Developing of real time reverse transcription polymerase chain reaction for rapid detection of Tomato brown rugose fruit virus and its comparison with other techniques

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Abstract

Background. *Tomato brown rugose fruit virus* (ToBRFV) is a highly infectious tobamovirus that causes a severe disease in tomato (*Solanum lycopersicum* L.) crops. In Sicily, the first outbreak occurred in 2018 in different Sicilian provinces represents a serious threat for tomato crops.

Methods. ~~For that, in this work a~~ molecular and biological characterization of ToBRFV isolate named ToB-SIC01/19 found in Sicily were carried out, 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 procedures (immunocapture, total RNA extraction, direct crude extract and leaf-disk crude extract) for sample preparation were evaluated.

Results. The Sicilian isolate ToB-SIC01/19 (6,391 nt) showed a strong sequence identity with isolates TBRFV-P12-3H and TBRFV-P12-3G from Germany, Tom1-Jo from Jordan and TBRFV-IL from Israel and was successfully transmitted by mechanical inoculations, in *Solanum*

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lycopersicum L. and *Capsicum annuum* L., while no transmission occurred in *Solanum melongena* L. The developed real time RT-PCR using a primer set designed from conserved sequences in ORF 3 enabled reliable quantitative detection and strong discrimination of ToBRFV with other viruses belonging to the genus *Tobamovirus*, minimizing false negative results. Comparing real-time RT-PCR to RT-PCR end point, both give the same results with immunocapture and total RNA extraction procedure, while, using direct crude extract and leaf-disk crude extract the RT-PCR end point was unfit to provide a reliable result. This real-time quantitative RT-PCR assay developed with high sensibility and specificity will be particularly valuable tool for early ToBRFV diagnosis, optimizing procedures in terms of costs and time.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural crops with a worldwide production of over 182 million tons in 2017 (*FAO*, *2017*). China is the world's largest 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 the tomato production is more located in Sicily, where it is one of the most economically horticultural crops, with the yield of approximately 40% of the total Italian production in greenhouses (*Agri ISTAT*, *2017*). In the last years, emerging viral diseases have been an important limiting factor in many crop production systems, such as tomato crops, causing considerable economical losses (*Hanssen et al*., *2010*). In fact, greenhouse tomato production in Italy is affected by important losses, 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* (TYLCV) – *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and recombinants of both (*Davino et al*., *2009*, *2012*, *Panno et al*., *2018*) and at the end of 2018 *Tomato brown rugose fruit virus* (ToBRFV) (*Panno et al*., *2019b*).

The international trade globalization and the free commodities movement in the area of the free trade for ~~Countries~~ countries facing the Mediterranean Basin makes more difficult to control pathogens. In this context, the control of pathogens transmitted by seeds is extremely difficult. This is the case of the recent ToBRFV outbreak in Sicily, which is very likely introduced into the island either through infected seeds or through infected fruits and their subsequently manipulation.

ToBRFV is a member of the genus *Tobamovirus*, family *Virgaviridae*. Tobamoviruses are the only members of the family to have an undivided genome. It is easily the largest genus in the family for numbers of species (*King et al*., *2011*). ToBRFV has a single stranded positive-sense RNA (gRNA) of ~ 6,400 nucleotides (nt), with a typical organization of tobamoviruses that consists in 4 open reading frames (ORFs) encoding two replication-related proteins of 126 and 183 kDa in which the second 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) that are expressed via 3'-coterminal sub-genomic RNAs (sgRNAs) (*Salem et al*., *2016*).

ToBRFV was first described in 2016 in Jordan by *Salem and co-workers* (*2016*) in tomato plants grown in greenhouses and in 2017 in Israel in tomato plants harboring the *Tm-2*^{2} gene (*Luria et al.*, *2017*), later in Mexico in tomato and pepper crops (*Cambrón-Crisantos et al*., *2018*) and subsequently it spread in 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*).

