UNIVERSITAT POLITÈCNICA DE VALÈNCIA

COMAV
Instituto de Conservación y Mejora de la Agrodiversidad Valenciana

DOCTORAL THESIS

First Report of Cucurbit Chlorotic Yellows Virus (CCYV) And Tomato Leaf Curl New Delhi Virus (ToLCNDV) In Algeria and Lack of Evidence for Seed Transmission of ToLCNDV in Melon and Pumpkin

Presented by:

Amina Kheireddine

Directors:

Carmelo López Del Rincón

Cristina Sáez Sánchez

Valencia, July 2023

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CONSIDER: that the report entitled "First report of Cucurbit Chlorotic Yellows Virus (CCYV) and Tomato Leaf Curl New Delhi Virus (ToLCNDV) in Algeria and lack of evidence for seed transmission of ToLCNDV in melon and pumpkin" presented by Amina Kheireddine, to apply for the degree of Doctor by the Universitat Politècnica de València, has been carried out under her direction at the University Institute of Conservation and Improvement of the Valencian Agrodiversity of the Universitat Politècnica de València, and meets the appropriate conditions to constitute her doctoral thesis, and therefore AUTHORIZE the interested party for its presentation.

Valencia, July 2023

Carmelo López del Rincón

Cristina Sáez Sánchez

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TABLE OF CONTENT

ABBREVIATIONS

AAP: acquisition access period.

AWMV: *Algerian watermelon mosaic virus.*

BCTIV*:* *Beet curly top Iran virus*

BCTV*: Beet curly top virus.*

BLAST: basic local alignment search tool.

BnYDV*: Bean yellow disorder virus.*

BPYV: *Beet pseudo yellow virus.*

CABMV*: Cowpea aphid borne mosaic virus.*

CABYV: *Cucurbit aphid-borne yellows virus.*

CCYV: *Cucurbit chlorotic yellows virus.*

CGMMV: *Cucumber green mottle mosaic virus.*

CMV: *Cucumber mosaic virus.*

CO2: carbon dioxide.

CocMoV: *Coccinia mottle virus*.

CP: coat protein.

CPm: minor coat protein.

CPSMV: *Cowpea severe mosaic virus.*

CR: common region.

CRISPR: clustered regularly interspaced short palindromic repeats.

CTAB: cetyltrimethyl ammonium bromide.

CTV: *Citrus tristeza virus*.

CVYV: *Cucumber vein yellowing virus.*

CYSDV: *Cucurbit yellow stunting disorder.*

dap: days after planting.

DAS: double antibody sandwich.

DNA: desoxyribonucleic acid.

dpg: days post-germination

dpi: days post-inoculation.

DPO: dual priming oligonucleotide.

DTBIA: direct tissue blot immunoassay.

EDTA: ethylenediaminetetraacetic acid.

ELISA: enzyme-linked immunosorbent assay.

EPPO: European and Mediterranean plant protection organization.

FAO: food and agriculture organization of the United Nations.

GFP: green fluorescent protein.

HR: hypersensitive response.

HTS: high throughput sequencing.

IAP: inoculation access period.

ICA: immunochromatographic assay.

IC-PCR: internal control PCR.

ICTV: International Committee on Taxonomy of Viruses.

IPM: integrated pest management.

LAMP: loop-mediated isothermal amplification.

LCV: *Lettuce chlorosis virus.*

LFA: lateral flow assay.

LIYV: *Lettuce infectious yellows virus*.

LSD: least significance difference.

MADR: Ministry of Agriculture and Rural Development of Algeria.

MEAM: middle East Asian minor.

MED: Mediterranean.

MGB: minor groove binding.

MNSV: *Melon necrotic spot virus.*

MNSV: *Melon necrotic spot.*

MP: Movement protein.

mRNA: microRNA.

MWMV: *Moroccan watermelon mosaic virus.*

MYMV *Mungbean yellow mosaic virus.*

MYSV: *Melon yellow spot virus.*

NASH: nucleic acid spot hybridization.

NGS: next-generation sequencing.

NSP: nuclear shuttle protein.

nt: nucleotides.

NW: new world.

ORF: open reading frame.

PCR: polymerase chain reaction.

PepGMV*: Pepper golden mosaic virus*

PepMV: *Pepino mosaic virus.*

PepYLCIV*: Pepper yellow leaf curl Indonesia virus.*

PHYVV: *Pepper huasteco yellow vein virus*

PLYV: *Papaya lethal yellowing virus.*

PPV*: Plum pox virus.*

PRSV: *Papaya ringspot virus.*

PS*:* Piñonet Piel de Sapo.

PTA-ELISA: plate-trapped antigen enzyme-linked immunosorbent assay.

PTGS : post-transcriptional gene silencing.

qPCR: quantitative PCR.

QTL: quantitative trait loci.

RCA: rolling-circle amplification.

RCR: rolling-circle replication.

RdRp: RNA-dependent RNA polymerase.

REn: replication enhancer.

Rep: replication initiator protein.

RNA: ribonucleic acid.

RNAi: ribonucleic acid interference.

RNase: ribonuclease.

RPA: recombinase polymerase amplification.

rpm: revolutions per minute.

RT-LAMP: reverse transcription loop-mediated isothermal amplification.

SLCV: *Squash leaf curl virus.*

SNP: single nucleotide polymorphism.

SPLCV: *Sweet potato leaf curl virus.*

SqMV: *Squash mosaic virus.*

SqVYV: *Squash vein yellowing virus.*

TBIA: tissue blot immunoassay.

TGS: transcriptional gene silencing.

TICV: *Tomato infectious chlorosis virus*.

TILLING: targeting induced local lesions in genomes.

TLCD: *Tomato leaf curl Gujarat virus*.

toCV: *Tomato chlorosis virus.*

ToLCNDV: *Tomato leaf curl New Delhi virus.*

ToLCNDV-ES: Spanish isolate of *tomato leaf curl New Delhi virus.*

ToLCV: *Tomato leaf curl virus.*

ToLCV-India*: Indian tomato leaf curl virus.*

TP: tissue-print.

TrAP: transcription activator of protein.

TYLCD*: Tomato yellow leaf curl disease.*

TYLCSV*: Tomato yellow leaf curl Sardinia virus.*

TYLCV: *Tomato yellow leaf curl virus.*

UV-B: ultraviolet B-rays.

VSRs: viral suppressors of RNA silencing.

WMV: *Watermelon mosaic virus.*

ZYMV: *Zucchini yellow mosaic virus.*

SUMMARY

RESUM

RESUMEN

SUMMARY

Emerging plant viruses in the Mediterranean Basin have arisen in the last decades becoming an important threat to crop production in this region. The spread of new viral diseases has become one of the main factors limiting agronomic and economic yields of cultivated species of *Cucurbitaceae* family, the second in importance after *Solanaceae*. A wide cluster of elements modulates and favors the emergence of these pathogens, including biological, ecological, social, and cultural factors. Global warming favors vector movement to new geographies, while global commercial networks lead to germplasm trade among countries, allowing the long-distance dissemination of viruses. Moreover, the intensive agricultural model, in which prevail genetically homogeneous cultivars, prompts pathogens outbreaks within new geographic areas at a fast rate. One of the key components to develop control strategies, and secure horticultural production, is to identify the type and distribution of viruses present in crops. However, such information in Algeria is scarce and very limited. The objective of this Doctoral Thesis was to identify and determine the prevalence and distribution of the most common viruses on cultivated cucurbit plants (zucchini, cucumber, and melon), and get information about their genetic variability and mode of dispersion.

In this work, cucurbit chlorotic yellows virus (CCYV) was first detected infecting cucumber and zucchini in Algeria. CCYV (genus *Crinivirus*, family *Closteroviridae*) is part of a complex of whitefly-transmitted viruses that cause yellowing disease in cucurbits. Determination of the complete CP, and partial RdRp and Hsp70 sequences of an Algerian CCYV isolate was conducted to unveil the evolutionary relationships with the published isolates in databases. The phylogenetic analysis showed that the Algerian isolate clustered into group I together with the majority of the reported CCYV isolates. The genetic variation within this group was very low (nucleotide identity higher than 99.5%) despite their extensive and discontinuous geographical distribution, plant species (cultivated or weeds) and year of collection. This low genetic divergence could be due to the recentness of this virus that did not face major changes for the present time. In our case, the presence of CCYV was only detected in mixed infection with tomato leaf curl New Delhi virus (ToLCNDV). In future works, it will be interesting to check the likely synergistic or antagonistic interaction between these two viruses that may affect the symptoms development and spread between crops.

ToLCNDV (genus *Begomovirus*, family *Geminiviridae*) was also first detected infecting cucurbit plants in Algeria. ToLCNDV is a bipartite begomovirus that causes destructive epidemics on economically valuable cucurbit and solanaceous crops. The complete DNA-A and DNA-B genomic sequences of a ToLCNDV isolate from cucumber (ToLCNDV-Biskra) were obtained. Alignments of the nucleotide sequences of DNA-A and DNA-B revealed identities of 98.7 and 97.6%, respectively, with the corresponding segments of the isolates from the Mediterranean Basin, whereas the identity with isolates from Asia was around 90 and 81% for DNA-A and DNA-B, respectively. Mediterranean isolates are monophyletic and form a single group, while the isolates of the Asian strain present a higher genetic variability and they are gathered in several clades. The sequence of different Algerian ToLCNDV isolates showed very low nucleotide diversity. Only a duplication of 17-nucleotides was observed in the DNA-B of some isolates, resulting in a putative movement protein 53 amino acids longer, although the functionality of tthis putative protein remain unknown. Also, despite the 17-nucleotide duplication, no recombination events among Algerian ToLCNDV isolates were detected. The low genetic variation of the isolates could preclude the detection of recombinants.

Recent studies have focused on identifying the main factors conducting the emergence of endemic plant viruses in new distal regions. Indeed, seed-transmission of viruses is considered a primary source of inoculum allowing long-distance propagation of plant diseases. Recent studies have reported begomoviruses being transmitted from the progeny to the offspring through seeds in different pathosystems, what has generated a new debate about this mode of transmission. In this Doctoral Thesis, it was characterized how ToLCNDV is distributed in leaves and flowers of melon (*Cucumis melo* L.) and pumpkin (*Cucurbita moschata* D.) accessions, studying the ability of the virus to reach the reproductive organs, including anthers and pistils. Moreover, seeds obtained from ToLCNDV-infected plants were dissected to separate external and internal tissues, determining that ToLCNDV is seed-borne transmitted in both cucurbit species. Nevertheless, in seedlings germinated from infected seeds, real seed-transmission of ToLCNDV was not identified. Even though viral replicative forms were detected in cucurbit leaves, flowers and seeds, this detection did not happen when the leaves of seedlings of the offspring were analyzed. These results suggest that ToLCNDV lacks the capability to replicate in seedlings whether the virus reached the embryo of infected mother seeds. Similar behavior was observed when we evaluated commercial cucurbit seeds of zucchini (*Cucurbita pepo* L.), melon, cucumber (*Cucumis sativus* L.) and watermelon

(*Citrullus lanatus* T.). Our findings contribute to increasing knowledge in terms of ToLCNDV epidemiology, which result useful when is needed to establish protocols for healthy germplasm certification and to account for quarantine policies that aim for healthy plant preservation.

RESUMEN

Los virus emergentes que afectan a las plantas en la cuenca Mediterránea se han convertido en una grave amenaza para la producción agrícola de esta región. La propagación de nuevas enfermedades causadas por virus constituye uno de los principales factores que limitan los rendimientos agrícolas de las especies cultivadas de la familia *Cucurbitaceae*, la segunda en importancia después de la familia *Solanaceae*. Numerosos factores modulan y favorecen la aparición de estos patógenos, incluyendo factores biológicos, ecológicos, sociales y culturales. El calentamiento global favorece el movimiento de vectores hacia nuevas áreas, mientras que el comercio global de plantas y semillas permite la diseminación a larga distancia de los virus. Además, el modelo agrícola de producción intensiva, en el que predominan las variedades homogéneas genéticamente, promueve la aparición de patógenos en nuevas áreas geográficas a un ritmo acelerado. Uno de los aspectos clave para desarrollar estrategias de control, y garantizar la producción hortícola, consiste en identificar el tipo y la distribución de los virus presentes en los cultivos. Sin embargo, esta información es escasa y muy limitada en Argelia. El objetivo de esta Tesis Doctoral consistió en identificar y determinar la prevalencia y distribución de los virus más comunes en plantas cultivadas de cucurbitáceas (calabacín, pepino y melón), y obtener información sobre su variabilidad genética y modo de dispersión.

En este trabajo, se detectó por primera vez el virus de la clorosis amarilla de las cucurbitáceas (CCYV, cucurbit chlorotic yellows virus) infectando pepino y calabacín en Argelia. El CCYV (género *Crinivirus*, familia *Closteroviridae*) forma parte de un complejo de virus transmitidos por la mosca blanca *Bemisia tabaci*, que causan enfermedades de amarillamiento en las cucurbitáceas. Se obtuvo la secuencia completa del gen que codifica la proteína de la cápside (CP) y secuencias parciales de los genes RdRp y Hsp70 de un aislado argelino de CCYV y se compararon con las secuencias presentes en las bases de datos. El análisis filogenético agrupó al aislado de Argelia en el grupo I junto con la mayoría de aislados de CCYV. La variación genética de este grupo fue muy baja (identidad de nucleótidos superior al 99,5%), a pesar de su amplia y discontinua distribución geográfica, especies de plantas a las que infecta (cultivadas o malas hierbas) y año de recolección. La baja diversidad genética podría deberse a la reciente aparición de este virus, por lo que todavía no ha acumulado cambios importantes. En nuestro caso, la presencia de CCYV solo se detectó en infecciones mixtas con el virus de la hoja rizada del tomate de Nueva Delhi (ToLCNDV, tomato leaf curl New Delhi virus). En trabajos futuros, será interesante comprobar si existe una interacción

sinérgica o antagonista entre estos dos virus que pueda afectar a la propagación y al desarrollo de síntomas entre los cultivos.

ToLCNDV (género *Begomovirus*, familia *Geminiviridae*) también se detectó por primera vez infectando plantas de cucurbitáceas en Argelia. ToLCNDV es un begomovirus bipartito que causa importantes epidemias en cultivos económicamente valiosos de las familias *Solanaceae* y *Cucurbitaceae*. Se obtuvo la secuencia genómica completa de un aislado de ToLCNDV de pepino de Argelia (ToLCNDV-Biskra). La alineación de las secuencias de nucleótidos de los segmentos de ADN-A y ADN-B reveló identidades del 98,7% y 97,6%, respectivamente, con los segmentos de los aislados de la cuenca del Mediterráneo, mientras que la identidad con los aislados de Asia fue de aproximadamente del 90% y 81%. Los aislados presentes en la cuenca mediterránea son monofiléticos y forman un único grupo, mientras que los aislados de la cepa asiática presentan una mayor variabilidad genética y forman varios grupos. Todos los aislados de Argelia mostraron una variación de nucleótidos muy baja. Solo se observó una duplicación de 17 nucleótidos en el ADN-B de algunos aislados que daría lugar a una proteína de movimiento 53 aminoácidos más larga, aunque la funcionalidad de esta supuesta proteína es desconocida. A pesar de esa duplicación, no se detectaron eventos de recombinación entre los aislados secuenciados. La baja variación genética podría dificultar la detección de recombinantes.

La transmisión de virus a través de las semillas permite su dispersión y propagación a larga distancia. En estudios recientes se ha publicado que la transmisión de begomovirus a través de las semillas es posible en diferentes patosistemas, lo que ha generado un nuevo debate sobre este modo de transmisión. En esta Tesis Doctoral, se analizó cómo se distribuye ToLCNDV en hojas y flores de accesiones de melón (*Cucumis melo* L.) y calabaza (*Cucurbita moschata* D.). Además, se diseccionaron semillas procedentes de plantas infectadas y se comprobó que el virus está presente en las semillas de ambas especies. Sin embargo, en las plántulas germinadas a partir de semillas infectadas, no se observó una transmisión real del virus. Aunque se detectaron formas replicativas del virus en hojas, flores y semillas, la detección no fue posible cuando se analizaron las hojas de las plántulas descendientes. Estos resultados sugieren que ToLCNDV no es capaz de replicarse en las plántulas, incluso si el virus alcanza el embrión de las semillas. Se observó un comportamiento similar al evaluar semillas comerciales de cucurbitáceas como calabacín (*Cucurbita pepo* L.), melón, pepino (*Cucumis sativus* L.) y sandía (*Citrullus lanatus* T.). Estos hallazgos contribuyen a aumentar el conocimiento en términos de epidemiología del ToLCNDV. Esta información resulta

útil cuando se necesita establecer protocolos para la certificación de semillas y para implementar políticas de cuarentena que buscan preservar la sanidad vegetal.

RESUM

En les últimes dècades, les virosis emergents que afecten plantes en la conca del Mediterrani s'han convertit en una greu amenaça per a la producció agrícola d'aquesta regió. La propagació de noves malalties causades per virus s'ha convertit en un dels principals factors que limiten els rendiments agrícoles de les espècies cultivades de la família *Cucurbitaceae*, la segona en importància després de la família *Solanaceae*.

Un ampli conjunt d'elements modula i afavoreix l'aparició d'aquests patògens, incloent-hi factors biològics, ecològics, socials i culturals. L'escalfament global afavoreix el moviment de vectors cap a noves zones geogràfiques, mentre que el comerç global de plantes i llavors permet la disseminació a llarga distància dels virus. A més, el model agrícola intensiu, en el qual prevalen les cultivars genèticament homogènies, promou l'aparició de patògens en noves àrees geogràfiques a un ritme accelerat. Un dels components clau per a desenvolupar estratègies de control i garantir la producció hortícola és identificar el tipus i la distribució dels virus presents en els cultius. No obstant això, a Algèria aquesta informació és escassa i molt limitada. L'objectiu d'aquesta Tesi Doctoral va consistir a identificar i determinar la prevalença i distribució dels virus més comuns en plantes cultivades de cucurbitàcies (carabasseta, cogombre i meló), i obtindre informació sobre la seua variabilitat genètica i manera de dispersió.

En aquest treball, es va detectar per primera vegada el virus de la clorosi groga de les cucurbitàcies (CCYV, cucurbit chlorotic yellows virus) infectant cogombres i carabassetes a Algèria. El CCYV (gènere *Crinivirus*, família *Closteroviridae*) forma part d'un complex de virus transmesos per mosca blanca que causen malalties d'esgrogueïment en les cucurbitàcies. Es va realitzar la determinació de la seqüència completa de la proteïna de la càpsida (CP) i seqüències parcials dels gens RdRp i Hsp70 d'un aïllat algerià de CCYV i es van comparar amb les seqüències publicades en les bases de dades. L'anàlisi filogenètica va agrupar l'aïllat algerià en el grup I juntament amb la majoria dels aïllats de CCYV. La variació genètica d'aquest grup va ser molt baixa (identitat de nucleòtids superior al 99.5%), malgrat la seua àmplia i discontínua distribució geogràfica, espècies de plantes a les que infecta (cultivades o males herbes) i any de recol·lecció. Aquesta baixa diversitat genètica podria ser deguda a la recent aparició d'aquest virus, pel que encara no ha acumulat canvis importants. En el nostre cas, la presència de CCYV només es va detectar en infeccions mixtes amb el virus de de la fulla arrissada de la tomaca de Nova Delhi

(ToLCNDV , tomato leaf curl New Delhi virus). En treballs futurs, serà interessant verificar si existeix una interacció sinèrgica o antagonista entre aquests dos virus que puga afectar la propagació i el desenvolupament de símptomes entre els cultius.

ToLCNDV (gènere *Begomovirus*, família *Geminiviridae*) també va ser detectat per primera vegada infectant plantes de cucurbitàcies a Algèria. ToLCNDV és un begomovirus bipartit que causa importants epidèmies en cultius econòmicament valuosos de les famílies *Solanaceae* i *Cucurbitaceae*. Es va obtindre la seqüència genòmica completa d'un aïllat de ToLCNDV de cogombre d'Algèria (ToLCNDV-Biskra). La alineació de les seqüències de nucleòtids del segments d'ADN-A i ADN-B van mostrar identitats del 98.7% i 97.6%, respectivament, amb els segments corresponents dels aïllats de la conca del Mediterrani, mentre que la identitat amb els aïllats d'Àsia va ser d'aproximadament el 90% i 81%. Els aïllats presents a la conca del Mediterrani són monofilètics i formen un únic grup, mentre que els aïllats de la soca asiàtica presenten una major variabilitat genètica i s'agrupen en diversos grups. Tots els aïllats algerians van mostrar una variació de nucleòtids molt baixa. Només es va observar una duplicació de 17 nucleòtids en d'ADN-B d'alguns aïllats que lonaria lloc a una proteïna de moviment 53 aminoàcids més llarga, encara que la funcionalitat d´aquesta suposada proteïnaés desconeguda. A més, malgrat la duplicació, no es van detectar esdeveniments de recombinació entre els aïllats seqüenciats. La baixa variació genètica podria dificultar la detecció de recombinants.

La transmissió de virus a través de les llavors permet la seua dispersió i propagació a llarga distància. En estudis recents s'ha publicat que la transmissió de begomovirus a través de les llavors és possible en diferents patosistemes, la qual cosa ha generat un nou debat sobre aquesta manera de transmissió. En aquesta Tesi Doctoral, es va analitzar com es distribueix ToLCNDV en fulles i flors d'accessions de meló (*Cucumis melo* L.) i carabassa (*Cucurbita moschata* D.). A més, es van disseccionar llavors procedents de plantes infectades i es va comprovar que el virus estava present en les llavors de totes dues espècies. No obstant això, en les plàntules germinades a partir de llavors infectades, no es va observar una transmissió reial del virus. Encara que es van detectar formes replicatives del virus en fulles, flors i llavors, la detecció no va ser possible quan es van analitzar les fulles de les plàntules descendents. Aquests resultats suggereixen que ToLCNDV no és capaç de replicar-se en les plàntules, fins i tot si el virus va aconseguir l'embrió de les llavors mare infectades. Es va observar un comportament similar en avaluar llavors comercials de cucurbitàcies com ara carabasseta (*Cucurbita pepo* L.), meló, cogombre (*Cucumis sativus* L.) i meló d'Alger

(*Citrullus lanatus* T.). Les nostres troballes contribueixen a augmentar el coneixement en termes de l'epidemiologia de ToLCNDV. Aquest fet resulta útil quan es necessita establir protocols per a la certificació de llavors i per a implementar polítiques de quarantena que busquen preservar la salut de les plantes.

GENERAL INTRODUCTION

1. The family of *Cucurbitaceae*

1.1. Economic importance

The *Cucurbitaceae* family, known as "cucurbits", with 130 genera and 800 species make up one of the most diverse and remarkable botanical families in horticulture, due to their wide consumption and cultivation and for being adapted to different geographical regions around the world. Cucurbit species from at least 20 genera are grown for culinary purposes, medicinal applications and as ornamentals (Schaffer & Paris, 2016), about 150 species are cultivated expansively, and 30 of these are essential for the global food production (Rolnik & Olas, 2020; Wintermantel et al., 2019).

The cultivated species of this family generate high economic profits at a global level, they have great genetic variability in fruit and plant traits, as well as a valuable nutritional profile rich in carbohydrates, fiber, vitamins, minerals (calcium, iron, phosphorus, etc.) and antioxidants (lycopene, β-carotene, etc.). Among the most important crops within this family are melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.), zucchini and pumpkins (*Cucurbita* spp.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), with a world production in 2021 that exceeded 28, 93, 23 and 101 million tons respectively, according to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2021).

Although China leads the world production of cucurbits, the Mediterranean Basin is one of the main regions where these crops are cultivated. In 2018, at least 923,078 ha were dedicated to cucurbit crops in the Mediterranean countries, generating a production of around 27 million tons, almost 30% of the world production if Chinese harvests are excluded (FAOSTAT, 2018).

In Algeria, melons and watermelons are cultivated at different locations of the country, constituting the second and third most produced crops in Algeria, respectively, after potato. Productions of both crops are mainly concentrated in the western regions of the country (Ain defla, Tlemcen, Sidi bel abbas), in extensive open-field plots. However, in the southeast regions of Algeria (Biskra, El oued and Ouargla), melon and watermelons are produced in both outdoor and under greenhouses. Instead, most of the Algerian production of cucumber, zucchini and pumpkin comes from protected cultivations in the regions of Biskra, Mostaganem and Tipaza (MADR, 2021). Within *Cucurbita* genera, Zucchini (*C. pepo*) production stands out, whose cultivation has

prevailed over that of pumpkins (*C. moschata*) in the country. Most of the production of cucurbits in Algeria is carried out in the region of Biskra, which is known as Algeria's vegetable garden providing 40% of national agricultural production. In addition to its economic and commercial importance, the production of these four crops has a high social impact due to its ability to generate employment, concentrating a large workforce within the agricultural sector (Bessaoud et al., 2019).

1.2. Main limitations of *Cucurbitaceae* cultivation

Cucurbits crops faces adverse disease and insect pressures, besides climate changes and water-related issues, that limits the profitability of the crops and engender critical environmental consequences (Grumet et al., 2021). One of the major aspects that have changed the perspectives of agriculture nowadays is the global development of new growing and production practices. Some examples are the indoors cultivations (greenhouses, tunnel farming), the digitalization of agricultural exploitations or the use of commercial hybrid seeds, which allow several cultivation cycles to be carried out in the same season by increasing yields and reducing inputs (Pitrat, 2012; Singh et al., 2021). Regardless of the quality and cost-effective benefits that have generated these new technologies, the negative consequences are numerous. In fact, this global exchange of techniques and plant materials, in addition to the dominance of monoculture, have raised new challenges in the agricultural production of cucurbits (Lecoq & Katis, 2014).

Moreover, the changing climate of the planet presents a major constraint to crop production, as to the relative incidence and impacts of insect pests, plant pathogens and weeds are relatively influenced by climatic and environmental conditions (Oerke, 2006; Juroszek & Von Tiedemann, 2000). It was proven that global warming, the disproportional ratio of precipitations and the elevation in the CO_2 and UV-B level have adverse effects on plant physiology and resistance, that occasionally, leads to plant disease epidemics and outbreaks of pests. To mitigate the effects occasioned by these complex biological interactions, integrated solutions and international coordination are required to ensure the global food security (Bastas, 2022; Chakraborty & Newton, 2011; Ghini et al., 2012).

1.2.1 Pests

According to Boote et al., (1983), pests (Table 1) and diseases play a crucial role in the decline of crop productivity in various manners that can be classified into different categories:

- **Stand reducers**: which are also known as damping-of fungi.
- **Photosynthetic rate reducers:** (fungi, bacteria, viruses) that directly affect the rate of carbon uptake in the plant host tissues.
- **Leaf senescence accelerators:** (pathogens) remove leaf and petiole mass and thereby reduce light interception.
- **Light stealers:** (weeds, some leaf pathogens) by inducing necrotic lesions that interfere with photosynthesis.
- **Assimilate sappers:** (nematodes, pathogens, sucking arthropods) that remove soluble assimilate from host cells.
- **Tissue consumers:** (chewing insects, necrotrophic pathogens) that feed on the plant tissue (foliage, roots, reproductive tissues).
- **Turgor reducers:** are root feeders such as nematodes that affect plant water balance.

In addition to weeds that trigger competition with the crop plants on inorganic nutrients, in detriment to the productivity.

Table 1. The most devastating pests that affect *Cucurbitaceae* (Brown, 2015; Messelink et al., 2020)

Pests	Scientific names	Additional information
Spider mites/ Two-spotted mites	*Tetranychus urticae (*Koch) *Oligonychus mexicanus (*McGregor and Ortega)	- Feed on the underside of the leaves. - Cause mottled leaves that can prematurely fall from the plant.
Whitefly	*Bemisia tabaci* (Gennadius) *Trialeurodes vaporariorum* (Westwood)	- Phloem feeding insects efficient in transmitting viruses. - Excrete honeydew that attract sooty mold fungi that reduce photosynthesis capacity of the plant.
Aphids	*Aphis gossypii* (Glover) *Myzus persicae* (Sulzer)	- Cause leaf and tip curl, distortion, and premature death of the leaf. - Intense secretion of honeydew, which are a substrate for sooty mold fungi that contaminate fruit and leaves.
Thrips	*Frankliniella occidentalis* (Pergande) *Thrips palmi* (Karny) *Frankliniella occidentalis* (Pergande) *Thrips palmi*	- Reduce production by feeding on the plant. - Act as transmission vectors of some viruses.
Caterpillars	*Chrysodeixis chacites* (Esper) *Chrysodeixis includens* (Walker) *Helicoverpa assulta* (Guenée) *Spodoptera exigua* (Hübner) *Trichoplusia ni* (Hübner)	- Feed on leaves and/or fruits.
Nematodes	*Meloidogyne* spp. *Xiphinema americanum* (Cobb) *Xiphinema rivesi* (Dalmaso)	- Cause disease-like symptoms by feeding on the roots of plants.

1.2.2. Fungal diseases

Fungal diseases cause the greatest losses in crops. The most common fungal diseases that severely affect cucurbits are:

- Powdery Mildew: (*Podosphaera xanthii* U. Braun and N. Shish. and *Golovinomyces orontii* Heluta)
- Downy Mildew: *Pseudoperonospora cubensis* (Berk. & Curt.) Rost
- Fruit rot fungus: *Phytophthora capsici Leonian*
- Didymella: *Didymella bryoniae* (Auersw.) Rehm
- Alternaria Leaf Blight: *Alternaria cucumerina* (Ellis & Everh.) J. A. Elliot
- Anthracnose: *Colletotrichum orbiculare* Damm, P.F. Cannon & Crous
- Mycosphaerella: *Mycosphaerella citrullina* (C.O. Smith) Grossenbacher
- Fusariosis: (*Fusarium oxysporum f. sp. melonis* (Leach & Currence) W.C. Snyder & H.N. Hansen; *F. oxysporumf. sp. niveum* (E.F. Smith) W.C. Snyder & H.N. Hansen; *F. oxysporumf. sp. Cucumerinum* J.H. Owen; *F. solani (*Mart.*) Sacc. f.sp. cucurbitae* W.C. Snyder and H.N. Hansen). Fusarium wilt engender devastating damages in cucurbits by causing lesions in the root and neck that can lead to the collapse and death of the plant (Egel et al., 2022; González et al., 2020).

However, powdery mildew is the most prevalent foliar fungal disease in cucurbit crops cultivated in both greenhouses and open fields. Powdery Mildew affects the correct development of the plant, severely affecting the quality of the fruit and causing yield losses. Nonetheless, several studies have reported genetic resistance against this disease in several species of cucurbits (He et al., 2013; Wang et al., 2016; Shnaider et al., 2022; López-Martín et al., 2022).

1.2.3. Viruses affecting the *Cucurbitaceae* crops

Viruses represent the second major biological threats for cucurbit production, as they can affect both yield and quality of crops. In natural conditions, more than 90 viruses have been identified infecting cucurbits. Viruses cause a variety of symptoms, which severity may be increased by mixed infections of multiple viruses in a single plant. Cucurbit infecting viruses are portrayed as a

highly dynamic system. Each year, new viral species initially found in other hosts are discovered to infect cucurbits, in addition to their expansion to new locations or new hosts for prevalent viruses (Desbiez, 2022).

Globally, the most important viruses are watermelon mosaic virus (WMV), papaya ringspot virus (PRSV), zucchini yellow mosaic virus (ZYMV) and cucumber mosaic virus (CMV), which can lead to complete crop losses and a significant decrease in the commercial value of production (Pozzi et al., 2020). Other viruses have a more limited geographic distribution, such as squash vein yellowing virus (SqVYV) or melon yellow spot virus (MYSV), which have less economic impact (Chikh-Ali et al., 2019; Hervé Lecoq & Katis, 2014). The number of viral species described as infecting cucurbits continue rising rapidly: around 20 species were described in 1980 (Lovisolo, 1980), 35 in 1998, 59 in 2012 (Lecoq and Desbiez, 2012), and more than 90 in 2019 (Desbiez, 2022).

Hemipteroid insects are the main vectors for plant viruses, including phloem-feeding aphids, whiteflies, plant hoppers, and leafhopper (Fiallo-Olivé et al., 2020), and they are considered a serious threat due to their high destructive capacity in these horticultural crops. In some cases, other types of transmission also generate significant economic losses in cucurbits. Thus, viruses of the *Tobamovirus* genus (*Virgaviridae* family) are transmitted by direct contact or by seeds (Cheng et al., 2018). And among them, the cucumber mottled green mosaic virus (CGMMV) has led to serious losses in watermelon crops in different parts of the world, due to the exchange of contaminated seeds or rootstocks between countries (Boubourakas et al., 2004).

1.2.3.1. Virus transmission by aphids

In nature, plant viruses often need a biological vector for horizontal propagation. Insects are the most prevalent and significant biological carriers of plant viruses, that enable them to spread by one of three ways, depending on the lowest time needed for acquisition and the maximum duration of retention of the virions (Ng & Falk, 2006). Non-persistent and semi-persistent viruses are typically transmitted via the stylet and the foregut, respectively. Whereas, persistent viruses travel from the foregut to the mid- and hindgut, where they are transported to the hemolymph and the most distant salivary glands before being released into plant tissues, persistent viruses either replicate (propagative) or do not replicate (circulative) in the insect vector (Fiallo-Olivé et al.,

2020). Among the viruses transmitted by aphids in a non-persistent manner, those of the genus *Potyvirus* (family *Potyviridae*) are widely distributed over the world and affect a broad range of species. There are at least 20 different species of potyviruses that have been described to infect cucurbit crops (Desbiez, 2022; Lecoq & Desbiez, 2012; Perotto et al., 2018). Viruses such as WMV, ZYMV and PRSV generate devastating epidemics in the main economic crops of cucurbits, due to their genetic diversification rate (Desbiez et al., 2007; Kaldis et al., 2018; Lecoq et al., 1998). CMV is another common and widespread virus belonging to the genus *Cucumovirus* (family *Bromoviridae*), symptoms typically include mosaics and deformations of the leaves and fruits of cucurbit plants, the virus has been described worldwide and is known to have the largest host ranges. CMV is transmitted in a non-persistent manner by more than 60 aphid species (Desbiez, 2022; Jacquemond, 2012).

In contrast, aphids can also transmit viruses to cucurbits in a persistent manner, such as cucurbit aphid-borne yellows virus (CABYV) derived from the genus *Polerovirus* (family *Luteoviridae*). CABYV was first described infecting cucurbit crops outdoors in France (Lecoq et al., 1992). Subsequently, the disease has been described in cucurbits on a global scale, affecting the yield of infected plants, but having no effect on fruit quality (Lecoq & Katis, 2014; Pitrat, 2012).

1.2.3.2. Virus transmission by whiteflies

Regardless of the damaging effects that whiteflies cause when feeding on plants, the main concern is their ability to transmit viruses. The most important viruses transmitted by whiteflies on cucurbits belong to the next genera:

- ***Begomovirus*** (family *Geminiviridae*) are persistently transmitted by whiteflies from the *Bemisia tabaci* complex. Virus acquisition occurs within several hours of insect feeding, and the insects remain virulent for the rest of their lives. Begomoviruses are a group of plant viruses that have appeared in recent decades as major threats to the production of many vegetables, root, and fiber crops throughout the world's tropical, subtropical, and temperate zones (Navas-Castillo et al., 2011). In cucurbits, the begomoviruses identified so far have a bipartite genome (Luria et al., 2019; Rojas et al., 2018).
- ***Ipomovirus*** (family *Potyviridae*) are transmitted by whiteflies in a semi-persistent manner; their acquisition and transmission take a few hours. Three ipomoviruses are known to infect cucurbits: cucumber vein yellowing virus (CVYV), squash vein yellowing virus (SqVYV)

and coccinia mottle virus (CocMoV) (Baker et al., 2008; Egel et al., 2022; Louro et al., 2004).

- ***Crinivirus*** (family *Closteroviridae*) are single-stranded bipartite RNA viruses that have a filamentous flexuous particle. They are transmitted by whiteflies in a semi-persistent manner. Four criniviruses have been described infecting cucurbits: beet pseudo-yellows virus (BPYV), lettuce infectious yellows virus (LIYV), cucurbit yellow stunting disorder virus (CYSDV) and cucurbit chlorotic yellows virus (CCYV). CYSDV has been the most destructive to cultivated cucurbits worldwide (Abrahamian & Abou-Jawdah., 2014; Orfanidou et al., 2019).

1.2.3.3. Virus transmission by contact, seeds, and soil

Other viral species do not require a specific biotic vector, but rather they are transmitted by contact on surfaces (in plant debris, water) that remain infectious for long period. For instance, *Tobamovirus* genera includes positive-sense single-stranded RNA viruses that infect a wide range of plant species and are mechanically transmited. They were the first plant viruses to be studied, well known for their high stability when infecting plants, which enables them to disseminate efficiently and are difficult to control (Dombrovsky et al., 2017). The most important tobamovirus that infect cucurbits is CGMMV, which is seed-transmited and was firstly described in England in 1935 (Dombrovsky et al., 2017). Other viruses such as squash mosaic virus (SqMV, genus *Comovirus*) are widely distributed due to their seed transmissibility (Lecoq et al., 1998). Melon rugose mosaic virus (MRMV, genus *Tymovirus*) is seed transmitted in melon, and is restricted to the Red Sea region (Mahgoub et al., 1997). Melon necrotic spot (MNSV, genus *Carmovirus*) is soil-transmitted in the presence of the soil fungus *Olpidium bornovanus*. Also several *Nepovirus* are transmitted by soil nematodes and infect cucurbits, however they have a limited geographic distribution with low agronomic impact (Desbiez, 2022; Lecoq et al., 1998).

1.2.4. Mixed infections of viruses in *Cucurbitaceae*

The current knowledge in the within-host viral population dynamics has been principally studying single viral infections, either in experimental conditions or by reviewing and analyzing a virus population when outbreaks happen (Acosta-Leal et al., 2011; Alcaide et al., 2020). Thus, the

interaction among viruses and their effects on genetic diversity and evolution of viral populations is still misunderstood. Co-infections in a plant host with different viruses is a common phenomenon that occur in nature (Moreno & López-Moya, 2020; Syller, 2012). It can be a simultaneous infection of several viruses or subsequent infection of one virus following another, where viruses can be different strains of the same viral species or from completely different species (Mcleish et al., 2019).

