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Nondestructive Raman Spectroscopy as a Tool for Early Detection and Discrimination of the Infection of Tomato Plants by Two Economically Important Viruses

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(Article begins on next page)

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11 4 **Early detection and discrimination of two different viruses**  
12 **infecting tomato by non-destructive Raman spectroscopy**  
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19 7 Luisa Mandrile<sup>†</sup>, Silvia Rotunno<sup>‡</sup>, Laura Miozzi<sup>‡</sup>, Anna Maria Vaira<sup>‡</sup>, Andrea M. Giovannozzi<sup>†</sup>,  
20  
21 8 Andrea M. Rossi<sup>\*\*</sup>, Emanuela Noris<sup>\*‡</sup>  
22  
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25  
26 10 <sup>†</sup>Istituto Nazionale di Ricerca Metrologica, Strada delle Cacce, 91 10135 Torino, Italy  
27

28 11 <sup>‡</sup>Institute for Sustainable Plant Protection, National Research Council of Italy, Strada delle Cacce  
29 12 73, 10135, Torino, Italy  
30  
31 13

32 14 Corresponding Authors

33  
34 15 \*E-mail: [emanuela.noris@ipsp.cnr.it](mailto:emanuela.noris@ipsp.cnr.it)

35 16 \*E-mail: [a.rossi@inrim.it](mailto:a.rossi@inrim.it)  
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## Abstract

Global population forecasts dictate a rapid adoption of multifaceted approaches to fulfill increasing food requirements, ameliorate food dietary value and security using sustainable and economically feasible agricultural processes. Plant pathogens induce up to 25% losses in vegetable crops and their early detection would contribute to limit their spread and economic impact. As an alternative to time-consuming, destructive and expensive diagnostic procedures, such as immunological assays and nucleic acid-based techniques, Raman spectroscopy (RS) is a non-destructive rapid technique that generates a chemical fingerprinting of a sample at low operating costs. Here, we assessed the suitability of RS combined to chemometric analysis to monitor the infection of an important vegetable crop plant, tomato, by two dangerous and peculiarly different viral pathogens, *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato spotted wilt virus* (TSWV). Experimentally inoculated plants were monitored over 28 days for symptom occurrence and subjected to RS analysis, alongside with the evaluation of virus amount by quantitative real-time PCR. RS allowed to discriminate mock-inoculated (healthy) from virus-infected specimens reaching accuracy >70% after only 14 days after inoculation for TYLCSV and >85% only after 8 days for TSWV, demonstrating its suitability for early detection of virus infection. Importantly, RS highlighted also spectral differences induced by the two viruses, providing specific information on the infecting agent.

**Keywords:** Raman Spectroscopy; plant virus; biotic stress; precision agriculture; tomato; virus diagnosis

## 38 INTRODUCTION

39 Intelligent and sustainable agricultural practices represent an outstanding need in view of the  
40 world's population outreach of 9 billion of individuals within 2050 <sup>1</sup>. Vegetable crops are  
41 fundamental for the human diet and economically remunerative for the agricultural sector, but  
42 according to FAO estimates, transboundary plant pests provoke up to 25% of crop failures, entailing  
43 significant economic losses and contributing to food scarcity. Therefore, innovative strategies for  
44 disease prevention and for limiting pathogen spread are mandatory to reduce failures.

45 Tomato (*Solanum lycopersicum* L.) is the most economically important vegetable crop, with a total  
46 harvested area of 4.7 million ha, a production of 180 million tons  
47 (<http://faostat3.fao.org/home/index.html>) and a consumption exceeding 20 kg/person/year, with a  
48 still expanding trend <sup>2</sup>. Tomato suffers from attacks caused by >200 pests and diseases <sup>2</sup>, currently  
49 controlled by chemical treatments, generating negative side effects such as pesticide resistance,  
50 environmental risks, and health issues for farmers and consumers. Among tomato pathogens,  
51 viruses play a significant role; two of them, *Tomato spotted wilt virus* (TSWV) and *Tomato yellow*  
52 *leaf curl virus* (TYLCV), are among the most ten destructive viruses of vegetables <sup>3</sup>.

53 TSWV (Family *Tospoviridae*), with a tripartite single-stranded (ss) RNA genome <sup>4</sup>, infects up to  
54 900 plant species <sup>5,6</sup>, causing over 1 billion \$ losses worldwide <sup>7,8</sup>. On tomato, it induces inward  
55 cupping and drooping of leaves, unilateral plant growth, stunting and ultimately death; if present,  
56 fruits display chlorotic/necrotic ring spots and become unmarketable. TYLCV and its relative  
57 *Tomato yellow leaf curl Sardinia virus* (TYLCSV) belong to the *Geminiviridae* family, with a  
58 circular ssDNA genome encapsidated in geminated particles, causing huge economic losses on food  
59 and cash crops <sup>9</sup>. TYLCSV and TYLCV are responsible for the tomato leaf curl disease and are  
60 transmitted by whiteflies in a circulative persistent manner <sup>10</sup>.

