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Spread of Tomato Brown Rugose Fruit Virus in Sicily and Evaluation of the Spatiotemporal Dispersion in Experimental Conditions

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Abstract: *Tomato brown rugose fruit virus* (ToBRFV) is an emerging pathogen that causes severe disease in tomato (*Solanum lycopersicum* L.) crops. The first ToBRFV outbreak in Italy occurred in 2018 in several Sicilian provinces, representing a serious threat for tomato production. In the present work, the spatiotemporal displacement of ToBRFV in Sicily was evaluated, analyzing a total of 590 lots of tomato seed, 982 lots of plantlets from nurseries and 100 commercial greenhouses. Furthermore, we investigated the ToBRFV spreading dynamic in a greenhouse under experimental conditions. Results showed several aspects related to ToBRFV dispersion in protected tomato crops. In detail, an important decrease of the ToBRFV-infected seed and plantlet lots was detected. Regarding the examined commercial greenhouses, ToBRFV still appears to be present in Sicily, although there has been a decrease during monitoring. In experimental conditions, it was demonstrated that the presence of few infected plants are sufficient to damage the entire crop in a short time, reaching almost 100% of infection.

Keywords: emerging pathogen; ToBRFV; epidemiology; dispersion

1. Introduction

Tomato (*Solanum lycopersicum* L., family *Solanaceae*), is one of the most important and extensively grown horticultural crops worldwide. According to the latest data reported, the global tomato production, in the decade between 2008 and 2018, has increased by over 40 million tonnes [1], with a total worldwide production of more than 182 million tonnes in 2018 [1]. China is the world's leading producer, with over 61 million tonnes, followed by India, United States and Turkey. Italy and Spain are the major tomato producers in Europe, with over 5,7 million and 4,7 million tonnes, respectively [1]. Many vegetable crops, such as tomato, are constantly exposed to different biotic factors. Among these, viruses play a fundamental role in crop management, especially when sudden and rapid outbreaks of a viral disease occur in different areas. In recent years, one of the most recent and dangerous disease outbreaks on tomato crops worldwide is represented by *Tomato brown rugose fruit virus* (ToBRFV).

Tomato brown rugose fruit virus belongs to the genus *Tobamovirus*, family *Virgaviridae*. Unlike other members of this family, tobamoviruses have an undivided genome [2]. In detail, ToBRFV has a typical genome organization of the genus *Tobamovirus*. The single-stranded positive-sense RNA (ssRNA+) of approximately 6.400 nucleotides (nt) contains four open reading frames (ORFs) encoding: two replication-related protein complexes of 126 and 183 kDa, respectively, with the second protein expressed by the partial suppression of the stop codon (ORF1a and ORF1b), the movement protein (MP) of *ca.* 30 kDa (ORF2), and the coat protein of about. 17.5 kDa (ORF3), expressed via the 3'-coterminal sub-genomic RNAs [3].

The first evidence of the presence of a new tobamovirus was reported in 2014 on tomato plants harboring the *Tm-2*^{2} gene in Israel [4]. Subsequently, Salem and coworkers [3] reported the presence of this new tobamovirus in Jordan in the spring of 2015, and proposed the name of *Tomato brown rugose fruit virus* (ToBRFV). Afterward, ToBRFV was detected on tomato plants in Mexico [5], Germany [6], Turkey [7], United Kingdom [8], China [9], and Holland [10]. It was also detected in United States (California) [11], but to date seems to be eradicated [12]. Regarding the countries facing the Mediterranean basin, ToBRFV was reported in Italy [13], Palestine [14], Greece [15], Spain [16], and France [17]. Furthermore, it has also been reported in sweet pepper plants grown under plastic houses in Jordan [18] and in a greenhouse located in Sicily (Italy) [19]. The reports at today's date are probably underestimated and will tend to grow with new reports in different Countries where tomato and sweet pepper crops are grown, due to the ability of ToBRFV to move by seeds and contaminated fruits [4,20].

On tomato plants, ToBRFV infection causes leaves interveinal yellowing and deformation, severe mosaic, young leaves deformation and necrosis, sepal necrosis and deformation, fruits discoloration and marbling, young fruits discoloration, deformation and necrosis. In sweet pepper plants, the main symptoms caused by ToBRFV consist of slight mosaic, discoloration and vein clearing of young leaves, browning of the stem with strong necrosis located in the intersection of the secondary branches leading to plant growth inhibition, partial necrosis of the vegetative apex and marbling, mosaic and distortion of the fruits [4,19].

In order to clearly understand the ToBRFV dispersion and epidemiology, it is important to describe and consider the different transmission pathways of this virus. Like other common tobamoviruses, ToBRFV transmission is mainly mechanical, but it can also be transmitted via contaminated seed/fruits over long distances. It is capable of being mechanically transmitted within crops through direct plant-to-plant contact [3], propagation material (grafts, cuttings) and bumblebees [21]. Transmission can also occur through infected sap by adherence to different surfaces, such as human body, clothing, pots, packaging, people bringing in tomatoes to consume on site, transportation materials, working tools, and nutrient solutions [22]. It is important to note that the ToBRFV inoculum can remain, after harvesting, in different surfaces and materials in a greenhouse, such as wires, glass, concrete and soil [20].

