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Virome analysis of melon with yellowish symptoms reveals mixed infections of known and emerging viruses in Southern Italy

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Summary. New viruses and virus strains have emerged with increasing frequencies in cucurbit cropping systems, and surveillance for these pathogens requires effective techniques. Virus diversity was assessed in a melon leaf sample from one field in the Campania region of Southern Italy. Four viruses were identified in melon presenting yellowish symptoms, including: cucurbit chlorotic yellows virus (CCYV), cucumis melo endornavirus (CmEV), tomato leaf curl New Delhi virus (ToLCNDV), and cucurbit aphid-borne yellows virus (CABYV). De novo assemblies reconstructed near-complete genomes of these viruses, and using data available in GenBank, whole genome phylogenetic relationships were determined. Based on the HTS results, a virus survey was then carried out in the same melon field, using 108 symptomatic and non-symptomatic leaf samples and RT-PCR/PCR for specific virus detections. Results obtained confirmed presence of the four viruses, with incidences of 78% for CmEV, 78% for ToLCNDV, 69% for CCYV and 52% for CABYV in the field. Most samples had mixed infections, with from two to four viruses in individual samples. Multiple infections comprised 41% infected with CABYV + ToLCNDV + CCYV + CmEV, 28% with ToLCNDV + CCYV + CmEV, 9% with CABYV + ToLCNDV and 2 % of CMV+CABYV. ToLCNDV and CmEV were the most abundant virus detected. This record of CCYV and CmEV is the first for these two viruses in Italy, with CCYV detected with high incidence. CABYV also had high incidence compared to previous reports for this virus. Further research is required, including with other cucurbit hosts, to determine incidence of emerging virus complexes causing yellowing disease in melon.

Keywords. Cucumis melo, whitefly-transmitted viruses, HTS, CCYV, CmEV.

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INTRODUCTION

Melon (*Cucumis melo* L.) is one of the most economically important vegetable crops in Italy, covering approx. 14,000 ha, mainly (88%) in open fields,

with total annual production of approx. 500,000 tons (Utili, 2023). During the last 20 years there has been a steady contraction both in the areas of melon cultivated and in melon production, especially for the protected crops (ISTAT, 2024). The main causes of reduced production are most likely associated with climate changes, which expose plants to multiple abiotic stresses, and have favoured the spread of new diseases in the Mediterranean region. Spread of new viruses or new variants of known viruses may threaten cucurbit production, especially in Southern Italy, reducing yields and organoleptic quality of crop production, including melon.

Several viruses have been reported in melon in Italy, but cucurbit production has become difficult due to high populations of whiteflies (*Bemisia tabaci* complex) and associated high incidence of whitefly-transmitted viruses. This occurs mainly in summer and autumn crops, even at higher latitudes than in the past, due to the recent northward movement and stabilization of *B. tabaci* (Bertin *et al*., 2018; Bertin *et al*., 2021).

Beet pseudoyellows virus (BPYV), causing leaf yellowing in melon and transmitted by the whitefly *Trialeurodes vaporariorum*, was detected in Sardinia (Southern Italy) by Tomassoli *et al*. (2003), while cucurbit yellow stunting disorder virus (CYSDV, genus *Crinivirus*) was the first *B. tabaci* transmitted virus detected in melon in Italy (Manglli *et al*., 2016). Almost at the same time the cucurbit cultivations in central-southern Italy were shown to be infected by the Mediterranean variant of tomato leaf curl New Delhi virus (ToLCNDV-ES), efficiently transmitted by the local populations of *B. tabaci* (Parrella *et al*., 2018; Panno *et al*., 2019; Luigi *et al*., 2019; Parrella *et al*., 2020). This variant had different infectivity in cucurbit crops compared to the Southern Asian tomato leaf curl New Delhi virus (ToLCNDV-In) (Vo *et al*., 2022a; Vo *et al*., 2022b). Several viruses can induce similar symptoms on cucurbits, and since each virus is often identified using a specific method, the presence of unsuspected or novel viruses is unknown.

Analyses of virus populations in plant samples using high throughput sequencing (HTS) has become established for detecting and identifying plant viruses and viroids introduced into agroecosystems (Barba *et al*., 2014; Maina *et al*., 2024). This method can potentially detect all viruses and viroids present in a plant sample, including those that are unknown. Thus, HTS is a powerful tool for plant disease management, because it can detect and characterize many viruses in individual plant samples.

The present study included a survey carried out in summer 2021 in a melon growing area of the Campania region (south Italy), and viruses present in a representative plant sample were identified by HTS. Viruses were then identified in several plant samples using specific PCR or RT-PCR. Near complete genome sequences of detected viruses were assembled from HTS data, and their characteristics and phylogeny were determined. Using unbiased small RNA sequencing and de-novo assemblies, aphid- and whitefly-transmitted viruses infecting melon in Campania were identified, showing high incidence of mixed infections with up to four viruses in some samples. Some of these viruses had not been previously reported in Italy.

MATERIALS AND METHODS

Sample collection

In July 2021, during field monitoring for cucurbit viruses, a *c.a.* 1000 m^2 melon field of the "Rodrigues" variety, located in Caserta province (Campania region, south Italy), was noticed, because of the intense yellowing of the leaves on almost all plants in the crop (Figure 1A). A single representative plant, labelled Cume-533-21, was chosen for HTS analysis (Figure 1B). Further, a small survey was conducted in the same melon field on one hundred eight leaf samples (around 20% of total plants). In detail, one plant was collected within each of fifty-four 1 m^2 plots on diagonals transect across the 1000 m^2 melon field.