61 Early detection of plant pathogens is fundamental to prevent disease spread, limit crop damages  
62 and regulate proper pesticide use, under sustainable crop management practices. Identification of  
63 pathogens by visual assessment must be supported by objective and “non-operator dependent”  
64 diagnostic techniques. Immunological assays are commonly used for tospovirus diagnosis <sup>11</sup>, while  
65 microarrays allows virus detection and species identification <sup>12</sup> and quantitative PCR (qPCR) is  
66 suitable for species identification and virus quantification <sup>11,13</sup>. For tomato leaf curl disease viruses,  
67 molecular hybridization and PCR are preferred diagnostic techniques <sup>14,15</sup>, and qPCR allows  
68 absolute and relative quantifications <sup>16,17</sup>. Although these traditional assays are sensitive, accurate,  
69 and effective to confirm visual inspection, they are unsuitable for rapid large scale monitoring of  
70 plants before symptom onset, as they are destructive and require detailed sampling procedures,  
71 expensive infrastructures, and skilled personnel. Therefore, innovative, non-invasive, and non-

destructive methods for disease detection have been proposed, based on fluorescence, volatile sensors and imaging, but these are still at an experimental stage<sup>18</sup>. Spectroscopic techniques have interesting applications and, unlike other methods, are simple, rapid and affordable<sup>18,19</sup>. Among them, Raman spectroscopy (RS) has been applied in the biomedical sector to whole cells, tissues, and fluids<sup>20</sup>. In the agricultural fields, RS was considered for species-specific analysis for feed safety and traceability<sup>21</sup> and contaminant detection<sup>22</sup>. RS provides specific biochemical fingerprints reflecting detailed chemical and structural alterations<sup>23</sup>, possibly representing sensitive and phenotypic markers of a disease. In plant pathology, RS was proposed to differentiate pepper plants infected by tobamoviruses<sup>24</sup> and *Abutilon* spp. plants infected by the geminivirus *Abutilon mosaic virus*<sup>25</sup>, concluding that carotenoid content was a discriminatory variable. More recently, RS was also used for early diagnosis of a phytoplasma on sweet orange<sup>26</sup> and for fungal pathogen detection on maize kernels<sup>27</sup>.

Here, we applied RS for the early and discriminative detection of two important viruses of tomato, in a dynamic infection time-frame. Tomato plants inoculated with TSWV and TYLCSV were visually inspected for symptoms for up to 28 days after inoculation, tested for relative viral accumulation by qPCR, and subjected to non-destructive RS analysis on leaves. Beside RS peaks assignment, a chemometric approach for data analysis was adopted to overcome the difficulties to interpret chemical information contained in the spectra of whole biological samples. Principal Components Analysis (PCA)<sup>28</sup> helped to determine systematic differences in the profiles of healthy and infected plants. Moreover, the sensitivity, precision and accuracy of RS to detect plant infection by partial least square-discriminant analysis (PLS-DA) were assessed, obtaining satisfactory classification rates starting from 8 days after inoculation for TWSV and from 14 days after inoculation for TYLCSV, time points at which viral symptoms were visually undetectable.

## EXPERIMENTAL SECTION

**Plant growth and treatments.** Tomato plants ( $n = 3$ ) grown in soil at 23 °C were inoculated with TYLCSV or with TSWV (see Supporting Information). Symptoms, relative virus amount, and RS were monitored weekly from 0 to 28 days post inoculation (dpi), using mock-inoculated plants as control. For TSWV, plants were also tested at 2, 5 and 8 dpi.

**Nucleic acid (NA) extraction.** NA were extracted from the basal leaflets of the second true leaf from the apex of each plant. For TYLCSV-infection, total DNA was extracted by the dot-blot method<sup>16</sup> and used for qPCR, while for TSWV, RNA was extracted with Trizol® and processed for qRT-PCR.

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3 105 **Quantitative Real-Time PCR for relative virus quantification.** TYLCSV was quantified  
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5 106 using tomato ascorbate peroxidase (APX) as housekeeping gene (Table S1), while the ubiquitin-  
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7 107 conjugating enzyme (UBC) was used as housekeeping transcript for TSWV. Reactions were  
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9 108 performed with iTaq™ Universal SYBR® Green Supermix (10- $\mu$ l volume) and the CFX Connect™  
10 109 Real-Time PCR Detection System (three technical replicates; see Supporting Information). The  
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12 110 relative viral amount was calculated by the comparative threshold cycle ( $\Delta C_t$ ) method, where  $\Delta C_t$  is  
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14 111  $|C_{t \text{ virus}} - C_{t \text{ housekeeping gene}}|$ .

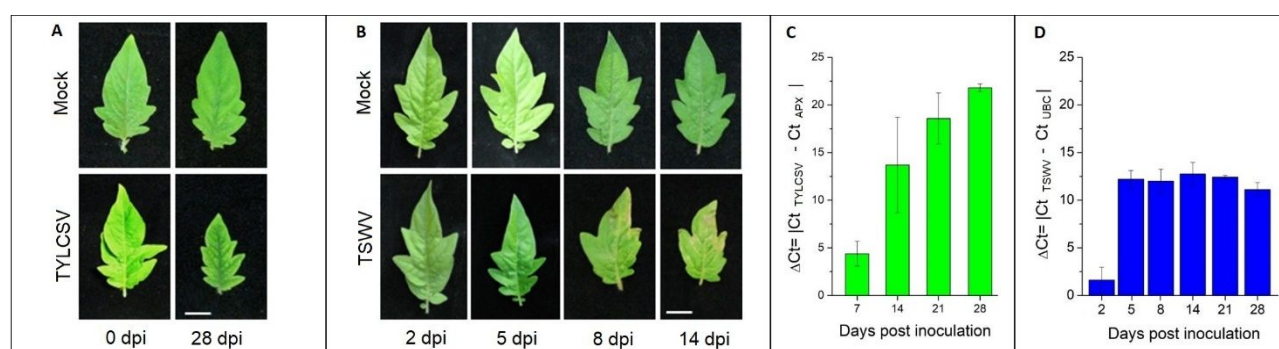
15 112 **Raman spectroscopic measurements.** Three Raman spectra were acquired from the apical  
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17 113 leaflets of the second leaf counting from the apex, immediately prior to sample collection for virus  
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19 114 quantification. Leaves were stored in plastic bags at 4 °C until spectra acquisition, occurring within  
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21 115 the following four hours. Spectra (400-3100  $\text{cm}^{-1}$ ; 5  $\text{cm}^{-1}$  resolution) were obtained with a  
22 116 Dispersive Raman Spectrometer DRX (780 nm excitation laser, 10X microscope objective, 2  $\mu$ m  
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24 117 laser spot diameter, 2 mW laser power, 5 sec/15 scans acquisition time). The spectrometer was  
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26 118 weekly calibrated using a certified white light for intensity and neon gas lines for frequency.  
27 119 Moreover, a Si standard was measured before each session, to guarantee consistency within  
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29 120 measurements and avoid differences due to instrument performances.

