

Seasonal detection of pome fruit viruses by RT-PCR in apple mother trees used for scion production

Saisonaler Nachweis von Kernobstviren mittels RT-PCR in Apfelbäumen, die für die Produktion von Pfropfreisern verwendet werden

Abstract

The detection of *Apple chlorotic leafspot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) was investigated with infected apple mother trees over a period of 17 months. Relative virus concentrations were estimated once a month in leaf samples and in bark with buds from May to November and January to April, respectively, in a serial dilution series of sap extracts using the silica nucleic acid extraction method and detection by reverse transcription PCR (RT-PCR). The greatest dilution limits, indicating the highest virus levels were found most frequently in the period from January to April, but these were different for the individual viruses. The values were highest for ApMV followed by ACLSV, ASPV and ASGV. In the remaining times a heterogeneous course of virus concentration was recorded for the viruses, also influenced by the year of sampling. Nevertheless, each virus was always detectable with the lowest dilution level of 1:10 (w/v) in plant extracts regardless if leaves or bark with buds were examined. From January to April all viruses were also reliably detected in dilutions up to 1:100, potentially enabling the use of pooled samples from different trees.

Key words: *Apple chlorotic leafspot virus*, ACLSV, *Apple mosaic virus*, ApMV, *Apple stem grooving virus*, ASGV, *Apple stem pitting virus*, ASPV, apple, detectability, seasonal course, RT-PCR, scion mother tree

Zusammenfassung

Die Nachweisbarkeit des *Chlorotischen Blattfleckenvirus des Apfels* (ACLSV), *Apfelmosaikvirus* (ApMV), *Stammfurchungsvirus des Apfels* (ASGV) und *Stammnarbenvirus des Apfels* (ASPV) wurde an infizierten Reiser Mutterbäumen von Apfel über einen Zeitraum von 17 Monaten untersucht. Die relativen Viruskonzentrationen wurden einmal monatlich in Blattproben (Mai–November) bzw. in Rinde mit Augen (Januar–April) mittels Silica-Nukleinsäureextraktion und einer Verdünnungsreihe des Saftextraktes sowie der konventionellen RT-PCR als Nachweismethode bestimmt. Die höchstmöglichen Verdünnungen, welche die höchsten Viruskonzentrationen kennzeichnen, fanden sich im Jahresverlauf von Januar bis April, waren aber für die einzelnen Viren unterschiedlich hoch. Die höchsten Werte wurden bei ApMV gemessen, gefolgt vom ACLSV, ASPV und ASGV. In den übrigen Zeiten wurde ein heterogener Verlauf der Viruskonzentrationen für die Viren verzeichnet, die auch vom Jahr der Probenahme beeinflusst wurden. Nichtsdestoweniger war jedes Virus immer mit der niedrigsten Verdünnungsstufe von 1:10 (w/v) im Pflanzenextrakt nachweisbar, unabhängig davon, ob Blätter oder Rinde mit Knospen verwendet wurden. Von Januar bis April konnten jedoch alle Viren auch in einem Verdünnungsverhältnis bis 1:100 zuverlässig nachgewiesen werden, was potentiell die Verwendung von Mischproben verschiedener Bäume ermöglichen würde.

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Stichwörter: Chlorotisches Blattfleckenvirus des Apfels, ACLSV, Apfelmosaikvirus, ApMV, Stammfurchungsvirus des Apfels, ASGV, Stammnarbungsvirus des Apfels, ASPV, Apfel, Nachweisbarkeit, Jahresverlauf, RT-PCR, Reiser Mutterbaum

Introduction

Apple chlorotic leaf spot virus (ACLSV), *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV) und *Apple stem pitting virus* (ASPV) are widely distributed pome fruit viruses with economic relevance. Therefore they are subject to testing within the certification guidelines of the European Union for healthy fruit planting material (ANONYMOUS, 2014). Although the detectability of these viruses by PCR and other methods has already been investigated for distinct dates or periods of sampling, dilution limits or tissue types of some apple varieties by different authors (e.g. ARTNJEN and JELKMANN, 2013, FUCHS et al., 1988, KUNDU et al., 2003, MENZEL et al., 2002, WINKOWSKA et al., 2016), hardly any investigations were made with vegetative propagation material used for certification programmes. Therefore some uncertainties still exist concerning a reliable virus test of apple mother trees for scion wood production, particularly regarding the most suited sampling time(s) of the year. It might be possible, that detection limits for single viruses could fall below a threshold at certain times even when tests are performed by sensitive PCR, as shown for ACLSV by HILBERBODMER et al. (2003) with nuclear stock trees, or with dilutions due to pooled samples. Sometimes large tree numbers have to be tested, or retested in case of virus infections, and the use of pooled samples would be advantageous due to limited staff for sampling or laboratory work and efficiency. The aim of this investigation was to monitor the relative concentration of four viruses in apple trees used as stock for scion production over at least a complete season to determine the favourite or potentially “critical” sampling time(s) for a reliable testing by RT-PCR.