Since the first identification in 2014, ToBRFV currently seems to have, as its main hosts, species belonging to the *Solanaceae* family, in particular tomato (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum annuum* L.). Under natural conditions, as just been reported above, ToBRFV can infect tomato and sweet pepper plants [19], especially in greenhouses with temperatures above 30 °C, as reported by Luria and coworkers [4]. In December 2017, another economically important solanaceous species, eggplant (*Solanum melongena*), was reported as a new host of ToBRFV. In this case only one positive sample was reported in the State of Sinaloa (Mexico) [23]. Regarding this new host, it is important to underline that divergent results were reported. In fact, under experimental conditions two different research groups failed to transfer the virus in this new host [4,24].

Regarding the host range, different studies were conducted in order to better understand the potential ToBRFV hosts. In laboratory conditions, inoculation experiments have shown that the virus has been successfully transmitted on different tomato and sweet pepper commercial cultivars with *Tm-2*^{2} and *L*^{1,3,4} resistance genes, respectively, showing the typical ToBRFV symptomatology. It was also artificially transmitted on several species of *Nicotiana* (N. *tabacum*, *N. benthamiana*, *N. clevelandii*, *N. glutinosa*), *Solanum nigrum* and common weeds, such as *Chenopodium murale*, *C. amaranticolor*,

C. quinoa, and *Petunia hybrida* [4]. In this context, common weeds could compromise the cultivated crop, acting as a reservoir for the virus. Furthermore, it was demonstrated that potato (*Solanum tuberosum* L.) is not the host of ToBRFV [4].

The aim of the current work was to analyze the criticalities of the tomato production cycle from seed companies, to nurseries, and farms. Furthermore, it was studied the ToBRFV spreading dynamic in controlled conditions, simulating the typical tomato production cycle.

2. Materials and Methods

2.1. Spatiotemporal Displacement of Tomato Brown Rugose Fruit Virus

2.1.1. Seed Sampling

During the period November 2018–January 2020, a total of 590 commercial lots of tomato seed were analyzed. Seeds were collected directly in the different nursery involved in the subsequent sampling. Seeds collected belonged to 10 companies that have their headquarters in Europe, while the seed production was carried out in the following countries: Israel, China and Mexico. Sampling was conducted by collecting seeds in the same nurseries subjected to the subsequent studies (see Section 2.1.2). Each sample was constituted by 3000 seeds collected by a single lot. A lot is defined as an identifiable, homogeneous and of certain origin group of seeds. In order to have a higher probability to identify even a few infected seeds, each sample was subsequently divided into 10 sub-samples consisting of 300 seeds. Each sub-sample was assigned an identification code and subjected to RNA extraction. Total RNA was extracted using 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 the RNA concentration was measured twice with a UV-Vis Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), adjusted to approximately 10 ng/µL and stored at −80 ºC until use. Subsequently, total RNA was used for Real Time RT-PCR analysis [24].

2.1.2. Analysis in Nurseries

Analysis in nurseries were carried out using the methodology described in the "International standards for phytosanitary measures" (ISPM) No. 31 [25]. A statistical approach was used for sampling. Samples size was based on the hypergeometric formula (sample size for small lots) adapted for lots having a number of plantlets ranging from 10,000 to 20,000. A confidence level P = 95% was chosen to derive the sample size and a product "level of detection × efficacy of detection" equal to 5%. From November 2018 to January 2020, a total of 982 samples were collected from six different nurseries, in Agrigento and Ragusa provinces, with a sampling peak during August–September 2019 (480 samples collected). Sampling was carried out each month, for a total of 15 samplings over the period. Each sample was composed of 60 leaves collected from 60 different plants of the same lot (L), where a lot is a group of homogeneous plants of identified origin, constituted by a number of plants ranging from 10,000 to 20,000. Each collected sample was subsequently divided into 3 sub-samples of 20 leaves. Sub-samples obtained were transferred into Bioreba extraction bags (BIOREBA AG, Reinach, Switzerland) and 15 mL of extraction buffer (sodium sulphite anhydrous 1.3 g, polyvinylpyrrolidone MW 24-40,000 20 g, powdered egg (chicken) albumin, Grade II 2 g, Tween-20 20 g in one L of distilled water, pH 7.4) were added. After homogenization, 100 µL of crude extract was incubated for 2 h into Real Time PCR tube, which was previously coated with TMV-polyclonal antibody (AGDIA, Elkhart, IN, USA) for 1 h at 37 °C. After three washing steps of 5 min each, sub-samples were analyzed by immunocapture (IC)-Real Time RT-PCR assay [24]. A total of 2946 analyses were carried out.