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31 121 **Chemometric analysis of Raman spectra.** Chemometric analysis was conducted using the PLS  
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33 122 Toolbox (Eigenvector Research, Inc., Manson, WA) for Matlab R2015a (Mathworks, Natick, MA).  
34 123 We used PCA to find correlations between measurements and the effect of virus infection and  
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36 124 calculated PLS-DA models<sup>29</sup> to determine RS sensitivity for early virus infection. Models were  
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38 125 cross-validated with the “leave-one group-out”, using subsets of samples constituting the sets for  
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40 126 cross-validation corresponding to one plant at a time. To compare classification performances at  
41 127 different plant ageing levels (0-28 dpi), we calculated Sensitivity [True Positive/(True  
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43 128 Positive+False Negative)], Specificity [True Negative/(True Negative+False Positive)], Accuracy  
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45 129 (correctly classified samples/total samples), and Classification Error (1-Accuracy). Spectra were  
46 130 pre-processed by Savitsky-Golay smoothing (31 points, polynomial order 2), removing Random  
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48 131 shift of the baseline offset by weighted least squares baseline correction (polynomial order 2)<sup>30</sup>.  
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50 132 Multiplicative scattering correction and mean centering were used. The same preprocessing was  
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52 133 adopted for PCA and PLS-DA.

## 53 54 134 **RESULTS AND DISCUSSION**

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56 135 **Visual symptom and virus quantification.** Viruses induce a variety of systemic symptoms on  
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58 136 plants that increase with time progression depending on the pathogen, the plant species, the time of  
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60 137 infection, and the environment. In our experimental conditions, TYLCSV symptoms consisting of

leaf yellowing and curling became evident at 28 dpi (Figure 1A), while for TSWV, chlorotic spots appeared on newly emerging leaves since 8 dpi (Figure 1B). qPCR showed that TYLCSV progressively increased from 7 to 28 dpi, following a logarithmic scale ( $R^2 = 0.9984$ ,  $P < 0.001$ ) (Figure 1C). Instead, TWSV accumulated faster, as its steady-state level was reached at 5 dpi (Figure 1D), in line with the more precocious symptom onset. Noteworthy, TSWV amount remained stable up to 28 dpi (Figure 1D) while symptoms progressively deteriorated, indicating that cumulative effects due to cell metabolism perturbation<sup>31</sup> rather than virus accumulation could be responsible for symptom progression.

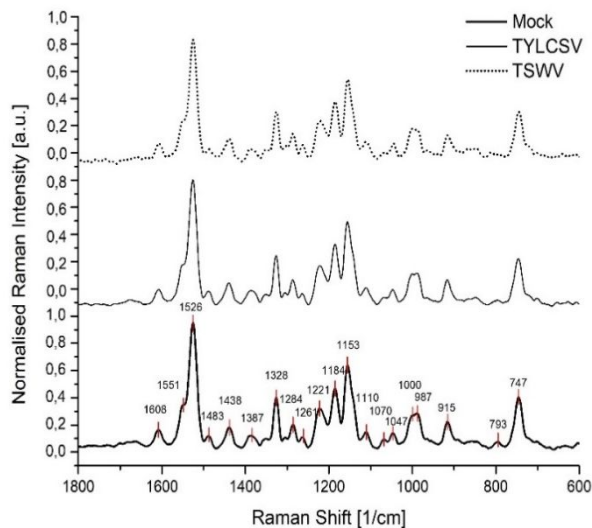


**Figure 1.** Infection of tomato plants by TYLCSV and TSWV. Images of leaflets collected before (0 dpi) and at different time points after inoculation with TYLCSV (A) or TSWV (B). Images of TSWV-infected leaves after 14 dpi are not shown, as they were clearly symptomatic. Bar = 1 cm. Relative accumulation of TYLCSV (C) and TSWV (D) in leaf tissue, at different times post-inoculation. Results are expressed as  $\Delta Ct$  values, representing the difference between the threshold cycle ( $C_t$ ) of each sample and that of the reference gene (APX for TYLCSV) or of the reference transcript (UBC for TSWV). Bars are the mean value of three plants, while vertical lines on each bar are the standard error.