Materials and Methods

Eight years old apple trees of a commercial mother orchard for scion wood production were examined in a first step to identify naturally virus-infected plants. Trees of the following cultivars were found to be infected (viruses in brackets): ‘Stina Lohmann’ (ACLSV, ASPV), ‘Idared’ (ASGV) and ‘Roter von Simonffi’ (ACLSV, ASPV), all grafted on rootstock ‘Bittenfelder Seedling’. ApMV was not found, therefore artificially virus-infected trees of the cultivar ‘Bittenfelder Seedling’ (not grafted) from another trial at a different location were used which were grown like mother trees for scion production. These trees had been inoculated by bark chips, double infected with ACLSV and ApMV, as one year old trees and were about 26 months old when the investigation started. Tests three

months post inoculation revealed a successful infection by both viruses. Four trees of each cultivar were selected for this study. In the case of a double viral infection, only one virus was pursued during the experiment. Sampling was carried out in monthly intervals. Five leaves (May to November) or shoot cuttings (January to April) were randomly sampled from one tree and pooled, except for February 2015, where only one shoot per tree was available for the three naturally infected cultivars from the commercial propagation stock. No samples were available for December 2014. The plant material was stored at +8°C after sampling and processed within 1 to 12 days. Extraction of total nucleic acids from the mixed sample, either leaf stalks with some leaf tissue or bark chips containing phloem and buds, was performed using the silica capture method described by MENZEL et al. (2002), except 900 mg of tissue was used for extraction at a ratio of 1:10 (w/v) with grinding buffer instead of 100 mg.

Virus titres in the plant tissue were determined as relative concentrations by progressive tenfold dilutions of the original extract up to 1:100,000 for ACLSV, ApMV and ASPV, still revealing a visual PCR amplification product in the agarose gel after electrophoresis. Tissue from a healthy control tree, was similarly extracted as from virus infected trees and used as diluter. For ASGV, dilutions of 1:25, 1:50, 1:75, 1:100 and 1:1,000 were used, because preliminary investigations clearly showed lower titres for this virus than for the other viruses. RT-PCR was conducted according to the method described by MENZEL et al. (2002), except PCR reactions were run individually for each virus and not as multiplex assay.

Results

The course of relative concentrations for each virus in apple trees monitored over a period of 17 months (May 2014 until September 2015) are shown in Figures 1–4. Peak titres were most frequently recorded for each of the viruses from January to April 2015, in which bark with buds was used as examination material. Comparing the number of positive PCR results at high extract dilution, ApMV samples performed best with dilutions up to 1:100,000, followed by ACLSV, ASPV and ASGV, the latter being positively detected only in a few cases in dilutions greater than 1:100.

Regarding the results with leaf samples (May to November 2014 and May to September 2015), titres for ApMV and ACLSV remained widely stable with some fluctuations from May to autumn in 2014, but dropped from May to September in 2015 to a level similar to the previous year (Fig. 1 and 2). The virus titre of ASPV remained nearly stable for those times in both years with a slightly higher level in 2014 for some of the trees (Fig. 3). In contrast, the concentration of ASGV showed a decrease in the summer 2014 but not in 2015 (Fig. 4). Dilution factors of 1:100 or 1:1,000 with positive PCR result were obtained for the viruses in most cases, although higher dilution factors were recorded for some trees. Positive

