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Mixed infections of Tomato yellow leaf curl New Delhi virus and a '*Candidatus* Phytoplasma asteris' strain in zucchini squash in Italy

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Summary. A new disease syndrome of zucchini squash was observed in Southern Italy, in 2018 and again in 2020. Affected plants were severely stunted and leaves were bent downwards, small, stiff, thick, leathery, and had interveinal chloroses. In addition, flowers were virescent and fruits were deformed and often cracked. Disease incidence was 20 and 30% in two different zucchini cultivations in Campania region (Southern Italy). Tomato yellow leaf curl New Delhi virus (ToLCNDV) was detected in eight samples, by loop-mediated isothermal amplification–based (LAMP) kit and by PCR and Sanger sequencing of the AV1 gene. Phytoplasmas were detected in the same samples using nested PCR assays with primer pairs P1/P7 and R16F2n/R16R2. Phytoplasma associations in plant samples were confirmed using specific primers for the multilocus genes *SecY*, *tuf* and *rp*. Sequence comparison of multilocus genes and phylogenetic analyses of the 16S rDNA gene confirmed the association of a phytoplasma strain closely related to '*Candidiatus* Phytoplamsa asteris'. This is the first report of mixed infections of ToLCNDV and a putative '*Ca*. Phytoplamsa asteris' strain in zucchini, associated with a new Squash-Phytoplasma-Begomovirus (SqPB) disease syndrome.

Keywords. Aster yellows phytoplasma, ToLCNDV, *Cucurbita pepo,* mixed infection.

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INTRODUCTION

Tomato leaf curl New Delhi virus (ToLCNDV), a bipartite begomovirus, was first reported to infect tomato in 1995 in India (Padidam *et al.*, 1995). During the last 20 years ToLCNDV has emerged as an important pathogen, and has spread rapidly in *Cucurbitaceae* in the Mediterranean area, where zucchini crops have been most affected. The disease caused by ToLCNDV is often epidemic, thanks to the presence of its efficient vector, the whitefly *Bemisia tabaci* (Bertin *et al.*, 2018; Parrella *et al.*, 2018; Panno *et al.*, 2019; Bertin *et al.*, 2021). ToLCNDV-infected zucchini squash plants showed typical symptoms, including stunting, severe leaf-curling, yellow mosaic and vein swelling of young leaves, rough skin and reduced size of fruit. Some of these symptoms are similar to those associated with phytoplasma infections.

Figure 1. The previously unreported Squash-Phytoplasma-Begomovirus (SqPB) disease syndrome that was observed on zucchini plants doubly infected with tomato leaf curl New Delhi virus (ToLCNDV) and a phytoplasma related to 16Sr-IB strain. A) yellowish young leaves and virescence of the flowers; B) deformed and cracked fruits with virescent flowers, compared to a healthy fruit and flower (on the left).

In 2018, during field monitoring for cucurbit viruses, an unusual syndrome was observed on zucchini squash plants in a field in Campania region (southern Italy). This syndrome was observed again in 2020, in a zucchini field located approx. 100 km from the 2018 observation. Symptoms consisted of severe stunting of the affected plants, while leaves were bent downwards, stiff, thicker than normal, with leathery texture, and showed interveinal chloroses and reduced leaf area. Young leaves were slightly chlorotic (yellowish) (Figure 1A). These symptoms were like those previously associated with ToLCNDV on zucchini in southern Italy (Panno *et al.*, 2019). However, additional symptoms were observed during fruit set including reductions in fruit size, and fruits were also deformed and often cracked. Flowers were virescence, which could be attributed to phytoplasma infections (Figure 1B). These field observations implied simultaneous infections of ToLCNDV and an unknown phytoplasma. This syndrome was first observed on zucchini plants in Italy, which is referred to as Squash-Phytoplasma-Begomovirus (SqPB).

MATERIALS AND METHODS

Two 1000 m^2 zucchini fields, one in 2018 and one in 2020, both with diseased plants and located in the Campania region of southern Italy, were selected for sample collection and analysis of disease incidence. The two fields were 100 km apart. The percentage incidences of SqPB was assessed by counting the number of plants with SqPB symptoms out of total number of plants observed in each field, using the following formula:

$$\% \text{ disease incidence} = \frac{\text{No. of symptomatic plants}}{\text{No. of plants observed}} \times 100$$