2.1.3. Analysis in Production Greenhouses

During the period October 2018–January 2020, different tomato greenhouses were inspected in order to understand the spreading of ToBRFV in the most important horticultural Sicilian protected

crop areas. Inspection was not done at the same time for all greenhouses, due to the unexpected emergence of ToBRFV in Sicily. Particularly, the inspections started in the greenhouse of the first outbreak, then in the following months the investigation area was gradually enlarged. At the end of the investigations, 100 greenhouses located in the provinces of Siracusa, Ragusa, Caltanissetta and Agrigento were examined. In Supplementary Figure S1 is reported the geographic location of the analyzed greenhouses. Visual inspection and sampling collection were carried out every month, except July, August, and September 2019, for a total of 14 months. Every month, for each greenhouse, two samples were collected. Each sample was constituted by 20 leaves collected from 20 different plants (one leaf per plant). There was a total of 2800 samples. Afterward, obtained samples were transferred into Bioreba extraction bags (BIOREBA AG, Reinach, Switzerland) and 15 mL of extraction buffer (sodium sulphite anhydrous 1.3 g, polyvinylpyrrolidone MW 24-40,000 20 g, powdered egg (chicken) albumin, Grade II 2 g, Tween-20 20 g in one L of distilled water, pH 7.4) were added. After homogenization, 5 µL of sap extract were spotted in a one cm^2 of Hybond®-N+ hybridization membrane (GE Healthcare, Chicago, IL, USA), and dried at room temperature. This step was carried out directly in a greenhouse; the different membranes were stored in laboratory. Subsequently, each membrane of 1 cm^2 obtained was 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). Tubes were vortexed for 30 s and heated at 95 °C for 10 min. After incubation, 3 µL of the obtained suspension were directly used for subsequent Real Time RT-PCR assay [24].

2.2. Evaluation of Tomato Brown Rugose Fruit Virus Epidemiology in Experimental Conditions

To carry out this experiment, an experimental greenhouse was used. The greenhouse structural characteristics were a 20 m width, 25 m depth, with a summit height of 6 m. The roof was covered with plastic, while the sides of the greenhouse were covered with insect-proof net. Inside was placed a micro-flow irrigation system and plastic mulching film. Each plant was placed in a 20 × 20 cm pot, in order to avoid any contact with the soil. The layout of the crop has included a total of 12 rows, each consisting of 40 plants, for a total of 480 plants. During July 2018 and for about 80 days, the soil was subjected to solarization by humid heat to disinfect soil from soil-borne diseases and pests. In the same month, two tomato plants of a commercial cultivar, harboring the *Tm-2*^{2} gene, kindly provided by a company, were inoculated in the laboratory with ToBRFV [24].

About 200 mg of fresh leaf tissue from a ToBRFV-infected plant were ground in a mortar with 6 mL of phosphate buffer pH 7 (0.2 M NaH_2PO_4, 0.2 M $Na_2HPO_4 \times 7H_2O$). The homogenate was subsequently distributed by rubbing on the leaf surface of the plants, previously sprinkled with Carborundum (320 mesh), in order to cause micro-lesions and facilitate the passage of virions.

Inoculated plants were grown on a sterilized soil in an insect-proof glasshouse, with a 14 h photoperiod of light, and a target air temperature set at 28/20 °C day/night. Thirty days post-inoculation (dpi), two young leaves per inoculated plants were tested by IC-Real Time RT-PCR [24], in order to confirm the successful transmission of the virus.

In the third week of September 2018, 478 healthy plants of the same cultivar used for mechanical inoculation, kindly prepared by a nursery, plus the 2 inoculated plants previously prepared, were transplanted into the greenhouse. The two infected plants (IP) were spaced by 5 rows and 5 columns, exactly, the IP were transplanted in row No. 3 position 16 and row No. 9 position 10. The transplant date represents the zero time for the start of the experiment. Plants were treated following the normal cultivation operations, adding two hives of 30 bumblebees each (*Bombus terrestris* L.) within the cultivation. All the workers were provided with disposable lab coats, gloves and boots in order to avoid a new introduction of ToBRFV from the external environment. Every 30 days, starting from transplanting to the end of May 2019, one young leaf per plant was collected and subjected to subsequent IC-Real Time RT-PCR analysis [24] as previously described. In total, 8 sample collections were carried out. The collected data have been processed by Matlab version 2019b, in order to generate the figures. Data measurements have been imported to the Matlab environment as spreadsheet files, and basic source code for plotting the data.

3. Results

3.1. Spatiotemporal Displacement of Tomato Brown Rugose Fruit Virus

3.1.1. Seed Sampling

A total of 590 lots of tomato seeds were analyzed by Real Time RT-PCR, during the period November 2018–January 2020. In detail, each sample was constituted by 3000 seeds and subsequently divided into 10 sub-samples of 300 seeds; each sub-sample was analyzed individually. All the sub-samples of each lot gave the same result, so the results are reported referring to the individual seed lots. As reported in Table 1, the analysis showed that 43 out of the 590 lots (7.28%) were positive to ToBRFV. For each seed company, at least one ToBRFV-positive seed lot was detected. In detail, the lots resulted positive were found exclusively within November 2018–May 2019, with a strongly downward trend. In fact, the percentage of infected lots went from 30% on a total of 20 lots analyzed, during November 2018, to 6% on a total of 50 lots analyzed, during April–May 2019. Afterward, in the remaining sampling period (June 2019–January 2020), no positive ToBRFV lots were reported. In Figure 1, the number of lots analyzed per month (blue color line) and related percentage of infected lots (orange color line) are reported.

Table 1. Number of analyzed seed lots per month and number of detected infected seed lots.

