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Detection of Infectious *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed

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Developed by ISHI-Veg

Detection of Infectious *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed

Crop: Tomato (*Solanum lycopersicum*), Pepper (*Capsicum annuum*)

Pathogen(s): *Tomato brown rugose fruit virus* (ToBRFV)

Version: 1.5 (November 2020)

PRINCIPLE

Detection of *Tomato brown rugose fruit virus* (ToBRFV) in tomato and pepper seed by a seed extract qPCR assay (SE-qPCR). If no virus is detected the seed lot is considered free from ToBRFV.

As the ToBRFV-specific PCR assay detects both infectious virions and non-infectious virus particles, a positive PCR is followed by a bioassay to confirm the absence of infectious ToBRFV. Leaves of indicator plants *Nicotiana tabacum* cv. Xanthi NN or *Nicotiana glutinosa* are inoculated with tomato or pepper seed extract. Infectious virions cause typical local lesions that demonstrate viability of the virus.

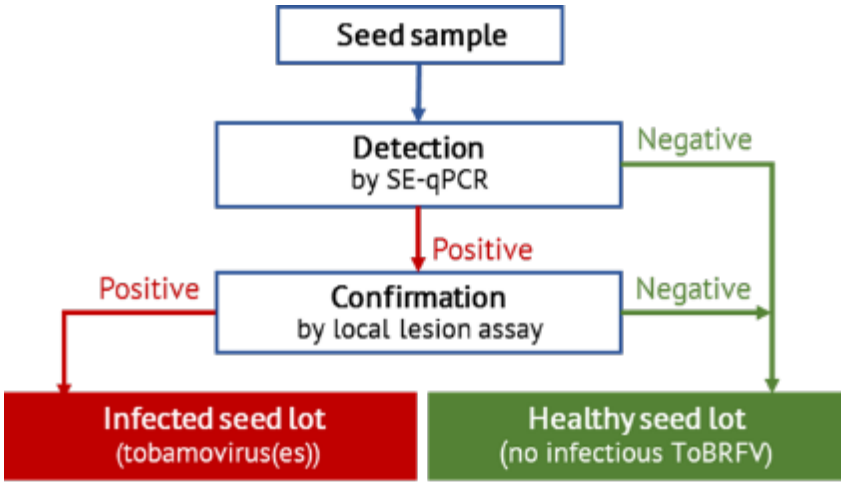

Figure 1. Method process flow

METHOD VALIDATION

The local lesion assay has been in use by seed companies for many years and is a seed industry standard. The method for detecting infectious *Tobacco mosaic virus* and *Tomato mosaic virus* in *Solanum lycopersicum* (tomato) seed by the local lesion assay (indexing) on *Nicotiana tabacum* plants is an ISTA Rule (7-028) since 2011 (www.seedtest.org). The US National Seed Health System (NSHS) (http://seedhealth.org) has approved the method as a Standard B.

In ISHI-Veg's test *Detection of Infectious Tobamoviruses in Tomato / Pepper Seed*, an ELISA pre-screen is used for the detection of tobamoviruses including ToBRFV, however antibodies usually react with several tobamoviruses and it is not possible to identify them specifically.

The SE-qPCR has been validated. However, the report is an interim one till it is complemented with a full comparative test. Contact the ISF Secretariat for all inquiries.

RESTRICTIONS ON USE

This method is suitable for testing untreated seed.

It is also suitable for testing seed that has been treated using physical or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for such antagonism and/or inhibition by analysis, sample spiking, or experimental comparisons.

It is not validated for seed treated with protective chemicals or biological substances. If treated seed is tested, the user is responsible for determining empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.

METHOD EXECUTION

When testing a seed lot using the SE-qPCR assay, it is strongly recommended that the best practices developed by ISHI-Veg for *PCR Assays in Seed Health Tests* (see http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/) be followed to ensure process standardization and that the results are valid.

Note 1: This protocol can also be used to identify ToBRFV after a positive ELISA in ISHI-Veg's tests *Detection of Infectious Tobamoviruses in Tomato / Pepper Seed* and using the same (ELISA) seed extract.