One SqPB diseased plant was randomly collected within each of four 1 m^2 plots on a diagonal transect across each of the two 1000 m^2 zucchini fields. Thus, four symptomatic SqPB plants and four asymptomatic plants were chosen from each field for laboratory analyses. Samples (one leaf per plant) were analyzed by double antibody sandwich ELISA with a commercial kit (Bioreba AG) for cucumber mosaic virus (CMV) and by indirect plate trapped antigen ELISA for potyviruses (potygroup test). ToLCNDV testing was carried out first, using a specific commercial loop-mediated isothermal amplification–based (LAMP) kit (Enbiotech). Total DNA was extracted from virescent flowers of the eight symptomatic plants following previously described methods (Parrella *et al.*, 2008). Nucleic acid samples diluted in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] to give a final concentration of 20-60 ng μL^{-1} were employed in PCR reactions, as described by Schaff *et al.* (1992). For ToLCNDV detection by PCR, the specific primer pair for the AV1 gene was used, as described by Parrella *et al.* (2018). For phytoplasmas detection, direct PCR amplification was carried out using the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) to amplify the 16S rDNA, the spacer region and part of the 23S region, followed by nested-PCR with primers R16F2n/R16R2 (Gundersen and Lee, 1996) on P1/P7 amplicons diluted 1:30, and following procedure described previously (Parrella *et al.*, 2008; Parrella *et*

al., 2014). The AYsecYF1/AYsecYR1 primers (Lee *et al.*, 2006) were used to amplify the *secY* gene, the fTufu/rTufu primers (Schneider and Gibb, 1997) to amplify the *tuf* gene, and the rpF1/rpR1 (Lim and Sears, 1992) to amplify the *rp* gene, in direct PCR assays. Positive and negative controls, including a no template control, were included in all ELISA and PCR assays. The potyvirus-positive control were the isolate PAC-1 of BYMV (Parrella and Lanave, 2009), and the phytoplasma positive control was the isolate CATA-IT1 of Catharanthus roseus 16Sr-IB phytoplasma (Parrella *et al.*, 2008). Amplicons of the ToLCNDV AV1 gene and of each of the four phytoplasma genes from each positive plant sample were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), and were then directly Sanger sequenced twice on both directions. Multiple sequence alignments of 16S rDNA from different *Candidatus* Phytoplasma species (*Ca.* P.) and of the AV1 gene from ToLCNDV-ES/ToLCNDV-In isolates were conducted using Muscle (Edgar, 2004) implemented in MEGA11. Phylogenetic trees were constructed using the best fit model for each alignment, using the maximum likelihood (ML) method in the MEGA11 (Tamura *et al.*, 2021) with 500 bootstrap replicates. The trees were drawn to scale, with branch lengths measured as the number of substitutions per site.

RESULTS AND DISCUSSION

Incidence of the SqPB was estimated at 20% in the field inspected in 2018, and at 30% in the second field inspected in 2020. No positive reactions were observed for all the samples analyzed by ELISA for the search of CMV and potyviruses. All eight SqPB syndrome leaf samples tested positive to ToLCNDV by LAMP, while the eight symptomless samples tested negative for ToLCNDV. The presence of ToLCNDV was further confirmed in the eight positive samples, using the ToLCNDV AV1 gene PCR assay. The nucleotide sequences of AV1 gene amplicon

