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SHORT COMMUNICATION

Detection and biocontrol of *Tobamovirus tabaci* infecting tomato in Iraq

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ABSTRACT

The antiviral activity of leaf extracts from *Datura stramonium* and tomato plants inoculated with TMV, combined with 20% skimmed milk, was investigated. A TMV isolate was confirmed using bioassay, serological, and molecular approaches and subsequently used to inoculate plants. Tomato plants, both pre- and post-inoculated with TMV, were sprayed with leaf extracts from either TMV-free or infected plants, alone or mixed with 20% skimmed milk. Enzyme-linked immunosorbent assay (ELISA) using tobamovirus-specific antibodies and local lesion tests were conducted to assess antiviral activity based on virus concentration and infectivity in treated plants. The experiment followed a completely randomized design (CRD), and the Least Significant Difference (LSD) test was applied to evaluate ELISA optical density (OD) values. OD data revealed that the combination treatment (inoculated tomato leaf extract + 20% skimmed milk) inhibited TMV in tomato plants by up to 56%, showing the highest antiviral activity. This study is the first to investigate the antiviral potential of leaf extracts from TMV-infected plants.

Key words: *Datura metel*, phylogeny, RT-PCR, tobamoviruses

INTRODUCTION

Tomato (*Solanum lycopersicum* L). is a cash crop grown worldwide. It is a major vegetable crop in Iraq, cultivated nationwide in both protected environments and open fields throughout the year (CSO, 2023; FAO, 2023).

Tomato fruit production can be affected by several biotic and abiotic factors, including viruses (Hančinský et al., 2020; Panno et al., 2021; Rivarez et al., 2021). Viral diseases have been reported to impact tomato production in Iraq, causing severe losses of up to 100% in both quality and quantity (Adhab & Alkuwaiti, 2022). Tobacco mosaic virus (TMV), a member of the genus *Tobamovirus*, is one of the major viruses affecting tomatoes globally (ICTV, 2024). This virus and other members of the genus pose a significant threat to tomato cultivation in both protected environments and open fields (Caruso et al., 2022). They can be transmitted mechanically through contaminated tools, clothing, direct plant-to-plant contact, human hands, and seeds. Tobamoviruses can survive for several weeks in plant debris (Ishibashi et al., 2023). The presence of tobamoviruses in Iraqi tomato crops has been reported based on biological and serological tests, however their occurrence has not yet been confirmed using molecular techniques (Adhab et al., 2021; Mheedi & Ali, 2023; Obaid & Adhab, 2025).

Due to the high incidence and spread of tobamoviruses, the development of rapid, simultaneous, and reliable diagnostic tests is essential for effective disease management (Alon et al., 2021). Biochemical treatments, including plant extracts and milk, have been explored as promising eco-friendly approaches to controlling various viral diseases (Andayanie et al., 2020; Putri & Damayanti, 2020), including those caused by tobamoviruses (Chanda et al., 2021; Monjezi et al., 2023).

This study aims to confirm the presence of tobamoviruses in Iraq using molecular approaches to provide sequence data. Additionally, it seeks to evaluate the effectiveness of biochemical treatments, including a combination of infected plant extracts and skimmed milk, in controlling tobamoviruses affecting tomato plants.

MATERIALS AND METHODS

Research Site. The study was conducted at the Department of Plant Protection, College of Agricultural Engineering Sciences, University of Baghdad, Iraq.

Virus Source and Identification. Leaf samples from symptomatic tomato plants were used as a source of

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tobamoviruses. Sap inoculation was performed on indicator plants. Tobamoviruses were detected using an enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Agdia, USA) following the manufacturer's standard protocol. The tobamovirus species was confirmed using molecular techniques.

Total RNA was extracted from tomato leaves using the TransZol Up Plus RNA commercial kit (TransGen Biotech, China) following the manufacturer's standard protocol. cDNA was synthesized using the cDNA Synthesis SuperMix commercial kit (TransGen Biotech, China) according to the manufacturer's recommended protocol. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the PCR SuperMix EasyTaq kit (TransGen Biotech, China).

Two primer sets were used to amplify different genomic regions of the tobamovirus: TobUni1(ATTTAAGTGGASGGAAAAVCAT)/ TobUni2(GTYGTTGATGAGTTCRTGGA) targeting the coat protein (CP) and movement protein (MP) (Letschert et al., 2002) and F-3666 (ATGGTACGAACGGCGGCAG)/R-4718 (CAATCCTTGATGTGTTTAGCAC) targeting the RNA-dependent RNA polymerase (RdRp) region (Luria et al., 2017).

Polymerase chain reaction (PCR) was performed with the following thermal conditions: Initial denaturation at 94 ºC for 1 min (1 cycle), 35 cycles of denaturation at 94 ºC for 30 s, annealing at 52 ºC (for TobUni1/ TobUni2) or 56 ºC (for F-3666/R-4718) for 30 s, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 5 min.

Amplified DNA fragments were visualized using 1% agarose gel electrophoresis (Sambrook & Russell, 2006) and sequenced (Macrogen, South Korea). Sequence analyses were performed using MEGA 11 (Tamura et al., 2021) and SDTv1.3 (Muhire et al., 2014). The obtained sequences were submitted to the GenBank database, National Center for Biotechnology Information (NCBI), under accession numbers PP032058–PP032061.

