/or insect, insecticides and integrated pest management. Characterizing transmission for viruses infecting *Allium* crops is critical to better understand eriophyid vector transmission mechanisms and their influence on the epidemiology of viruses.

It is important to bear in mind that plant viruses can modify insect vector behavior directly, and indirectly by manipulating plant hosts, leading to enhanced transmission efficiency, and spread. The understanding of the virus-vector relationship and the interaction with the host opens avenues for interference and control of the vector populations as well as virus transmission. Direct and indirect effects on vector behavior in relation to the infected plant host is another important research area impacting on virus acquisition and dissemination that once better understood, to adoption of novel control strategies. Our knowledge of viruses will evolve with our insights into vector biology.

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# 10 SUPPLEMENTS

**Table 12** Subgenera of the collected *Allium* species

| **Subgenera** | ***Allium* species** |
|---|---|
| *Allium* | *Allium caeruleum, A. caesium, A. carinatum, A. callimischon, A. flavum, A. fuscum, A. longicuspis, A. oleraceum, A. porrum, A. rotundum, A. sativum, A. scorodoprasum, A. sphaerocephalon, A. vineale* |
| *Amerallium* | *A. cernuum, A. haematochiton, A. insubricum, A. moly, A. narcissiflorum, A. neapolitanum, A. platycaule, A. paradoxum, A. runyonii, A. roseum, A. ursinum, A. zebdanense* |
| *Anguinum* | *A. microdictyon, A. victorialis* |
| *Butomissa* | *A. ramosum, A. tuberosum* |
| *Cepa* | *A. altaicum, A.cepa, A. fistulosum, A. ledebourianum, A. pskemense, A. schoenoprasum, A. thunbergii, A. ×proliferum* |
| *Cyathophora* | *A. cyathophorum* |
| *Melanocrommyum* | *A. atropurpureum, A. bucharicum, A. cristophii, A. giganteum, A. hollandicum, A. hybridum, A. karataviense, A. nigrum, A. schubertii, A. stipitatum* |
| *Nectaroscordum* | *A. bulgaricum* |
| *Polyprason* | *A. ericetorum, A. macrorrhizum, A. macrostemon, A. suaveolens* |
| *Porphyroprason* | *A. oreophilum* |
| *Reticulatobulbosa* | *A. scabriscapum, A. strictum, A. strictum, A. przewalskianum* |
| *Rhizirideum* | *A. angulosum, A. anisopodium, A. bidentatum, A. flavescens, A. mongolicum, A. nutans, A. senescens* subsp. *montanum* |

**Table 13** Oligonucleotide primers used for detection of different viruses infecting *Allium* spp.

| Virus | Primer | Nucleotide Sequence (5´-3´) | Ta (°C) | Authors |
|---|---|---|---|---|
| GarV-A | ACPF | ATGTCGAATCCAACTCAGTCG | 54 | Chodorska *et al*., 2014 |
| | ACPR | AGACCATGTTGGTGGCGCG | | |
| GarV-B | BCPF | TGACGGGCAAACAGCAGAATAA | 50 | |
| | BCPR | ATATAGCTTAGCGGGTCCTTC | | |
| GarV-C | CCPF | TTGCTACCACAATGGTTCCTC | 52 | |
| | CCPR | TACTGGCACGAGTTGGGAAT | | |
| GarV-D | DCPF | AAGGAGCTACACCGAAGGAC | 52 | |
| | DCPR | TAAAGTCGTGTGGATGCATCAGA | | |
| ShVX | ShVXF | ACCGAAATCACAGTTAACTCCTTTGG | 54 | |
| | ShVXR | TCTACGGTTGTCGATTTTGTGCGT | | |
| GarV-X | XF | GCGGTAATATCTGACACGCTCCA | 55 | |
| | XP | ACGTTAGCTTCACTGGGGTAGAATAT | | |
| GarMbFV | + | ATGTCAGGTTCCACAAGT | 50 | |
| | - | TCAGAACGTAATCATGGGA | | |
| GarV-E | EF2 | TTGCTAGACCACCTCAGTATTGAGAA | 55 | |
| | ER2 | TAT TGG GCG TAC ATC GGT GAC TGT | | |
| SYSV | SYS-UP | TTCGGATCCATRTGAGCTTCCTTCGC | 52 | Der Vlugt *et al*., 1998 |
| | SYS-DW | CTGGATCCGCAGTKCGATAYCAAG | | |
| SLV | SL-N30 | TATGGCTAACGAAGAAGAAGAACTC | 54 | Nam *et al*., 2015 |
| | SL-C10 | CGTTCACGCTAGACAATTCAGACAT | | |
| LYSV | LYS-N10 | CGCATATGCAGTGATGTTTCGGTT | 54 | Nam *et al*., 2015 |
| | LYS-C15 | ATCAAATTCAGGCTGCTTATACAC | | |
| IYSV | IY1 | ATGGCTACCGTTAGGG | 55 | Cortêz *et al*., 1998 |
| | IY2 | TTAATTATATCTATCTTTCTTGG | | |
| INSV | INSV-F3 | GCAAAGATTACCAAGGAG | 55 | Lebas *et al*., 2004 |
| | INSV-R2 | TCCCAAAATCAATAGTAGC | | |
| GCLV | GCL-N30 | GCACCAGTGGTTTGGAATGA | 54 | Nam *et al*., 2015 |
| | GCL-C40 | AGCACTCCTAGAACAACCATTA | | |
| OYDV | F | CGAAGCAAATTGCCAAGCAG | 57 | Mahmoud *et al*., 2007 |
| | R | CGATTAGCTGCCCCTCTAAC | | |

**Figure 16** *Allium* flower bud of some of the collected species during the survey: **(a)** *A. ampeloprasum*, **(b)** *A. rotundum*, **(c)** *A. schoenoprasum*, **(d)** *A. vineale*. (Taken by Dr. Martin Duchoslav)

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**Mansouri, F**., Richert-Pöggeler, K.R., Lewandowski, M., Ryšánek, P., 2021. Transmission characteristics of allexiviruses by the eriophyid mite, *Aceria tulipae* (Keifer) (Acari: Eriophyidae) from naturally mixed infected garlic (*Allium sativum* L.). *European Journal of Plant Pathology* 160, 789-796.

**Mansouri, F**., Krahulec, F., Duchoslav, M. and Ryšánek, P., 2021. Newly identified host range of viruses infecting species of the genus *Allium* and their distribution in six habitats in the Czech Republic. *Plant Pathology* 70, 1496-1507.

## Publications of abstracts in conferences

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