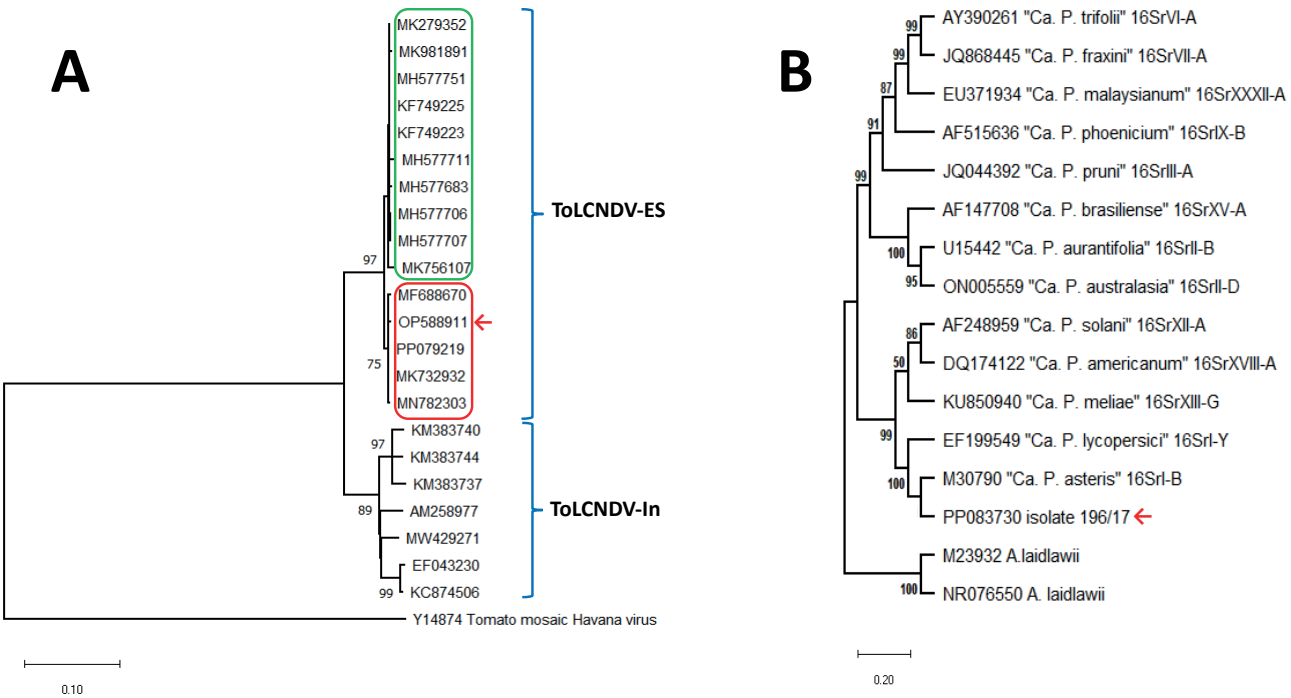

Figure 2. A) Phylogenetic analyses based on ToLCNDV coat protein gene of different virus isolates from the Mediterranean area (ToLCNDV-ES) and Asia (ToLCNDV-In). Evolutionary analyses were conducted in MEGA 11 (Tamura *et al.*, 2021), using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993), with 500 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches for values >70%. The ToLCNDV isolate from double-infected zucchini (indicated in the tree with a red arrow) groups together with four other isolates identified in the same Italian region (Campania) belonging to the subgroup I (red box), as described by Troiano and Parrella (2023), and within the ToLCNDV-ES major clade. The green box groups the ToLCNDV isolates belonging to subgroup II (isolates from Spain and Lazio, central Italy) within the ToLCNDV-ES major clade. B) Phylogenetic tree based on phytoplasma 16S rDNA, using the Maximum Likelihood method and Tamura-3 parameter model (Tamura and Nei, 1993), with 500 bootstrap replicates. The tree shows relationships among '*Candidatus* Phytoplasma' species (*Ca.* P.). GenBank accession numbers are specified in the tree together with ribosomal group or subgroup indications (the position of the phytoplasma isolate is indicated with a red arrow).

obtained from the eight symptomatic plants were identical. A BLASTN search showed that they were also identical (100% nucleotide similarity) to the sequence of the ToLCNDV Italian isolate Caa-164/16 from pepper (MK732932), which belongs to the ToLCNDV-ES subgroup I (Troiano and Parrella, 2023). This was confirmed by the phylogenetic analyses (Figure 2A). The 1049 bp sequence was submitted to NCBI (GenBank Acc. No. PP079219).

Amplicons of the expected size for the 16S rDNA, *tuf*, *SecY* and *rp* gene assays were produced only from all eight plants showing the SqPB syndrome. Sanger sequences were 100% identical among each gene and 99-100% identical to the related sequences of '*Ca*. P. asteris' strain RP166 (CP055264), a phytoplasma in the 16SrI-B Aster yellows group and associated with rapeseed phyllody (Cho *et al*., 2020). This was confirmed by the phylogenetic analyses (Figure 2B). Sequences of each gene were submitted to NCBI (accession nos. PP083730 for 16S rDNA; PP079220 for *SecY*; PP079221 for *tuf*; PP079222 for *rp*).

Mixed infections of phytoplasmas and begomoviruses have been reported in different vegetable crops from other countries. These reports include: in tomato and pepper in Mexico (Cardenas-Conejo *et al*., 2010; Lebsky *et al*., 2011); in chickpea, eggplant, tomato, pepper and *Withania somnifera* in India (Swarnalatha and Reddy, 2014; Singh *et al*., 2015; Venkataravanappa *et al*., 2018; Reddy *et al*., 2021; Tiwari *et al*., 2022); in common bean in Cuba (Zamora *et al*., 2021); in tomato in Saudi Arabia (Sohrab *et al*., 2016); and in many ornamental crops (Ahmad and Khan, 2021). The present study results represent the first evidences of mixed infections of the whitefly-transmitted *Begomovirus* ToLCNDV and a 16SrI-B group related phytoplasma in diseased zucchini squash, and in Italy. The presence of both pathogens in zucchini squash in Campania it not surprising, since ToLCNDV infects zucchini in the region and '*Ca.* P. asteris' is widespread in Italy (Panno *et al*., 2019).

