Seasonal fluctuation and host species affect Tobacco ringspot virus detection

Eunice E Beaver-Kanuya (✉ eunice.kanuya@wsu.edu)

Washington State University   https://orcid.org/0000-0003-1771-3317

S.J. Harper

Washington State University College of Agricultural Human and Natural Resource Sciences

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Abstract

Tobacco ringspot virus (TRSV) is a pathogen with a wide host range that can cause disease in many perennial species. Yet detection of this virus can be insistent and unreliable, complicating disease management. In this study we asked whether seasonal effects changed the titer and distribution of the virus in *Vitis vinifera*, *Malus domestica*, and *Prunus armeniaca.* We developed an RT-qPCR assay and used it to monitor TRSV accumulation in leaves, stems, and roots over the course of a year. TRSV titer, and the tissues in which it accumulated, changed throughout the year in a host-specific manner which may be due to host plant growth patterns and temperature. These data will aid in determining optimal sampling time, and which tissues to collect to have confidence in diagnostic results.

Introduction

Management of viruses in perennials relies on sensitive, accurate detection, particularly when the viruses in question either do not produce significant symptoms or take many years to do so. However, the efficacy of virus detection is often impacted by factors including tissue tropism of the infected host [13, 29] and the seasonal variability in viral titer [17].

One such case is Tobacco ringspot virus (TRSV). This nematode-transmitted subgroup-A nepovirus infects a wide host range of field crops, ornamentals, weeds, and perennials [23, 25, 20]. TRSV causes a number of diseases of economic importance including bud blight disease of soybean [23] blueberry necrotic ringspot disease on blueberry [20] and decline on grapevines [28]. This virus has also been associated with foliar chlorotic ringspots or lesions on some stone fruit cultivars, and union necrosis in grafted apples, although infection is often latent and asymptomatic in both pome and stone fruit [32, 18, Harper unpublished). Because of its wide host range and the potential for disease, TRSV is regarded as a pathogen of concern for the certification, distribution, and maintenance of "virus-tested" material [10].

Yet TRSV detection is complicated by the virus being unevenly distributed throughout the host plant [8], and as is common with many viruses, fluctuating due to seasonal or environmental effects [20]. This in turn reduces confidence in diagnostic results, leading us to ask whether a negative plant is truly negative, or did sampling occur at the wrong time, or was the wrong tissue sampled?

Therefore, in this study we aimed to answer this question for three major perennial host genera: *Vitis*, *Malus*, and *Prunus*, for host-specific effects on virus titer are poorly studied within single host genera [13], let alone between plant genera or families. This particularly is true of perennial species that are not considered as the major or primary host of a given virus but are to some degree impacted by it. Here we developed and optimized RT-qPCR assay for the detection of TRSV and used it to follow TRSV titer in plants of these three genera over the course of one year. While we found notable differences in virus titer and distribution between the three genera, there was a general pattern of virus titer over time that suggested seasonal effects play a major role in how and where TRSV accumulates.

Materials And Methods

Assay development and optimization

To study the effect of seasonal fluctuation of TRSV titer and distribution it was first necessary to select an assay suitable for the project. As there was, at the time the project commenced, a paucity of real-time RT-qPCR assays available for this virus, we elected to design a new assay, as described below.

An alignment (Supplementary data 1) was made from near-complete sequences of TRSV capsid protein present in the NCBI database using ClustalW [30], and primers and Taqman probe (Table 1) were designed in silico using the Biosearch Real-Time Design online software program (LGC Biosearch Technologies, Novato, CA) with default parameters. The designed primers and probes were then analyzed for predicted melting temperature, self-dimer and heterodimer formation

using the Oligo Analyzer (IDT Technologies, Coralville, IA) online software, and BLASTn searches conducted to determine that they did not match non-target viruses or potential host genomic sequences.

