Real-time reverse transcription polymerase chain reaction development for rapid detection of Tomato brown rugose fruit virus and comparison with other techniques

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Background. Tomato brown rugose fruit virus (ToBRFV) is a highly infectious tobamovirus that causes severe disease in tomato (Solanum lycopersicum L.) crops. In Italy, the first ToBRFV outbreak occurred in 2018 in several provinces of the Sicily region. ToBRFV outbreak represents a serious threat for tomato crops in Italy and the Mediterranean Basin.

Methods. Molecular and biological characterisation of the Sicilian ToBRFV ToB-SIC01/19 isolate was performed, and a sensitive and specific Real-time RT-PCR TaqMan MGB probe method was developed to detect ToBRFV in infected plants and seeds. Moreover, four different sample preparation procedures (immunocapture, total RNA extraction, direct crude extract and leaf-disk crude extract) were evaluated. Results. The Sicilian isolate ToB-SIC01/19 (6,391 nt) showed a strong sequence identity with the isolates TBRFV-P12-3H and TBRFV-P12-3G from Germany, Tom1-Jo from Jordan and TBRFV-IL from Israel. The ToB-SIC01/19 isolate was successfully transmitted by mechanical inoculations in Solanum lycopersicum L. and Capsicum annuum L., but no transmission occurred in Solanum melongena L.. The developed Real-time RT-PCR, based on the use of a primer set designed on conserved sequences in the ORF3, enabled a reliable quantitative detection. This method allowed clear discrimination of ToBRFV from other viruses belonging to the genus Tobamovirus, minimising false-negative results. Using immunocapture and total RNA extraction procedures, Real-time RT-PCR and end-point RT-PCR gave the same comparable results. Using direct crude extracts and leaf-disk crude extracts, end-point RT-PCR was unable to provide a reliable result. This developed highly specific and sensitive Real-time RT-PCR assay will be a particularly valuable tool for early ToBRFV diagnosis,
optimising procedures in terms of costs and time.
Real-time reverse transcription polymerase chain reaction development for rapid detection of Tomato brown rugose fruit virus and comparison with other techniques

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Abstract

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Results. The Sicilian isolate ToB-SIC01/19 (6,391 nt) showed a strong sequence identity with the isolates TBRFV-P12-3H and TBRFV-P12-3G from Germany, Tom1-Jo from Jordan and TBRFV-IL from Israel. The ToB-SIC01/19 isolate was successfully transmitted by mechanical
inoculations in Solanum lycopersicum L. and Capsicum annum L., but no transmission occurred in Solanum melongena L.. The developed Real-time RT-PCR, based on the use of a primer set designed on conserved sequences in the ORF3, enabled a reliable quantitative detection. This method allowed clear discrimination of ToBRFV from other viruses belonging to the genus Tobamovirus, minimising false-negative results. Using immunocapture and total RNA extraction procedures, Real-time RT-PCR and end-point RT-PCR gave the same comparable results. Using direct crude extracts and leaf-disk crude extracts, end-point RT-PCR was unable to provide a reliable result. This developed highly specific and sensitive Real-time RT-PCR assay will be a particularly valuable tool for early ToBRFV diagnosis, optimising procedures in terms of costs and time.

Introduction

Tomato (Solanum lycopersicum L.) is one of the most important horticultural crops worldwide. Indeed in 2017, more than 182 million tons of tomato have been produced (FAO, 2017). China is the most important tomato producer (59 million tons), followed by India, Turkey and United States, while Italy and Spain are the major tomato producers in Europe (over 6 million and 5 million tons, respectively) (FAO, 2017). In Italy, greenhouse tomato production is mainly developed in the southern regions, and Sicily produces about 40% of national production (Agri ISTAT, 2017). In the last years, emerging viral diseases have been an important limiting factor for many crop production systems, such as tomato crops, causing considerable economic losses (Hanssen et al., 2010). Greenhouse tomato production in Italy is affected by important losses that are caused by several different viruses: Pepino mosaic virus (PepMV) (Davino et al., 2008; Davino et al., 2017a; Tiberini et al., 2011), Tomato mosaic virus (ToMV), Tomato spotted wilt virus (TSWV) (Panno et al., 2012), Tomato leaf curl New Delhi virus (ToLCNDV) (Panno et al., 2019a), Tomato yellow leaf curl virus (TYLCSV) – Tomato yellow leaf curl Sardinia virus (TYLCSV) and their recombinants (Davino et al., 2009, 2012, Panno et al., 2018) and Tomato brown rugose fruit virus detected at the end of 2018 (ToBRFV) (Panno et al., 2019b).

The international trade globalisation and the free commodities movement complicate the control of pathogens in the free trade area of the Mediterranean Basin. In this context, the control of seed-transmitted pathogens is extremely difficult. This is the case of the recent ToBRFV outbreak in Sicily, which was probably introduced into the island either through infected seeds or through infected fruits and their subsequent manipulation.