**Unsupervised data analysis of Raman spectra during virus infection.** The vibrational bands obtained from the normalized average RS recorded on tomato leaves (Figure 2) were assigned to corresponding functional groups and biochemical species; the majority of peaks were attributed to carotenoids, chlorophyll and carbohydrates (Table 1). Specifically, carotenoids generated bands at 1526, 1153, and 1000  $\text{cm}^{-1}$ , due to C=C and C–C stretching and to in-plane  $\text{CH}_3$  rocking modes<sup>32,33</sup>, as well as bands at 1387, 1328, and 1184  $\text{cm}^{-1}$ <sup>34,35</sup>. The structure of carotenoids shows various  $\text{CH}_3$  groups attached to C=C responsible for the band at 1387  $\text{cm}^{-1}$ , but the band linked to this bending was relatively weak. Other bands associated with =CH rocking are visible at 1330–1250  $\text{cm}^{-1}$ , whereas the band at 1184  $\text{cm}^{-1}$  is again assigned to a C–C stretching mode. The weak bands at 1353, 1287 and 915  $\text{cm}^{-1}$  and the shoulders at 1551 and 987  $\text{cm}^{-1}$  associated to chlorophyll<sup>36</sup> partially overlapped with those of certain carotenoids around 1350–1280  $\text{cm}^{-1}$ <sup>33</sup>. Other components identified in the spectra represented cellulose and hemicellulose, with limited contributions<sup>37</sup>, and

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lignin (characteristic peak at  $1608\text{ cm}^{-1}$ ; C-C stretching)<sup>27</sup>. The contribution of monomeric sugars and starch was observed at  $1153\text{ cm}^{-1}$  and in the region  $940\text{-}850\text{ cm}^{-1}$  (C-O-C vibration, typical of starch), while bands at  $1110$ ,  $1070$ ,  $1047$  and  $1026\text{ cm}^{-1}$  are associated to C-OH, related to monomeric sugars.



**Figure 2.** Mean Raman spectra of tomato leaves obtained from plants mock-inoculated (healthy) and infected by either TYLCSV or TSWV. Spectra of all tested plants were averaged to obtain the three spectra shown.

**Table 1.** Vibrational bands and their assignments for tomato leaf samples.

Band	Vibrational assignment
1608	m $\nu$ (phenyl ring) (phenolics and lignin) <sup>38</sup>
1551	m br chlorophyll - central 16-membered-ring vib.+ $\nu$ (C=C) (pyrrole ring)
1526	s $\nu$ 1(C-C) (carotenoids) <sup>39</sup>
1438	m $\nu$ (phenyl ring) (phenolics) <sup>38</sup>
1483	m $\delta$ (CH <sub>2</sub> ) and $\delta$ (CH <sub>3</sub> )
1328	m $\delta$ (CH). $\nu$ (CN) (pyrrole ring br. - chlorophylls) <sup>40</sup>
1353	w undefined (chlorophylls)
1387	w $\delta$ (CH <sub>3</sub> ) ( $\beta$ -carotene and luteine) <sup>25</sup>
1284	m $\delta$ (phenyl-OH) (phenolics) <sup>38</sup> + - $\delta$ (CH). $\nu$ (CN) (chlorophyll) <sup>25</sup>
1261	w ro(=CH) (carotenoids) <sup>25</sup>
1222	m $\delta$ (CH). $\delta$ (CH <sub>2</sub> ) (chlorophyll) <sup>40</sup>
1125-1185	ms $\nu$ (CC). $\gamma$ (CH) (chlorophylls) <sup>40</sup> + $\delta$ (CH phenyl) phenolics) <sup>38</sup>
1153	s $\nu$ 2(C\C) (carotenoids) <sup>41</sup>
1144	m sh $\nu$ (CN). $\delta$ (CNC) (chlorophyll) <sup>40</sup>



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3	1110	$\delta$ (C-OH) (carbohydrates) <sup>27</sup>
4	1070	$\delta$ (C-OH) (carbohydrates) <sup>27</sup>
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6	1047	$\nu$ (C-O)+ $\nu$ (C-C)+ $\nu$ (C-OH)
7		(carbohydrates) <sup>27</sup>
8	1000	m $\delta$ (C-CH <sub>3</sub> ) (carotenoids) <sup>41</sup>
9		
10	987	m undefined (chlorophylls)
11	915	m undefined (chlorophylls)
12		
13	747	m-s ring br. Mode (aromatics)
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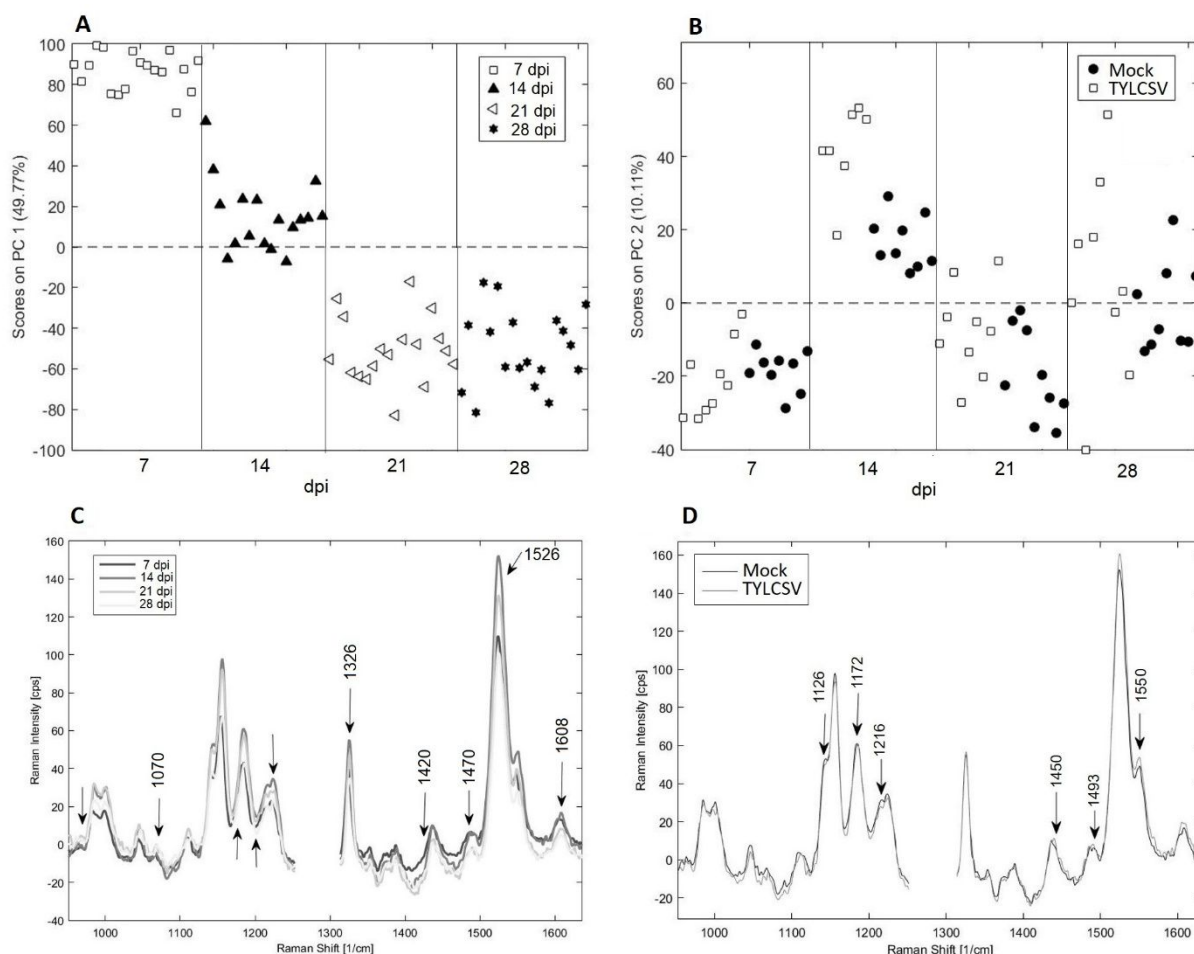
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 m=medium w=weak ms= medium strong

