Comparative performance evaluation of double-stranded RNA high-throughput sequencing for the detection of viral infection in temperate fruit crops

Armelle Marais, Pascal Gentit, Yoann Brans, Jean Philippe Renvoisé, Chantal Faure, Anne Saison, Pascaline Cousseau, Julie Castaing, Fabien Chambon, Angélique Pion, et al.

Comparative performance evaluation of double-stranded RNA high-throughput sequencing for the detection of viral infection in temperate fruit crops

Armelle Marais[1*], Pascal Gentit[2*], Yoann Brans[3], Jean Philippe Renvoisé[4], Chantal Faure[1], Anne Saison[2#], Pascaline Cousseau [2], Julie Castaing[3], Fabien Chambon[4], Angélique Pion[4], Grégory Calado[4], Marie Lefebvre[1], Soraya Garnier[4], François Latour[3], Kévin Bresson[3], Nathalie Grasseau[3] and Thierry Candresse[1+]

[1]INRAE, Univ. Bordeaux, UMR Biologie du fruit et Pathologie, CS20032, 33882 Villenave d'Ornon Cedex, France

[2]ANSES, Plant Health Laboratory, Unité de Bactériologie, Virologie et détection des OGM, 7 rue Jean Dixméras, 49044 Angers Cedex 01, France

[3]CTIFL, Laboratoire de virologie fruitière, Centre de Lanxade, 24130 Prigonrieux, France

[4]ANSES, Plant Health Laboratory, Unité de Quarantaine, 63370 Lempdes, France

[*] Equally contributed to this work

[+]Corresponding author: T. Candresse; thierry.candresse@inrae.fr
ORCID 0000-0001-9757-1835

in memoriam

Running title: dsRNA HTS performance evaluation

Abstract: 245 words
Text (Introduction + M&M + Results + Discussion + Acknowledgements): 5363 words
2 Tables + 3 Figures

The nucleotide sequences reported here have been deposited in GenBank under accession numbers OR513892-OR513895

ABSTRACT

There is limited information on the compared performance of currently used biological, serological and molecular assays with high-throughput sequencing (HTS) for viral indexing in temperate fruit crops. Here, using a range of samples of predetermined virological status, we compared two performance criteria (inclusivity and analytical sensitivity) of ELISA, molecular hybridization, RT-PCR, and double-stranded RNA (dsRNA) HTS for the detection of a total of 14 viruses (10 genera) and four viroids (three genera). Using undiluted samples from individual plants, ELISA had the lowest performance, with an overall detection rate of 68.7%, followed by RT-PCR (82.5%) and HTS (90.7%, and 100% if considering only viruses). The lower performance of RT-PCR reflected the inability to amplify some isolates as a consequence of point mutations affecting primer-binding sites. In addition, HTS identified viruses that had not been identified by other assays in close to two-thirds of samples. Analysis of serial dilutions of fruit tree samples allowed to compare analytical sensitivity for various viruses. ELISA showed the lowest analytical sensitivity, but RT-PCR showed higher analytical sensitivity than HTS for a majority of samples. Overall, these results confirm the superiority of HTS over biological indexing in terms of speed and inclusivity and show that while the absolute analytical sensitivity of RT-PCR tends to be higher than that of HTS, PCR inclusivity is affected by viral genetic diversity. Taken together, these results make a strong case for the implementation of HTS-based approaches in fruit tree viral testing protocols supporting quarantine and certification programs.

Keywords: Fruit tree, Prunus, Malus, Pyrus, virus, viroid, Indexing, High-throughput sequencing, dsRNA.

INTRODUCTION

The control of plant viruses for vegetatively propagated species, such as temperate fruit crops, relies heavily on the use of virus-indexed planting materials (Martin et al. 2000; Hadidi et al. 2011). Efficient and sensitive diagnostic techniques constitute a key pillar of control efforts, including quarantine measures and certification programs aiming to make available to growers certified or virus-tested propagation materials (Martin et al. 2000, 2016; Reed and Foster 2011). Biological indexing by grafting on sensitive woody indicators (Thompson et al. 2011) has been extensively used for quarantine and certification programs for temperate fruit crops. However, serological and molecular diagnostic assays, such as ELISA, molecular hybridization, or RT-PCR (Martin et al. 2000; Mumford et al. 2006) have been gradually integrated for virus diagnosis. Because of its broad scope (inclusivity) and perceived analytical sensitivity, biological indexing is still routinely used in association with available laboratory tests, despite its long turnover and high costs linked to the months or years sometimes needed for symptoms expression (Rowhani et al. 2005; Thompson et al. 2011, Al Rwahnih et al. 2015; Rott et al. 2017).

Since their first application for plant virus discovery and characterization in 2009 (Kreuze et al. 2009: Adams et al. 2009; Al Rwahnih et al. 2009) high-throughput sequencing (HTS) approaches have demonstrated a remarkable potential and indeed revolutionized several fields, including virus discovery, etiology and, more broadly, metagenomics and virus ecology (Malmstrom et al. 2011; Roossinck et al. 2015: Maliogka et al. 2018; Zhang et al. 2019; Villamor et al. 2019; Maclot et al. 2020). The potential of HTS in the diagnostics field was recognized very early (Adams et al. 2009; Massart et al. 2014) and stems from several factors, including its ability to provide an unbiased and potentially complete picture of the sanitary status of a sample in a single experiment (Olmos et al. 2018; Maree et al. 2018). It was also

recognized that a number of hurdles would need to be overcome for HTS widespread adoption in routine diagnostic laboratories, including the availability of internationally recognized guidelines, robust protocols and standard operating procedures, reliable bioinformatics tools for sequence data analysis, and strategies to define positive detection thresholds (Massart et al. 2014; Maree et al. 2018; Olmos et al. 2018). Many of these challenges have now been addressed, including the definition of international standards or recommendations (Lebas et al. 2021; Massart et al. 2022; OEPP/EPPO, 2022) or the development and benchmarking of appropriate bioinformatics pipelines (Massart et al. 2018). Another key element for the adoption of HTS in diagnostic laboratories that was identified early is the availability of information on the performance of HTS-based diagnostics and direct comparisons with currently used biological, serological, or molecular assays (Massart et al. 2014; Maree et al. 2018).

A few such direct comparisons are available for some herbaceous hosts (see, for example, Hagen et al. 2012; Rolland et al. 2017; Santala and Valkonen 2018). However, low viral titers, uneven virus distribution, and the potential presence of inhibitory substances frequently complicate virus detection in woody hosts (Wetzel et al. 1992; Knapp et al. 1995, Maliogka et al. 2018, Wright et al. 2021), so that direct comparisons of HTS with existing assays is specifically needed in such hosts. Comparisons with biological indexing are available for grapevine (Al Rwahnih et al. 2015), small fruits (Villamor et al. 2022) or temperate fruit crops such as apple (*Malus*), pear (*Pyrus*) or *Prunus* (apricot, peach, plum or cherry) (Rott et al. 2017). Broadly speaking, these studies concluded that HTS is superior to biological indexing for detection purposes due to its speed, lower cost and comprehensiveness, and to HTS ability to detect novel agents. For small fruits, the comparison also included RT-PCR assays, but no attempts were made to compare analytical sensitivities (Villamor et al. 2022). The results

obtained showed that for the testing of individual plants, HTS and RT-PCR provided nearly parallel results but that HTS allowed the detection of a broader range of isolates.

As a further step towards the widespread adoption of HTS assays, we report here the results of a direct comparison of the performance criteria (OEPP/EPPO 2021) of biological indexing, ELISA, RT-PCR and double-stranded RNA (dsRNA) based HTS, for the detection of a wide range of viruses and viroids in temperate fruit trees, including a comparison of the limits of detection for the latter three approaches.

MATERIALS AND METHODS

Plant samples and virus isolates

A total of 36 fruit tree samples belonging to six species [apricot (*Prunus armeniaca*), sweet cherry (*P. avium*), plum (*P. domestica*), peach (*P. persica*), apple (*Malus domestica*) and pear (*Pyrus communis*)] were included in the present study. For each species, one uninfected control sample (sampled from certified germplasm collections) and between three and six samples infected by various viruses and/or viroids were used Table 1. With the exception of five peach samples, which were obtained following chip-budding inoculation on GF305 peach seedlings, all other samples were obtained from trees that are part of the collections of viral isolates held by the various partners. Depending on their regulatory status, plants were either grown in high-containment greenhouses or under insect-proof screenhouses. Quarantine pests were held and shared between partners under the appropriate letter of authority. For all infected samples, between six and ten grams of leaves were collected in spring 2017 (except for one apricot sample growing in a greenhouse, which was collected in early spring 2018). Leaves were cut in small slivers using scissors to homogenize the sample and aliquots (1g fresh weight) lyophilized before being distributed to partners. Healthy samples were similarly processed, except that a total of 25g of leaves were processed for each healthy sample.

For the determination of analytical sensitivity, a sample was selected for each of the six fruit species. For each of these samples, serial ten-fold dilutions in a healthy control homogenate of the same species were prepared and tested as described below.