Note 2: In case of a positive result in the local lesion assay, the presence of infectious ToBRFV can be confirmed by a qPCR on the lesions.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 3,000 seeds with a maximum subsample size of 250 seeds

Note that extracts of four subsamples are combined for SE-PCR (§3.1) consequently for SE-PCR three datapoints are generated, each representing a subsample of 1,000 seeds.

Laboratories can also choose to perform directly the SE-PCR on three subsamples of 1,000 seeds ground in 40 and 80 ml of PBS for tomato and pepper, respectively (§ 2.2). From these extracts 100 µl is then taken from each subsample for RNA purification (§3.1) and further execution of the SE-PCR steps. If extraction of the virus from the seed was run directly on 3 subsamples of 1,000 seeds, a positive PCR result must be followed up with a bioassay on a new sample of 3,000 seeds in subsamples of 250 seeds, which is the maximum subsample size for the bioassay.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1	April 2019	
1.1	May 2019	In Table 4 nM corrected to µM.
1.2	May 2019	Note on primer name added in Table 2.
1.3	September 2019	Seed extraction buffer volume for pepper adjusted from 15-20 to 20 ml in § 2.2 of the section on Seed Extract qPCR.
1.4	March 2020	Text in *Principle* adjusted for more clarity, paragraphs on ELISA pre-screen and the validation report added to *Method Validation* and note 1 rewritten for more clarity in *Method Execution*.
1.5	November 2020	Option for testing 3 x 1,000 seeds with the SE-qPCR more explicitly described.

Protocol for detecting infectious ToBRFV in tomato and pepper seed

SEED EXTRACT qPCR

Material

Seed extraction buffer	RNA purification kit and equipment
TaqMan RT-qPCR mix, primers and equipment	1.5 ml RNase Free tube
Spike solution	RNAse free water
Controls	Centrifuge

Seed extraction buffer

Table 1. Phosphate Buffered Saline (PBS) - pH 7.2 – 7.4 per liter

Sodium chloride (NaCl)	8.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Add de-ionized water up to 1 liter, adjust pH and autoclave buffer at 121 °C, 15 psi for 15 minutes.	

Note: If a different seed extraction buffer is used, it must be verified in a comparison using uniform positive control material that it does not lead to a reduction in the number of lesions obtained.

Primers

Table 2. Primers, their sequences and source

Name	Sequence	Source
CaTa28 Fw	5' - GGT GGT GTC AGT GTC TGT TT - 3'	Enza Zaden B.V. Netherlands
CaTa28 Pr	5' - 6FAM - AGA GAA TGG AGA GAG CGG ACG AGG - BHQ1 - 3'	
CaTa28 Rv	5' - GCG TCC TTG GTA GTG ATG TT - 3'	
CSP1325[1] Fw	5' - CAT TTG AAA GTG CAT CCG GTT T - 3'	CSP Labs USA
CSP1325 Pr	5' - VIC - ATG GTC CTC TGC ACC TGC ATC TTG AGA - BHQ1 - 3'	
CSP1325 Rv	5' - GTA CCA CGT GTG TTT GCA GAC A - 3'	
BaCV-F	5' - CGA TGG GAA TTC ACT TTC GT - 3'	Naktuinbouw Netherlands
BaCV-R	5' - AAT CCA CAT CGC ACA CAA GA - 3'	
BaCV-P	5' - TxR - CAA TCC TCA CAT GAT GAG ATG CCG - BHQ2 - 3'	

[1] The name CSPtbrfv101 is also used for the primer sequence

Spike solution

The spike solution is prepared by taking a leaf from a plant infected by *Bacopa chlorosis virus* (BaCV) and making an extract of it in PBS. The extract is diluted to obtain a suitable concentration and aliquots are stored at -80 °C.

Note: Other organisms such as *Dahlia latent viroid* (DLVd) and *Squash mosaic virus* (SqMV) may also be used and should be shown to be compatible with the ToBRFV primers in a multiplex PCR.