Proportional incidence of symptomatic plants, showing generalized yellowing disease (YD), was assessed by counting the number of plants with symptoms out of the total number of plants observed in the field, using the following formula:

$$\% \quad \text{disease} \quad \text{incidence} = \frac{\text{No.} \quad \text{of} \quad \text{symptomatic} \quad \text{plants}}{\text{No.} \quad \text{of} \quad \text{plants} \quad \text{observed}}$$

Collected samples were kept on ice and brought to the Portici section of the Institute for Sustainable Plant Protection of National Research Council (IPSP-CNR). All samples were annotated in the IPSP-CNR virus collection and kept both in calcium chloride at 5°C and at –80 °C until further analysis. In addition, the presence of *B. tabaci* was verified, and individual adult insects were randomly collected from different plants, with the aim to identify the vector genotype associated with the melon crop, using the method described by Parrella *et al*. 2012.

RNA isolation and library preparation

The Cume-533-21 sample was chosen for HTS analysis, using a bulk of leaf disks (each approx. 1 cm diam.

Figure 1. A, Melon field showing yellowing symptoms on almost all the plants (sampling site: 40°58'28''N 14°07'28''E). B,)The plant, subsequently designated Cume-533-21, was chosen for HTS virus analysis.

and taken from the top, middle and basal portion of the plant. Extraction of total RNA was carried out with the NucleoSpin RNA Plant Kit (Macherey-Nagel), using 100 mg of fresh leaves to obtain enough RNA to submit to RT-PCR and HTS analysis. RNA samples were checked for their quality and quantity using a NanoDrop 2000c UV-vis spectrophotometer (Thermo Fisher Scientific Inc.). One microgram total RNA was used for library preparation. Ribosomal RNA was depleted from the purified RNA using the RiboMinus Plant Kit for RNA-Seq (Thermo Fisher Scientific), and sequencing libraries were prepared by using TruSeq stranded total RNA of the RiboZero Plant kit (Illumina). High-throughput sequencing was carried out by Macrogen (Republic of Korea), using the Illumina NovaSeq 6000 platform with 101 nt paired-end chemistry.

High Throughput Sequencing (HTS) analyses

The bioinformatics analysis of the obtained raw data was carried out using the CLC Genomics Workbench (Qiagen). Reads were trimmed and filtered (reads with Q ≤ 0.05 and shorter than 15 bp were discarded). Host transcripts were filtered using collection reads that were not mapped to the host genome (GCF_025177605.1). Filtered sequencing reads were assembled into contigs using *de novo* assembly, with a minimum contig length cutoff of 200 bp, and using the following for the graph parameters: word size = 20, bubble size = 50. To map the reads back to contigs the parameters were: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.5 and similarity fraction = 0.8. Assembled contigs were subjected to BLASTn and tBLASTx analyses against the NCBI online database and viral RefSeq (November 2023). BLASTn *E*-value was set as default (1e-5), and for tBLASTx, the parameter was adjusted for optimization. Contigs that mapped with the individual viral genome sequences from the NCBI database were used to identify candidate viruses present in analyzed melon samples. Scaffolds were finally assembled by mapping each specific virus contig and residual reads on the reference genomes. The consensus viral genomes were deposited in NCBI GenBank, and were used for further analyses. The open reading frames (ORFs) of the identified viruses were found using the ORF finder NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The schematic representation of the workflow followed for the entire virome analysis, consisting of the three main steps of HTS, data processing, and in-depth data analysis, is reported in Supplementary Figure S1A.

Confirmation of identified viruses using RT-PCR

Specific pairs of primers were also designed on the viral sequences obtained by HTS, to confirm the presence of the viruses within the infected melon plant (sample Cume-533-21) (Supplemetary Table S1). The cDNAs were produced as described above, while PCR amplifications were carried out using the proofreading Platinum SuperFi II DNA Polymerase (Thermo Fisher Scientific Inc.) under the following cycling conditions: initial denaturation at 94°C for 4 min; 35 cycles, each at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final extension step at 72°C for 10 min. The amplicons were controlled on 1% agarose gel and were directly sequenced in both orientations with the primers used for PCR (Microsynth, Seqlab GmbH).

RT-PCR determination of field incidence of identified viruses

Additional leaf samples (n = 108) from distinct melon plants of the same field were analyzed for the presence of zucchini yellow mosaic virus (ZYMV; Family *Potyviridae*), watermelon mosaic virus (WMV; Family *Potyviridae*), papaya ringspot virus (PRSV; Family *Potyviridae*), poleroviruses, cucurbit chlorotic yellows virus (CCYV; Family *Closteroviridae*), cucurbit yellow stunting disorder virus (CYSDV; Family *Closteroviridae*), and cucumber mosaic virus (CMV; Family *Bromoviridae*), through virus-specific RT-PCR assays (Supplementary Table S2).

cDNA was synthesized from the extracted RNAs using the ImProm-II reverse transcriptase system (Promega), according to the manufacturer's instructions. Reactions were performed at 42°C for 60 min, followed by incubation at 70°C for 5 min. References for the specific PCR conditions and cycles of each virus are reported in Supplementary Table S2. The amplicons of all samples shown to be positive with the poleroviruses generic primers were sequenced by Sanger sequencing, to determine the virus species present.

Pairwise and phylogenetic analysis

The assembled virus genomes identified in this study from HTS were subjected to Blastn search of the NCBI database. Only the near complete genomes that mapped with the assembled virus genomes found in the present study were used to determine the pairwise identity and phylogenetic relationships. The genomes were trimmed to equal sizes and aligned using the ClustalW multiple alignment program in BioEdit version 7.2.5 (Hall, 1999). Pairwise nucleotide identities were calculated using the sequence demarcation tool (SDTv1.2) (Muhire *et al*., 2014), and identity scores were generated using colour coded matrix. The maximum likelihood method was used to establish phylogenetic relationships among the aligned sequences, with 1000 bootstrap replications, and adopting the nucleotide substitution models with the lowest Bayesian Information Criterion (BIC) scores to describe best substitution patterns implemented in the MEGA X software (Kumar *et al*., 2018).