In mixed infections, within-host viruses can interact with each other in different manners. Neutralism is when the viruses infecting the plant do not interfere with the replication, accumulation, and transmission of each other. However, this neutral interaction might develop to antagonism, which happen when a virus decreases the infection or accumulation of another virus, or to synergism where symptom expression is enhanced, and virus accumulation levels are increased due to the co-infection (Alcaide et al., 2020; Syller, 2020). Synergic interactions are widely found in host plants infected with a combination of potyviruses and viruses from other genera, leading to a competitive dynamic between them within the plant host. For instance, recent studies show that the presence of potyviruses in mixed infections is highly prevalent in cucurbits crops (Khanal et al., 2021).

In cucurbits, the synergistic effect of mixed infections between viruses of different genera have been widely studied. Taking for instance the co-infection of CMV and two potyviruses (ZYMV and WMV), which has been characterized in melon, zucchini, watermelon, and squash (*C. maxima* Duchesne), a change in the concentration levels of these viruses in the plant was observed (Barbosa et al., 2016; Zhu et al., 2019). In another report, it has been found that co-infections with CVYV enhance the titer of CYSDV in cucumber (Gil-Salas et al., 2012). Viral co-infections may lead to the intensification of symptoms, alter viral titers, and produce novel recombinant strains (Syller, 2012). Commercially grown cucurbits have been reported to have mixed CYSDV and CCYV infections (Abrahamian et al., 2012). Moreover, because they are two closely related cucurbit-infecting criniviruses with similar symptomology, single and multiplex RT-PCR were developed revealing that CYSDV and CCYV titers decreased in double infected plants (Abrahamian et al., 2013). Furthermore, Abrahamian et al., (2015) determined an increasement in the severity of symptoms in cucumber after mixed infections of criniviruses and begomoviruses. In another report, the prevalence of mixed infections in cucurbits was found to be high, with the majority of the plants examined carrying at least two viruses, mostly of CCYV and CYSDV (Kavalappara, et al., 2021).

As viruses have the readiness to move easily from reservoir hosts to new hosts and to adapt to new conditions easily, when a new plant is introduced into a new environment, their associated viruses are prone to move to neighboring hosts, which leads to mixed infections either through physical contact and/or vectors (Singhal et al., 2021). Taking the example of pepino mosaic virus (PepMV), which was originally found in South America infecting pepino and then detected in tomato crops of a greenhouse in Netherlands in 1999, eventually, the virus has evolved and differentiated to distinct strains engendering mixed infections, which created a severe outbreak in Europe (Rojas & Gilbertson, 2008). More recently, Domingo-Calap et al., (2021) have described how co-infection of CYSDV and WMV in melon benefits both viruses, favoring and maintaining their transmission.

1.2.5. Incidence and virus control in *Cucurbitaceae*

Emerging cucurbit viruses have increased in recent years, on one hand, due to the rapid dissemination of their natural vectors, and on the other, to the improvement in diagnostic methods that have allowed their identification (Lecoq & Desbiez, 2012). Insects are the main vector of emerging viruses, and whiteflies stands out among them (Fiallo-Olivé et al., 2020). The increase in whitefly populations in recent decades is correlated with the development of resistance to pesticides and with global warming, which favors the migration of insects to new warm areas of the planet (Navas-Castillo et al., 2011). The intensification of agriculture and the trend towards the simplification of ecosystems also favor outbreaks of new viruses, facilitated by the introduction of viruses and exotic pests through trade of different plant materials (Navas-Castillo et al., 2011). Cucurbits require specific climatic conditions for their cultivation. However, the increasingly widespread use of hybrid seeds and the prolongation of the warm seasons make it possible to continue cultivation during the year in a greenhouse or in extensive cultivation in the open air, favoring survival and overlap of generations of virus-transmitting vectors (Panno et al., 2019; Sagar et al., 2020).

In spite of the large diversity of plant viruses and their modes of action and transmission, control measures are the same for most of them, relying primarily on cultural practices and in the use of host genetic resistance if available (Lecoq & Katis, 2014). Viral diseases cannot be tackled by chemical treatments, as it is the case with pests, fungi or bacteria, which makes it difficult for them to spread (Loebenstein & Katis, 2014; Messelink et al., 2020).

According to Desbiez et al., (2020), good cultural practices involve: 1) limiting virus sources and virus vectors in the cultivated plants and their surroundings by the removal of weeds before planting, which helps diminishing primary virus inoculum in crops; 2) avoid overlapping crops to reduce the infection of younger plantlets; 3) production of seeds in regions where seed-borne viruses are not widespread; or 4) growing mother plants in protected conditions. Monitoring and analyzing seed lots using serological or molecular testing is primordial to limit virus dissemination. Virus eradication for persistent seed borne viruses such as CGMMV, using chemical or heat treatment. Restricting Long-distance movements of plant material to minimize the chances of virus circulation.

Moreover, effective control of viruses transmitted by insect vectors needs to be established using insect-proof nets that are more efficient in greenhouses, whereas in field, insecticides or biological controls of the vectors are commonly used (Lecoq & Katis, 2014). The application of insecticides is generally found to be inefficient in limiting the spread of non-persistent aphid-transmitted viruses (Perring et al., 1999). Biocontrol methods are gaining progress and provided noticeable results in the reduction of virus propagation (Messelink et al., 2008).

However, the most practical way to combat viral diseases remains the use of cultivars that are genetically resistant to viruses (Lefebvre et al., 2020; Martín-Hernández & Picó, 2020). This might be conferred through different practices, the conventional methods includes; grafting desired susceptible cultivar onto a virus-resistant rootstock against soil-borne viruses in commercial cucurbits (Cohen et al., 2007; Huitrón-Ramírez et al., 2009; Louws et al., 2010). Cross-protection, is a reverse method that consist of inoculating a virus strain with mild symptoms into the plants to protect them against severe strains of the same virus, this method was applied in several commercials crops against severe viruses like ZYMV, WMV, CMV, CGMMV, PRSV (Lecoq & Katis, 2014). Nonetheless, the use of genetically resistant varieties against viruses is the simplest, most efficient, form of control to combat viral diseases in horticultural crops in addition to the economic and ecological properties of this technology (Lecoq et al., 1998; Lecoq & Katis, 2014).

1.2.6. Virus evolution in cucurbit crops

Plant viruses are well known for their rapid multiplication and their ability to generate variants through mutation, which is the primary factor that influence genetic diversity (Figure 1) (Domingo

et al., 1996; Duffy et al., 2008; Escriu, 2017). RNA viruses are prominent to have the highest mutation rate due to the lack of proofreading activity of their RNA polymerases (Acosta-Leal et al., 2011). One of the forms of evolution is natural selection from which fewer fit variants are selected by decreasing their frequency in the population, which is also called negative selection. In the other hand, positive selection will act on the increasement of the frequency of the fittest variant that will carry genetic traits that will adapt to the new conditions set (Chare & Holmes, 2006; Rubio et al., 2020; Schneider & Roossinck, 2001). Genetic draft is also a selective phenomenon that produce changes in the frequency of a gene variant in a population due to random sampling of haplotypes, which will eventually cause a change in the genetic structure of a population (Escriu, 2004). Gene flow is another factor that need to be considered in genetic variation of population, it is described as the migration between viral populations from distinct geographic areas, as high migration rate promotes genetic homogeneity among populations and decrease global genetic diversity (Escriu, 2004; Rubio et al., 2020).

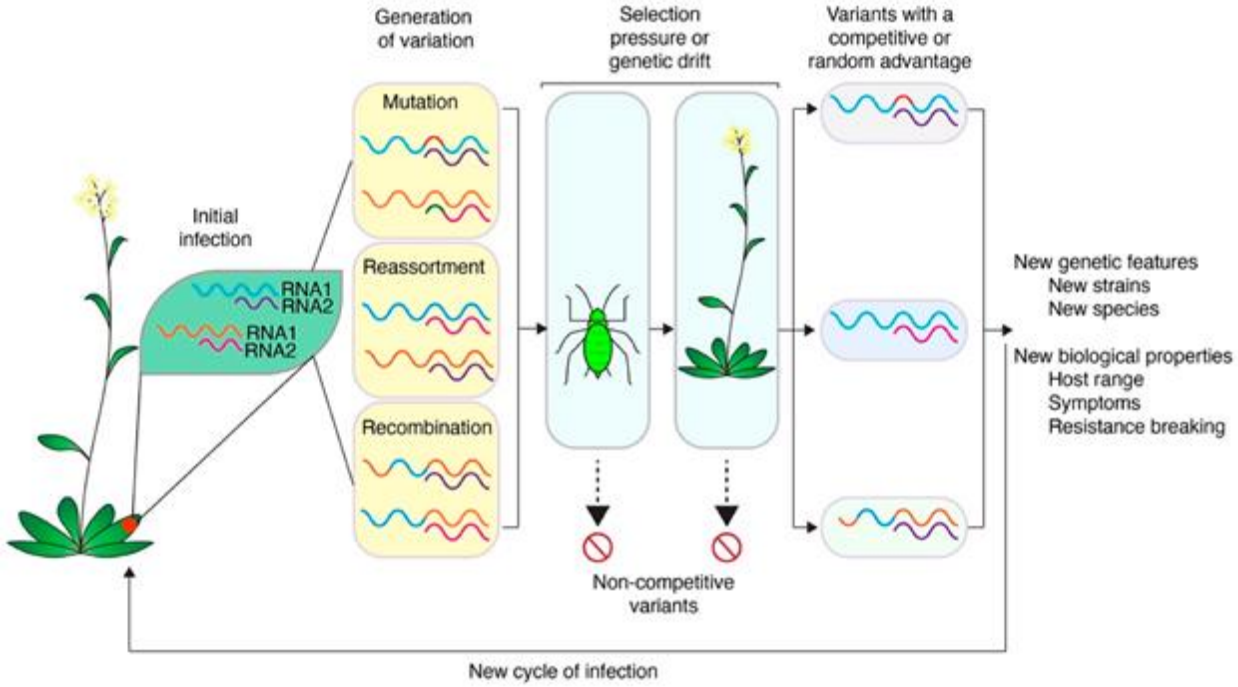

Figure 1. Mutation, reassortment, and recombination all result in the creation of genetic variants. Following infection, multiple genomes coexist within the same host and can mutate, reassort, or recombine. These variants are subject to genetic drift and selective pressure from host and vector factors. Random or competing variants can be inherited, become established within populations, and emerge as new strains or species with new biological properties. Retrieved from LaTourrette & García-Ruiz (2022).

In contrast, recombination may occur when there is an exchange of genetic information between distinct variants infecting the same cell, from which segments of nucleotide strands shuffle between them, creating mosaic genomes, this event happens mostly during mixed infection in a plant host (Figure 1) (Escriu, 2017; García-Arenal et al., 2001; Posada et al., 2003; Rubio et al., 2020). Previous studies have described recombination in the evolution of plant viruses (Díaz-Pendón et al., 2019; Ferriol et al., 2014; Kraberger et al., 2015; Rubio et al., 2013). Reassortment or also called pseudo-recombination differs from recombination, whereby entire components of multipartite viruses that are the most common genome structure found in nature, are exchanged between two related viruses (Chen et al., 2009; LaTourrette & García-Ruiz, 2022; Lee et al., 2018). Reassortment also includes the association of viral satellite components with other viral genomes (Briddon et al., 2002).

From an evolutionary perspective, viral diversity should be studied at the population level rather than the individual level (García-Arenal et al., 2001). In that matter, changes in population size may have a great impact on genetic variation. Genetic bottleneck causes severe reduction in population size, decreasing genetic polymorphism of the population, which may halter the transmissibility of the fittest variants due to the small population size (Escriu, 2004). These bottleneck events can also lead to the accumulation of deleterious mutations and consequently fitness losses, this phenomenon is called the "Muller's ratchet" (Iglesia & Elena, 2007; Simon-Loriere & Holmes, 2011). Vector transmission, environmental conditions, hosts susceptibility and the spread of the virus within individual plants and genome integration are involved in the decrease of virus genetic diversity (Acosta-Leal et al., 2011; Escriu, 2017). "The founder effect" is also a type of genetic drift entailing that a new population is started from a small number of variants selected randomly from the original population, resulting in a low genetic diversity within a population that originated from a larger diversified population (Elena et al., 2014; LaTourrette & García-Ruiz, 2022). All these factors mentioned above play crucial roles in shaping the genetic structure, the fitness and the virulence of viruses between populations, which may lead to the extinction or the emergence of viruses (LaTourrette & García-Ruiz, 2022).

In viruses affecting cucurbits, recombination events were reported. For CABYV, a new recombinant CABYV-M1strain has appeared and spread in Brazil. CABYV-M1 was incompetent to infect several cucurbits (*C. lanatus*, and *C. sativus*), due to a recombination event that have triggered the change in vector transmission to the whitefly *Bemisia tabaci* MEAM1 rather than its

previous vector *A. gossipii*. The recombination has also conferred resistance to a melon strain (*C. melo* 'TGR 1551) that has been reported to be resistant to common CABYV, ultimately the virus was renamed "cucurbit whitefly borne yellow virus" (Costa et al., 2019; 2020). Recombination and reassortment between subgroups of CMV (IA, IB and II) were detected under natural conditions in several reports (Ben Tamarzizt et al., 2013; Hasiów-Jaroszewska et al., 2017; Ouedraogo et al., 2019; Pita et al., 2015). Since 1999, new strains of WMV were found to be the direct consequence of recombination (Desbiez et al., 2011). It was also suggested by Fortes et al., (2016) that begomoviruses are prone to recombination, suggesting that the occurrence of tomato leaf curl New Delhi virus (ToLCNDV) and tomato yellow leaf curl disease (TYLCD)-associated viruses in mixed infection represent a plausible danger to both tomato and cucurbits crops. A virulent pseudo-recombinant was also reported, between two distinct species of begomoviruses; ToLCNDV and tomato leaf curl Gujarat virus (TLCD) infecting tomato in India (Chakraborty et al., 2008). The study of genetic variability and virus evolution mechanism are primordial to understand virus epidemiology for a better detection and characterization of emergent viruses with the purpose to develop efficient and durable virus control schemes (Acosta-Leal et al., 2011; Rubio et al., 2013).

2. Diagnostic methods for identification and detection of plant viruses

Many diagnostic procedures with improved sensitivity and specificity for the detection of common and/or unidentified plant viruses are continuously being developed because of advances in biochemical and molecular biology techniques (Mehetre et al., 2021). Several diagnostics approaches have been commercialized. Yet, the actual implementation of each widely used technique depends on a number of variables, including the cost, sensitivity, speed, instrumentation, and disease stage (Martinelli et al., 2015). A rapid and accurate diagnosis of the underlying cause is crucial for disease management. When a virus infects a plant sample, diagnosis involves placing it in a category of viruses with similar characteristics, known as a species. However, the specificity of the diagnosis can also extend to lower taxonomic levels, such as strain or variant, or higher taxonomic ones, such as genus or family (Rubio et al., 2020).

2.1. Serological detection methods

Enzyme-linked Immunosorbent Assays (ELISA) are frequently used in the diagnosis of plant viruses, they are based on the specific interaction of viral proteins with antibodies (Clark & Adams, 1977). These tests have the capacity to measure the pathogen biomass in plant tissues and other matrices with excellent sensitivity, simplicity of use, and rapidity (Mehetre et al., 2021). ELISA-based methods, such as direct tissue blot immunoassay (DTBIA), double antibody sandwich (DAS) ELISA, and tissue-print (TP) ELISA are the most widely used method for identifying viruses. Other ELISA methods were developed like the plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA), which was used in detecting several plant viruses, such as the squash mosaic virus (SQMV), cowpea severe mosaic virus (CPSMV), CMV, cowpea aphid borne mosaic virus (CABMV), ZYMV, and papaya lethal yellowing virus (PLYV) (Nascimento et al., 2017). The development of Lateral Flow Assay (LFA) is an immunochromatographic assay (ICA) performed for the early and fast detection of viral infections, in which plant extracts are dropped on a chromatographic test strip and move capillary. When virions bound with labeled antibodies or viral nucleic acids with labeled DNA or RNA probes, a stained band appears, indicating the presence of the virus (Carter & Cary, 2007; Koczula & Gallotta, 2016). LFA has facilitated the use of virus diagnostics immediately in the field by keeping track of viruses in seeds and plant materials (Byzova et al., 2009; Hema & Konakalla, 2021; Mehetre et al., 2021).

2.2. Molecular identification methods

2.2.1. Detection method based on molecular hybridization

The basis of nucleic acid hybridization tests is complementarity between the base pairs of the target pathogen's nucleic acid sequence that needs to be detected and the suitably labeled probe with a complementary sequence. Attached markers based on fluorescent dyes, enzymes that produce colorimetric or chemiluminescent reactions, radioactivity, or other substances are employed to visualize these binding events (Hema & Konakalla, 2021). In hybridization assays, probes are usually labeled with either a radioactive (^{32}P) or nonradioactive (biotin/digoxigenin) reporter group. Probes can be synthesized from DNA (DNA probes) or RNA molecules (riboprobes). RNA probes are created via *in vitro* transcription, whereas DNA probes can be labeled using nick translation, random primer labeling, or PCR (Pallás et al., 2018). As majority of

plant viruses have RNA as their genome, RNA probes are frequently employed. RNA:RNA hybrids are more stable than RNA:DNA or DNA:DNA hybrids. Use of highly stringent hybridization conditions is possible because of the stability of RNA:RNA hybrids, resulting in enhancement of probe specificity and reduction in background problems due to interference of plant sap. However, the main drawbacks of RNA probes are their expensive cost and risks of degradation due to RNase contamination during hybridization (Hema & Konakalla, 2021). There are several types of hybridization methods such as the dot-blot, slot-blot and squash-blot hybridization assay (Naidu & Hughes, 2001).

Even though it is regarded to be a relatively accurate and reliable method, molecular hybridization has some flaws. For instance, in some pathogen/host combinations, erroneous hybridization signals may be seen as a result of host RNAs that share sequence similarities with the plant virus or viroid that is expected to be identified (Pallás et al., 2018). By incubating the membranes with RNase at a high ionic strength after hybridization, the potential false positives can be eliminated (Pallás et al., 2018). Yet, it is interesting to point out that molecular hybridization can readily be adjusted to simultaneously detect multiple pathogens in a single hybridization assay (James et al., 2006). Polyvalent assay using a single polyprobe to detect several combinations of plant viruses and/or viroids that damage various crops has revolutionized molecular hybridization (Aparicio et al., 2009; Herranz et al., 2005; Sánchez-Navarro et al., 2018).

2.2.2. Detection method based on PCR

One of the most fundamental techniques for molecular detection of several infections is polymerase chain reaction (PCR). The PCR-based analysis of viral detection has undergone substantial improvements (Rubio et al., 2020). In fact, the sensitivity and effectiveness of viral detection are greatly increased by PCR, which can duplicate a single DNA strand up to 10^9-fold after multiple amplification cycles, and these copies are typically seen by electrophoresis or by hybridization with fluorescent probes. PCR can use complementary DNA produced by reverse transcription (RT) of viral RNA or genomic DNA as a template. Because it can identify viruses at low titer or concentration levels, RT-PCR is the most popular technique for diagnosing plant viruses (Mekuria et al., 2003; Olmos et al., 2007). By calculating the DNA concentration following each amplification step in the PCR process, the qPCR, also known as real-time PCR, can determine the viral titer level in the samples, it can also greatly shorten the detection time, enabling diagnosis

even when the target gene concentration is low (Jeong et al., 2014; Taylor et al., 2010). Monitoring accumulation of amplicon in real-time PCR is possible by labeling primers, probes (TaqMan technologies), and amplicon with fluorophore molecules (SYBR Green, Taqman probes, molecular beacons, or the minor groove binding [MGB] probes). Real-time PCR technology additionally offers reliable evidence by accurately distinguishing between closely related organisms and is thus used to detect a wide range of plant pathogens, including viruses and viroids (Martinelli et al., 2015).

Significant advances in molecular methods involving the implementation of multiplex detection methods have become cost-effective in the last decade, as multiple viruses can be detected with increased efficiency. Multiplex PCR (for DNA viruses) and multiplex RT-PCR (for RNA viruses) have been utilized successfully to detect up to nine pathogens simultaneously in a single assay (Pallás et al., 2018). The sensitivity of this technique, however, is influenced by the number of targets to be detected, primarily due to the number of different primer pairs present in the cocktail rather than the total amount of primers (Sánchez-Navarro et al., 2005). Some of these limitations have been overcome by improving the quality of the nucleic acid extraction procedures, the products obtained, and/or the PCR different parameters, magnetic nano beads, dual priming oligonucleotide (DPO) primers, and nested reactions have all increased specificity and sensitivity (Hema & Konakalla, 2021; Pallás et al., 2018).

2.3. Detection method based on isothermal amplification

The first isothermal amplification method developed was rolling circle amplification (RCA) that amplify circular DNA by binding to a Phi29 DNA polymerase with strand displacement activity (Fire & Xu, 1995). RCA is a widely used method mainly to diagnose small DNA genomes such as geminiviruses, nanoviruses and associated satellite DNAs (Ivanov et al., 2021; Jeske, 2018).

The loop-mediated isothermal amplification (LAMP) technique is a well-established diagnostic method based on auto-cycling and high DNA strand displacement activity induced by Bst polymerase from *Geobacillus stearothermophilus* under isothermal temperature ranging from 60°C to 65°C (Panno et al., 2020). The technique is highly sensitive and cost-effective. Plant viruses can be easily diagnosed in field using this method. LAMP amplification demands only an isothermal condition, removing the need for specialized thermal cycler device. Though, to ensure

the specificity of the LAMP amplification reaction, a set of four or six specifically designed primers are used to recognize six different sections of the target gene (Martinelli et al., 2015; Peng et al., 2021). LAMP technique has been widely used to detect prominent DNA viruses, such as *Squash Leaf Curl Virus* (SLCV) (Kuan et al., 2010). in RNA viruses including CCYV (Okuda et al., 2015), CMV (Bhat et al., 2013) ZYMV (Kuan et al., 2014), and CGMMV (Li et al., 2013). Similarly, Recombinase Polymerase Amplification (RPA) is a portable real-time assay for rapid isothermal detection of nucleic acids in plants (Zhang et al., 2014).

2.4. Detection methods based on High-Throughput Sequencing tools

High throughput sequencing (HTS) analysis of viral populations in plant samples has become a standard method for detecting and identifying plant viruses and viroid's introduced into an agroecosystem, as well as verifying the virus-free status of planting materials and screening germplasm collections for virus diversity and detection of latent viruses (Kavalappara et al., 2021; Zhou et al., 2022). Next-generation sequencing (NGS), also referred to as massively parallel sequencing or deep sequencing, is a powerful sequencing technology that is highly sensitive and has the potential to detect the full spectrum of viruses infecting a given host, including known and unknown viruses (Hema & Konakalla, 2021; Pallás et al., 2018). It allows the generation of large amounts of sequencing data (up to three billion reads per run) with read lengths varying between 35 to 800 nucleotides depending on the platform (Rott et al., 2017). NGS-based techniques share a few common processes, such as total nucleic acid (DNA or RNA) extraction from infected host plants samples followed by DNA fragmentation for library preparation. By incorporating a set of synthetic DNA adapters and primers to the fragmented DNA, different sequencing chemistries and platforms can be used for analysis (Mehetre et al., 2021). The development of single-molecule sequencing technologies also called third generation sequencing are promising technologies, such as Nanopore sequencing that has several advantages, including a low cost when compared to other HTS technologies, high mobility due to the small size of the sequencer, and rapid sample processing without the need for reverse transcription for RNA viruses (Rubio et al., 2020). Recently, this platform was applied to detect *plum pox virus* (PPV) and TYLCV (Badial et al., 2018; Chalupowicz et al., 2019). HTS have revolutionized virus diagnostics by using metagenomics approaches in the detection of viruses, a recent application involve metagenomics

analysis in cucumber RNA genome that have revealed aphid lethal paralysis virus (Maina et al., 2017). Specifications of plant virus detection and diagnostic techniques are shown in Figure 2.

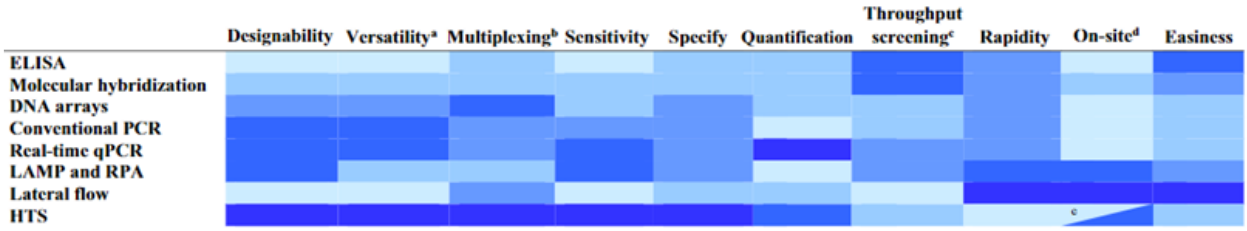

Figure 2. Specifications of plant virus detection and diagnostic techniques. The intensity of the color is proportional to the degree of positive qualification. ELISA, enzyme-linked immunosorbent assay, PCR, polymerase chain reaction; qPCR, quantitative PCR, LAMP, loop-mediated isothermal amplification, RPA, recombinase polymerase amplification; and HTS, high-throughput sequencing. [a]the potential to detect viruses at different levels (family, genus, species, strain or isolate). [b]The ability to simultaneously analyze several viruses. [c]The capability to analyze multiple samples at once. [d]The possibility to detect viruses on field with portable devices. [e]Among the HTS techniques, only nanopore sequencing is portable (Rubio et al., 2020).

3. Cucurbit chloric yellow virus (CCYV)

3.1. Origin and geographical distribution

In 2004, an unidentified disease-causing chlorotic yellow on the leaves of melon was observed in Japan. Later, it was confirmed that the agent responsible of the disease was the *Crinivirus* cucurbit chloric yellow virus (CCYV) (Gyoutoku et al., 2009). Since then, the virus has been detected in different countries of eastern Asia such as Taiwan (Huang et al., 2010), China (Zeng et al., 2011), Sri Lanka (Abeykoon et al., 2018), Philippines (H. Y. Chang et al., 2021), South Korea (Kwak et al., 2021), India (Ashwini Kumar et al., 2022), Takijstan (Chan et al., 2022). In the Middle East has been detected in Sudan (Hamed et al., 2011), Iran (Bananej et al., 2013), Saudi Arabia (Al-Saleh et al., 2015), Turkey (Orfanidou et al., 2017b), and Jordan (Salem et al., 2020). The virus was reported in countries of the Mediterranean Basin infecting cucumber, melon, watermelon and zucchini crops in Lebanon (Abrahamian et Sobh., 2012), Greece (Orfanidou et al., 2014), Cyprus (Orfanidou et al., 2019), Egypt (Amer, 2015), Algeria (Kheireddine et al., 2020), and Spain (Chynoweth et al., 2021; Alfaro-Fernández et al., 2022). It was also reported in the new world in different states such as California (Wintermantel et al., 2019), Georgia (Kavalappara et al., 2021), Alabama (Mondal et al., 2021), Texas (Hernandez et al., 2021), and Florida (Jailani et al., 2022) leading to severe outbreaks in these areas (Figure 3).

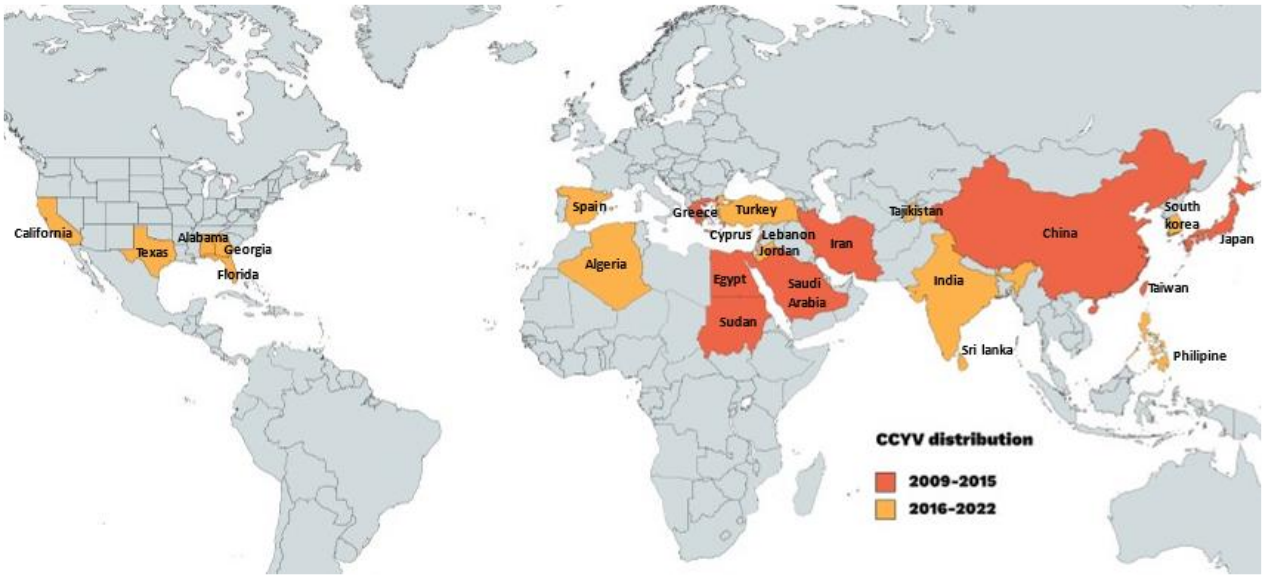

Figure 3. Geographical distribution of CCYV in the world according to its temporal propagation (created with mapchart.net).

CCYV has been identified mainly in cucurbit crops, such as melon, watermelon, cucumber, and zucchini. However, alfalfa plants were discovered to be naturally infected with CCYV and may serve as an important reservoir host for this virus (Maliogka et al., 2019; Orfanidou et al., 2017a). Also, additional hosts belonging to the families *Asteraceae*, *Chenopodiaceae*, *Convolvulaceae*, and *Solanaceae* are some of the botanical groups included in its experimental host range (Okuda et al., 2010; Orfanidou et al., 2017). More recently, wild radish was found to serve as a reservoir host for CCYV in the USA (Kavalappara et al., 2022). This leaves a clear knowledge gap regarding the virus's actual host range (Orfanidou et al., 2017a).

3.2. Classification, genomic structure, and genetic diversity of CCYV

CCYV is a member of the genus *Crinivirus* , in latin "*Crini*", refers to "hair" due to their segmented genomes that are separately encapsidated in long, filamentous particles (Kiss et al., 2013). CCYV is a positive sense single stranded RNA virus that is classified within the family *Closteroviridae* (Maliogka et al., 2019). Its bipartite genome comprises an RNA1 and an RNA2 with a size of 8607 nucleotides (nt) and 8041 (nt), respectively. RNA1 contain four open reading frames (ORF) that encode for proteins involved in replication, while RNA2 encodes for eight

proteins responsible for virus movement, encapsidation, and transmission (Figure 4) (M. Okuda et al., 2010).

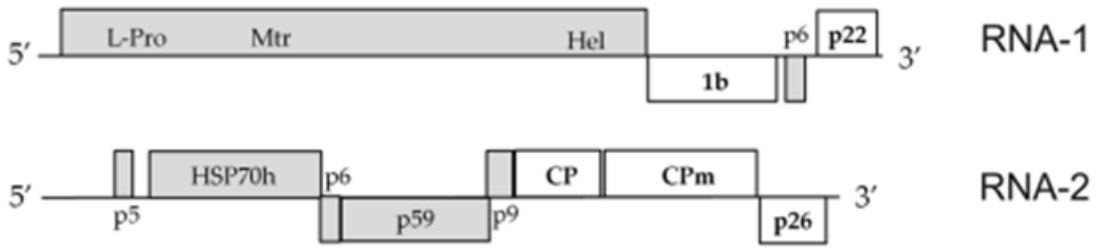

Figure 4. Genome organization of cucurbit chlorotic yellows virus (CCYV). The CCYV ORFs encodes the follow: 1b, RNA-dependent RNA polymerase; p22, 22-kDa protein; CP, major coat protein; CPm, minor coat protein; p26, 26-kDa protein (Orfanidou et al., 2019).

CCYV was found to be genetically related to lettuce chlorosis virus (LCV), bean yellow disorder virus (BnYDV), and CYSDV, according to phylogenetic analysis. CCYV should be classified as a distinct crinivirus species based on amino acid sequence similarities of representative proteins with these viruses (Keshavarz et al., 2014; M. Okuda et al., 2010). Furthermore, from previous reports, phylogenetic analysis of the CP gene of CCYV isolates were clustered in: “group Ia” including isolates from China, Lebanon, Japan, Sudan, Taiwan and Greece, “group Ib” represent Saudi Arabia isolates, while the Iranian CCYV isolates were placed in "group II" (Keshavarz et al., 2014; Orfanidou et al., 2019). The different CCYV isolates share significant similarities from Asian and European countries, implying that this virus was likely transmitted across these countries via seeds and whiteflies (Amer, 2015), in addition to the lastly reported CCYV isolates from the United States of America which present 99% genetic identity with several respective CCYV isolates from Asia and Africa (Wintermantel et al., 2019).

Several studies on the tomato infectious chlorosis virus (TICV), CYSDV, BPYV and tomato chlorosis virus (ToCV) from various plant hosts demonstrated substantial nucleotide sequence similarity, even when viral isolates originated from geographically distant areas, indicating that criniviruses display low genetic variation (Orfanidou et al., 2017a). Because the majority of criniviruses, including CCYV, infect annual herbaceous hosts that complete their biological cycle in a few months, the time available for virus-host interaction is quite limited (Maliogka et al., 2019). In accordance, sequence analysis of several criniviruses revealed low intraspecies genetic

variability (Aguilar et al., 2003; Orfanidou et al., 2017a; Rubio et al., 2013), this may be partially due to the high specific interactions between criniviruses and their whitefly vectors (Rubio et al., 2013).

3.3. Visual evaluation of CCYV symptomology in *Cucurbitaceae*

As host plant latency varies depending on the host plant and the age, at which the plant becomes infected, symptoms typically appear 3 to 4 weeks after infection (Okuda et al., 2010). CCYV is one of the most damaging criniviruses and on cucurbit plants, symptoms begin as chlorotic spots on the leaves of infected plants and rapidly develop into yellowing, mottling, and interveinal chlorosis, which progresses from older leaves near the base or crown to the plant's growing point (Okuda et al., 2010; Wintermantel et al., 2019). In some cases, brittle, thickened leaves, necrotic patches, and upward curling of the leaves accompany the yellowing. The entire plant may eventually turn yellow or chlorotic (Maliogka et al., 2019). Although no symptoms are showing, the fruit is often fewer and smaller as a consequence of the reduction of the plant's photosynthetic activity, that eventually lowers the yield (Orfanidou et al., 2019). Infection affects the quality of melons and watermelons, Brix grades, a measure of the sugar content, may be reduced in fruits of infected plants. Symptoms on infected plants are easily misinterpreted as nutritional or physiological disorders, such as magnesium and/or manganese deficit, or pesticide toxicity (Wisler & Duffus, 2001). The application of laboratory-based detection techniques are essential for the differentiation of the symptoms caused on by CYSDV, CCYV,CABYV, and other criniviruses as they are all relatively similar (Amer, 2015).

In recent investigation that study the effect of CCYV in cucumber plant and in its virulent whitefly vector, RT-PCR was used to identify CCYV in infected cucumber leaves that show slight yellowing symptoms at 7 days post infection (dpi). However, the yellowing symptoms became more severe at 15 dpi, whereas the leaves of healthy plants and of whitefly-damaged plants remained green (Figure 5) (Zhang et al., 2022).

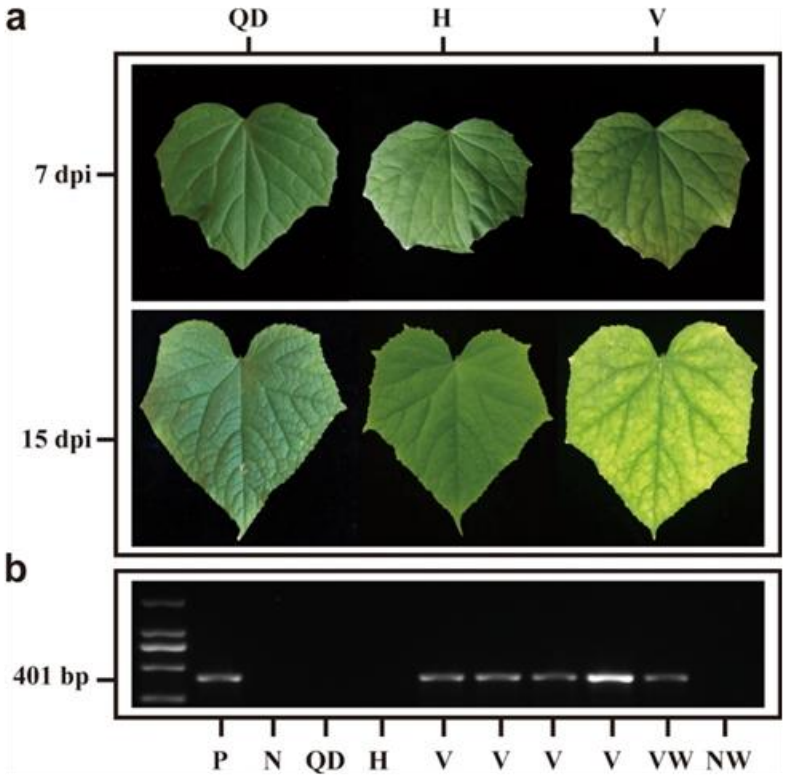

Figure 5. Identification of CCYV-infected cucumber plants and viruliferous whiteflies using RT-PCR. (a) Cucumber leaves with CCYV symptoms at 7 and 15 dpi. (b) RT-PCR gel; QD: Q-biotype damaged plant, H: healthy plants, V: CCYV-infected plants, N: negative control, P: positive control, NW: non-viruliferous whiteflies, VW: viruliferous whiteflies. Retrieved from Zhang et al., (2022).

3.4. Transmission of CCYV

Several whitefly-transmitted criniviruses have been introduced into cucurbit crops, rapidly spreading to other continents over the past 20 years, and generating a detrimental effect on agricultural production and significant economic losses (Navas-Castillo et al., 2011). The circulation of infected plant material through international trade, the trend toward large-scale monoculture in many affected regions of the world, uniformity in the use of genetic resources, and the intensive use of insecticides are all presumed to be directly connected to this emerging virus-vector complex (Orfanidou et al., 2019).