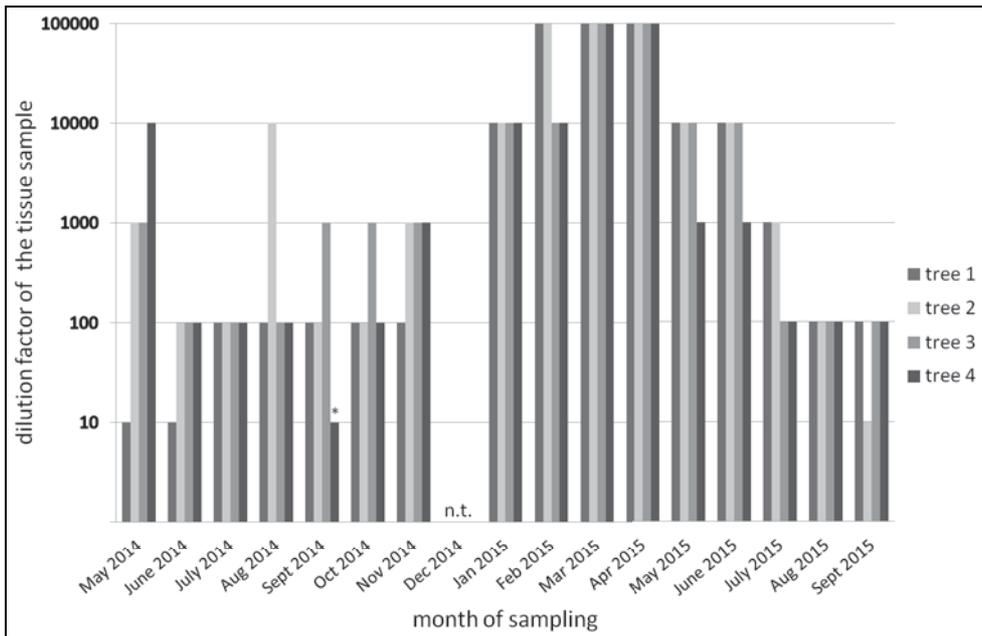


Fig. 1. Relative concentrations of *Apple mosaic virus* in apple trees of the cultivar ‘Bittenfelder Seedling’ with four replications in the seasonal course, expressed by logarithmic dilution series of the tissue samples (leaves from May to November, bark with buds from January to April). Undiluted tissue samples were not examined. n.t. = not tested, * = faint band of the PCR-amplification product on the agarose gel.

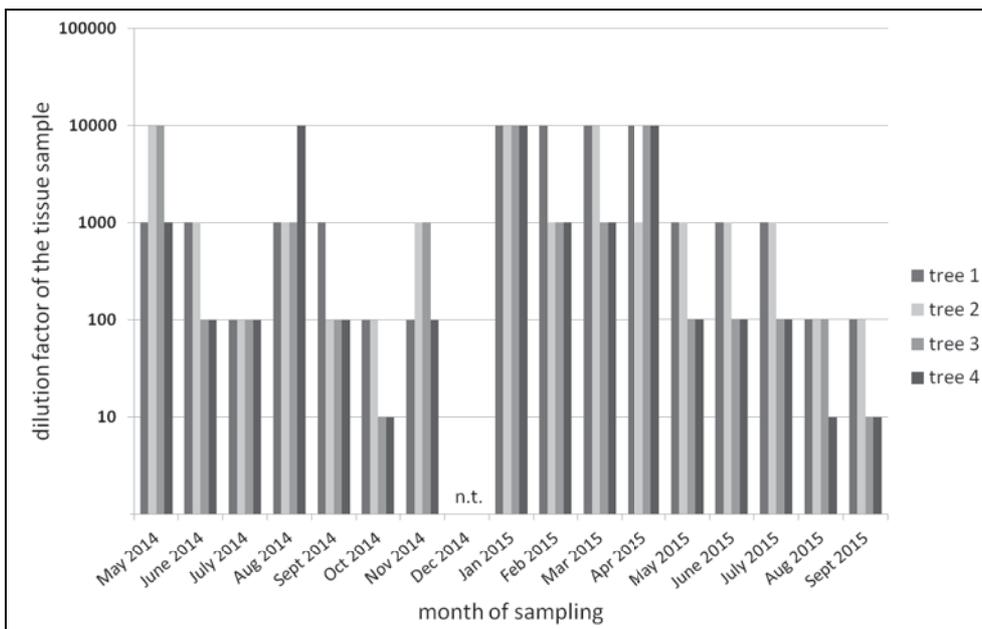


Fig. 2. Relative concentrations of *Apple chlorotic leaf spot virus* in apple trees of the cultivar ‘Stina Lohmann’ with four replications in the seasonal course, expressed by logarithmic dilution series of the tissue samples (leaves from May to November, bark with buds from January to April). Undiluted tissue samples were not examined. n.t. = not tested

results for the viruses were obtained throughout the year in plant tissue extracts diluted 1:10, regardless if leaves or bark with buds were examined. This is most clearly shown with the ASGV November 2014 samples (Fig. 4).