Double-stranded RNA purification, HTS analysis and bioinformatic sequence data analysis

Double-stranded RNAs (dsRNAs) were purified by a first round of CC41 cellulose chromatography followed by nucleases treatment and a second round of cellulose chromatography as described (Marais et al. 2018). Purified dsRNAs were used for cDNA synthesis, followed by a random PCR amplification step (Marais et al. 2018). In order to limit intersample cross-talk, individual libraries were prepared for each sample, which were then sequenced on an Illumina HiSeq 3000 (2x150 nt) at the GetPlaGe (INRAE Toulouse, France) platform. Following quality trimming using CLC Genomics Workbench (CLC-GW version 15 or later, Qiagen), HTS reads obtained from the various samples were analyzed using CLC-GW and the VirAnnot pipeline (Lefebvre et al. 2019). If needed, number of reads for different samples was normalized by random reads sampling using CLC-GW. Reads were mapped using CLC-GW against reference genomes downloaded from GenBank for all viruses known to infect the tested fruit tree species. This mapping was performed at a reduced stringency level (length fraction 100%, minimal similarity fraction 75%) in order to take viral intraspecific diversity into account so that reads from divergent isolates would still be identified.

In addition, following *de novo* assembly performed with CLC-GW (word size, 50; bubble size, 300), contigs were annotated by BlastN and BlastX analyses against the GenBank database to detect novel viruses or highly divergent variants of known viruses.

To take into account inter-sample crosstalk due to index jumping (Illumina 2017; van der Valk et al. 2019), a threshold of positive detection was computed for each viral molecule by calculating the average plus 3 standard deviations (SD) of background virus reads observed in

libraries generated for the six healthy samples. Assuming a normal distribution of background reads, the use of such a positivity threshold would provide a <1% risk of reporting a false positive detection (https://en.wikipedia.org/wiki/68%E2%80%9395%E2%80%9399.7_rule).

Testing of fruit tree samples using ELISA assays

All ELISA tests were performed using commercial kits and following the recommendations of the kit providers. For apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), prune dwarf virus (PDV), prunus necrotic ringspot virus (PNRSV), plum pox virus (PPV), strawberry latent ringspot virus (SLRSV) and tomato ringspot virus (ToRSV), BIOREBA kits (Reinach, Switzerland) were used, while a kit from AGDIA (Elkhart, IN, USA) was used for American plum line pattern virus (APLPV). Each sample was assayed using two ELISA wells and the analysis was repeated twice. For the interpretation of the results, samples were considered positive if their average absorbance value at 405 nm was above two times the average absorbance of the uninfected control of the same plant species.

Testing of fruit tree samples by molecular hybridization

Peach latent mosaic viroid (PLMVd) was detected by molecular hybridization using non-radioactive *in vitro* transcribed RNA probes labeled with digoxygenin. The protocol used broadly follows that of Turturo et al. (1998). Each sample was spotted in duplicate on the hybridization membrane.

Testing of fruit tree samples using RT-PCR assays

Testing of fruit tree samples by RT-PCR was performed using RT-PCR conditions and primers that are either according to published protocols or have been developed in the frame of the present study. A detailed list of the protocols used is provided in Supplementary Table S1.

RESULTS

Detection of viruses and viroids in temperate fruit tree samples using various techniques

Infection by a range of viruses and viroids in a total of 36 temperate fruit tree (apricot, peach, sweet cherry, plum, apple, and pear) samples, including one healthy control per tree species, were tested in parallel by ELISA, RT-PCR, and double-stranded RNA (dsRNA) based high-throughput sequencing (HTS). The virological status of the samples had previously been determined by biological indexing using recommended protocols and woody indicators (Jelkmann 2003) and/or by targeted serological or molecular analyses combined with symptoms observation. The samples (from three to six per species) were selected to cover a broad diversity of fruit tree viruses and viroids, including agents of quarantine or certification significance in single or mixed infections. In total, the 30 infected samples analyzed represent 14 viruses belonging to 10 genera and four viroids belonging to three genera (Table 1).

For each virus, only samples previously found infected by a given virus and negative controls were tested using the corresponding ELISA assay. Detection of peach latent mosaic viroid (PLMVd) was similarly performed by molecular hybridization using digoxygenin-labeled RNA probes on healthy controls and on two PLMVd-infected peach samples (Table 1).

The presence of all viruses and viroids, with the exception of PLMVd, was similarly assessed in controls and in relevant samples using virus-specific validated RT-PCR or qRT-PCR assays or using assays specifically developed for the present project (Supplementary Table S1). Lastly, all samples were indexed by the Illumina HTS analysis of randomly amplified cDNAs produced from highly purified dsRNAs (Marais et al. 2018) (on average 2.58M +/-1.27M trimmed reads/sample).

Overall, of the 46 infections expected in the 30 infected samples, only three were not confirmed by any of the assays. This concerns a peach sample (F586) in which an expected tomato ringspot virus (ToRSV) infection was not detected by ELISA, RT-PCR and HTS and two pear samples (PO13357 and PO1492) in which apple chlorotic leaf spot (ACLSV) infection was not

confirmed by ELISA, RT-PCR and HTS (Table 1). Since spring is the recommended sampling period for these two viruses, these results might reflect the irregular distribution of the viruses in the sampled tree, a loss of a viral isolate during propagation or possibly a misidentification of a viral infection during biological indexing resulting from the mixed infection status of some of the analyzed samples. In any case, due to the convergent results obtained using several techniques, the three samples concerned were considered as free of infection by the relevant virus/viroid and therefore not considered in all further data analyses. No virus or viroid detection was obtained by any of the techniques for the negative controls.

Altogether, a positive ELISA detection result was obtained for 22 of the 32 expected infections tested (68.7%), the 10 expected infections not detected concerning ACLSV [two infections in plum (PR72 and PR3473) and two in apple (LX86 and X259)], apple stem pitting virus (ASPV) [one infection in apple (LX86) and two in pear (PO13357 and PO161)] and all three tested American plum line pattern virus (APLPV) infections in peach and plum (Table 1). The three APLPV infections missed by ELISA were confirmed by both RT-PCR and HTS, together with one ACLSV infection (X259 apple) and one ASPV infection (LX86 apple), while the other ACLSV or ASPV infections not detected by ELISA were only confirmed by HTS.

RT-PCR assays confirmed 33 infections out of the 40 tested (82.5%). The seven negative results concern a strawberry latent ringspot virus (SLRSV) infection in peach (LS27) confirmed by ELISA and HTS, an ACLSV infection in peach (PR3473) similarly confirmed by ELISA and HTS and three ACLSV infections (PR72 and PR3473 plums, LX86 apple) plus two ASPV infections (PO13357 and PO161 pears), only confirmed by HTS.

Finally, HTS confirmed 39 of 43 tested infections (90.7%). The four infections not identified by HTS concern three viroids confirmed by RT-PCR [apple scar skin viroid, (ASSVd) in X259 apple, hop stunt viroid (HSVd) in Roma1 apricot and PR72 plum, and pear blister canker viroid

194 (PBCVd) in PO2098 pear], so that the performance of HTS if considering only infections by
195 viruses reaches 100% detection (39/39).

196 **Unexpected viral infections detected by dsRNA-based HTS**

197 Of the 30 tested samples, 19 (63%) were found to be infected by viruses that had not been
198 previously detected by biological indexing or by targeted assays. Five of those 19 samples had
199 mixed infections involving two unforeseen viruses. All these infections detected by HTS and
200 that had not been previously identified by biological indexing were confirmed by specific RT-
201 PCR assays (Table 1).

202 Six of the unforeseen infections detected by HTS concern cherry virus A (CVA) in peach,
203 cherry and plum, a virus for which there is currently no known biological indicator. Other
204 detected infections for which no biological indexing assay is available concern mostly recently
205 described viruses such as Asian prunus virus 3 (APV3) in peach, nectarine stem pitting-
206 associated virus (NSPaV) in peach, plum bark necrosis stem pitting-associated virus
207 (PBNSPaV) in peach and plum, peach leaf pitting-associated virus (PeLPaV) in plum, prunus
208 virus F (PrVF) in cherry, citrus concave gum-associated virus (CCGaV) in apple and citrus
209 virus A (CiVA) in pear (Table 1). They also concern viruses whose presence in temperate fruit
210 trees was not expected, such as cucumber mosaic virus (CMV) in plum or grapevine cryptic
211 virus (GCV) in apricot. However, eight of the detected infections concern well-known fruit tree
212 viruses that should have been detected by biological indexing, such as little cherry virus 1
213 (LChV1) in apricot and cherry, apple stem grooving virus (ASGV) in apple and pear, ACLSV
214 in apple, ASPV in pear or apple rubbery wood viruses 1 and 2 (ARWV1 and ARWV2 in apple
215 and pear, respectively) (Table 1).

216 In parallel, several infections were identified by HTS but not confirmed by RT-PCR. Some of
217 these represent clear instances of false positive detection because the viruses involved are not
218 known to be able to infect the host in which they were identified. This concerns, for example,

the detection of plum pox virus (PPV) in a pear sample or the detection of ASPV in a peach and a plum sample. In some other cases, the infections detected by HTS could not be discounted on such biological bases. However, when analyzed, the isolates identified by HTS and not confirmed by RT-PCR were found to be identical to other isolates included in the experiment and that had yielded particularly high read counts. For example, the PPV isolate identified in the PO13357 pear is identical to the GS apricot PPV isolate, which yielded in excess of 1.7 million (M) reads (65.7% of total reads), while that identified from the LS27 peach is identical to the one that yielded over 1.2M reads (48.4% of total reads) from the D peach sample. These comparisons thus indicate that HTS detections not confirmed by RT-PCR should be considered as false positives resulting from laboratory or sequencing platform experimental contaminations or inter-sample crosstalk during sequencing.