17 185 Overall, the spectral profiles for healthy and for TYLCSV- or TSWV-infected plants showed  
 18 similar patterns (Figure 2); in both cases, signals due to chlorophylls were remarkably weaker than  
 19 186 those assigned to carotenoids and even weaker in infected plants, as previously described <sup>25</sup>,  
 20 187 providing a clear indication of metabolic changes occurring during infection <sup>42</sup>.

24 189 Contrary to a recent RS analysis on plants subjected to abiotic stress <sup>43</sup>, it was difficult to identify  
 25 single Raman bands strongly altered by virus infection. Therefore, to straightforwardly interpret  
 26 190 data and emphasize systematic spectral variations, a chemometric analysis was applied, a process  
 27 191 that could be unnecessary for plants subjected to abiotic stresses, eliciting more uniform and  
 28 192 synchronous metabolic responses in all cells, as opposed to the progressive changes on newly  
 29 193 emerging tissue occurring during systemic pathogen infection.

34 195 At first, we investigated by PCA non-random variability associated with virus infection,  
 35 obtaining a systematic grouping of spectra linked to the progression of both plant ageing and virus  
 36 196 infection. To determine the effect of each virus, two separate PCA models were calculated. For  
 37 197 TYLCSV, the most informative spectral regions within 950-1250 cm<sup>-1</sup> and 1320-1640 cm<sup>-1</sup> were  
 38 198 considered (Figure 3).  
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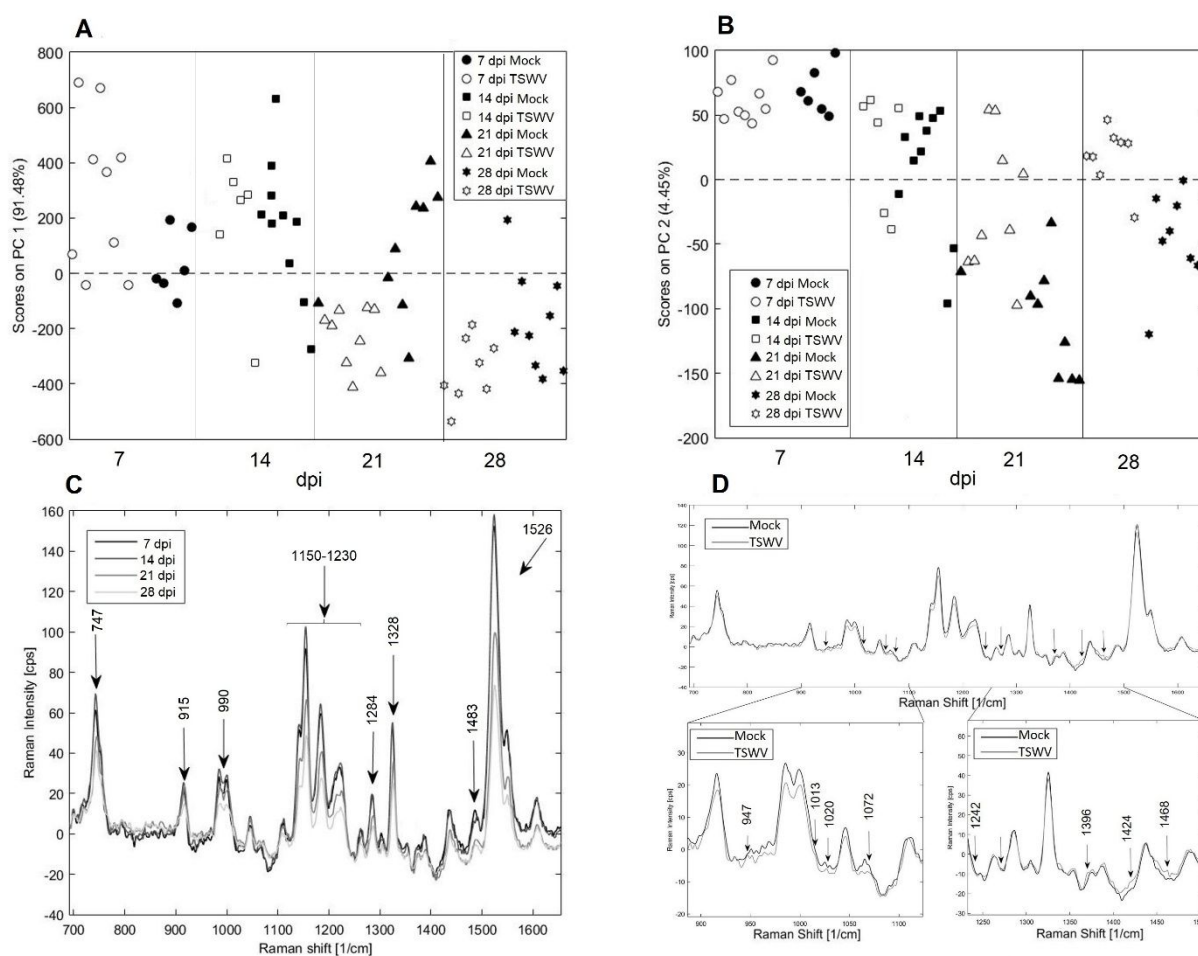
**Figure 3:** PCA model for TYLCSV-inoculation experiment. (A, B) PCA score plots as a function of time for the two first components. (C) Average tomato leaf spectra for each time point of analysis; arrows indicate the bands mainly represented in PC1, mostly correlated with time progression. (D) Average tomato leaf spectra of all mock-inoculated and all TYLCSV-infected plants; arrows indicate bands mainly represented in PC2, mostly correlated with TYLCSV infection.