The effects of possible interactions between viruses and phytoplasmas have not yet been studied in detail in mixed infections. Phytoplasmas belonging to the aster yellows group are probably one of the most diverse and widespread groups (Lee and Davis, 2000). ToLCNDV has also become an endemic virus on cucurbits grown in countries in the Mediterranean Basin, partly due to the invasion into new areas of its efficient vector, the whitefly *Bemisia tabaci* (Panno *et al*., 2019; Bertin *et al*., 2018). Generalized yellowing caused by ToLCNDV infections in zucchini could be attractive to different sucking insects, including the leafhoppers that transmit phytoplasmas (Shi *et al*., 2020). Conversely, if '*Ca*. P. asteris' causes yellowing in zucchini squash, this could further attract ToLCNDV infectious whiteflies (Johnston and Martini, 2020). Mixed infections may enhance disease severity and yield losses compared to those of single infections. Hence, interactions between phytoplasmas and viruses should be studied in more detail, especially in vegetable crops and from epidemiological and pathogens interaction viewpoints.

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LITERATURE CITED

Ahmad A., Khan A.A. 2021. Association of phytoplasma and begomovirus with some ornamental plants: a review. *Plant Archives* 21: 350–355.

Bertin S., Luigi M., Parrella G., Giorgini M., … Tomassoli L. 2018. Survey of the distribution of *Bemisia tabaci* (Hemiptera: Aleyrodidae) in Lazio region (Central Italy): a threat for the northward expansion of the Tomato leaf curl New Delhi virus (*Begomovirus*: Geminiviridae) infection. *Phytoparasitica* 46: 171–182.

Bertin S., Parrella G., Nannini M., Guercio G., Troiano E., Tomassoli L., 2021. Distribution and genetic variability of *Bemisia tabaci* cryptic species (Hemiptera: Aleyrodidae) in Italy. *Insects* 12: 521.

Cardenas-Conejo Y., Arguello-Astorga G., Poghosyan A., Hernandez-Gonzalez J., Lebsky V., … Vega-Peña, 2010.First report of *Tomato yellow leaf curl virus* co-infecting pepper with *Tomato chino La Paz virus* in Baja California Sur, Mexico. *Plant Disease*, 94: 1266.

Cho S.T., Zwolińska A., Huang W., Wouters R.H.M., Mugford S.T., … Kuo C.H. 2020. Complete genome sequence of "*Candidatus* Phytoplasma asteris" RP166, a plant pathogen associated with rapeseed phyllody disease in Poland. *Microbiology Resources Announcement* 9: e00760-20.

Deng S., Hiruki C. 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiology Methods* 14: 53–61.

Edgar R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Research* 32: 1792–1797.

Gundersen D.E., Lee I.M. 1996. Ultrasensitive detection of Phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea* 35: 114–151.

Johnston N., Martini X. 2020. The influence of visual and olfactory cues in host selection for *Bemisia tabaci* biotype B in the presence or absence of *Tomato Yellow Leaf Curl Virus*. *Insects* 11: 115.

Lebsky V., Hernández-González, Arguello-Astorga G., Cardenas-Conejo Y., Poghosyan A. 2011. Detection of phytoplasmas in mixed infection with begomoviruses: a case study of tomato and pepper in Mexico. *Bulletin of Insectology* 64 (Supplement): S55–S56.

Lee I.M., Davis R.E. 2000. Aster yellows. In: *Encyclopedia of Plant Pathology (*Maloy OC, Murray TD., ed.), New York, Wiley, pp 60–63.

Lee I.-M., Zhao Y., Bottner K.D. 2006. SecY gene sequence analysis for finer differentiation of diverse strains in the aster yellows phytoplasma group. *Molecular and Cellular Probes* 20: 87–91.

Lim P.O., Sears B.B. 1992. Evolutionary relationships of a plant-pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *Journal of Bacteriology* 174: 2606–2611.

Padidam M., Beachy R.N., Fauquet C.M. 1995. Tomato leaf curl geminivirus from India has a bipartite genome and coat protein is not essential for infectivity. *Journal of General Virology* 76: 25–35.