Tomato Virus Biocontrol Treatment. Crude leaf extracts were prepared from virus-inoculated and non-inoculated *Datura stramonium* and tomato plants. Fresh leaf tissue was homogenized in distilled water at a 1:1 (W/V) ratio using a pre-cooled porcelain pestle and mortar. The extracted plant sap was filtrated, and the final volume was adjusted to 1 L using distilled water. Skimmed milk powder was mixed with certain plant extracts at a 20% concentration (W:V).

Tomato plants grown in pots were divided into two treatment groups: 1) Post inoculated treatment (Ta) includes plants were first inoculated with the virus and then sprayed with the treatment; 2) Pre-inoculated treatment (Tb) includes plants were first sprayed with the treatment and later inoculated with the virus.

A 1-L hand sprayer was used for each treatment. Three replicates were used per treatment, and the plants were maintained under greenhouse conditions. Spraying was conducted for one month at two-week intervals, after which leaf samples were collected.

The antiviral activity of each treatment was assessed through bioassay and ELISA. The following treatments were applied:

1) Pre-inoculation treatment (Tb):
Tb1 = Non-inoculated Datura leaf extract,
Tb2 = TMV-inoculated Datura leaf extract,
Tb3 = TMV-inoculated Datura leaf extract + 20% skimmed milk,
Tb4 = TMV-inoculated tomato leaf extract,
Tb5 = TMV-inoculated tomato leaf extract + 20% skimmed milk

2) Post-inoculation treatment (Ta):
Ta1 = Non-inoculated Datura leaf extract,
Ta2 = TMV-inoculated Datura leaf extract,
Ta3 = TMV-inoculated Datura leaf extract + 20% skimmed milk,
Ta4 = TMV-inoculated tomato leaf extract,
Ta5 = TMV-inoculated tomato leaf extract + 20% skimmed milk

Statistical Analysis. The GenStat software package (VSN International, UK) was used for statistical analysis. The experiment followed a Complete Randomized Design (CRD). ELISA optical density (OD) values were compared using the Least Significant Difference (LSD) test ($p \leq 0.05$).

Virus Inhibition Percentage Calculation. The virus inhibition percentage was calculated based on ELISA absorbance values of each treatment at 405 nm using the following formula:

$$\text{Inhibition \%} = \frac{+\text{ve OD} - \text{Treatment OD}}{+\text{ve OD}} \times 100$$

RESULTS AND DISCUSSION

Necrotic local lesions were observed on inoculated *Datura stramonium, D. metal* and *Phaseolus vulgaris* var. Pinto (Figure 1A-C), while tomato

exhibited mosaic symptoms (Figure 1D), indicating the presence of TMV in symptomatic samples (Jeżewska et al., 2018; Ara et al., 2012; Zaitlin, 2000). RT-PCR confirmed tobamovirus infection by successfully amplifying DNA fragments from tested tomato samples (Figure 2A & B). Sequence comparison confirmed that ~1052 bp and ~750 bp DNA fragments were amplified from the RdRp and CP/MP regions of the TMV genome, scoring 98% and 97% maximum nucleotide sequence identities, respectively, compared to equivalent NCBI sequences (Figure 3A & B). Similarly, deduced amino acid sequences shared 99%, 93%, and 98% maximum identities with the RdRp, CP, and MP genes, respectively, from NCBI reference sequences (Figure 4A-C).

Phylogenetic analyses of DNA fragments amplified from the TMV genome confirmed their relatedness, as TMV isolated grouped with equivalent sequences retrieved from NCBI (Figure 5A-E). The RdRp gene isolate showed high relatedness to NCBI isolates from Japan (Acc. No. D78608.1), the UK (Acc. No. KY810785.1), Germany (Acc. No. MT737799.1), France (Acc. No. OQ953825.1) and the USA (Acc. No. NC 001367.1) in both nucleotide and amino acid

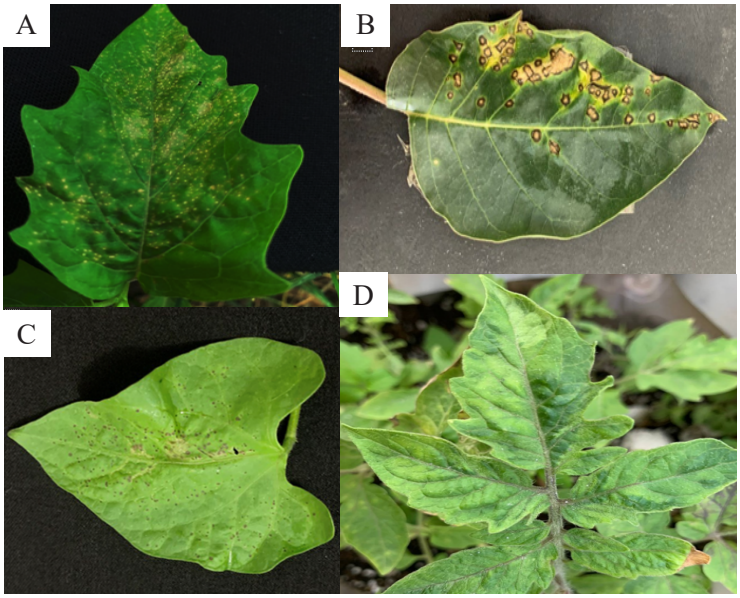

Figure 1. Symptoms on assay hosts inoculated with TMV. A. Necrotic local lesions on *D. stramonium* 4 days after inoculation; B. Necrotic local lesions on *D. metel* 7 days of inoculation; C. Pin-point necrotic spots on *Phaseolus vulgaris* var *Pinto* 3 days of inoculation; D. Mosaic symptoms on *Solanum lycopersicum* 14 days of inoculation.