The assay was optimized against TRSV isolates from *Malus, Vitis,* and *Prunus* cultivars held in the Clean Plant Center Northwest's positive control collection at Washington State University, Prosser, WA. Total RNA was extracted from each source plant as per [3]. Reaction conditions for this assay were optimized, using Invitrogen SuperScript™ III Platinum™ One-Step qRT-PCR reagents (Thermo-Fisher Scientific, Waltham, MA), across the following ranges: primer concentration (400 to 600 nM), probe concentration (100 to 300 nM), $MgSO_4$ concentration (3 to 6 mM), and annealing/extension temperature (57°C to 70°C) on a BioRad CFX96 real-time thermocycler (BioRad Laboratories, Hercules, CA). Conditions that gave the lowest Cq values across three technical replicates for each parameter on the test samples were selected.

Once optimized, the assay was assessed for exclusivity against: 1) potential host species using TRSV-free samples of *Vitis vinifera, Malus domestica, Pyrus communis, Pyrus pyrifolia, Prunus avium, Prunus armeniaca, Prunus persica, Vaccinium corymbosum,* and *Vaccinium vitis-idaea*. 2) other nepoviruses that may be present in these host species, using positive controls of Tomato ringspot virus, (ToRSV), Grapevine fanleaf virus (GFLV), Arabic mosaic virus (ArMV), Raspberry ringspot virus (RpRSV), Tomato black ring virus (TBRV), Cherry leaf roll virus (CLRV) and 3) against unrelated viruses and viroids commonly found to co-infect these hosts, Apple chlorotic leaf spot virus (ACLSV), Apple stem pitting virus (ASPV), Apple mosaic virus (ApMV), Apple rubbery wood virus 1 and 2 (ARWaV-1 and -2), Little cherry virus-2 (LChV-2), Grapevine leafroll-associated virus 3 (GLRaV-3), Pear blister canker viroid (PBCVd), and Peach latent viroid (PLMVd). No detectable amplification signal from each of the above samples was considered as a successful result.

The sensitivity of the designed assay was examined using an artificial RNA standard of TRSV target region designed and produced as per [3]. Serial dilutions containing between 10^{10} through 10^1 copies of transcript per reaction, diluted in TRSV-free total RNA extract from *Vitis vinifera* to account for the effect of plant inhibitors, were created, tested in triplicate, and the resulting data used to determine detection limit of the assay. The reaction efficiency of the assay was calculated from this dilution series using the CFX96 manager software (Bio-Rad).

Finally, the performance and inclusivity of the newly designed assay was compared to two RT-PCR assays, performed as described in the respective publications (15, 8], against a panel of 7 *Malus*, 1 *Cydonia* (quince), 3 *Vitis*, 5 *Prunus* and 2 *Vaccinium* samples positive for TRSV. The amplification products from the two RT-PCR assays were visualized using capillary gel electrophoresis on a QIAxcel Advanced system (Qiagen, Hilden, Germany). Given the difference in numbers of samples detected by the assays, a serial 10-fold dilution series of a TRSV positive extract from *Prunus armeniaca* was created down to 1:10,000,000 and compared across all three assays as above.

Seasonal TRSV accumulation in perennial hosts

The RT-qPCR assay developed above was used to study the seasonal accumulation of TRSV in three different perennial hosts: *Vitis vinifera*, *Malus domestica*, and *Prunus armeniaca*. A total of two plants per species, naturally infected with TRSV that had been identified by RT-PCR using the method of [8] and confirmed by sanger sequencing (Genewiz Inc., South Plainfield, NJ) (NCBI numbers: MW574103, MW574104, BMW574105) were used for this study. These plants were maintained in 2-gallon root-training pots, in contained screenhouse conditions and subject to ambient temperature fluctuations throughout the course of the observation period (August 2019 through August 2020).