Panno S., Caruso A.G., Troiano E., Luigi M., … Davino S. 2019. Emergence of tomato leaf curl New Delhi virus in Italy: estimation of incidence and genetic diversity. *Plant Pathology* 68: 601–608.

Parrella G., Lanave C. 2009. Identification of a new pathotype of Bean yellow mosaic virus (BYMV) infecting blue passion flower and some evolutionary characteristics of BYMV. *Archives of Virology* 154: 1689–1694.

Parrella G., Paltrinieri S., Botti S., Bertaccini A. 2008. Molecular identification of phytoplasmas from virescent ranunculus plants and from leafhoppers in Southern Italian crops. *Journal of Plant Pathology* 90: 537–543.

Parrella G., Paltrinieri S., Contaldo N., Vitale M.R., Bertaccini A. 2014. Characterization of 'Candidatus Phytoplasma asteris' strains associated with periwinkle virescence in Southern Italy. *Phytopathogenic Mollicutes* 4: 53–58.

Parrella G., Troiano E., Formisano G., Accotto G.P., Giorgini M. 2018. First report of *Tomato leaf curl New Delhi virus* associated with severe mosaic of pumpkin in Italy. *Plant Disease* 102: 459–460.

Reddy M.G., Baranwal V.K., Sagar D., Rao G.P. 2021. Molecular characterization of chickpea chlorotic dwarf virus and peanut witches' broom phytoplasma associated with chickpea stunt disease and identification of new host crops and leafhopper vectors in India. *3 Biotech* 11: 112.

Schaff D., Lee I.-M., Davis R.E. 1992. Sensitive detection and identification of mycoplasma-like organisms by polymerase chain reaction. *Biochemical and Biophysical Research Communications* 186: 1503–1509.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC. 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology* (Razin S., Tully J.G., ed.), San Diego, CA, Academic press, pp. 369–380.

Schneider B., Gibb K.S. 1997. Sequence and RFLP analysis of the elongation factor *Tu* gene used in differentiation and classification of phytoplasmas. *Microbiology* 143: 3381–3389.

Shi L, He H, Yang G, Huang H, Vasseur L, You M. 2020. Are yellow sticky cards and light traps effective on tea green leafhoppers and their predators in Chinese tea plantations? *Insects* 12: 14.

Singh J., Singh A., Kumar P., Rani A., Baranwal V.K., Sirohi A., 2015. Evidence of a mixed infection of Candidatus Phytoplasma trifolii and a Begomovirus in Eggplan (*Solanum melongena*). *Journal of Pure and Applied Microbiology* 9: 663–670.

Sohrab S.S., Yasir M., El-Kafrawy S.A., Abbas A. T., Mousa M.A.A., Bakhashwain A.A. 2016. Association of tomato leaf curl sudan virus with leaf curl disease of tomato in Jeddah, Saudi Arabia. *VirusDisease* 27: 145–153.

Swarnalatha P., Reddy M. K. 2014. Duplex PCR for simultaneous detection of Begomovirus and Phytoplasma from naturally infected tomato. *Pest Management in Horticultural Ecosystems* 20: 59–68.

Tamura K., Nei M. 1993. Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees. *Molecular Biology and Evolution* 10: 512–526.

Tamura K., Stecher G., Kumar S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution* 38: 3022–3027.

Tiwari N.N., Jain R. K., Prajapati M.R., Singh J., Srivastava S., Tiwari A.K., Marcone C. 2022. Evidence of mixed infection of phytoplasma and begomovirus associated with *Withania somnifera* and *Capsicum annum* plants from Uttar Pradesh, India. *Archives of Phytopathology and Plant Protection* 55: 2146–2157.

Troiano E., Parrella G. 2023. First report of tomato leaf curl New Delhi virus in *Lagenaria siceraria* var. longissima in Italy. *Phytopathologia Mediterranea* 62: 17–24.

Venkataravanappa V., Prasanna H.C., Lakshminarayana C.N., Reddy M.K. 2018. Molecular detection and

characterization of phytoplasma in association with begomovirus in eggplant. *Acta Virologica* 62: 246–258.

Zamora L., Quiñones M., Acosta K., Piñol B., Santos M.E., … Arocha Y. 2021. Molecular characterization of '*Candidatus* Phytoplasma asteris' and a *Bean golden yellow mosaic virus* isolate present in mixed infection in common bean in Cuba. *Revista de Protección Vegetal* 36: 1–9.