RESULTS

Incidence of symptomatic plants, and identification of the Bemisia tabaci *genotypes*

Symptoms of YD were observed on almost 80% of the plants inspected. Seven *B. tabaci* adults collected from symptomatic melon plants and analysed by the method of Parrella *et al*., (2012) were all of the MED Q2 mitotype (Supplementary Figure S4).

HTS results from one melon plant

The RNA extracted from the pooled leaves of the Cume-533-21 plant passed the quality control (QC) showing an RNA integrity number (RIN) of 5.3. To guarantee reliability of the data, QC was assessed at each step of the procedure. A total of 16.9 GB of sequence data was obtained from Illumina sequencing of constructed cDNA libraries, with 69,898,104 raw reads and 14,119,417,008 bases. After trimming, 61,111,634 trimmed reads and 5,605,609,577 bases were obtained, with quality scores of Q30 and 49% GC content. Of these, 3,100,867 were related to viruses (Supplementary Figure S1B). Overall, the raw data with average length of 101 bp became of 91.73 bp after trimming. The *de novo* assembly and BLASTn results generated nine contigs related to partial/near-complete genomes of these viruses: two related to CCYV, one related to cucumis melo alphaendornavirus (CmEV, genus *Alphaendornavirus*), two to ToLCNDV, and four to CABYV (Supplementary Figure S2). The near complete genome of one isolate of CCYV (named Cume-533-21-CY), one isolate of CmEV (named Cume-533-21-Alpha), one isolate of ToLCNDV (named Cume-533-21-ND), and one isolate of CABYV (named Cume-533-21-CAB), were assembled. A quantitative analysis of viral reads was carried out by mapping the trimmed reads with over 99% similarity to each assembled virus genome. The most abundant was Cume-533-21-CY with 543,277 reads for RNA1 and 1,706,894 for RNA2, followed by Cume-533-21-Alpha with 724,271 reads, Cume-533-21-ND with 59,181 reads for DNA-A and 66,353 for DNA-B, and only 891 reads for Cume-533-21-CAB. The classification results showed that most of virus sequences were derived from CCYV, representing 66% of total viral reads. CmEV was 23% of the reads, ToLCNDV was 11%, and CABYV was 1 % of the reads (Supplementary Figure S1C). The CABYV genome was then definitely assembled to complete genome by the overlapping contigs obtained by HTS, with amplicons sequences obtained by Sanger sequencing and using a primer set for genome walking (Table S3). The genomic sequences were subsequently deposited in the GenBank database, with accession numbers OQ190463 (RNA1) and OQ190464 (RNA2) for Cume-533-21-CY; PP977196 for Cume-533-21-Alpha; PQ115115 (DNA-A) and PQ115116 (DNA-B) for Cume-533-21-ND; and PV165535 for Cume-533-21-CAB (of 5,672 nt).

Genome organization, pairwise nucleotide comparisons, and phylogenetic analysis of detected viruses

The entire sequence of RNA1 and RNA2 of isolate Cume-533-21-CY of CCYV were of lengths, respectively, 8,606 and 8,032 nucleotides. Overall, the genome organization was consistent to that described for CCYV (Okuda *et al*., 2010; Supplementary Figure S2). In particular, the lengths of the 5' untranslated region (UTR) for RNA1 was 73 nt, and for RNA2 was 1,026 nt, both showing individual high similarity, and the lengths of the 3' UTR were 250 nt for RNA1 and 221 nt for RNA2, Four ORFs were found in Cume-533-21-CY RNA1. These were: ORF1a, with predicted methyltransferase and helicase motifs; ORF1b, with predicted RNA dependent RNA polymerase (RdRp) motif; ORF2, encoding a predicted protein of 6.04 kDa (p6), and ORF3, encoding a predicted protein of 22.11 kDa (p22). The ORF1a could be fused with ORF1b via a ribosomal frameshift, as reported for other criniviruses (Karasev, 2000). The RNA2 encoded eight ORFs (designated ORF1 to ORF8, from 5' to 3' end), and the relative proteins are shown in Supplementary Figure S2. The BLASTn analysis showed that the RNA 1 of Cume-533-21-CY had the greatest nucleotide similarity with isolates Beijing (Acc. No. JQ904628) from China and TW (Acc. No. JN641883) from Taiwan, with 99.8% similarity and 100% genomic cover for both isolates. The RNA2 had the greatest nucleotide similarity with a Japanese isolate (Acc. No. AB523789), with 99.8% of similarity 100% of genomic cover. The pairwise nucleotide identity of Cume-533-21-CY with all the other complete genomic sequences of CCYV isolates (n = 32) available in GenBank (October 2024) ranged from 99.8% to 99.4%, for both RNA1 and RNA2 (Supplementary Figures S3A and S3B). Consequently, phylogenetic trees obtained for both RNA1 and RNA2 did not detected any clear evolutionary relationships among the CCYV isolates (Figure 2, A and B).