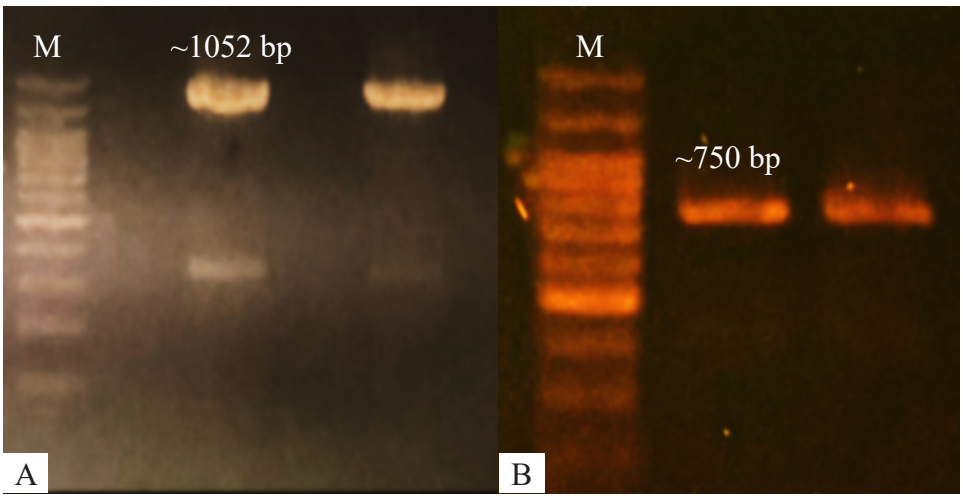

Figure 2. Ethidium bromide agarose gel electrophoresis pattern stained with red safe dye showing ~1052 bp. A. and ~ 750 bp; B. DNA fragments amplified from symptomatic tomato leaf samples using F-3666/R-4718 and TobUni1/TobUni2 tobamovirus specific primer sets. M: 100 bp DNA marker (TransGen Biotech, China).