Sampling per Month	No. Lots Analyzed	No. Infected Lots
November 2018	20	6
December 2018	30	6
January 2019	40	9
February 2019	40	8
March 2019	40	8
April 2019	50	3
May 2019	50	3
June 2019	50	0
July 2019	40	0
August 2019	30	0
September 2019	40	0
October 2019	50	0
November 2019	50	0
December 2019	30	0
January 2020	30	0
Total	**590**	**43**

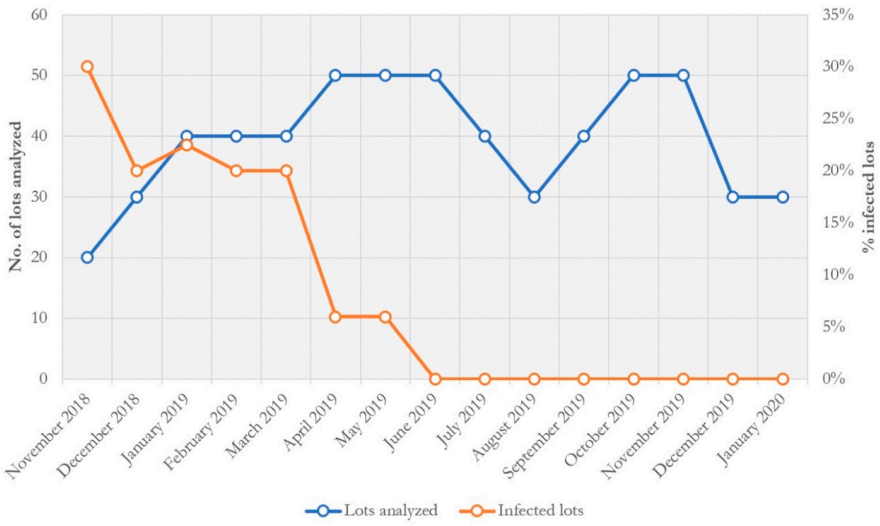

Figure 1. Number of lots of tomato seeds analyzed per month, during the period November 2018–January 2020 and percentage of ToBRFV-infected lots.

3.1.2. Analysis in Nurseries

The IC-Real Time RT-PCR analysis showed that 14 out of 982 tomato-plantlet lots analyzed (1.4%) were infected with ToBRFV, during the period November 2018–January 2020. For each nursery, at least one ToBRFV-positive plantlet-lot was detected. As reported in Materials and Methods, each lot was divided into three sub-samples, which gave the same result. In this case, a relatively stable trend of the disease was observed. In fact, as reported in Table 2, leaving out the three detected peaks (30% of 10 infected lots analyzed in November 2018, 6.25% in February 2019 and May 2019 out of 16 analyzed), with increasing sampling, the percentage of infected lots was very low. In detail, the largest number of lots was analyzed between August and September 2019 (480 lots), of which only two lots were positive (Figure 2). Lastly, in the period October 2019–January 2020, no positive ToBRFV lots were detected out of a total of 208 lots analyzed.

Table 2. Number of analyzed plantlet-lots per month and number of detected infected plantlet-lots in nursery.

Sampling per Month	Lots Analyzed	No. of Infected Lots
November 2018	10	3
December 2018	80	3
January 2019	80	3
February 2019	16	1
March 2019	16	0
April 2019	20	0
May 2019	16	1
June 2019	16	0
July 2019	40	1
August 2019	240	1
September 2019	240	1
October 2019	16	0
November 2019	32	0
December 2019	80	0
January 2020	80	0
Total	**982**	**14**

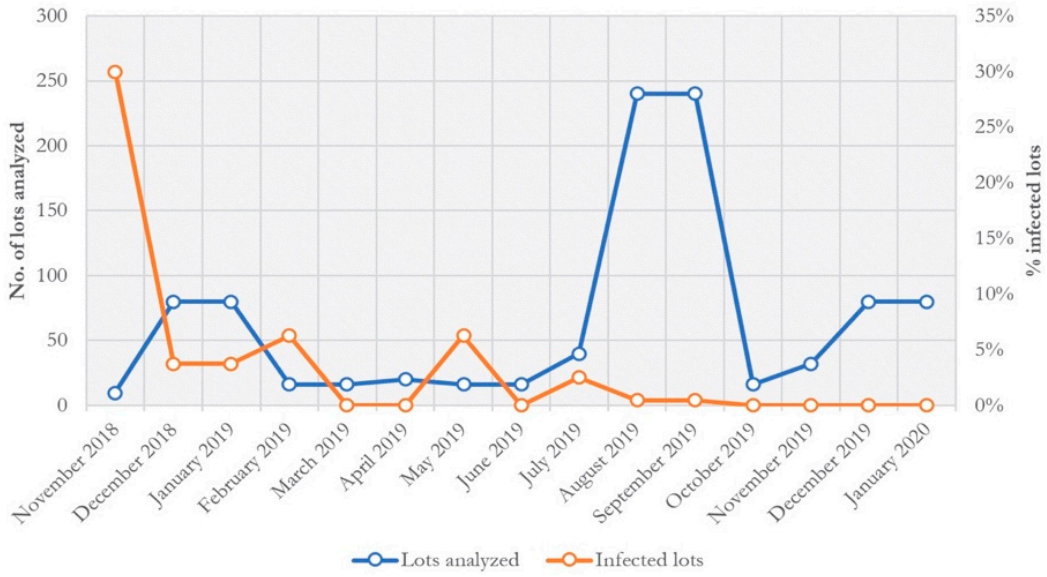

Figure 2. Number of lots of tomato plantlets analyzed per month, during the period November 2018–January 2020 and percentage of ToBRFV-infected lots.