The near full genome size of Cume-533-21-Alpha isolate of CmEV was of 15,074 nt, while the ORF comprising 1,5035 nt, which encodes a predicted polyprotein of 5,011 aa (Supplementary Figure S2). The two untranslated regions (UTR) at the 5' and 3' ends of genomic RNA consisted, respectively, of 3 nt and 35 nt. The greatest nucleotide identity of 99% was shared with the sequence of isolate MVU2/21 (Acc. No. OL957252) from

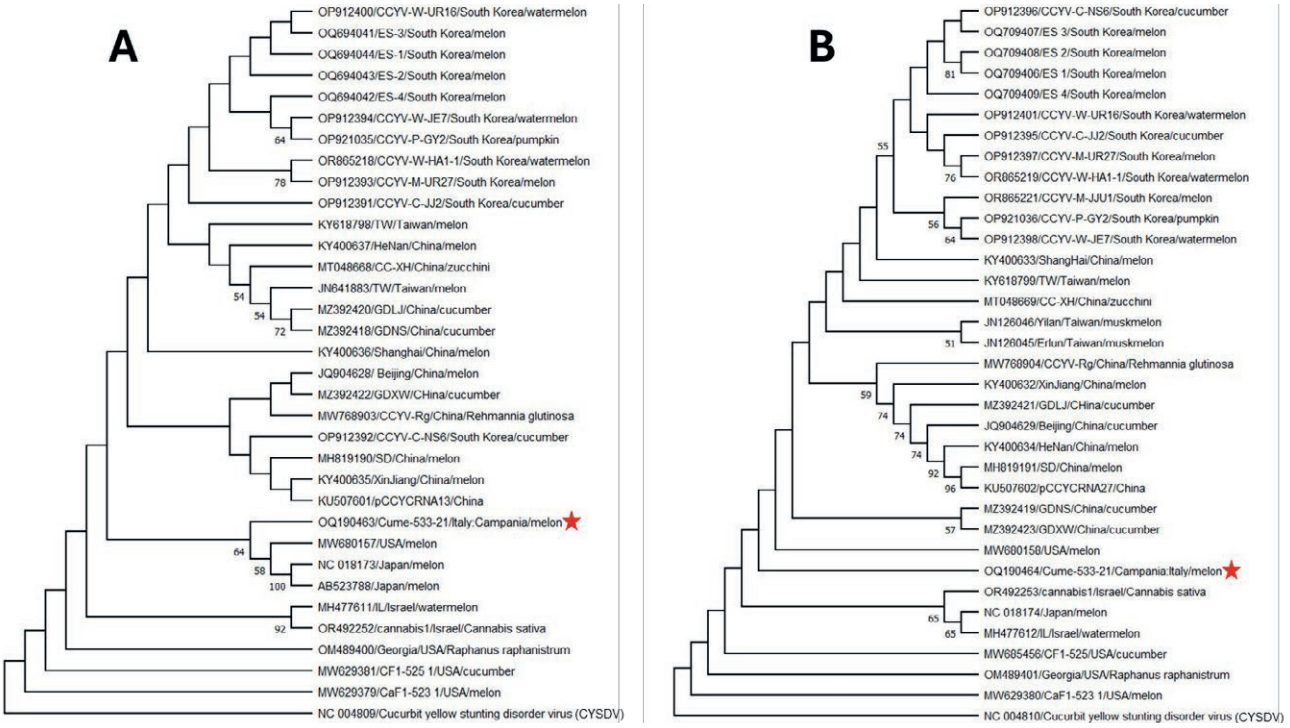

Figure 2. Maximum-likelihood trees obtained for complete RNA1 (A) and RNA2 (B) of CCYV sequences retrieved from GenBank. Based on the best substitution model obtained, the Hasegawa-Kishino-Yano (1985) model of nucleotide substitutions was used. The tree with the greatest log likelihood (-24,028.46) is shown. Percentages of replicate trees (1,000 replicates) are shown next to the branches. The sequence corresponding to the Italian isolate from the present study survey is marked with a red star. Cucurbit yellow stunting disorder virus (CYSDV) was used as the outgroup.

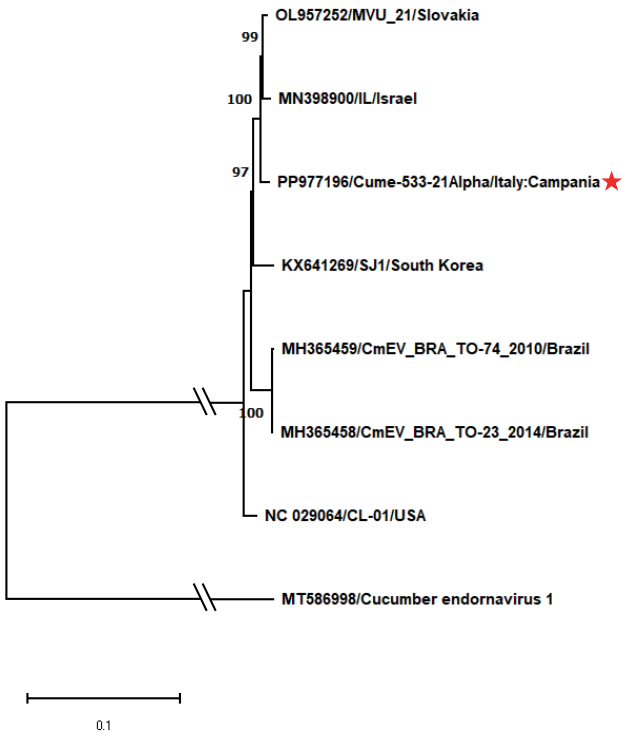

Figure 3. Phylogenetic tree obtained for CmEV complete sequences using the Maximum Likelihood method and General Time Reversible model (Nei and Kumar, 2000) of nucleotide substitutions. The tree with the greatest log likelihood (-38,882.00) is shown. The bootstrap values (1,000 replicates) above 75% are shown next to the branches. The sequence corresponding to the Italian isolate from the present study survey is marked by a red star. Cucumber endornavirus 1 was used as the outgroup.

