# ISHI-veg develops new, specific **detection** method





**ISHI-veg's test for *Tobamoviruses* in tomato and capsicum is in use by the seed industry for many years and it detects all *Tobamoviruses*, including ToBRFV. However, to facilitate trade a new test that provides an immediate conclusion on the identity of ToBRFV, if present in the seed lot, has now been developed.**

**Tomato and capsicum** (fresh and chilli peppers) are grown worldwide and are among the world's most consumed vegetables. According to FAO global production of tomatoes in 2017 was 182 million tons and another 36 million tons of capsicum. Asia has the highest production share of tomatoes by region at 61.1%, followed by Europe (13.5%) and the Americas (13.4%)[1]. It is estimated that the market value of tomato fruits is roughly US$ 510 billion.

The *Tobamovirus* genus comprises multiple economically important and contagious pathogens that infect solanaceous crops. They are considered to be the most stable and infectious viruses known, and are readily transmitted mechanically by workers, tools and equipment during plant handling. *Tobamoviruses* can also be spread via fruits and insects. They survive in soil, water and infested debris from previous crops. They can be seed borne, however, infection and spread most likely occurs mechanically.

To protect their investment in fresh produce production, producers and plant raisers have access to a toolbox of mitigation measures, such as clean seed, resistant varieties and hygiene measures during crop growth. The use of resistant varieties has been the most effective control strategy in last decades for growers. Non-resistant varieties when accompanied with strict hygiene management have also allowed growers to produce healthy solanaceous crops.

The seed industry plays an important role in providing resistant varieties and healthy seed for the start of a clean and profitable fresh produce production cycle. As incorporation of disease resistance genes in new vegetable varieties is a long multiyear process, the industry uses more direct mitigation measures at every stage of seed production until shipping to end user, to mitigate the effects of seed borne diseases. Measures include application of hygiene protocols, field and crop inspection, seed health testing and (preventive) seed sanitation treatments.