ToBRFV is a member of the genus Tobamovirus, family Virgaviridae. Tobamovirus is probably the largest genus of this family for numbers of species (King et al., 2011). Tobamoviruses are the only members of this family that have an undivided genome. ToBRFV has a single-stranded positive-sense RNA (gRNA) of ~ 6,400 nucleotides (nt), with a typical tobamoviruses organisation that consists in 4 open reading frames (ORFs) encoding two replication-related proteins of 126 and 183 kDa, in which the second protein is expressed by the partial suppression of the stop codon (ORF1 and ORF2), the movement protein (MP) of 30 kDa (ORF3) and the coat
protein (CP) of 17.5 kDa (ORF4), which are expressed via the 3’-coterminal sub-genomic RNAs (sgRNAs) (Salem et al., 2016).

ToBRFV was described in 2016 for the first time in Jordan by Salem and co-workers (2016) on greenhouse tomato plants, and in 2017 in Israel on tomato plants harbouring the Tm-2² gene (Luria et al., 2017). Afterwards, ToBRFV was detected in Mexico in tomato and pepper crops (Cambrón-Crisantos et al., 2018) and after it spread to Germany, United States (California), Palestine, Italy and Turkey (Menzel et al., 2019; Ling et al., 2019; Alkowni et al., 2019; Panno et al., 2019b; Fidan et al., 2019).

ToBRFV symptoms are typical of the tobamoviruses infection and consist of tomato leaves interveinal yellowing and deformation, mosaic staining, young leaves deformation and necrosis, sepal necrosis and deformation, young fruits discolouration, deformation and necrosis. For this reason ToBRFV represents a very dangerous problem for tomato crops in all the regions where tomato is cultivated, due to the ability of the virus to be transmitted by contact through contaminated tools, hands, clothing, direct plant-to-plant contact, propagation material (grafts, cuttings), bumblebees and seeds (Levitzky et al., 2019).

The aim of the present study was to characterise the virus found in Italy and to develop a sensitive, specific and economical method to detect ToBRFV in infected plants and seeds.

**Materials & Methods**

**Source of viral material**

In October 2018, virus-like symptoms typical of tobamovirus infection were observed in tomato greenhouses in the Ragusa province (Sicily – Italy). These symptoms were very similar to those described by Salem and co-workers (2016) who identify a new tobamovirus, named Tomato brown rugose fruit virus, which overcomes the Tm-2² resistance gene. The symptoms consisted of severe mosaic, young leaves deformation and necrosis, fruits discolouration and marbling and sepals’ necrosis (Figure 1).

The samples used in this study were collected in four different areas of Sicily, in the provinces of Agrigento, Caltanissetta, Ragusa and Siracusa. 15 samples were collected from two different greenhouses of each area, according to the following scheme: 3 plants rows were selected from 60 total rows (one row every 20) and five samples were taken from each selected row (one sample every 10 plants) (Panno et al., 2019b). Sampling was repeated three times: the first sampling in October 2018, the second in December 2018 and the third at the end of February 2019. A total of 360 samples were collected and marked by GPS using PLANTHOLOGY mobile application (Davino et al., 2017b).

**Screening of ToBRFV using end-point RT-PCR**

One-step end-point RT-PCR was performed in 25 μl (final volume) containing 2 μl of total RNA extract, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 0.4 mM dNTPs, 1 mM of the forward primer ToBRFV-F-5722 and 1 mM of the reverse primer ToBRFV-R-6179 (Panno et al., 2019b), 4U of RNaseOut, 20 U of superscript II reverse transcriptase-RNaseH and 2U of Taq
DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RT-PCR was carried out in a MultiGene OptiMax thermal cycler (Labnet International Inc, Edison, NJ, USA) according to the following cycling conditions: 42 °C for 45 min, 95 °C for 5 min; 40 cycles of 30 sec at 95 °C, 30 sec at 55 °C, 30 sec at 72 °C and a final elongation of 10 min at 72 °C. The obtained DNA products of expected size were confirmed by electrophoretic separation in a 1.5% agarose gel and staining with Sybrsafe (Thermo Fisher Scientific, MA, USA).

Mechanical transmission
The Sicilian ToBRFV ToB-SIC01/19 isolate was mechanically inoculated into three plants of different hosts (Solanum lycopersicum L., Solanum melongena L. and Capsicum annuum L.) for subsequent biological characterisation. Plants were grown on a sterilised soil in an insect-proof glasshouse, with a photoperiod of 14 h of light and a target air temperature set at 28/20 °C day/night. The symptoms were reported weekly and the presence of ToBRFV was evaluated at 30 dpi by RT-PCR as previously described.