Slovakia, while pairwise nucleotide identity of isolates Cume-533-21-Alpha with the other CmEV isolates available at GenBank (n = 7; February 2025) ranged from 97.6% to 99% (97–100% of genomic cover). The phylogenetic analysis showed a similar trend as indicated by pairwise analysis. The isolate Cume-533-21-Alpha clustered with the MVU2/2 isolate from Slovakia and the IL isolate from Israel (Acc. No. MN398900) (Figure 3 and Supplementary Figure S3F).

The DNA-A and DNA-B of Cume-533-21-ND isolate were of lengths, respectively, 2738 nt and 2686 nt. The genomes exhibited typical organization of Old World bipartite begomoviruses (Brown *et al*., 2015). The DNA-A contained the AV1 and AV2 genes, in the virion sense orientation, which encode, respectively, the coat protein (CP) and pre-coat protein. In the complementary sense orientation, AC1 encoded the replication-associated protein (Rep), AC2 the transcriptional activator protein (TrAP), AC3 encoded the replication enhancer protein (REn), and AC4 encoded the viral effector. The DNA-B encoded the BV1 gene, in the virion sense orientation which functions as a nuclear shuttle protein (NSP), and the BC1 gene which functions as a movement protein (MP) (Supplementary Figure S2). Both DNAs clustered within the subgroup I of ToLCNDV-ES isolates (the pink clades in Figure 4, A and B) (Panno *et al*., 2019; Troiano and Parrella, 2023). The DNA-A showed greatest nucleotide similarity (99.8%) with isolate Z366 (Acc. No. PP526249), and the DNA-B had 99.7% similarity with isolate Cum-45/16 (Acc. No. MF688671).

The near full-length genome of the CABYV isolate Cume-533-21-CAB was composed of 5,672 nt, and had genomic organization typical of poleroviruses, consisting of single positive-strand RNA, organized in two regions separated by a non-coding internal region (IR) of 200 nucleotides (Supplementary Figure S2). The two genomic regions each had three overlapping open reading frames (ORFs) encoding, respectively, the P0, P1, P1-P2 and the MP, CP, P3-P5 proteins (Supplementary Figure S2) (La Tourrette *et al*., 2021). Phylogenetic relationships based on the near full-length genome of non-recombinants CABYV isolates (n = 85), showed three main groups, according with the geographic origins of isolates: Brazil, the Mediterranean region, and Asia. The Cume-533-21-CAB isolate clustered within the Mediterranean group (Figure 5). Isolates of the Mediterranean group were further grouped into two clades: one composed of Spanish and also isolates from other Mediterranean regions, including the Italian isolate from the present study (Figure 5, pink box), and the other only with Spanish isolates (Figure 5, green box). The Brazilian group (Figure 5, yellow box) was composed only of isolates from *Passiflora* spp., and was most related to the Mediterranean group. The Asian group included two sister clades: a monophyletic clade containing mostly Korean isolates (Figure 5, orange box) and two isolates from China and Japan, and the second, more basal (Figure 5, blue box) containing isolates from different Asian countries.

Confirmation of identified viruses by RT-PCR and PCR

Viruses identified by HTS were confirmed by RT-PCR and PCR (for ToLCNDV) using specific primers for each virus against the same sample used for RNA-Seq. Four virus-specific primers designed were used (Supplementary Table S2). Presence of all four viruses was confirmed, with expected amplicon sizes of 509 bp for CCYV, 581 bp for CmEV, 632 bp for ToLCNDV, and 787bp for CABYV. RT-PCR/PCR amplified virus-derived fragments were assessed on agarose gel (Supplementary Figure S5), and further confirmed by Sanger sequencing. The sequencing results showed 100% similarities with