Controls

Table 3. Controls and their purpose

Negative Process Control (NPC)	Tomato or pepper seed free of ToBRFV
Positive Amplification Control (PAC)	ToBRFV RNA aiming for a Cq (Cycle quantification) value between 28 and 32
	ToBRFV oligo DNA (oligonucleotide (single-stranded DNA) for all ToBRFV target sequences) aiming for a Cq value between 28 and 32
	ToBRFV cDNA aiming for a Cq value between 28 and 32
Positive Extraction Control (PEC)	Spike solution added to the sample aiming for a Cq value between 28 and 32 The PEC serves as an Internal Amplification Control (IAC)
Inhibition Control (IC)	Dilution of the PEC in a non-infected seed extract aiming for a Cq value between 28 and 32 Note: a non-infected seed extract is preferred over a seed extraction buffer, as a strongly diluted infected leaf extract may lead to a relatively high loss of RNA in the purification process (i.e. no carrier RNA present)
Negative Template Control (NTC)	Contains all PCR reagents but no target or spike DNA, RNA or PEC nucleic acids

1. General Requirements

– Seed extracts and controls must be prepared at the same time, under the same laboratory conditions and stored under the same conditions.

– Seed extracts and all controls must be stored at 4°C until the assay begins. It is strongly recommended to perform the local lesion assay within 24 hours following seed extraction.

– The final results of the local lesion assay must be validated through a comparison of the results given by both controls.

2. Extraction of the virus from the seed

2.1. Add PEC to the extraction buffer.

2.2. Grind seeds of each subsample, 250 seeds, in 10 ml of the seed extraction buffer PBS for **tomato** seed, *or* in 20 ml of PBS buffer for **pepper** seed.

Alternatively, grind 3 subsamples of 1,000 seeds directly in 40 and 80 ml of PBS for tomato and pepper, respectively. Note: In case of a positive PCR result when using this option, a fresh seed extract from a new sample of 3,000 seeds in subsamples of 250 seeds is required for the local lesion assay.

2.3. Store seed extracts at 4°C.

3. RNA extraction

3.1. In the case of 250 seed subsamples, combine 25 µl of 4 seed extract subsamples into a 100 µl combined sample. Use all three combined samples for further analysis.

In the case of 1,000 seed subsamples, use 100 µl from each subsample for further analysis.

3.2. Add RNA extraction buffer within 4 hours after grinding.

3.3. Use a commercial RNA extraction kit for RNA extraction. Process the three samples according to the supplier's instructions.

3.4. Use the eluted RNA for RT-qPCR using a commercial TaqMan RT-qPCR kit.

4. Preparation of the TaqMan RT-qPCR mix

4.1. Prepare the RT-qPCR mix with the components described in Table 4.

Note: Good results have been obtained by ISHI-Veg member laboratories with the TaqMan RT-qPCR mix: UltraPlex™ 1-Step ToughMix (QuantaBio). Any commercial PCR mix that is suitable for multiplex TaqMan RT-qPCR applications can be used as long as suitability and performance have been demonstrated in an in-lab validation study. The RT-qPCR parameters (Tables 4 and 5) may need to be adjusted when using a different commercial TaqMan RT-qPCR kit.

Table 4: RT-qPCR ToBRFV mix

ToBRFV/BaCV (PEC)	Target	Final concentration (µM)	Volume (µl)
UltraPlex 1-Step ToughMix (4x)			6.25
CaTa28 Fw (forward)	ToBRFV	0.3	
CaTa28 Pr (probe)		0.2	
CaTa28 Rv (reverse)		0.3	
CSP1325 Fw (forward)	ToBRFV	0.3	
CSP1325 Pr (probe)		0.2	
CSP1325 Rv (reverse)		0.3	
BaCV-F (forward)	BaCV	0.3	
BaCV-P (probe)		0.2	
BaCV-R (reverse)		0.3	
RNAse free water		adjust accordingly	
Subtotal PCR-mix			20.00
RNA			5.00
Total			25.00