Full genome sequencing
Full genome sequencing was performed using the primer walking strategy (Knippers and Alpert, 1999) with the specific and overlapping primers reported by Luria and coworkers (2017). Four pairs of primers targeting ToBRFV conserved sequences were included to obtain the full genome sequence: FTobGEN, RTobGEN, F1-R1572, F1534-R3733 and F4587-R6392. RT-PCR was performed in a MultiGene OptiMax thermal cycler (Labnet International Inc, Edison, NJ, USA) according to the conditions reported by Luria et al. (2017). The obtained products were confirmed by electrophoretic separation in a 1.5% agarose gel and visualized using Sybrsafe staining. The products were subsequently purified with the Ultraclean 96 PCR Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and cloned using the TA-cloning system (Promega, WIS, USA). The five obtained plasmids were sequenced in both directions using an ABI PRISM 3100 DNA sequence analyser (Applied Biosystems, CA, USA).
To obtain the 3’ and 5’ terminal region sequences, a rapid amplification of cDNAs RACE was performed with the following primers: R-Ex-480, R-In-408, F-Ex-5931 and F-In-6041 (Luria et al., 2017), using a SMARTer® RACE 5’/3’ Kit (Takara Bio, USA) according to the manufacturer’s instructions. The obtained DNA products were cloned using the TA-cloning system (Promega, WIS, USA) and the five obtained plasmids were sequenced. All the obtained sequences of each region were assembled using the program Contig implemented in Vector NTI Advance 11.5 software (Invitrogen, CA, USA) to obtain the de novo full-length sequence of ToBRFV genome.

Primer and Taqman® MGB probe design
Four full-length genomic sequences of Tomato brown rugose fruit virus retrieved from GenBank (accession nos. MK133095, MK133093, KT383474 and KX619418) and a sequence assembled
in our lab during this work were aligned using ClustalX2 program (Larkin et al., 2007) in order
to design 5 primer pairs targeting only conserved sequences.
The obtained primers were tested in vitro with the Primer-BLAST algorithm
(https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) to evaluate the possibility of specific
hybridisation with other organisms. The same primers were also tested using Vector NTI
Advance 11.5 software (Invitrogen, CA, USA) with the complete sequence of other
tobamoviruses to understand their affinity percentages. The included tobamoviruses were: Bell
pepper mottle virus (1 sequence), Brugmansia mild mottle virus (1 sequence), Obuda pepper
virus (2 sequences), Paprika mild mottle virus (2 sequences), Pepper mild mottle virus (13
sequences), Rehmannia mosaic virus (6 sequences), Tobacco mild green mosaic virus (7
sequences), Tobacco mosaic virus (10 sequences), Tomato mottle mosaic virus (5 sequences) and
Tomato mosaic virus (10 sequences).
The obtained five primer pairs were tested by Real-time RT-PCR with SYBR green, in order to
understand which primer pair has the lowest Ct value. Real-time RT-PCR was performed in a
Q2plex HRM Platform Thermal Cycler (Qiagen, Hilden, Germany). The mixture consists in a 20
µl final volume containing 1 µl of total RNA extract (ToB-SIC01/19) with ∼10 ng RNA/µl
concentration, 1 µl of 1mM of each primer, 10 µl of Master mix QuantiNOVA XX (Qiagen,
Hilden, Germany) and H₂O DEPC to reach the final volume.
Healthy tomato plant RNA and water were used as control samples. Each sample was analysed
twice. Cycling conditions included reverse transcription at 48 °C for 10 min, incubation at 95 °C
for 5 min, 45 cycles of 95 °C for 2sec and 60 °C for 20 sec and fluorescence was measured at the
end of each cycle. Melting curve steps were added at the end of RT-PCR as following: 95 °C for
1 min, 40 °C for 1 min, 70 °C for 1 min and a temperature increase to 95 °C at 0.5 °C/s to record
the fluorescence.
A specific ToBRFV TaqMan® MGB probe (Eurofins Genomics, Luxembourg) was designed in
a conserved domain within the region encompassed by the primers. The probe, with a length of
22 nucleotides, was 5’-labelled with the reporter dye FAM (6-carboxyfluorescein) and 3’-
labelled with a non-fluorescent quencher (MGB NFQ). TaqMan MGB probes include a minor
groove binder (MGB) moiety at the 3’ end, which increases the melting temperature (Tm) of the
probe and stabilises the probe–target hybrids. Consequently, MGB probes can be significantly
shorter than traditional probes, providing better sequence discrimination and flexibility to
accommodate more targets. The predicted Tm values for ToBRFV primers and probe were 59-60
°C and 67 °C, respectively, calculated with the prediction tool provided by Primer Express
Software v3.0.1. (Thermo Fisher Scientific, MA, USA). The sequences included in this study are
reported in Table S1.
ToBRFV genotype-specific Real-time RT-PCR assay with TaqMan MGB probe
The Real-time RT-PCR assay with TaqMan MGB probe was performed in a Rotor-Gene Q2plex
HRM Platform Thermal Cycler (Qiagen, Hilden, Germany) in a reaction mix of 12 µl final
volume, containing 1 µl of total RNA extract with the concentration of ∼10 ng RNA/µl, 0.5 µM
of the forward primer ToB5520F and the reverse primer ToB5598R (the primer set that yielded
the most sensitive detection for all ToBRFV isolates in any type of tissue providing the lowest Ct
values), 0.25 μM of TaqMan MGB probe, 0.5 μl of RNase Inhibitor (Applied Biosystems), 6 μl
of 2x QuantiNova Probe RT-PCR Master Mix, 0.2 μl of QN Probe RT-Mix and H2O DEPC
water to reach final volume.