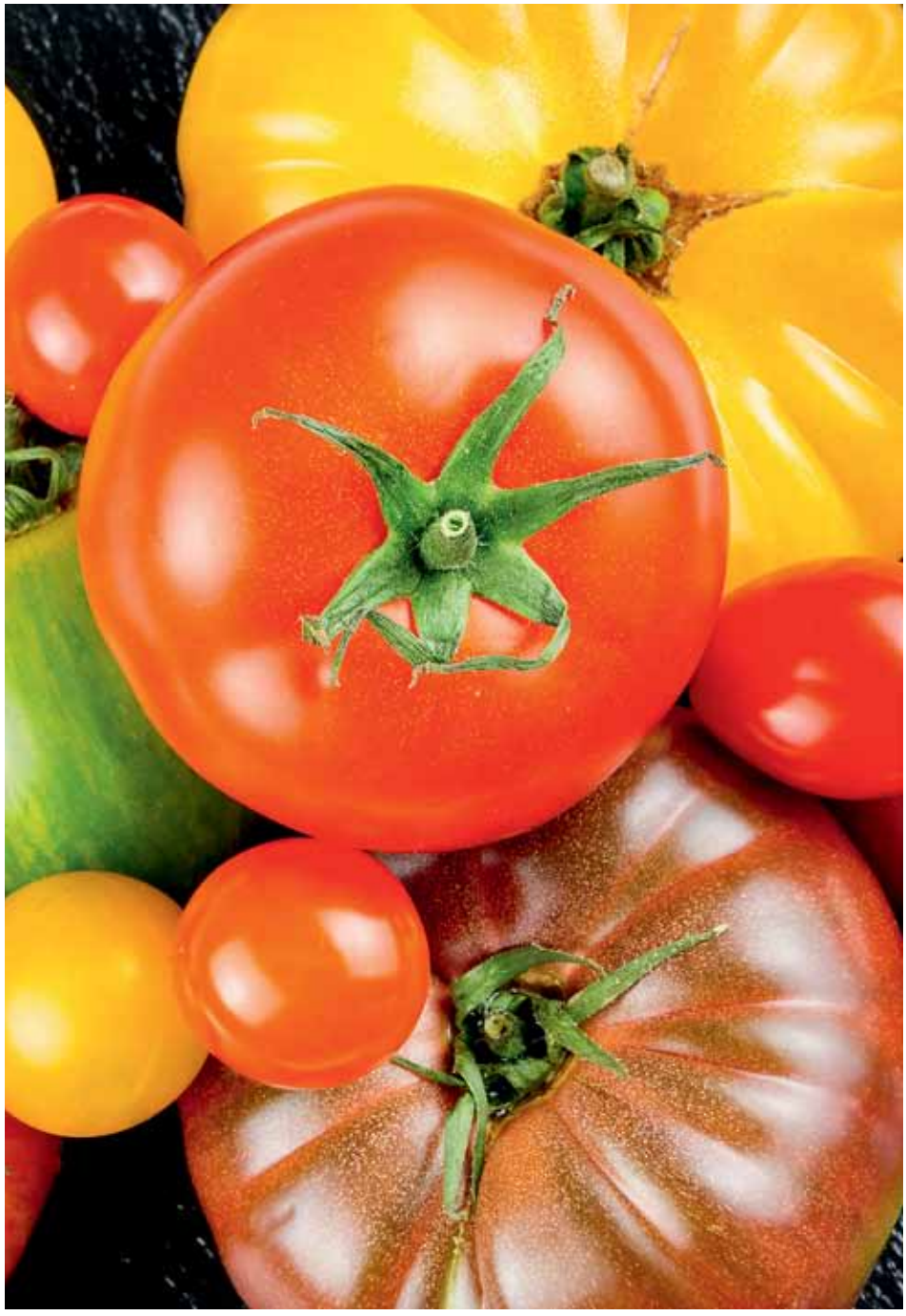
## A new virus

*Tomato brown rugose fruit virus* (ToBRFV) is a new *Tobamovirus* isolated from tomato plants grown in greenhouses in Jordan in 2015 [2]. An outbreak of a new disease infecting resistant tomato cultivars grown in net houses observed in 2014 in Southern Israel was caused by an Israeli isolate of ToBRFV that had a high genomic sequence identity to the Jordan isolate. More recently, ToBRFV was detected in tomato plants in production fields in Mexico, Germany, USA and Italy according to EPPO[3] but wider spread is likely.

*Tobamoviruses* infecting tomato crops are of great concern in general, but ToBRFV outbreaks are particularly worrisome because of its ability to overcome resistance rendered by the Tm-2² gene. Disease symptoms on tomato include chlorosis, mosaic and mottling accompanied occasionally by narrowing of leaves and yellow spotted rugose fruit,

**Table 1a.** Sequences of ToBRFV-specific and the Positive Extraction Control (PEC) primers

| Primer target | Primer Name | Sequence |
|---|---|---|
| ToBRFV | CaTa28-Fw | 5' – GGT GGT GTC AGT GTC TGT TT – 3' |
| ToBRFV | CaTa28-Probe | 5' – 6FAM – AGA GAA TGG AGA GAG CGG ACG AGG – BHQ1 – 3' |
| ToBRFV | CaTa28-Rv | 5' – GCG TCC TTG GTA GTG ATG TT – 3' |
| ToBRFV | CSP1325-Fw | 5' – CAT TTG AAA GTG CAT CCG GTT T – 3' |
| ToBRFV | CSP1325-Probe | 5' – VIC – ATG GTC CTC TGC ACC TGC ATC TTG AGA – BHQ1 – 3' |
| ToBRFV | CSP1325-Rv | 5' – GTA CCA CGT GTG TTT GCA GAC A – 3' |
| PEC | BaCV-Fw (PEC) | 5' – CGA TGG GAA TTC ACT TTC GT – 3' |
| PEC | BaCV-Rv (PEC) | 5' – AAT CCA CAT CGC ACA CAA GA – 3' |
| PEC | BaCV-Probe (PEC) | 5' – TxR – CAA TCC TCA CAT GAT GAG ATG CCG – BHQ2 – 3' |



**Call for cooperation**

**ISHI-Veg invites researchers seeking to develop ToBRFV-specific detection methods to use its primer sets and harmonise methods used for phytosanitary certification.**



**Table 1b.** Thermal cycler run conditions for the amplification of RNA-fragments of ToBRFV

| | |
|---|---|
| **RT reaction** | |
| 10 min 50°C | |
| **Denaturation** | |
| 180 sec 95°C | |
| **Cycling** | |
| 10 sec 95°C<br>60 sec 60°C 40x | |

making fruit unmarketable. Fruit may also mature irregularly. On susceptible capsicum, EPPO describes symptoms including foliar deformation, yellowing and mosaic while fruits are deformed with yellow or-brown areas, or green stripes.