4.2. Take 5 µl of the RNA sample as input for the PCR.

4.3. In each run, include an NTC and at least one PAC that give a Cq value between 28 and 32.

4.4. Run the TaqMan RT-qPCR according to the following program.

Table 5: RT-qPCR program

RT reaction	10 min 50°C
Denaturation	3 min 95°C
Cycling	10 sec 95°C 60 sec 60°C 40x

5. Evaluation of the test results

5.1. It is the responsibility of the user to determine a Cq threshold, positioned just above background fluorescence, for each of the fluorophores. A fixed threshold should be checked and modified if necessary to remain just above background fluorescence in each test.

5.2. Check for exponential amplification, indicated by an S-shaped amplification curve, for ToBRFV positive samples. Compare with the PAC.

5.3. A negative test result for the SE-qPCR assay means that the sample does not contain ToBRFV.

6. Validity of test results

6.1. Results are only valid if the positive amplification control (PAC) gives a clear signal < Cq 32.

6.2. Results are only valid if the Cq in the negative control samples (IC and NTC) is above a cut off value defined by the laboratory during validation.

6.3. The Cq of the PEC must be within a 3-cycle range of the Cq of the IC in all samples. If it isn't, loss of ToBRFV or inhibition during amplification may have occurred. The Cq of the IC itself should be between 28 and 32.

Note: If DNA is used as a PAC for ToBRFV, the BaCV Cq is also a check of proper reverse transcription. A BaCV PAC is recommended to distinguish between RT-PCR failure (mix/program) and inhibition.

LOCAL LESION ASSAY

Material

Assay plants (*Nicotiana glutinosa* or *Nicotiana tabacum* cv. Xanthi NN)	Carborundum (320 mesh grit powder, Fisher Scientific or equivalent)
Controls	Seed extraction buffer (in Table 1)
Gloves or alkaline soap and water	Protective mask

Controls

Table 6: Description of controls

Positive Process Control (PPC)	A well-characterized positive seed lot
	Pea seed flour and powdered ToBRFV infected leaves from a systemic host ground together in a seed extraction buffer (5 mL per 0.5 gr). The mix should be ground into a fine flour for efficient virus extraction
	Liquid extract of ToBRFV infected leaves of Solanaceous hosts sufficiently diluted in PBS seed extraction buffer
Negative Process Control (NPC)	Seed extraction buffer
	Tomato or pepper seed free of ToBRFV

1. Controls

1.1. Choose one of the PPCs and NPCs listed.

1.2. Depending on the amount of infectious virus in the PPC, adjust the quantity used to induce development of countable local lesions.

2. Inoculation and incubation of *Nicotiana* assay plants

2.1. Use the 12 original seed extract subsamples from the SE-qPCR assay.

If storage conditions for the seed extract were not met or the SE-PCR was performed on subsamples of 1,000 seeds, take a new seed sample and grind 12 subsamples of 250 seeds as in § 2.2 of the section on Seed Extract qPCR.

2.2. Raise assay plants (*Nicotiana glutinosa* or *Nicotiana tabacum* cv. Xanthi NN) under sufficient light intensity at a temperature of 20 – 25°C.

- Choose plants with 4-5 true leaves (6-7 weeks after sowing) with high turgor. Do not use flowering or old plants (Takahashi, 1975); see Figure 2.
- The primary leaf (oldest true leaf) should not be used as it is different in shape/texture/thickness and therefore less susceptible.
- Leaves of tobacco plants affected by downy mildew are significantly less sensitive. Inoculated plant must have no visible symptoms and be free of disease (Koenraadt H. personal communication).

2.3. Dust the leaves with the appropriate quantity of carborundum (320 mesh grit powder, Fisher Scientific or equivalent) so that there is a very fine layer on the leaf surface. Wear a protective mask.

2.4. The entire surface of two (nearly) fully expanded consecutive leaves of two plants should be inoculated with each seed extract, a total of 4 leaves for each subsample.