The detectability of the viruses often differed at the same sampling date between the individual trees of one group after a tenfold dilution. This situation was recorded for all viruses and a difference of up to a 1000-fold was found in maximum (Fig. 1, ApMV May 2014), but a 10-fold difference was registered in most cases. However, these fluctuations did not compromise the detectability of all four viruses during the trial period. For a simultaneous testing of all four viruses the months from January to

April were most favoured, because a dilution of the sample extracts up to 100-fold was possible to detect each of the viruses.

Discussion

The detection of pome fruit viruses has been investigated by many authors employing different extraction procedures and detection methods like immunosorbent enzyme-linked assays (ELISA) or nucleic acid based techniques like PCR for different tissues, sampling times and apple varieties, which led to numerous recommendations how

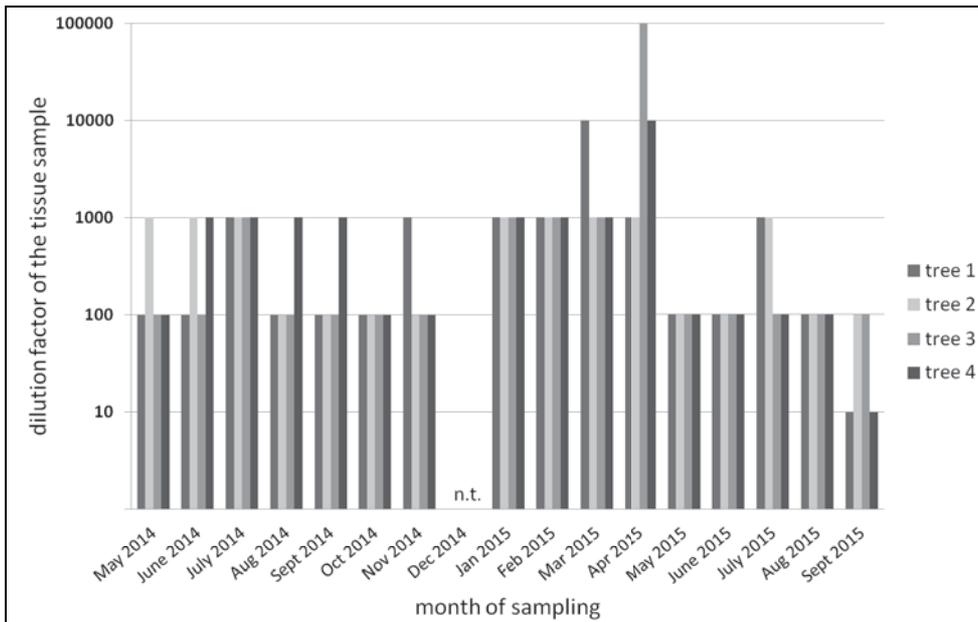


Fig. 3. Relative concentrations of *Apple stem pitting virus* in apple trees of the cultivar 'Roter von Simonffi' with four replications in the seasonal course, expressed by logarithmic dilution series of the tissue samples (leaves from May to November, bark with buds from January to April). Undiluted tissue samples were not examined. n.t. = not tested