3.1.3. Analysis in Production Greenhouses

A different situation was observed in tomato greenhouses; in this case, a higher percentage of the virus was detected. Analyzing the results in detail, it is easy to understand that the presence of ToBRFV is much higher than in nurseries (plantlets or seed lots) (Table 3). After the first month (October 2018), in which a single greenhouse was tested and resulted positive, that corresponding to the first outbreak of ToBRFV in Italy, the number of analyzed greenhouses increased to a total of 100 greenhouses in May-June 2019. In particular, during the period between November 2018 and June 2019, the percentage of infected greenhouses ranged from 58% (December 2018) to 39% (May 2019) (Table 3). After a temporary break from sampling in the period July-September 2019, due to the end of the cultivation and preparation for subsequent production cycle, a slightly lower percentage of infected greenhouses (out of a total of 100 greenhouses analyzed) was observed, ranging between 37% in October 2019 and 26% in January 2020 (Figure 3) with a decrease of 11%.

Table 3. Number of analyzed farms (A.F.) and number of detected infected farms (I.F.) per month in the different provinces investigated.

Sampling per Month	Province			
	Siracusa	Ragusa	Caltanissetta	Agrigento
	A.F./I.F.	A.F./I.F.	A.F./I.F.	A.F./I.F.
October 2018	1/1	0/0	0/0	0/0
November 2018	4/2	3/2	0/0	0/0
December 2018	6/3	6/4	0/0	0/0
January 2019	10/5	10/7	5/3	5/0
February 2019	15/7	20/11	6/3	6/0
March 2019	20/7	35/19	6/4	6/1
April 2019	21/8	55/21	6/5	6/3
May 2019	24/9	62/24	8/4	6/2
June 2019	24/9	62/28	8/2	6/2
July 2019	0/0	0/0	0/0	0/0
August 2019	0/0	0/0	0/0	0/0
September 2019	0/0	0/0	0/0	0/0
October 2019	24/8	62/28	8/1	6/0
November 2019	24/8	62/20	8/1	6/0
December 2019	24/6	62/20	8/1	6/0
January 2020	24/5	62/20	8/1	6/0

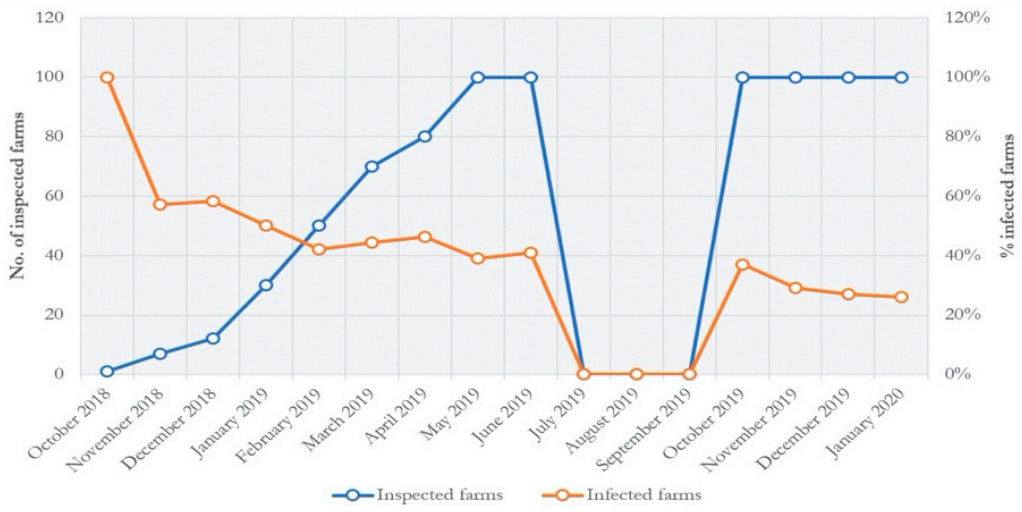

Figure 3. Number of tomato greenhouses inspected per month, during the period October 2018–January 2020 and percentage of ToBRFV-infected greenhouses.

3.2. Evaluation of Tomato Brown Rugose Fruit Virus Epidemiology in Experimental Conditions

An experimental greenhouse was used as the model, in order to evaluate the dynamic of ToBRFV in a monitored environment, on a total of 480 tomato plants (478 healthy plants + 2 inoculated plants), in presence of bumblebees over nine months (from September 2018 to May 2019). At the end of May 2019, as reported in Table 4, the IC-Real Time RT-PCR analysis showed that a total of 475 (473 positive plants + 2 artificially inoculated plants) out of 480 plants were ToBRFV-infected (starting from only two artificial infected tomato plants, placed inside the greenhouse). In detail, the eight sample collections carried out during the experiment, showed a particularly accelerated increase in the spread of the disease, in terms of infected plants. The highest disease increase was recorded between December 2018 and January 2019; in fact, there was an exponential increase in the number of infected plants (+109 and +158 infected plants respectively) with an increase in percentage of almost 490% and 215%, respectively). In the last three months of the test, the increase was more moderate, with a total of 89 plants tested positive for ToBRFV due to reaching saturation (Figure 4). As regards the increase of infection (%), it progressed from 1.45% in October 2018 to almost 100% at the end of May 2019, when the test ended. It is interesting to note that, the higher increase in the infection incidence occurred in only three months, between the end of November 2018 (5.83%) and the end of February 2019 (80%).