The scores of the first two components plotted against time progression indicated significant differences among samples along the entire experimental period. In particular, PC1 mainly captured changes correlated with plant ageing, with scores progressively decreasing from 7 to 28 dpi (Figure 3A), while PC2 caught spectral modifications due to virus infection, as infected leaves showed scores lower than healthy ones, at the corresponding time points (Figure 3B). The loadings of the model and the histogram of the variance of spectral frequencies captured by each component (Figure S1) show the bands or the spectral regions responsible for sample grouping on the scores plot. Specifically, at 7 dpi (very early stage of infection), all signals displayed a relatively low intensity that increased at 14 dpi (early infection) and gradually decreased thereafter. However, the general intensity was not as informative as the differences in the intensity ratio and the shape of bands. The signals represented in PC1 (marked by arrows in Figure 3C) are mainly related to plant ageing and derive in particular from the carotenoid peak (1526 cm<sup>-1</sup>) that underwent a slight

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3 218 frequency shift and a bandwidth modification over time. A non-negligible contribution arose also  
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5 219 from the  $1470\text{ cm}^{-1}$  peak and the small shoulder at  $1420\text{ cm}^{-1}$  that decreased from 7 to 28 dpi,  
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7 220 together with the chlorophyll band at  $1326\text{ cm}^{-1}$ . Other important contributions to PC1 arose also by  
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9 221 the triplet bands between  $1250\text{-}1100\text{ cm}^{-1}$ , again related to carotenoids and chlorophylls; on the  
10 222 contrary, an increase in the  $1070\text{ cm}^{-1}$  peak (C-OH in carbohydrates) was recorded. In conclusion,  
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12 223 the RS analysis of TYLCSV-infected plants identified a decrease in signals related to  
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14 224 carotenoid/chlorophylls along with an increase in carbohydrates accumulation.

15 225 Interestingly, complementary bands represented in PC2 resulted more related to the virus  
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17 226 presence (Figure 3D). Here, the peak shoulder at  $1150\text{ cm}^{-1}$ , associated with the pyrrole ring  
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19 227 stretching of chlorophylls, played a relevant role, together with the two smaller bands between  
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21 228  $1500\text{-}1400\text{ cm}^{-1}$  related to phenolic compounds. PC2 also captured signals with significant variance  
22 229 at  $1216, 1172\text{ cm}^{-1}$  and the shoulder at  $1126\text{ cm}^{-1}$  probably due to chlorophylls. In conclusion,  
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24 230 although single Raman bands affected by TYLCSV infection could not be identified, the entire  
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26 231 spectral profile showed modifications in the regions associated to chlorophylls and phenolic  
27 232 compounds, at least in the period of infection considered. Interestingly, the early identification of a  
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29 233 decrease in chlorophyll content in TYLCSV-infected plants is in line with the reported  
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31 234 transcriptional perturbation of this metabolic pathway <sup>44</sup>.