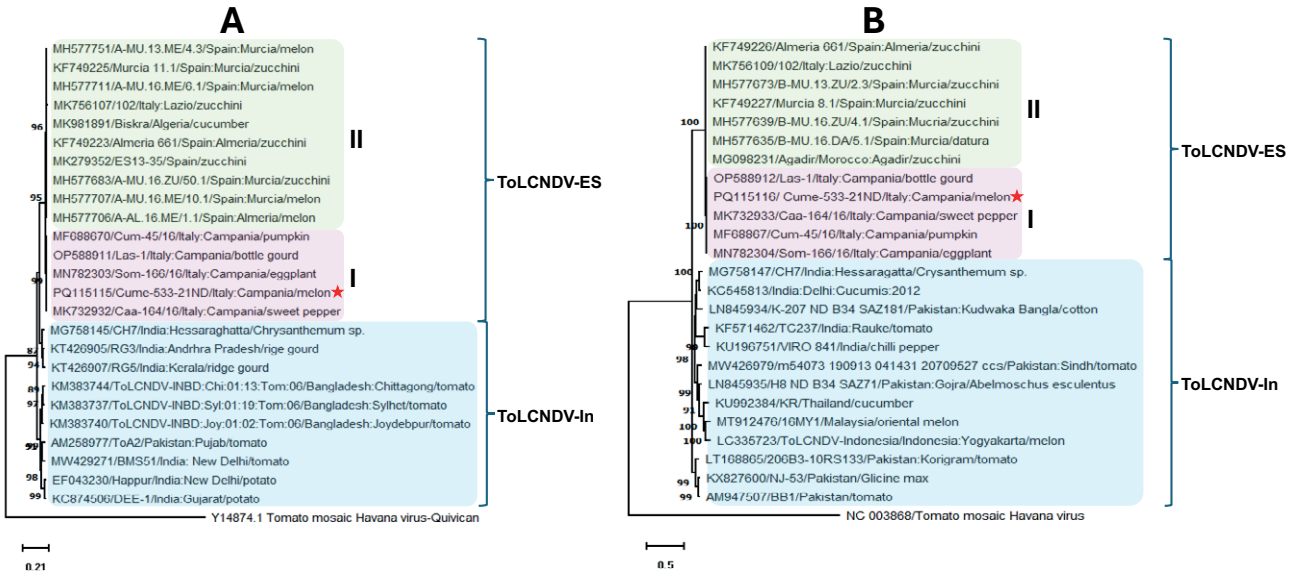

Figure 4. Phylogenetic analyses based on the complete nucleotide sequences of DNA-A (A) and DNA-B (B) components of different ToLCNDV isolates from the Mediterranean region (ToLCNDV-ES) and Asia (ToLCNDV-In). Bootstrap values >75% (n = 1,000 bootstraps) are indicated for each node. In both trees, the ToLCNDV isolate Cume-533-21-ND (red star) groups together with the other four isolates identified in the Italian region Campania, forming a distinct subgroup (pink box), within the ToLCNDV-ES major clade. The green box groups ToLCNDV isolates belonging to subgroup II (from Spain and central Italy) within the ToLC-NDV-ES major clade. The blue box highlights the ToLCNDV-In clade, including isolates from Asia.

the contig sequences derived from the HTS data. The RT-PCR/PCR and HTS results both indicated that all the viruses identified by HTS were present in the melon sample Cume-533-21 used for HTS sequencing.

RT-PCR/PCR-based incidence of identified viruses

Approximately 80% of collected plants showed symptoms (i.e. 86 plants). Ninety-six of the 108 samples were positive for viral pathogens, with CmEV present in 84 (78%) of the samples, ToLCNDV in 84 (78%), CCYV in 74 (69%) and CABYV in 56 (52%) of the samples. CMV was detected only in two plants, while WMV, ZYMV and PRSV were not detected in any of the sampled plants. Of the 22 asymptomatic plants, ten were infected by CmEV and twelve were negative to all the viruses assayed.

Most of the symptomatic samples showed positive reactions for more than one virus, and mixed infections were present in all the symptomatic samples. The mixed infection rate was greatest among CABYV, ToLCNDV, CCYV and CmEV, with 44 samples (41%) having these quadruple infections. Thirty plants (28%) were infected by ToLCNDV + CCYV + CmEV. Double infections by CABYV + ToLCNDV were identified in ten samples (9%), and by CMV + CABYV in two samples (2%) (Figure 6). CABYV, ToLCNDV and CCYV were detected in all of the mixed infections.

DISCUSSION

Spring-summer production of melons in Southern Italy has been severely affected by heavy infestations of whiteflies and aphids, probably associated with ongoing climate changes, which favour thermophilic virus vectors such as *B. tabaci*, and with the increasing difficulties in controlling insects. This has resulted in increased field incidence of symptoms probably caused by viruses, and emergence of yellows symptoms associated with decline of melon crops in Southern Italy. However, the causal agents responsible for melon yellows in the main growing areas of Italy had not been previously identified.

Data presented here, based on HTS results from one melon plant and a following survey in the same field using RT-PCR/PCR with virus-specific primers, showed that whitefly-transmitted viruses, particularly ToLCNDV and CCYV, with this record of CCYV being the first in Italy, were probably more established in the field inspected than viruses transmitted by aphids. CABYV, with incidence of 52% was the most prevalent aphid-transmitted virus in the assessed field. The other aphid-transmitted viruses (WMV, ZYMV and PRSV), except for CMV found in two

