HIGH THROUGHPUT SEQUENCING TESTING OF SMALL RNAs EMPOWERS THE SIMULTANEOUS DETECTION OF CITRUS VIRUSES AND VIROIDS

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Introduction and Aims

Citrus are affected by a relevant number of **viruses and viroids** routinely tested by bioindexing and molecular assays. Anyway, the selection of virus free mother trees candidate for the foundation block requires a process of shoot tip grafting and biological indexing in greenhouse and molecular assays.

Recent papers have shown the **High Throughput Sequencing (HTS) technology** is effective for the simultaneous detection and identification of viruses and viroids in Citrus and other crops, as pre-screening to conventional detection methods to solve ambiguous results [1].

To explore the reliability of an HTS-based virus detection protocol in association to bioinformatic strategies, we developed a pilot testing of different field trees in parallel with molecular and biological methods. **Three sweet orange trees (Q7, N1, N3) and two alemow seedlings (M55, M1A)** previously indexed by conventional methods **were re-analyzed by HTS of small RNAs.** In this study, we evaluated the advantages of HTS according to EU phytosanitary regulation and more.

13 Virus And 6 Viroids

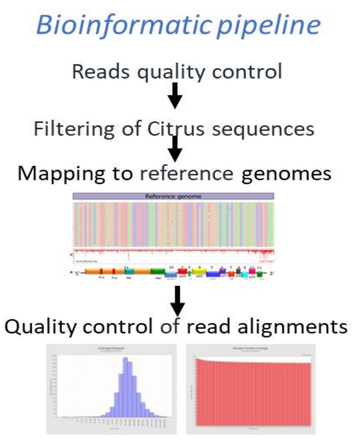
- *Citrus tristeza virus, CTV (6 genotypes)*
- *Citrus leaf blotch, CLBV*
- *Citrus concave gum virus, CCGaV*
- *Citrus psorosis virus, CPV*
- *Citrus leaf rugose virus*
- *Citrus satsuma dwarf virus*
- *Citrus tatter leaf virus, CTLV*
- *Citrus yellow vein clearing virus, CYVCV*
- *Indian citrus ringsport virus, ICRSV*
- *Citrus variegation virus, CVV*
- *Citrus leprosis virus, CiLVC*
- *Citrus vein enation, CVEV*
- *Citrus virus A, CiVA*
- *Citrus exocortis viroid, CEVd*
- *Hop stunt viroid, HSVd*
- *Citrus bent leaf viroid, CBLVd*
- *Citrus bark cracking viroid, CBCVd*
- *Citrus viroid V, CVd-V*
- *Citrus viroid VI, CVd-VI*

HTS Sequencing And Bioinformatic Analysis

• Small interfering RNAs (siRNA) of 21-24 nt were extracted from young bark and sequenced by Illumina technology. High-quality reads, depleted of the *Citrus sinensis* genome, were aligned according to the "**map reads to reference**" bioinformatic approach [2].

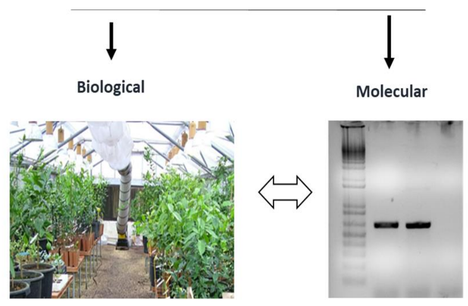
• Four metrics generated by Qualimap [3] were analysed: **read counts**, **percentage of reads count**, **percentage of genome fraction coverage** (GFC) at 50X and **genome coverage** > 90% of reference genome.

•The number of transcripts per million (TPM) was calculated to make the results comparable.



Biological and molecular validation

The presence/absence of viruses and viroids was validated by a parallel biological testing on indicator plants and molecular RT-PCR assays. The presence of CTV-VT isolates in all samples has been analysed by real time RT-PCR [4].



Virome HTS analysis

Here we show the virome HTS analysis of N1 plant as revealed by Genome fraction coverage chart elaborated by Qualimap software displaying the coverage values of reads along the genomes of each pathogen reference.

• The virome profile of the five plants was obtained after alignment of the total small RNA reads with each pathogen reference genome. Libraries depth ranged from 12 to 21 million of reads. A genome coverage of at least 90% of the reference length at 50x was assumed indicative of the presence of a specific virus or viroid.

• All the plants resulted infected by CTV isolates belonging to VT genotype. In addition, M1A was infected also by a CTV-T30 strain. CEVd, CDVd and HSVd viroids have been detected only in N1 whereas CDVd also in N3 sweet orange plants. No virus of quarantine or non-quarantine concern as well as not yet regulated, have been detected.

• Assuming a cut off > 90% of the reference length at 50x coverage, the pathogens with green flag were considered infecting the sample. Non-target viruses showed no reads mapping or coverage below 30% of reference genome.

• Viruses and viroids with coverage of at least 1x for 90-100% of the length represent "alert" results that have to be confirmed by molecular and biological assays.

Stacked column chart displays the transcripts per million (TPM) ratio for each pathogen reference analyzed. It refers to the number of reads aligned to the reference for each and converted in TPM in order to make the samples comparable. The number of reads aligned to CTV reference genome is significantly higher than the other virus and viroids.

Validation assays

Molecular tests by RT-PCR assays allowed to uniquevocally define target and non-target viruses and viroids. HTS positive and negative signals have been confirmed. For most of the suspect pathogens, molecular detection gave negative results, whereas few of them have been confirmed as positive.

Biological indexing allow to uniquivocally discern between target and non-target viruses, although in a longer time and with labour.

RT-PCR tests

	CTV	CEVd	HSVd	CDVd	CPSV	CCGaV	CiVA
N1	+	+	+	+	-	+	-
N3	+	-	-	+	-	-	-
M1A	+	-	-	-	-	-	-
M55	+	-	-	-	-	-	-
Q7	+	-	-	-	-	-	-

CONCLUSIONS

- Based on our results and similar investigation on fruit trees, HTS is highly effective for a fast and smart identification of any virus and viroid affecting host plants.
- The number of mapped reads and the genome coverage is strictly linked to the titer of the virus or viroid.
- Library depth, sequencing technology, bioinformatic pipelines and assembly strategies as well as age of the plant and its biological status could significantly influence the results.
- Despite the cost, the use of HTS could provide a comprehensive phytosanitary status of citrus samples, thus reducing the greenhouse footprint, labor, time, and costs.
- Further comparisons, including those aimed at determining limits of detection, are evidently needed to clarify the analytical sensitivity required in HTS focused on surveillance surveys and to define specific details of the protocol. This will further contribute to improve the procedure, and the use will help to validate it.

References

[1] Bester et al., 2022 *Plants*, 11, 1939. [2] Licciardello *et al.,* 2021, *Agriculture* 11, 400; [3] Okonechnikov *et al.,* 2016 *Bioinformatics*, 32, 292–294; [4] Ruiz Ruiz *et al.,* 2009 *Virol Methods* 160, 57-62.

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