32 235 Similar experimental evidence was also recorded in the PCA model obtained for TSWV on  
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34 236 spectra between  $700\text{ and }1800\text{ cm}^{-1}$  (Figure 4). In the long-term TSWV-infection analysis (7 to 28  
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36 237 dpi), both PC1 and PC2 resulted related to plant ageing and virus infection, contrary to the results  
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38 238 with TYLCSV. In fact, TSWV -infected plants showed higher scores on PC1 at 21 and 28 dpi  
39 239 (Figure 4A) and, concurrently, the scores on PC2 of TSWV-infected plants were generally lower  
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41 240 than control plants, at the corresponding time points (Figure 4B).  
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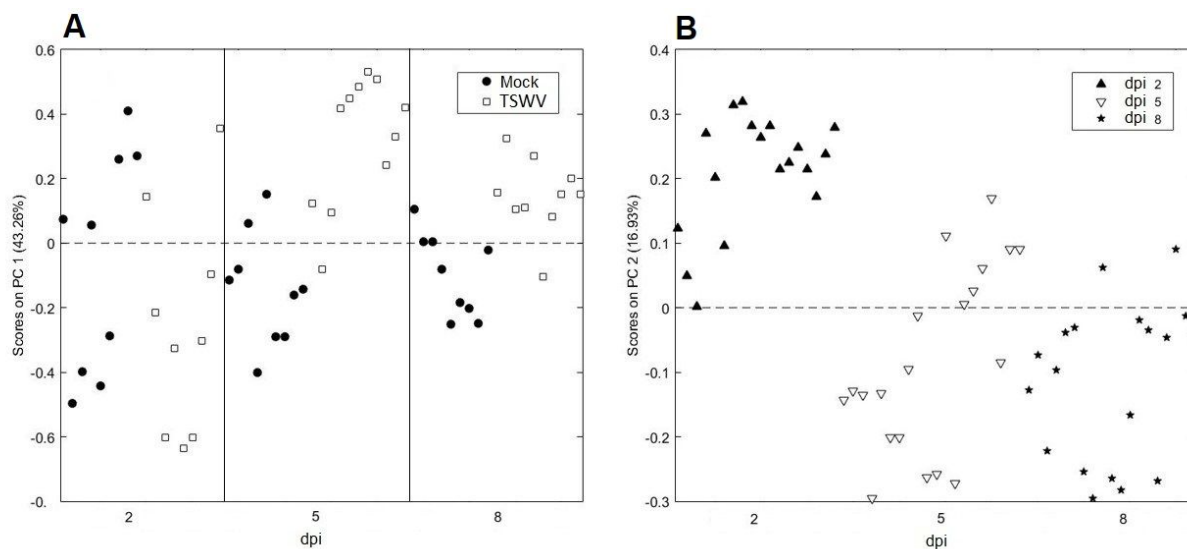


**Figure 4:** PCA model for TSWV inoculation experiment. (A) PCA score plots as a function of time for PC1. (B) PCA score plots as a function of time for PC2. (C) Average tomato leaf spectra for each time point of analysis; arrows indicate bands mainly represented in PC1, mostly correlated with time. (D) Average tomato leaf spectra of all mock and all TSWV-infected plants; arrows indicate bands mainly represented in PC2, mostly correlated with TSWV infection, with a magnification of the Raman spectral regions with major differences between control samples (Mock) and TSWV.

Therefore, the variations induced by TSWV are masked by changes linked to plant ageing arising from the same spectral bands, supporting the concept that TSWV provokes premature ageing signals. In particular, based on the model loadings and the percent of variance captured by each PC (Figure S2), TSWV induced a general decrease of the entire spectrum intensity (Figure 4C), accompanied by rising of the low-intensity peaks at 1468, 1424, 1396, and 1242  $\text{cm}^{-1}$  (Figure 4D). Small, but statistically significant differences between mock-inoculated and TSWV-infected plants were also revealed for bands at 1072, 1020, 1013, and 974  $\text{cm}^{-1}$  (Figure 4D).

Interestingly, when we analyzed plants in the first week after TSWV inoculation (short-term experiment), similar conclusions were reached. Here, the PCA model calculated with spectra collected at 2, 5 and 8 dpi highlighted non-random variations due to TSWV infection. Most

information was captured by the first two components that correlated with virus infection (PC1) and plant ageing (PC2) (Figure 5 A, B).



**Figure 5:** PCA scores plots of TSWV early-infected plants. (A) Scores of PC1 plotted against dpi and colored by infection conditions. (B) Scores of PC2 plotted against dpi and colored by time of analysis.

Therefore, in this case, the spectral changes due to virus infection prevail compared to the ageing effects occurring during one week. In particular, infected samples showed on average higher scores on PC1, already since 5 dpi, whereas the scores on PC2 gradually decreased from 2 to 5 dpi, confirming that PC2 captures information related to plant aging. The loadings of PC1 and PC2 of the model calculated for this short-term experiment exhibit several bands responsible for the differences between healthy and infected samples, but the most relevant variance was associated with the carotenoid peak at  $1526\text{ cm}^{-1}$ , that relatively decreased in virus infected samples (Figure S3).

**Supervised data analysis of Raman spectra during virus infection.** To gather indications about the sensitivity, specificity and accuracy of RS in the identification of infected plants, PLS-DA classification models were calculated. These results were validated by the “leave-one group-out” cross-validation using one plant at a time as cancellation group, the most robust procedure when a separate external validation set is not available. For both viruses, an increasing trend of all figures of merit along the time frame considered (Table 2) was obtained, showing that mock-inoculated and experimentally infected samples can be distinguished with >70% accuracy since 14 dpi for TYLCSV. Moreover, RS provided positive results also for the very early detection of TSWV, i.e. within the first 8 dpi, with an accuracy >85%. Therefore, RS allowed to recognize infected plants since the early stages of infection, when symptoms are visually undetectable.

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283 **Table 2.** Identification of virus infection by PLS-DA classification  
284 in “leave-one-plant-out” cross-validation.