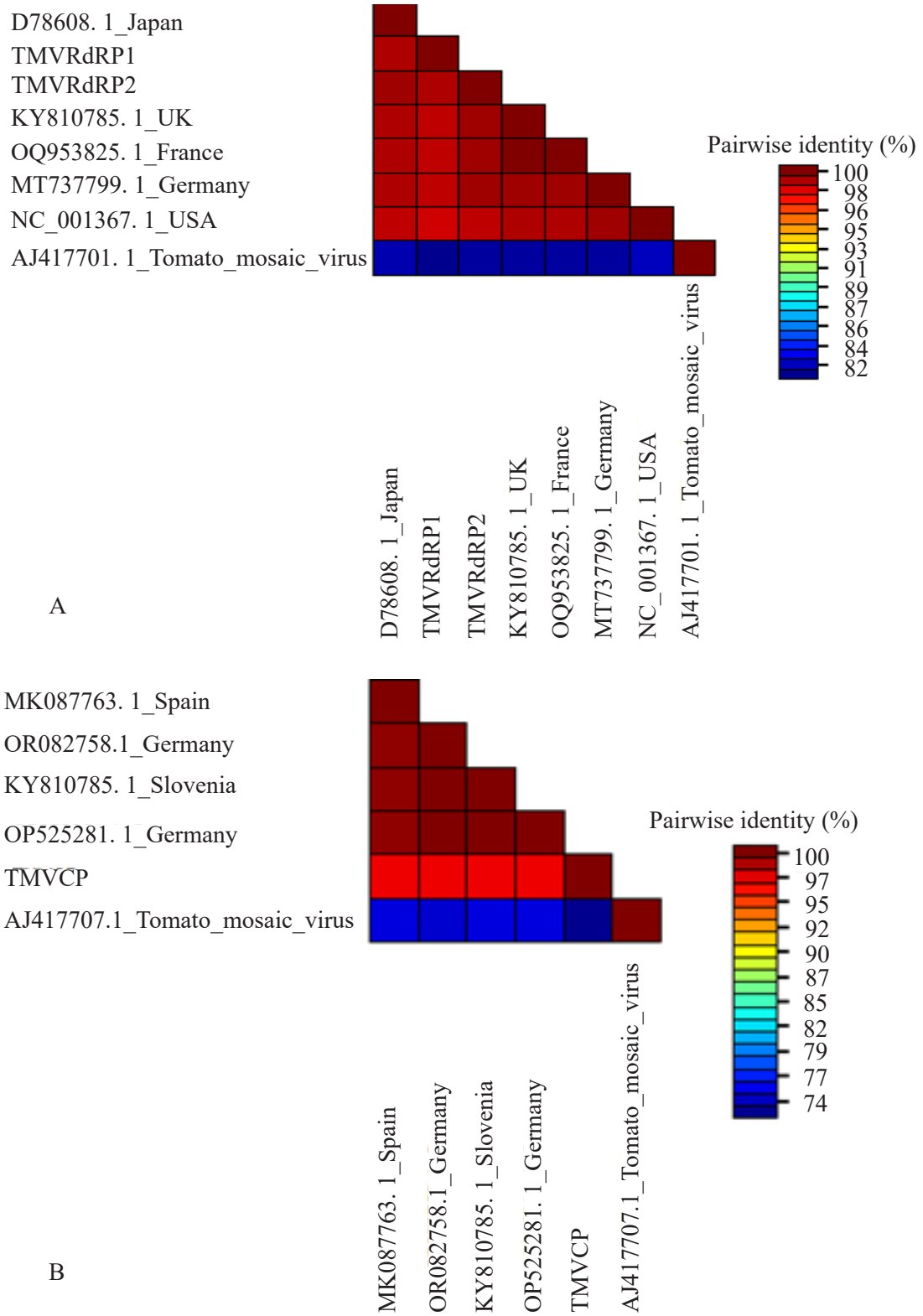

Figure 3. Nucleotide sequence identity percentages interpreted into colored matrix generated from RNA dependent RNA polymerase RdRp. A. Coat Protein/Movement Protein CP/MP; B. Open Reading Frames (ORFs) in *Tobamovirus tabaci*. The comparison included amplified in this study from symptomatic tomato plants using F-3666/R-4718 primers (referred to as TMVRdRp1 and 2) and TobUni1/TobUni2 primers (referred to as TMVCP) and the equivalent GenBank sequences. Tomato mosaic virus *Tobamovirus tomatotessellati* was included as an outgroup comparison. The nucleotide sequences were analyzed using ClustalW alignment methods within SDT v1.3 software package (Muhire et al., 2014).