Similarly, to the transmission pathway of CYSDV, CCYV is transmitted exclusively in a semi-persistent manner by the Mediterranean (MED, formerly biotype Q) and Middle East-Asia Minor 1 (MEAM1, formerly biotype B) cryptic species of *Bemicia. tabaci* (Gennadius) complex (Gyoutoku et al., 2009; M. Okuda et al., 2010). However, Comparative transmission studies have

demonstrated that biotype Q is more effective at virus transmission than biotype B, as a result of the strongest impacts of CCYV on biotype Q feeding behavior by extending the period of phloem salivation and sap ingestion (Lu et al., 2019). Moreover, it has been shown that weeds are critical to sustaining the virus, particularly during crop-free periods. Actually, CCYV has been discovered to naturally infect thirteen weed species from eleven families (Orfanidou et al., 2017a).

According to research on other plant viruses, the efficient transmission of a particular virus in a certain agroecosystem is impacted by acquisition access period (AAP) and inoculation access period (IAP). Li et al., (2016) have successfully reached a rate of 95% positive whiteflies during a time lapse of 24 h AAP of *B. tabaci* MED on CCYV cucumber plants and were able to detect CCYV in *B. tabaci* MED after12 days post-acquisition by RT-qPCR. Nevertheless, given the significant decline in the number of positive whiteflies by day 6, researchers assume that *B. tabaci* MED may not be able to transmit the virus after 3-5 days according to studies on other criniviruses, as reported by Orfanidou et al., (2017a) where the retention period of CCYV in *B. tabaci* MED was of 4 days. Other findings revealed that a single whitefly could transmit the virus to healthy seedlings. It was also discovered that the vector needed 90 minutes to acquire and inoculate the virus. Furthermore, the vector was found to be capable of retaining the virus for up to 6 days after acquisition. The effectiveness of cucumber and *D. stramonium* plants as a source of CCYV inoculum for virus acquisition by *B. tabaci* was compared, and the results revealed that cucumber was a more effective source of virus infection than *D. stramonium* (Shakeel et al., 2018). Although the transmissibility of *B. tabaci* biotype Q may be influenced by its host preference, *C. melo* and *C. sativus* appeared to be more vulnerable to CCYV than *C. pepo* and *Luffa cylindrica* (Okuda et al., 2010).

Recent researches have shown that plant viruses can alter host metabolites to attract insect vectors for feeding (Pan et al., 2021). In a latest investigation, CCYV induced the reduction of terpenoids in cucumber plants to potentially protect its vector whiteflies. During the inoculation period, CCYV infection suppressed the defensive flavonoids and terpeneoids metabolism while activating the lipids, amino acids, and nucleotide metabolism. This finding implies that CCYV infection could influence cucumber plants to be more vulnerable to whitefly intrusion and CCYV infection. The decrease in defensive biomolecules and increase in amino acids may be responsible for whitefly feeding preferences toward CCYV-infected hosts (Zhang et al., 2022). In other

findings they suggest that CCYV can have a significant impact on the biological features of its vector, *B. tabaci*, assuming that CCYV and *B. tabaci* have formed a typical mutualist relationship mediated by host plants (He et al., 2021).

3.5. Diagnostic Methods

It is challenging to diagnose disease in crinivirus infected plants since different virus species might cause identical symptoms and delay the detection of certain viruses. According to Wintermantel et al., (2019), CCYV was likely introduced to the Imperial Valley approximately at the same time that squash vein yellowing virus (SqVYV) was first identified in California (Batuman et al., 2015). However, because of its resemblance to CYSDV in terms of symptoms on cucurbits and vector transmission, CCYV was likely undetected (Wintermantel et al., 2019). Diagnosis requires molecular or serological protocols. In a study, Gyoutoku et al., (2009) developed a reliable RT-PCR test for the CCYV detection. To detection by ELISA methods, antibodies have been developed against a number of criniviruses using viral proteins expressed in bacteria. (Hourani & Abou-Jawdah, 2003; M. Jacquemond et al., 2009; Kubota et al., 2011; Steel et al., 2010). In most reports, due to the symptoms similarity between CCYV and CYSDV, DAS-ELISA was used for a first evaluation and RT-PCR for an accurate detection of both CYSDV and CCYV (Abrahamian et al., 2012; Al-Saleh et al., 2015; Bananej et al., 2013; Orfanidou et al., 2014; Zeng et al., 2011). Several immunoassays, including immunoelectron microscopy, tissue blot (TBIA), and ELISA, were successfully employed to detect both viruses using antibodies produced against recombinant coat proteins of these viruses (Kubota et al., 2011).

For the accurate identification of criniviruses, molecular methods are presently applied extensively. A universal degenerate primer set targeting the RdRp was used to create a general RT-PCR for the identification of both known and unknown criniviruses. Furthermore, multiplex RT-PCR assays targeting the RdRp have been developed for the simultaneous identification and differentiation of viruses infecting the same host group (Wintermantel & Hladky, 2010). Real time RT-PCR assay are also widely used for their reliability and accuracy as well as for quantification of plant viruses in their hosts and vectors. Another detection method in use is the reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays which was developed to detect CCYV (M. Okuda et al., 2015; Z. Wang et al., 2014). As such molecular detection methods

become more widely available, RT-LAMP and RT-qPCR assays have the potential to provide rapid and efficient detection of criniviruses (Maliogka et al., 2019).

3.6. Genetic resistance to CCYV

One of the main strategies used to minimize the threat of virus infection is the identification of genetic resistance (Okuda et al., 2013). Resistance to cucumber and melon has been identified for CYSDV, which is genetically related to CCYV (Mccreight & Wintermante, 2011; Pérez-De-Castro et al., 2020; Tamang et al., 2021; Wintermantel et al., 2017). As CCYV is a new emerging virus, genetic resistances studies have just start expanding. Okuda et al., (2013) found five melon accessions of interest for development of resistant varieties, specifically the accession "JP 138332," which offers a promising CCYV resistance trait that may be associated to virus multiplication inhibition (Okuda et al., 2013). Subsequently, another study has reported that CCYV resistance trait of JP 138332 was found to be recessive, and the first QTL analysis for resistance to CCYV in melon revealed a major locus for the resistance located on Chromosome 1 (Kawazu et al., 2018).

RNA silencing acts as an antiviral system in plants; thus, virus infection involves suppression of gene silencing. By encoding viral suppressors of RNA silencing (VSRs), viruses have developed mechanisms to counteract the plant host defense (Lakatos et al., 2004). In this respect, RNA silencing suppression activity of several proteins encoded by the RNA1 (RdRp, p22) and RNA2 (CP, CPm, and p26) of CCYV was investigated through co-agroinfiltration on *Nicotiana benthamiana* plants. The results reveled that p22 is a suppressor of local RNA silencing, but it does not disrupt systemic silencing or the transmission of RNA silencing signals between cells. Moreover, after comparing CCYV p22 suppression activity with two other well-known crinivirus suppressors (CYSDV p25 and ToCV p22), it appeared that CCYV p22 is a weaker suppressor of local RNA silencing than the other two proteins (Orfanidou et al., 2019). Other finding has confirmed the weakness of CCYV p22 and its implication in the suppression of RNA silencing via RNA binding (Salavert et al., 2020). Additionally, in a latest study, the cucumber ribosomal-like protein CsRPS21 was found to interact with the p22 protein both *in vitro* and *in vivo*. CsRPS21, a p22-interacting ribosomal protein, was discovered to play a role in early viral replication and silencing suppressor activity (Yang et al., 2021).

As new advanced applications evolve to undercover genetic resistance in plants, nanotechnology tools were used to suppress CCYV symptoms via inhibition of viral replication and systemic movement using *N. benthamiana* plants with GFP-tagged CCYV via carbon-based Nano materials (Al-Zaban et al., 2022).

4. Tomato Leaf Curl New Delhi Virus (ToLCNDV)

4.1. Origin and geographical distribution

Tomato leaf curl New Delhi virus (ToLCNDV) was first described in 1995 (Srivastava et al., 1995) in tomato (*Solanum lycopersicum* L.) crops in New Delhi (India). It was initially named as Indian tomato leaf curl virus (ToLCV-India), but after cloning and sequencing of its genome (Padidam et al., 1995; Srivastava et al., 1995), it was renamed ToLCNDV, according to the nomenclature of the International Committee on Taxonomy of Viruses (ICTV). Currently, ToLCNDV is one of the predominant diseases in tomato production in India (Moriones et al., 2017; Zaidi et al., 2017), but it also infects an extensive range of host species in more than 15 Old World countries, including economically important crops (Zaidi et al., 2017). In the first years after its identification, the virus was detected in Thailand, infecting not only solanaceae but also cucurbits such as melon, cucumber, and bottle gourd (Ito et al., 2008), and in Pakistan on tomato (Figure 6) (Zaidi et al., 2017).

Between 2000 and 2012, ToLCNDV spread to other countries in the Indian subcontinent and neighboring countries in South Asia. It was described in a diverse range of hosts, such as in tomato, watermelon, bitter gourd (*Momordica charantia* L.) and chili (*Capsicum annuum* L.) in Pakistan (Hussain et al., 2004). In tomato and melon in Iran (Fazeli et al., 2009; Yazdani-Khameneh et al., 2013). In melon cultivar 'Silver light' (*C. melo* makuwa group) in Taiwan (Chang et al., 2010). In tomato and bitter gourd in Bangladesh (Maruthi, Rekha, Cork, et al., 2007; Tiwari et al., 2010). In cucumber and chili in Indonesia (Mizutani et al., 2011; Zaidi et al., 2017), and in pumpkin in the Philippines (Figure 6) (Phaneendra et al., 2012).

The host range of ToLCNDV and its geographic distribution is in continuous expansion. Recently, in Pakistan it has been identified infecting potato (Hameed et al., 2017), soybean (*Glycine max* L) (Jamil et al., 2017) and cotton (*Gossypium hirsutum* L.) (Zaidi et al., 2016) and in Sri Lanka in bitter gourd, ridge gourd (*Luffa acutangular* L.), pumpkin and cucumber (Bandaranayake et al.,

2014). ToLCNDV was identified in the Seychelles Islands in tomato (Scussel et al., 2018) and more recently in Nepal in three cryptic species of *Bemisia tabaci* found in *Cucurbitaceae* and *Solanaceae* host plants (Acharya et al., 2021), and in China infecting tomato(Figure 6) (Li et al., 2022).

ToLCNDV was limited to Asian countries until 2012, when it was detected in Spain for the first time and became one of the most important emerging viral diseases in cucurbit crops in the Mediterranean basin. *Begomovirus* infection symptoms were observed in zucchini crops firstly in Murcia and later in 2013 in Almería (Juárez et al., 2013), in the same year ToLCNDV was identified as the pathogen causing the disease (Juárez et al., 2014). Since then, ToLCNDV has become the main disease of viral etiology affecting cucurbits in the region (Rodríguez & Janssen, 2019). Watermelon has been described as a natural host of ToLCNDV in Spain; however, severe infections have not occurred in this crop, even in regions where inoculum pressure was high and other cucurbits did become infected (Juárez et al., 2019).

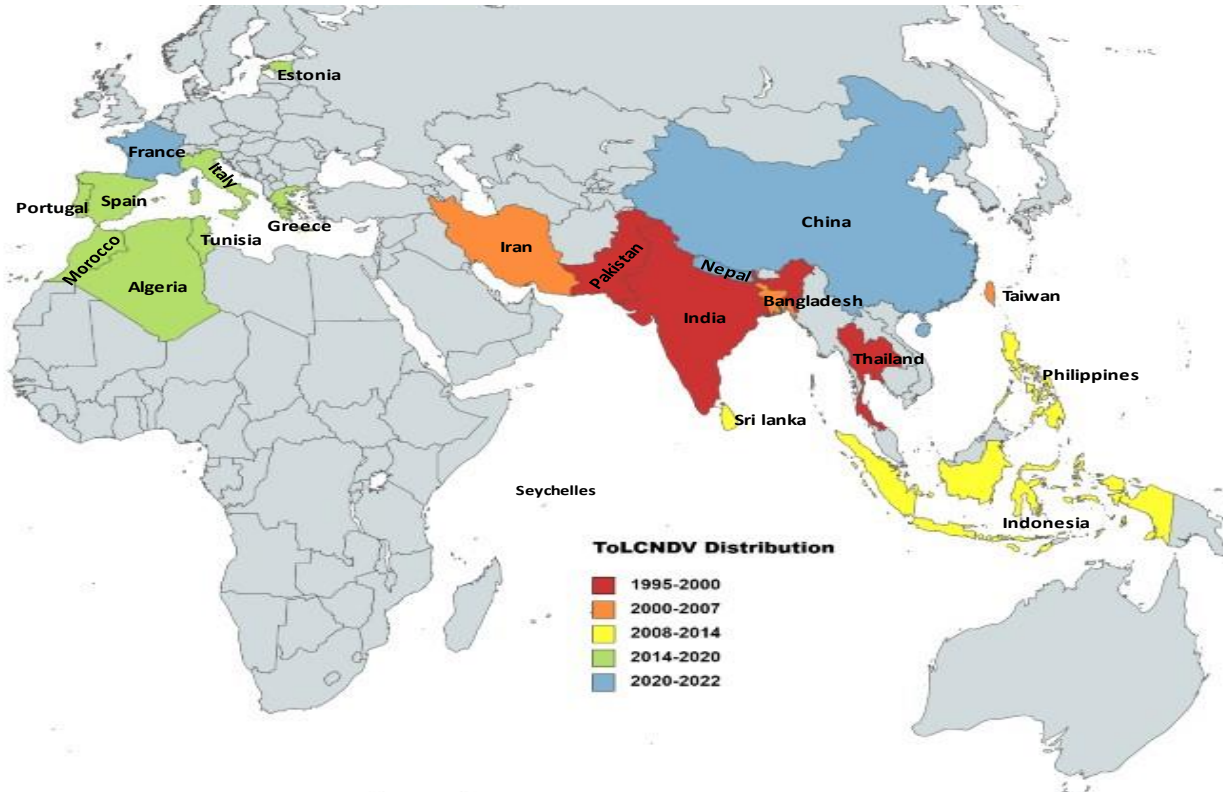

Figure 6. Geographical distribution of ToLCNDV in the world according to its temporal propagation (created with mapchart.net)

In 2015, the Spanish isolate of ToLCNDV was detected also in Tunisia infecting melon, cucumber, zucchini, tomato and pepper (*Capsicum annuum* L.) (Mnari-Hattab et al., 2015; Mnari-Hattab et al., 2022; Zammouri et al., 2017), in Italy infecting melon, squash and zucchini (Panno et al., 2019; Panno et al., 2016; Parrella et al., 2017), in Morocco in zucchini, melon, and tomato (Radouane et al., 2018; Radouane et al., 2022; Sifres et al., 2018), in Greece in zucchini (Orfanidou et al., 2019), in Algeria in melon, cucumber, and zucchini (Kheireddine et al., 2019), in Portugal in zucchini (EPPO, 2019), in France also infecting zucchini (Desbiez et al., 2021) and in Estonia in cucumber and tomato plants(Figure 6) (Just et al., 2022). ToLCNDV has also been identified in *Ecballium elaterium* (L.) A. Rich., *Datura stramonium* L., *Sonchus oleraceus* L., and *Solanum nigrum* in these Mediterranean regions, which may serve as inoculum sources (Juárez et al., 2019).

ToLCNDV-ES, a ToLCNDV isolate described in Spain, is better adapted to cucurbit infection and is very limited to solanaceae. In the Mediterranean basin countries where this virus has been described, zucchini is the crop that has suffered the most damage and losses (Panno et al., 2019). However, the virus has been found in tomato plants, though at a low frequency and without causing symptoms that prevent cultivation (Fortes et al., 2016; Juárez et al., 2019; Ruiz et al., 2015). In Italy, the ToLCNDV-ES isolate from the Mediterranean basin has been found infecting eggplant and bell pepper (Luigi et al., 2019; Parrella et al., 2020). Although no symptoms were observed on these species' fruits, their presence in these crops represents a new reservoir for the virus, a new reservoir for the spread of ToLCNDV and a threat to the production of not only cucurbits but also solanaceae.

4.2. Classification, and genomic structure of ToLCNDV

ToLCNDV is a single-stranded DNA virus. According to its genomic characteristics, transmission vector and host range, it is classified in the genus *Begomovirus*, the largest within the *Geminiviridae* family, as a bipartite begomovirus. Its genome consists of two circular single-stranded DNA molecules of approximately 2.7 and 2.6 kb respectively, named DNA-A and DNA-B (Zerbini et al., 2017). Each fragment of the genome is enclosed in a T=1 icosahedron, which contains 110 capsid protein molecules arranged into 22 pentameric capsomeres (Brown et al., 2015) (Figure 7). These viruses make the most of their genome by using a mechanism for bidirectional transcription and genetic overlap (Rojas et al., 2005). Their name deriving from the

Latin "*geminis*" meaning twin, referring to their structural twinned icosahedral virions (Cortés & López, 2011; Rojas et al., 2005).

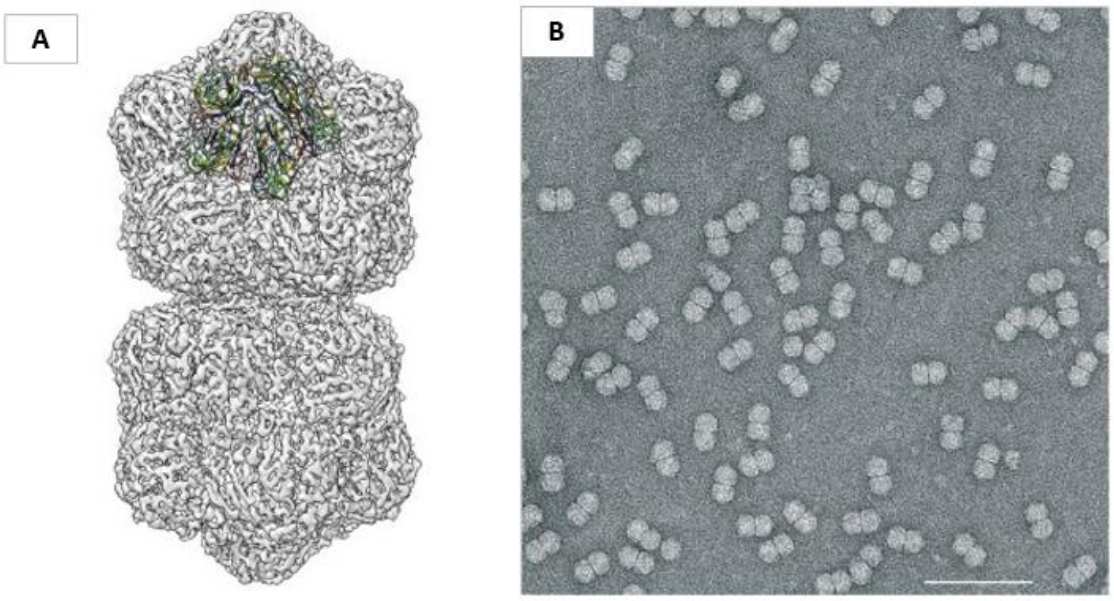

Figure 7. Characteristic capsid of geminiviruses. A) Cryo-electron microscopic reconstruction of a geminivirus capsid (Hipp et al., 2017). B) Image of purified particles of begomovirus with electron microscopy (Bar represent 100 nm) (Glick et al., 2009).

The geminiviruses have few genes and require host proteins, such as primases and nuclear DNA polymerases, to carry out replication. In the ToLCNDV genome, there are eight open reading frames (ORFs) that encode the proteins necessary for the virus to carry out its replication and infection cycle (Fondong, 2013). DNA-A ORFs overlap with each other, even integrating within each other, resulting in the regulation of the expression and function of adjacent proteins (Moriones et al., 2017). The ORFs of DNA-B, on the other hand, do not overlap (Figure 8). In fragment A there are six ORFs, four encoding in the antisense direction of the virus DNA strand (AC1, AC2, AC3, AC4) and two encoding the sense direction (AV1 and AV2). AC1 codes for the replication initiator protein (Rep), which introduces a nick in the nucleotide sequence of the virus to initiate the circular replication cycle of the viral genome (Kushwaha et al., 2017; Rojas et al., 2018). AC2 is expressed at the onset of infection and encodes a multifunctional protein, transcription activator of protein (TrAP), which activates the expression of late genes, such as the capsid protein and genes encoded in DNA-B (Cantú -Iris et al., 2019). AC2 also acts as a pathogenicity factor and is involved in the suppression of RNA silencing (RNAi) (Voinnet et al., 1999). AC3 code for a viral DNA replication enhancer (REn) (Pradhanet al., 2017). AC4 ORF is integrated into AC1 and is involved

in symptom development as well as RNAi suppression (Fondong, 2013). AV1 encodes the capsid protein (CP), which is required for viral DNA encapsidation and recognition by the transmission vector, and overlaps with AV2, which encodes the pre-capsid protein and is involved in transcriptional and post-transcriptional RNAi suppression (Luna & Lozano-Durán, 2020; Y. Wang et al., 2019).

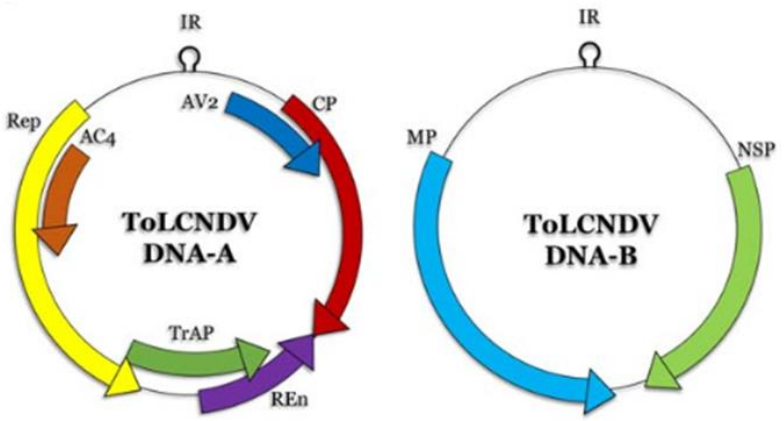

Figure 8. Genomic organization of ToLCNDV. ORFs and direction in which they encode the different proteins in the DNA A and B strands. Rep: replication initiator protein, TrAP: transcription activator protein, REn: viral DNA replication enhancer, CP: capsid protein, NSP: nuclear shuttle protein and MP: virus cell-to-cell movement protein. Retreived from (Zaidi et al., 2017).

The two ORFs of fragment B; BC1 on the antisense strand of the virus and BV1 on the sense strand are necessary for symptom development and virus movement in the plant. BV1 encodes the nuclear shuttle protein (NSP), responsible for the transport of viral DNA between the cytoplasm and the nucleus, and necessary for the host to recognize the virus and initiate the defense response. BC1 encodes the protein required for cell-to-cell movement of the virus (MP) (Lee et al., 2020) (Padidam et al., 1996; Hussain et al., 2005; Jeske, 2009; Pratap et al.,2011; Khan et al.,2012; Lee et al., 2020). In the intergenic non-coding region of both genomic particles there is a common region (CR) of 163 nt that includes bidirectional promoters to activate the expression of sense and antisense genes on the DNA strand (Cantú -Iris et al., 2019). In the CR there are 30 nt that create a hairpin structure with a conserved nucleotide sequence (TAATATTTAC) where the origin of replication is located (Hanley-Bowdoin et al., 2013; Padidam et al., 1995; S. Panno et al., 2019)

In some isolates of ToLCNDV, betasatellites have been described (Malathi et al., 2017), which are circular DNA strands of approximately 1.3 kb that are separately encapsidated and rely on

genomic DNA-A and DNA-B segments for replication and encapsidation. They encode a single protein, βC1, which acts as a major pathogenicity factor, increasing symptomatology by accumulation of viral DNA, suppresses RNAi silencing, and may favor host range extension (Jyothsna et al., 2013; Rojas et al., 2018). The ToLCNDV-ES isolate does not present betasatellites as DNAs A and B are sufficient for infection to take place and for symptom induction (Juárez et al., 2019; Ruiz et al., 2017).

4.3. Genetic diversity of ToLCNDV

To study the diversity of emerging ToLCNDV isolates in Mediterranean countries, several genetic analyses have been performed, comparing the genomes identified in cucurbits, solanaceae and adventitious plants from this region with each other and, in addition, with the sequences of Asian isolates. ToLCNDV isolates from the Mediterranean basin constitute a genetically homogeneous group, with no host, geographic location or year differences, but distinct from Asian population of the virus (Fortes et al., 2016; Juárez et al., 2019; Moriones et al., 2017; Panno et al., 2019). Over 500 DNA-A full-length sequences of ToLCNDV are provides by the GenBank dataset. Mediterranean isolates present 91%-94% DNA-A similarity to other ToLCNDV isolates, based on the phylogenetic analysis (Vo et al., 2022; Yamamoto et al., 2021). According to the taxonomic rule for begomoviruses, they should be classified as a different strain and named as ToLCNDV-ES (Brown et al., 2015; Fortes et al., 2016). With a common origin and a single fairly recent introduction in the Mediterranean area. ToLCNDV-ES arose from several recombination events favoring its adaptation to cucurbit infection, but with low pathogenicity in solanaceae (Fortes et al., 2016; Moriones et al., 2017).

Juárez et al., (2019) have detected a higher level of genetic variability of the virus in *Datura stramonium* than in cultivated varieties, which has increased over the years, and could manifest the evolutionary capacity of ToLCNDV and its potential threat to increase its pathogenicity or expand the infection of new hosts. In addition to this source of genetic variability, the ToLCNDV-ES strain has been detected in mixed infection with other bipartite begomoviruses and with viruses from different families have also been detected in Asia (Moriones et al., 2017; Sharma et al., 2015), resulting in recombinant isolates on different hosts (Ashwathappa et al., 2020; Venkataravanappa et al., 2019, 2020). Recently, ToLCNDV-ES isolate was found in double infection with TYLCV in tomato plants with an infection ratio of up to 50% occurring in natural and experimental

conditions. qPCR results indicated an enhanced infectivity of ToLCNDV in synergy with TYLCV (Vo et al., 2022).

4.4. Mode of transmission of ToLCNDV

4.4.1. Whitefly transmission

Begomoviruses are naturally transmitted by the tobacco whitefly complex's species *Bemisia tabaci*. ToLCNDV is a phloem-limited plant virus that is persistently transmitted by this vector in a circulative manner (Fondong, 2013; Rojas et al., 2018; Rosen et al., 2015; Zaidi et al., 2017). The virus capsid protein binds to the vector and facilitates transmission when the fly sucks sap from an infected plant to feed (Rosen et al., 2015). Following this interaction, the virus travels through the insect's alimentary canal and from the midgut membrane leaks into the part of the circulatory system, called the hemocoel, responsible for directly bathing the organs with hemolymph, in this way the virus reaches the primary salivary gland (Gray et al., 2014). When the viruliferous fly feeds on a healthy host plant, it inoculates the virus and infection occurs, in a process that can take 15 minutes (Janssen et al., 2017). Virus acquisition occurs within several hours of insect feeding, and the insects remain virulent for the rest of their lives (Desbiez, 2022). In another assay, the retention of ToLCNDV in adult vectors was established to be between 7- and 14-day (Janssen et al., 2022). Until recently, transovarial begomovirus transmission in whiteflies had not been observed. However, it was proven that in TYLCV vectors could generate at least two generations of virus transmission without the need for an inoculum source. In fact transovarial virus transmission from female vectors to offspring may be highly important in maintaining a source of infection and thus has significant epidemiological significance (Guo et al., 2019; Wei et al., 2017). The MEAM biotype in India and the MED-Q1 haplotype in Spain have so far been identified as the transmitters of ToLCNDV among the three begomovirus-transmitting biotypes of *B. tabaci*: Middle East Asia and Asia minor (MEAM) biotype or B, and Q Biotype or Mediterranean (MED) (Janssen et al., 2017; Maruthi & Muniyappa, 2007). In Italy, MED-Q2 has also been identified as a viruses transmitter (Bertin et al., 2021). Its diffusion throughout the Mediterranean basin countries has been facilitated by extremely high temperatures and intensive farming methods, further to that, biotype Q has proven to have a greater insecticide resistance compared to the MEAM biotype (Rodríguez et al., 2019).

4.4.2. Mechanical transmission

Mechanical sap transmission is an efficient solution for biological studies of plant viruses and for the identification of sources of tolerance against them (López et al., 2015). Begomoviruses, much like most phloem-limited viruses, including other geminiviruses and closteroviruses, are rarely mechanically transmissible by rub or sap inoculation (Lee et al., 2020). However, it has been shown that some isolates of ToLCNDV can indeed be mechanically transmitted (Sohrab et al., 2016). ToLCNDV was sap transmitted to *N. benthamiana* Donim and potato plants in India, causing yellow mosaic and leaf distortion symptoms (Usharani et al., 2003). ToLCNDV was also reported to be mechanically transmitted in ridge gourd, sponge gourd and *N. benthamiana* (Sohrab et al., 2016). Another finding showed that ToLCNDV-OM isolate, causing mosaic, leaf curl and puckering in oriental melon, can infect oriental melon (*C. melo* subsp. agrestis (Naudin) Greb. var. makuwa) in Taiwan, in addition to pickling melon, bottle gourd, cucumber, zucchini, and luffa via mechanical sap inoculation (Chang et al., 2010). In a recent work, Lee et al., (2020) reveled that a mutation at amino acid 19 of the N-terminal end of the MP protein (encoded in DNA-B) is responsible for the mechanical transmission of the ToLCNDV isolate identified in oriental melon, excluding the nuclear shuttle protein's involvement in this type of transmission as previously suggested. In contrast, despite similarities with the genomes of potato and oriental melon isolates, a ToLCNDV isolate infecting cucumber could not be mechanically transmitted to its natural host (Lee et al., 2020; Padidam et al., 1995; Samretwanich et al., 2000).

The ToLCNDV-ES strain has proven to be mechanically transmissible in *Cucurbitaceae*, as demonstrated by López et al., (2015) where they efficiently transmitted this strain to different species of the genera *Cucumis, Cucurbit* and *Citrullus* by mechanical inoculation. The strain was also confirmed to be transmitted by mechanical inoculation to *C. pepo*, and *C. melo* of the inodorus and cantalupensis groups, although they could not transfer it to *C. sativus*, *S. lycopersicum*, *S. melongena* and *C. annuum*. (Panno et al., 2019). In any case, the mechanical transmissibility of ToLCNDV is an important element in breeding programs searching for resistance sources. Natural inoculation with viruliferous whitefly populations or agroinoculation of large germplasm collections presents numerous challenges, including reproducibility issues, selective inoculation by vector preference, high cost, and execution time. Mechanical inoculation, on the other hand, provides reproducibility, speed, controlled conditions, and a low cost (Lefebvre et al., 2020).

4.4.3. Seed transmission

The spread of new viral diseases has been linked to the exchange of potentially infected seeds between countries (Dombrovsky, Smith, et al., 2017). To date, it is unproven how ToLCNDV was introduced into the Mediterranean region from India or other Asian countries (Kil et al., 2020). Previous seed transmission reports on other begomoviruses have raised the possibility of ToLCNDV seed transmission. The first case was in sweet potato (*Ipomoea batatas* L.) Lam) plants where seed-borne transmission of sweet potato leaf curl virus (SPLCV) was proven (J. Kim et al., 2015). One year later, TYLCV and mungbean yellow mosaic virus (MYMV) were also detected in tomato and black bean seedlings, respectively, germinated from infected seeds (Kil et al., 2016; Kothandaraman et al., 2016). Seed-borne transmission of TYLCV was also observed in 2017 in white soybean (Kil et al., 2017) and in 2018 in sweet bell pepper (Kil et al., 2018). In contrast, other recently published work has not been able to confirm seed transmission of TYLCV in *N. benthamiana* or in tomato (Pérez-Padilla et al., 2020; Rosas-Díaz et al., 2017).

As for ToLCNDV, seed-borne transmission of an isolate in chayote and another in bitter gourd has been described in India (Manivannan et al., 2019; Sangeetha et al., 2018), and recently, seed-borne transmission of a ToLCNDV-ES isolate from Italy has also been confirmed in zucchini plants (Kil et al., 2020). This mode of transmission could be another route by which ToLCNDV was introduced from Asia into the Mediterranean region (Juárez et al., 2019; Kil et al., 2020).

Traditional seed transmissibility tests include serological tests on seeds, what often greatly overestimate the real seed transmission and observation of symptoms on plantlets after germination tests. However, in some cases plants infected from seed are asymptomatic and have very low virus titers that can be detected only by molecular methods (Dombrovsky et al., 2017).

Using molecular approaches, seed transmission was observed for viruses considered as non-seed-transmissible, including begomoviruses. The agronomic impact of cryptic infections after seed transmission is not well known, but since in some cases vector transmission to new plants has been demonstrated from asymptomatic plants infected from seeds, these plants can constitute primary inoculum to start epidemics. The importance of seed transmission for long-distance spread of plant viruses has probably long been underestimated, and particular care should be taken during seed trade (Sangeetha et al., 2018).

4.5. Visual evaluation of ToLCNDV symptomatology in cucurbits

The characteristic symptomatology that begomoviruses generate in their hosts mainly includes yellowing, mosaic, curling and leaf curling (Sagar et al., 2020). Symptoms caused by the ToLCNDV-ES strain on cucurbits are similar to those caused by Asian isolates (Malathi et al., 2017), with leaf curling, intense mosaic, yellowing in leaves, vein thickening in young leaves, internodal shortening, dwarfing, and inhibition of plant development (Panno et al., 2016). The most severe symptoms occur in zucchini, generating high stress in the plant that prevents its development, flowering and fruiting, especially if the infection occurs when the plant is in young stages (Font San Ambrosio & Alfaro Fernández, 2014). If infection occurs after fruit set, grooves, deformations and spots appear on the fruit surface, reducing the size considerably, which eventually reduce fruit quality, and might even prevent their commercialization (Juárez et al., 2014).

According to the visual evaluation of the severity of the ToLCNDV-ES symptoms on cucurbits, López et al., (2015) classified the symptoms. For this purpose, they established a numerical scale with five levels: no symptoms (0), mild symptoms (1), moderate symptoms (2), severe symptoms (3), and very severe symptoms or plant senescence (4). This scale enables semi-quantitative phenotyping of the response to virus infection in cucurbit germplasm collections.

In recent studies, a comparative analysis of the infectivity of Mediterranean and Southern Asian ToLCNDV isolates in cucurbit crops demonstrated that zucchini plants infected with ToLCNDV-India present more adverse symptoms compared to those infected with ToLCNDV-ES, assuming that ToLCNDV-India had higher pathogenicity in this species (Vo et al., 2022; Yamamoto et al., 2021). Categorized as a different strain than Asian ToLCNDV, the. ToLCNDV-ES isolated from Spain showed different pathogenicity on some plants, such as tomato presenting lower incidence in the crop, in correlation to a reduced transmission efficiency in this plant (Fortes et al., 2016; Janssen et al., 2022; Juárez et al., 2019; Panno et al., 2019).

4.6. Diagnostic methods

To evaluate new germplasm in breeding programs and identify resistant varieties, highly sensitive and effective detection tools are needed. For the detection of ToLCNDV, a number of tools (ELISA, PCR, NASH, IC-PCR, Real-time PCR, and LAMP) have been optimized (Naganur

et al., 2019; Venkataravanappa et al., 2020; Venkatasalam et al., 2011). Due to its ease of use, sensitivity, accuracy, and affordability, serological techniques are widely used such as the different ELISA immnoassays and the Lateral Flow Assay (LFA) has been frequently used for standard virus detection.(Alfaro-Fernández et al., 2016; Naganur et al., 2023). Nucleic acid hybridization are also commonly used for the detection of ToLCNDV, the technique is economical, and allows the processing of a large number of samples, carrying out field surveys to determine the presence of ToLCNDV (Alfaro-Fernández et al., 2016; Hameed et al., 2017; Jamil et al., 2017).

As for molecular detection techniques, the PCR allows the amplification of one or more regions of the ToLCNDV genome using specific DNA primers. PCR is the most sensitive, specific and versatile method for the detection of ToLCNDV, in addition to being the most widely used technique for its diagnosis (Alfaro-Fernández et al., 2016; Islam et al., 2011; López et al., 2015). Quantitative PCR (qPCR) is also used as a real-time detection of ToLCNDV accumulation in infected tissue using nucleotides or fluorophore-labeled probes. The detection of ToLCNDV genes by qPCR was approximately 10^4 to 10^6 times more sensitive than tissue-print hybridization (Simón et al., 2018).

4.7. Control and management of ToLCNDV

It is difficult to predict epidemics and/or assess field incidents in many virus pathosystems until it is too late to prevent economic loss. As a result, prophylactic sprays are routinely employed (Perring et al., 1999). Chemical control has conventionally been the dominant strategy in *B. tabaci* management. As a result, in the field, the pest has acquired resistance and cross-resistance to a broad range of insecticides (Rodríguez et al., 2019). With the raising concern related to the excessive use of pesticides, integrated pest management (IPM) is an alternative interdisciplinary strategy, that aim to control agricultural pest's populations using biological control, such as the use of predatory mite *Amblyseius swirskii* that have been proven to be efficient in zucchini crops (Tellez et al., 2017). Moreover, good cultural practices are essential for the control of whitefly population in protected cultivations such as the use of chromotropic traps, sealed greenhouses, fly-free seedlings, etc.). These practices are used together with biological control, combining both with the application, to a lesser extent, of chemical treatments, compatible with natural enemies (Rodríguez et al., 2019). To prevent the whitefly from entering the greenhouses, structural measures can be implemented, such as maintaining airtightness of greenhouses, covering young

plants with thermal blankets, installing insect-repellent nets on window sashes and windows, and installing a double entrance door to the facilities. Subsequently, to avoid virus reservoirs, maintain hygiene in greenhouses and plots by immediately removing and destroying plant debris, infected plants, and adventitious plants in the crop environment (Lecoq & Katis, 2014).

4.8. Genetic resistance to ToLCNDV

The understanding of viral pathogenicity and host responses in antiviral resistance has been enriched by the recent developed techniques in plant virology on RNA silencing, such as virus-induced gene silencing, large-scale genomic analysis, and epigenetic analysis. R gene-mediated resistance, recessive resistance, and antiviral RNA silencing are examples of plant antiviral activities. R gene-mediated resistance, the most extensively studied resistance mechanism against bacteria and fungi, commonly associated with hypersensitive response (HR), is also effective in viruses (Akhter et al., 2021).