400 mg samples of leaf midrib and lamina, current-year growth stem, and secondary root tissues were collected from each of the plants on a monthly basis, and total RNA extracted as above. TRSV titer present in each of the tissues was determined using the optimized TRSV-specific RT-qPCR assay developed in this study, performed as above with 2 µl of total RNA for each sample. Normalization of samples was carried out using a combination of NAD5 [21, 5], with EF1 [26] for *V. vinifera*, with Tubulin [34] for *M. domestica*, and with TEF2 [34] for *P. armeniaca*, amplified as described in the

respective references. All samples were tested in technical replicates of three, and efficiency values calculated from a 10-fold dilution series for each reference gene (Supplementary Figure 1).

Prior to quantification of the experimental samples, the standard deviation of the respective reference genes in the three tissue types was calculated. All showed values of less than 1.0 (Supplementary Table 1), indicating stability and suitability for use to normalize TRSV expression [6]. Relative quantification (RQ) values for TRSV titer at each timepoint and sample were calculated using the formula described by [24], then log-transformed (base 10) to normalize the non-parametric values. All statistical analysis was performed using the R project for statistical computing (R core team, 2017).

Results

Assay development and optimization

To study the effect of seasonal fluctuation of TRSV titer and distribution we first developed and optimized a real-time RT-qPCR assay. Optimized conditions were 400 nM each of the forward and reverse primers, 100 nM of the FAM-labelled probe, 3 mM $MgSO_4$, 0.4 µl Superscript III / Platinum Taq enzyme mix, and 2 µl of total RNA in a final reaction volume of 20 µl. One-step thermocycling conditions were 50 °C for 10 minutes and 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 10 seconds, and 58 °C for 45 seconds.

The optimized assay was capable of detecting as low as 10^1 copies of TRSV RNA transcript target with an efficiency of 132.4% (Supplementary Figure 2) and demonstrated exclusivity against non-target host and potential related and/or coinfecting virus species (Table 2). Comparison of the assay against two conventional RT-PCR assays [15, 8] using a panel of 18 TRSV isolates from different host species and sources showed that the newly developed real-time RT-qPCR assay could detect TRSV at a lower titer than the conventional assays (Table 3). This was further confirmed by serial dilution of a high concentration sample in water, where the real-time RT-qPCR assay could amplify down to a dilution of $1:10^7$ versus $1:10^4$ for the two conventional assays (Supplementary Figure 3).

Seasonal TRSV accumulation in perennial hosts

Having developed a reliable RT-qPCR assay we next turned to an examination of the seasonal variation of TRSV titer in three perennial plant hosts, *V. vinifera*, *M. domestica*, *P. domestica,* and P.*armeniaca*. Leaf, stem, and secondary root tissue was collected from each of the monitored plants on a monthly basis, where available, and TRSV titer estimated by relative quantification against host-specific reference genes (Figure 1). TRSV titer was generally low for all three host species throughout the experiment, with virus titer dropping below the limit of detection at several timepoints for specific tissues.

Plotting quantified TRSV titer as a timecourse from the beginning of the observation period (August 2019) to the end (August 2020) revealed considerable fluctuation in virus titer in all three species over time and in different tissues (Figure 1). Examining each in turn, we found that in grapevine, TRSV titer was low throughout much of the late summer through to winter dormancy where it became undetectable in all three tissue types, with a peak in spring that then declined by 1-2 orders of magnitude (log 10) during early summer. Titer in stem tissue showed the greatest degree of fluctuation, followed by leaf (when tissue was available), and root tissues (Figure 1a). A similar pattern was observed in apple, although root tissue consistently contained higher relative titer than stem. Interestingly, titer in leaf tissue remained relatively constant throughout the observation period, while roots and stem showed fluctuations of 3-4 orders of magnitude (log10) (Figure 1b). Finally, TRSV in plum was broadly comparable to the pattern observed in apple, though both stem and root tissue were much more comparable across the seasons (Figure 1c).