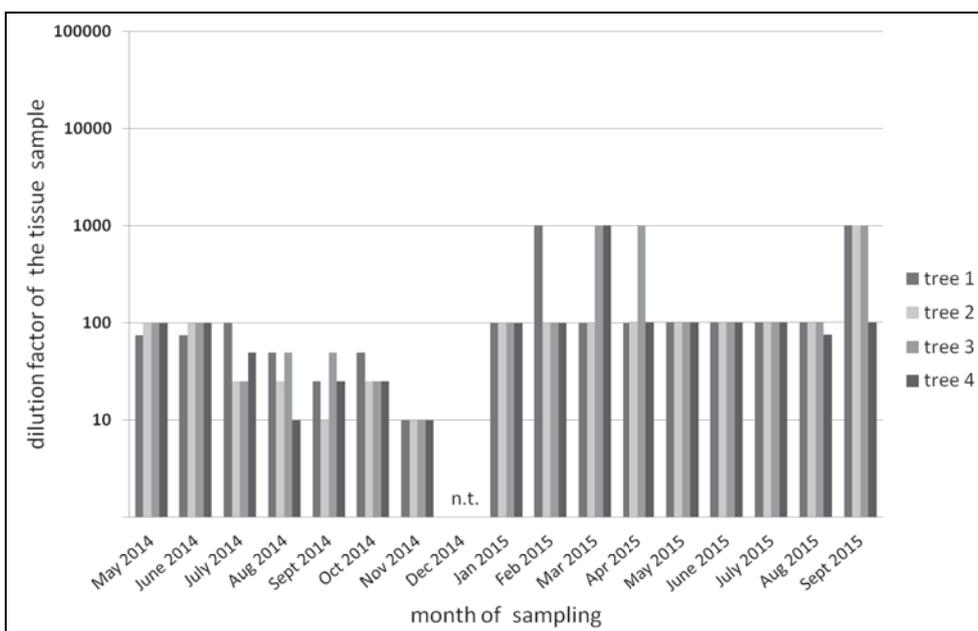


Fig. 4. Relative concentrations of *Apple stem grooving virus* in apple trees of the cultivar 'Idared' with four replications in the seasonal course, expressed by logarithmic dilution series of the tissue samples (leaves from May to November, bark with buds from January to April). Undiluted tissue samples were not examined. n.t. = not tested

to perform analyses. Procedures have been summarized for single viruses by GRIMOVÁ et al. (2016), JELKMANN and PAUNOVIC (2011), MASSART et al. (2011), PETRZIK and LENZ (2011) and YAEGASHI et al. (2011). For the purpose of this investigation it seemed to be adequate to analyse serially diluted plant extracts with RT-PCR for the determination of relative virus concentrations. However, the correlation between the graduation of extract dilutions and the determined relative virus concentrations may be influenced by some factors in the course of the year. Thus, fluctuating concentrations of inhibitors (phenolics, polysaccharides etc.) could affect the efficiency of the RT-PCR or changing water contents of the plant material could

alter the concentration of extracted nucleic acids. However, resulting correlation degrees between the tenfold dilution steps of the extracts and the virus concentrations or titers, respectively, are assumed to be sufficient to detect substantial changes of virus titers in the course of the year. Some other authors used this extract dilution method to follow the change of plant virus concentrations over time (SVOBODA and POLÁK, 2010, WINKOWSKA et al., 2016). Leaves were used instead of bark with buds for the testing as long as being available in the vegetation period, since sampling and processing of leaf material is easier and faster with large stocks in mother orchards for scion wood production. However, the virus concentration

at various times in different types of tissue must be considered as a known influencing factor for the determination of the virus titre. Different recommendations exist for the most suitable tissue type when using molecular detection techniques. MENZEL et al. (2002) recommended the use of bark chips rather than leaf material as the amount of PCR product was reduced when leaf material was used, and for some isolates of ApMV the detection failed completely with leaf material. In contrast, KUNDU et al. (2003) found leaf material of apple to be the most suitable tissue for a sensitive detection of ASGV compared to bark or petals using RT-PCR. ARTNJEN and JELKMANN (2010) considered phloem tissue to be a reliable source for the detection of ASPV throughout the year for several apple varieties, whereas MATHIOUDAKIS et al. (2009) reliably detected the same virus in leaves and bark of apple by nested RT-PCR at all sampling dates during the year.

The results presented here demonstrate, that ACLSV, ApMV, ASGV and ASPV were reliably detected in leaf or bark with bud samples at the lowest dilution level of 1:10 (w/v) in plant extracts by RT-PCR throughout the year using the silica nucleic acid extraction method. Samples were not available for December 2014, but based on the virus levels of the adjacent months it can be assumed that the viral titres were equally high for that month. It would not be advisable to test pooled leaf samples from several trees collected during summer and autumn, because a reliable detection of some viruses could fall below a critical limit due to the dilution effect of the sap from virus-poor or virus-free trees.

Pome fruit virus titres have been investigated by many researchers, utilising various tissue types at different sampling times, mostly based on results of serological methods (e.g. FUCHS, 1982, FUCHS et al., 1988, GRÜNTZIG et al., 1994, MENZEL et al., 2002, KUNDU et al., 2003, PADUCH-CICHAL and TOMALA, 2007, SVOBODA and POLÁK, 2010). However, only a few authors investigated the detection of pome fruit viruses by PCR methods in the seasonal course. Regarding the results obtained for leaf extracts, WINKOWSKA et al. (2016) investigated the accumulation of ACLSV, ApMV, ASGV and ASPV in different apple tissues from March to December in two subsequent years. They found dilution limits from 1:50–1:1,000 with leaf tissue by RT-PCR from spring to autumn in the first year, which is in line with results of this study, but failed to detect ACLSV reliably in leaf samples from June and August in the second year with the same method. In contrast, the authors found that the virus was always reliably detected by reverse transcription real-time PCR (RT-qPCR) during the sampling times. Also HILBER-BODMER et al. (2003) reported about a failure in detection of ACLSV by RT-PCR in leaves from nuclear stock apple trees during summer time.