To subsist, virus proteins have evolved to act as suppressors of plant defense mechanisms, inhibiting the plant's response to viral infection. The main defense mechanism against DNA viruses is RNA interference (RNAi)-mediated silencing machinery. Several virus-encoded proteins have been implicated in suppressing host defenses, especially the silencing machinery. AC2 was the first discovered viral protein to act as a suppressor of silencing activity in both posttranscriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) (Sharma & Prasad, 2017). Specific mechanisms such as R gene-mediated resistance, RNA silencing, or recessive resistance, as well as non-specific cellular mechanisms such as transcription factor regulation, autophagy, or ubiquitination, are used by plants to generate a defense response in geminivirus infection (Alcaide-Loridan & Jupin, 2012; Haxim et al., 2017; N. Sharma & Prasad, 2017).

The use of genetically resistant crop cultivars or varieties is an alternative virus control strategy. These resistant genotypes possess a heritable trait or set of traits that prevent the spread of viruses, even in environments that are conducive to virus infection in the species. The majority of crop species have virus resistance traits, and numerous cultivars with varying levels of resistance have become commercially successful (Gómez et al., 2009)**.** The Ty genes for TYLCV resistance (Ty-1, Ty-2, Ty-3, Ty-4, Ty-5, and Ty-6) have received the most attention in geminivirus resistance research. Different molecular markers have been developed for their introgression into commercial

varieties, enabling assisted selection in breeding programs (Jung et al., 2015; M. Kim et al., 2020). These genes have been transferred to different commercial varieties (Rojas et al., 2018; Sagar et al., 2020). Although the resistance provided by each may differ depending on the growing environment, temperature, virulence of each isolate, or their capacity to provide immunity to begomoviruses with a single-partite genome, while failing to do so for those with a bipartite genome (Rojas et al., 2018). Pyramiding of Ty genes is the most efficient method to achieve durable resistance to TYLCV (Kumar et al., 2019). In the case of ToLCNDV, transgene-based or marker-assisted breeding-based approaches were developed for the identification of a potential gene conferring ToLCNDV tolerance in tomato cultivars (Prasad et al., 2022). In a simpler approach Mastrochirico et al., (2023) proposed to use grafting to induce tolerance rather than resistance to airborne virus infection such as ToLCNDV. The grafted *C. melo* with cv. Barattiere was selected as the most tolerant to ToLCNDV, showing no symptoms and very low levels of viral DNA accumulation.

The inheritance regulation of ToLCNDV resistance in cucurbits has been investigated in sponge gourd (*Luffa cylindrica* M. Roem.), and has been determined to be monogenic dominant (Islam et al., 2010, 2011). In *C. melo*, López et al., (2015) identified resistance to ToLCNDV-ES in five accessions from India, three belonging to the momordic group and two to the kachri group. Later on, the resistance to ToLCNDV of these accessions was also confirmed by Romay et al., (2019). ToLCNDV resistance in four accessions of melon belonging to the subsp. agrestis var. momordica and to the wild agrestis group was also identified to have a major QTL region in chromosome 11 that controls resistance to TolCNDV along with two modifier regions located in chromosomes 2 and 12 (Sáez et al., 2017). In another study conducted by Román et al., (2019), resistant momordica genotypes were used for the molecular analysis of ToLCNDV resistance in *C. melo*, confirming that the level of ToLCNDV accumulation and the severity of symptom development are related to the accumulation of transcripts from modified QTL located in chomosome 2.

In *Cucurbita* ssp. resistance has been identified in *C. moschata* from different origins, namely the US, India, Japan, Nigeria, and Spain (Sáez et al., 2016). Genetic studies on pumpkin accessions of an American improved cultivar Large Cheese (PI 604506) and an Indian landrace (PI 381814) have revealed in both cases, a major recessively inherited gene located in chromosome 8 that

regulate resistance in intraspecific crosses. This finding adds to the previous identification of ToLCNDV resistance, which was fond to be syntenic to the major QTL region in chromosome11 that controls resistance to the same virus in melon (Sáez et al., 2020). Interestingly, in another work, one *C. moschata* accession, BSUAL-252, from Japan, was identified as highly resistant to ToLCNDV, that was conferred by a single dominant gene after crossing with a susceptible accession of *C. moschata*. This gene is not associated with the genomic region on chromosome 8 that holds the previously identified recessive gene for ToLCNDV resistance (Romero-Masegosa et al., 2021). As for cucumber, a monogenic recessive genetic control and a QTL in chromosome 2 was associated with ToLCNDV resistance in three Indian accessions (CGN23089, CGN23423, and CGN23633) (Sáez et al., 2021).

Overall, the possibility of pyramiding these genes to create commercial cultivars with improved and long-lasting resistance to ToLCNDV is made possible by the availability of various ToLCNDV resistance genes (Romero-Masegosa et al., 2021). Also taking part in the development of marker-assisted breeding programs for ToLCNDV resistance and to study the mechanisms involved in this recessive resistance (Sáez et al., 2017, 2020, 2021). The availability of large marker collections, high-throughput genotyping tools, and high-resolution mapping populations will provide effective marker-based selection procedures, pyramiding of resistance genes and comparative analysis of syntenic regions, etc. With the purpose to study gene function and to introduce resistance genes in elite cultivars, thanks to new tools, such as gene editing by CRISPR/Cas and TILLING, which will be helpful for the generation and introduction of resistances in species where transformation is not possible (Martín-Hernández & Picó, 2020)

OBJECTIVES

OBJECTIVES

Cucurbits are cultivated from temperate to tropical climatic regions of the world since ancient times and are essential to human nutrition. In Algeria, three major cucurbit genera are cultivated: *Cucumis* (cucumbers, melons), *Cucurbita* (pumpkins and squash), and *Citrullus* (watermelons). Diseases and pests are one of the major limiting factors for cucurbits production, of which viral are the most ravaging for crops resulting in severe damages and economic losses worldwide. Since investigations on the incidence and distribution of viral diseases are an important step in developing diagnostic tools as well as appropriate control measures, the objectives of this thesis were the next:

1. Determine the incidence and distribution of viruses infecting cucurbits in one of the regions with more agronomic importance in the southeast of Algeria.
2. Analyze the genetic variability of tomato leaf curl New Delhi virus (ToLCNDV-ES) occurring on cucurbit plants in Algeria.
3. Evaluate whether ToLCNDV-ES can be transmitted through seeds in cucurbit species.

CHAPTER I

First report of cucurbit chlorotic yellows virus infecting cucumber and zucchini in Algeria

INTRODUCTION

The family *Cucurbitaceae* includes many fruit and vegetable species cultivated in temperate and tropical regions that supply essential vitamins and minerals to current diets in countries around the world. The most economically cultivated cucurbit crops in the Mediterranean region are cucumber (*Cucumis sativus* L.), zucchini (*Cucurbita pepo* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* Thumb.) and *Cucurpita* spp. These crops have become important income sources providing export and local consumption commodities in many Mediterranean countries including Algeria (Radouane et al., 2021). According to the Food and Agriculture Organization of the United Nations (FAO), during the year 2020 Algeria produced 2,286,809 tons of melon and watermelons on 61,000 ha, being the fifth producer worldwide. This country also produced 435,327 tons of zucchini on 15,558 ha, and 184,362 tons of cucumbers on 4,470 ha (FAOSTAT, 2023).

Increased area of cucurbits has led to the emergence of several viral diseases and over 30 viruses are currently threatening cucurbit crop production in the Mediterranean region (Desbiez et al., 2020; Hervé Lecoq & Desbiez, 2012; Radouane et al., 2021). The most common viruses that affect cucurbits in this region are the aphid-transmitted: watermelon mosaic virus (WMV), zucchini yellow mosaic virus (ZYMV), cucumber mosaic virus (CMV), cucurbit-aphid borne yellows virus (CABYV), Moroccan watermelon mosaic virus (MWMV) and papaya ring spot virus (PRSV). Melon necrotic spot virus (MNSV), mainly transmitted by the soil fungus *Olpidium bornovanus* (Riviere et al., 1989), and cucumber green mottle mosaic virus (CGMMV), transmitted by contact and seeds (Dombrovsky et al., 2017) are also found in several Mediterranean countries. Furthermore, whitefly-transmitted viruses such as cucurbit yellow stunting disorder virus (CYSDV), cucumber vein yellowing virus (CVYV), tomato leaf curl New Delhi virus (ToLCNDV), and cucurbit chlorotic yellows virus (CCYV) are currently emerging in several countries in the Mediterranean basin (Desbiez et al., 2020; Hervé Lecoq & Desbiez, 2012; Moriones et al., 2017).

In Algeria, studies in cucurbit crops to understand virus evolution and emergence have never been conducted. To date, only the aphid-transmitted viruses CMV, CABYV, ZYMV, and Algerian watermelon mosaic virus (AWMV), and recently the whitefly-transmitted ToLCNDV have been described infecting cucurbits (Kheireddine et al., 2019; Lecoq & Desbiez, 2012; Radouane et al.,

2021; Yakoubi et al., 2008). CABYV (*Luteoviridae, Polerovirus*) is a phloem restricted virus, which was first reported in France in 1992 (Lecoq et al., 1992) and later in numerous Mediterranean countries (Lecoq & Desbiez, 2012). CMV (*Bromoviridae*, *Cucumovirus*) was first reported in 1916 in the USA, and the virus has since spread to several countries. CMV is very common in cucurbit crops grown in temperate and Mediterranean areas (Lecoq & Desbiez, 2012). ZYMV (*Potyviridae*, *Potyvirus*) was first observed in zucchini plants in Italy in 1973 (Lisa, 1981), and then identified in all continents within a decade. In Algeria, this virus was detected in melon plants in 1989 (Belkhala and Lecoq, 1990). AWMV (*Potyviridae*, *Potyvirus*) is a virus closely related molecularly as well as biologically to MWMV, which has been found only in Algeria so far (Yakoubi et al., 2008). Finally, the presence of ToLCNV in Algeria was detected in 2018 in zucchini, melon and cucumber plants (Kheireddine et al., 2019).

CCYV is a virus species of the genus *Crinivirus* within the *Closteroviridae* family (Gyoutoku et al., 2009) implicated in cucurbit yellows disease (CYD). The typical symptoms of CYD, characterized by mottling, chlorosis and interveinal yellowing of leaves, are indistinguishable from those caused by nutrition deficiencies or by other criniviruses such as CYSDV or CABYV (Orfanidou et al., 2017a). Since its first identification in Japan in 2004 (Gyoutoku et al., 2009), CCYV has spread rapidly to various countries of Asia, including Taiwan (Huang et al., 2010), China (Gu et al., 2011), Philippines (Chang et al., 2021), South Korea (Kwak et al., 2021), India (Kumar et al., 2022), and Pakistan (Nouman et al., 2022); Middle East, including Iran (Bananej et al., 2013), Turkey (Orfanidou et al., 2017b), and Saudi Arabia (Shakeel et al., 2018); Africa, including Sudan (Hamed et al., 2011); Mediterranean basin, including Lebanon (Abrahamian et al., 2012), Israel (Luria et al., 2019), Jordan (Salem et al., 2020), Greece and Cyprus (Orfanidou et al., 2014), Egypt (Amer, 2015), Algeria (Kheireddine et al., 2020), and Spain (Chynoweth et al., 2021; Alfaro-Fernández et al., 2022). Further, CCYV was also reported in the New World in different states of the United States of America, including California (Wintermantel et al., 2019), Georgia (Kavalappara et al., 2021), Alabama (Mondal et al., 2022), and Florida (Jailani et al., 2022).

The CCYV genome consists of two single-stranded RNA segments designated as RNA1 and RNA2, which are 8607 nucleotides (nt) and 8041 nt in length, respectively (Okuda et al., 2010), being among the largest single-stranded positive-sense RNA viruses (Martelli et al., 2002). RNA1

encodes proteins involved in replication such as papain-like protease, methyltransferase, RNA helicase 1, and RNA-dependent RNA polymerase (RdRp) motifs. While RNA2 encodes genes implicated in virus movement, encapsidation, and transmission by *Bemisia tabaci* such as coat protein (CP), minor coat protein (CPm), a homolog of the cellular 70-kDa heat-shock protein (HSP70h), the 59-kDa protein, and several other small proteins (Okuda et al., 2010; Orfanidou et al., 2017a). The amino acid sequence similarities of CP, CPm, and HSP70h are used to assign the molecular criteria for species demarcation (Okuda et al., 2010).

CCYV infects systematically under natural conditions cucurbit crops such as melon, watermelon, cucumber, zucchini, and luffa, and numerous non-Cucurbitaceous and weed species, which may also be an important reservoir host for this virus. Besides, it infects many plant species, such as *Nicotiana benthamiana* and *Datura stramonium*, under experimental conditions (Okuda et al., 2010; Orfanidou et al., 2017a), and its experimental host range extends to at least four botanical families (*Asteraceae, Chenopodiaceae, Convolvulaceae,* and *Solanaceae*) (Okuda et al., 2010; Orfanidou et al., 2017). In general, symptoms develop between 3 to 4 weeks post infection causing severe chlorotic yellow lesions that usually progress from older basal leaves to upper new foliage, accompanied with brittleness of the leaves. As a result, the whole plant will turn yellow, leading to a reduction in its photosynthetic capacity which leads to yield reduction, presenting fewer and smaller fruits (Maliogka et al., 2019). The yellowing symptoms are usually indistinguishable to those occasioned by nutritional deficiencies or by infections with other criniviruses, such as CYSDV or beet pseudo yellows virus (BPYV), or with the begomovirus squash leaf curl virus (SLCV) making the detection process more elaborate (Orfanidou et al., 2019). As point out by Abrahamian et al., (2015), in single infections, the symptoms induced by CCYV are less severe than those induced by the crinivirus CYSDV. Symptoms induced by dual infections with CYSDV or SLCV in any combination were like those of singly infected plants, while triple infections resulted in higher increase in symptom severity and resulted in greater yield reductions in contrast to a single or double infection.

CCYV shares a similar transmission pathway with CYSDV, because it is transmitted in a semipersistent manner by the Mediterranean (MED, formerly biotype Q) and Middle East-Asia Minor 1 (MEAM1, formerly biotype B) of the sweet potato or tobacco whitefly, *Bemisia tabaci* (Gennadius). Studies on comparative transmission of CCYV by both species revealed that MED is

more effective in transmission of the virus than MEAM1 (Lu et al., 2017). CCYV is a virus phloem restrictive, and *B. tabaci* MED whiteflies require a minimum 1 h of acquisition access period (AAP) to successfully acquire CCYV virions. The transmission efficiency depends mainly on the number of vectors (Li et al., 2016). It is presumed that criniviruses such as CCYV are held in the foreguts of whiteflies as a consequence of their semipersistent transmission traits (Li et al., 2016). For CCYV detection, antibodies prepared against the recombinant coat proteins of CCYV have been used by using immunoassays such as tissue blot (TBIA) and ELISA (Kubota et al., 2011). Reverse transcription polymerase chain reaction (RT-qPCR) and more recently, reverse transcription loop-mediated isothermal amplification (RT-LAMP) methods have also been developed for sensitive and rapid detection of CCYV in plant hosts or in whitefly vectors (Kil et al., 2015; Wang et al., 2014; Zhao et al., 2015).

To secure horticultural production, effective strategies to control viral diseases are important for farmers. One of the key components to develop control strategies is to identify the type and distribution of viral diseases in horticultural crops. However, such information in Algeria is very limited. The objective of this study was to identify and determine the prevalence and distribution of the most common viruses on cultivated cucurbit plants (zucchini, cucumber, and melon) in some regions of the southeast of Algeria during 2018 and 2019. Detection was carried out by molecular nonradioactive hybridization using a specific single probe for each viral sequence (one membrane for each virus detection). Reverse transcription-polymerase chain reaction (RT-PCR) or PCR and DNA sequencing was used on positive samples, to determine diversity of the virus detected.

MATERIAL AND METHODS

Field survey and sample collection

The survey was conducted in June of 2018 and 2019. Zucchini, melon, and cucumber plants showing virus-like symptoms such as foliar yellowing and vein clearing, were observed in the agricultural region of Biskra, southeast of Algeria. The presence of whiteflies was observed in all the investigated fields in the region. A total of 68 symptomatic leaf samples (42 from zucchini, 18 from cucumber and 8 from melon) were collected from four agricultural areas in the city of Biskra and its surroundings (Biskra, El ghrouss, Sidi okba and Zribat eloued), and 13 asymptomatic

samples from pumpkin (*Cucurbita maxima*) were collected from a field located in Sidi-Okba (Table 1). Samples were directly pressed onto different nylon membranes for tissue-printing analysis and then placed in plastic bags and dried using anhydrous silica gel and sent to the Centro de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV-UPV, Valencia, Spain). Two replicates from each sample were prepared in Eppendorf tubes and stored at -80°C for virus diagnosis.

Table 1. Table representing the collected samples from Algeria dating from 2018 to 2019.

Crop	Region of collection	Year of collection	No. of samples tested	No. of samples infected with CCYV
Zuchinni (*Cucumis Pepo* L.)	Sidi-Okba	2018	30	2
	Leghrouss	2019	6	1
	Biskra	2019	6	0
Cucumber (*Cucumis sativus* L.)	Sidi-Okba	2018	12	2
	Sidi-Okba	2019	6	0
Melon (*Cucumis melo* L.)	Zribet el oued	2018	8	0
Pumpkin (*Cucurbita maxima* D.)	Sidi-Okba	2019	13	0
		Total	**81**	**5**

Virus detection in tissue printing analysis

For tissue printing analysis, freshly cut leaf petioles from symptomatic and asymptomatic samples were directly pressed onto positively charged nylon membranes (Hybond-N, Amersham). Membranes were air dried, fixed by UV irradiation (700 × 100 mJ/cm^2) in a cross-linker, and hybridized with individual digoxigenin-labeled RNA riboprobes, corresponding to the CP gene of the next whitefly transmitted viruses: the begomovirus ToLCNDV (family *Geminiviridae*), the ipomovirus CVYV (family *Potyviridae*), and the criniviruses CCYV and CYSDV (family *Closteroviridae*). Positive and negative controls were also added into the membranes. To construct the riboprobes, total nucleic acids from zucchini plants infected with each virus (CVYV, CCYV, and CYSDV) were extracted from 0.1 g of fresh leaf tissue using TRIzol reagent (Thermo Fisher

Scientific, Carlsbad, CA, USA) following the manufacturer's protocol. The design of the primers was based to amplify the complete CP gene from each virus. Reverse transcription and PCR reactions were carried out using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen) following the manufacturer's instructions. Amplified products corresponding to the three viral CP genes were cloned individually into a pTZ57R/T vector (MBI Fermentas). For each virus, CP clones in the orientation pTZ/CP:minus-strand were selected, linearized with the appropriate restriction enzyme and the riboprobes were generated by transcription with T7 RNA polymerase from the corresponding pTZ/CP:minus-strand, following the manufacturer's instructions (Roche Diagnostics). The riboprobe to detect ToLCNDV had previously been synthesized in the laboratory following the same strategy. Prehybridizations, hybridizations and washing of the membranes were conducted as described previously (Aparicio et al., 2009; Sáez et al., 2021). Hybridizations with the single probes were conducted at 60°C for DNA-RNA hybridizations (to detect the presence of ToLCNDV) and at 68°C for RNA-RNA hybridizations (to detect the presence of CVYV, CCYV, and CYSDV). Chemiluminiscent detection using CSPD reagent as substrate was performed as recommended by the manufacturer (Roche Diagnostics). Films were exposed to the membranes at room temperature for 30–60 min.

CCYV detection by reverse transcription polymerase chain reaction (RT-PCR)

To confirm the presence of CCYV by RT-PCR in the collected samples that tested positive in the tissue printing analysis, total RNA extracts were obtained with TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer's protocol. The resulting pellet was resuspended in 50 µL of sterile deionized water and stored at -80°C. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted with sterile deionized water to a final concentration of 1.5 µg/mL. RNAs were reverse-transcribed by incubation at 42°C for 60 min, in a reaction mixture (20 µl) containing: 1 x RT buffer (50 mM Tris-HCl, pH 8.3; 50 mM KCl; 4 mM MgCl2; and 10 mM DTT); 1 mM of each of the four dNTPs; 1 µL of random hexamer primer; 20 U of RiboLock RNase inhibitor (Thermo Scientific, U.S.A); and 1 mL (200 U) of RevertAid RT (Thermo scientific, U.S.A). For synthesis of second-strand cDNA, an aliquot (1/20) of this preparation was PCR-amplified in a reaction mixture (50 µl) containing: 1 x PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; and 1.5 mM $MgCl_2$); 1 µL of DreamTaq™ DNA polymerase (5 U/µL) (Thermo Scientific, U.S.A.); 0.2 mM dNTPs; and 0.2 µM of each

CCYV forward and reverse primers. Based on the complete CCYV sequence (accession numbers AB523788 and AB523789 for RNA1 and RNA2, respectively) (Okuda et al., 2010), three pairs of primers were designed. RdRp-up (5´-CCTAATATTGGAGCTTATGAGTAC-3´)/RdRp-do (5´-CATACACTTTAAACACAACCCCCT) was expected to amplify a portion of the RNA dependent RNA polymerase (RdRp) region (754 bp) of RNA1; whereas Hsp-up (5´-TGCGTATGTCAATGGTGTTATG-3´)/Hsp-do (5´-ATCCTTCGCAGTCAAAAACC-3´) and CP-up (5´-ATGGAGAAGACTGACAATAAAC-3´)/CP-do (5´-TTATTTACTACAACCTCCCGGTGC-3´) were anticipated to amplify, respectively, a portion of the heat shock protein 70 homologue (Hsp70h) region (462 bp) and the complete coat protein (CP) gene (753 bp) of RNA2. The PCR cycling conditions consisted of an initial step at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 50 s, with a final extension step at 72°C for 10 min. The resulting PCR products were analyzed by electrophoresis in 1% agarose gels in TAE buffer, followed by ethidium bromide staining according to Sambrook et al., (1989).

Sequence analysis

PCR products of the expected size were directly sequenced in both directions by Sanger sequencing at the Instituto de Biología Molecular y Celular de Plantas (Universitat Politècnica de València, Valencia, Spain). The nucleotide sequence of the inserts was determined with an ABI PRISM DNA Sequencer 377 (Perkin-Elmer). For phylogenetic assessment, the sequences were compared with sequences available at NCBI GenBank using BLAST analysis. Multiple sequence alignments were carried out using Clustal Omega. The Phylogenetic trees were generated with MEGA software using the Neighbour-Joining method (Kumar & Gadagkar, 2000) and a Bootstrap value of 1000 replicates.

RESULTS

Virus detection by tissue printing hybridization

During virus surveys in the growing season of 2018 and 2019, symptoms of foliar yellowing and vein clearing (Figure 1A and B), were observed in zucchini, melon, and cucumber plants in

four independent areas of Biskra, southeast of Algeria (Figure 1C). In all cases, *Bemisia tabaci* populations were observed on infected plants (data not shown). The symptomology observed was like that caused by whitefly-transmitted virus. When the 81 collected leaf samples were tested by tissue printing for the presence/absence of ToLCNDV, CYSDV, CVYV and CCYV, most of them were positive for ToLCNDV (see chapter II), while five (6.2%) were positive for CCYV (three from zucchini and two from cucumber). None of the samples tested positive for CVYV and CYSDV (Figure 2 and Table 1).

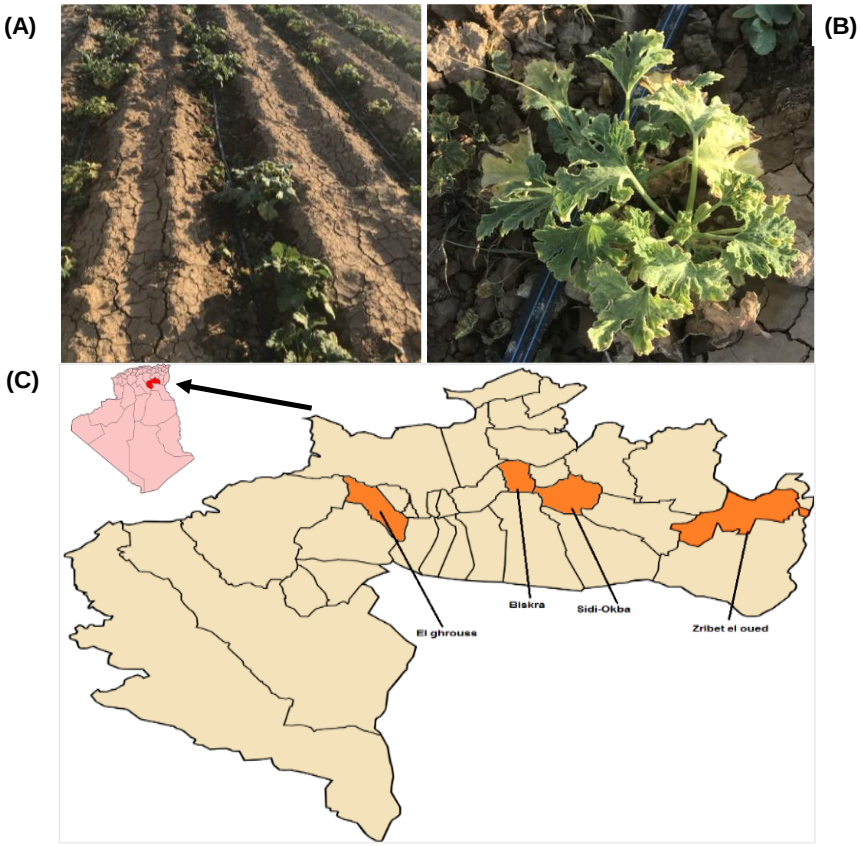

Figure 1. (A) General view of a field with zucchini (*Cucurbita pepo*) plants in Biskra. (B) Zucchini plant showing virus-like symptoms. (C) Locations of the collection fields in the geographical map of Biskra (Algeria).

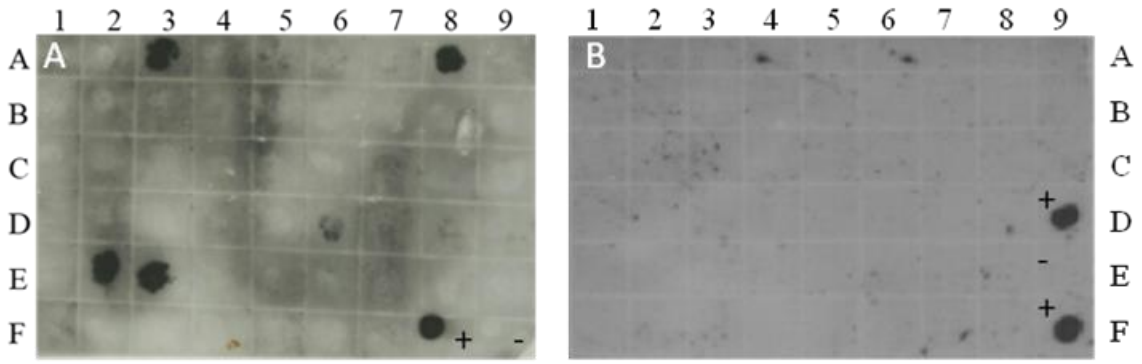

Figure 2. Tissue-printing approach for the detection of CCYV and CYSDV. Freshly cut leaf petioles from cucurbit plants were directly pressed onto nylon membranes and hybridized with a riboprobe corresponding to the complete CP gene of the CCYV (A) and CYSDV (B). (A) Positive (F8) and negative (F9) controls were included. Samples A3, A8, D6, E2 and E3 were CCYV-infected. (B) Positive (D9 and F9) and negative (E9) controls were included. None of the samples was CVYV-infected.

CCYV was detected in all cases in mixed infections with ToLCNDV, which was also recently reported for the first time in this region (Kheireddine et al., 2019). This mixed CCYV-ToLCNDV infection occurred in one sample from El ghrouss (of zucchini) and in four samples from Sidi-Okba (two of zucchini and two of cucumber). Although the number of samples collected in the four areas and in the three symptomatic crops varied, the high incidence of CCYV was found in Sidi-Okba, both in cucumber and zucchini samples. In contrast, none of the melon samples collected were positive for CCYV. Mixed infection with two or more viruses is a common phenomenon in samples collected from the field. Although plants infected with multiple viruses generally display diverse symptoms, no differences were observed between symptoms shown by plants only infected with ToLCNDV or plants infected with the combination ToLCNDV and CCYV (Figure 3). The results show that relying on symptoms alone is insufficient for identifying the viruses present in a plant. Additionally, to increase the yield and quality of cucurbits in this area of Algeria, strategies to manage multiple virus infections are required.

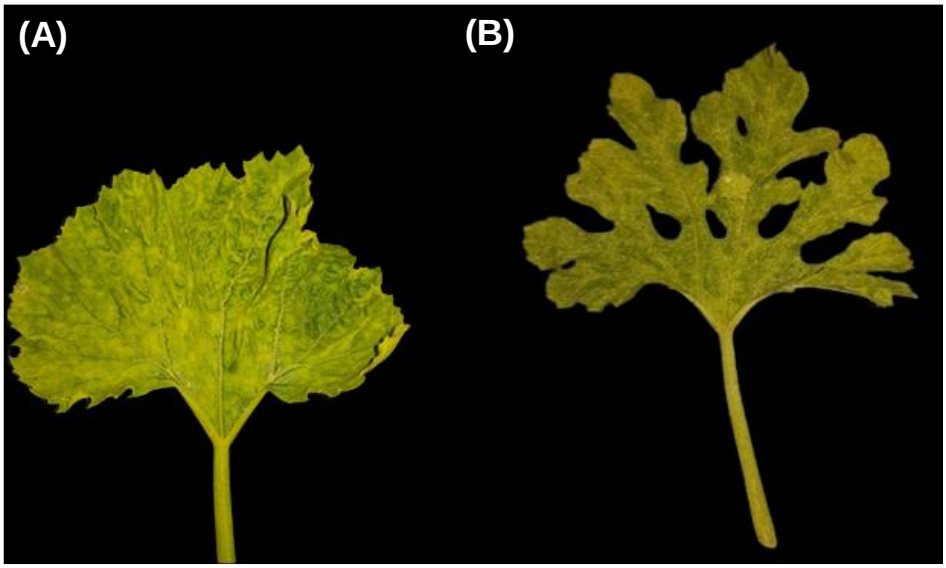

Figure 3. Foliar yellowing and vein clearing on zucchini leaf (A) doubly infected with the virus cucurbit chlorotic yellows virus (CCYV) and tomato leaf curl New Delhi virus (ToLCNDV), (B) single infection with ToLCNDV.

CCYV detection by RT-PCR and sequencing

To confirm CCYV identification, total RNA extracts were obtained with TRIzol reagent from the original zucchini (three) and cucumber (two) samples and from asymptomatic samples (two) and reverse transcribed using random primers. Based on the complete CCYV sequence (accession numbers AB523788 and AB523789 for RNA1 and RNA2, respectively) (Okuda et al., 2010), three pairs of primers were designed to amplify a portion of the RNA dependent RNA polymerase (RdRp) region (754 bp) of RNA1, and two portions of the RNA2; a portion of the heat shock protein 70 homologue (Hsp70h) region (462 bp) and the complete coat protein (CP) gene (753 bp). PCR products of the expected sizes were obtained from the five symptomatic samples, but not from the asymptomatic controls (Figure 4).

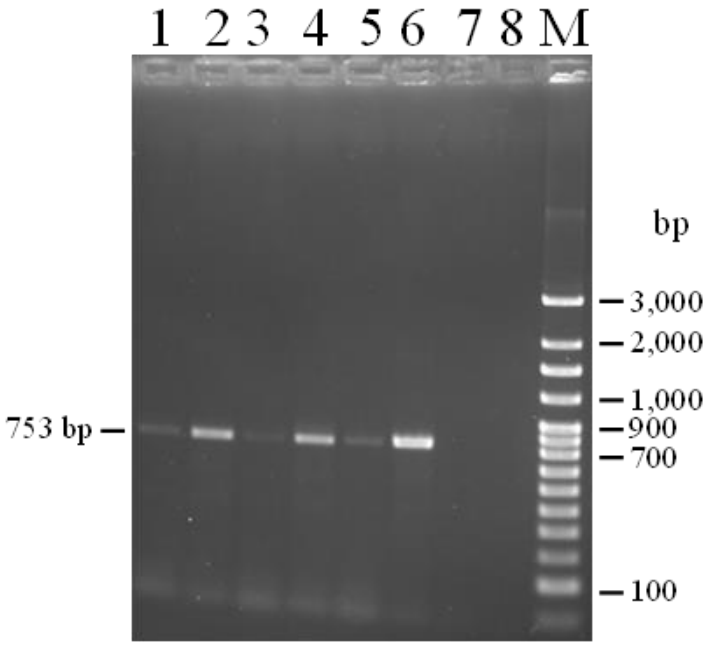

Figure 4. RT-PCR detection of CCYV using primers CP-up/CP-do in the five plants (lanes 1-5) that were positive for CCYV by tissue-printing. Lane 6 positive control from a CCYV-infected plant and lanes 7-8 negative controls from asymptomatic plants. M, molecular size marker.

BLASTn analysis indicated that the five symptomatic samples shared identical amplicon sequences, and therefore only the sequences of a CCYV isolate from zucchini (CCYV-Sidi) were deposited in GenBank under the accession numbers MN529558 (RdRp), MN529559 (Hsp70h) and MN529560 (CP). BLASTn analysis of the complete CP (segment RNA2) showed high nucleotide identity, greater than 99.5% with isolates from different geographical origins and hosts such as China (KY400633-melon, MZ392423-cucumber, MT048669-zucchini, KJ735450-*Luffa cylindrica*, MZ542496-*Rehmannia glutinosa* and ON101252-*Dioscoreae rhizome*), Greece (LT716000-cucumber, LT716003-*Chenopodium album*, and LT716004-*Ecballium elaterium*), Taiwan (JF502222-melon), Israel (MH477612-watermelon), Japan (AB523789-melon), Georgia (MW685461-squash, MW629380-melon, MW685456-cucumber, and OM489401-*Raphanus raphanistrum*), Florida (MZ325848-watermelon), California (MW680158-melon), Texas (MW584336-melon), Sudan (JF807053-melon), South Korea (OP921036-pumpkin, MW033301-cucumber, OP912401-watermelon, and OP912397-melon), Spain (OK562588-watermelon and OK562589-zucchini), Saudi Arabia (KT946810-cucumber), and Lebanon (JX014262-cucumber); about 98% with two Saudi Arabian isolates (KT946809 and KT946816, both from cucumber); and

about 95% with one Indian (MZ318672 from pumpkin) and three Iranian isolates (KC577201 and KC577202 from melon and KC577203 from cucumber). The partial CP sequence of two Turkish isolates from melon (MH883789 and MK129538) also showed nucleotide identity of about 95%. These genetic relationships were confirmed with a phylogenetic tree (Figure 5) where the Algerian isolate clustered in group I together with the most of isolates, whereas a second group (group II) consisted of the Indian and Iranian isolates. A further subdivision was observed, as the Saudi Arabian isolates formed a different clade (subgroup Ib) from the other sequences (subgroup Ia) (Orfanidou et al., 2017a).

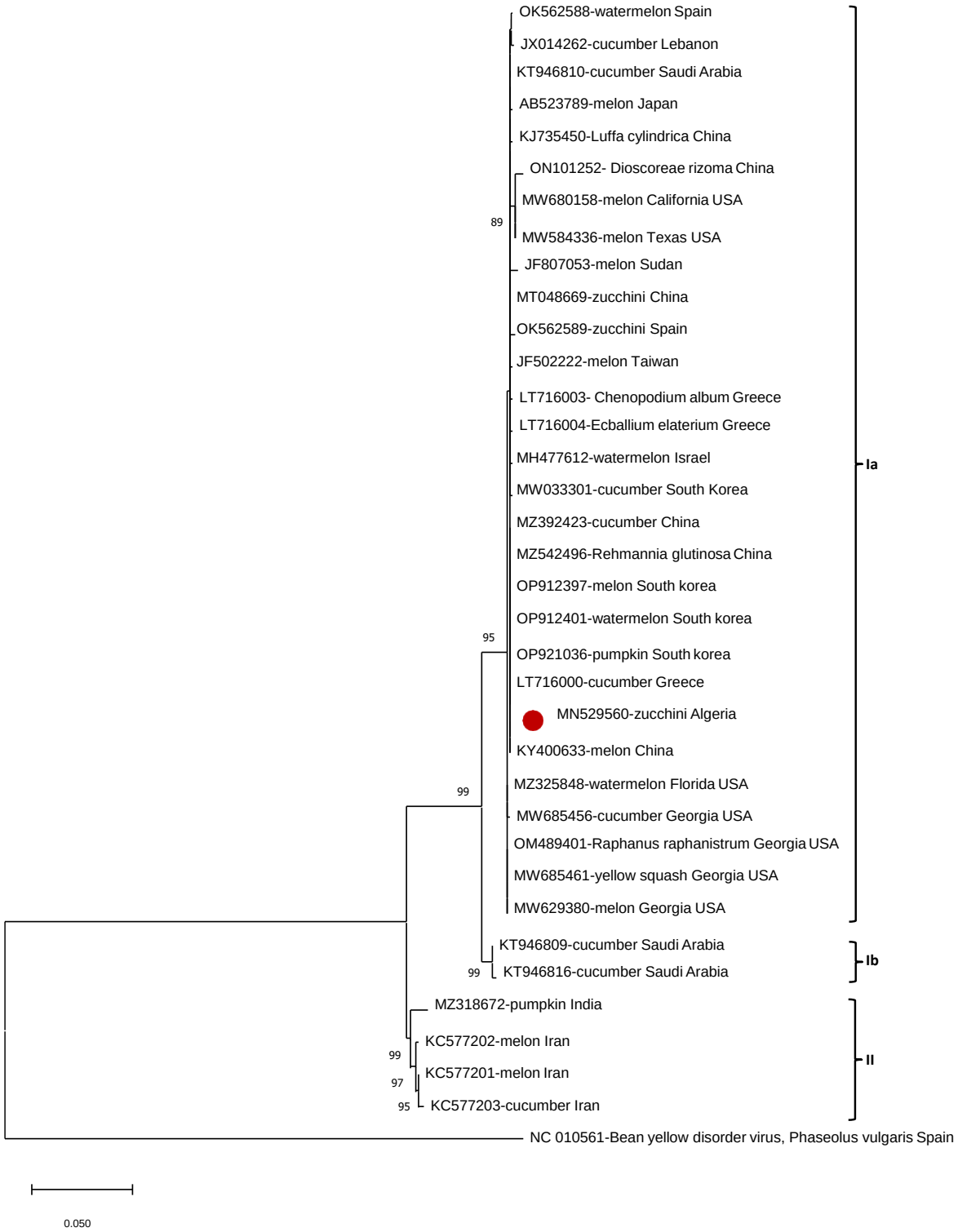

Figure 5. Unrooted maximum likelihood phylogenetic tree of the entire coat protein gene sequence of cucurbit chlorotic yellows virus determined in this study (marked with a red circle) and published in the NCBI databases from different geographic origins and hosts. All isolates are reported with the accession number, host, and country origin. Been yellow disorder virus (NC_010561) is used as outgroup. Branch lengths are proportional to the genetic distances and bootstrap values are indicated.