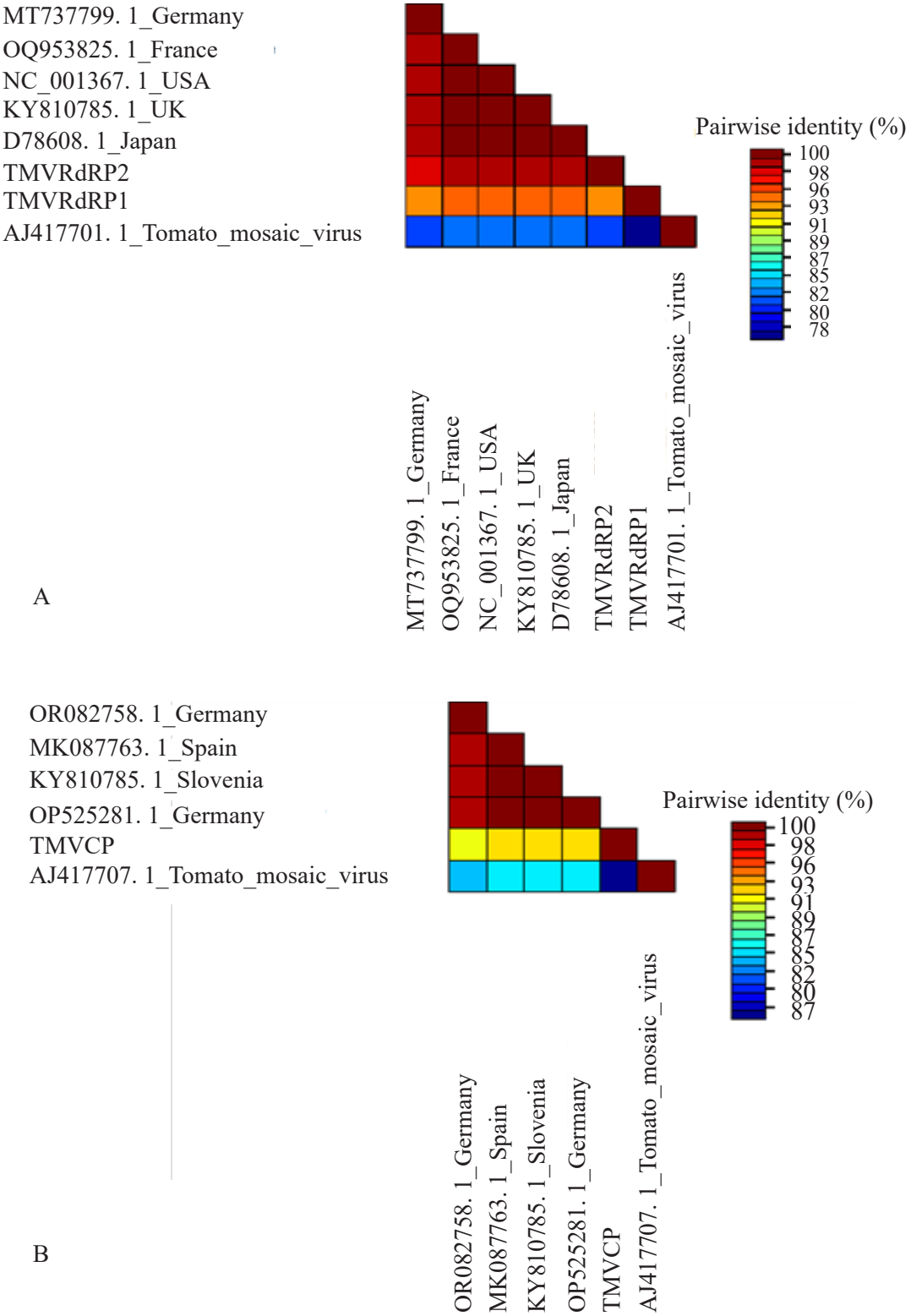

Figure 4. Deduced amino acid sequence identity percentages interpreted into colored matrix generated. A. RNA dependent RNA polymerase RdRp; B. Coat Protein/Movement Protein CP/MP. Open Reading Frames (ORFs) in *Tobamovirus tabaci*. The comparison included amplified in this study from symptomatic tomato plants using F-3666/R-4718 primers (referred to as TMVRdRp1 and 2) and TobUni1/TobUni2 primers (referred to as TMVCP) and the equivalent GenBank sequences. Tomato mosaic virus *Tobamovirus tomatotessellati* was included as an outgroup comparison. The nucleotide sequences were analyzed using ClustalW alignment methods within SDT v1.3 software package (Muhire et al., 2014).

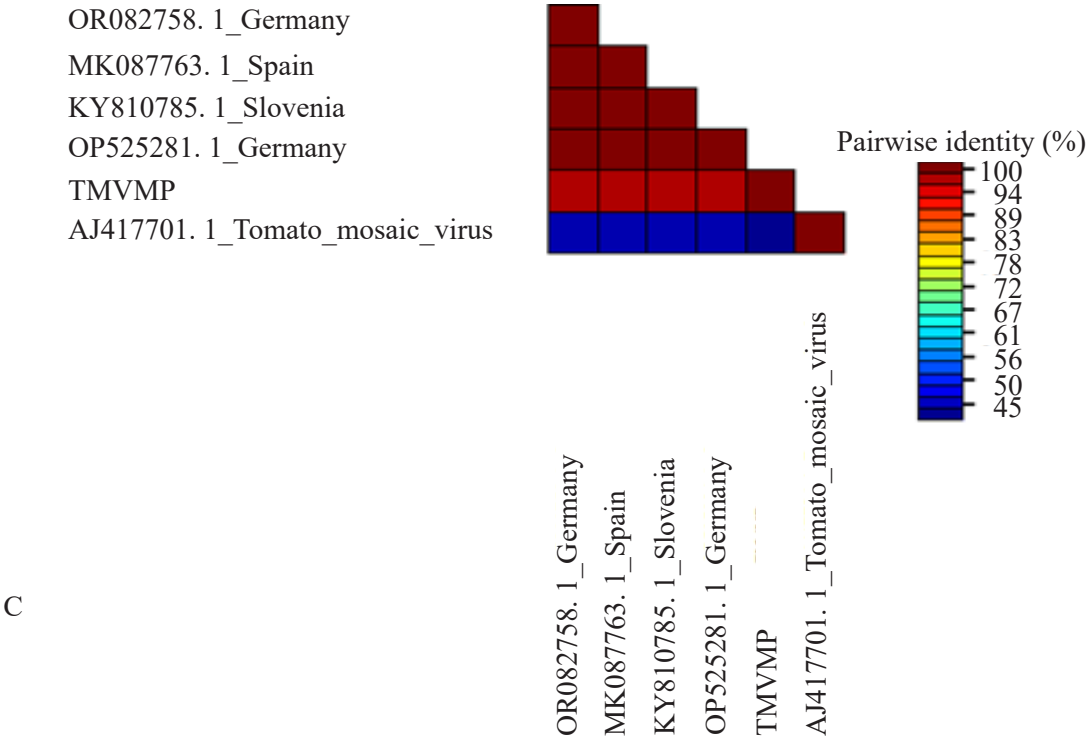

Figure 4. (Continued). Deduced amino acid sequence identity percentages interpreted into colored matrix generated. A. RNA dependent RNA polymerase RdRp; B. Coat Protein/Movement Protein CP/MP. Open Reading Frames (ORFs) in *Tobamovirus tabaci*. The comparison included amplified in this study from symptomatic tomato plants using F-3666/R-4718 primers (referred to as TMVRdRp1 and 2) and TobUni1/TobUni2 primers (referred to as TMVCP) and the equivalent GenBank sequences. Tomato mosaic virus *Tobamovirus tomatotessellati* was included as an outgroup comparison. The nucleotide sequences were analyzed using ClustalW alignment methods within SDT v1.3 software package (Muhire et al., 2014).

sequences. In contrast, the CP and MP genes isolates were closely related to those from Germany (Acc. Nos. OR082758.1 & OP525281.1), Spain (Acc. No. MK087763.1), and Slovenia (Acc. No. KY810785.1), suggesting a common origin.