Point mutations in primer-binding sites explain the failure to detect some viral isolates by RT-PCR

Given its known analytical sensitivity, the failure of RT-PCR to detect some viral isolates found positive by ELISA and HTS came as a surprise and prompted efforts to see whether mutations affecting primer-binding sites might be responsible for these results. Indeed, for several viral isolates, such mutations could be identified. A particularly clear-cut case involves the SLRSV isolate in peach LS27, which was detected by ELISA and by HTS (>35,000 reads above threshold, nearly complete genome assembled) and yet was not detected by RT-PCR. Inspection of the assembled genome revealed, respectively, four and five mutations affecting the binding sites of primers SLRSV-F and SLRSV-R (Postman et al. 2004) (Figure 1A). In particular, one of the mutations affects the 3' end of primer SLRSV-R, which is very likely to limit amplification of the LS27 isolate. The assembled genome of this highly divergent isolate has been deposited in GenBank (OR513894-OR513895) and shows an overall 29-32% nt divergence with other GenBank SLRSV isolates. For ACLSV and ASPV isolates not detected

by RT-PCR and for which HTS genome coverage allowed the analysis of primers binding sites, a similar pattern was observed (Figure 1B and 1C). In the case of ACLSV, respectively six and five mismatches were identified with primer A52 (Candresse et al. 1995), including a mutation affecting the primer 3' end, for isolates PR3473 from peach and LX86 from apple (Figure 1B), while the corresponding region was not covered by HTS reads for pear isolates PO13357, PO1492 and PO161. Between two and three mismatches were also found to affect the binding site of primer A53 for all non-detected isolates (Figure 1B). It should be noted that the detection of isolates PR3473 and LX86 by HTS was unambiguously positive, with respectively >69,000 reads and 1,390 reads above the threshold and genome coverages of 89% and 74%, respectively (GenBank OR513892 for the PR3473 isolate, the fragmented assembly of the LX86 isolate was not deposited).

In the case of ASPV, while no sequence information could be obtained for pear isolate PO161, analysis of the reads for pear isolate PO13357 showed the binding sites for primers ASPV-F and ASPV-R (Menzel et al. 2002) to be respectively affected by three- and four-point mutations, including an insertion in the case of ASPV-R (Figure 1C). Similar to the situation with ACLSV, the detection by HTS of ASPV in PO13357 was unambiguous, with 21,500 reads above the threshold and ~86% of genome coverage (GenBank OR513893).

Comparative analytical sensitivity evaluation of ELISA, RT-PCR and HTS on fruit tree samples

In a second step, using serial dilutions of a set of temperate fruit tree samples, we tried to assess the analytical sensitivity of the three techniques by means of the limit of detection (LOD) determination using a set of serial dilutions. For this experiment, we selected one sample for each of the six fruit species addressed in the present work. For each of those samples, 10-fold serial dilutions of a homogenate were prepared in a healthy control homogenate of the same species. The serial dilutions were tested directly by ELISA or used for total nucleic acids

extraction prior to RT-PCR assays or for dsRNA extraction prior to HTS analysis, which yielded an average of 2.06 +/-0.76M trimmed reads per sample.

The results obtained are presented in Table 2. Of the 10 viruses thus analyzed, three (CVA, ARWV1 and ARWV2) could not be assayed by ELISA because no commercial kits are available for their detection. Only three of the seven tested viruses could be detected by ELISA in diluted samples: PNRSV in plum with a LOD of 10^{-1}, PDV in peach with a 10^{-2} LOD and PPV in apricot, which was still weakly detected at the 10^{-3} dilution. In addition, ASPV was detected with a LOD of 10^{-2} in apple but was only detected in pear in the undiluted sample. All other viruses (ApMV, ACLSV, ASGV) could only be detected in undiluted samples. In two cases (plum PR3473 and apple LX225), ACLSV was not detected by ELISA, even in undiluted samples (Table 2).

Although there was a lot of variability in the LOD for different viruses and for different virus/host combinations, RT-PCR generally proved much more sensitive than ELISA. LODs of 10^{-5} to 10^{-7} were, for example, obtained for ACLSV (in apple LX225), CVA, PDV, ApMV, PPV, and ASPV (in apple LX225). The lowest analytical sensitivity was observed for ARWV2 and ACLSV in cherry (LOD of 10^{-1}) and ARWV1, ASPV in pear and PNRSV (LOD of 10^{-2}).

For its part, dsRNA-based HTS generally showed an analytical sensitivity intermediate between ELISA and RT-PCR. Two viruses, ARWV1 and ARWV2 could only be detected from undiluted samples. ACLSV showed a LOD of 10^{-1} to 10^{-3} depending on the host/sample and ApMV a LOD of 10^{-2}, while the other six viruses (CVA, PDV, PNRSV, PPV, ASGV and ASPV) showed LODs of 10^{-3} or better, the most sensitive detection being observed for PPV with a LOD of 10^{-5}. In the case of PDV and PNRSV, analytical sensitivity higher by a 10 to 100-fold factor was observed if considering RNA3 reads than those for RNA1 or RNA2 (Table 2).

Since sequencing depth is obviously a factor affecting analytical sensitivity using HTS approaches (Bester et al. 2021; Schönegger et al. 2023), HTS datasets for the various samples and controls were randomly subsampled at different depth (1M, 300K, 100K, 33K and 10K reads) and detection performance evaluated by mapping the reads on reference genomes. The same threshold (average of negative controls plus three standard deviations) was used to determine positive detections and all resamplings were performed three times in order to evaluate repeatability of results. For this analysis, the detection of CCGaV in the LX225 apple sample, of CiVA in the PO1492 pear sample and of PLMVd in the Gotheron 94/6 peach sample were also taken into consideration so that the overall rate of detection of 13 agents (12 viruses plus one viroid) as affected by sequencing depth and sample dilution is shown in Figure 2.

As expected, performance evaluated in terms of the proportion of viral agents detected was generally reduced as sequencing depth decreased, whatever the tested samples' dilution rate. Similarly, for a given sequencing depth, the fraction of viral agents detected was reduced as more and more diluted samples were analyzed (Figure 2).

Detection of all 13 tested viruses and viroids was only achieved using undiluted samples, with the agents most frequently lost when resampling the 10^{-1} dilution dataset being PLMVd and the four negative-stranded viruses ARWV 1 and 2, CCGaV and CiVA. On the other hand, using undiluted samples, all viral agents could be reproducibly detected using as little as 300K reads, while variability in performance was observed when using only 100K reads (Figure 2), with a failure to detect, in some of the resampling, one or two of the agents frequently lost using the 10^{-1} dilution.

DISCUSSION

Very few studies have attempted so far to directly compare the performance of currently used biological, serological, or molecular assays with that of HTS-based approaches for woody plant materials (Al Rwahnih et al. 2015; Rott et al. 2017; Villamor et al. 2022). Viral diagnosis in

318 woody perennial hosts often poses specific difficulties that are linked to the uneven distribution
319 and low titers observed for many viruses in these hosts (Knapp et al. 1995; Maliogka et al.
320 2018; Morvan and Castelain 1976; Wetzel et al. 1992; Wright et al. 2021). These problems can
321 also be compounded by the frequent presence in these hosts of inhibitory substances, such as
322 polysaccharides or polyphenolics, that may interfere with detection assays. Using samples of
323 the six most common temperate fruit crops, we report here efforts at a direct performance
324 comparison of a dsRNA-based HTS approach with not only biological indexing as done in
325 previous publications but also ELISA and RT-PCR.
326 The results reported here confirm previous efforts in woody perennials, showing that the main
327 advantage of HTS is its ability to detect a broader range of viruses than biological indexing (Al
328 Rwahnih et al. 2015; Rott et al. 2017; Villamor et al. 2022). Three infections expected from
329 previous biological indexing or targeted assays were not identified using HTS, but these
330 infections were also not detected using ELISA or RT-PCR, which suggests that they either
331 represent erroneous anterior indexing results, are the results of virus loss during virus source
332 propagation or are due to uneven viral distribution in the sampled trees. All other infections
333 expected from biological indexing results (43/46, Table 1) were confirmed by one or more
334 assays.
335 On the other hand, a total of 24 unexpected infections were detected in 19 of the 30 tested fruit
336 tree samples (63%), indicating that biological indexing had failed to identify a viral infection
337 in almost two-thirds of samples. Remarkably, eight of these unexpected infections (33%)
338 involved well-known viruses for which biological indexing assays are available (LChV1,
339 ASGV, ASPV, rubbery wood viruses). It should, however, be stressed that not all samples had
340 gone through a complete biological indexing scheme as used in certification programs and
341 which involves the use of multiple indicators and samples over an extended period. In this
342 respect, it is noteworthy that no infections were detected in the healthy samples obtained from

certified germplasm collections, which rules out, for example, the possibility that detection of these agents might represent pollen contamination of the samples rather than real viral infections. None of these four viruses has known vectors or is known to be mechanically transmissible in woody hosts and their transmission is therefore believed to be mostly through vegetative propagation of their hosts (Jelkmann and Eastwell 2011; Massart et al. 2011; Jelkmann and Paunovic 2011; Yaegashi et al. 2011). This makes it highly unlikely that their detection may reflect new infections of sampled hosts that developed between biological indexing and sampling. Likewise, the fact that all these infections were confirmed by RT-PCR rules out the possibility that they may represent false positive HTS detections. On the other hand, symptom observations can be difficult, particularly in situations of mixed infection, such as in many samples used for this study. Together with the fact that not all samples used here had gone through a complete biological indexing scheme involving grafting on multiple indicators to extend the range of viruses detected, the impact of mixed infections on symptoms observation and the lack of biological indicators for some viruses likely explain the results reported here.