Comparative analysis of the partial Hsp70h sequence (segment RNA2) also showed nucleotide identity greater than 99% of the Algerian isolate with isolates from China, Taiwan, Greece, South Korea, Japan, Georgia, Florida, California, Sudan, Egypt, Israel, Lebanon, Philippines, Saudi Arabia and one from Iran (isolate MH660532 from melon), and about 94% identity with six Iranian isolates; one from cucumber (KC559449) and five from melon (MH939354, MH939355, MH939357, MH939358 and MH939359). When these Iranian isolates were included in the phylogenetic analysis formed a different clade from all CCYV sequences (Figure 6).

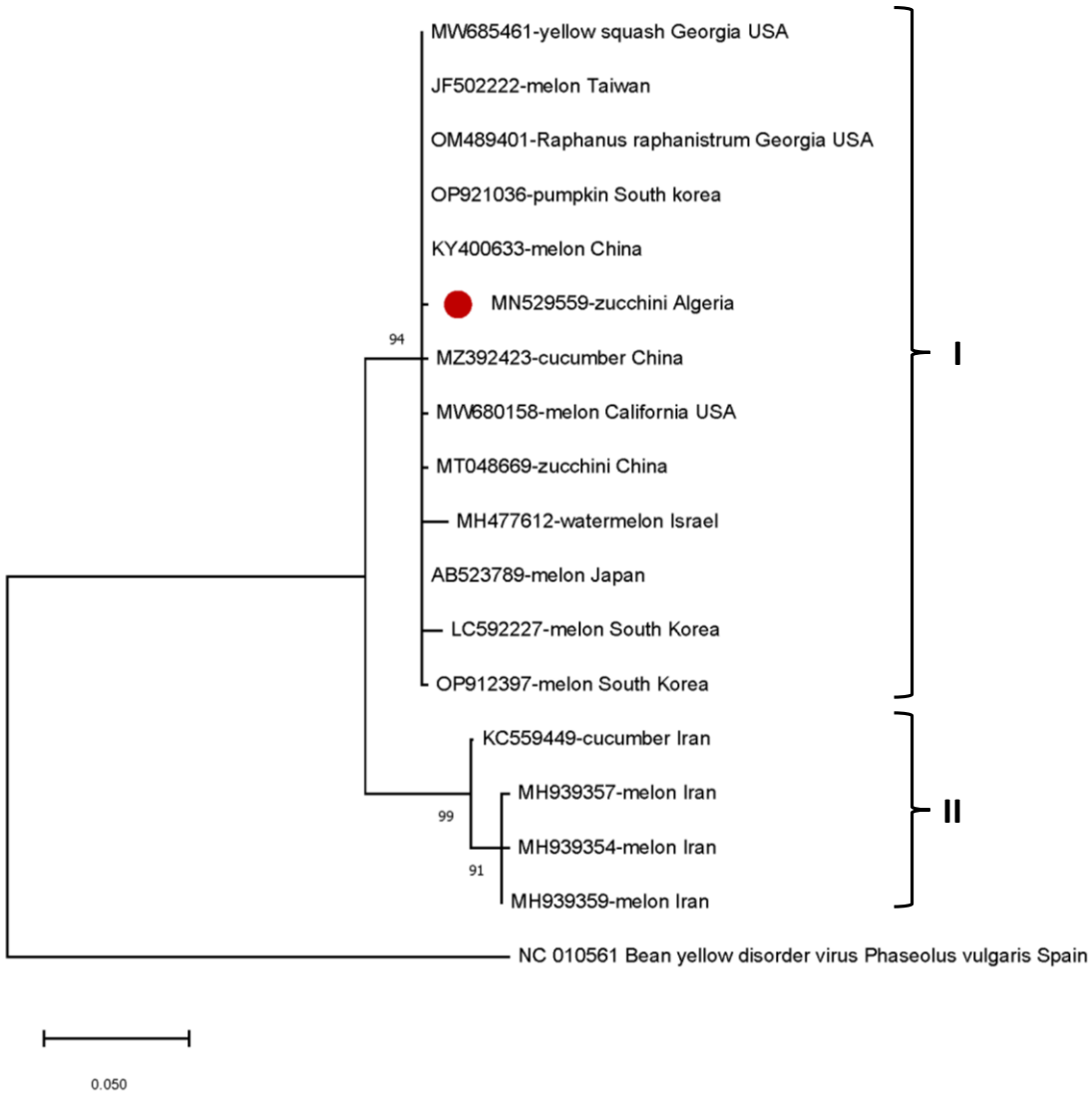

Figure 6. Unrooted maximum likelihood phylogenetic tree of the partial Hsp70h sequence (segment RNA2) of cucurbit chlorotic yellows virus determined in this study (marked with a red circle) and published in the NCBI databases from different geographic origins and hosts. All isolates are reported with the accession number, host, and country origin. Been yellow disorder virus (NC_010561) is used as outgroup. Branch lengths are proportional to the genetic distances and bootstrap values are indicated.

BLASTn analysis of the partial sequence of the RdRp gene (segment RNA1) from Algeria showed nucleotide identity greater than 99% with most worldwide CCYV isolates, about 98% with two Saudi Arabian isolates from cucumber (LM653109 and LM653110), about 95% with one Pakistani isolate from cucumber (MN723535), and about 91% with one Indian isolate from pumpkin (MZ318673). These genetic relationships were confirmed with a phylogenetic tree inferred with the Maximum Likelihood method with 1000 bootstrap replications using Kimura-2 parameter (Figure 7).

Figure 7. Unrooted maximum likelihood phylogenetic tree of the partial RNA-dependent RNA polymerase gene sequence (segment RNA1) of cucurbit chlorotic yellows virus determined in this study (marked with a red circle) and published in the NCBI databases from different geographic origins and hosts. All isolates are reported with the accession number, host, and country origin. Been yellow disorder virus (NC_010561) is used as outgroup. Branch lengths are proportional to the genetic distances and bootstrap values are indicated.

DISCUSSION

Cucurbit chlorotic yellows virus (CCYV) belongs to the genus *Crinivirus* and is part of a complex of whitefly-transmitted viruses that cause yellowing disease in cucurbits. The disease has rapidly spread around the world as a consequence of the whitefly population expansion and the movement of infected propagative material. In the last thirty years, a large incidence of whiteflies and viruses transmitted by them, including CCYV, CVYV, CYSDV and ToLCNDV, have been observed in the Mediterranean basis, especially during spring and summer (Desbiez et al., 2020; Hervé Lecoq & Desbiez, 2012; Moriones et al., 2017; Radouane et al., 2021). CCYV is often found in co-infections with other whitefly-transmitted viruses, which induce similar yellowing symptoms and share the same vectors (Abrahamian et al., 2013; 2015). Thus, CCYV has been found in Greece and other countries infecting cucumber, watermelon and melon both in single and mixed infections with CYSDV (Orfanidou et al., 2014). In our case, the presence of CCYV was only detected in mixed infection with ToLCNDV, which was found in all the five positive samples collected from two distinct sites. However, attempts to detect CYSDV or CVYV in samples were unsuccessful. It would be interesting to check if there is a synergistic or antagonistic interaction between these two viruses (CCYV and ToLCNDV) that may affect the spread and symptoms between crops.

CCYV causes severe economic losses in cucurbit crops from East Asia: China, Japan, Taiwan, Phillipines, and South Korea; North America (the U.S. states of Florida, California, Texas, Georgia, and Alabama); the Middle East: Sudan, Saudi Arabia, and Iran; and the Mediterranean basin: Egypt, Israel, Lebanon, Turkey, Greece, Algeria, and Spain. Also, natural infection of CCYV has been detected in field samples of several annual and perennial weed species from at least 12 families such *Amaranthaceae, Asteraceae, Boraginaceae, Brassicaceae, Caparaceae, Chenopodiaceae, Convolvulaceae, Cucurbitaceae, Fabaceae, Malvaceae, Solanaceae* and *Zygophyllaceae* (Okuda et al., 2010; Orfanidou et al., 2017a). Furthermore, the amount of CCYV accumulated in weeds like in wild radish has been shown to be sufficient for efficient acquisition and successful transmission of the virus to recipient hosts. Consequently, wild radish and possible other weeds are likely good reservoirs for virus survival when the cucurbit crops are not present in the field (Kavalappara et al., 2022) and they play an important role in plant virus epidemiology (Orfanidou et al., 2017a). In the case of viruses not transmitted by seed, such as criniviruses, weeds are especially important for virus spread and overwintering between crop seasons (Orfanidou et al., 2017a).

RNA viruses have a potential for high genetic variation due to the absence of proofreading activity of their RNA replicases, their large populations and rapid replication (Domingo & Holland, 1997; Holland et al., 1982). However, comparative analysis of CCYV sequences have revealed limited genetic diversity and the variability within CCYV isolate sequences from cucurbits was not different from that of isolates from weed species. In general, criniviruses show limited genetic diversity as several studies on tomato chlorosis virus (ToCV), tomato infectious chlorosis virus (TICV), CYSDV, and beet pseudo-yellows virus (BPYV) from different hosts revealed, even when virus isolates originated from geographically distant locations (Akhter et al., 2016; Orfanidou et al., 2014; 2017a; Orílio & Navas-Castillo, 2009; Rubio et al., 2001). In the present study, determination of the complete CP, and partial RdRp and Hsp70 sequences of an Algerian CCYV isolate was conducted to unveil the evolutionary relationships with the published isolates in EMBL-EBI database. Sequencing analysis of five Algerian samples that tested positive to CCYV, originated from cucumber and zucchini, revealed 100% homology both in nucleotide and amino acid levels. The phylogenetic analysis based on the complete CP and partial RdRp nucleotide sequence showed that the Algerian isolate clustered into group I together with the majority of the published CCYV isolates. The genetic variation within this group was very low (nucleotide identity higher than 99.5%) despite their extensive and discontinuous geographical distribution, plant species (cultivated or weeds) and year of collection. Spatial and temporal genetic stability have also been reported for citrus tristeza virus (CTV), another member of the family *Closteroviridae,* where sequences separated in time and space were essentially identical (Albiach-Martí et al., 2000). The main group was further divided into 2 subgroups; subgroup Ia containing most of isolates and subgroup Ib including only two isolates from Saudi Arabia (Figures 5 and 7), which showed a nucleotide identity about 98%. In RNA2 segment a second group (II) was observed, which consisted of one isolate from India and three from Iran (phylogenetic tree of the entire CP gene sequence, Figure 5) and the four isolates from Iran (phylogenetic tree of the partial Hsp70h gene sequence, Figure 6), which were clearly most distant and showed nucleotide identity between 94-95%. This population division was previously observed for CCYV (Orfanidou et al., 2017a) and for CYSDV when a portion of the HSP70 homologue gene was analyzed (Rubio et al., 2001).

In RNA1 segment, a similar population division was found when a portion of the CCYV RdRp gene was analyzed: subgroup Ia containing most of isolates and subgroup Ib including only two isolates from Saudi Arabia (showing a nucleotide identity about 98%) and group II with one

Pakistani and one Indian isolate with nucleotide identity between 91 and 95%. Thus, from the genetic viewpoint, the CCYV isolates collected until now could be divided in two groups or subpopulations where most isolates clustered in one main group, while isolates from Middle East including Iran, India, Turkey, and Pakistan which showed about 5% of variability, clustered in a different branch of the tree. This analysis indicates a poorly genetic variability present within CCYV species. This low genetic divergence could be due to the recentness of this virus that did not face major changes for the present time. However, given that in Middle East the number of isolates analyzed is very low, we must consider the possible existence of more diverged isolates in this area.

To conclude, the spread of CCYV to neighboring countries in Africa and other countries can be expected due to the large whitefly vector populations and its rapid dissemination worldwide. Clearly, stringent management is needed to minimize the losses caused by this virus. To our knowledge, this is the first report of CCYV in Algeria, and its first reported presence in mixed infection with ToLCNDV.

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CHAPTER II

First report and the genetic variability of tomato leaf curl New Delhi virus occurring on cucurbit plants in Algeria

INTRODUCTION

Cucurbits are cultivated from temperate to tropical climatic regions of the world since ancient times and are essentials to human nutrition. Three major cucurbit genera are cultivated worldwide: *Cucumis* (cucumbers, melons), *Cucurbita* (pumpkins, squash, gourds), and *Citrullus* (watermelons). Diseases and pests are one of the major limiting factors for cucurbits production, of which viral are the most ravaging for crop plants resulting in severe damages and economic losses worldwide, mainly due to the rapid evolution of viruses through mutations and genetic recombination (Panno et al., 2019). Cucurbit crops are affected by more than 70 virus species, belonging to the major plant virus genera and transmitted by a diversity of vectors (Lecoq and Katis 2014). Among the virus diseases that cause major yield losses, and hat have emerged in the Mediterranean basin over the last decades, most are caused by viruses transmitted by the whiteflies of the species *Bemisia tabaci* (Navas-Castillo et al., 2011). Among them, tomato leaf curl New Delhi virus (ToLCNDV, genus *Begomovirus*, family *Geminiviridae*) causes significant yield losses in crop plants throughout the tropical and subtropical parts of the world. ToLCNDV consists of two circular single-stranded DNA molecules of about 2.7 kb in size each, referred to as DNA-A and DNA-B, which encodes all virus functions required for DNA replication, gene expression, plant movement and insect transmission (Hanley-Bowdoin et al., 1999). DNA-A includes six ORFs; the coat protein (CP), which forms the viral capsid and mediates vector transmission and the AV2 protein, which function as anti-defence protein to inhibit post-transcriptional gene silencing (PTGS) in the virion-sense orientation, and the replication-associated protein (Rep; a rolling circle replication initiator protein), the replication enhancer protein (REn), the transcriptional activator protein (TrAP) and the C4 protein (counteracts PTGS) in the complementary-sense orientation, which are responsible for viral encapsidation, replication and production of virions. In the other hand, DNA-B contains two ORFs; the nuclear shuttle protein (NSP) and the movement protein (MP) in the virion- and complementary-sense, respectively. While DNA-A is able to replicate autonomously, DNA-B is dependent on DNA-A for its replication and it is required for systemic infection and symptoms expression, nuclear localization, and systemic movement (Hanley-Bowdoin et al., 2013; Sanderfoot & Lazarowitz, 1996). In many cases they are accompanied by circular ssDNA alphasatellites and betasatellites, the replicate and spread via the helper virus. Betasatellites are known to encode for βC1 protein, which is associated

with ToLCNDV infections (counteracts with transcriptional gene silencing) (Jyothsna et al., 2013; Venkataravanappa et al., 2019).

ToLCNDV was first reported on tomato (*Solanum lycopersicum* L.) in India in 1995 (Srivastava et al., 1995) and subsequently, the virus was reported in other Asian countries on several plant species mainly crops of the *Solanaceae* and *Cucurbitaceae* families (Sohrab et al., 2003; Ito et al., 2008; Singh et al., 2009; Chang et al., 2010; Roy et al., 2013; Moriones et al, 2017, Zaidi et al., 2017; Zeng et al., 2023; Gu et al., 2023). The virus also affects crops of other plant families, such as *Malvaceae, Papaveraceae, Fabaceae,* and *Acanthaceae* (Srivastava et al., 2016; Zaidi et al., 2016; Jamil et al., 2017; Sundararaj et al., 2019). In addition, numerous weed species have been found to be hosts of the virus (Moriones et al., 2017; Zaidi et al., 2017; Juárez et al., 2019), being able to act as reservoirs during the whole year. ToLCNDV was confined to the Asian continent until 2012, when a new strain of the virus, named ToLCNDV-ES, was detected in different Mediterranean and European countries, first in Spain (Juárez et al., 2014), and later in Tunisia (Mnari-Hattab et al., 2015), Italy (Panno et al., 2016), Morocco (Sifres et al., 2018), Greece (Orfanidou et al., 2019), Algeria (Kheireddine et al., 2019) and more recently in Portugal, Estonia (Just et al., 2022). The ToLCNDV-ES strain evolved from ToLCNDV isolates of Asian origin and adapted to infect cucurbit crops, causing important economic damage (Panno et al., 2016; Moriones et al., 2017). In zucchini, the virus causes severe stunting of plants, which exhibit upward and downward curling of the leaves, severe mosaic, and fruit skin roughness (Juárez et al., 2014). Infected plants often present partial or complete yield loss and fruits with lower market value. The virus is naturally transmitted by the whitefly *Bemisia tabaci* biotypes MEAM1 and MED in a persistent-circulative manner, targeting the phloem of infected plants (Rosen et al., 2015), although some isolates are also mechanically sap-transmitted to different hosts (Usharani et al., 2004; Chang et al., 2010b; Sohrab et al., 2013; López et al., 2015).

To control the spread of begomoviruses, most affected countries focus on vector management measures. However, this strategy is not effective on the long term since other variants have to be taken in consideration, such as the occurrence of multiple infections of several viruses, as well as genetic recombination events and mutations that will ultimately give rise to new virus strains that are more resistant and adaptive to changing environmental conditions (Román et al., 2019). Previous studies have suggested that the mutation and the recombination are key factors to the evolution of geminiviruses, which enable viruses to colonize new niches at a higher rate, leading

to epidemic outbreaks (Duffy et al., 2008; Lefeuvre and Moriones, 2015; Seal et al., 2006). Something similar has happened with the emergence of the isolate ToLCNDV-ES, representing a typical case of a founder effect associated with a population bottleneck during the transmission of ToLCNDV to a new area (Fortes et al., 2016; Moriones et al., 2017). Accordingly, all ToLCNDV isolates characterized from the Mediterranean Basin are genetically very homogeneous and clearly different from Asian virus populations. Similar cases of low genetic diversity have been previously identified in other begomoviruses, where the genetic variation was restricted in genomic regions regardless of geographical, temporal agro-ecological and host differences (García-Andrés et al., 2007; Sánchez-Campos et al., 2007). Therefore, to control ToLCNDV spread, genetic resistance remains the most promising strategy. In cucurbits, resistance to ToLCNDV has been described in sponge gourd (*Luffa cylindrica* M. Roem.) (Islam et al., 2010), in melon (Román et al., 2019; Sáez et al., 2017; López et al., 2015), in *Cucurbita moschata* (Sáez et al., 2016, 2020), and more recently in cucumber (Sáez et al., 2021).

In a survey for cucurbit viruses in Algeria, several cucurbit plants had conspicuous virus disease-like symptoms. ToLCNDV was detected by tissue printing and polymerase chain reaction (PCR), in some cases in complex with the crinivirus CCYV. This is the first report of ToLCNDV in Algeria (Kheireddine et al., 2019). Besides, we describe here its genetic variability and phylogenetic relationship with other isolates reported from different parts of the world.

MATERIALS AND METHODS

Survey and samples collection

Surveys were carried out in 2018, 2019 and 2020 at four agricultural regions of Biskra, which are Leghrouss, Sidi Okba and Zribet el oued (Figure 1), and where cucurbit plants showing virus-like symptoms such as vein clearing, foliar yellowing, leaf deformation and curling (Figure 2) were observed. A total of 95 leaf samples of zuchinni (*Cucurbita pepo* L. subsp. *pepo*), melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.) and squash (*Cucurbita maxima* Duchesne) (Table 1) were collected from open fields and greenhouses of these regions. The collected leaves were dried and preserved in silica gel until their arrival to the laboratory, where they were immediately frozen and stored at -80°C for analysis.

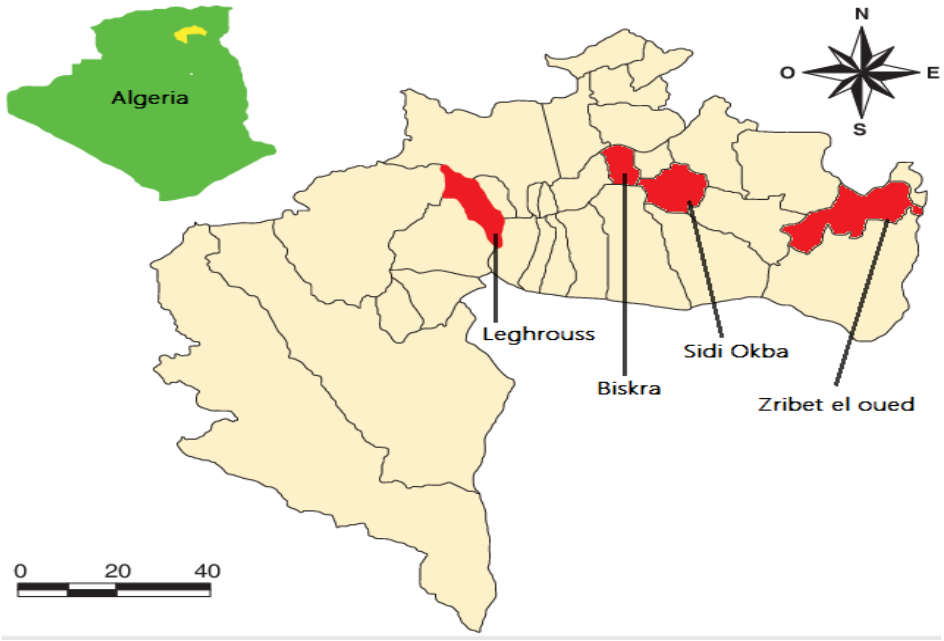


Figure 1. Geographical map of the region of Biskra with collections sites indicated.

Table 1: Table representing the collected samples from Algeria dating from 2018 to 2020.

Crop	Region of collection	Year of collection	No. of samples tested	No. of samples infected with ToLCNDV (%)
Zuchinni (*Cucumis Pepo* L.)	Sidi-Okba	2018	31	27 (87.1%)
	Leghrouss	2019	6	1 (16.7%)
	Biskra	2019	6	3 (50%)
	Biskra	2020	8	4 (50%)
Cucumber (*Cucumis sativus* L.)	Sidi-Okba	2018	12	12 (100%)
	Sidi-Okba	2019	6	6 (100%)
Melon (*Cucumis melo* L.)	Zribet el oued	2018	8	8 (100%)
	Biskra	2020	4	4 (100%)
	Leghrouss	2020	1	1 (100%)
Squash (*Cucurbita maxima* D.)	Sidi-Okba	2019	13	0 (0%)
		Total	**95**	**66 (69.5%)**

Figure 2: (a) Zucchini plants from open fields (a and b) and melon plants from greenhouses (c and d) in Sidi Okba showing virus-like symptoms.

ToLCNDV detection by nucleic acid dot spot hybridization

To investigate the etiology of the disease, leaf extracts (~ 0.1 g) were subjected to total RNA extraction with TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer's protocol. The resulting pellet was resuspended in 50 µL of sterile deionized water and stored at -80°C. Total RNA corresponding to 2 mg (1 µL) of tissue of each sample were blotted, in a total volume of 5 µL, onto nylon membranes and then air dried, fixed by UV irradiation (700

× 100 mJ/cm^2) in a cross-linker, and hybridized using individual specific riboprobes for tomato leaf curl New Delhi virus (ToLCNDV), cucurbit yellows stunting disorder virus (CYSDV), cucumber vein yellowing virus (CVYV), cucumber mosaic virus (CMV), watermelon mosaic virus (WMV), Moroccan watermelon mosaic virus (MWMV), zucchini yellow mosaic virus (ZYMV) and cucumber green mottle mosaic virus (CGMMV). Positive and negative controls were also added into the membranes. Also, we evaluated the possible use of the tissue-printing as a sample preparation technique applied to routine diagnosis of cucurbit plants. For tissue-printing analysis different petioles were detached from each plant with a razor blade and the cross-sections were directly blotted onto nylon membranes. Membranes were air-dried, the nucleic acids were covalently fused by UV cross-linking (700×100 J/cm^2) and the hybridization with the riboprobe was carried out as in the previous case. To construct the riboprobes, total nucleic acids from zucchini plants individually infected with each virus were extracted from 0.1 g of fresh leaf tissue using TRIzol reagent. The design of the primers was based to amplify a portion of CP gene from each virus (Table 2). Reverse transcription and PCR reactions were carried out using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen) following the manufacturer's instructions. Amplified products corresponding to the viral CP genes were cloned individually into a pTZ57R/T vector (MBI Fermentas). For each virus, CP clones in the orientation pTZ/CP:minus-strand were selected, linearized with the appropriate restriction enzyme and the riboprobes were generated by transcription with T7 RNA polymerase from the corresponding pTZ/CP:minus-strand. Prehibridizations and hybridizations with the single probes were conducted at 68°C as described previously (Aparicio et al., 2009; Sáez et al., 2021). Chemiluminiscent detection using CSPD reagent as substrate was performed as recommended by the manufacturer (Roche Diagnostics). Films were exposed to the membranes at room temperature for 30–60 min. A hybridization signal was observed for some symptomatic leaf samples with the ToLCNDV riboprobe, whereas no signal was observed for samples from zucchini plants grown in a virus-free facility. Moreover, the presence of any of the other abovementioned viruses was not observed in any of the analyzed samples.

Table 2. Primers pairs used for polymerase chain reaction amplifications.

Virus	Primer sequence 5'-3´	Location in CP gene	Accession[a]
ToLCNDV	ATGTCGAAGCGACCAGCAGATATC GATCCGGATTTTCAAAGTAGC	279-1022	KF749225
CYSDV	ATGGCGAGTTCGAGTGAGAATAAAACTTCC TCAATTACCACAGCCACCTGGTGC	4927-5682	AY242078
CVYV	GAGAATGCCACTACGGGGAGGTGG TCATTCAATCATCGCTCCACCATAA	8716-9514	MZ130935
CMV	ATGGACAAATCTGAATCAACCAGTGC TCAGACTGGGAGCACTCCAGATGTGGG	1257-1913	D10538
WMV	TCAGGAAAAGAAAAAGAAACGGTGG TTACTGCGGTGGACCCATACCCAAC	8929-9780	MH469650
MWMV	GATCTTGCCTAGAGTCAGAG CACTTACGCATGCCCAGGAG	8892-9430	EF579955
CGMMV	GAAGAGTCCAGTTCTGTTTC ACCCTCGAAACTAAGCTTTC	5735-6258	KY115174
ZYMV	TCAGGCACTCAGCCAACTGTGGC TTACTGCATTGTATTCACACCTAG	8542-9381	EF062583

[a] Accession numbers correspond to nucleotide sequences from where primers were designed.

ToLCNDV detection by PCR amplification

To confirm the identity of the virus, DNA was isolated from leaf samples with the CTAB method. Genomic DNA was quantified by NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted to a final concentration of 50 ng · μL^{-1}. ToLCNDV infection was confirmed by standard Polymerase Chain Reaction (PCR), using 1 µL of total DNA (50 ng) in a final volume of 25 µL. Each reaction contained 1 µL of DreamTaq™ DNA polymerase (5 U/µL) in 10x Green buffer (Thermo Scientific, U.S.A.), 0.2 mM dNTPs and 0.2 µM of To-A1F/A1R and To-B1F/B1R primer pairs (Table 3) (Sáez et al., 2016). Cycling conditions consisted of incubation at 94°C for 5 min and 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s, with a final extension of 10 min at 72°C. The resulting PCR products were separated by electrophoresis in 1% agarose gels in TAE buffer or in 5% polyacrylamide gels in TAE buffer. Randomly selected PCR fragments of each host were bi-directionally sequenced and confirmed as ToLCNDV.

Table 3. Primers used for polymerase chain reaction amplification, and DNA sequencing.

Name	Polarity	Primer sequence (5´to 3´)	Positions	Accession number[a]
To-A1F	+	GGGTTGTGAAGGCCCTTGTAAGGTGC	476-501	KF749225
To-A1R	-	AGTACAGGCCATATACAACATTAATGC	954-980	
To-B1F	+	GAAACACAAGAGGGCTCGGA	637-656	KF749228
To-B1R	-	GCTCCACTATCAAAGGGCGT	1294-1313	

[a] Accession numbers correspond to nucleotide sequences from where primers were designed.

Further tests conducted by PCR with conserved primers for DNA alphasatellites and betasatellites (Briddon et al., 2002; Bull et al., 2003) were negative, and tests by PCR using degenerate primers designed to amplify a fragment of different species of the genus *Begomoviruses* (Alfaro-Fernández et al., 2016) did not detect any other begomoviruses (Table 4).

Table 4. Primer pairs used in the PCR assays for the detection of DNA satellites and begomovirus species.

Primer ID	Primer sequence (5´to 3´)[a]	DNA target	Reference
PGI	GCCYATGWAYMGRAAGCC	Begomovirus specific	Alfaro-Fernández et al., 2016
PGII	GTTWGAVGCATGHGTRCAWG		
UN101	AAGCTTGCGACTATTGTATGAAAGAGG	Alphasatellite molecules	Bull et al., 2003
UN102	AAGCTTCGTCTGTCTTACGAGCTCGCTG		
Beta01	GGTACCACTACGCTACGCAGCAGCC	Betasatellite molecules	Briddon et al., 2002
Beta02	GGTACCTACCCTCCCAGGGGTACAC		

[a] Redundancy code: V represents A, C or G; R represents A or G; Y represents C or T; V represents A, C or G; H represents A, T or C; M represents A or C; and W represents A or T.

Biological characterization of ToLCNDV isolates from Algeria

Samples that tested positive for ToLCNDV (dehydrated tissue mixture from four zucchini samples) were used as a source of inoculum to inoculate five zucchini plants (accession MU-CU-16), five *Nicotiana benthamiana* plants and one cucumber plant to check the infectivity of the Algerian isolate. Mechanical inoculation was carried out as described in (López et al., 2015) by grounding 1 g of infected zucchini leaf tissue in inoculation buffer in a 1:4 (w:v) proportion. The resultant homogenate was used to inoculate one cotyledon and one fully expanded leaf of each plant, previously dusted with carborundum (600 mesh), by gently rubbing with cotton-bud sticks

soaked in homogenate. Plants were then kept under controlled conditions in an insect-proof glasshouse at 20–27ºC (night–day) temperature. Ten days post inoculation, a standard PCR assays was performed to check the presence of ToLCNDV using (To-A1F/ToA1R) and (To-B1F/ToB1R) primers pairs (Table 3). The cycling conditions were the same as those described in the previous section.

Genome sequencing and phylogenetic analysis

PCR-amplified products obtained with different combination of primers corresponding to DNA-A and DNA-B (Table 5) were purified and the nucleotide sequence of the inserts was determined in both directions by means of an ABI PRISM DNA Sequencer 377 (Perkin-Elmer) at the Instituto de Biología Molecular y Celular de Plantas (Universitat Politècnica de València, Valencia, Spain).

Table 5: Designed primers used for sequencing of complete genome A and B of ToLCNDV.

Primer ID	5′–3′ sequence	Genome[a]	
		Position[b]	Segment
ND SeqA2-5F	TGGATATGCGGAGACCCATC	2408-578	
ND SeqA-R	TGCCTCGAGTAACATCACTAACA		
NDA1-D	GGTTGTGAAGGCCCTTGTAAGGTGC	476-981	
NDA1-R	AGTACAGGCCATATACAACATTAATG		
NDSeqA-F	AGGCCGGCAAGTATGAGAAT	942-1421	A
ToLCNDVR1	GGATCGAGAAGAGAGTGGCG		
ToLCNDVF1	AATGCCGACTACACCAAGCAT	1145-1746	
ND Seq-3AR	GACTGGCAAAGCAACACAAA		
ND SeqA2-4F	GCCTCCTGCGAATGTTCTT	1505-2443	
ND SeqA2-4R	AGAGAGCTTCACGAGGATGG		
NDB-Seq4-D	GAACCGGATTGGTGTCTCTC	2538-780	
NDSeqB-R	ACGTTCCAGATATGCTCAAACTC		
ND-B1d	AACACAAGAGGGCTCGGA	638-1314	
ND-B1r	GCTCCACTATCAAAGGGCGT		
NDSeqB-F	TTCAAAGACGAAGAAGATGG	1094-2089	B
NDB-Seq3-R	ACACTCGGAGCGAGTTGAGT		
NDSeqB2-F	ATTCCGCTTGCTTTGTGTCT	1862-78	
NDB-Seq5-R	TCGTGTGAACAAACACGTCA		
ND-Arg F	AGCATCGTTCGTGAGAGCAT	2049-2238	
ND-Arg R	AAGCAATTGCCTTCTTTGCT		

[a] Primers were designed from the ToLCNDV Spanish isolate Murcia 11.1 (GenBank accession No. KF749225 and KF749228 for DNA-A and DNA-B, respectively). [b] Numbered from 5′-terminus of each segment.

The complete DNA-A and DNA-B genomic sequences of a ToLCNDV isolate from cucumber (ToLCNDV-Biskra) were assembled using CAP3 Sequence Assembly Program (Huang and Madan, 1999) and deposited in GenBank (accessions numbers MK981891 and MK981892). The nucleotide sequences were analyzed using BLAST for database searching (https://blast.ncbi.nlm.nih.gov); translate to obtain amino acid sequences (https://web.expasy.org/translate/); and MUSCLE (Multiple Sequence Comparison by Log-Expectation, https://www.ebi.ac.uk/Tools/msa/muscle/) for multiple sequence alignment. Phylogenetic relationships were represented by means of a tree built using the neighbor joining method based on the Tamura-Nei model with 1000 bootstrap replicates available in the Molecular Evolutionary Genetics Analysis (MEGA, https://www.megasoftware.net/) version X (Tamura et al., 2021). Pairwise nucleotide identity comparisons were calculated using the Sequence Demarcation Tool program (SDT; Version 1.2) with the MUSCLE option for sequence alignment. Additionally, we used Recombination Detection Program (RDP) Version 4.39, to analyze the genetic recombination events of isolates of ToLCNDV retrieved from NCBI Genbank.

RESULTS

Detection of ToLCNDV

During years 2018, 2019 and 2020, cucurbit plants showing virus-like symptoms such as foliar yellowing and vein clearing were observed in the agricultural region of Biskra (Biskra, Leghrouss, Sidi Okba and Zribet el oued) (Figures 1 and 2). Due to the frequent occurrence of mixed infections, it was difficult to describe the virus using symptomatology under field conditions (Figure 3). A total of 82 symptomatic leaf samples were collected: 51 of zucchini, 18 of cucumber, and 13 of melon. Thirteen asymptomatic samples of squash were also collected in Sidi-Okba in 2019 (Table 1).

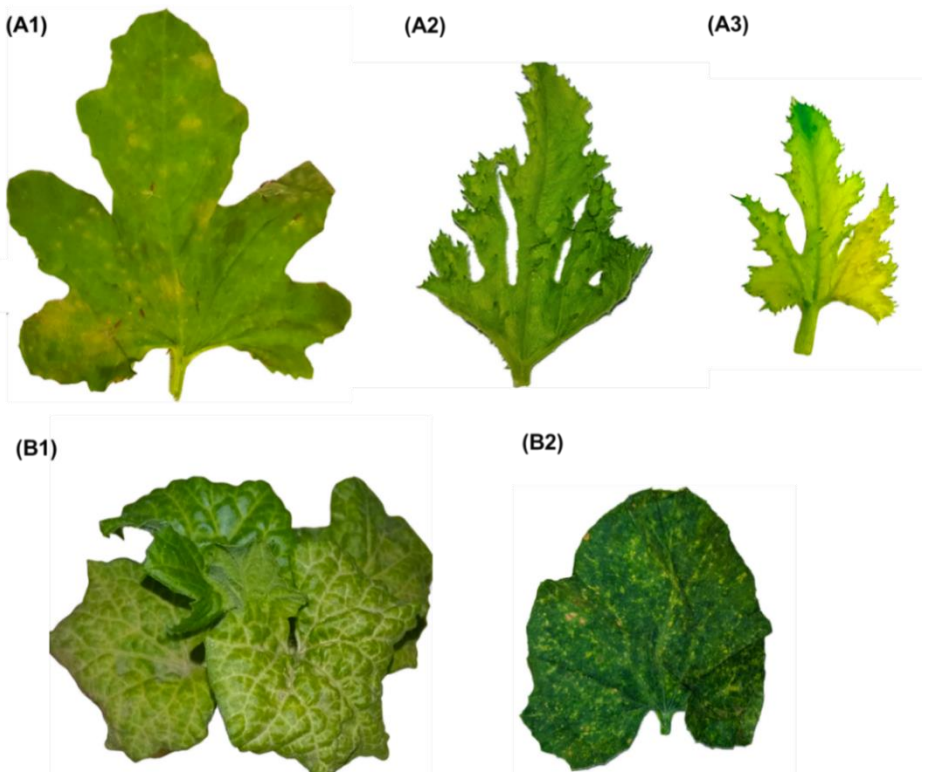


Figure 3. Leaf samples showing different virus-like symptoms. (A) symptomatic leaves from zucchini plants showing chloric spots (A1), leaf curling (A2), leaf curling and yellowing (A3). (B) symptomatic leaves from melon plants showing vein clearing and curling (B1) and chloric spotting (B2).

To investigate the etiology of the observed disease, leaf extracts were subjected to total RNA extraction with TRIzol reagent. Virus identification was carried out by nucleic acid spot hybridization using specific riboprobes for ToLCNDV, CYSDV, CVYV, CMV, WMV, MWMV, ZYMV, and CGMMV. A hybridization signal was observed for most symptomatic leaf samples with the ToLCNDV riboprobe (Figure 4B), whereas no signal was observed for samples from zucchini plants grown in a virus-free facility and for squash asymptomatic samples (data not shown). Moreover, the presence of any of the other abovementioned viruses was not observed in any of the analyzed samples (Figure 4A). In order to simplify the sample handling procedure, several healthy and infected cucurbit plants tested positive in blot analysis (Figure 4B) were analyzed by the tissue-printing method (Aparicio et al., 2009). As shown in Figure 4C the ToLCNDV riboprobe showed a high specific detection of plants infected with ToLCNDV discriminating between healthy and infected samples. This result validates the tissue-printing and

dot blot procedures as simple and reliable methods for sample preparation to detect ToLCNDV in plants by non-radioactive molecular hybridization with the riboprobe.