The total RNA extracts used to perform this assay were obtained from five plants infected with
ToBRFV and from eight tomato plants infected with Cucumber green mottle virus (CGMV),
Paprika mild mottle virus (PaMMV), Pepper mild mottle virus (PMMV), Tobacco mild green
mosaic virus (TMGMV), Tobacco mosaic virus (TMV), Tomato mottle mosaic virus (ToMMV),
Tomato mosaic virus (ToMV) and Zucchini green mottle mosaic virus (ZGMMV).

Each sample was analysed in duplicate in two independent Real-time RT-PCR assays. The
control samples in each run included total RNA from a healthy tomato plant, water instead of
sample and at least two RNA transcript dilutions of the standard curve (see below). The probe
annealed specifically in an internal region of the PCR product amplified with primers
ToB5520F-ToB5598R. After binding, the probe is cleaved by the 5’ exonuclease activity of the
DNA polymerase, which releases the reporter molecule away from the quencher, allowing the
reporter dye to emit its characteristic fluorescence. The TaqMan MGB probes incorporate an
NFQ to absorb the (quench) signal from the fluorescent dye label at the 3’ end of the probe.

The properties of the NFQ combined with the length of the MGB probe result in lower
background signal than with no-MGB NFQ probes. Lower background means increased
sensitivity and precision. The cycling conditions consisted in reverse transcription at 45 °C for
10 min, enzyme denaturation at 95 °C for 10 min, and 45 cycles of 95 °C for 5 sec and 60 °C for
60 sec with fluorescence measured at the end of each cycle. The mean (X) Ct value and the
standard deviation (SD) for each tomato sample were calculated from the four Ct obtained
values.

**Standard curve**

An external standard curve was generated to determine the sensitivity of the Real-time RT-PCR
protocol with the TaqMan MGB probe. Serial dilutions of an *in vitro* synthesised positive-sense
RNA transcript of the selected gRNA region were amplified using the Real-time RT-PCR
TaqMan MGB assay. The template for the *in vitro* transcription was obtained by conventional
RT-PCR amplification using total RNA extract from a tomato petiole infected with the just
characterised ToBRFV ToB-SIC01/19 isolate. The obtained DNA product was cloned in the
commercial pGEM-T vector, linearized with SalI enzyme and transcribed in vitro with the T7
RNA polymerase (New England Biolab, MA, USA) following the manufacturer’s instructions.
Transcripts were purified with RNaid Spin kit (Bio101, CA, USA), treated twice with RNase
free DNase (Turbo DNA-free from Ambion) and their concentration was determined in duplicate
with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA).

Ten-fold serial dilutions of the transcript in healthy tomato tRNA extract (10 ng/μl) containing
10^{10} to 10^{1} copies were used in the Real-time RT-PCR TaqMan MGB probe assay. The assay
was performed with and without the reverse transcriptase, to ensure the absence of DNA template in transcript preparations.

The RNA transcript concentration (pmol) in each dilution was calculated with the formula:

\[
\text{micrograms of transcript RNA} \times (106 \text{ pg/1 } \mu\text{g}) \times (1 \text{ pmol/340 pg}) \times (1/\text{number of bases of the transcript}),
\]

and the number of RNA copies was calculated using this concentration value and Avogadro’s constant. The standard curve was obtained plotting the threshold cycle (Ct) values from two independent assays with four replicates per standard dilution versus the logarithm of the RNA concentration dilution. The amplification efficiency was calculated from the slope of the corresponding curve using the formula \(10^{(-1/\text{slope of the standard curve})}\), or the same formula \(\times 100\) (when given as a percentage value).

**Different methods for sample preparation and comparison of different ToBRFV detection techniques**

Four different procedures of sample preparation were evaluated using 40 samples from the 360 samples that were analysed previously by end-point RT-PCR (10 per province), the just characterised isolate ToB-SIC01/19 and a negative-tomato control plant. The obtained results were also compared by DAS-ELISA and end-point RT-PCR.