Symptoms of ToBRFV are typical of the tobamoviruses infection and consist in interveinal yellowing and deformation on tomato leaves, mosaic staining, deformation and necrosis on young leaves, necrosis and deformation of sepals and discoloration, deformations and necrosis on young fruits. For that, represents a very dangerous problem for tomato crops in all regions where tomato is grown, due to the ability of the virus to be transmitted by contact, such as 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 characterize the virus found in Italy and to develop a sensitive, specific and economic method to detect ToBRFV in infected plants and seeds.

Materials & Methods

Source of viral material

In October 2018, virus-like symptoms typical of tobamovirus infections were observed in tomato greenhouses in Ragusa province (Sicily – Italy). These were very similar to those described by *Salem and co-workers* (*2016*) where they identify a new tobamovirus, named *Tomato brown rugose fruit virus*, that overcame the *Tm-2*^{2} resistance gene. Symptoms consisted in severe mosaic, deformation and necrosis on young leaves, discoloration and marbling on fruits and necrosis on sepals (Figure 1).

Samples included in this study were collected in four different areas of Sicily, within the provinces of Agrigento, Caltanissetta, Ragusa and Siracusa. For each area, 15 samples were collected from two different greenhouses, according to the following scheme: out of 60 rows of plants, 3 were selected (one every 20 rows) and then, on each selected row, a sample was taken every 10 plants, for a total of five samples per row (*Panno et al*., *2019b*). Sampling was repeated three times: first sampling in October 2018, second in December 2018 and 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 RT-PCR end point

RT-PCR end point in one-step format 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, $MgCl_2$, 0.4 mM dNTPs, 1 mM of primer forward ToBRFV-F-5722 and 1 µM of primer reverse 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 and 30 sec at 72 °C; and a final elongation of 10 min at 72 °C. DNA products of the expected size obtained were confirmed by electrophoretic separation in a 1.5% agarose gel and staining with Sybrsafe (Thermo Fisher Scientific, MA, USA).

Mechanical transmission

The ToBRFV isolate ToB-SIC01/19 from Sicily was mechanically inoculated into three plants per host (*Solanum lycopersicum* L., *Solanum melongena* L. and *Capsicum annuum* L.) for subsequently biological characterization. Plants were grown in sterilized soil in an insect-proof glasshouse, with a photoperiod of 14 h light and a target air temperature set at 28/20 °C day/night. 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 using the primer walking strategy (*Knippers and Alpert*, *1999*) were carried out with specific and overlapping primers reported by *Luria and coworkers* (*2017*). Four pairs of primers targeting conserved sequences in ToBRFV were included to obtain the full genome sequence: FTobGEN, RTobGEN, F1-R1572, F1534-R3733, F4587-R6392.
RT-PCR was carried out in a MultiGene OptiMax thermal cycler (Labnet International Inc, Edison, NJ, USA) according to the condition reported in *Luria et al*. (*2017*). Products obtained were confirmed by electrophoretic separation in 1.5% agarose gel and visualized using Sybrsafe staining. Subsequently the products were purified with the Ultraclean 96 PCR Cleanup Kit (Qiagen, Hilden, Germany) according with the manufacture's instruction and cloned using the TA-cloning system (Promega, WIS, USA). Five plasmids obtained were sequenced in both directions using an ABI PRISM 3100 DNA sequence analyzer (Applied Biosystems, CA, USA). To obtain the 3' and 5' terminal region sequences a rapid amplification of cDNAs RACE were carried out with the following primers: R-Ex-480, R-In-408, F-Ex-5931, F-In-6041 (*Luria et al*., *2017*), using a SMARTer® RACE 5'/3' Kit (Takara Bio, USA) according with the manufacture's instruction. The DNA products obtained were cloned using the TA-cloning system (Promega, WIS, USA) and five plasmids obtained were sequenced. To obtain de novo the full-length sequence of ToBRFV genome, all the obtained sequences of each region were assembled using the program Contig implemented in Vector NTI Advance 11.5 software (Invitrogen, CA, USA).