2.5. Place a drop of inoculum (100-200 µl) onto the leaf. Smear the drop with gloved fingers by applying constant, slight pressure but avoid damaging the leaf.

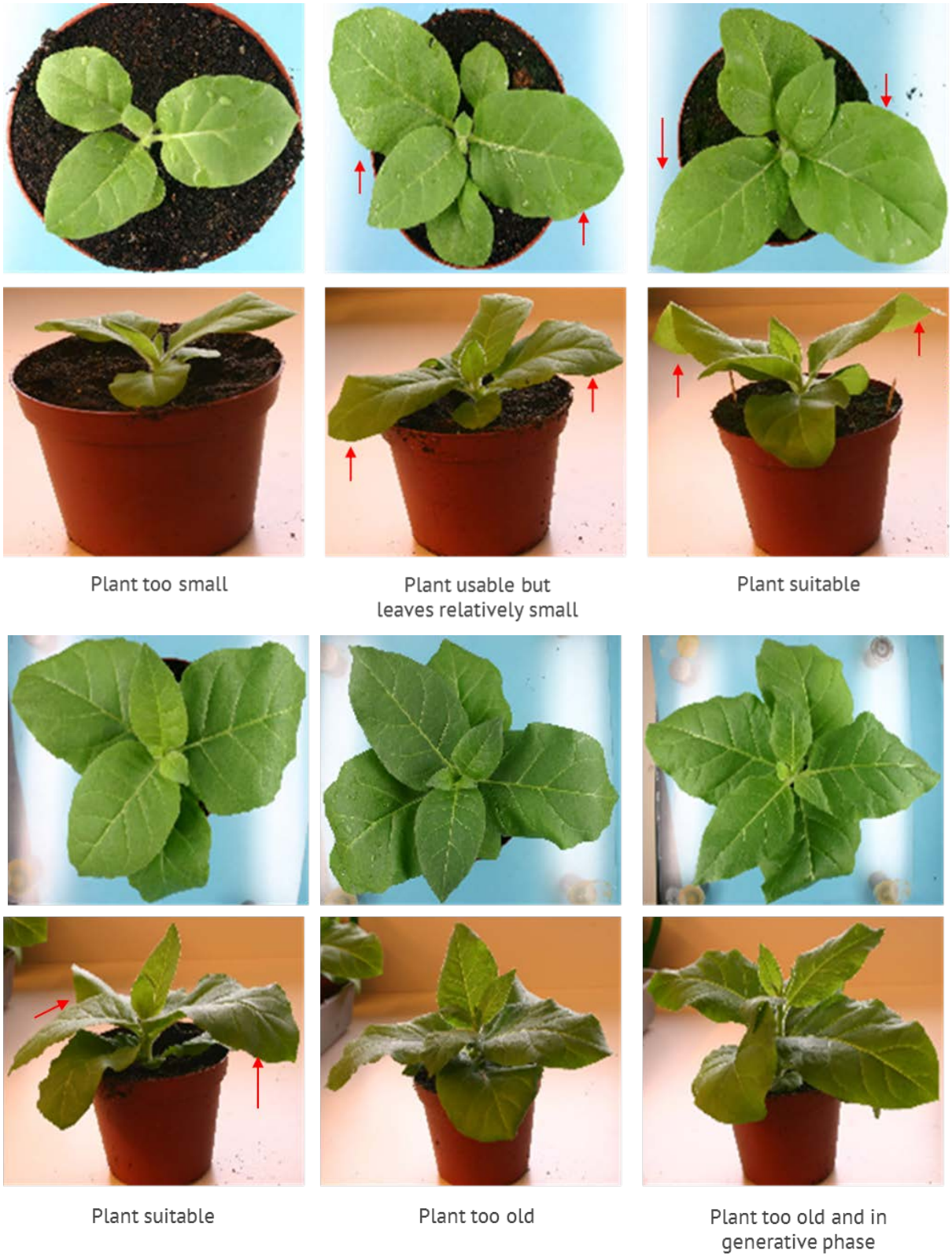

Figure 2. Overview of *Nicotiana tabacum* cv. Xanthi NN plants in different growth stages (two pictures taken per plant; pot diameter 14 cm)

Note: Work with gloves and change them between samples or clean hands thoroughly between samples by using alkaline soap or equivalent and then rinse with water to remove residues.