ELISA OD values at 405 nm showed that all treatments reduced TMV concentration in tomato plants compared to the infected control, indicating the antiviral activity of the tested plant extracts (Figure 6). Additionally, growth in all treated tomato plants improved compared to the untreated infected control (Figure 7A-D). Furthermore, bioassays showed that *D. stramonium* and Pinto bean plants did not develop local lesions when inoculated with treated tomato leaves. Statistical analysis revealed that the post-inoculation combination treatment Ta5 (TMV-inoculated tomato leaf extract + 20% skimmed milk) exhibited the highest virus inhibition (56.6%), followed by Ta3 (TMV-inoculated *Datura* leaf extract + 20% skimmed milk) (Figure 6). Meanwhile, the highest inhibition among pre-inoculation treatments was observed in Tb3 (TMV-inoculated *Datura* leaf extract + 20% skimmed milk), with a 27% virus inhibition rate (Figure 6).

The antiviral activity of leaf extracts from TMV-pre-inoculated tomato and *D. stramonium* plants was investigated. The TMV isolate was tested on *Datura* spp. to confirm that this isolate could trigger resistance mechanisms by inducing local lesions. Additionally, tomato plants were inoculated with the TMV isolate tto assess their susceptibility, as most tomato cultivars contain the TMV resistance gene *Tm-2* (Ishibashi et al., 2023; Zheng et al., 2024). The two tobamovirus-specific primer sets, targeting RdRp and CP, combined with sequence analysis, indicated that the tested TMV isolate was a distinct variant (Luria et al., 2017). The high variability of the TMV CP isolate may be host-related. However, ELISA csuccessfully detected the virus under study, as the TMV commercial kit used was designed to detect a wide range of tobamoviruses, according to the manufacturer (Agdia, USA).

Skimmed milk was included to enhance the antiviral activity of the tested plant extracts through two pathways. First, it minimized virus infectivity in these extracts, as they originated from TMV-infected