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, KX619418) and one more assembled in our

lab during this work were aligned using ClustalX2 program (*Larkin et al*., *2007*) and carried out in order to design 5 primer pairs targeting only in conserved sequences.
The primers obtained were in vitro tested with the Primer-BLAST algorithm (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) to evaluate the possibility of a specific hybridization with other organisms. The same primers were also tested using Vector NTI Advance 11.5 software (Invitrogen, CA, USA) with the following complete sequences of tobamoviruses in order to understand the percentage of affinity with them. The tobamoviruses included 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).
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'-labeled with the reporter dye FAM (6-carboxyfluorescein) and 3' with a non-fluorescent quencher (MGB NFQ). TaqMan MGB probes include a minor groove binder (MGB) moiety at the 3' end that increases the melting temperature (Tm) of the probe and stabilizes probe–target hybrids and for that 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). Sequences included in this study are reported in Table S1.

ToBRFV genotype-specific RT-qPCR assay with TaqMan MGB probe

The real-time RT-PCR with TaqMan MGB probe assay 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 at the concentration of ~10 ng RNA/µl, 0.5 µM of primer forward ToB5520F and primer reverse ToB5598R (the primer set that yielded the most sensitive detection for all ToBRFV isolates in any tissue providing the lowest Ct values, data not showed), 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 H_2O DEPC water to reach final volume.
To carried out this assay, total RNA extracts from five ToBRFV-infected plants and eight from 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) were included.
Each sample was analyzed in duplicate in two independent real-time RT-PCR assays. Control samples in each run included total RNA from 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 and then they were cleaved by the 5' exonuclease activity of the DNA polymerase, which released the reporter molecule away from the quencher, thus allowing the reporter dye to emit its characteristic fluorescence. TaqMan MGB probes incorporate an NFQ to absorb (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 non-MGB NFQ probes. Lower background means increased sensitivity and precision. The cycling conditions consisted of 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 values obtained.

Standard curve

To determine sensitivity of the real-time RT-PCR protocol with the TaqMan MGB probe, serial dilutions of in vitro-synthesized, positive sense RNA transcript of the selected gRNA region, was amplified using the real-time RT-PCR TaqMan MGB assay to generate an external standard curve. The template for the in vitro transcription was obtained by conventional RT-PCR amplification using total RNA extract from tomato petiole infected with the just characterized ToBRFV isolate ToB-SIC01/19, cloned in the commercial pGEM-T vector, linearized with *Sal*I 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 determined in duplicate with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA).
Ten-fold serial dilutions of each transcript in healthy tomato tRNA extract (10 ng/µl) containing 1010 to 101 copies were used in real-time RT-PCR TaqMan MGB probe assay with and without reverse transcriptase, to ensure the absence of DNA template in transcript preparations.
The transcript RNA concentration (pmol) in each dilution was calculated with the formula: micrograms of transcript RNA × (106 pg/1 µg) × (1 pmol/340 pg) × (1/number of bases of the transcript), and the number of RNA copies using this concentration value and Avogadro's constant. 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 × 100 (when given as a percentage value).

Different method for sample preparation and comparison of different techniques for ToBRFV detection

To select the best method for sample preparation, 40 samples from the 360 previously analyzed by RT-PCR end point (10 per province), the just characterized isolate ToB-SIC01/19, and a negative-tomato control plant were used in order to evaluate four different procedures of sample preparation. The results obtained were also compared with DAS-ELISA and RT-PCR end point.

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 in 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. Then 100 µl of sap extract, obtained by grinding the petioles in extraction buffer (Sodium sulfite 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) were added. After 1 h of incubation at room temperature the multiwell was washed with standard washing buffer, dried and prepared for subsequently analysis.

2) Total RNA extraction: Total RNA (RNAt) was extracted from 0.1 g of 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 petiole of each sample were directly placed in a 1.5 ml tubes 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 subsequently analysis.

4) Leaf-disk crude extract: Five fresh cut petioles were impressed in a 1 cm^2 of Hybond®-N+ hybridization membrane (GE Healthcare. IL, USA), dried at room temperature for 5 min and placed in a 1.5 ml tubes 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 subsequently steps.