1) **Immunocapture in Real-time PCR tubes:** Multiwell plates for Real-time PCR were incubated at 37 °C for 1 h with 100 μl of polyclonal antibody for *Tobacco mosaic virus* (TMV) (AGDIA, IN, USA) diluted 1:200 in a coating buffer (sodium carbonate anhydrous 1.59 g, sodium bicarbonate 2.93 g, sodium azide 0.2 g in 1 L of distilled water, pH 9.6). As reported by manufacture’s protocol, this antibody reacts with a variety of viruses from the *Tobamovirus* genus, such as *Cucumber green mild mottle virus* (CGMMV), *Kyuri green mottle mosaic virus* (KGMMV), *Pepper mild mottle virus* (PMMoV), *Tobacco mosaic virus* (TMV), *Tomato brown rugose fruit virus* (ToBRFV) and *Tomato mosaic virus* (ToMV). After incubation, three washing steps were performed. 100 μl of sap extract were obtained grinding the petioles in extraction buffer (sodium sulphite anhydrous 1.3 g, polyvinylpyrrolidone (PVP) MW 24-40,000 20 g, powdered egg (chicken) albumin, Grade II 2 g, Tween-20 20 g in 1 L of distilled water, pH 7.4). After 1 h of incubation at room temperature, the multiwell was washed with standard washing buffer, dried and prepared for subsequent analysis.

2) **Total RNA extraction:** Total RNA (RNAt) was extracted from 0.1 g of the petiole, with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instruction. RNA extracts were re-suspended in 30 μl of RNase-free water and adjusted to approximately 10 ng/μl using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA).

3) **Direct crude extract:** A slice of 0.4 mm of the petiole of each sample was directly placed in a 1.5 ml tube containing 0.5 ml of Glycine buffer (EDTA 1 mM, NaCl 0.05 M, Glycine 0.1 M), vortexed for 30 sec and heated at 95 °C for 10 min. Three microliters were used for subsequent analysis.

4) **Leaf-disk crude extract:** Five fresh-cut petioles were impressed in a 1 cm² of Hybond®-N+ hybridisation membrane (GE Healthcare. IL, USA), dried at room temperature for 5 min and
placed in a 1.5 ml tube containing 0.5 ml of glycine buffer. Tubes were vortexed for 30 sec and
heated at 95 °C for 10 min. Three microliters were used for the subsequent steps.
Finally, the 42 sample preparations were tested by DAS-ELISA using a commercial kit of
toxic antibodies for *Tobacco mosaic virus* (TMV), which can also detect ToBRFV
(AGDIA). The same samples were also tested by end-point RT-PCR using the primers ToBRFV-
F-5722 and ToBRFV-R-6179, and by Real-time RT-PCR-MGB- probe based-method according
to the protocols described previously.

In order to understand if the samples with high Ct value were really positive or false positives,
the products obtained with the samples named 1A, 3C, 7R, 8R and 9R using the methods No. 3
and No. 4 of sample preparation were analysed in 2% agarose gel. The products were purified
with the Ultraclean 96 PCR Cleanup Kit (Qiagen, Hilden, Germany) according to the
manufacturer’s instruction and sequenced in both directions using an ABI PRISM 3100 DNA
sequence analyser (Applied Biosystems, CA, USA).

**Results**

**Screening of ToBRFV using end-point RT-PCR**
The end-point RT-PCR analysis showed that 129 of the 360 analysed tomato samples (35.83%) were positive for ToBRFV. Table 1 reports the number of infected plants per province and data collection. As reported in Table 1, the incidence of ToBRFV was higher in the greenhouses in the provinces of Ragusa and Siracusa, with an infection percentage of 93.3% and 70%, respectively, than in the greenhouses of the provinces of Agrigento and Caltanissetta that showed a percentage of infected plants of 16.6% and 6.66%, respectively. Analysing the three surveys performed in the 4 provinces, the virus showed a downward trend. Indeed, Ragusa decreased from 93.3% of the first sampling to 63.3% of the third sampling, Siracusa from 70% to 40% and Agrigento and Caltanissetta passed from 16.6% and 6.66%, respectively, to zero.

**Mechanical transmission**
Mechanical inoculations successfully transmitted ToB-SIC01/19 in all the plants of *Solanum lycopersicum* and *Capsicum annuum*, while no transmission occurred in *Solanum melongena.* The tomato-inoculated plants did not show any symptom until 22 dpi. Starting from the day 22, tomato plants showed symptoms that consist in interveinal yellowing, deformation and mosaic in young leaves. In pepper-inoculated plants, the symptoms started at 23 dpi and consisted in slight interveinal yellowing on young leaves and necrosis on the stem. RT-PCR performed at 30 dpi confirmed the presence of ToBRFV in tomato and pepper plants and its absence in eggplants (Table 2).