2.6. Rinse the plants with tap water a few minutes after inoculation.

2.7. Inoculate plants with the PPC and NPC in a similar manner to the ones used for the seed extracts.

2.8. Incubate the plants for 5-7 days under controlled conditions at 20 – 25 °C and provide at least 12 hours of light.

Note: The hypersensitive reaction of tobacco plants, the development of local lesions, may not be expressed at temperatures higher than 28°C (Samuel, 1931; Takahashi, 1975; Weststeijn, 1981 and Kiraly et al., 2008) and may vary for different tobamoviruses.

3. Evaluation of inoculated plants

3.1. Evaluate the inoculated leaves for local lesions (see Figure 3).

3.2. If there are many local lesions, a comparison with the PPC and NPC will readily reveal if most, or all, the lesions observed are authentic virus lesions or might be caused by, for instance, rubbing (rubbing damage).

3.3. Optional: If the number of lesions is low, confirm they are a true virus infection and not an artifact (leaf damage during mechanical inoculation, use of pesticides, etc.) by cutting out the suspect lesion, crushing it in a small amount of the seed extraction buffer and inoculating two leaves of two assay plants again. Lesions caused by virus infection contain sufficient amounts of infectious virus to produce multiple lesions in this confirmation test (Hadas, 2000).

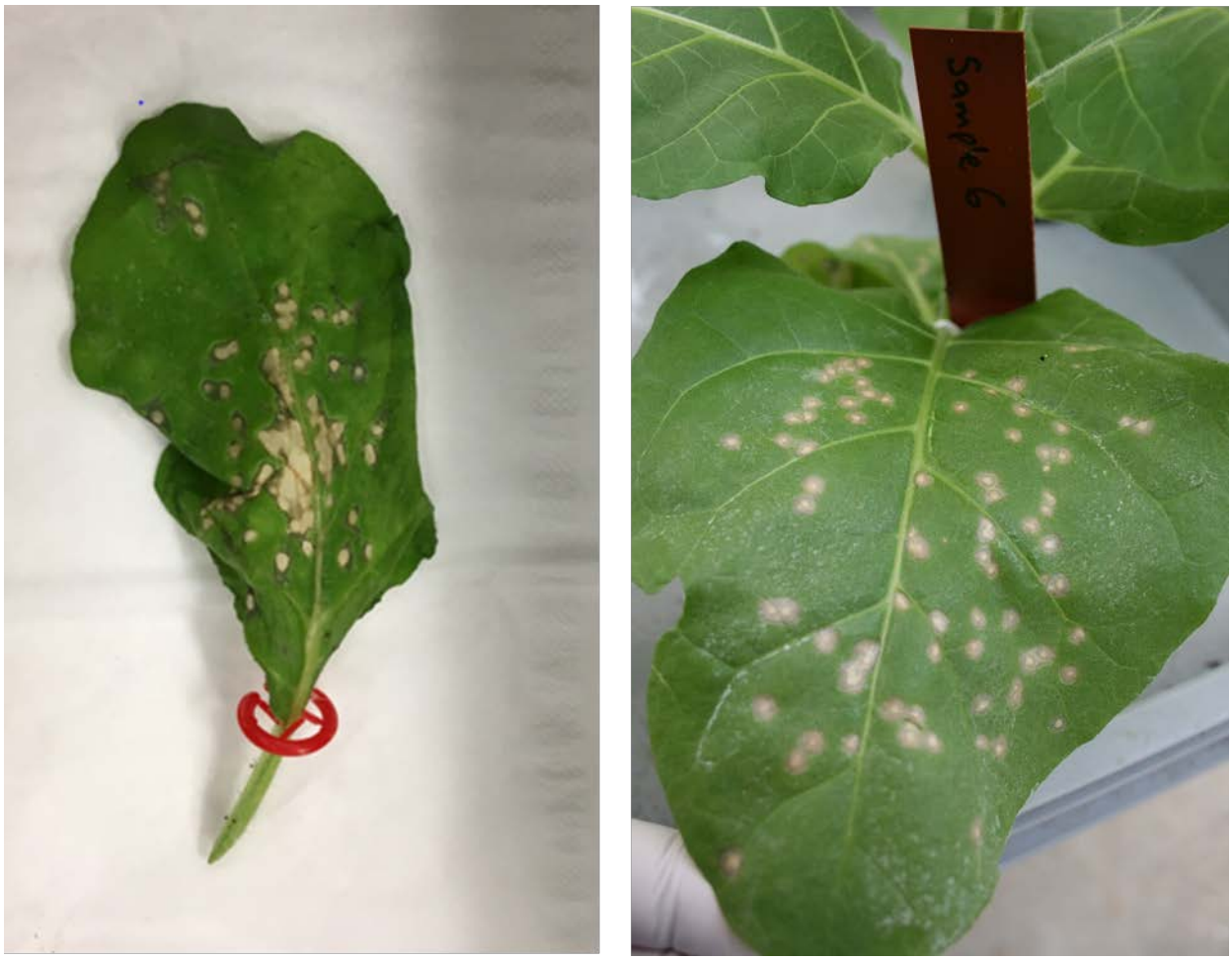

Figure 3. Lesions on a tobacco leaf inoculated with ToBRFV-infected tomato leaf (left) and tobamovirus-infected seed extract (right)

REFERENCES

Hadas, R. (2000). A report of comparative test for Tobamoviruses in pepper seeds. ISHI-Veg Research Report 1-2000-pepper-tobamo. Nyon, Switzerland: International Seed Federation.