Finally, the 42 sample preparations were tested by DAS-ELISA using a commercial kit of polyclonal antibodies for *Tobacco mosaic virus* (TMV), that have the ability to detect also ToBRFV (AGDIA), by RT-PCR end point using primers ToBRFV-F-5722 and ToBRFV-R-6179, and by RT-qPCR-MGB- probe based-method according to the protocols previously described.

Results

Screening of ToBRFV using RT-PCR end point

The RT-PCR end point analysis showed that 129 out of the 360 tomato samples analyzed (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 object of the study in provinces of Ragusa and Siracusa, with a percentage of infection of 93.3% and 70%, respectively, than the greenhouses located in the provinces of Agrigento and Caltanissetta, with a percentage of infected plants of 16.6% and 6.66%, respectively. Analyzing the three surveys performed in the 4 provinces, the virus showed a downward trend. Exactly, 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 pass from 16.6% and 6.66%, respectively, to zero.

Mechanical transmission

Mechanical inoculations successfully transmitted ToB-SIC01/19 in all plants of *Solanum lycopersicum* and *Capsicum annuum*, while no transmission occurred in *Solanum melongena*. The tomato-inoculated plants do not show symptoms until 22 dpi. From the day 22 on going, tomato plants showed symptoms that consist in interveinal yellowing, deformation and mosaic in young leaves. In pepper-inoculated plants, symptoms starting from 23 dpi and they consisted in slight interveinal yellowing in young leaves and necrosis in the stem. RT-PCR carried out at 30 dpi confirmed the presence of ToBRFV in tomato and pepper plants and its absence in eggplants.

Full genome sequencing

The gRNA of the ToBRFV isolate named ToB-SIC01/19 from Sicily was completely sequenced in this study. To avoid sequencing of different templates, the RT-PCR synthesis using the genome walking strategy of overlapping fragments for each adjacent amplified product was designed (*Luria et al*., *2017*). Nucleotide sequences comparison of five different clones of each amplicon assured accuracy in sequencing a pure isolate. The assembled sequences constructed with the program Contig implemented in the Vector NTI Advance 11.5 software (Invitrogen, CA, USA) of the ToB-SIC01/19 was deposited in GenBank under the Acc. No. MN167466. The genome length of ToB-SIC01/19 consist in 6,391 nucleotides and it was organized 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 sequences 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 optimization

The *in vitro* analysis using Primer-BLAST algorithm of the five primers pairs designed targeting the Movement Protein (MP) gene showed no relevant match with other organisms. All primer pairs obtained were tested in RT-qPCR with SYBR in order to know the primer pair with highest sensibility. In Table 2,was reported the primer pair with the highest sensibility was reported and used in this work, named ToB5520F and ToB5598R.

In vitro hybridization 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 identify (see Table S1).

ToBRFV specific RT-qPCR assay with TaqMan MGB probe

To determine the specificity of RT-qPCR assay with TaqMan MGB probe, 17 different samples were analyzed. As reported in Table 3, the two RNA transcript gave the most sensitive signal with a Ct value that range from 5.0 to 5.1 in the four different assays. Total RNA derived from artificial ToBRFV-infected plants give also positive signal with a Ct value that range from 12.2±0.2 to 18.1±0.1, while the other tobamoviruses utilized as outgroup do not give any signal.

Standard curve

In order to calculate the number of RNA copies and sensibility threshold of the technique developed, a standard curve was generated using as template a ten-fold serial dilutions of RNA in vitro transcripts (from 10^{10} to 10^{1} copies) from ToBRFV isolate ToB-SIC01/19 in healthy tomato RNAt. 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 its amplification efficiency was 100% (Figure 2 A and B). This real-time RT-PCR assay and standard curve enabled detection of as few as 10^{1} ToBRFV RNA copies in tomato extracts and was used to determine the number of RNA copies in total RNA extracts from 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 method for sample preparation and comparison of different techniques for ToBRFV detection

To select the best method for sample preparation, four different procedures were evaluated. 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 samples preparation were compared with each other and with DAS-ELISA and RT-PCR end point (Table 4). 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. Analyzing the four different procedures, the Ct value obtained for immunocapture range from 16 to 28, for total RNA extraction range from 14 to 23, for leaf-disk crude extract range from 17 to 35 and for direct crude extract range 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; in fact, 11 samples that give negative results in DAS-ELISA were positive in RT-qPCR (Table 4). Comparing real-time RT-PCR to RT-PCR end point, the two techniques give the same results with immunocapture and total RNA extraction procedure, while, using direct crude extract and leaf-disk crude extract the RT-PCR end point was unfit to provide a reliable result.