In this study the time of the highest virus accumulations (January to April) coincided with the use of bark with buds as sample material, possibly resulting from higher virus titres in this tissue type than in leaf material, which was used before and after this period. In contrast, WINKOWSKA et al. (2016) found mostly lower virus titres in

bark than in leaves when they compared absolute virus concentrations for inner bark and leaf tissue in March for four apple cultivars. In June and September this was still true for ACLSV and ASGV, but not for ApMV and ASPV, which showed mostly higher titres for inner bark compared to leaves.

Regarding the dilution limits obtained with bark and buds, MENZEL et al. (2002) found lower values for ACLSV, ASGV and ASPV (up to 1:40) and for ApMV (up to 1:100) compared to this study with bark material from apple infected simultaneously by four viruses. However, samples in the MENZEL et al. (2002) study were collected during the summer months. WINKOWSKA et al. (2016) recorded dilution limits between 1:50 and 1:1,000 for buds and inner bark from winter to springtime for the same viruses with RT-PCR. ACLSV and ApMV could not be detected by this method with inner bark tissue in spring and winter in the first year and not reliably in March and December, respectively, in the second year using the same tissue. However, the viruses were always detected by RT-qPCR. With respect to the order of highest titres obtained for the various viruses in the study presented here (ApMV > ACLSV > ASPV > ASGV), it should be considered that the virus accumulation was determined for each of the viruses in a different apple variety, which might have led to this succession. WINKOWSKA et al. (2016) investigated the titres for the same viruses in four apple cultivars at different seasons. Based on RT-PCR and serial dilution of extracts from buds and inner bark sampled in March their results showed the highest titre for ASPV, followed by ApMV and ASGV, and the lowest titre for ACLSV. However, when the authors investigated the same tissue types with RT-qPCR in the following year, highest titres were obtained for buds infected by ApMV, followed by ASGV, ACLSV and ASPV. For the inner bark samples the order was reversed for ASGV and ApMV.

In this study a detection of all four viruses was possible up to a dilution of 1:100 in the period from January to April. Potentially, this would allow the use of pooled samples from different trees. Processing samples at that time would also be an advantage in regard to reduce the workload in peak times in the laboratory, which often occur during the summer months. Except ASGV, all other apple viruses examined were detectable in even higher dilutions during this period and would allow to pool even more samples for testing individual viruses. In practice, the manner of pooling samples will depend on the preferences of the analysing laboratory. Mixing individually prepared nucleic acid extracts of trees would mean, that only single extracts have to be retested to identify a virus-infected tree in the case of a positive PCR result. In case tissues from different trees are pooled for extraction, this has to be made up for each individual tree represented in the sample in case of a positive result. However, the latter method saves time and expenses in situations where only a few infections are suspected.

Testing samples in the period of the highest virus accumulation should always be preferred to render tests most sensitive, reliable and minimize unfavorable factors.

Attention, however, should be paid to the results of this study because even samples taken at the same time, from individual trees of the same age, variety and location, exhibited great differences in virus accumulation. A similar observation was reported by SVOBODA and POLÁK (2010) who repeatedly tested leaf samples of two varieties from the same location for the relative concentration of ApMV with ELISA. The reasons therefore are unknown. In times of high virus titres this effect will be of little importance for a reliable virus detection but should be considered in times of low virus levels. Furthermore, the present virus isolates might influence the virus titre. This effect is known for different isolates of other viruses (e.g. GRAY et al., 1991, SYLLER and GRUPA, 2014).

Regarding the divergent results obtained by the various authors, it should be kept in mind that the detection of pome fruit viruses is influenced by numerous factors like climatic conditions, tree growth, apple cultivars, the kind of plant tissue examined and performance of analysis. All factors together influence the detectability, complicating the comparability of results. Furthermore, it can't be excluded that the virus titre is even influenced by the simultaneous infection with multiple viruses or by the way the tree is used. A strongly pruned scion mother tree, grafted on a strongly growing rootstock might have a divergent physiology compared to a fruit producing tree grafted on a weakly growing rootstock.

This study clearly reveals that apple virus titres and hence their detection can be distinctly influenced amongst other factors by the year of sampling and even by individual trees of the same cultivar growing under the same conditions. For successful testing, studies should be performed as described here, in particular when working with new apple cultivars, pooled samples, different tissue types or detection methods.

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