Interestingly, when these data were compared to the average monthly temperatures for Prosser, WA, during the observation period, we noted that in all hosts TRSV titer was highest during late spring to early summer temperatures (~12-17 °C, Figure 1), and lower when outside of this range. This may be related to seasonal growth patterns for all three hosts, where in January 2020 mild winter conditions led to early bud break followed by an unexpected cold weather event in February which led to some dieback of emerging tissues, evidenced by the rise then decline in TRSV titer in all three species examined.

Optimal detection

Based on the results of the seasonal observation and real time RT-qPCR Cq values optimal sampling times and tissue selections were identified (Table 4). These are: 1) *Vitis* January (roots, stems, leaves), March (leaves), April (leaves, stems), May (leaves), 2) *Malus* December (roots), January (leaves, stems, roots), May (leaves, roots), June (roots), and 3) *Prunus* (September (roots), January (roots), March (stems), April (leaves, stems), May (leaves, stems, roots), June (leaves, stems). Based on these data, sampling outside of optimum times increases the risk of false negatives.

Discussion

In this study we aimed to determine whether there were seasonal fluctuations in TRSV titer, driven in small part by frequent observations of individual plants testing positive one season, and negative the next (Beaver-Kanuya, unpublished). Fluctuation of virus titer over time, and in different tissues is a common phenomenon of plant viruses from many different genera, and host species [33, 29, 11, 14]. What drives this phenomenon is speculative, with virus-host interactions, plant growth state, and temperature, being proposed as potential causes.

How, and the extent to which a virus interacts with its host, is the result of a complex interplay of host structures and defenses such as silencing on one hand, and whether a virus possess compatible gene products, how adapted they are to the given host and whether the virus can evade or suppress the host's defenses [22, 4]. Nepoviruses such as TRSV are impacted by host hypersensitive or SA-mediated responses as well as RNA silencing, leading in some cases, to symptom recovery but not clearance of the virus [9]. It is unclear whether nepoviruses possess viral-derived suppressors of silencing, although the ToRSV coat protein has been demonstrated to interact with AGO1 proteins to potentially prevent translation repression [16].

Beyond overall fitness in a given host, a virus may not be able to infect all tissues equally. Here we observed that while TRSV consistently accumulated to higher relative titer in grapevine stem and leaf tissues, a phenomenon reported for other nepoviruses in grapevine [27, 11], it accumulated to higher relative titer in the roots of both apple and plum. Higher virus titer, or more frequent detection of viruses in roots, has been frequently reported [2, 19, 13], including for TRSV in blueberry [8] which may indicate that roots are more permissive to viral infection. This may be due to reduced silencing [1] or more effective suppression thereof by the virus [2, 19]. Whether this is the cause for our observations in these two species remains speculative, although a root-biased tropism would be advantageous for a nematode transmitted virus [9] such as TRSV.

Fluctuations in titer may be correlated with changing patterns of plant growth, with replication and accumulation slowed during dormancy, and accelerated as the plant produces seasonal flush growth [14]. Here we observed significant decreases in relative TRSV titer in all three host species tested throughout autumn and winter, rising as dormancy broke in early spring then falling again as growth slowed in summer. This pattern has been similarly observed for a diverse range of viruses including ilarviruses in pome and stone fruit [31] potyviruses in garlic [7], Turnip mosaic virus in Arabidopsis [14], and Little cherry virus-2 in sweet cherry [34].

However, temperature may also play a significant role in seasonal fluctuation of TRSV titer in the three species. Here we observed that TRSV titers were lower during months with average temperatures below 4°C and above 20°C, suggesting an

optimum range for accumulation of this virus. Nepoviruses have been reported to be sensitive to higher temperatures where RNAi is more active [12], while cold temperatures can reduce virus titer significantly [14], perhaps due to reduced replicase activity. As roots are below-ground and hence insulated from the extremes of temperature fluctuation, as well as being cooler on average than aerial parts of the plant it is not unexpected that virus titer would remain higher than in other tissues, as we observed here.