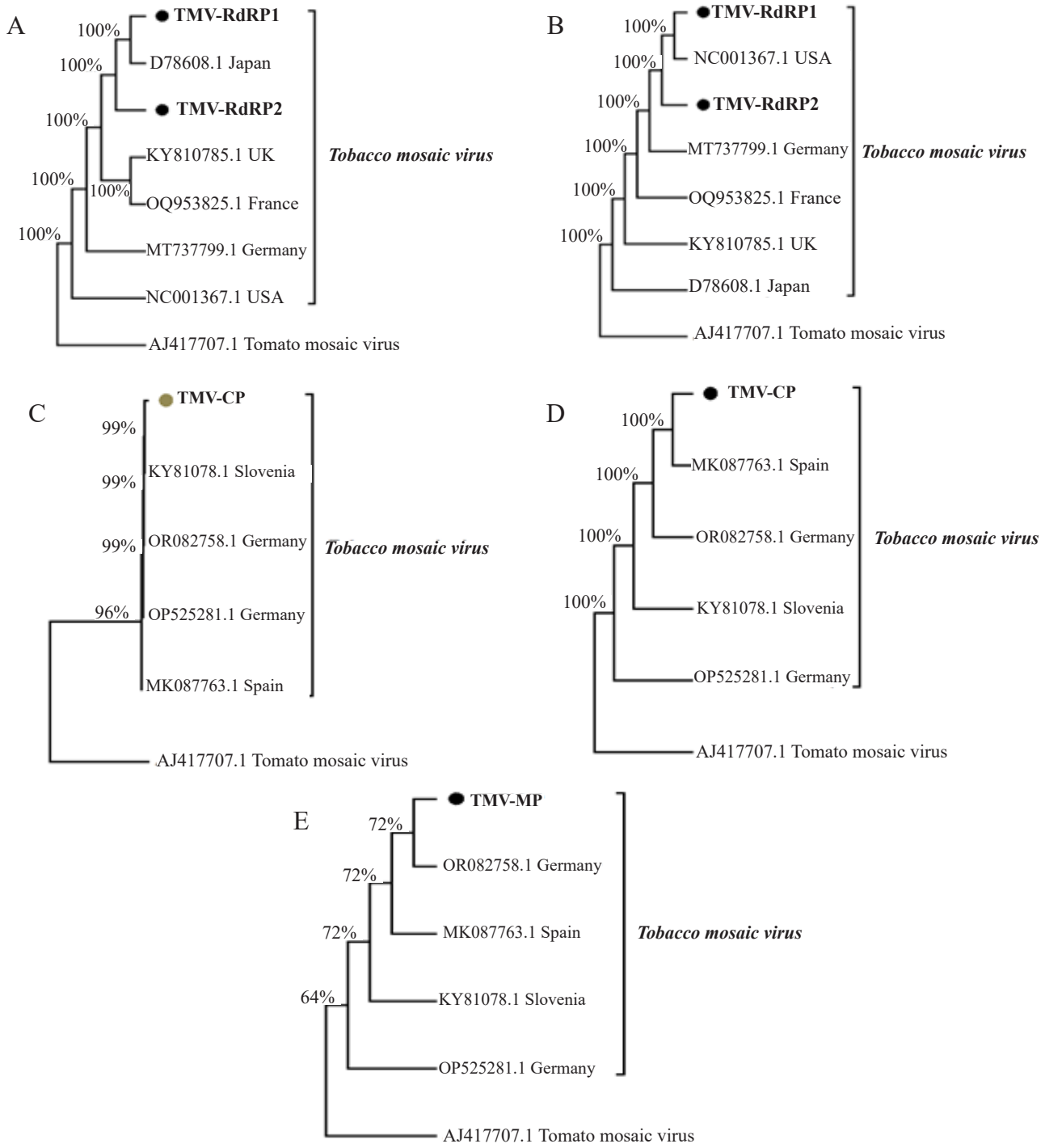

Figure 5. Neighbor-Joining phylogenetic trees constructed. A. RNA dependent RNA polymerase RdRp nucleotide; B. Deduced amino acid; C. Coat Protein CP nucleotide; D. Deduced amino acid; E. Movement Protein MP deduced amino acid. Partial sequences of TMV isolated (marked with ●) and equivalent GenBank sequences. Tomato mosaic virus Tobamovirus tomatotessellati was included as an outgroup comparison. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965) and are in the units of the number of nucleotide and amino acid substitutions per site. The pairwise deletion option was applied to all ambiguous positions for each sequence pair. Evolutionary analyses were conducted in MEGA11(Tamura et al., 2021).

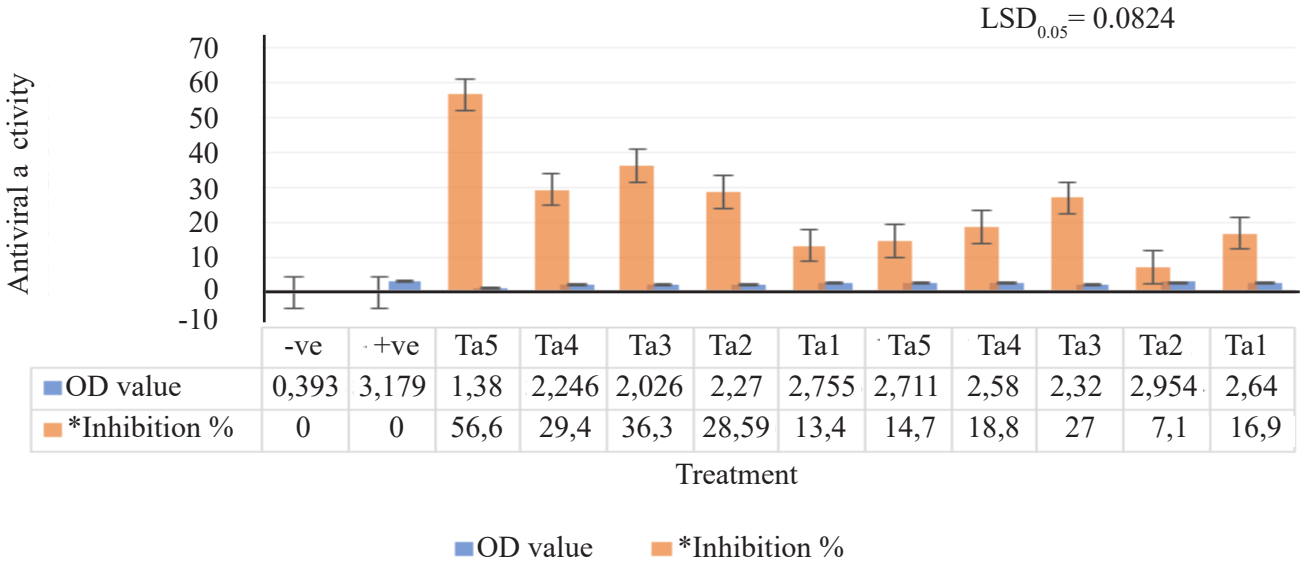

Figure 6. Optical density (OD) values and inhibition percentages of TMV inoculated tomato plants treated with different plant extracts. The virus concentration was estimated by ELISA. OD values were measured at 405 nm. Tb1= non-inoculated Datura leaf extract, Tb2= TMV inoculated Datura leaf extract, Tb3= TMV inoculated Datura leaf extract + 20% skimmed milk, Tb4= TMV inoculated tomato leaf extract, Tb5= TMV inoculated tomato leaf extract + 20% skimmed milk. Ta1= Non-inoculated Datura leaf extract, Ta2= TMV inoculated Datura leaf extract, Ta3= TMV inoculated Datura leaf extract + 20% skimmed milk, Ta4= TMV inoculated tomato leaf extract, Ta5= TMV inoculated tomato leaf extract + 20% skimmed milk. Ta(n)= post-inoculated treatments. Tb(n)= pre-inoculated treatments. +ve: ELISA TMV negative control (Agdia USA), -ve: ELISA TMV negative control (Agdia USA).