**Full genome sequencing**
The gRNA of the ToBRFV isolate named ToB-SIC01/19 of Sicily was completely sequenced in this study. An RT-PCR synthesis was designed using the genome walking strategy of
overlapping fragments for each adjacent amplified product to avoid the sequencing of different templates (Luria et al., 2017). The accuracy of pure isolate sequencing was assured by the nucleotide sequences comparison of five different clones of each amplicon. The ToB-SIC01/19 sequence was assembled with the program Contig implemented with the Vector NTI Advance 11.5 software (Invitrogen, CA, USA) and deposited in GenBank under the Acc. No. MN167466. The genome length of ToB-SIC01/19 consists in 6,391 nucleotides and it was organised as just reported for ToBRFV (Luria et al., 2017). ToB-SIC01/19 showed a percentage identity of 99.8%, 99.7%, 99.7% and 99.7% with the sequences of TBRFV-P12-3H (Germany; Acc. No. MK133095), TBRFV-P12-3G (Germany; Acc. No. MK133093), Tom1-Jo (Jordan, Acc. No KT383474) and TBRFV-IL (Israel, Acc. No KX619418), respectively.

**Primer, Taqman® MGB probe design and protocol optimisation**

The *in vitro* analysis using Primer-BLAST algorithm of the five primers pairs designed to target the Movement Protein (MP) gene showed no relevant match with other organisms. All the obtained primer pairs were tested by Real-time RT-PCR with SYBR green to identify the primer pair with the lowest Ct value. Table 3 reports the five designed primer pairs. Between the 5 tested primer pairs, 2 did not give any signal (ToB5461F/ToB5592R and ToB5461F/ToB5593R), while the primer pair ToB5520F/ToB5598R showed the lowest Ct value and was used for the subsequent analyses (Figure 2A and 2B).

*In vitro* hybridisation analysis of the primer pair ToB5520F and ToB5598R against other tobamoviruses was carried out with the program Vector NTI 11.5 program (Invitrogen, CA, USA) and results showed that no relevant matches were identified (see Table S1).

**ToBRFV specific Real-time RT-PCR assay with TaqMan MGB probe**

To determine the specificity of the Real-time RT-PCR assay with TaqMan MGB probe, 17 different samples were analysed. As reported in Table 4, the two RNA transcripts gave the most sensitive signal with a Ct value ranging from 5.0 to 5.1 in four different assays. The total RNA derived from artificially ToBRFV-infected plants gave also positive signal with a Ct value that ranged from 12.2±0.2 to 18.1±0.1, while the other tobamoviruses used as outgroups did not give any signal.

**Standard curve**

In order to calculate the number of RNA copies and the sensibility threshold of the developed technique, a standard curve was generated using ten-fold serial dilutions of RNA *in vitro* transcripts (from $10^{10}$ to $10^1$ copies) of ToBRFV ToB-SIC01/19 isolate in healthy tomato RNAat. The standard curve covered a wide dynamic range (10 units of concentration) and showed a strong linear relationship, with a correlation coefficient of 0.9997 and 100% amplification efficiency (Figure 3A and 3B). The Real-time RT-PCR assay and the standard curve enabled the detection of as few as $10^1$ ToBRFV RNA copies in tomato extracts and were used to determine the number of RNA copies in the total RNA extracts from the tomato samples collected in four
different areas of Sicily, within the provinces of Agrigento, Caltanissetta, Ragusa and Siracusa. The average Ct values were within the dynamic range of the standard curve and ranged from 14 to 23.

**Different methods for sample preparation and comparison of different ToBRFV detection techniques**

Four different procedures were evaluated to identify the best method for sample preparation. Forty-two samples (10 per province, ToB-SIC01/19 and a healthy tomato plant) were included. The results obtained with the four different methods of sample preparation were compared with each other and with DAS-ELISA and end-point RT-PCR (Table 5). All the four different methods for sample preparation (immunocapture, total RNA extraction, direct crude extract and leaf-disk crude extract) were effective for ToBRFV detection by Real-time RT-PCR. Analysing the four different procedures, the Ct value obtained with immunocapture ranged from 16 to 28, with total RNA extraction from 14 to 23, with leaf-disk crude extract from 17 to 35 and with direct crude extract from 17 to 37.

Regarding the comparison between Real-time RT-PCR and other techniques, Real-time RT-PCR showed more sensitivity than DAS-ELISA; indeed, 11 samples that gave negative results by DAS-ELISA were positive with Real-time RT-PCR (Table 5). Comparing Real-time RT-PCR to end-point RT-PCR, the two techniques gave the same results with immunocapture and total RNA extraction procedure, while, using direct crude extract and leaf-disk crude extract the end-point RT-PCR was unfit to provide a reliable result.

To confirm the absence of false positives in the Real time RT-PCR assay, the amplification products of the samples named 1A, 3C, 7R, 8R and 9R were analysed in 2% agarose gel. The samples 7R, 8R and 9R gave the expected fragment while the samples 1A, 3C, healthy plant (HP) and H₂O did not show amplification (Figure 4). Sequencing of the samples 7R, 8R and 9R with the sample preparation methods No. 3 and No. 4 confirmed the presence of ToBRFV.