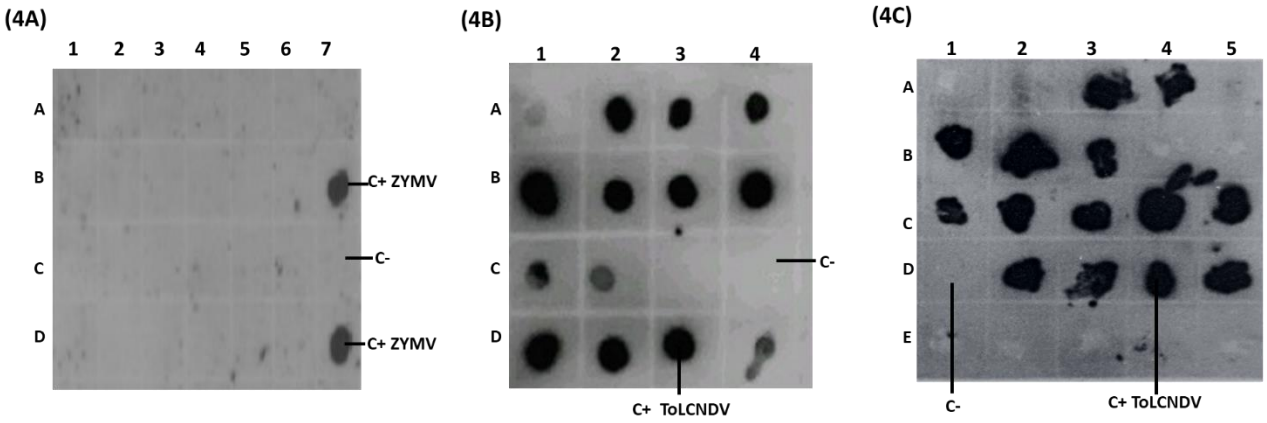


Figure 4. Virus diagnostics via dot-blot and tissue-printing procedure. **4A:** Hybridization membrane for the detection of ZYMV; C+ and C-, ZYMV positive and negative controls. **4B:** dot-blot for the detection of ToLCNDV in zucchini plants. **4C:** Tissue-printing for the detection of ToLCNDV in symptomatic zuchinni plants. ToLCNDV positive and negative controls are indicated.

To confirm the identity of the virus, samples positive as determined by non-isotopic molecular hybridization were analyzed using polymerase chain reaction (PCR) with virus-specific primers. Initially, DNA was isolated from 24 symptomatic samples (12 of zucchini, six of melon, and six of cucumber) with the CTAB method and was subsequently analyzed by PCR with two ToLCNDV-specific primer pairs (To-A1F/R from DNA-A, and To-B1F/R from the DNA-B) (Sáez et al., 2016) to amplify 505- and 677-bp fragments of viral DNAs A and B, respectively. PCR products of the expected size were observed on 1% agarose gel for all symptomatic samples (Figure 5). Two randomly selected PCR fragments of each host (zucchini, cucumber, and melon) were bidirectionally sequenced and confirmed as ToLCNDV. The nucleotide sequences of these amplicons were essentially identical irrespective of the host and BLAST analysis showed nucleotide identity higher than 99% with sequences from ToLCNDV isolates of Spain. Finally, all the samples of this survey were tested by PCR using specific primers for ToLCNDV detection. All samples recorded as positive by nucleic acid hybridization (dot blot or tissue printing) for ToLCNDV were also positive by PCR. However, seven samples detected as positives by PCR were negative by hybridization, which suggests a virus concentration below the nucleic acid hybridization detection limit. Of the 82 symptomatic samples, 80.5% (66 of 82) were ToLCNDV positive and only 16 samples of zucchini resulted negative, independently of the primers used,

suggesting a strong relationship between disease symptoms and the presence of ToLCNDV. However, no ToLCNDV-positive samples were detected in the 13 asymptomatic samples of squash by PCR. These results suggest that ToLCNDV is distributed widely in cucurbits crops (mainly in zucchini, cucumber, and melon) in Sidi-Okba, Leghrouss, Biskra and Zribet el oued.

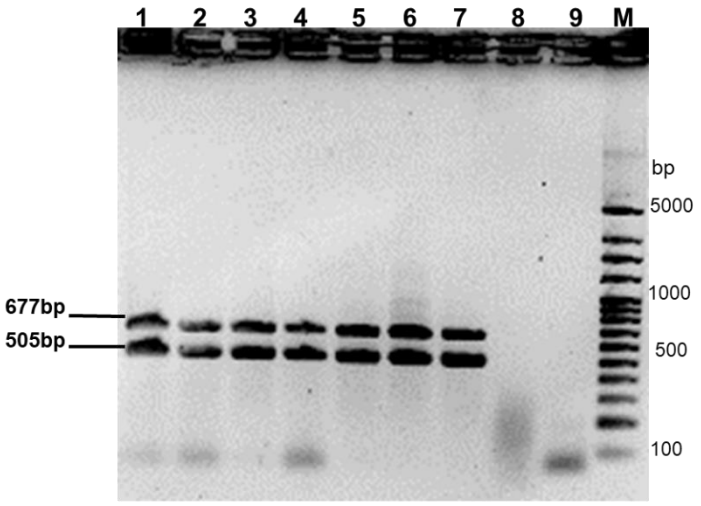


Figure 5. Amplified products obtained from ToLCNDV positive samples with primers: To-A1F/R from DNA-A, and To-B1F/R from DNA-B. lanes (1-4), zucchini samples; lanes (5-6), melon samples; lane (7), positive control from a ToLCNDV-infected plant; lanes (8-9) negative controls from healthy plant (8) and water (9). M: molecular size markers. The PCR products were separated in a 1% agarose gel and stained with ethidium bromide.1

Genomic characteristics of the Algerian ToLCNDV isolates

The complete DNA-A and DNA-B genomic sequences of a ToLCNDV isolate from cucumber (ToLCNDV-Biskra) were obtained as described by Sifres et al., (2018), and deposited in GenBank under accession numbers MK981891 and MK981892. The two DNA segments have the typical genome organization of ToLCNDV and translation of the nucleotide sequence into an amino acid sequence yielded predicted protein products of similar sizes. DNA-A segment of Biskra isolate was 2,738 nucleotides in length, containing the six open reading frames (ORFs) typical of other ToLCNDV, including in viral sense two ORFs, AV1 (771 nt), and AV2 (339 nt), and in complementary sense four ORFs, AC1 (1,086 nt), AC2 (405 nt), AC3 (411 nt), and AC4 (177 nt) (Chang et al., 2010a). DNA-B segment of Biskra isolate was 2,684 nucleotides in length, containing the typical two ORFs, including in viral sense the ORF BV1 (807 nt) and in complementary sense the ORF BC1 (846 nt). Tests conducted by PCR with conserved primers for betasatellites and alphasatellites (Briddon et al., 2002; Bull et al., 2003) were negative, and an additional test by PCR with conserved primers for begomoviruses (Alfaro-Fernández et al., 2016) did not detect any other begomovirus.

Biological characterization

Because the Mediterranean Basin isolates are mechanically transmitted (López et al., 2015), experiments were conducted using dehydrated tissue of some of the zucchini plants naturally infected with ToLCNDV as a source of inoculum to inoculate seedlings of zucchini accession MU-CU-16, highly susceptible to this virus, as well as plants of *N. benthamiana* and cucumber. Virus accumulation and symptoms varied, depending on the plant considered. According to the visual evaluation on a scale from 0 (no symptoms) to 4 (very severe symptoms) (López et al., 2015), symptoms were variable in zucchini and *N. benthamiana*, with some plants symptomless (score 0; Figure 6 A1 and B1) and others with mild symptoms (score 1-2; Figure 6 A2 and B2). The only inoculated plant of cucumber was practically asymptomatic (data not shown). In all cases, at 15 days post inoculation symptomatic plants showed vein clearing and yellowing and curling on younger leaves.

Figure 6. Symptoms exhibited at 13 days after mechanical inoculation of ToLDCNV from Algeria in different species. A1, A2: *Nicotiana benthamiana* plants with curling symptoms on young leaves. B1, B2: zucchini plants exhibiting vein clearing and yellowing on young leaves.

Ten days postinoculation, ToLCNDV infection was confirmed by PCR amplification, and additionally confirmed by DNA sequencing. A positive correspondence between symptoms and virus titer was found, as plants with mild symptoms had low to intermediate virus amounts, and asymptomatic plants where negative or in some cases had very low virus titers (Figure 7). The

transmission rate was 81% (9 infected plants out 11 inoculated plants), indicating that this is a cucurbit-infecting isolate with high mechanical transmission efficiency, even though dehydrated tissue was used.

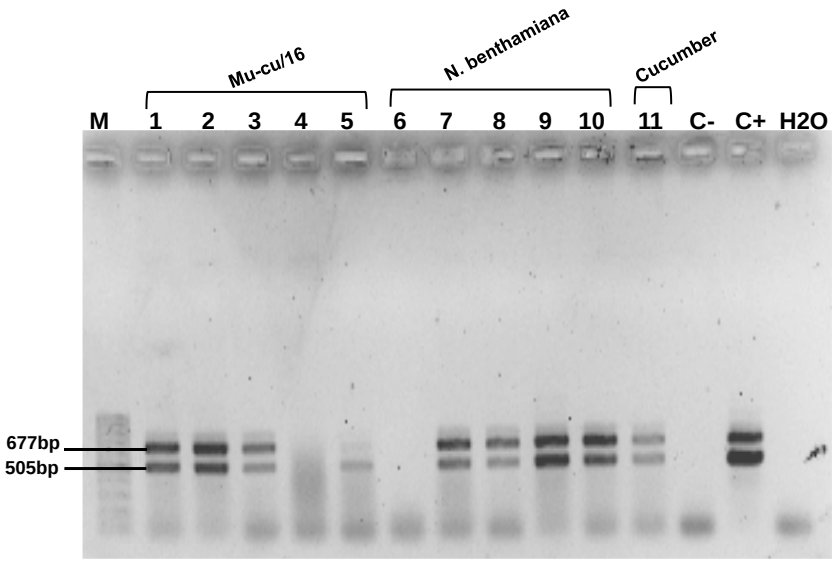


Figure 7. Amplified products obtained from ToLCNDV inoculated plants with primers: To-A1F/R from DNA-A, and To-B1F/R from DNA-B. Lanes 1-5, plants of zucchini (MU-CU/16); lanes 6-10, plants of *N. benthamiana*; lane 11, cucumber. C- and C+, negative and positive controls from non-inoculated zucchini plants and the original source of inoculum of ToLCNDV, respectively. H_2O, negative control with water. The PCR products were separated in a 1% agarose gel and stained with ethidium bromide.

Phylogenetic analysis

Sequence identity analysis were performed using BLASTn by comparing the sequences of the isolate ToLCNDV-Biskra with sequences available in the GenBank database. BLASTn analysis showed that sequence of DNA-A was 100% identical to the Indian isolate Kodinar (accession number MT316387) from *Solannum lycopersicum* (collected in Gujarat, India in 2019), and shared >99% nucleotide identity with the corresponding segment of the isolates from the Mediterranean basin, whereas the identity with the rest of isolates from Asia was around 90%. According to the International Committee on Taxonomy of Viruses (ICTV) (http://www.ictvonline.org/virusTaxonomy.asp), the best nt sequence identity cutoff value to separate strains of *Begomovius* is 94%. Therefore, ToLCNDV is a virus species with two strains; the ToLCNDV-ES or European strain, reported from Spain and other countries of the Mediterranean Basin, and the Asian strain (Fortes et al., 2016; Zaidi et al., 2017). The phylogenetic

tree, generated by MEGA X software using the neighbor-joining method (Kumar et al., 2018), indicated that isolate Biskra from Algeria is grouped with isolates of the European strain. These isolates are monophyletic and form a single group (regardless of the plant host, year of collection and geographical origin), while the isolates of the Asian strain present a higher genetic variability and they are gathered in several clades (Figure 8). This pattern suggests that the European strain was originated by a single isolate of the Indian subcontinent (Fortes et al., 2016; Rauniyar et al., 2023).

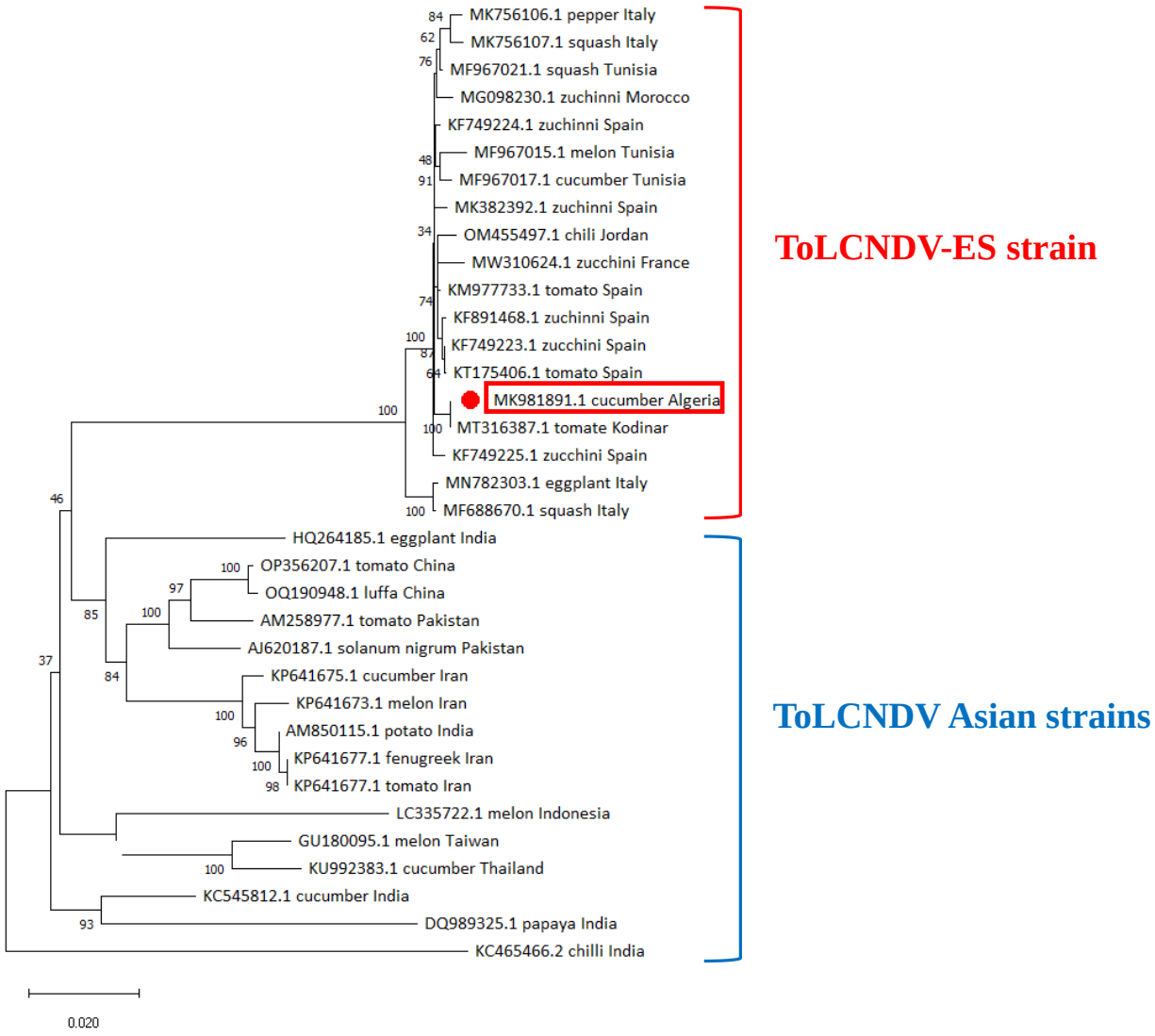


Figure 8. Phylogenetic tree of the complete nucleotide sequence of the DNA-A segment determined in this study (marked with a red circle and boxed) and published in the NCBI databases from different geographic origins and hosts. All isolates are reported with the accession number, host and country origin. Phylogenetic tree was constructed by neighbor joining method in Mega X software. The bootstrap values corresponded to 1,000 replications. Branch lengths are proportional to the genetic distances and bootstrap values are indicated.

Similar results were obtained in studies of viruses through heat map (Figure 9) using the Muscle method in SDT (Sequence Demarcation Tool) Version 1.2 (Muhire et al., 2014). The Alignments obtained of the sequence of DNA-A showed a higher genetic identity in between isolates belonging to the European strain with identities higher than 98%, whereas the identity with isolates from Asian strain does not exceed a percentage of 91% (Figure 9).

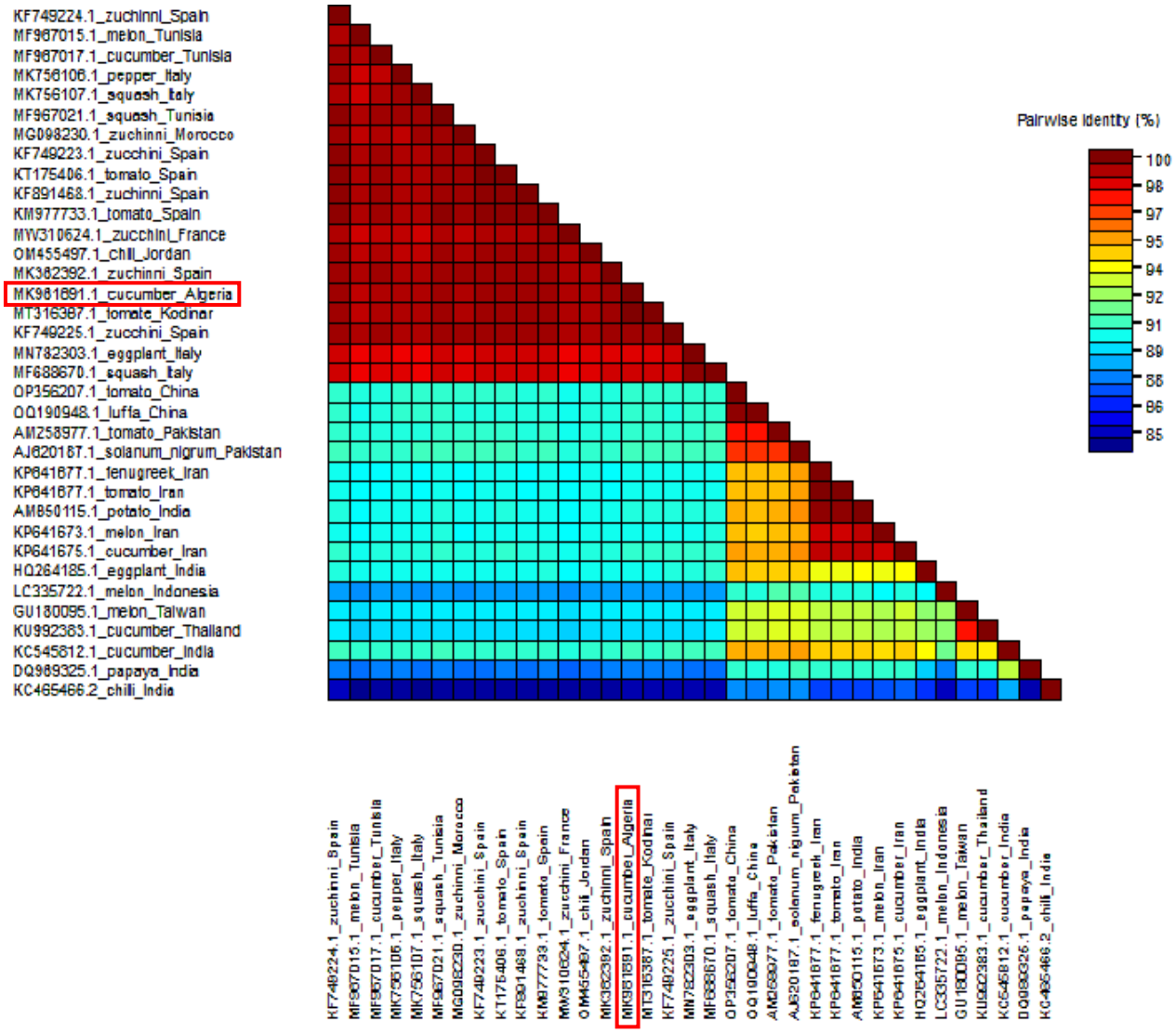


Figure 9. The pairwise identity of DNA A sequences of ToLCNDV were aligned using Clustal W software and visualized by the SDT. On the colored heat map, the colors on the scale denote the pairwise identities. The accession of this study is marked boxed in red.

BLASTn analysis showed that sequence of DNA-B shared >98% nucleotide identity with the corresponding segment of the isolates from the Mediterranean basin, whereas the identity with isolates from Asia was around 81-82%. In this case, the Indian isolate Kodinar (accession number MT295300) also showed a high percentage of identity (99%) with the Algerian isolate (ToLCNDV-Biskra, accession number MK981892). The phylogenetic tree constructed based on DNA-B segments showed that isolate Biskra is grouped with the monophyletic isolates of the European strain (Figure 10). Newly, the isolates of the Asian strain present a higher genetic variability and they are gathered in several clades, however they share the same ancestral lineage (Figure 10).

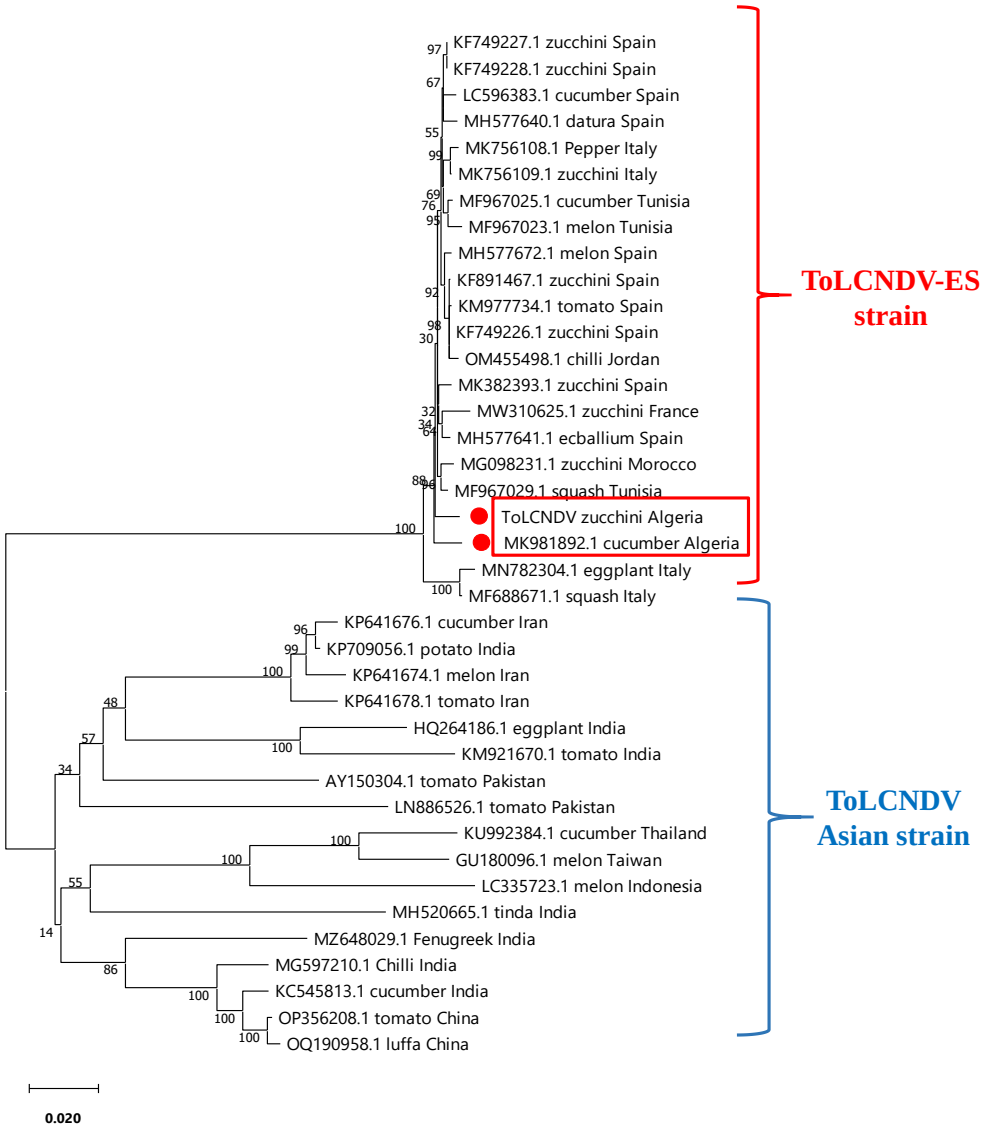


Figure 10. Phylogenetic tree of the complete nucleotide sequence of the DNA-B segment determined in this study (marked with a red circle and boxed) and published in the NCBI databases from different geographic origins and hosts. All isolates are reported with the accession number, host and country origin. Phylogenetic tree was constructed by neighbor joining method in Mega X software. The bootstrap values corresponded to 1,000 replications. Branch lengths are proportional to the genetic distances and bootstrap values are indicated.

The pairwise identity obtained of the sequence of DNA-B through heat map using the Muscle method in SDT (Figure 11) showed a higher genetic identity in between isolates belonging to the European strain with identities ranged between 96-100%. The Asian cluster showed a genetic identity between isolates that ranged between 81-96%. The genetic variation between the two main clusters have shown a pairwise identity lesser than 81%.

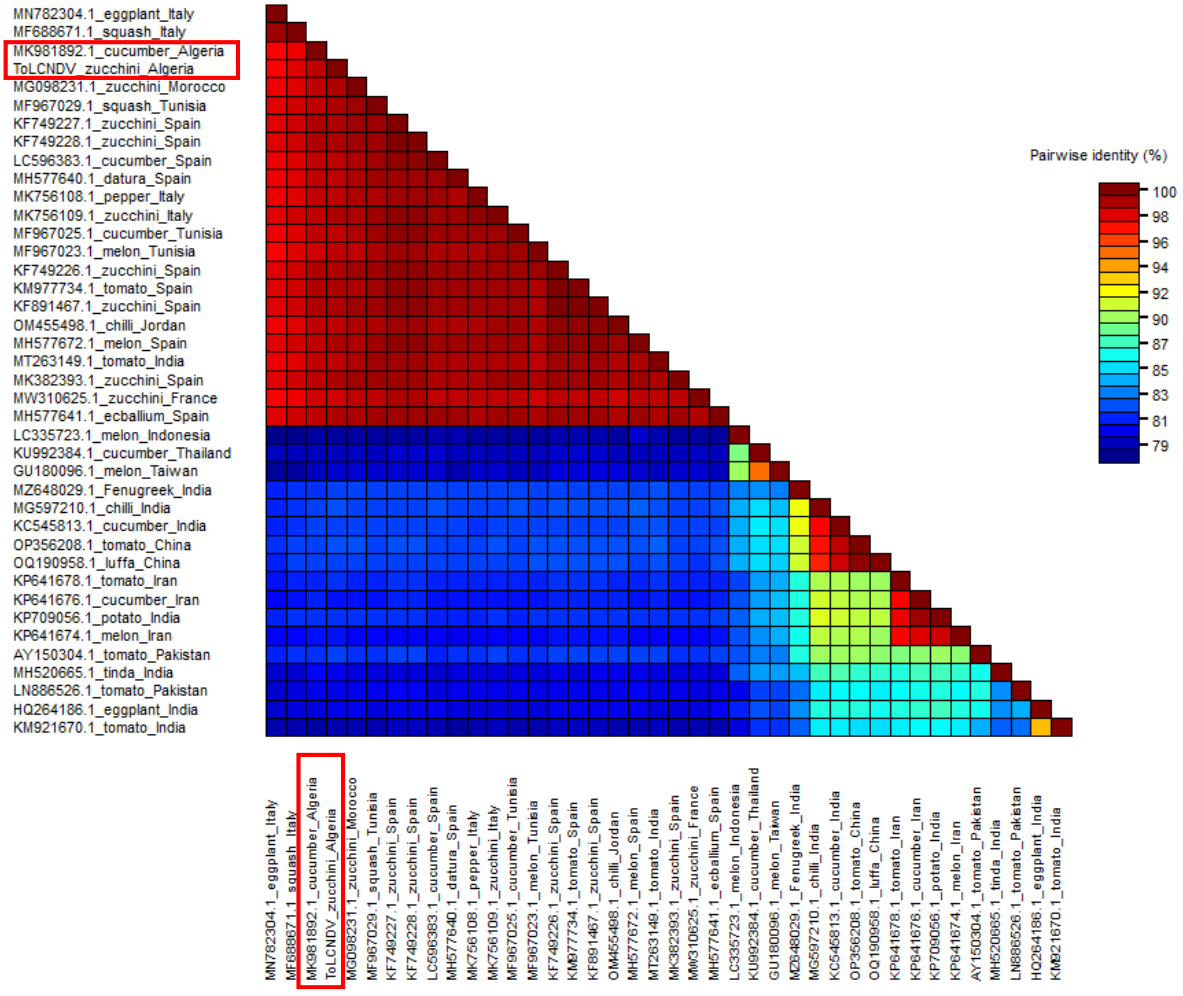


Figure 11. The pairwise identity of DNA B sequences of ToLCNDV were aligned using Clustal W software and visualized by the SDT. On the colored heat map, the colors on the scale denote the pairwise identities. The accession of this study is marked boxed in red.

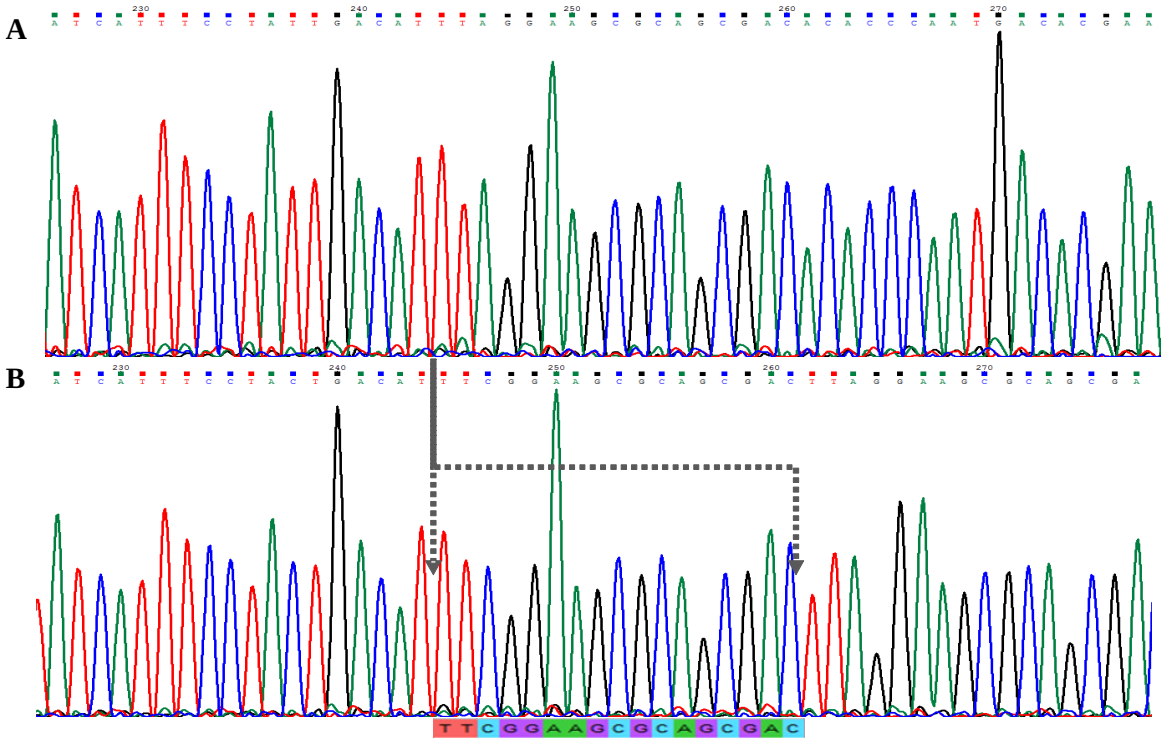
Genetic variability of Algerian ToLCNDV isolates

To increase knowledge of the molecular variability among ToLCNDV isolates from Algeria, in this study, the complete DNA-A genomic sequence of four isolates (two of zucchini, one of melon, and one of cucumber) and the partial sequences of other 40 isolates (23 of zucchini, 11 of cucumber, and six of melon) were obtained. On the other hand, the complete DNA-B genomic sequence of 17 isolates (10 of zucchini, three of melon, and four of cucumber) and the partial sequences of other 25 isolates (18 of zucchini, four of melon, and three of cucumber) were also obtained (Table 6). When these sequences were compared with the corresponding segments of the ToLCNDV-Biskra isolate, the nucleotide sequences exhibited only minor differences or were 100% identical (data not shown) irrespective of the host and year of collection. A low nucleotide diversity of all ToLCNDV isolates characterized from the Mediterranean Basin had been previously described (Fortes et al., 2016; Juárez et al., 2019).

Table 6. Isolates sequenced in this study. The number of isolates sequenced from each crop, and the DNA segments sequenced are indicated.

Crops	**DNA-A segment**		**DNA-B segment**	
	Isolates sequenced	**Isolates partially sequenced**	**Isolates completely sequenced (No. of isolates with insertion)**	**Isolates partially sequenced (No. of isolates with insertion)**
Zucchini	**2**	23	10 (5)	18(2)
Melon	1	6	3 (1)	4
Cucumber	1	11	4	3

In some samples, a 17-nucleotide insertion was detected at position 2,136 of DNA-B (Figure 12A-B). Of the 17 nucleotides of the insertion, 16 are identical to the adjacent ones, so it really is a duplication of nucleotides (Figure 12C). The insertion was located just before the start of the gene BC1, so in principle the expression of the MP protein should not be affected. However, a start codon for the movement protein (MP) synthesis is found at a different position than the other ToLCNDV isolates (nt 2,294 vs 2,135), resulting in a MP 53 amino acids longer (Figure 12D and supplementary material). Sequencing of more isolates and biological assays should be performed to verify the biological and epidemiological implications of this putative major MP.



C

INSERTION (nt 2136-2152)

CATTT**C**GGAAGCGCAGCGAC TT**A**GGAAGCGCAGCGAC

nt 2133 nt 2169

D

```
MKSIINACSPFSAKQLPSLLGFLLLLLLIGVRCTIFFVSLGVSLRFLSRCASEMS**V**GNDSLGLGVGGYI**Q**SERVEYALTNDAAEVTM
M----------------------------------------------------------**I**GNDSLGLGVGGYI**H**SERVEYALTNDAAEVTM

SFPSMFEQKISQLRNRCMKIDHVLLEYRSQVPINAVGHVVIEIHDMRLTEGDTKQAEFTIPIRCNCNLHYYSSSFFSLKDKNPWRVE
SFPSMFEQKISQLRNRCMKIDHVLLEYRSQVPINAVGHVVIEIHDMRLTEGDTKQAEFTIPIRCNCNLHYYSSSFFSLKDKNPWRVE

YRVENANVVNGVHFCKMLGKLKLSSAKHSTDVEFRPPKIEIQTKEYTVNDIDFWSVGSKPQTRRLVDGSRLIGHSSRSLRVPYMAIG
YRVENANVVNGVHFCKMLGKLKLSSAKHSTDVEFRPPKIEIQTKEYTVNDIDFWSVGSKPQTRRLVDGSRLIGHSSRSLRVPYMAIG

PNESWASRSEVGHSSFTTRPYKNLSGLDDSAIDPGPSASQAGSITREDITDIITKTVEQCIRSNVNAPLSKGV  334
PNESWASRSEVGHSSFTTRPYKNLSGLDDSAIDPGPSASQAGSITREDITDIITKTVEQCIRSNVNAPLSKGV  281
```

Figure 12. A-B: Nucleotide sequence comparison of Biskra (A) and BZ (B) isolates. The 17-nucleotides insertion of the BZ isolate is between dashed lines. C: nucleotide sequence of the region before the start codon of the BC1 gene of the Algerian BZ isolate. The ATG start codon of the BC1 gene (complementary polarity) is indicated in bold. The 17-nucleotide insertion is displayed in a bracket, which is a duplication of the boxed sequence, except for the nucleotide marked in red. D: Protein sequence alignment of the putative MP of the Algerian BZ isolate (234 amino acids) and Biskra isolate (281 amino acids). Different amino acids are marked in bold.

To check if the insertion was a general characteristic of the Algerian ToLCNDV isolates, the 66 samples that tested positive for ToLCNDV were analyzed by PCR. PCR amplification with primers ND-Arg F/ ND-Arg R generated a 190-bp DNA fragment from 58 isolates tested, whereas that in eight samples collected in 2018 (seven of zucchini and one of melon) generated a 207 bp DNA fragment. Thee isolates (Figure 12, lines 3-5) yielded amplification of both fragments, while in the remaining four the fragment of 207 bp was the only amplification product (Fig 13, lane 6).

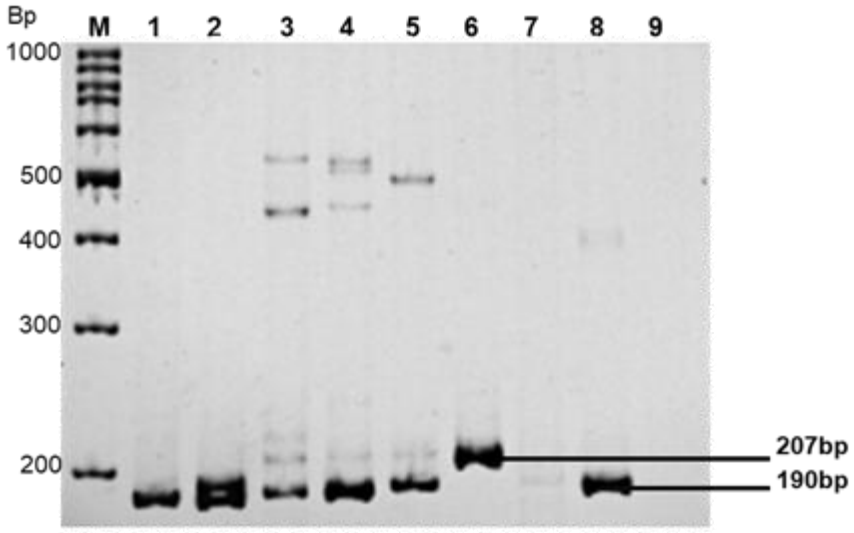


Figure 13. Amplification products obtained from the DNA-B segment by PCR with the primers ND-Arg F and ND-Arg R (see Table 5). The PCR products were separated in a 5% polyacrylamide gel and stained with ethidium bromide. M molecular markers with their size in bp indicated on the left. lane 1: positive control from a ToLCNDV-infected plant without insert. Lane 9: negative control of PCR. Lanes 2-8 PCR products from seven zucchini infected plants showing a 190-bp DNA fragment and/or a 207 bp DNA fragment.