Virus	dpi	Sensitivity	Specificity	Accuracy	Class. Error %
TYLCSV	7	0.71	0.64	0.67	33
	14	0.80	0.67	0.71	29
	21	0.78	0.78	0.78	22
	28	0.80	0.88	0.83	17
TSWV	2	0.67	0.33	0.50	50
	5	0.91	0.56	0.75	25
	8	0.80	1.00	0.89	11
	14	0.67	1.00	0.87	13
	21	0.78	1.00	0.90	10
	28	0.67	1.00	0.83	17

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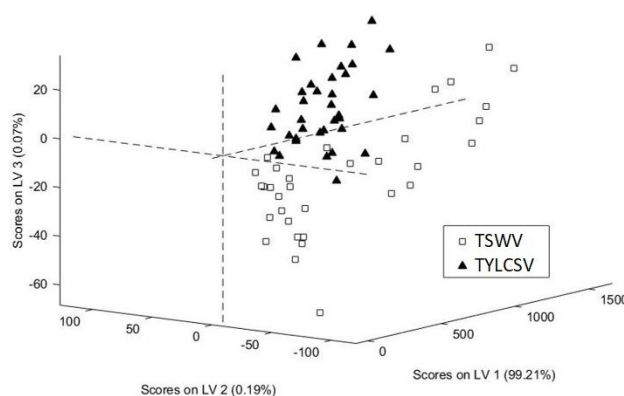
22 285 When comparing the loadings of the above models for TYLCSV and TSWV, differences in the  
23 relevant spectral regions for virus detection were revealed, suggesting that different viruses provoke  
24 286 specific modifications. To confirm this evidence and possibly distinguish the effect of the two  
25 287 viruses, a PLS-DA model was calculated to classify TYLCSV- vs. TSWV-infected samples. To  
26 288 highlight differences specifically due to each virus, independently from plant ageing, all the spectra  
27 289 of infected plants at the different time points were used as training set. Noteworthy, we detected a  
28 290 remarkable class separation between TYLCSV- and TSWV-infected plants (Figure 6) with three  
29 291 latent variables (LVs) (the loadings of the considered LVs are shown in Figure S4).

32 291 **Table 3.** Virus discrimination (TYLCSV vs. TSWV) by PLS-DA classification in “leave-one-plant-  
33 292 out” cross-validation (CV). The first two lines represent the confusion matrix in CV.

	Pred. TSWV	Pred. TYLCSV
True TSWV	25	8
True TYLCSV	8	27
Sensitivity	0.76	0.77
Specificity	0.77	0.76
Accuracy	0.76	0.76
Class. error %	24	24

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295 **Figure 6:** Classification model for the discrimination of TYLCSV vs. TSWV in tomato plants. LS-  
296 DA scores on the first three of the model.



297 Moreover, the “leave-one plant-out” cross-validation indicated that sensitivity, specificity and  
298 accuracy values were > 75% (Table 3), confirming that specific spectral features can be associated  
299 to each virus, according to the previous unsupervised data elaboration. This result could be linked to  
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3 301 the different nature of the two viruses, TSWV infecting all plant tissues and rapidly invading the  
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5 302 plant, TYLCSV restricted to few phloem cells and slowly colonizing the plant <sup>45</sup>.

## 6 7 8 303 **CONCLUSIONS**

9 304 The current research contributes to develop alternative, automated and non-destructive  
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11 305 technologies for plant disease and stress evaluation, helpful to reach objective, reliable and quick  
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13 306 plant diagnostic procedures required in a new era of precision agriculture <sup>18,27,43,46-49</sup>. RS represents  
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15 307 a rapid, specific and sensitive tool to realize practical and cheap methods for large-scale disease  
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17 308 monitoring in real time, alternative to molecular and traditional techniques.

18 309 In this work, we applied for the first time RS to monitor the spectral changes occurring during  
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20 310 virus infection of an important crop plant, in a time frame including early (asymptomatic) and late  
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22 311 infection stages, monitoring in parallel the amount of infecting virus using quantitative molecular  
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24 312 approaches. A coherent modification of the entire spectral profile in virus-infected plants was  
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26 313 detected in regions associated with carotenoids, chlorophylls, carbohydrates, and phenolic  
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28 314 compounds. The more informative spectral regions related to the early onset of virus infection were  
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30 315 identified by PCA. Unexpectedly, such analysis also revealed differences related to plant ageing,  
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32 316 even if samples always consisted of newly emerged leaves. Noteworthy, RS captured not only the  
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34 317 effect of infection since early and still asymptomatic infection stages for both tested viruses, but  
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36 318 could also differentiate TYLCSV and TSWV, identifying relevant and specific spectral variations  
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38 319 between them.

37 320 Conclusively, RS could be applied in plant breeding programs for disease resistance monitoring,  
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39 321 under phytosanitary surveillance screenings, particularly relevant for the viruses here considered for  
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41 322 which the most effective control strategies rely on selection of resistant genotypes <sup>6,50</sup>. Nonetheless,  
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43 323 this study provides interesting clues to screen other species of interest exposed to different biotic or  
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45 324 abiotic stimuli.

## 46 47 325 **ORCID**

48 326	Luisa Mandrile	0000-0002-5597-2590
49 327	Laura Miozzi	0000-0003-0410-8230
50 328	Anna Maria Vaira	0000-0001-6702-1162
51 329	Andrea M. Giovannozzi	0000-0001-6646-5052
52 330	Andrea M. Rossi	0000-0001-5638-7978
53 331	Emanuela Noris	0000-0001-8656-8841

## 54 332 55 333 **Author Contributions**

1  
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3 334 EN, LMandriale, LMiozzi, and AMR designed the experiments; LMandriale, SR, EN performed the  
4 experiments; LMandriale, LMiozzi, SR, AMV, AMG, AMR analyzed the data, EN and LMandriale  
5 335 wrote the manuscript. All authors approved the final version of the manuscript.  
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## 9 337 **Notes**

10  
11 338 The authors declare no competing financial interest.  
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