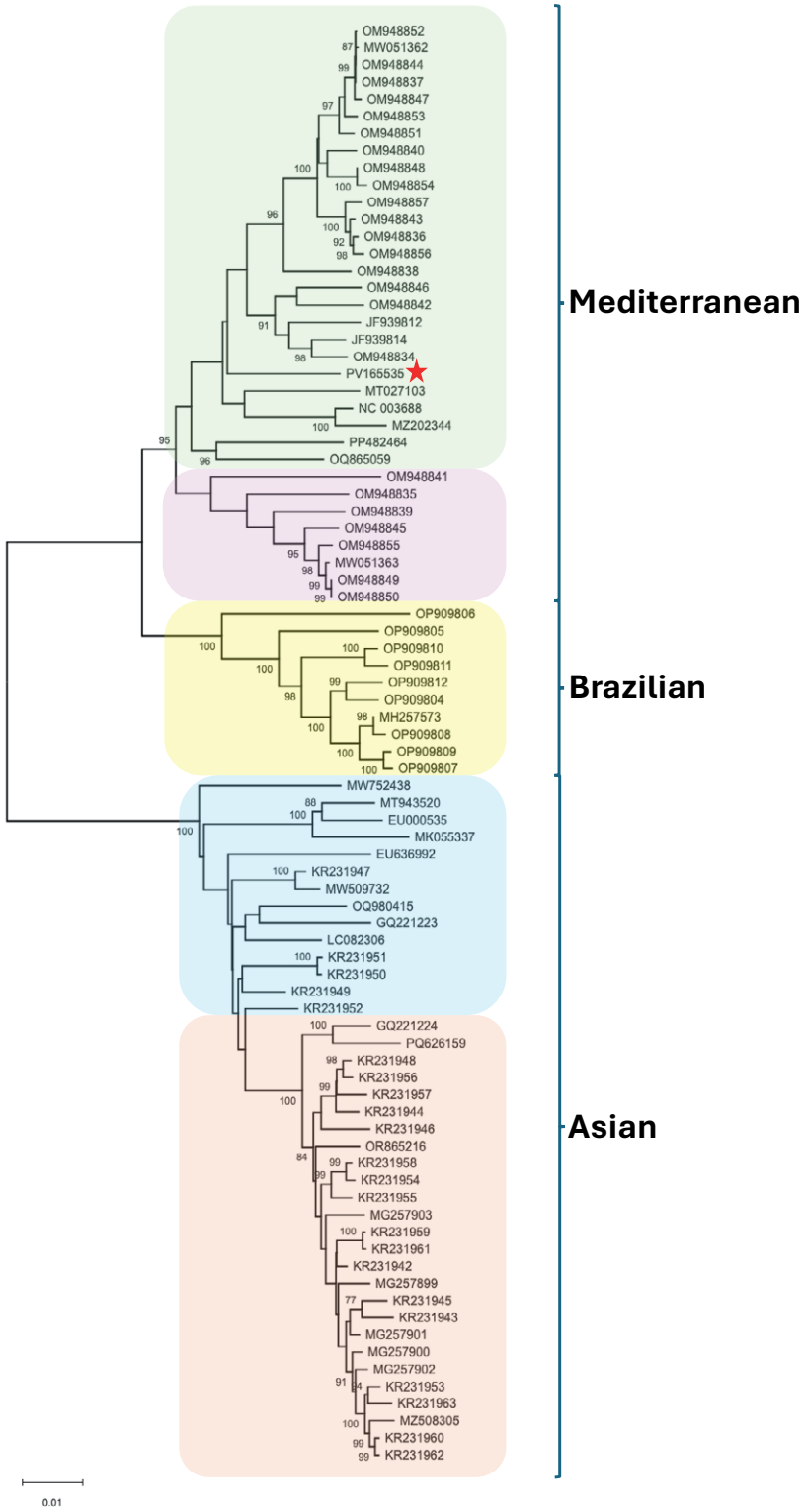

Figure 5. Maximum likelihood tree obtained with the whole genome of CABYV international isolates. Bootstrap values (n = 1000 replicates) >75% are indicated. Phylogeny reconstruction showed that Mediterranean clade was composed of a major (green box) and a minor (pink box) subgroup. Similarly, the Asian clade was composed by two sister subclades (blue and orange boxes). The Brazilian clade (yellow box) was related to the Mediterranean clade. The CABYV isolate Cume-533-21-CAB (PV165535) (red star) clustered within the major subgroup (green box) of the Mediterranean clade.

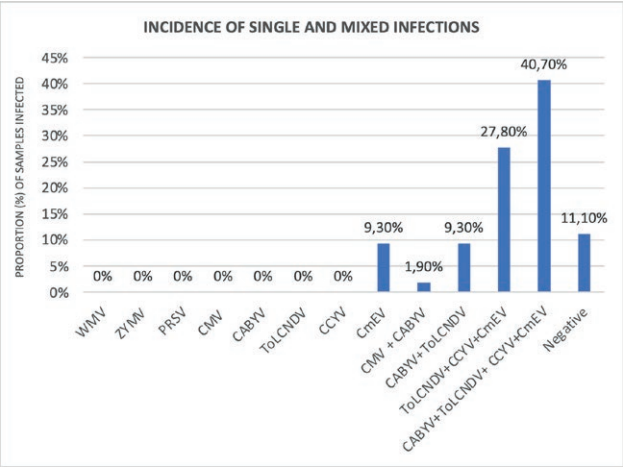

Figure 6. Proportions of 108 sampled melon plants that were infected by four viruses, as shown by RT-PCR results. WMV, watermelon mosaic virus; ZYMV, zucchini yellow mosaic virus; PRSV, papaya ring spot virus; CMV, cucumber mosaic virus; CABYV, cucurbit aphid-borne yellows virus; ToLCNDV, tomato leaf curl New Delhi virus; CCYV, cucurbit chlorotic yellows virus; CmEV, cucumis melo alphaendornavirus.

plants, were not detected (Figure 6). Since only one melon field was sampled, these results may not reflect virus prevalence in melon crops in the Campania region.

ToLCNDV has been considered the main emerging virus in cucurbit cultivations of the Mediterranean region (Moriones *et al*., 2017; Vo *et al*., 2025), and this was found to be the most prevalent virus (78% of the tested samples) in the present study. The MEAM1 and MED genotypes of the *B. tabaci* complex are involved in the transmission and spread of the ToLCNDV-ES strain in the Mediterranean region (Moriones *et al*., 2017; Janssen *et al*., 2017; Bertin *et al*., 2018). From the phylogenetic point of view, the subgroup I and II of this virus have been described in ToLCNDV-ES populations. Based on CP phylogeny, subgroup II comprises isolates from Spain, Italy and other Mediterranean countries, while subgroup I comprises only Italian isolates from the central and southern Italy (Panno *et al*., 2019; Troiano and Parrella, 2023). Phylogenetic relationships of the ToLCNDV-ES isolate from Campania region, based on both DNA components, showed that the Cume-533-21-ND isolate grouped within subgroup I (Figure 4, A and B). Using an infectious clone of a ToLCNDV-ES isolate identified from Campania region and belonging to subgroup I (Parrella *et al*., 2018; Troiano and Parrella, 2023), Vo *et al*. (2022b) demonstrated that this isolate was able to infect only cucurbit hosts, although reported that infection in tomato could be enhanced by tomato yellow leaf curl virus (TYLCV). The continuous overlapping of cucurbit cultivations, particular courgette, from spring until late autumn, which occurs in some cultivation areas of the Campania region, may have resulted in biological adaptation of subgroup I to cucurbits, although further evidence is required to support this hypothesis.

The consistent detection of ToLCNDV on symptomatic cucurbits led to the assumption that this was the only virus responsible for losses on cucurbits in southern Italy. However, two poleroviruses, CABYV and recently PABYV, have been detected on cucurbits, responsible of YD in Italy. CABYV has been present for at least 20 years (Tomassoli and Meneghini, 2007) and has been recently detected with high incidence in the Campania region, associated with yellowing of the older leaves of courgette (Desbiez *et al*., 2023), whereas Parrella *et al*. (2023) detected PABYV at low frequency in the same area. In the present study we also detected CABYV infecting melon. The CABYV isolate Cume-533-21-CAB, here identified by HTS analysis, was completely sequenced, and it grouped within the Mediterranean clade (Figure 5). The incidence of CABYV was high (52% of the sampled plants), and always found in mixed (double or quadruple) virus infections (Figure 6). CABYV is persistently transmitted by cucurbit-colonizing aphids (e.g., *Aphis gossypii*, *Myzus persicae*). Since the number and frequency of use of insecticides allowed for a sustainable crop management have been reduced in recent years, this could lead to increased incidence of persistent-transmitted viruses in susceptible crops including cucurbits.