Kiraly, L., Hafez, Y. M., Fodor, J. and Kiraly, Z. (2008). Suppression of tobacco mosaic virus-induced hypersensitive-type necrotization in tobacco at high temperature is associated with down regulation of NADPH oxidase and superoxide and stimulation of dehydroascorbate reductase. *Journal of General Virology* 89, 799-808.

Li, R., Gao, S., Fei, Z. and Ling, K.S. (2013). Complete genome sequence of a new tobamovirus naturally infecting tomatoes in Mexico. Genome announcements, 1(5): e00794-13.

Luria, N., Smith, E., Reingold, V., Bekelman, I., Lapidot, M., Levin, I., Elad, N., Tam, Y., Sela, N., Abu-Ras, A. and Ezra, N. (2017). A new Israeli Tobamovirus isolate infects tomato plants harboring Tm-2^2 resistance genes. *PloS one*, 12 (1), p.e0170429.

Salem, N., Mansour, A., Ciuffo, M., Falk, B.W. and Turina, M. (2016). A new tobamovirus infecting tomato crops in Jordan. *Archives of virology*, 161 (2), pp. 503-506.

Samuel. G. (1931). Some experiments on inoculating methods with plant viruses and on local lesions. *Annals of Applied Biology* 18, 494-507.

Sui, X., Zheng, Y., Li, R., Padmanabhan, C., Tian, T., Groth-Helms, D., Keinath, A.P., Fei, Z., Wu, Z. and Ling, K.S. (2017). Molecular and Biological Characterization of Tomato mottle mosaic virus and Development of RT-PCR Detection. *Plant disease*, 101 (5), pp. 704-711.

Takahashi, T. (1975). Studies on viral pathogenesis in plant hosts VIII. Systemic virus invasion and localization of infection on "*Samsun NN*" tobacco plants resulting from tobacco virus infection. *Phytopathology*, Z. 84, 75-87.

Weststeijn, E. A. (1981). Lesion growth and virus localization in leaves of *Nicotiana tabacum* cv. Xanthi nc after inoculation with tobacco mosaic virus and incubation alternately at 22°C and 32°C. *Physiological Plant Pathology* 18, 357-368.