This study demonstrates the need for empirical examination of virus accumulation and tissue specificity prior to conducting diagnostic surveys, for we found that relative TRSV titer fluctuated markedly through one year of observation and was undetectable in some tissues during dormancy. It also differed between host species, with the distribution in grapevine differing markedly from the pattern observed in both apple and plum. It is to be hoped that this work will increase diagnostic accuracy and confidence by identifying optimal times and tissues in these three different perennial genera.

Declarations

Conflicts of interest/Competing interests (Not applicable)

Availability of data and material (Not applicable)

Code availability (Not applicable)

Authors' contributions (**Eunice Beaver-Kanuya:** Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Review & Editing. **Scott Harper:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition)

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals

Ethics approval (Not applicable)

Consent to participate (Not applicable)

Consent for publication (Not applicable)

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Tables

Table 1. Primers and probes developed in this study for the amplification of TRSV by RT-qPCR. T7 promoter sequences was added in the forward primer used for TRSV sequence and same primer set was used to generate RNA standards.

Assay	Primer name	Orientation (5'-3')	Sequences	Binding site
TRSV RT-qPCR	TRSV-RT-F	+	CCTGGGCACAAGTGAAATGTTG	1832-1853
	TRSV-RT-R	-	GCTACCAGAAACAACGGTCTAAC	1878-1900
	TRSV-RT-P	+	6FAM-CGTTGCATAGTGAAGCGCACGA-BHQ	1856-1877
TRSV RNA standards	TRSV-ST-F	+	TAATACGACTCACTATAGGGATGGTCCGGCTCTTAGGTGA	1712-1733
	TRSV-ST-R	-	ACAGCAAATAGCAAAGCAGCT	1927-1948

 Table 2. Examination of the inclusivity and exclusivity of the assay developed in this study against, A: common TRSV host species, B: related virus species, C: coinfecting viruses and viroids commonly found to co-infect with TRSV.