Discussion

Sicily is one of the Mediterranean Basin regions with the most important tomato productions and, due to its geographical position, it represents the main access point for plant material to European countries. This situation greatly increases the risk of introducing new pathogens into our environments and this entails a serious risk related to biosecurity for agriculture and food production, jeopardizing the future of Italian horticulture. The outbreak of *Tomato brown rugose fruit virus* (ToBRFV) represent a threat due to its multiple transmission methods and the absence of tomato and peppers resistant varieties. Furthermore, many hybrid tomato varieties presenting *Tomato mosaic virus* (ToMV) and *Tobacco mosaic virus* (TMV) *Tm-1*, *Tm-2* and *Tm-2*^{2} resistance genes (*Pelham*, *1966*), can be severely affected by ToBRFV. This can lead to a rapid spread of the virus in all areas of tomato cultivation. So, with regard to this pathogen, to date the only two tools available are: early diagnosis and the implementation of preventive measures in crop management, which can be a valuable aid in reducing introduction and subsequent spread of ToBRFV in cultivation environments. Consequently, today there is the need to develop alternative diagnostic methods, which are at the same time sensitive and very reliable, for the diagnosis of plant viruses, which are compromising the tomato cultivations 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 characterized, and was developed a sensitive, specific, rapid and economic method for its detection in infected plants and seeds.

The biological characterization of the Sicilian isolate ToB-SIC01/19 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 could simplify genetic improvement activities, in order to constitute new tolerant/resistant germplasm towards ToBRFV. Molecular characterization showed that the ToBRFV Sicilian isolate ToB-SIC01/19 presents a percentage identity of 99% with 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 Central America, the very low level of variability found among isolates support the hypothesis of the recent introduction in Italy, probably occurred by infected seeds.

The ability of the virus to transmit plant-by-plant by contact, by manipulation and, above all, by seeds, in addition to overlapping cycles of crops, such as tomato intensive cultivation in greenhouse, facilitate the rapid spread of the virus.

For that, we have developed a quick detection procedure for ToBRFV diagnosis based on real-time RT-PCR TaqMan MGB probe. This method detects ToBRFV in different sample preparation procedures. Additionally, the direct crude extract and leaf-disk crude extract were successfully used to avoid total RNA extraction, which shorted the processing time, allowed simultaneous analysis of multiple samples and finally, and drastically reduce the total cost for single analysis. Its high sensitivity is relevant as a criterion to minimize false negatives and to obtain a correct discrimination of ToBRFV with other viruses belonging to the genus

Tobamovirus. Furthermore, the developed technique, associated with direct crude extract sample preparation, can be used to make in-field diagnosis, using a portable device, allowing a considerable saving of time and could also be used by non-technical personnel.

Conclusions

The method based on real-time RT-PCR TaqMan MGB probe, developed in this work, could represent a good and reliable tool to be included in certification programs. Currently, in some cases, for plant virus diagnosis, immuno-enzymatic methods are used, such as DAS-ELISA, but due to the low viral titer present in nursery plants or at early infection, or due to the absence of specific antibodies, these tests may ~~lead to~~ not be very reliable ~~results~~ones, obtaining "false negatives" (*Jacobi et al.*, *1998*). In conclusion, to avoid ToBRFV spreading to other Italian and European regions, more restrictive management is required, such as a correct crop management, more restrictive measures at international borders based on rapid, sensitive and economic tools for diagnosis and, more important, the developing of tomato-resistant-cultivar that have the ability to tolerate the disease.

Comment [P1]: To be revised

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