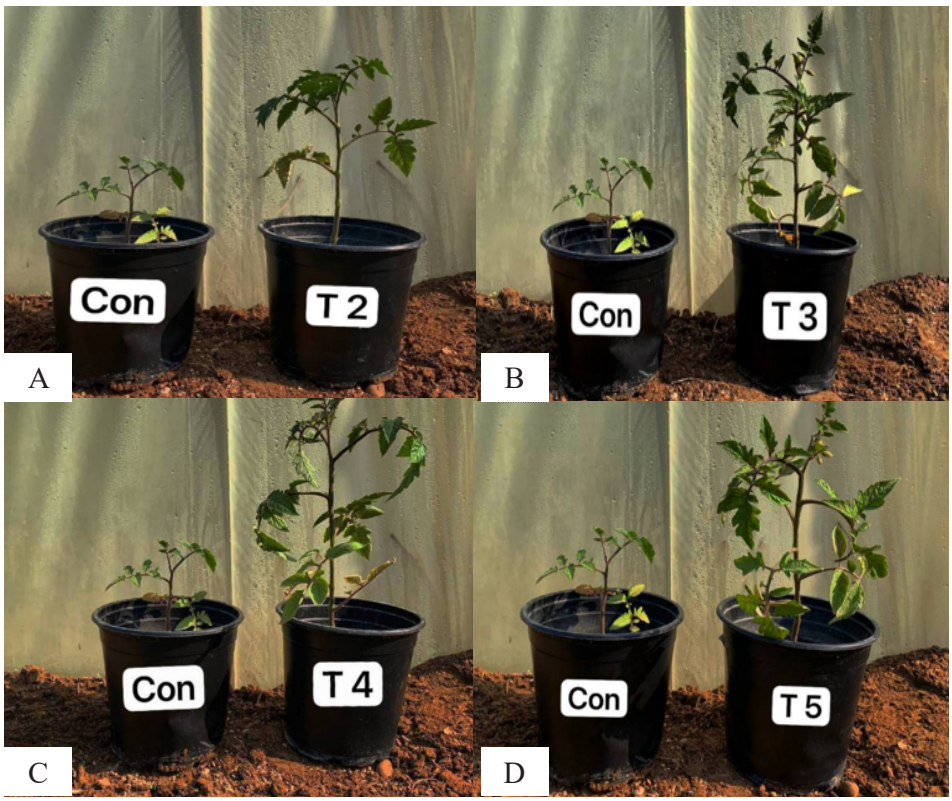

Figure 7. Treated tomato plants showing growth improvement compared to TMV infected control. A. TMV inoculated *Datura* leaf extract; B. TMV inoculated *Datura* leaf extract + 20% skimmed milk; C. TMV inoculated tomato leaf extract; D. TMV inoculated tomato leaf extract + 20% skimmed milk. Con: Untreated TMV infected control.

plants (Lewandowski et al., 2010; Rodríguez-Díaz et al., 2022). Second, the proteins in skimmed milk may act as nanoparticle carriers, adsorbing antiviral components produced by inoculated plants and delivering virus inhibitors into plant cells (Dutta et al., 2022). Additionally, skimmed milk may have functioned as a biofertilizer by improving the growth of treated tomato plants and enhancing soil microbial activity (Balla et al., 2022).

Various plant-derived products have been applied against tobamoviruses worldwide (Hamdi et al., 2020; Monjezi et al., 2023). When extracting leaves from TMV-inoculated or non-inoculated tomato and *Datura* plants, numerous antiviral compounds can be released. The antiviral activity of *D. stramonium* leaf extract may be attributed to its nitrogen-like proteins, which were induced through TMV inoculation and triggered defense mechanisms in treated tomato plants (Mheedi & Ali, 2023; Zheng et al., 2024). Moreover, TMV infection could enhance the production of other antiviral compounds, including phenols and alkaloids, which may contribute to reducing TMV concentration in treated plants (Mihálik et al., 2022).

Additionally, spraying tomato plants with extracts from TMV-infected tomato plant materials may have inhibited TMV by delivering antiviral compounds specifically induced against TMV. These include gene silencing intermediates, signaling chemicals, antiviral-related proteins, and other pathogenicity-related compounds (Akbar et al., 2022; Zheng et al., 2024). Since these compounds originated from the same tomato cultivar, they could be recognized by the immune system of treated tomato plants, potentially inducing resistance against TMV.

This study provides the first molecular conformation of *Tobamovirus tabaci* infecting tomato in Iraq. Furthermore, it introduces a novel approach to managing *T. tabaci* infection in tomato through the application of leaf extracts from TMV-infected plants to trigger plant immunity against this virus.

CONCLUSION

Plant treatment with infected plant extracts combined with skimmed milk presents a promising approach for controlling viral diseases, as investigated for the first time in this study. Additionally, this study provides the first molecular investigation of TMV in Iraq. The use of group-specific primers and antibodies can serve as a valuable tool for studying tobamovirus control strategies. However, a key challenge that remains is identifying the antiviral mechanism of action. This can be addressed through molecular approaches such as transcriptome and virome analyses. The application of Next-Generation Sequencing (NGS) can further enhance our understanding of antiviral interactions and the sources of immunity in treated plants.

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AUTHORS' CONTRIBUTIONS

NA conceived and designed the experiment. HJ conducted the field and laboratory work. NA performed the data analysis and interpretation. HJ and NA prepared the manuscript. Both authors contributed to discussions on research progress, data analysis, and manuscript refinement. All authors have read and approved the final manuscript.

COMPETING INTEREST

The authors declare no competing interests regarding this publication.

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