**Discussion**

Sicily is one of the Mediterranean Basin regions with the most important tomato production and, due to its geographical position, it represents the main access point for plant material to the European countries. This situation considerably increases the risk of introducing new pathogens into our environments and entails a serious risk for agriculture biosecurity and food production, jeopardising the future of Italian horticulture. The outbreak of *Tomato brown rugose fruit virus* (ToBRFV) represents a threat due to its multiple transmission methods and to the absence of tomato and peppers resistant varieties. Furthermore, several hybrid tomato varieties presenting *Tomato mosaic virus* (ToMV) and *Tobacco mosaic virus* (TMV) *Tm-1, Tm-2* and *Tm-2²* resistance genes (*Pelham, 1966*), can be severely affected by ToBRFV, leading to a rapid virus spread in all those areas where tomato is cultivated. To date, the only two available tools to contain ToBRFV worldwide are early diagnosis and the implementation of preventive measures in crop management, which can be a valuable aid in reducing the introduction and subsequent
ToBRFV spread in other countries. Consequently, today there is the need to develop alternative, sensitive and very reliable diagnostic methods for the diagnosis of plant viruses, which are compromising the tomato crops in various Italian and international areas (Hanssen et al., 2010; Puchades et al., 2017; Ferriol et al., 2015, Panno et al., 2014). In the present work a Tomato brown rugose fruit virus isolate, recently found in Sicily (Panno et al., 2019b), was characterised, and a sensitive, specific, rapid and economical method for its detection in infected plants and seeds was developed.

The biological characterisation of the Sicilian ToB-SIC01/19 isolate demonstrated the possibility of mechanical transmission on tomato and pepper plants, as previously reported by Luria and co-workers (2017), while on eggplant it cannot be transmitted. Mechanical transmission is very important because it could simplify genetic improvement activities, in order to constitute new tolerant/resistant germplasm towards ToBRFV. Molecular characterisation showed that the ToBRFV Sicilian isolate ToB-SIC01/19 presents a percentage identity of 99% with the sequences retrieved in Germany, Jordan and Israel (Menzel et al., 2019; Salem et al., 2016; Luria et al., 2017). Since ToBRFV spread in a few years within the Mediterranean Basin countries and in Central America, the very low level of variability found among isolates supports the hypothesis that the recent introduction in Italy probably occurred through infected seeds.

The ability of the virus to transmit through plant-to-plant contact, manipulation and especially by seeds in overlapping crops cycles, such as tomato intensive cultivation in the greenhouses, facilitated the rapid spread of the virus.

For these reasons, we have developed a quick detection procedure for ToBRFV diagnosis based on Real-time RT-PCR TaqMan MGB probe. This method detects ToBRFV in samples obtained by different preparation procedures. Additionally, the direct crude extracts and leaf-disk crude extracts were successfully used to avoid total RNA extraction, shortening the processing time, allowing the simultaneous analysis of multiple samples and drastically reducing the total cost for single analysis. Its high sensitivity is relevant to minimise false negatives and to obtain correct discrimination of ToBRFV with other viruses belonging to the genus Tobamovirus. Furthermore, the developed technique, associated with the direct crude extract sample preparation, can be used to make in-field diagnosis with a portable device, allowing a considerable saving of time and it could also be used by non-technical personnel.

Conclusions
The method developed in this work is based on the use of a Real-time RT-PCR TaqMan MGB probe and could represent a good and reliable tool to be included in certification programs. Currently, immuno-enzymatic methods, such as DAS-ELISA, are used in some cases for plant virus diagnosis, but these tests may not be very reliable and give “false negatives”, due to the low viral titre of nursery plants and early infections, and to the absence of specific antibodies (Jacobi et al., 1998).

Moreover, the method developed in the present study requires short time and allows the analysis of a great number of samples at the same time, when associated with the use of leaf-disk crude
and direct crude extracts. For this reason, this method could be used as a routine test in the laboratories of vegetable diagnosis. In conclusion, to avoid ToBRFV spread to other Italian and European regions, phytosanitary actions are required, such as correct crop management, more restrictive measures at international borders using rapid, sensitive and economical tools for diagnosis and, more importantly, the development of tomato resistant cultivars that have the ability to tolerate the disease. To date, new tomato cultivars that are tolerant/resistant to ToBRFV are not available. For this reason, the only way to contain this disease globally is the use of a sensitive and economical diagnostic tool such as the Real time RT-PCR method that was developed and described in this paper.