When comparing the performance of ELISA, RT-PCR, and HTS on undiluted plant samples in the first experiment, ELISA had an overall detection rate of 69% (22/32), RT-PCR of 82.5% (33/40) and HTS of 90.7% (39/43) (Table 1). However, if considering only viruses, the HTS detection rate increases to 100% (37/37, Table 1). The low performance with viroids (only PLMVd detected out of the four viroids tested) of the dsRNA purification protocol used here (Marais et al. 2018) is already known and possibly results from the nuclease treatment included between the two cellulose chromatography steps. Indeed, PLMVd was only detected with low sensitivity since it was among the agents that could not be reliably detected from 10-fold diluted samples. The efficient detection of PLMVd using a dsRNA-based HTS approach has, however, been reported previously (Kesanakurti et al. 2016; Rott et al. 2017).

The overall low performance of ELISA should not come as a surprise since some low titer viruses. such as ASGV, ASPV, or ACLSV are notoriously difficult to detect by this technique in woody hosts (Massart et al. 2011; Yaegashi et al. 2011). Isolate variability, which is known to be large for these viruses, may further complicate detection of all isolates. On the other hand, the relatively poor performance of RT-PCR, with an overall detection rate of 82.5%, appears to be mostly (if not fully) linked to point mutations affecting primer binding sites in some isolates rather than to a lack of analytical sensitivity of the technique or other artefactual causes. Despite the use of validated assays known to show broad analytical specificity (Candresse et al. 1995; El Morsy et al. 2017 ; Menzel et al. 2002; Postman et al. 2004; Tang et al. 2013), these limits in the ability of the RT-PCR assays to amplify all the isolates of a given virus resulted in an overall better performance of HTS if considering only viruses. It is, however, clear that the use of different primer pairs may have modified the ability to detect some isolates (Katsiani et al. 2018a). An added benefit of HTS is that by providing access to the genomic sequence of viral isolates, it offers a direct route to the evaluation and, if needed, the improvement of the inclusivity of detection primers (Katsiani et al. 2018a; Marais et al. 2014).

When comparing the three techniques using 10-fold dilutions of samples, ELISA proved once again to be the technique with the lowest analytical sensitivity (Figure 3), while RT-PCR outperformed HTS by allowing the detection of all viruses tested using a 10^{-1} dilution of samples. This indicates that the dsRNA-based HTS approach used should not be performed on pooled samples unless a sequencing depth significantly deeper than 1M reads per sample is used (Figure 2). In particular, the performance of this approach on the minus-stranded RNA viruses analyzed (ARWV1 and 2, CCGaV, and CiVA) was not as good as on positive RNA viruses. This confirms other observations on a lower performance of dsRNA-based approaches with negative-stranded viruses (Schönegger et al. 2023). If needed, the use of a different nucleic acids target population, such as ribodepleted total RNAs, could provide for more efficient

393 detection of minus-stranded viruses and viroids but would necessitate deeper sequencing of
394 samples since it does not benefit from the enrichment of viral sequences brought by dsRNA
395 purification.
396 Significant variability of the LOD was observed between viruses but also between fruit tree
397 samples infected by the same virus, despite the fact that the sampling was performed during the
398 spring period, which is generally regarded as the most appropriate for virus detection (Cambra
399 et al. 2011; Katsiani et al. 2018b). This might reflect a range of factors, including uneven virus
400 distribution, the variability of viral isolates, their level of adaptation to different fruit tree
401 species, or the impact of mixed infections on viral concentrations. This implies that LOD values
402 reported here should be considered as providing indications limited to the analyzed samples
403 that should not be generalized without caution. There similarly was variability in the compared
404 performance of RT-PCR and HTS, with RT-PCR having a lower LOD in 61.5% of comparisons
405 (8/13) but HTS showing the same or a lower LOD than RT-PCR in 38.5% of comparisons (2/13
406 equal performance and 3/13 better performance, Table 2). This might reflect viral variability
407 and the impact of mutations in primer-binding sites, possibly reducing amplification efficiency.
408 In this context of viral variability, it is interesting to notice that as little as 300K reads per single
409 plant sample allowed the detection of all viruses, thus providing a baseline for future use of this
410 technique for fruit tree indexing by sequencing.
411 Overall, the results reported here confirm previous findings showing the superiority of HTS
412 approaches over biological indexing in terms of speed and inclusivity (Al Rwahnih et al. 2015;
413 Rott et al. 2017; Villamor et al. 2022). They also show that while the absolute analytical
414 sensitivity of RT-PCR assays tends to be higher, their inclusivity is often incomplete as a
415 consequence of viral variability and of mutations affecting primer-binding sites. Taken
416 together, these elements make a strong case for the implementation of HTS-based approaches

417 in fruit tree diagnostics protocols supporting quarantine and certification programs for which
418 the speed and inclusivity of these approaches represent clear benefits.

419 **Acknowledgments:** This research was funded by the French Ministry of Agriculture to
420 CASDAR project VirValid C-2016-09 n°2102017800. The authors thank the INRAE
421 GenoToul Platform (Toulouse, France) for high throughput sequencing, CEP and CTPS
422 (Comité technique permanent de la sélection) for their support and participation in designing
423 the project, and Benoit Remenant (ANSES, Plant Health Laboratory, Angers, France) for
424 technical assistance with some aspects of the project.

425

LITERATURE CITED

Adams, I.P., Glover, R.H., Monger, W.A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M. and Boonham, N. 2009. Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. Mol. Plant. Pathol. 10:537-545.

Al Rwahnih, M., Daubert, S., Golino, D. and Rowhani, A. 2009. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology 387:395-401.

Al Rwahnih, M., Daubert, S., Golino, D., Islas, C. and Rowhani, A. 2015. Comparison of next-generation sequencing versus biological indexing for the optimal detection of viral pathogens in grapevine. Phytopathology 105:758-763.

Bester, R., Cook, G., Breytenbach, J.H., Steyn, C., de Bruyn, R. and Maree, H.J. 2021. Towards the validation of high-throughput sequencing (HTS) for routine plant virus diagnostics: measurement of variation linked to HTS detection of citrus viruses and viroids. Virology J. 18:1-19.

Cambra, M., Boscia, D., Gil, M., Bertolini, E. and Olmos, A. 2011. Immunology and immunological assays applied to the detection, diagnosis and control of fruit tree viruses. In "Virus and virus-like diseases of pome and stone fruits". Hadidi, A., Barba, M., Candresse, T. and Jelkmann, W. Eds, APS, St Paul (MN), USA, pp 303-313.

Candresse, T., Lanneau, M., Revers, F., Grasseau, N., Macquaire, G., German, S., Malinovsky, T. and Dunez, J. (1995) An immunocapture-PCR assay adapted for the detection and the analysis of the molecular variability of apple chlorotic leaf spot trichovirus. Acta Horticulturae 386:136-147.

448 El-Morsy, S.I., el-Sheikh, M.A., Abd el-Razik, R.A., Youssef, S.A. and Shalaby, A.A. 2017.
449 Molecular identification of Strawberry latent ring spot virus (SLRSV) in Egypt. J. Basic
450 Environ. Sci. 4:24-33.

451 Hadidi, A., Pasquini, G., Barba, M., Spiegel, S., Zaidi, A. and Crescenzi, A. 2011. Strategies
452 for control of systemic pathogens of fruit trees. In "Virus and virus-like diseases of pome
453 and stone fruits". Hadidi, A., Barba, M., Candresse, T. and Jelkmann, W. Eds, APS, St Paul
454 (MN), USA, pp 373-375.

455 Hagen, C., Frizzi, A., Gabriels, S., Huang, M., Salati, R., Gabor, B. and Huang, S. 2012.
456 Accurate and sensitive diagnosis of geminiviruses through enrichment, high-throughput
457 sequencing and automated sequence identification. Arch. Virol. 157:907-915.

458 Illumina, 2017. Effects of index misassignment on multiplexing and downstream analysis.
459 https://www.illumina.com/content/dam/illumina-marketing/documents/products/
460 whitepapers/index-hopping-white-paper-770-2017-004.pdf

461 Jelkmann, W. 2003. International working group on fruit tree viruses. Detection of virus and
462 virus-like diseases of fruit trees. Acta Hortic. 657:575-596.