Table 4. Trend of Tomato brown rugose fruit virus infection during the 9 months of investigation.

Sampling per Month	No. of Infected Plants	Increase of Infected Plants	Disease Increase (%)	Percentage of Infection
September 2018	2 *	-	-	-
October 2018	7	+5	350	1.45
November 2018	28	+21	400	5.83
December 2018	137	+109	489.30	28.54
January 2019	295	+158	215.30	61.46
February 2019	386	+91	130.80	80.41
March 2019	433	+47	112.20	90.20
April 2019	458	+25	105.80	95.41
May 2019	475	+17	103.70	98.96

* artificial inoculated plants used to start the experiment.

Figure 4. Trend of ToBRFV diffusion (No. of infected tomato plants) during the nine months inside the greenhouse.

In order to understand how the virus spread inside the greenhouse, the obtained data showed that in the first three months the virus has spread in the plants adjacent to the two inoculated plants along the same row (Figure 5), almost certainly favored by direct plant-to-plant contact. Subsequently, as previously described, the increase in infected plants has been exponential, affecting all the rows of the greenhouse, with only five healthy plants at the end of the nine months of monitoring. In Figure 6

it is shown the spatiotemporal progression of controlled infections inside the greenhouse and the viral infection intensity of each plant, where it is possible to find the two infected plants that originated the induced infection (row No. 3 position 16 and row No. 9 position 10), from which the virus subsequently spread to other plants. This was mainly caused by transmission occurred by plant-to-plant contact between contaminated and adjacently planted uncontaminated tomato plants but, probably, also by the presence of bumblebees, which can transfer the ToBRFV viral particles in the abdomen.

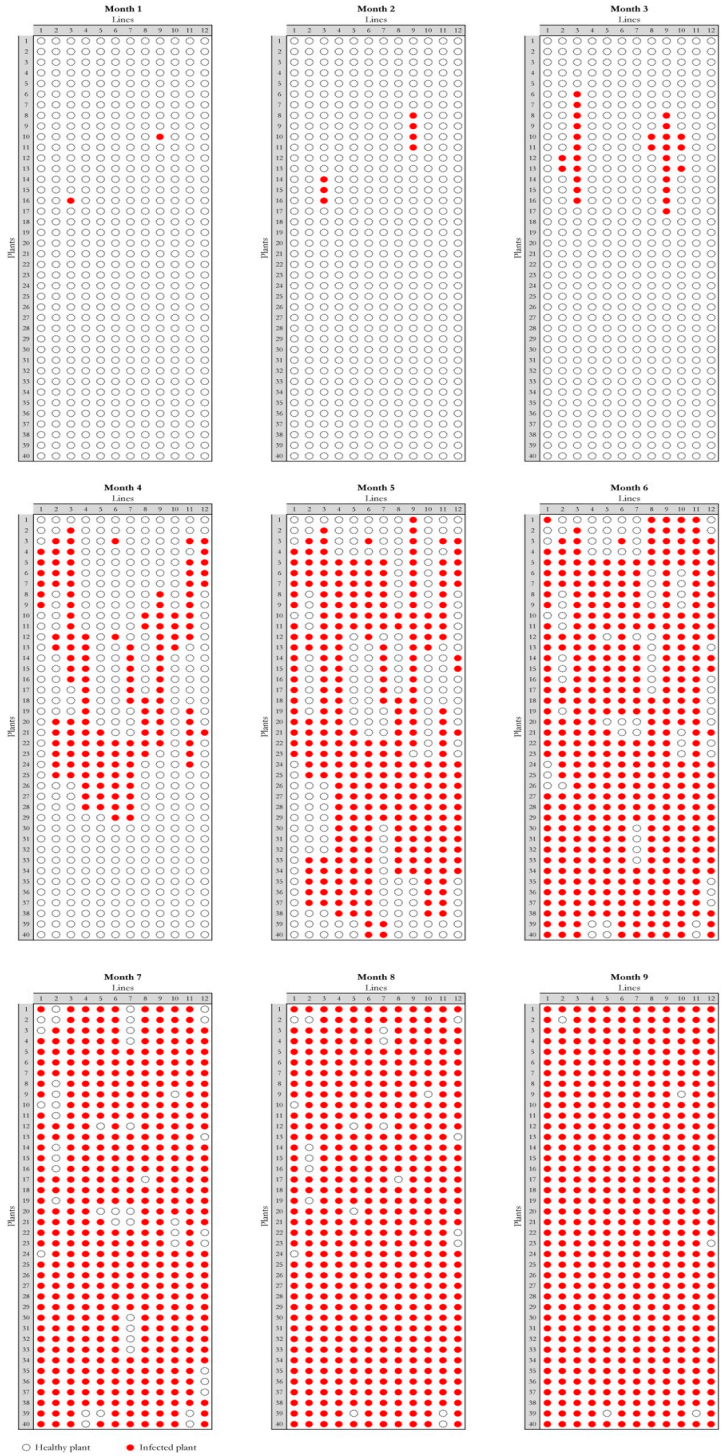

Figure 5. Visualization of ToBRFV spreading in a greenhouse during the nine-month monitoring, starting from two ToBRFV infected plants and two bumblebees hives. Red dots represent the infected plants; white dots represent healthy plants.