References


Table 1 (on next page)

Number of ToBRFV-infected tomato plants detected by endpoint RT-PCR.
<table>
<thead>
<tr>
<th>Province</th>
<th>No. ToBRFV-infected plants / No. Collected plants</th>
<th>2018-October</th>
<th>2018-December</th>
<th>2019-February</th>
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<tbody>
<tr>
<td>Agrigento</td>
<td>2/30</td>
<td>0/30</td>
<td>0/30</td>
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<td>Caltanissetta</td>
<td>5/30</td>
<td>0/30</td>
<td>0/30</td>
<td></td>
</tr>
<tr>
<td>Ragusa</td>
<td>28/30</td>
<td>22/30</td>
<td>19/30</td>
<td></td>
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<tr>
<td>Siracusa</td>
<td>21/30</td>
<td>20/30</td>
<td>12/30</td>
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</tbody>
</table>
Table 2 (on next page)

Mechanical inoculations and symptoms caused by ToB-SIC01/19 isolate on different herbaceous species

iy: interveinal yellowing, myl: mosaic in young leaves, dyl: deformation in young leaves, siyl: slight interveinal yellowing in young leaves, ns: necrosis in the stem.
<table>
<thead>
<tr>
<th>Species</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>RT-PCR</th>
<th>% infected plants</th>
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<tr>
<td><em>Solanum lycopersicum</em></td>
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<td>-</td>
<td>iy, myl</td>
<td>iy, myl, dyl</td>
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<tr>
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<td>-</td>
<td>siy</td>
<td>siyl, ns</td>
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<td>100%</td>
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Table 3 (on next page)

Forward, reverse primers and probe designed for quantitative RT-qPCR for the detection of *Tomato brown rugose fruit virus* (ToBRFV).

n.a.: not amplification
<table>
<thead>
<tr>
<th>Name</th>
<th>Genomic position</th>
<th>Referring sequence</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon Size (bp)</th>
<th>Ct value</th>
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<td>ToB-probe</td>
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Table 4 (on next page)

Real time RT-PCR assay with TaqMan MGB probe. Comparison between ToBRFV and other tobamovirus.
<table>
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<th>Virus isolate</th>
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<td>ToB-SIC1/19-P1</td>
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<td>ToB-SIC1/19-P2</td>
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<td>PaMMV</td>
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Table 5 (on next page)

Analysis of 42 samples using different methods of sample preparation and comparison of real time RT-PCR against DAS-ELISA and endpoint RT-PCR

1* Immunocapture in RT-qPCR multiwell; 2* Total RNA extraction; 3* Leaf-disk crude extract; 4* Direct crude extract
<table>
<thead>
<tr>
<th>Province</th>
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<tr>
<td>healthy plant</td>
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</tbody>
</table>
Figure 1

Symptoms of Tomato brown rugose fruits virus

Severe mosaic (A), deformation and necrosis on young leaves (B); discolouration and marbling on fruits (C); necrosis on sepals (D).
Manuscript to be reviewed
Figure 2

Real time RT-PCR using the SYBR green method with different primer pairs

**Panel A:** Amplification curves of real time –RT-PCR using SYBR green with the following primer pairs: p1 (ToB5520F/ToB5598R), p2 (ToB5498F/ToB5683R), p3 (ToB5461F/ToB5683R), p4 (ToB5461F/ToB5592R) and p5 (ToB5461F/ToB5593R). Primer pairs p4 and p5 did not show amplification, while primer pair A showed the lowest Ct value. **Panel B:** Melting curves of the amplification curves previously obtained with the 5 different primer pairs.
Figure 3

Standard curve and linear regression of real time RT-PCR.

**Panel A**: Standard curves prepared with ten-fold serial dilutions of in vitro-synthesised RNA transcripts from ToBRFV ToB-SIC01/19 isolate using real time RT-PCR with TaqMan MGB probe. **Panel B**: Curves were generated by linear regression analysis, plotting the Ct value in the Y axis vs the logarithm of the starting RNA dilutions in the X axis. Each plotted point represents the mean Ct value that was calculated from the four different experiments with two replicates. The calculated correlation coefficient ($R^2$) and amplification efficiency ($E$) values are indicated in each curve.
**A**

Graph showing multiple curves with increasing cycles.

**B**

Graph with the equation:

\[ y = -3.2817x + 1.9378 \]

- \( R^2 = 0.9997 \)
- \( E = 1.00 \)

Graph with y-axis labeled as Threshold cycles (Ct) and x-axis labeled as Log concentration sample.
Figure 4

Comparison of samples showing high Ct value in real time RT-PCR with electrophoretic gel

**Panel A:** Amplification curves obtained with the primer pair ToB5520F/ToB5598R and ToBprobe-5558 probe of the samples reported in table No. 5, which are named: tRNA1, 1A, 3C, HP (Healthy Plant), H2O, 7R, 8R and 9R. The 3* and 4* indicated the different samples preparation methods that are reported in table No. 5. **Panel B:** Electrophoretic 2% agarose gel of real-time RT-PCR products. M: 100 bp marker (Thermo Fischer Scientific).
Manuscript to be reviewed