Genetic recombination analysis of ToLCNDV isolates

To identify recombination events in the Algerian isolate, complete genome sequences of the DNA-B segment from numerous isolates of ToLCNDV were evaluated for likely recombination events using Recombination Detection Program (RDP) Version 4.39 (Figure 13). Recombination events were found among Asian isolates, but the isolate from Algeria was observed to be non-recombinant. Although the insertion of the Algerian isolate was clearly detected in this genetic analysis, it was not recognized as a recombination event as it is regarded as an intragenic mutation in a single site of the genome, whereas a recombination induces genetic changes all along the genome. Nonetheless, it is plausible that future variations may occur on the ToLCNDV isolates of Mediterranean countries as it has happened in the Asian isolates cluster. In accordance with Panno

et al., (2019), the genetic intragroup of Mediterranean countries (Spanish, Italian and Tunisian groups) exhibit very low differentiation in between them implying a common origin, whereas there are indicators of genetic differentiation developed amongst isolates from Asian countries. The genetic variations in between isolates from Asian continent were already identified in genome A (Fortes et al., 2016; Moriones et al., 2017).

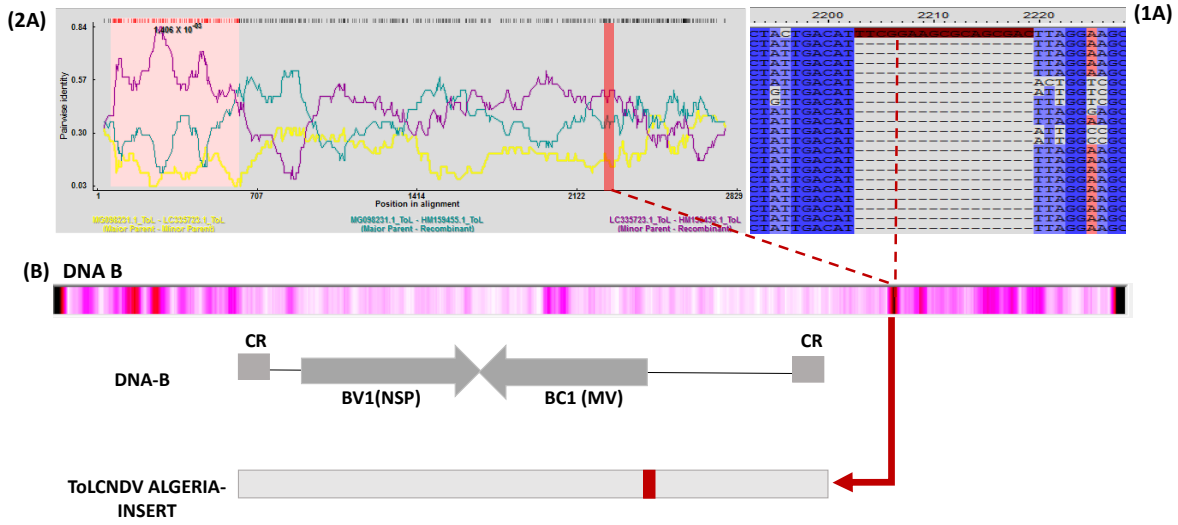


Figure 14. Putative recombination events of isolates of ToLCNDV using Recombination Detection Program (RDP) Version 4.39. **1A)** multiple alignment of DNA-B sequences of ToLCNDV isolates, the insertion on the Algerian isolate is colored in dark. **2A)** RDP graph present the pairwise identity between isolates analyzed, the insertion position is colored in red and the Asian recombination event in pink. **B)** Schematic representation of DNA-B of ToLCNDV and the location of the insert in the genome of Algeria.

DISCUSSION

Cucurbits are widely cultivated among tropical, subtropical, and temperate countries of the world, and their crop is affected for several viral diseases, with different economic effects depending on the region the crop and the year. However, there are not studies on the incidence of specific viruses in Algeria, and this is the first report of a survey carried out using molecular procedures to determine the prevalence and distribution of viruses that affect cucurbit crops in open field melon, zucchini, cucumber, and squash crops in the agricultural region of Biskra, southeast of Algeria. All samples collected from melon, zucchini, and cucumber were leaf samples of plants that showed symptoms of viral infection, i.e., yellowing, mosaics, and leaf deformations and were

analyzed by hybridization to detect the aphid-transmitted viruses: CMV, ZYMV, WMV, and MWMV; the virus CGMMV transmitted by contact and seeds; and the whitefly transmitted virus: ToLCNDV. In addition, most of samples were also analyzed to detect the presence of other whitefly transmitted virus such as cucurbit yellow stunting disorder virus (CYSDV), cucumber vein yellowing virus (CVYV), and cucurbit chlorotic yellows virus (CCYV) (see chapter I). ToLCNDV was the most frequently detected in samples of the three symptomatic crops (80.5%). CCYV was present with lower incidence, always identified in mixed infections with ToLCNDV (Kheireddine et al., 2019). None of the samples tested positive for the remaining viruses. Although the aphid-transmitted viruses CMV, CABYV, and ZYMV were reported previously in Algeria infecting several crops (Lecoq et al., 1992; Yakoubi et al., 2008; Lecoq and Desbiez 2012; Radouane et al., 2021), in the present survey were not detected. The lack of virus detection in samples showing virus-like symptoms might be due to different causes such as low virus concentration, unidentified viruses, or nutritional deficiency that may induce symptoms like those caused by viruses.

Throughout the last decades, a notable rise in the emergence of begomoviruses in new agricultural areas has been observed, creating epidemical outbreaks around the world, actuated principally by the rapid expansion of their vector the whitefly *B. tabaci* (Rosen et al., 2015), and by the movement of infected propagative material. Therefore, environmental changes and human impact are direct consequences of the widespread of begomoviruses (Seal et al., 2006). ToLCNDV belongs to this genus and is part of a complex of whitefly-transmitted viruses that cause destructive epidemics on economically valuable crops such as *Solanaceae* and *Cucurbitaceae*. Today, the lack of effective control measures is alarming. Nonetheless, analysis advanced of genetic diversity and evolution of its populations in a specific area are crucial to understand the epidemiology of ToLCNDV and develop effective methods for virus detection and to establish efficient and durable measures to prevent further dissemination (Moriones et al., 2017). ToLCNDV was first reported on tomato (*Solanum lycopersicum* L.) in India (Padidam et al., 1995; Srivastava et al., 1995). Since then, its host range has increased and it is now the most predominant and economically significant disease affecting cucurbit and solanaceous crops in the Indian subcontinent, East and Southeast Asia, and the Middle East (Moriones et al., 2017). In the Mediterranean basin, ToLCNDV is considered as an emerging threat in the production of cucurbit crops since their first report in 2012 in Spain (Juárez et al., 2014; Mnari-Hattab et al., 2015;, Panno et al., 2016;, Sifres et al., 2018;

Orfanidou et al., 2019; Kheireddine et al., 2019). Phylogenetic analyzes have revealed that the Mediterranean virus population is genetically very homogeneous, composed of isolates belonging to the new genotype named ToLCNDV-ES strain (Fortes et al., 2016; Qureshi et al., 2022), with no clustering pattern, and clearly different from Asian virus populations, named Asian strain (Juárez et al., 2019). This pattern strongly suggests that ToLCNDV-ES strain evolved of a single virus originated from the Indian subcontinent by recombination and point mutations, which are the driving force behind the evolution of ToLCNDV (Lefeuvre and Moriones 2015; Moriones et al., 2017). This strain infects cucurbits very efficiently, but poorly infect other hots, including solanaceous crops (Fortes et al., 2016; Moriones et al., 2017). Recombination events have been found among Asian isolates, but the low genetic variation of Mediterranean isolates has precluded the detection of recombinants. We analyzed possible recombination events in our isolate, since it has a duplication of 17 nucleotides, but no recombination events among DNA B segments of different ToLCNDV isolates were detected. The insertion of the Algerian isolate was clearly detected in genetic analysis, but it was not recognized as a recombination event as it is regarded as a mutation in a single site of the genome. The randomly accurance of insertion or deletions in genomes is commonly known as replication slippage, or slipped strand mispairing. DNA polymerase pauses and detach from the DNA strand during replication which enable the growing strand to split from the template and reanneal upstream or downstream a distinct homologous region (Viguera et al., 2001). The insertion was detected at position 2,136 of DNA-B, resulting in a putative MP 53 amino acids longer, even though the functionality of this putative protein remains unknown. The MP is required for virus cell-to-cell and long-distance movement, through its cooperative interaction with the Nuclear Shuttle Protein (NSP) (Ward et al., 1997). Previous studies have demonstrated that the adaptive evolution of proteins is not only affected by changes in amino acid composition, but also by size change. Then, sequencing of more isolates and biological assays should be performed to verify the biological and epidemiological implications of this putative MP longer.

In conclusion, this study highlights the prevalence of ToLCNDV in Algeria which has recently been detected (Kheireddine et al., 2019) using different diagnostic tools such as tissue printing, dot blot hybridizations, and PCR. The riboprobe/tissue-printing strategy to detect virus has shown to be a reliable alternative detection method to the dot-blot hybridization. Only one synthesis reaction of the riboprobe and one hybridization assay is needed to detect the virus, whereas the handling

and manipulation of the samples is reduced to a minimum. This approach could be a very useful tool as a first detection-step to determine the phytosanitary status of cucurbit plants in routine diagnosis. The virus was detected in all investigated regions infecting zucchini, melon, and cucumber, expect for squash. The incidence of the virus in the region provided a clear indicator that it has been present for a longer period. To measure the severity of the virus, plant symptoms might not be an accurate indication, in fact, several factors can influence the severity of the symptoms such as nutritional deficiencies, susceptibility of the cultivar, environmental conditions, as well as potential mixed infections with other plant viruses (Juárez et al., 2019). It should be mentioned that CCYV was detected in five samples in coinfection with ToLCNDV (Kheireddine et al., 2020). It is well known that mixed infection is a common phenomenon occurring in nature, however the effect of such interactions might greatly influence the genetic variability of a virus population, where the vectors are the major connectors for those mixed infections playing a pivotal role in plant host transmissibility (Moreno & López-Moya, 2020). ToLCNDV has been reported in Spain, Italy, Greece, Portugal Tunisia, and Morocco infecting different cucurbit crops, and this is the first report of the presence of ToLCNDV in Algeria. The international trades of seeds, seedlings and agricultural materials possibly has facilitated the long-distance spread of the virus and their insect vector (Rojas et al., 2015). The mechanism of infection of the virus remain unknown and further research should be undertaken to understand the epidemiology of the virus and the ecological factors influencing genetic variability of ToLCNDV and its evolution in view of the ever-changing conditions.

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SUPPLEMENTARY MATERIAL

Tomato leaf curl New Delhi virus isolate BZ segment DNA-B, complete sequence

ACCGAAAGGCCGCGAAAATTTTTGCCCTCTCATCCTGACCGTTAATTTAAAAGAAGATGA
CGTGTTTGTTCACACGATAAATTACACGTGTATCCCACTAACAGACTTTATGCTAATAAA
TGTCTGAGATGTTTCGATAAAATGCACCTGTGGTCCCCATCTTTATCATCTCTTACATAT
ATTATTGGTCCCCCCACACTACATCAATTATTAATTTTGTCCATCCGGCGATATCTATAA
TATCAGTTTTGAACGGTCGAGATCAAAACAAATACCATACCAACTTTCCTGTCATTCGAT
CATATCATATTGGCTTCAATTCAAACAATAATTGTTTGAATTTATACATATTATACAAGA
ATAGTATATAAACATTCGACATATATTCTACTACGTGGTGTTCTATTTGCCATTCGTTAT
CTTTTGTAGATGGCTTTTCCTTCTCCTTATTCCACTCCTCGCCGGTCCGGTTACCCATTG
TACAGAACCTACAACGGAAACAAGAGTTTACGCTTGTGGAAGTCACGGAAGTTTCAAAAC
TGGAGGCGCCACCGCATCTCAAATTCCGTCACACGTTCTCCAACCGAATTGTTTGGCGAT
CCAATCTCCAAACAATACACGCGTAAGGAAATATGTGAAACACAAGAGGGCTCGGAATAT
GTCCTCCACAACAATCGTTATAGGACGTCATATGTCACATATCCAGCCAAAACCCGAACC
GGAACGAACAACCGCGTTCGTTCCTACATCAGGTTAAAGAGTTTGAGCATATCTGGAACG
TTTGCTGTTCGTAAGTCGGACTTGATGACAGAAGTTGTTCAAACAAATGGACTTTACGGA
GTGATGTCTGTTGTAGTAGTCCGCGATAAATCGCCAAAGATTTATTCTGCGACCCAACCG
CTAATACCATTTGTTGAAATGTTTGGATCCGTGAATGCCTGCAGGGGTAGTCTTAAAGTG
GCAGAACGTCATCAAGAACGTTTTGTATTACTGAATCAAACATCCATCGTCGTCAATACT
CCACATCCGACTGCTATCAAGAAGTTCTGCATTCGTAACTGCATACCAAGAACGTACACA
ACGTGGGTAACGTTCAAAGACGAAGAAGAAGATGGCTGTACTGGACTATACTCCAACACG
CTCCGAAATGCAATTATTATATATTATGTATGGTTAAGCGATATATCATCACAAGTGGAT
ATTTATAGCAATGTAATTCTTAATTACATTGGCTAATCATTAAAGTCATTACAAACAATT
TATTTATCTATAAATTGCAGTAGTTTTTATTACACGCCCTTTGATAGTGGAGCATTTACA
TTAGATCTTATACATTGTTCTACTGTTTTCGTAATTATATCCGTAATATCTTCCCTGGTA
ATACTCCCAGCCTGTGATGCCGATGGACCTGGATCGATTGCCGAATCATCTAATCCGCTC
AGGTTTTTGTATGGTCTGGTAGTGAAGGAAGAATGTCCGACCTCCGATCTGCTAGCCCAT
GACTCGTTCGGACCTATAGCCATATAGGGTACCCTTAACGATCTTGAACTATGTCCAATC
AATCGTGAACCATCTACAAGACGTCGTGTTTGTGGTTTCGAACCTACGGACCAGAAATCA
ATGTCGTTCACGGTGTATTCTTTAGTTTGTATTTCTATTTTCGGGGGTCTGAATTCGACG
TCAGTCGAATGTTTGGCCGACGACAGCTTCAATTTTCCTAGCATCTTACAGAAGTGCACT
CCATTCACTACGTTTGCGTTCTCCACTCTGTATTCCACTCTCCAAGGATTCTTATCCTTT
AGAGAGAAAAATGAGGAAGAGTAGTAGTGGAGGTTACAGTTGCATCGGATCGGAATTGTG
AATTCCGCTTGCTTTGTGTCTCCCTCCGTCAATCTCATGTCGTGTATTTCTATAACTACA
TGTCCGACAGCATTAATTGGAACCTGACTGCGATATTCCAGTAATACGTGATCTATTTTC
ATGCACCTGTTCCTCAGCTGGCTAATTTTTTGTTCGAACATGGAAGGAAATGACATGGTT
ACCTCTGCAGCATCGTTCGTGAGAGCATACTCAACTCGCTCCGATTGTATATACCCACCC
ACTCCCAGACCCAGACTATCATTTCCTACTGACATTTCGGAAGCGCAGCGACTTAGGAAG
CGCAGCGACACACCCAATGACACGAAGAAGATCGTACAACGAACACCTATTAACAGAAGA
AGAAGAAGAAACCCTAGCAAAGAAGGCAATTGCTTTGCAGAAAATGGAGAACATGCATTT
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TTTTCTACGAAAAATGTATTGTGTAATTTTATCTGTTGATACTTGTTGCCTGATTTTATT
TGGACGCAGCCCAAATGTATTAATAATCTCGATGCATAAAGAAGAAGAAAAAGCCCAAGA
AATTGGTCCAGTTGCAAAGCCCATGTTAAAAATGCACCATTCCCGTCCATTTATTTCTAT
TAAAGTTAGAGAGAAGCTGCTGTTTCTCTCTCTAGAACCGGATTGGTGTCTCTCAACTTC
CTACATATAATTGGTGTCTGGAGTCCCATATATAGGTAAGACACTAAATGGCAGAATTGT
AATTTTGATAAAGAAAATTACTTTAATTCAAATTCCCAAAAGCGGCCTTTCGTATAATAT
T

Figure S1. Complete sequence of DNA-B from the ToLCNDV isolate BZ. The start codon (complementary polarity) of the movement protein of all ToLCNDV isolates is marked in red and the stop codon in blue. The putative start codon of the MP from the BZ isolate is marked in pink. The 17-nucleotide of the insertion are marked in yellow and the adjacent ones in green.

CHAPTER III

Further molecular diagnosis determines lack of evidence for real seed transmission of tomato leaf curl New Delhi virus in cucurbits

INTRODUCTION

Species of *Cucurbitaceae* family include major crop species that provide vegetables and fruits. Cucurbits are present in the diets of diverse cultures worldwide since they are rich in vitamins and minerals. Spain is one of the leading world producers of cucurbits and the first European exporting country (FAOSTAT, 2021). The production of these crops has been severely affected by viral diseases, with infections of aphid-borne viruses belonging to the *Potyviridae* family as the most widespread and damaging (De Moya-Ruiz et al., 2021; Pérez-De-castro et al., 2020). However, whitefly-transmitted viruses (*Geminiviridae* family). have been more recently discovered in different cucurbit species. The tomato leaf curl New Delhi virus (ToLCNDV), a member of the genus *Begomovirus*, family *Geminiviridae*, was introduced into Spain in 2012 and has spread rapidly in southern regions of this country but also to neighborhood countries in the Mediterranean basin, causing a significant impact with substantial losses in this horticultural region (Fortes et al., 2016).

ToLCNDV genome consists of two circular single-stranded DNA molecules of approximately 2.7 kb each (designated as DNA-A and DNA-B), both required for essential viral functions and encapsidated in geminate particles (Jyothsna et al., 2013; Padidam et al., 1995). ToLCNDV was first detected on tomato (*Solanum lycopersicum* L.) in north India in 1995 (Srivastava et al., 1995), and later, it has propagated to other Asian countries on several hosts, particularly vegetable species of the *Solanaceae* and *Cucurbitaceae* families (Moriones et al., 2017; Zaidi et al., 2017). The isolate emerged in the Mediterranean basin, designated as ToLCNDV-ES, presents a very low degree of genetic variability (Juárez et al., 2019; Panno et al., 2019), affects mainly cucurbits (preferentially zucchini [*Cucurbita pepo* L. subsp. *pepo*], melon [*Cucumis melo* L.], and cucumber [*Cucumis sativus* L.]) (Juárez et al., 2014; Kheireddine et al., 2019; Mnari-Hattab et al., 2015; Panno et al., 2016; Sifres et al., 2018). Symptoms include curling and severe mosaic of young leaves, shorted internodes and fruit of smaller size with skin deformation (Juárez et al., 2014), often resulting in a null or reduced yield and a lower market value.

The understanding of the epidemiology and evolution of viral diseases constitutes a fundamental strategy to control devastating viruses like ToLCNDV for cucurbit crops. Two main modes of transmission have been described for plant viruses: horizontal, in which the virus is transmited to hosts of a same generation, or vertical, in which viral transmission occurs from parent

to offspring (Simmons et al., 2011). Like most begomoviruses, ToLCNDV is horizontally transmitted only by the whitefly *Bemisia tabaci* (Gennadius) in a persistent manner (Chang et al., 2010; Islam et al., 2010; Jyothsna et al., 2013; Khan et al., 2012), although cloned genomic DNAs of this virus can be used as infectious constructions in *Agrobacterium*-mediated experimental transmission (agroinoculation). In addition, some isolates of this virus, including ToLCNDV-ES, can also be mechanically sap transmitted to a broad host range (Chang et al., 2010; López et al., 2015; Sohrab et al., 2013; Usharani et al., 2004). With respect to vertical transmission, many researches have described seed transmission of geminiviruses, among which is ToLCNDV, while others reported no evidence of this event.

Seed transmission of geminiviruses to the infected host progeny had been considered inefficient until the recent emergence of studies reporting this possibility (Renukadevi et al., 2022). The sweet potato leaf curl virus (SPLCV, genus *Begomovirus*) was detected in seeds from SPLCV-infected sweet potato (*Ipomoea batatas* (L.) Lam.) plants and the transmission level of SPLCV from seeds to seedlings was up to 15% (Kim et al., 2015). The mung bean yellow mosaic virus (MYMV, genus *Begomovirus*) was detected in seeds of black gram (*Vigna mungo* L. Hepper) plants naturally infected in the field and the virus was also detected in 32% of the seedlings of the offspring (Kothandaraman et al., 2016). The beet curly top virus (BCTV) and the beet curly top Iran virus (BCTIV) (both of genus *Curtovirus*) were detected in percentages of 38.2-78.0% and 8.8-18.5% of the seedlings developed from the seeds of a petunia cultivar (*Petunia x hybrida* hort. ex E. Vilm.) infected with BCTV and BCTIV, respectively (Anabestani et al., 2017). The tomato yellow leaf curl virus (TYLCV) was also detected in seeds of TYLCV-infected tomato plants, and the reported average transmission rate to seedlings arrived at 80% (Kil et al., 2016). Seed transmission of TYLCV was also verified in white soybean (*Glycine max* (L.) Merr.) and sweet pepper (*Capsicum annuum* L.) (Kil et al., 2017, 2018). However, evidence of seed transmissibility of TYLCV in *Nicotiana benthamiana* was not supported (Rosas-Díaz et al., 2017). Indeed, recent studies in tomato transmission of TYLCV and tomato yellow leaf curl sardinia virus (TYLCSV) through infected seed was not observed either after studies performed at different locations (Pérez-Padilla et al., 2020; Tabein et al., 2021). Furthermore, seed-transmission of SPLCV was not detected after a recent large-scale screening of sweet potato plants (Andreason et al., 2021).

ToLCNDV was reported as seed-transmitted for Indian isolates in chayote (*Sechium edule* L.) in Tamil Nadu, India (Sangeetha et al., 2018), in cucumber in Taiwan (Chang et al., 2022), in bitter

gourd (*Momordica charantia* L.) in Coimbatore, India (Gomathi Devi et al., 2023), and in sponge gourd (*Luffa cylindrica* L.) in Varanasi, India (Krishnan et al., 2023). Besides, seed- transmission in cucurbits of the Spanish isolate of ToLCNDV has also been investigated in Mediterranean regions. Kil et al., (2020) reported vertical-transmission of ToLCNDV-ES through seeds in zucchini plants in Italy, with transmission rates overcoming 60% in the evaluated offspring seedlings. Instead, the analysis of melon seedlings germinated from ToLCNDV-ES-infected seeds did not support vertical-transmission from seeds to the offspring (Fortes et al., 2023).

Elucidating and understanding the epidemiology of ToLCNDV-ES is crucial to develop and implement control strategies, and in the case of seed-transmission, essential to define import requirements. The EFSA Panel on Plant Health (2020) considered that even though transmission through seeds may be possible for ToLCNDV, production of commercial seeds contaminated with ToLCNDV is not very likely. Thus, the goal of our research was to evaluate whether ToLCNDV-ES can be transmitted through zucchini, melon, pumpkin, cucumber and watermelon (*Citrullus lanatus* (Thunb.) Matsumara & Nakai) seeds, assaying own and commercially produced seeds, which could serve as a primary source of inoculum for transmission by vectors in the field. Moreover, since the use of resistant host varieties is the most efficient viral disease management strategy in cucurbits (Martín-Hernández & Picó, 2020), we included the tolerant to ToLCNDV-ES accession Nigerian Local (*Cucurbita moschata* (Duchesne), original from Nigeria) (Sáez et al., 2016) in our trials, with the aim to likely detect reduced seed transmission of this virus, if it did arise. The results here obtained may contribute to shedding light and identifying a ToLCNDV-ES primary source of inoculum, with an impact on both global seed trade and germplasm conservation in gene-banks.

MATERIALS AND METHODS

Obtention of seeds from ToLCNDV infected cucurbit plants and sampling

Seeds obtained at COMAV-UPV greenhouse

To investigate seed transmission of ToLCNDV-ES in *Cucurbitaceae* family we tested the offspring of 27 genotypes belonging to *C. melo* and *C. moschata* species. The number of genotypes assayed and botanical classification are shown in Table 1.

Table 1. Accessions of *Cucurbitaceae* family infected with ToLCNDV used to obtain progeny.

Genus	Species	Subspecies	Group	Number of evaluated genotypes	Number of genotypes with fruit
Cucumis	*Melo*	*melo*	*inodorus*	18	15
			cantalupensis	3	1
			flexuosus	3	1
Cucurbita	*moschata*			3	3

Seeds were provided by the Institute for the Conservation and Breeding of Agricultural Biodiversity genebank (COMAV-Universitat Politècnica de València, Valencia, Spain). All *C. melo* genotypes had been previously reported as susceptible to ToLCNDV (López et al., 2015), whereas the three *C. moschata* plants evaluated were the pumpkin accession Nigerian local, known to be resistant to ToLCNDV under mechanical transmission conditions and tolerant when the virus is inoculated by whiteflies (Sáez et al., 2016), and two breeding lines with different levels of resistance derived of Nigerian local. Melon seed coats were slightly opened by forceps, to facilitate germination. All seeds of each genotype were disinfected by soaking them in a 10% solution of sodium hypochlorite for 3 min and washed for 5 min in distilled water. Germination was performed in Petri plates with moistened cotton at 37ºC for 48 h and seedlings were transplanted to pots in a growth room with controlled environmental conditions of 25ºC, 60% relative humidity, and a 16–8 h light/dark photoperiod. At four true-leaves growth stage, seedlings were transplanted to a leak-proof greenhouse.

For inoculation, a ToLCNDV-ES-infectious clone was agroinfiltrated by injection into petioles of MU-CU-16 zucchini (*C. pepo*) plants, as is described in (Sáez et al., 2016). After clear symptom

development of ToLCNDV infection, these plants and a population of whiteflies were stablished in the same greenhouse described above, constituting the source of inoculum and the natural vector to perform the transmission of ToLCNDV to the selected genotypes. At this time, healthy melon and pumpkin plants had arrived at the flowering stage of development. Plants were monitored for ToLCNDV infection by symptoms development and PCR as is described in (López et al., 2015). Differences in viral distribution through the plant were further investigated by quantitative PCR (qPCR) in leaf and flower tissues of selected genotypes as is described below. All plants were self-pollinated and fruits cultivated and harvested at maturity. Seeds were collected and dried to be preserved at 4ºC. To gain evidence about virus location, three seeds from infected fruits of selected melon genotypes were used to check viral infection separating the internal part of the seed (embryo and endosperm) from the coat. Viral titers of seeds were determined by qPCR comparing between not treated and surface-disinfected seeds with sodium hypochlorite as is described above. Finally, fifteen not surface-disinfected seeds per fruit were germinated and growth in a climatic chamber in three batches of five plants each to evaluate ToLCNDV seed-transmission to the progeny.

Analysis of seeds from a commercial greenhouse

Both environmental factors and host genotype influence seed-transmission of viruses (Dombrovsky & Smith, 2017; Montes & Pagán, 2019). To consider this effect, ToLCNDV seed-transmission was additionally evaluated using sixty seeds coming from ToLCNDV-diseased melon fruits cultivated in a commercial greenhouse located in La Mojonera (Almería, Spain). Four of these seeds were reserved to perform a PCR test of whole seeds or separating embryo-endosperm and coat to evaluate ToLCNDV accumulation. The remaining 56 seeds were germinated as is described above but avoiding previous disinfection. Seedlings were transplanted to pots and cultivated in a climatic chamber under the same conditions.

Analysis of seeds from a commercial nursery

Finally, a third assay was performed with commercial seeds to evaluate both whether it was possible to detect ToLCNDV in them, and in positive cases, testing seed transmission. Batches including fifteen seeds of 43, 17, 30 and 19 varieties of cucumber (*C. sativus* var. *sativus*), melon (*C. melo* subsp. *melo*), watermelon (*C. lanatus* var. *lanatus*) and zucchini (*C. pepo*), respectively, were bought in a commercial nursery. Five whole seeds of each variety were checked by PCR to

identify ToLCNDV contamination (as is described below). In case of ToLCNDV detection, the remaining seeds of the corresponding batch were used to evaluate vertical transmission of ToLCNDV to the offspring.

Evaluation of ToLCNDV transmission to the offspring through seeds

Avoiding previous disinfection, seeds obtained from infected plants were germinated and seedlings grown as is described above. All seedlings were weekly monitored to detect symptoms development. Thirty and 60 days after germination, the completely expanded youngest leaf of each seedling from the assays of the section "Seeds obtained at COMAV-UPV greenhouse" was sampled and used for DNA extraction followed ToLCNDV detection by conventional and quantitative PCR.

To assess additional temporal stages of development, the seedlings of the offspring from plants of the commercial greenhouse and from the seeds bought in a nursery were sampled weekly, since two to four weeks post-germination. In both studies, tissue from the apical leaf of each plant was collected and subsequently used for DNA extraction and qPCR.

DNA extractions from different plant tissues, seeds and seedlings for ToLCNDV diagnosis

Total genomic DNA for ToLCNDV diagnosis was extracted from parental leaf and flower (petals, stamens and pistils) tissues and from leaves of seedlings using the Cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Hortorium, 1991), To identify ToLCNDV infection in flower tissue, bulks of two or three flowers of the same plant were used for DNA extraction. DNA was quantified using a NanoDrop 1000 spectrophotometer and diluted with sterile distilled deionized water to a final concentration of 50 ng/µL.

To avoid co-precipitated polysaccharides and inhibitors of PCR reaction, DNA of whole seeds, coats and internal seed tissues (embryos and endosperms) were extracted using Zymo-Spin™ I columns (Zymo Research). Each seed or component of seed was mashed in an Eppendorf tube with stainless steel "UFO" beads of 3.5 mm (Next Advance, Inc., USA), which have sharper edges specific for resilient samples, in a Retsch (MM300) homogenizer for 1.5 min at 30 s^{-1}. Mashed samples were centrifuged and placed on ice. To each tube 1.4 ml of extraction buffer (4 M guanidine thiocyanate; 0.1 M sodium acetate, pH 5.5; 10 mM ethylenediaminetetraacetic acid

(EDTA), 0.1 M 2-mercaptoethanol) was added and mixed vigorously in a vortex. After 5 min centrifuging at 13000 rpm, 700 µL of supernatant were transferred to silica gel spin columns and then washed twice with 500 µl of washing buffer (70% ethanol, 10 mM sodium acetate, pH 5.5). Finally, total DNA was eluted loading 10 µl of 20 mM Tris-HCl, pH 8.5 and stores at -20°C.

Detection of ToLCNDV by PCR

The presence of ToLCNDV in the plant samples was determined by PCR reaction to detect the presence of both viral components using two specific pairs of primers. For this purpose, 1 µl aliquots of total DNA (50 ng) were used as templates in PCR reactions of 20 µl with 1 U of DreamTaq DNA polymerase (Thermo Scientific™), 1 µM of two different primer pairs (To-A1F/To-A1R, and To-B1F/To-B1R), 10X DreamTaq Buffer (Thermo Scientific™), 0.2 mM dNTPs and water up to final volume. The two primer pairs were derived from the Spanish isolate Murcia 11.1, one from the segment DNA-A, accession number KF749225, (To-A1F 5´-GGGTTGTGAAGGCCCTTGTAAGGTGC-3´, position 476-501, and To-A1R 5´-AGTACAGGCCATATACAACATTAATGC-3,´ position 954-979), and other one from the segment DNA-B, accession number KF749228, (To-B1F 5´-GAAACACAAGAGGGCTCGGA-3´, position 637-656, and To-B1R 5´-GCTCCACTATCAAAGGGCGT-3´, position 1294-1313). Cycling conditions consisted of incubation at 94ºC for 5 min and 45 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 45 s, with a final extension of 10 min at 72ºC. The resulting PCR products of 505 and 677 bp in length were analyzed by electrophoresis in 1.5 % agarose gels in TAE buffer (40 mM Tris, 20 mM sodium acetate and 1 mM EDTA, pH 7.2) and stained with ethidium bromide. To verify the identity of amplified fragments, PCR products of random samples were sequenced in both directions by means of an ABI PRISM DNA Sequencer 377 (Perkin–Elmer) using the same primers that for amplification reaction. The presence of intact DNA in negative seed samples was confirmed using PCR to amplify the β-actin gene in cucumber, melon and watermelon (CIACT-F 5'-CCATGTATGTTGCCATCCAG-3´, CIACT-R 5´-GGATAGCATGGGGTAGAGCA-3´) samples and CpACS7 in the case of zucchini seeds (CpACS7F 5´-GTGAGAGTGGCAAGAGGGAG-3´, CpACS7R 5´-CGGCATTGCAAAGAAAAAGCAG-3´).

Relative viral titre estimation by quantitative PCR (qPCR)

To estimate ToLCNDV titers in the different samples analyzed, relative quantification of viral loads by qPCR was also performed at 60 days after planting (dap) in selected positive samples of leaves and flowers from white-fly infected plants at COMAV-UPV. Seeds and seedlings samples that tested positive for the presence of ToLCNDV were also subjected to qPCR relative quantification. For this purpose, total DNA was diluted to a final concentration of 5 $ng \cdot \mu L^{-1}$. Equal amounts of genomic DNA (15 ng) were used as templates in qPCR reactions of 15 µL, containing 7.5 µl of 2 X iTaqTM universal SYBR® Green Supermix and 1.5 µl of each primer and 1.5 µl of H2O. Primers ToLCNDVF1 (5'-AATGCCGACTACACCAAGCAT-3', positions 1145–1169) and ToLCNDVR1 (5'-GGATCGAGCAGAGAGTGGCG-3', positions 1399–1418) were used for the amplification of a 274 bp fragment of viral DNA-A. As reference host genes, the single copy genes CmWIP1 (primers CmWIP1F (5'-TAGGGCTTCCAACTCCTTCCTCTT-3') and CmWIP1R (5'CTTGCAATTGATGGGTGTGATCTTCTTG-3′) and CpACS7 were amplified in melon and *C. moschata* samples, respectively. Quantification was performed in a LightCycler® 480 Instrument (Roche, Basel, Switzerland) under cycling conditions consisting on incubation step at 95 °C for 15 min, followed by 45 cycles of 95 °C for 5 s, 57 °C for 30 s, and 72 °C for 30 s. Finally, a melting curve was obtained to each sample to confirm and characterize the amplicons. Relative ToLCNDV accumulation was calculated to each sample applying the simplified expression ($2^{-\Delta Ct}$) of the method developed by (Schmittgen & Livak, 2008). Significant differences between samples were evaluated by ANOVA-multiple range tests (least significance difference (LSD)) using STATGRAPHIC 18 TM (Statgraphics Technologies, Inc., The Plains, VA, USA) statistic software.

Southern blot hybridization

To understand how the virus is transmitted through seed, we attempted to detect replicative forms of ToLCNDV by southern blot in those seedling samples in which viral load was detected. As controls, positive samples of leaves and flowers of parental ToLCNDV-infected plants were included. An amount of 15 µg of total DNA of each sample were loaded onto a 1% agarose gel. Electrophoresis was performed in 1x TAE buffer at 50 V for 3 h and then blotted onto a nylon membrane positively charged (Roche, Basel, Switzerland). Membranes were fixed by UV irradiation (700 × 100 mJ/cm^2), and hybridized with a digoxigenin-labeled RNA probe as is

described in (Sáez et al., 2021). Results were visualized in films exposed to the membranes for one hour at room temperature.

Roling cycle amplification (RCA)

Total DNA of samples of the offspring seedlings, positives for ToLCNDV infection, was diluted to 10 ng/µl and used as a template in rolling circle amplification (RCA) by resuspending 1 µl in 10 µl of sample buffer from a TempliPhi kit (Cytiva 25-6400-10, Sigma Aldrich) and incubated at 95ºC for 90 seconds. After freezing on ice, a mix of 5 µl of reaction buffer and 0.2 µl of the enzyme was added to each sample. Rolling circle amplification reactions were carried out at 30ºC for 18 hours and subsequently, the enzyme was heat inactivated at 65ºC for 10 minutes. The products were directly subjected to PCR amplification and electrophoresis separation in agarose gel as is described above. Samples of ToLCNDV-infected leaves and reproductive organs tissues were added as positive controls, while a healthy seedling sample was included as a negative control.

RESULTS

Symptoms development and ToLCNDV detection in vegetative and reproductive tissues of infected plants

All *C. pepo* plants agroinfiltrated with ToLCNDV clones developed typical symptoms of curly and mosaic in young leaves after 10-15 days post inoculation (dpi). As a consequence of the high incidence of the disease, zucchini plants experienced severe growth restriction and failed to produce any fruit, with all plants dead before fructification (data not shown). Three weeks following the establishment of the whiteflies, all melon plants showed typical curli ng, yellowing and severe mosaic symptoms of ToLCNDV infection in youngest leaves (Fig. 1A). At late stages of the disease, both male and female melon flowers presented petal discoloration and deformation and floral ovaries were cracked in some plants (Fig. 1B-C). Despite the presence of cracked fruits with non-viable seeds (Fig. 1D), seventeen out of the 24 plants produced melons showing symptoms of skin roughness, but containing viable seeds (15 of inodorus group, one of cantalupensis group and one of flexuosus group) (Table 1) (Fig. 1E-G). Among *C. moschata* genotypes, Nigerian Local plant remained symptomless, and the two derived lines just showed

slight symptoms in leaves, generating each plant one fruit with well-formed seeds (Table 1) (Fig. 1H-K).

ToLCNDV was detected by polymerase chain reaction (PCR) in all leaf tissues of the assayed plants (Fig. 2, Table 2). As was expected, the viral load detected in *C. melo* was higher than in *C. moschata* genotypes. In almost all of both male and female melon flowers, ToLCNDV-specific bands were detected in petals, stamens and pistils (Table 2).