The present study is also the first to identify CCYV infecting melon in Italy. However, CCYV has been reported in the Mediterranean region for several years, particularly in Spain, Greece, Turkey, Algeria and Israel (EPPO, 2022), and this virus has probably also been present in Italy for some time. CCYV in melon produces symptoms that are very similar to those of caused by CYSDV and BPYV, consisting of yellowing of host plant leaves. CCYV, CYSDV and BPYV are criniviruses (or: belong to the genus *Crinivirus*), and are part of the complex of emerging whitefly-transmitted viruses causing yellows diseases in cucurbits responsible for severe losses (Kavalappara *et al*., 2021). Whereas BPYV is transmitted by *T. vaporariorum*, both MEAM1 and MED genotypes of *B. tabaci* transmit CCYV and CYSDV, although the MED genotype can transmit CCYV more efficiently than CYSDV (Lu *et al*., 2017). Since the MED genotype was found to be much more widespread than the MEAM1 genotype in Campania (Parrella *et al*., 2014 and the present study) this could partly explain the high incidence of CCYV found in melon in the present survey. From a phylogenetic viewpoint, the RNA 1 sequence of the new CCYV isolate clustered with sequences of isolates from the United States of

America (USA) and Japan, although this grouping was not well supported statistically (Figure 1A). For the RNA 2, no clear grouping was shown by the phylogenetic reconstruction (Figure 1B). This is not surprising since criniviruses show limited genetic diversity even among geographically distant isolates (Rubio *et al*., 2001; Orilio and Navas-Castillo, 2009). CCYV has already been shown to have low genetic variability in the RNA-dependent RNA polymerase, coat protein, and minor coat protein genes of different virus isolates (Orfanidou *et al*., 2017).

The present study is also the first to report CmEV in Italy, with high incidence (78%) among the viruses detected. This is not surprising, since endornaviruses are vertically transmitted, infecting host germ line, and remaining permanently associated with that host (Sabanadzovic *et al*., 2016). This is consistent with division of CmEV isolates into two phylogenetic groups based on partial sequences identified, respectively, in *Cucumis melo* subsp. *melo* and *C. melo* subsp. *agrestis* (Sabanadzovic *et al*., 2016). Nevertheless, this phylogenetic reconstruction was based on partial sequences of CmEV isolates. In the present study, the phylogenetic reconstruction shown in Figure 3 was based on the whole CmEV genomes available in Genbank, including the new isolate Cume-533-21-Alpha, and concerns only isolates from *C. melo* subsp. *melo*. Phylogenetic relationships among CmEV genomic sequences available in GenBank showed that the Italian isolate was more related to two isolates from the Mediterranean/European region (one from Israel and the other from Slovakia) compared to isolates from Brazil and the USA), suggesting possible phylogeographic distribution of CmEV isolates. Nevertheless, these data should be supported by additional large-scale CmEV phylogenetic reconstruction, including genomic sequences of geographically different isolates.

Plant viruses can influence behaviour of their vectors by increasing efficiency of their transmission, as reported in CCYV-*B. tabaci* interactions (Lu *et al*., 2019). Virus-virus interactions in mixed infections can also influence population dynamics of particular component viruses in their hosts (Wintermantel *et al*., 2008; Gautam *et al*., 2020). For example, for the CCYV-CYSDV pathosystem, CYSDV and CCYV tend to accumulate at lower level in zucchini plants infected with the two viruses than in singly infected plants (Orfanidou *et al*., 2021). Also in *Begomovirus-Crinivirus* pathosystem of cucurbit leaf crumple virus (CuLCrV)-CYSDV, *B. tabaci* acquires the same levels of the CuLCrV but reduced levels of CYSDV from double infected plants compared to plants infected by any one of these viruses (Gadhave *et al*., 2020; Gautam *et al*., 2020). This could partially explain the greater incidence of ToLCNDV compared to that of CCYV observed in melon in the present study.

Virus infections of melon can cause important economic losses. Field monitoring of major production areas is important for identifying emerging and spreading viruses. In the present study two previously unreported viruses (CCYV and CmEV) were deteted in an Italian melon crop. In addition to these viruses, most of the plants were doubly or multiply infected with other viruses including ToLCNDV and CABYV, confirming the trend for YD described in melon in some regions of Spain, where CmEV, CABYV, ToLCNDV as well as WMV were the most abundant viruses detected (Maachi *et al*., 2022), and in other cucurbit crops in Mediterranean region (Radouane *et al*., 2021; López-Martín *et al*., 2023). The results of the present study, although temporally and spatially limited, indicate that these emerging viruses, forming a complex in association with other known cucurbit viruses, represent new threats of YD to melon crops in southern Italy. However, future impacts of these diseases are likely to be influenced by success of global and national efforts to create effective surveillance and response systems, based on accurate diagnostic tools and regular field monitoring. Extensive and frequent surveys of winter and spring crops, of melon, other cucurbits and wild plants, will elucidate aspects of the epidemiology of the viral yellowing complex, including the identification of the hosts allowing virus overwintering and the role of the different vector genotypes in disease spread. This information will assist definition of useful strategies to mitigate the disease impacts of viruses on host crops.

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