463 Jelkmann, W. and Eastwell, K.C. 2011. Little cherry virus-1 and 2. In "Virus and virus-like
464 diseases of pome and stone fruits". Hadidi, A., Barba, M., Candresse, T. and Jelkmann, W.
465 Eds, APS, St Paul (MN), USA, pp 153-159.

466 Jelkmann, W and Paunovic, S. 2011. Apple stem pitting virus. In "Virus and virus-like diseases
467 of pome and stone fruits". Hadidi, A., Barba, M., Candresse, T. and Jelkmann, W. Eds, APS,
468 St Paul (MN), USA, pp 35-40.

469 Katsiani, A., Maliogka, V.I., Katis, N., Svanella-Dumas, L., Olmos, A., Ruiz-García, A.B.,
470 Marais, A., Faure, C., Theil, S., Lotos, L. and Candresse, T. 2018a. High-throughput

sequencing reveals further diversity of little cherry virus 1 with implications for diagnostics. Viruses 10:385.

Katsiani, A.T., Pappi, P., Olmos, A., Efthimiou, K.E., Maliogka, V.I. and Katis, N.I. 2018b. Development of a real-time RT-PCR for the universal detection of LChV1 and study of the seasonal fluctuation of the viral titer in sweet cherry cultivars. Plant Disease 102:899-904.

Kesanakurti, P., Belton, M, Saeed, H., Rast, H., Boyes, J. and Rott, M. 2016. Screening for plant viruses by next generation sequencing using a modified double strand RNA extraction protocol with an internal amplification control. J. Virol. Meth. 236:35-40.

Knapp, E., da Câmara Machado, A., Pühringer, H., Wang, Q., Hanzer, V., Weiss, H., Weiss, B., Katinger, H. and Laimer da Câmara Machado, M. 1995. Localization of fruit tree viruses by immuno-tissue printing in infected shoots of *Malus* sp. and *Prunus* sp. J. Virol. Meth. 55, 157-73.

Kreuze, J.F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I. and Simon, R. 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. Virology 388:1-7.

Lebas, B., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G.J., Blouin, A.G., Boonham, N., Candresse, T., Chandelier, A., de Jonghe, K., Fox, A., Gaafar, Y.Z.A., Gentit, P., Haegeman, A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutjnak, D., Landa, B., Liu, M., Maclot, F., Malapi-Wight, M., Maree, H.J., Martoni, F., Mehle, N., Minafra, A., Mollov, D., Moreira, A., Nakhla, M., Petter, F., Piper, A.M., Ponchart, J., Rae, R., Remenant, B., Rivera, Y., Rodoni, B., Roenhorst, J.W., Rollin, J., Saldarelli, P., Santala, J., Souza-Richards, R., Spadaro, D., Studholme, D.J., Sultmanis, S., van der Vlugt, R., Tamisier, L., Trontin, C., Vazquez-Iglesias, I., Vicente, C.S.L., Vossenberg, B.T.L.H, Wetzel, T., Ziebell, H. and

Massart, S. 2022. Facilitating the adoption of high-throughput sequencing technologies as a plant pest diagnostic test in laboratories: a step-by-step description. EPPO Bull. 52:394-418.

Lefebvre, M., Theil, S., Ma, Y. and Candresse, T. 2019. The VirAnnot pipeline: a resource for automated viral diversity estimation and operational taxonomy units assignation for virome sequencing data. Phytobiomes J. 3:256-259.

Maclot, F., Candresse, T., Filloux, D., Malmstrom, C.M., Roumagnac, P., van der Vlugt, R. and Massart, S. 2020. Illuminating an ecological black-box: using high throughput sequencing to characterize the plant virome across scales. Front. Microbiol. 11:578064.

Maliogka, V.I., Minafra, A., Saldarelli, P., Ruiz-García, A.B., Glasa, M., Katis, N. and Olmos, A. 2018. Recent advances on detection and characterization of fruit tree viruses using high-throughput sequencing technologies. Viruses 10:436.

Malmstrom, C.M., Melcher, U. and Bosque-Pérez, N.A. 2011. The expanding field of plant virus ecology: historical foundations, knowledge gaps, and research directions. Virus Res. 159:84-94.

Marais, A., Faure, C., Couture, C., Bergey, B., Gentit, P. and Candresse, T. 2014. Characterization by deep sequencing of divergent plum bark necrosis stem pitting-associated virus (PBNSPaV) isolates and development of a broad-spectrum PBNSPaV detection assay. Phytopathology 104:660-666.

Marais, A., Faure, C., Bergey, B. and Candresse, T. 2018. Viral double-stranded RNAs (dsRNAs) from plants: alternative nucleic acid substrates for high-throughput sequencing. Meth. Mol. Biol. 1746:45-53.

Maree, H.J., Fox, A., al Rwahnih, M., Boonham, N. and Candresse, T. 2018. Application of HTS for routine plant virus diagnostics: state of the art and challenges. Front. Plant Sci. 27:1082.

519 Martin, R.R., Constable, F.E. and Tzanetakis, I.E. 2016. Quarantine regulations and the impact
520 of modern detection methods. Annu. Rev. Phytopathol. 54:189-205.

521 Martin, R.R., James, D. and Lévesque, C.A. 2000. Impacts of molecular diagnostic
522 technologies on plant disease management. Annu. Rev. Phytopathol. 38:207-239.

523 Massart, S., Adams, I., Al Rwahnih, M., Baeyen, S., Bilodeau, G.J., Blouin, A., Boonham, N.,
524 Candresse, T., Chandelier, A., de Jonghe, K., Fox, A., Gaafar, Y.Z.A., Gentit, P., Haegeman,
525 A., Ho, W., Hurtado-Gonzales, O., Jonkers, W., Kreuze, J., Kutnjak, D. and Lebas, B.S.M.
526 2022. Guidelines for the reliable use of high throughput sequencing technologies to detect
527 plant pathogens and pests. Peer Comm. J. 2:e62.

528 Massart, S., Chiumenti, M., de Jonghe, K., Glover, R., Haegeman, A., Koloniuk, I., Komínek,
529 P., Kreuze, J., Kutnjak, D., Lotos, L., Maclot, F., Maliogka, V., Maree, H.J., Olivier, T.,
530 Olmos, A., Pooggin, M.M., Reynard, J.S., Ruiz-García, A.B., Safarova, D., Schneeberger,
531 P.H.H., Sela, N., Turco, S., Vainio, E.J., Varallyay, E., Verdin, E., Westenberg, M.,
532 Brostaux, Y. and Candresse, T. 2018. Virus detection by high-throughput sequencing of
533 small RNAs: large-scale performance testing of sequence analysis strategies.
534 Phytopathology 109:488-497.

535 Massart, S., Jijakli, M.H. and Kummert, J. 2011. Apple stem grooving virus. In "Virus and
536 virus-like diseases of pome and stone fruits". Hadidi, A., Barba, M., Candresse, T. and
537 Jelkmann, W. Eds, APS, St Paul (MN), USA, pp 29-33.

538 Massart, S., Olmos, A., Jijakli, H. and Candresse, T. 2014. Current impact and future directions
539 of high throughput sequencing in plant virus diagnostics. Virus Res. 188:90-96.

540 Menzel, W., Jelkmann, W. and Maiss, E. 2002. Detection of four apple viruses by multiplex
541 RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Meth.
542 99:81-92.

543 Morvan, G. and Castelain, C. 1976.Localization of the Sharka virus in peach trees in reference
544 to sanitary checking. Mitt. Biol. Bundesanst. Land-Forstwiztsch. 170:65-72.

545 Mumford, R., Boonham, N., Tomlinson, J. and Barker, I. 2006. Advances in molecular
546 phytodiagnostics - new solutions for old problems. Eur. J. Plant Pathol. 116:1-19.

547 OEPP/EPPO, 2021. PM 7/98(5) Specific requirements for laboratories preparing accreditation
548 for a plant pest diagnostic activity. EPPO Bull. 51:468-498.

549 OEPP/EPPO, 2022. PM 7/151(2) Considerations for the use of high throughput sequencing in
550 plant health diagnostics. EPPO Bull. 52:619-642.

551 Olmos, A., Bertolini, E., Gil, M. and Cambra, M. 2005. Real-time assay for quantitative
552 detection of non-persistently transmitted Plum pox potyvirus RNA targets in single aphids.
553 J. Virol. Meth. 128:151-155.

554 Olmos, A., Boonham, N., Candresse, T., Gentit, P., Giovani, B., Kutnjak, D., Liefting, L.,
555 Maree, H.J., Minafra, A., Moreira, A., Nakhla, M.K., Petter, F., Ravnikar, M., Rodoni, B.,
556 Roenhorst, J.W., Rott, M., Ruiz-García, A.B., Santala, J., Stancanelli, G., van der Vlugt, R.,
557 Varveri, C., Westenberg, M., Wetzel, T., Ziebell, H. and Massart, S. 2018. High-throughput
558 sequencing technologies for plant pest diagnosis: challenges and opportunities. EPPO Bull.
559 48:219-224.

560 Postman, J.D., Postman , I. E. Tzanetakis, I.E. and R. R. Martin, R.R. 2004. First report of
561 strawberry latent ringspot virus in a *Mentha* sp. from North America. Plant Disease 88:907.