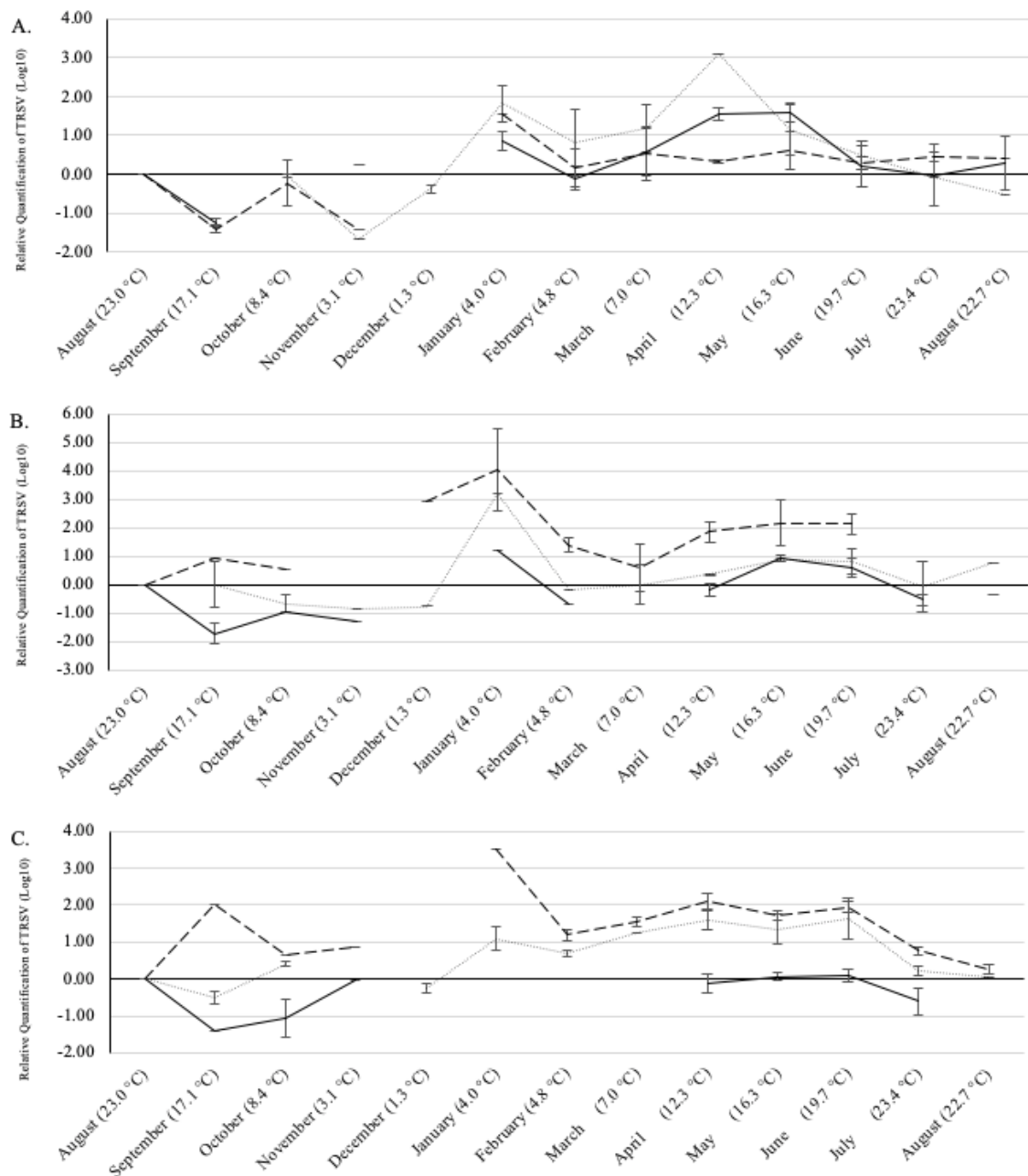| Group	Host species	Nad5	TRSV
A: Host Species	*Vitis vinifera*	17.55	-
	Malus domestica	18.08	-
	Pyrus communis	18.37	-
	Pyrus pyrifolia	16.52	-
	Prunus avium	17.99	-
	Prunus armeniaca	21.64	-
	Prunus persica	17.7	-
	Prunus persica var.nectarina	17.31	-
	Vaccinium corymbosum	17.69	-
	Vaccinium vitis-idaea	16.15	-
B: Related viruses	ToRSV	17.9	-
	GFLV	20.04	-
	ArMV	17.46	-
	RpRSV	16.26	-
	TBRV	17.83	-
	CLRV	16.03	-
C: Coinfecting Viruses	ACLSV	15.99	-
	ASPV	16.52	-
	ApMV	N/A	-
	ARWaV-1	15.78	-
	ARWaV-2	17.55	-
	PBCVd	18.08	-
	PLMVd	18.37	-
	LChV-2	16.52	-
	GLRaV-3	17.99	-

Table 3. A comparison between RT-qPCR developed in this study and two currently used RT-PCR (Fuchs et al.2010, Jossey & Babadoost 2006) for the detection of Tobacco ringspot virus (TRSV).

Sample No.	Species	Country of Origin	RT-qPCR	Fuchs et al. (2010)	Jossey & Babadoost (2006)
1	*Malus domestica*	USA	34.74	-	-
2			34.65	-	-
3			31.53	-	-
4			20.29	+	+
5			27.05	+	-
6			29.81	+	-
8	*Pyrus pyrifolia*	USA	32.53	-	-
8	*Cydonia oblonga*	Netherlands	25.49	+	+
9	*Vitis vinifera*	USA	35.55	-	-
10			36.58	-	-
11			35.43	-	-
12	*Prunus domestica*	France	34.92	-	-
13	*Prunus armeniaca*	Australia	31.59	-	-
14	*Prunus avium*	USA	25.14	+	+
15	*Prunus persica*	France	21.00	+	+
16			32.72	-	-
17	*Vaccinium corymbosum*	USA	38.08	-	-
18	*Vaccinium vitis-idaea*	USA	37.51	-	-