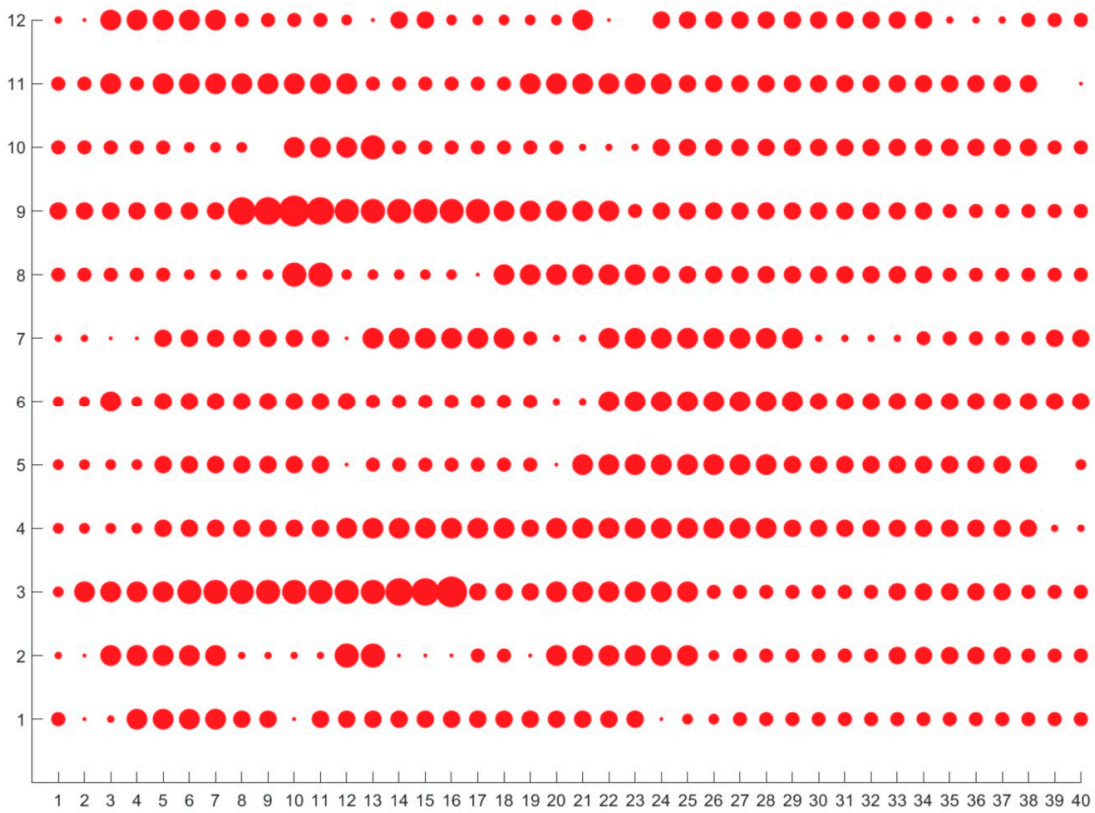

Figure 6. Spatiotemporal displacement of ToBRFV inside the greenhouse. The 12 × 40 grid shows the progression of controlled infections. Each round shows the viral infection intensity in the corresponding plant, located in the grid. No round means no infection. Conversely, the more extended the round, the longer the infection, with respect to the start of the induced infection. The most extended rounds represent the locations of the two infected plants that originated the induced infection (row No. 3 position 16 and row No. 9 position 10).

4. Discussion

Emerging infectious diseases caused by new viruses, such as *Tomato torrado virus* [26], *Pepino mosaic virus* [27], *Tomato leaf curl New Delhi virus* [28] and *Tomato brown rugose fruit virus* [13], or the re-emerging of old pathogens, such as *Cucumber mosaic virus*, that are capable of causing frequent spillover [29] and Tomato yellow leaf curl disease [30,31], have played a major role in the development of Sicilian horticulture, which has suffered huge losses in production and significant economic consequences, due to the negative impact caused by them.

For these reasons, it is crucial to immediately implement strict containment rules when a disease appears in a new area. It is important to operate in a context of plant disease surveillance and early diagnosis to deal with these threats, taking into account different parameters, such as genetic characteristics, environment, transmission efficiency of vectors involved [32] and agronomic practices. In this context, the development of different phytopathological diagnosis protocols for the early detection of plant viruses [24,33,34] and the study of different mechanisms involved in the viral diseases dispersion, represents one of the most important steps in order to contain a new epidemic.

The study of epidemiology is considered the basis to develop efficient disease management strategies [35]. As reported by [36], plant virus epidemiology focuses on the complex association between the virus and its host plant resulting in disease, and the factors influencing spread within the host plant population. Furthermore, epidemiology of a plant virus population is strictly associated with the ecological factors that define the environment in which plant virus interactions occur. For example, the level of human intervention in the ecosystems is considered a relevant ecological factor for both plant virus evolution and epidemiology [37,38].