562 Reed, P.J. and Foster, J.A. 2011. Exclusion of pome and stone fruit viruses, viroids, and
563 phytoplasmas by certification and quarantine. In "Virus and virus-like diseases of pome and
564 stone fruits". Hadidi, A., Barba, M., Candresse, T. and Jelkmann, W. Eds, APS, St Paul
565 (MN), USA, pp 381-388.

566 Rolland, M., Villemot, J., Marais, A., Theil, S., Faure, C., Cadot, V., Valade, R., Vitry, C.,
567 Rabenstein, F. and Candresse, T. 2017. Classical and next generation sequencing approaches
568 unravel Bymovirus diversity in barley crops in France. PLoS ONE 12:e0188495.

569 Roossinck, M.J., Martin, D.P. and Roumagnac, P. 2015. Plant virus metagenomics: advances
570 in virus discovery. Phytopathology 105:716-727.

571 Rott, M., Xiang, Y., Boyes, I., Belton, M., Saeed, H., Kesanakurti, P., Hayes, S., Lawrence, T.,
572 Birch, C., Bhagwat, B. and Rast, H. 2017. Application of next generation sequencing for
573 diagnostic testing of tree fruit viruses and viroids. Plant Dis. 101:1489-1499.

574 Rowhani, A., Uyemoto, J.K., Golino, D.A. and Martelli, G.P. 2005. Pathogen testing and
575 certification of Vitis and Prunus species. Annu. Rev. Phytopathol. 43:261-278.

576 Santala, J. and Valkonen, J.P.T. 2018. Sensitivity of small RNA-based detection of plant
577 viruses. Front. Microbiol. 9:939.

578 Schönegger, D., Moubset, O., Margaria, P., Menzel, W., Winter, S., Roumagnac, P., Marais,
579 A. and Candresse, T. 2023. Benchmarking of virome metagenomic analysis approaches
580 using a large, 60+ members, viral synthetic community. J. Virol. 97:e01300-23.

581 Tang, J., Ward, L.I. and Clover, G.R.G. 2013. The diversity of Strawberry latent ringspot virus
582 in New Zealand. Plant Disease 97:662-667.

583 Thompson, D., Howell, W.E. and Kolber, M. 2011. Biological indexing. In "Virus and virus-
584 like diseases of pome and stone fruits". Hadidi, A., Barba, M., Candresse, T. and Jelkmann,
585 W. Eds, APS, St Paul (MN), USA, pp 299-302.

586 Turturo, C., Minafra, A., Ni, H., Wang, G., di Terlizzi, B. and Savino, V. 1998. Occurrence of
587 peach latent mosaic viroid in China and development of an improved detection method.
588 Journal of Plant Pathology, 80:165-169.

van der Valk, T., Vezzi, F., Ormestad, M., Dalén, L. and Guschanski, K. 2019. Index hopping on the Illumina HiseqX platform and its consequences for ancient DNA studies. Mol. Ecol. Resour. 20:1171-1181.

Villamor, D.E.V., Ho, T., al Rwahnih, M., Martin, R.R. and Tzanetakis, I.E. 2019. High throughput sequencing for plant virus detection and discovery. Phytopathology 109:716-725.

Villamor, D.E.V., Keller, K.E., Martin, R.R., Tzanetakis, I.E. 2022. Comparison of high throughput sequencing to standard protocols for virus detection in berry crops. Plant Dis. 106:518-525.

Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M. and Dunez, J. 1992. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. J. Virol. Meth. 39, 27-37.

Wright, A.A., Shires, M.K. and Harper, S.J. 2021. Titer and distribution of little cherry virus 2 in *Prunus avium*. Arch. Virol. 166:1415-1419. https://doi.org/10.1007/s00705-021-05015-z

Yaegashi, H., Yoshikawa, N. and Candresse, T. 2011. Apple chlorotic leaf spot virus in pome fruits. In "Virus and virus-like diseases of pome and stone fruits". Hadidi, A., Barba, M., Candresse, T. and Jelkmann, W. Eds, APS, St Paul (MN), USA, pp 17-21.

Zhang, Y.Z., Chen, Y.M., Wang, W., Qin, X.C. and Holmes, E.C. 2019. Expanding the RNA Virosphere by Unbiased Metagenomics. Ann. Rev. Virol. 6:119-139.

TABLE 1. List of *Prunus*, *Malus* and *Pyrus* viral sources used in the present study, with the date of biological indexing and the viruses expected to be present, together with results of testing by serological ELISA assays, molecular hybridization, RT-PCR assays and dsRNA-based high-throughput sequencing (HTS). The additional viruses identified by HTS and confirmed by RT-PCR are also indicated.

Prunus species	Source name	Previous testing	Viruses present[%]	ELISA	Molecular hybridization	RT-PCR	HTS	Additional HTS viruses
Apricot	GS	2017	PPV	+	nt	+	+	-
	Roma1	2015	HSVd	nt	nt	+	-	LChV1
	16.334	2017	PPV	+	nt	+	+	GCV
	Negative control	-	-	-	-	-	-	-
Peach	D	2016	PPV	+	nt	+	+	APV3, PBNSPaV
	S5476	2016	APLPV	-	nt	+	+	-
			PLMVd	nt	+	nt	+	
	Gotheron 94/6	2017	PDV	+	nt	+	+	NSPaV
			PLMVd	nt	+	nt	+	
	PR3473	2017	PNRSV	+	nt	+	+	CVA
			ACLSV	+	nt	-	+	
	LS27	2017	SLRSV	+	nt	-	+	-
	F586	2017	ToRSV	-	nt	-	-	CVA
	Negative control	-	-	-	-	-	-	-
Cherry	Balaton1	2016	ACLSV	+	nt	+	+	-
	V1927	2015	CNRMV	nt	nt	+	+	PrVF
	V319	2017	PDV	+	nt	+	+	-
	V642	2017	PNRSV	+	nt	+	+	LChV1
	F586c	2017	ToRSV	+	nt	+	+	CVA
	Negative control	-	-	-	-	-	-	-
Plum	#3	2016	APLPV	-	nt	+	+	CVA
	PR72	2015	ACLSV	-	nt	-	+	PeLPaV, PBNSPaV
			HSVd	nt	nt	+	-	
	PR3473	2017	ACLSV	-	nt	-	+	CVA
			PNRSV	+	nt	+	+	
	LPR495	2017	PDV	+	nt	+	+	-
	PR13025	2016	PBNSPaV	nt	nt	+	+	-
			AVCaV	nt	nt	+	+	

	Cerasifera#2	2017	APLPV	-	nt	+	+	CVA, CMV
	Negative control	-	-	-	-	-	-	-
Apple	LX86	2017	ACLSV	-	nt	-	+	-
			ASPV	-	nt	+	+	
	X259	2009	ACLSV	-	nt	+	+	ASGV
			ASSVd	nt	nt	+	-	
	LX225	2017	ACLSV	+	nt	+	+	ARWV1, CCGaV
			ASPV	+	nt	+	+	
			ASGV	+	nt	+	+	
			ApMV	+	nt	+	+	
	J2959	2017	ApMV	+	nt	+	+	-
	LX223	2017	ASPV	+	nt	+	+	ACLSV
			ASGV	+	nt	+	+	
	Negative control	-	-	-	-	-	-	-
Pear	PO13357	2016	ACLSV	-	nt	-	-	-
			ASPV	-	nt	-	+	
			ARW	nt	nt	nt	+	
	PO1492	2017	ACLSV	-	nt	-	-	ASGV, CiVA
			ASPV	+	nt	+	+	
			ARW	nt	nt	+	+	
	PO2098	2006	PBCVd	nt	nt	+	-	ASPV
	PO161	2013	ASPV	-	nt	-	+	-
	P160	2010	ASPV	+	nt	+	+	ARWV2
	Negative control	-	-	-	-	-	-	-

%: viruses expected to be present in the source based on previous biological indexing and virus testing (for example for CVA, AVCaV, PBNSPaV, HSVd). ACLSV: apple chlorotic leaf spot virus; APLPV: American plum line pattern virus; ApMV: apple mosaic virus; APV3: Asian prunus virus 3; ARW: apple rubbery wood virus; ARWV1: apple rubbery wood virus 1; ARWV2: apple rubbery wood virus 2; ASGV: apple stem grooving virus; ASPV: apple stem pitting virus; ASSVd: apple scar skin viroid; AVCaV: apricot vein clearing-associated virus; CCGaV: citrus concave gum-associated virus; CiVA: citrus virus A; CMV: cucumber mosaic virus; CNRMV: cherry necrotic rusty mottle virus; CVA: cherry virus A; GCV: grapevine cryptic virus; HSVd: hop stunt viroid; LChV1: little cherry virus 1; NSPaV: nectarine stem pitting-associated virus; PBCVd: pear blister canker viroid; PBNSPaV: plum bark necrosis stem pitting-associated virus; PDV: prune dwarf virus; PeLPaV: peach leaf pitting-associated virus; PLMVd: peach latent mosaic viroid; PNRSV: prunus necrotic ringspot virus; PPV: plum pox virus; PrVF: prunus virus F; SLRSV: strawberry latent ringspot virus; ToRSV: tomato ringspot virus. nt: not tested.