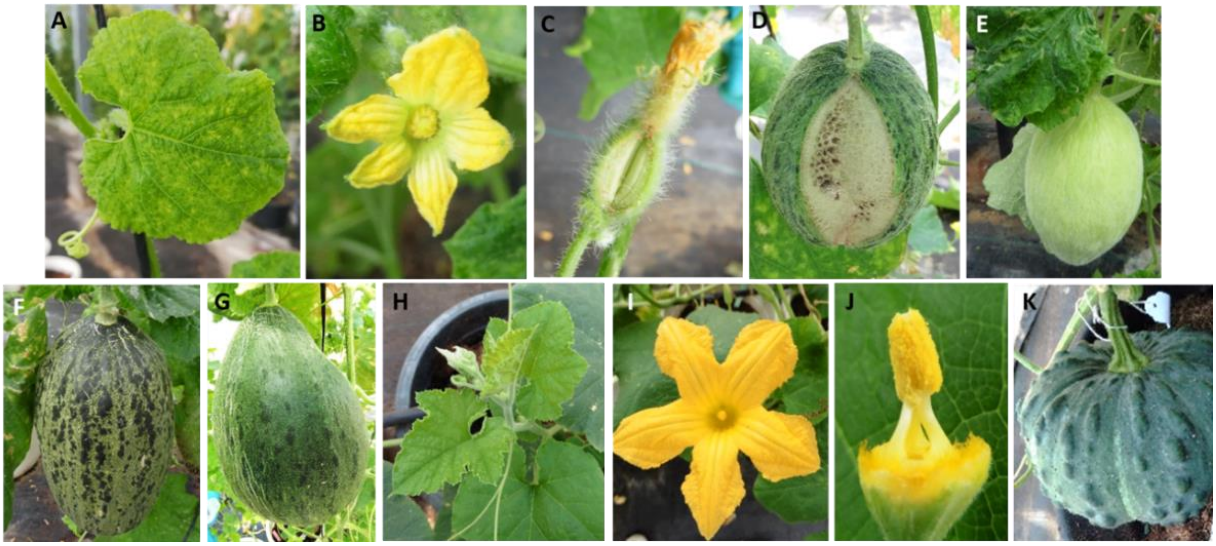


Figure 1. Symptoms development of ToLCNDV in vegetative and reproductive organs of *C. melo* and *C. moschata* plants. **A:** melon leaf infected with ToLCNDV displaying severe mosaic and mild curling. **B-C:** male and female flowers of *C. melo* infected plants with deformed petals and cracked ovary. **D:** immature melon fruit aborted as consequence of ToLCNDV infection. **E-G:** melon fruits with skin roughness in ToLCNDV infected plants that arrived to ripening stage and produced viable seeds. **H-K:** leaves, flowers, and fruit of asymptomatic *C. moschata* plants.

Even in the symptomless plants of *C. moschata*, ToLCNDV was detected in all floral tissues (Table 2). In all of those genotypes in which DNA was successfully extracted from seed coats, the presence of ToLCNDV was confirmed in this part of the seed, while the virus was not detected in endosperms of some of *C. melo* and in none of *C. moschata* seeds (Table 2). Sequencing of the PCR products confirmed the viral infection (data not shown).

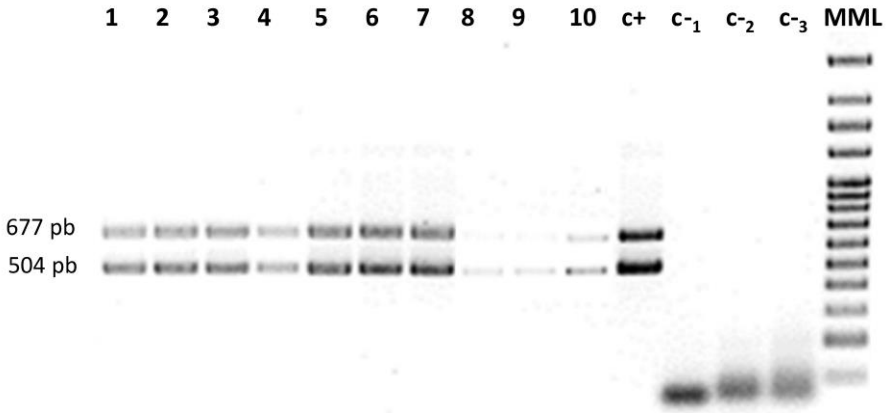


Figure 2. Detection of ToLCNDV-A and -B specific fragments by conventional PCR in symptomatic leaves of plants infected by white-fly transmission 21 days after vector and source of inoculum (MU-CU-16 zucchini plants) establishment. Lanes 1 to 5: *C. melo* inodorus group samples, lane 6: *C. melo* cantalupensis group sample, lane 6: *C. melo* flexuous group sample, lane 8: *C. moschata* Nigerian Local genotype, lanes 9 and 10: Nigerian Local derived breeding lines, c+: positive control, $c\text{-}_1$ and $c\text{-}_2$: healthy plants used as negative controls, $c\text{-}_3$: water negative control, MML: molecular weight marker DNA Ladder (NZYDNA Ladder VII, ranging from 100 to 3000 bp, NZYTECH, Lisbon, Portugal).

Table 2. Evaluation by conventional PCR of ToLCNDV presence in leaves, flowers and seed tissues of cucurbit plants. Cantalupensis group (Cant.), Flexuosus group (Flex.), Internal control not detected (nd).

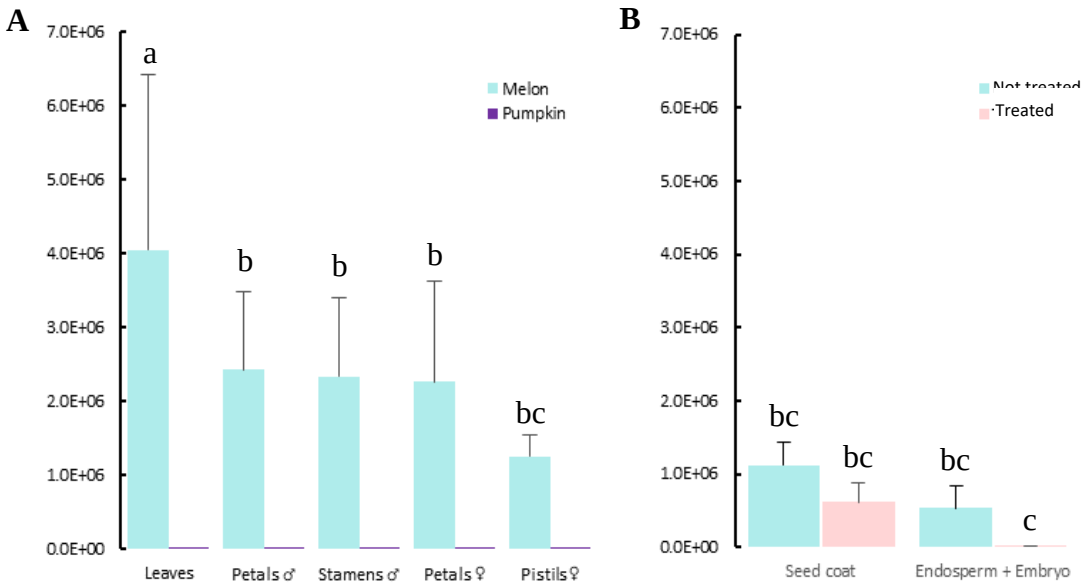
		C. melo* subsp. *melo																	***C. moschata***		
		var. *inodorus*															**Cant.**	**Flex.**			
Sample		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**1**	**1**	**1**	**2**	**3**
Leaves		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flowers	Petals ♀	+	+	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	+	+	nd	+
	Ovary-pistils	+	+	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	+	-	-	+
	Petals ♂	+	+	+	-	+	+	+	+	+	nd	+	nd	+	+	nd	+	+	+	-	+
	Anthers	+	+	+	+	+	+	+	+	+	nd	+	nd	+	+	+	+	-	+	+	+
Seeds	Coat	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	nd	nd	+	+	+
	Endosperm	+	-	-	+	-	+	-	+	+	+	+	-	+	+	+	-	+	-	-	-

ToLCNDV detection by quantitative PCR in different parts of inoculated plants, seeds and seedlings

To quantify differences in the distribution of ToLCNDV between the analyzed tissues in both *C. melo* and *C. moschata* plants, a quantitative PCR (qPCR) assay was performed. Leaves of *C. melo* exhibited the highest viral accumulation, with an average level three times higher than that identified in different floral organs (Figure 3A). ANOVA and LSD tests did not identify significant differences between the means of the viral accumulation in the floral tissues (Figure 3A). On

average, the relative viral load in leaves, petals and reproductive tissues of *C. moschata* was six magnitude orders lower than that quantified in the same tissues of *C. melo* (Figure 3A). Regarding seeds obtained by self-pollination, viral titers were detected in all parts of the seed (Figure 3B). However, a significant reduction of ToLCNDV accumulation was detected in bleach treated endosperm and embryo tissues compared with not treated seeds or treated seed coats (Figure 3B).

Figure 3. ToLCNDV relative accumulation ($2^{-\Delta Ct}$) quantified by qPCR on leaf tissue, petals and reproductive organs



of male (♂) and female (♀) flowers of infected plants at the green-house of COMAV-UPV (**A**) and in different parts of seeds obtained by their self-pollination with or without bleach treatment (**B**).

Evaluation of ToLCNDV presence in seedlings obtained from inoculated plants

None of the offspring seedlings derived from fruits of ToLCNDV-infected plants at COMAV-UPV developed symptoms during the assay. Tissue samples were collected from each seedling at 30 and 60 days post-germination (dpg) to assess the presence of ToLCNDV by PCR. Out of 255 *C. melo* plants evaluated, specific bands were detected in only 6 and 13 plants of inodorus group at 30 and 60 dpg, respectively. In none of the 6 plants that exhibited viral load at 30 dpg the presence of the virus was detected at 60 dpg. ToLCNDV was not detected in any plant of *C. melo* cantalupensis and flexuosus groups. On the other hand, out of 45 *C. moschata* evaluated seedlings, seven and 19 tested positive for ToLCNDV at 30 and 60 dpg, respectively. Only in four seedlings ToLCNDV was detected at both 30 and 60 dpg.

To further assess virus transmission from seed to the offspring, the viral load in all *C. melo* and *C. moschata* seedlings was quantified by conducting a qPCR analysis. On average, relative accumulation of ToLCNDV was lower in *C. melo* plants (means of $2^{-\Delta Ct}$ = 29.19±13.66 and $2^{-\Delta Ct}$ = 17.88±6.0 at 30 and 60 dpg, respectively) than in *C. moschata* plants (means of $2^{-\Delta Ct}$ = 191.64±68.92 and $2^{-\Delta Ct}$ = 96.14±40.11 at 30 and 60 dpg, respectively). Seedlings of *C. moschata* harbored similar viral loads than parental leaf tissues, while viral titers detected in *C. melo* were 10^5 times lower than in leaves of the progeny. In both species, ToLCNDV average accumulation decreased from 30 dpg to 60 dpg.

Seeds and seedlings from a commercial greenhouse

ToLCNDV was detected in three out four evaluated seeds when whole seed or separated coat where analyzed. Endosperm and embryos were negative. In six seedlings out the 56 germinated to assed vertical-transmission of ToLCNDV the virus was detected by both PCR and qPCR at four weeks after germination (wag), but in only one viral load was also detected one week later. In three additional seedlings ToLCNDV was detected at five wag.

Commercial seeds and seedlings

Among the commercial seeds bought in a nursery, ToLCNDV was not detected in watermelon seeds (Table 3). However, in one seed of cucumber and in one seed of zucchini, ToLCNDV could be amplified by PCR (Table 3). Furthermore, all analyzed seeds of one of the Piel de Sapo melon varieties tested positive for viral infection. After germinating the remaining seeds of each variety in which ToLCNDV was confirmed, all plants keep symptomless to the fifth week after germination. Viral titer was detected in only one melon plant (qPCR cycle threshold = 30.95).

Table 3. Evaluation of ToLCNDV presence in commercial seeds of watermelon (*C. lanatus*), melon *(C. melo*), cucumber (*C. sativus*) and zucchini (*C. pepo*). Type of cultivar, number of analyzed varieties, number of positive varieties where ToLCNDV was identified in seed, and proportion of positive out of analyzed seeds.

Species	Type	Varieties analyzed	Varieties Positive for ToLCNDV	Positive seeds/analyzed seeds	Offspring seedlings positive for ToLCNDV
Watermelon *(Citrullus lanatus)*	Mini	1	-		
	Black	10	-		
	Striped	19	-		
Cucumber *(Cucumis sativus)*	Short	10	-		
	Long	33	1	1/5	-
Melon *(Cucumis melo)*	Blanco	1	-		
	Amarillo	1	-		
	Cantalupo	2	-		
	Galia	8	-		
	Piel de Sapo	5	1	5/5	1
Zucchini *(Cucurbita pepo)*	Yellow	1	-		
	White	1	-		
	Round	1	1	1/5	-
	Dark green	7	-		
	Edium green	9	-		

Replicative forms of ToLCNDV are not present in any seedling of the offspring

Samples of those seedlings in which ToLCNDV was detected, coming from fruits obtained at COMAV-UPV or originated with commercial purposes, were subjected to Southern blot hybridization. Although a specific RNA-probe complementary to ToLCNDV coat protein was used, genomic forms of the viral DNA could not be detected (Figure 4). Conversely, in leaf, flowers and seed samples from tissues of plants of the progeny, characteristic open circular, linear, supercoiled and single strand viral replicative forms were identified (Figure 4).

To avoid detection sensitivity limitations, we included a second assay to just detect single- or double-stranded circular DNA of ToLCNDV. Thus, circular DNA was exponentially amplified as a previous step by a rolling circle amplification (RCA) reaction. The resulting products were used as template in conventional PCR assay to detect both A and B genomic particles. RCA products tested positive for ToLCNDV in samples of leaves, all the sampled parts of flowers and the different parts of seeds. Even in disinfected endosperm and embryo complex of melon seeds, both

DNA-A and DNA-B of ToLCNDV were detected. Instead, in leaves of seedlings germinated from infected seeds, integral genomic components of the virus were not possible to identify.

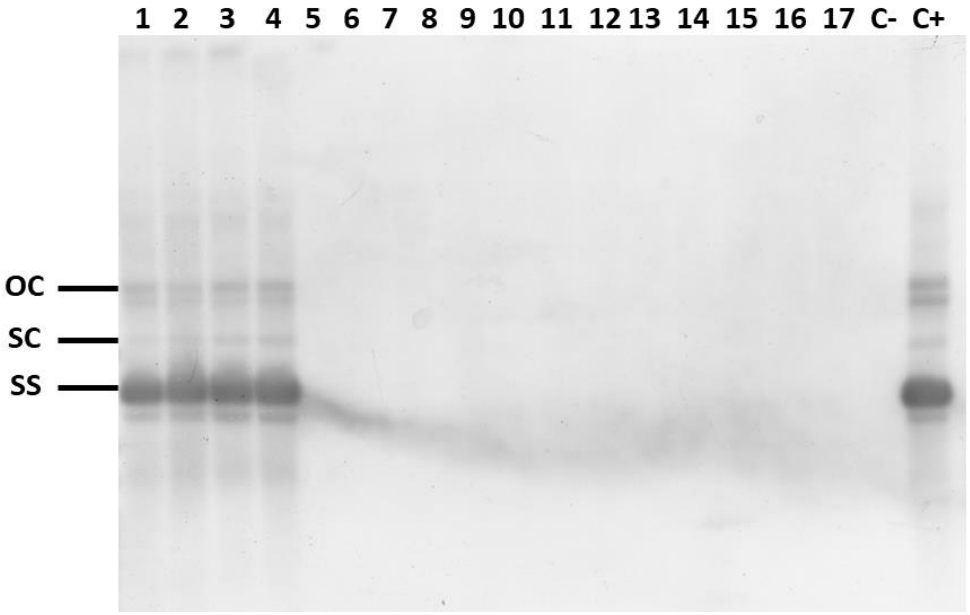


Figure 4. Southern blot analysis. Detection of open circular dsDNA (OC), supercoiled dsDNA (SC) and ssDNA (SS) forms of ToLCNDV in leaf (lane 1), flower tissues (lanes 2 and 3) and whole seed (lane 4) of melon plants cultivated at COMAV-UPV. None of the forms were detected in seedlings where the virus was identified by conventional or quantitative PCR (lanes 5 to 17). C-: a healthy melon plant was used as a negative control; C+: DNA of a MU-CU-16 agroinfiltrated plant was used as a positive control.

4. DISCUSSION

Plant viruses are intracellular parasites that may be introduced into plant cells when insects transmit them during the plant-feeding process, through injuries generated in agricultural management practices or through seeds. Around 80% of all known plant viruses are vector-borne horizontally transmitted (Hohn, 2007), while a least 25% of viruses infecting plants are vertically transmitted through seeds to seedlings of the offspring (Sastry, 2013). In vertical transmission, viruses remain in seeds for long periods of time, overcoming adverse conditions and entailing serious consequences to global trade and international germplasm exchanges (Kumar et al., 2021). Consequently, detecting viral presence in seeds and determining vertical transmission through gametes and embryos to seedlings of subsequent generations are increasingly gaining importance (Chalam et al., 2020; Pagán, 2019; 2022).

In diseases generated by geminiviruses, transmission of viral particles directly into the plant phloem through whiteflies has been described as the main epidemiological factor contributing to disease propagation. However, investigations into seeds as another source of geminivirus inoculum have resulted in finding some evidence for the possible seed-borne nature of some begomoviruses in different host plants (Just et al., 2017; Suruthi et al., 2018). However, real seed-transmission of these viruses has been questioned and is currently under revision (Pérez-Padilla et al., 2020). In real seed-transmission, viruses are transferred from the embryo of fertilized seeds to the seedlings of the next generation, whereas seed-borne viruses may invade any tissue of the seed. Both cases require that viruses arrive to reproductive organs of infected plants. Many studies have reported high geminiviral loads in plant flowers and fruits (Shahmohammadi et al., 2022), as well as the presence of these viruses in whole seeds (Devi et al., 2023). In accordance, in this work ToLCNDV was detected in different parts of male and female flowers and seeds. Despite sodium hypochlorite disinfection, virus contamination was identified in whole seeds, coats and the set of endosperms and embryos ensemble. Even so, the observed reduction in ToLCNDV accumulation from leaves to flowers and seeds evidences how plants rely on diverse barriers to avoid viral achievement of floral and seminal tissues. For instance, cell death of the suspensor structure that connects the embryo and maternal tissues is programmed once the embryo is properly formed (Liu et al., 2015), leading viruses a scarce time period to spread to this part of the seed. Reduced and sporadic cucumber green mottle mosaic virus (CGMMV) presence has been reported in ovaries and ovules of cucumber plants compared with other flower tissues, what was also associated to internal barriers limiting viral propagation (Shargil et al., 2019). The efficiency of these barriers to impair viral invasion of gametes and embryos is environmental, cultivar and viral isolate dependent (Sastry, 2013).

In the particular case of ToLCNDV, most reports describing transmission through seed were focused on Asiatic strains of the virus (Chang et al., 2022; Gomathi Devi et al., 2023; Krishnan et al., 2023; Sangeetha et al., 2018). Previous to this work, only two researches have studied seed-transmission of ToLCNDV-ES in cucurbits, of which only one described that this mode of transmission is possible in zucchini plants (Kil et al., 2020). The Spanish isolate of ToLCNDV is adapted to infect cucurbits better than solanaceous crops (Yamamoto et al., 2021), with *C. pepo* species as the most susceptible hosts to this viral disease (Sáez et al., 2016). Our results support the lack of evidence on ToLCNDV-ES seed-transmission in melon plants determined by (Fortes et

al., 2023) and agree with these authors on the seed-borne-nature of this virus in cucurbits. The high susceptibility of zucchini plants to ToLCNDV-ES might come determined by host genetic determinants likely also involved in viral seed transmission.

Some other ToLCNDV-ES modes of transmission have been described as cultivar-dependent (Janssen et al., 2022). For instance, the whitefly transmission of the Spanish strains from zucchini crops to plants of watermelon and wild cucurbit species has been reported as inefficient, even under high inoculum pressure (Farina et al., 2023; Juárez et al., 2019). Additionally, the Spanish isolate of this viral species resulted mechanically transmitted to cucurbits, but with different rates of success depending on the assayed species (López et al., 2015). In many pathosystems, horizontal transmission of parasites leads to an increment in virulence, while vertical transmission conduct to less virulent pathogens since, thus, they are transmitted to a higher proportion of the offspring as less damage to the host is produced (Stewart et al., 2005). Then, parasites strictly vertically transmitted tend to evolve towards slight or no virulence (Hamelin et al., 2016). In geminiviridae family, an isolate of yellow mosaic virus (YMV) vertically transmitted to greengram (*Vigna radiata* (L.) R. Wilczek) produced latent symptomless infections, with unsuccessful horizontal transmission by sap inoculation (Behera et al., 2022). On the basis of this hypothesis, in the only seed-transmission report of ToLCNV-ES in zucchini crops (Kil et al., 2020), all seedlings of the offspring were asymptomatic, even when plants of *C. pepo* species represent the hosts with the highest susceptible response to this isolate. However, althoug zucchini seeds used by (Kil et al., 2020) were harvested from fruits of plants open-field cultivated displaying viral-compatible symptoms, the only confirmation for viral infection was performed to ToLCNDV by PCR, but unknowing mots of the viral genomic sequence. Since mechanical transmission of a geminivirus come determined by a single amino acid substitution in the viral movement protein (Lee et al., 2020), vertical transmission of viral isolates might be also influenced by a few nucleotidic changes in the genomes. Therefore, whether ToLCNDV transmission in zucchini plants is associated with viral or host genetic determinants must be further investigated.

Although the sudden spread of ToLCNDV in cucurbit crops of the Mediterranean Basin is hardly explainable, this is not the first time that an Asian endemic begomovirus is spread to other distal niches in the world (Shukla, 1991; Domingo et al., 1996; Qureshi et al., 2022). Although both, through *B. tabaci* and contaminated germplasm propagations are the most plausible mode mediating ToLCNDV-ES outbreaks, the results obtained in this work do not support the

assumption that transmission to seedlings of subsequent generations through seeds is a likely event for this virus. Even so, the information here provided increases the epidemiological knowledge about ToLCNDV-ES disease in cucurbits, which may be considered to stablish phytosanitary policies that guarantee virus-free plant material.

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GENERAL DISCUSSION

CCYV emergence and incidence

The emergence and widespread of plant viruses are in continuous expansion causing severe damages to valuable crops of the *Cucurbitaceae* and *Solanaceae* families. In the Mediterranean region, whitefly-transmitted viruses such as those from genus *Begomovirus, Ipomoviruses* and *Criniviruses* present major challenges to the agriculture in the area. Its rapid spread can be attributed to a confluence of climatic and cultural practices, that facilitate the dissemination of its natural vectors and an increase in trade in plant material that allows for its long-distance spread (Desbiez, 2022). This affects plant-virus interactions that could lead to epidemics and insect vectors may intensify this complexity and pathogenicity. Recognizing the variables that lead to the emergence of virus diseases is the initial step in their management, by means of effective control and sophisticated diagnostics that are available nowadays. For that, understanding the epidemiology and biologic characteristics of the viruses and their vectors is essential for the establishment of virus control strategies (Radouane et al., 2021).

Cucurbit chlorotic yellows virus (CCYV) belongs to the genus *Crinivirus*, and like ToLCNDV is part of a complex of whitefly-transmitted viruses that cause yellowing disease in cucurbits. In the survey conducted in Algeria, ToLCNDV was the most frequently detected in samples of zucchini, melon, and cucumber (80.5%), while CCYV was also detected with lower incidence (6.2%) in two different regions: El ghrouss and Sidi-Okba. This was the first report of CCYV in Algeria (Kheireddine et al., 2020), and in North Africa in general, and it could represent a serious threat for valuable cucurbit crops in this and other countries of Mediterranean Basin. Recently, CCYV was reported infecting cucumber plants (Chynoweth et al., 2021) and watermelon plants (Alfaro-Fernández et al., 2022) in Spain. Also, it is plausible that it has already spread to other neighboring countries but remain unnoticed due to the symptom's similarity with other viruses, such as CYSDV. Nonetheless, CCYV is considered a newly emerging virus, which explain its low genetic diversity in between isolates of different cucurbit and weed species. Regardless of the geographical distribution, plant host and year of collection, CCYV is a highly homogenous population, that will probably change with time taking in consideration its rapid spread and the high efficacity of its vector *B. tabaci* in infecting plants.

CCYV is commonly found, in natural conditions, in mixed infections with other whitefly-transmitted viruses, which induces similar yellowing symptoms and shares the same vectors

(Abrahamian et al., 2015; Abrahamian et al., 2013). In Greece, CCYV was detected in cucurbits in mixed infections with CYSDV (Orfanidou et al., 2014), but we were not able to detect CYSDV in any of the analyzed samples. Interestingly, in our case, and for the first time, CCYV was detected in mixed infection with ToLCNDV. It would be interesting to check if there is a synergistic or antagonistic interaction between CCYV and ToLCNDV, that may affect the spread and symptoms between crops. These virus-virus interactions may modulate infection traits such as the virus multiplication level, the capacity for plant colonization and virulence. It has been reported that two geminiviruses with a previously described synergistic interaction, pepper huasteco yellow vein *virus* (PHYVV) and pepper golden mosaic virus (PepGMV), infected more vascular tissue cells in mixed than in single infections (Rentería-Canett et al., 2011).

ToLCNDV emergence and incidence in the Mediterranean Basin

Cucurbit's cultivations are widely affected by the infection of tomato leaf curl New Delhi virus (ToLCNDV) worldwide (Juárez et al., 2019). Since its emergence in the Mediterranean basin in 2012 in Spain, it has rapidly spread to neighboring countries (Portugal, France, Italia) and overseas in North Africa affecting mainly cucurbits in Tunisia, Morocco, and Algeria. This let us to deduce that this rapid dissemination is mainly due to the active trading of plant materials and agricultural goods export between these countries, favorizing the spread of its vector *B. tabaci,* which can cause epidemics with only a small number of individuals. Moreover, the genetic uniformity of the commercial varieties currently has weakened the genetic variability of crops (Grumet et al., 2021).

The occurrence of the new strain ToLCNDV-ES in the Mediterranean Basin was likely the result of a single recent introduction event, which explains the highly homogenous population within the ToLCNDV-ES isolates. This is a typical example of a population bottleneck that happen when the virus acquire a new host or colonize a new geographical area (Ruiz et al., 2017). The ToLCNDV isolate of Algeria showed a high sequence similarity with the Spanish isolates, which suggests that probably, was introduced directly or indirectly from Spain. The presence of associated satellites was not detected, similarly to the Spanish isolate (Ruiz et al., 2017). However, the presence of alphasatellite and betasatellite molecules were reported in association with a ToLCNDV isolate of Pakistan (Anwar, 2017). Still, not enough information is available on associated satellites of the virus and their effect on infection severity. Moreover, a little genetic variation was observed on segment B, since a 17-nucleotide insertion was found, just before the

start of the gene BC1. Insertions or deletions in genomes are commonly known as replication slippage, or slipped strand mispairing. The DNA polymerase pauses and detach from the DNA strand during replication, which enable the growing strand to split from the template and reanneal upstream or downstream of a distinct homologous region (Viguera et al., 2001). As a consequence, of the insertion a MP 53 amino acids longer could be synthesized. The MP is required for virus cell-to-cell and long-distance movement, through its cooperative interaction with the Nuclear Shuttle Protein (NSP) (Ward et al., 1997). Sequencing of more isolates and biological assays should be performed to verify the biological and epidemiological implications of this major MP. However, this let us to speculate that perhaps the ToLCNDV-ES strain in the western Mediterranean countries is starting to accumulate genetic variability. The evolution of the viruses depends on its constant interaction with its vector and plant host, which changes with time and geographical location during its dissemination.

Similarly, to other begomoviruses, recombination events are considered to be the major force driving the evolution and emergence of ToLCNDV (Fortes et al., 2016). In fact, genetic recombination enables parental viruses to transfer on genetic information to their offspring through parasexual reproduction. This mechanism is important in the evolution of many virus families and has been extensively documented for members of the *Geminiviridae* family (Lefeuvre & Moriones, 2015; Seal et al., 2006). Recombination events were found in Asian isolates, but the low genetic variation of Mediterranean isolates has precluded the detection of recombinants. We analyzed possible recombination events in the DNA-B segment of our isolate, but no were found. The insertion was clearly detected in genetic analysis, but it is regarded as a mutation in a single site of the genome.

In contrast to Indian isolates, it was proven that ToLCNDV-ES is more adaptable to infect cucurbits than tomato, which is its original plant host (Fortes et al., 2016). The ability of ToLCNDV to infect a specific plant species rely on specific genetic factors of the host and the efficiency of the virus to exploit plant machinery in its favor (Moriones et al., 2017). This could explain the inadequacy of ToLCNDV to infect pumpkin in our survey, when it is was found nearby other infected cucurbits crops, as virus-host plant interactions play an important role in the dissemination of the virus.

ToLCNDV and CCYV molecular detection

Visually it is difficult to identify the presence of ToLCNDV and/or CCYV in a symptomatic plant, since the symptoms are very similar, and other times are in mixed infections with different viruses. The advancement of diagnostic tools, during the past decade, has permitted a better understanding of the intricate viral pathosystems affecting cucurbit crops in the Mediterranean basin (Lecoq & Desbiez, 2012). Efficient detection methods are primordial to avoid virus dissemination and minimize economic losses in cucurbits crops. For that, numerous detection methods have been developed based on different parameters, such as rapidness, precision, adaptability for on-site detection, genetic specificities of viruses, simultaneous detection of several viruses (Pallás et al., 2018). Moreover, a real-time LAMP assay was recently developed for a rapid and sensitive detection of ToLCNDV in cucurbits (Caruso et al., 2023).

However, molecular detection methods are the most used techniques to identify plant viruses, PCR and RT-PCR demonstrated a fast and precise application in ToLCNDV and CCYV identification. In the other hand, hybridization method was adapted to detect several viruses in many samples as a first screening. In both methods, virus titer was not taken in consideration to the intensity of the signals in the membranes or the PCR bands in the gel electrophoresis.

Geminiviruses transmission pathways controversy

Understanding how plant viruses are transmitted is a long-standing goal of plant virology. Indeed, the ability to be transmitted is regarded as one of the most important determinants of the fitness of any pathogen, including viruses (Anderson & May, 1982). It is essential to understand the epidemiology and evolution of ToLCNDV to manage this severe crop disease. Viral transmission generally occurs in one of two ways: horizontally, which is the transmission of the virus between unrelated hosts, or vertically, which is the transmission of the virus from parent to offspring (Simmons et al., 2011). In the case of plant viruses, vertical transmission has a great epidemiological impact, since it allows them to survive in the seed as long as it remains viable, travel great distances, and finally emerge in a new host population where they can initiate damaging epidemics.

Like most of begomovirus, in nature ToLCNDV is horizontally transmitted only by the whitefly *B. tabaci*, although some isolates are also mechanically sap-transmitted to different hosts

(Chang et al., 2010; López et al., 2015; Sohrab et al., 2013; Usharani et al., 2004). Besides, the ToLCNDV-ES strain infects cucurbits very efficiently, but poorly infect other hosts, including tomato plants (Fortes et al., 2016; Moriones et al., 2017), so the host selection could be important by *B. tabaci* in horizontal transmission. Selective transmission of *B. tabaci* was determined by real-time PCR and it was observed a transmission rate of 20% and 0% from infected zucchini and infected tomato with ToLCNDV-ES strain, respectively, to virus-free zucchini (Janssen et al., 2022). Moreover, the incidence of infection of ToLCNDV-ES in tomato was found to be enhanced in case of associated infection with TYLCV (Vo et al., 2022).

The transmission of viruses by seeds plays a pivotal role in the dissemination of plant viruses from season to season (Johansen et al., 1994). Even extremely low seed transmission rates can initiate damaging epidemics (Maule & Wang, 1996; Sastry, 2013). For instance, it was demonstrated that a percentage of lettuce seeds infected by lettuce mosaic virus as low as 0.003% was enough to start an outbreak (Albrechtsen, 2006; Grogan, 1980). Seed transmission serves as a source of initial inoculum for many viruses, which are subsequently disseminated via insect vectors causing ravaging epidemics (Mahatma et al., 2021).

Transmission of viruses through seed has been reported for approximately 25% of plant viruses and can, even with low transmission rates, be important for virus perpetuation, overwintering and long-distance dissemination of viruses. Little is known on this mode of transmission in begomovirus. Traditionally, it has been considered that begomoviruses are not transmitted by seeds, since they are unable to penetrate the seed because of a lack of vascular connectivity, even though they are detected in seed coat, endosperm, cotyledon, and embryonic axis, but this is a debate that is still open (Kil et al., 2016, 2020; Kothandaraman et al., 2016). In 2015, was reported that the sweet potato leaf curl virus (SPLCV) was transmitted from seeds collected of SPLCV-infected potato plants to seedlings was up to 15% (Kim et al., 2015). The Mung bean yellow mosaic virus (MYMV) was also detected in 32% of the seedlings grown from seeds of black gram (*Vigna mungo L*. Hepper) plants naturally infected in the field (Kothandaraman et al., 2016). TYLCV-IL was transmitted from seeds of TYLCV-infected tomato plants to offspring with an average transmission rate up to 80% (Kil et al., 2016). In addition, seed detection and transmission of ToLCNDV has been reported in chayote, bitter gourd and zucchini (Gomathi Devi et al., 2023; Kil et al., 2020; Sangeetha et al., 2018). However, in other cases, evidences of seed transmission were discarded for TYLCV, TYLCSV, and SPLCV (Andreason et al., 2021; Pérez-Padilla et al., 2020;

Rosas-Díaz et al., 2017; (Andreason et al., 2021; Pérez-Padilla et al., 2020; Rosas-Díaz et al., 2017; Tabein et al., 2021). Recently, it has been reported that ToLCNDV in melon is seed-borne, but no seed transmitted to offspring. (Fortes et al., 2023). Seed-borne is the ability of a virus to be carried through seeds, which does not necessarily implicate to transmission to the next generation (Fortes et al., 2023).

Seed transmission involve the viral multiplication level in the reproductive tissues, the speed of systemic colonization and the germinability of infected seeds. There are several factors that are determinant for the efficiency of vertical transmission, such as the virus and host genotype variability, time of infection, age of the seed, seed desiccation before sowing and climatic conditions (Renukadevi et al., 2022). In this thesis, to provide new data around seed transmission of ToLCNDV-ES, we have evaluated the presence of ToLCNDV in floral tissues and seeds of infected plants of several species of *Cucumis* and *Cucurbita* (seed-borne) and the later transmission to plants germinated from the infected-seeds (seed transmission). ToLCNDV was clearly detected in all floral tissues, while the detection in mature seeds was variable; the virus was detected in the seed coat, in the endosperm in some *Cucumis* plants, but was not detected in all *C. moschata* species. On the other hand, all plants germinated from infected seed were completely asymptomatic and the virus was not detected by molecular techniques. Additionally, commercial seeds of several cucurbit species (zucchini, melon, cucumber, and watermelon) were negative for most samples. In sum, our results are in agreement with those published by Fortes et al., (2023) which suggest that in melon and pumpkin ToLCNDV is seed-borne but not seed transmitted, a process that currently remains poorly understood. Although seed transmission has not been confirmed, the seed-borne nature of begomoviruses has been validated, and is though relevant to epidemiological studies (Fortes et al., 2023; Pérez-Padilla, Fortes et al., 2020). Understanding plant viruses epidemiology and evolution will require deep insight on the internal journey of viruses to the seed. Lack of knowledge on this aspect of life of plant viruses is relatively hindered by the low virus titer measured in the seed, the complexity of the detection and the time-consuming experimentation required to achieve attainable results (Pagán, 2022).

CONCLUSIONS

1. CCYV was detected for the first time in Algeria infecting cucumber and zuchinni in two distinct areas and in mixed infection with ToLCNDV in all cases. A priori, the severity of the symptoms was not affected by the co-infection of the two viruses.

2. Partial RNA1 and RNA2 genomic sequences of a CCYV isolate from zucchini (CCYV-Sidi) were obtained and deposed in Genbank under accessions numbers: MN529558 (RdRp), MN529559 (Hsp70h) and MN529560 (CP).

3. BLASTn analysis of the complete CP (segment RNA2) showed high nucleotide identity, greater than 99.5% with isolates from different geographical origins and hosts, about 98% with two Saudi Arabian isolates, and about 95% with one Indian and three Iranian isolates. These genetic relationships were confirmed with a phylogenetic tree, where the Algerian isolate clustered in group I together with most of the isolates.

4. ToLCNDV was detected for the first time in Algeria infecting zucchini, cucumber, and melon. The highest incidence of ToLCNDV was found in the locality of Sidi-Okba mostly infecting zucchini plants. Given the virus's high prevalence, we could presume that it has been present in Algeria for years.

5. The complete DNA-A and DNA-B genomic sequences of a ToLCNDV isolate from cucumber (ToLCNDV-Biskra) were obtained and deposited in GenBank under accessions numbers MK981891 and MK981892.

6. Comparative analysis of the obtained sequences of DNA-A and DNA-B revealed identities of 98.7 and 97.6%, respectively, with the corresponding segments of the isolates from the Mediterranean basin, whereas the identity with isolates from Asia was around 90% and 81% for DNA-A and DNA-B, respectively.

7. A 17-nucleotide insertion was detected at position 2,136 of DNA-B in samples of zucchini and melon in two distinct areas. The insertion was located just before the start of the gene BC1, so the expression of the MP protein should not be affected. However, a start codon for the movement protein (MP) synthesis is found at a different position, resulting in a MP 53 amino acids longer. Sequencing of more isolates and biological assays should be performed to verify the biological and epidemiological implications of this putative major MP.

8. ToLCNDV was detected in all floral tissues, while the detection in mature seeds was variable. The virus was detected in the seed coat, and in the endosperm in some *Cucumis* plants, but was not detected in all *C. moschata* species. On the other hand, all plants germinated from infected seed were completely asymptomatic and the virus was not detected by molecular techniques. These results suggest that in melon and pumpkin ToLCNDV is seed-borne but not seed transmitted.

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