Table 4. Analysis of variance (ANOVA) and Tukey's honestly significant differences were used to calculate TRSV expression differences and to determine the best time and tissue to sample. The lowest Ct values in each group was underlined and compared to all other Ct values in the group. Ct values with the same subscripted letters (highlighted) were not significantly different. Ct values with different subscripted letters (not highlighted) were significantly different. L= leaves, S = stems, R = roots.

Timepoint	Vitis-L	Vitis-S	Vitis-R	Malus-L	Malus-S	Malus-R	Prunus-L	Prunus-S	Prunus-R
August	34.5_{bc}	37.83_a	35.11_{bcd}	34.25_{bc}	36.69_a	38.91_a	**33.85_b**	NA	38.61_a
September	38.26_a	NA	39.07_a	37.02_a	34.14_{abc}	34.89_{abc}	37.39_a	37.91_a	32.24_{def}
October	NA	36.75_a	36.32_b	36.68_{ab}	36.09_{ab}	36.2_{abc}	36.00_a	35.89_{abc}	35.84_{abcd}
November	36.35_{ab}	38.02_a	$39.69a$	36.87_a	37.27_a	NA	35.42_{ab}	NA	36.16_{abc}
December	TA	36.99_a	NA	TA	36.26_{ab}	31.96_{bcd}	TA	37.15_{ab}	NA
January	31.45_d	31.59_{de}	$\underline{31.31}_f$	$\underline{29.6}_e$	$\underline{25.85}_d$	$\underline{27.5}_d$	TA	33.79_{cd}	$\underline{28.07}_f$
February	35.52_{abc}	33.37_{bcd}	$35b_{cd}$	35.6_{ab}	35.37_{abc}	34.54_{bc}	TA	34.93_{bc}	34.95_{acde}
March	32.96_{cd}	32.78_{cd}	33.97_{de}	NA	33.85_{abc}	34.15_{bc}	TA	32.58_d	33.57_{cde}
April	$\underline{30.19}_d$	$\underline{29.23}_e$	34.14_{de}	34.21_{bcd}	33.93_{abc}	32.72_{bc}	33.55_{bc}	32.59_d	37.39_{cde}
May	30.45_d	32.63_{cd}	33.67_e	31.87_{de}	32.36_{bc}	31.76_{cd}	32.99_c	32.70_d	32.33_{def}
June	34.02_{bc}	34.24_{bc}	33.9_{5de}	32.08_{cde}	32.61_{bc}	32.45_{bc}	$\underline{32.98}_c$	$\underline{32.45}_d$	33.27_{cde}
July	34.84_{abc}	34.89_{ab}	35.63_{bc}	36.31_{ab}	33.88_{abc}	36.02_{abc}	35.49_{ab}	36.39_{ab}	36.39_{ab}
August	$33.8c_d$	38.01_a	$34.1c_{de}$	35.78_{ab}	32.31_{bc}	38.36_{ab}	NA	36.85_{ab}	37.76_{ab}
P-Value	$3.55e^{-8}$	$7.93e^{-13}$	$<2e^{-16}$	$1.06e^{-11}$	$1.02e^{-13}$	$3.81e^{-9}$	$3.03e^{-8}$	$2.38e^{-12}$	3.59^{e-13}

Figures

Figure 1

Timecourse of relative TRSV titer as quantified by RT-qPCR from August 2019 to August 2020, in a) V. vinifera, b) M. domestica, and c) P. armeniaca. Leaf tissues are represented by solid lines, stem tissue by dotted lines, and root tissue by dashed lines.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData1.docx
- SupplementaryFigure1.docx
- SupplementaryFigure2.docx

- SupplementaryFigure3.docx
- SupplementaryTable1.docx