TABLE 2. Comparative limit of detection of various viruses in 10-fold dilutions of temperate fruit tree samples using ELISA, RT-PCR and dsRNA-based HTS analysis. Positive detection is indicated by a grey shading and a bold X. -: negative detection; nt: not tested

Virus	Host	Isolate	Test	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
ACLSV	Plum	PR3473	ELISA	-	-	-	-	-	-	nt	nt	nt
			HTS	**X**	**X**	-	-	-	-	nt	nt	nt
			RT-PCR	-	nt	nt	nt	nt	nt	nt	nt	nt
	Cherry	Balaton1	ELISA	**X**	-	-	-	-	-	nt	nt	nt
			HTS	**X**	**X**	**X**	**X**	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	-	-	-	-	nt	nt	nt
	Apple	LX225	ELISA	-	-	-	-	-	-	nt	nt	nt
			HTS	**X**	**X**	**X**	-	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	-
CVA	Plum	PR3473	ELISA	nt	nt	nt	nt	nt	nt	nt	nt	nt
			HTS	**X**	**X**	**X**	**X**	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	**X**	**X**	**X**	**X**	-	nt
PNRSV	Plum	PR3473	ELISA	**X**	**X**	-	-	-	-	nt	nt	nt
			HTS	**X**	**X**	**X**[a]	**X**[a]	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	-	-	-	-	-	nt
PDV	Peach	Gotheron 96/4	ELISA	**X**	**X**	**X**	-	-	-	-	-	nt
			HTS RNA	**X**	**X**	**X**	**X**	**X**	**X**[a]	-	-	nt
			RT-PCR	**X**	**X**	**X**	**X**	**X**	**X**	**X**	-	nt
ApMV	Apple	LX225	ELISA	**X**	-	-	-	-	-	nt	nt	nt
			HTS	**X**	**X**	**X**	-	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	**X**	**X**	**X**	-	-	nt
PPV	Apricot	GS	ELISA	**X**	**X**	**X**	**X**	-	-	-	nt	nt
			HTS	**X**	**X**	**X**	**X**	**X**	**X**	-	nt	nt
			RT-PCR	**X**	**X**	**X**	**X**	**X**	**X**	**X**	-	-

ASGV	Apple	LX225	ELISA	**X**	-	-	-	-	-	nt	nt	nt
			HTS	**X**	**X**	**X**	**X**	**X**	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	**X**	**X**	-	-	nt	nt
	Pear	PO1492	HTS	**X**	**X**	**X**	**X**	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	**X**	-	-	-	nt	nt
ASPV	Apple	LX225	ELISA	**X**	**X**	**X**	-	-	-	nt	nt	nt
			HTS	**X**	**X**	**X**	**X**	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	**X**	**X**	**X**	-	-	nt
	Pear	PO1492	ELISA	**X**	-	-	-	-	-	nt	nt	nt
			HTS	**X**	**X**	**X**	**X**	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	-	-	-	-	-	nt
ARWV1	Apple	LX225	ELISA	nt	nt	nt	nt	nt	nt	nt	nt	nt
			HTS	**X**	-	-	-	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	**X**	-	-	-	nt	nt	nt
ARWV2	Pear	PO1492	ELISA	nt	nt	nt	nt	nt	nt	nt	nt	nt
			HTS	**X**	-	-	-	-	-	nt	nt	nt
			RT-PCR	**X**	**X**	-	-	-	-	nt	nt	nt

a: only PNRSV or PDV RNA3 detected at these dilutions

LEGENDS TO THE FIGURES

Fig. 1. Multiple nucleotide sequence alignments showing detection primers and their binding sites on the genome of strawberry latent ringspot virus (SLRSV, **A**), apple chlorotic leaf spot virus (ACLSV, **B**) and apple stem pitting virus (ASPV, **C**). Primers are indicated in bold italics and, for each, the reverse complement sequence is shown for reverse primers. For each virus, the sequence of a reference isolate known to be amplified is shown immediately below primer sequences with its GenBank accession number, followed by sequences for viral isolates that were not detected by RT-PCR but were detected by HTS. Mutations affecting primer-binding sites are in bold and highlighted in light grey. Dashes represent an insertion, while question marks are used to represent missing information not available from HTS sequencing reads.

Fig. 2. Overall rate of detection of 12 viruses and one viroid (PLMVd) in undiluted and in 10-fold serial dilutions of temperate fruit tree extracts as a function of Illumina sequencing depth. For each sequencing depth, three random subsamplings of the original dataset were performed. A detection is considered positive if the number of reads mapping on the reference genome used for each pathogen is superior to the average of mapped reads for the six negative controls plus three times the standard deviation. For viruses with a divided genome, mapped reads number above threshold for any one of the genomic RNAs were considered as positive detection.

Fig. 3. Percentage of detection of the viral agents present in the six tested fruit tree samples as a function of the rate of dilution of those samples in uninfected samples of the same species.

Figure 1

A

```
                    SLRSV-F                                  SLRSV-R
           CCTCTCCAACCTGCTAGACT    //...AGTTACACCTTCATGCGCTT
NC_006965  CCTCTCCAACCTGCTAGACT...//...AGTTACACCTTCATGCGCTT
X77466     CCTCTCCAGCCTGCTAGACT...//...AGTTACACCTTCATGCGCTT
LS27       CCTTTGCAACCTGCACGACT...//...GGTTACCGGTTCATGCGGTT
```

B

```
                    A52                                      A53
           TTCGACTTCAATAAGGGTCTG    //    GGCAACCCTGGAACAGA
M58152     TTTGATTTCAATAAGGGTCTG...//...GGCAACCCTGGAACAGA
PR3473     TTTGATTTTAACAAGGGCCTC...//...GGTAATCCTGGAACAGA
LX86       TTTGATTTTAATAAGGGCCTA...//...GGCAATTCTGGAACAGA
PO13357    ??????????????????????   //   GGTAATCCTGGAACAGA
PO1492     ??????????????????????   //   GGCAATCCTGGAACAGA
PO161      ??????????????????????   //   GGTAATCCTAGAACAGA
```

C

```
                    ASPV-F                                   ASPV-R
           ATGTCTGGAACCTCATGCTGCAA...//...TTATGCTTTTT-AGTAAAGTTGATCCCAA
KY490039   ATGTCTGGAACCTCATGCTGCAA...//...TTATGCTTTTG-AATAAAGTTGATCCCGA
P013357    ATGTTTGGAATCTGATGCTGCAA...//...TTATGCTTTTTAAATAAAGTTGATCCCGC
```