Tobamoviruses epidemiology is related to their rapid dissemination, because viral molecules are considerably stable, characterized by high persistence in soil, drainage or irrigation water [39]. The virions can remain infectious for prolonged periods, even after cultivation and in adverse conditions [40,41]. The transmission process is related to wounding on leaves, during cultivation operations, or to the root system of transplanted seedlings, and to the systemic movement of virions through the phloem system [42]. Other possible mediators of spread-by-contact transmission include root-to-root contact, nutrient solutions, and contaminated seeds or transplants.

In this context, understanding ToBRFV epidemiology is essential before the disease can be controlled effectively. To date, regarding ToBRFV, there are no secondary hosts reported in the literature, although artificial transmission has been successfully demonstrated on *Petunia hybrida*, *Solanum nigrum* and different species of *Nicotiana* and *Chenopodium* [4]. Seed transmission and secondary spread such as manipulation and plant-to-plant contact represent the most critical aspect for the rapid spread of ToBRFV.

Results obtained in this work have highlighted several aspects related to ToBRFV dispersion in protected tomato crops, and the necessity to produce ToBRFV-free propagation material (seeds and plantlets). The phytosanitary emergency caused by ToBRFV began in Italy during late 2018 in tomato crops [13], causing significant economic losses in the main areas of tomato cultivation. In detail, as regards tomato seeds, ToBRFV-positive samples went from a percentage of infected lots of about 30% in the first months (end of year 2018) of investigation to 0% for the last eight months. Most likely, this result is due to the strong and appropriate measures, such as different seed disinfection procedures before sale, undertaken by the seed companies once the threat was identified. The same scenario occurred in nurseries, where a significant decrease of infected plantlets by ToBRFV was reported; in particular, despite the first month of sampling (November 2018), when a percentage of infected lots of 30% was recorded out of a total of 10 analyzed lots, the percentage definitely decreased in the following months with the increase of analyzed lots, with a minimum of 0,42% infected lots out of 240 lots. In the last four months of sampling (October 2019–January 2020) the percentage dropped to zero. The important decrease of the ToBRFV-infected lots percentage just described, is related to several factors, such as, seeds checked against viruses (virus tested) and implementation of efficient phytosanitary practices (e.g., use of gloves, disposable gowns, hand sanitizers). These practices made it possible to completely reduce the incidence of the disease, also confirmed by the fact that in the last four months of sampling, no positive lots were detected.

The situation appears less encouraging as regards greenhouse production. It has been shown that, although there has been a decrease in the percentage of ToBRFV dispersion, it still appears to be present in the Sicilian territory, and this can represent a serious threat of further ToBRFV spread in other Sicilian or Italian production areas. In detail, starting from the first outbreak that occurred during October 2018 in a greenhouse located in Ragusa province, a monitoring process has started, as far as possible, in all areas where the main tomato production greenhouses are located. A total of 100 farms were inspected at May 2019; in the period May–June 2019, the highest peak of the disease (39% and 41%, respectively) was reached. The subsequent break in July–September 2019, corresponding to the end of the cultivation and preparation for subsequent tomato production cycle, with more restrictive cultivation practices, rapid diagnosis, soil solarization and ToBRFV-free seeds/plantlets, allowed to drop the percentage of infected farms to 26% in January 2020. This percentage, despite being significantly reduced compared to the past months, which indicates the phytosanitary measures have been appropriate, is still an indication of the presence of ToBRFV, even if minimally, in the production process, from seed to greenhouses.

The evaluation of *Tomato brown rugose fruit virus* epidemiology in experimental conditions carried out in the present work, confirm what described previously; in fact, in a confined environment like a greenhouse, even if ToBRFV is present in very low percentages, it can spread very quickly, damaging the entire crop in a short time. In this case, only two ToBRFV-infected plants were sufficient to quickly spread the infection to almost all the plants in the greenhouse. In detail, in October 2018, one month

after the start of the experiment, only 1.45% of the plants were infected but, after a further 4 months, more than 80% of the plants were positive to ToBRFV, up to about 100% of the infected plants at the end of cultivation, confirming the rapid spread and high danger of ToBRFV. This experiment demonstrates that it is mandatory that the entire tomato production chain works in synergy, because the phytosanitary actions taken can be effective only if all the steps of the production chain are checked, and moreover, if the phytosanitary practices are applied during all the entire tomato production cycle. In addition, in the near future, plant breeders will play a fundamental role in order to obtain new tomato varieties with resistance genes against ToBRFV. The results and data collected confirmed that a good approach has been put in place by Sicilian phytosanitary services, in order to contrast the rapid spread of ToBRFV. It is appropriate to manage tomato crops in Sicily in the rightest way, especially in greenhouses, for effective mitigation of ToBRFV impact and dispersion on production.

5. Conclusions

In conclusion, in order to produce healthy, reasonably ToBRFV-free crops and reduce the disease spreading, it is crucial to identify and apply a combination of simple phytosanitary practices in protected tomato crops, such as use of certified propagation material, rapid diagnostic tools, followed by the removal of positive plants, and continuing monitoring of cultural practices and human activities.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/6/834/s1, Figure S1: Geographic location of greenhouses for in-field ToBRFV epidemiologic study, conducted in Agrigento, Caltanissetta, Ragusa and Siracusa provinces, during October 2018–January 2020. The red dot represents a single greenhouse; the square dot represents the first outbreak of ToBRFV.

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