Figure 2

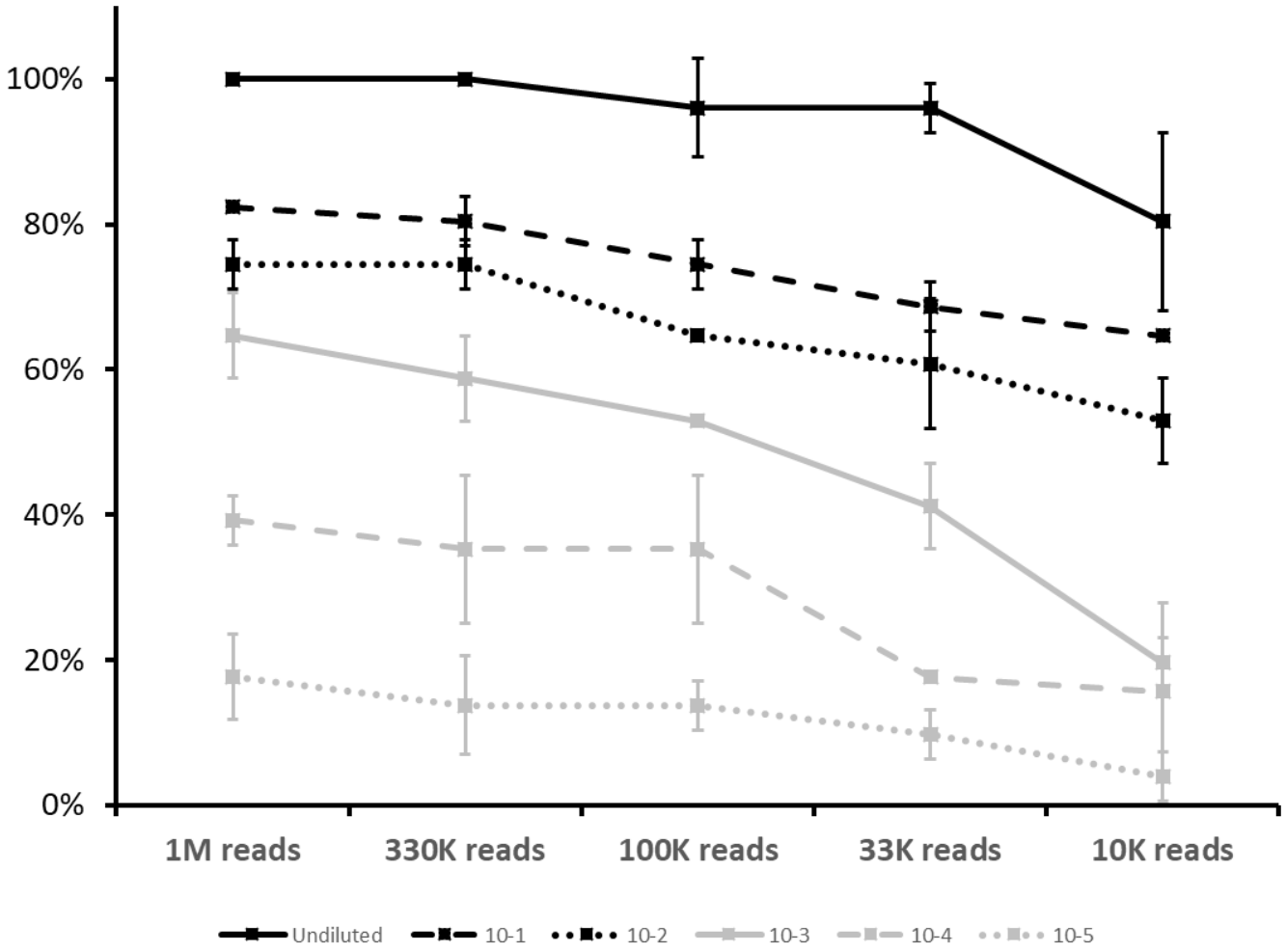
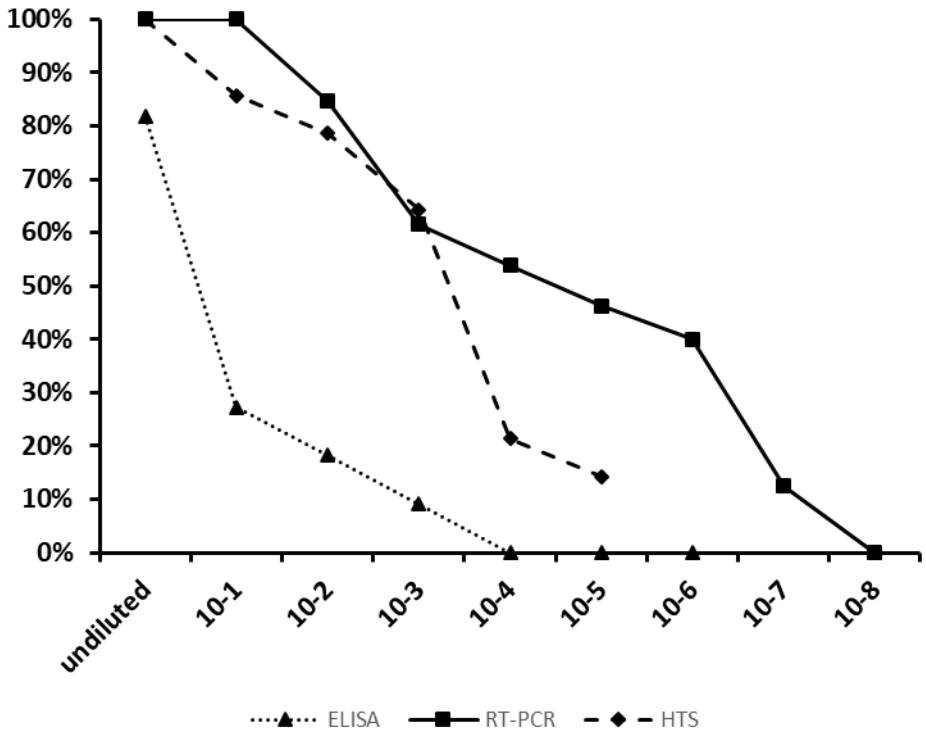

100%
80%
60%
40%
20%
0%
1M reads
330K reads
100K reads
33K reads
10K reads
Undiluted
10-1
10-2
10-3
10-4
10-5

Figure 3


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
undiluted
10-1
10-2
10-3
10-4
10-5
10-6
10-7
10-8
ELISA
RT-PCR
HTS

Supplementary table title

Supplementary TABLE S1. RT-PCR assays used for the detection of the various viruses in the present study.

Supplementary TABLE S1. RT-PCR assays used for the detection of the various viruses in the present study.

Targeted virus	Reference of the RT-PCR assay used or primer pair used
ACLSV	Candresse *et al*. (1994). Acta Hortic. 386, 136-147.
APLPV	Primers APLPV196 5GGTGCCCGTTTAATTCAGG3' and et APLPVc420 5'TATTGCTGCCTCACAAGTGG3' (Tm 55°C, amplicon 224 nt), Villamor (2012), PhD dissertation. https://rex.libraries.wsu.edu/esploro/outputs/99900581654701842
ApMV	Candresse *et al*. (1997). Acta Hortic. 472, 219-226.
APV3	Marais *et al*. (2006). Virus Res. 120, 176-183.
ARWV1	Rott *et al*. (2018). Plant Dis. 102, 1254-1263.
ARWV2	Rott *et al*. (2018). Plant Dis. 102, 1254-1263.
ASGV	Roy *et al*. (2005). J. Virol. Meth. 129, 47-55.
ASPV	Menzel *et al*. (2002). J. Virol. Meth. 99, 81-92.
ASSVd	This work. Primers ASSf 5'GAGAAAGGAGCTGCCAGC3' and ASSr 5'CCCGCCGCTGCGTCAAA3' (Tm 60°C, amplicon 193 nt)
AVCaV	Marais *et al*. (2015). PLoS One, 10, e0129469.
CCGaV	This work. Primers CCGaV-F1 5'CTAGAGATGGTGATGACGGGCAA3' and CCGaV-R1 5'CTGTTTGGCCCTCAGTAGACAAC3' (Tm 60°C, amplicon 327 nt).
CiVA	Svanella-Dumas *et al*. (2019). Plant Dis. 103, 2703.
CMV	Primers CMV-C1 5'CCAGGACGGCGTACT3' and CMV-C1' 5'CCTGATTCAGTCACGG3' (Tm 46°C, amplicon 300 nt). Eric Verdin (INRAE, Avignon, France) personnal communication.
CNRMV	Rott & Jelkmann (2001). Arch. Virol. 146, 395-401.
CVA	Marais *et al*. (2012). Plant Pathol. 61, 195-204.
GCV	This work. Primers GCryV-F 5'ACGGAACGACCCCGACAAGATG3' and GCryV-R 5'CAAACCCAATGGCTTGTGCCAT3' (Tm 60°C, amplicon 854 nt).
HSVd	Bernad & Duran-Vila (2006). Mol. Cell. Probes 20, 105-113.
LChV1	Katsiani *et al*. (2018). Plant Disease 102, 899-904.
NSPaV	Khalili *et al*. (2023). Phytopathology 113, 345-354.
PBCVd	This work. Primers PBCf 5'CCCCTGACCTGCRTTCC3' and PBCr 5'TTACTCACAGCCGCGCGC3' (Tm 60°C, amplicon 116 nt)
PBNSPaV	Marais *et al*. (2014). Phytopathology 104, 660-666.
PDV	Parakh *et al*. (1994). Acta Hortic. 386, 421-430.
PeLPaV	This work. Primers PeLPaV-F 5'GCACATGCATGAGCTTGTTAGA3' and PeLPaV-R 5'CACTCCTTCCAATGCACACAA3' (Tm 60°C; amplicon 347 nt).
PLMVd	Ambrós *et al*. (1998). J. Virol. 72:7397-7406.
PNRSV	Candresse *et al*. (1997). Acta Hortic. 472, 219-226.
PPV	Olmos *et al*. (2005). J. Virol. Meth. 128, 151-155.
PrVF	This work. Primers PrVF-F 5'TTGAGTGGCACTTGGAAACTC3' and PrVF-R 5'AGTCTGGTCTTAGGCTTACCA3" (Tm 58°C; amplicon 526 nt).
SLRV	Tang *et al*. (2013). Plant Dis. 97, 662-667.
ToRSV	Tang *et al*. (2014). J. Virol. Meth. 201, 38-43.

ACLSV: apple chlorotic leaf spot virus; APLPV: american plum line pattern virus; ApMV: apple mosaic virus; APV3: asian prunus virus 3; ARW: apple rubbery wood virus; ARWV1: apple rubbery wood virus 1; ARWV2: apple rubbery wood virus 2; ASGV: apple stem grooving virus; ASPV: apple stem pitting virus; ASSVd: apple scar skin viroid; AVCaV: apricot vein clearing-associated virus; CCGaV: citrus concave gum-associated virus; CiVA: citrus virus A; CMV: cucumber mosaic virus; CNRMV: cherry necrotic rusty mottle virus; CVA: cherry virus A; GCV: grapevine cryptic virus; HSVd: hop stunt viroid; LChV1: little cherry virus 1; NSPaV: nectarine stem pitting-associated virus; PBCVd: pear blister canker viroid; PBNSPaV: plum bark necrosis stem pitting-associated virus; PDV: prune dwarf virus; PeLPaV: peach leaf pitting-associated virus; PLMVd: peach latent mosaic viroid; PNRSV: prunus necrotic ringspot virus; PPV: plum pox virus; PrVF: prunus virus F; SLRV: strawberry latent ringspot virus; ToRSV: tomato ringspot virus.