Once the virus is introduced in an area, infected plants should be eliminated and strict hygiene protocols implemented. Inoculation experiments show that besides its main hosts, tomato and non-resistant capsicum, *Nicotiana benthamiana*, *N. glutinosa*, *N. sylvestris* and some *N. tabacum* (tobacco) were susceptible and developed initially necrotic lesions and later on mosaic symptoms. Weeds such as *Chenopodium murale* and *Solanum nigrum* may act as reservoirs for ToBRFV. Eggplant and potato did not show symptoms after inoculation of the virus and ToBRFV was not detected when the plants were subsequently tested by ELISA[4].

## Seed health test for Tobamoviruses

An outbreak of disease caused by PMMoV in Spain in 1992 in capsicum and the need to resolve the issue of diverging seed health test results based on different types of assays used by different labs led to establishment of the International Seed Health Initiative for Vegetable Crops (ISHI-veg). It is an industry led platform for development of seed health test methods [5].

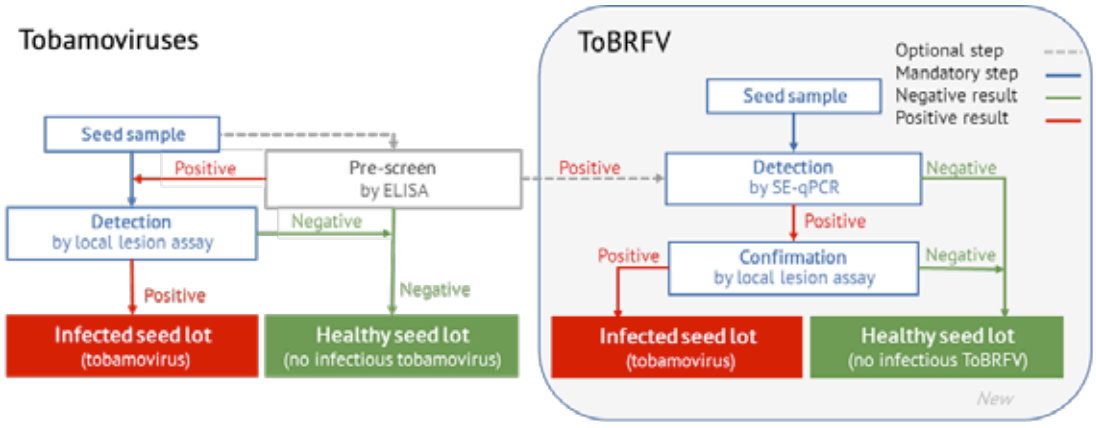
The current ISHI-veg method for detecting *Tobamoviruses* in tomato seed, an industry standard, is a local lesion assay that provides conclusive evidence of presence of viable and infectious *Tobamoviruses*, in other words a 'direct' test [6]. In the assay, leaves of indicator plant species *Nicotiana tabacum* cv. Xanthi NN or *Nicotiana glutinosa* are inoculated with an extract from seed of solanaceous crops. If the seed extract contains infectious virus they provoke development of small, necrotic local lesions on tobacco leaves. These clear necrotic lesions are typical for *Tobamoviruses*. For higher throughput, an ELISA can be used to pre-screen seed lots; a negative test result indicates that the tested seed lot is free from *Tobamoviruses*. An ELISA detects proteins that are specific to the target pathogen but does not demonstrate the presence of infectious virus. An ELISA, for instance, may also give a positive result when disinfected seed is tested. Inactivated virus fragments may still be present on or in seed. ELISA is, therefore, considered an 'indirect' test and is used as pre-screen; a positive ELISA result is confirmed by a direct test – the local lesion assay – to confirm viability and pathogenicity of *Tobamoviruses* (see ISF's Viewpoint on Indirect Seed Health Tests[7]).

## Detection of ToBRFV

ISHI-veg's local lesion assay for *Tobamoviruses* in

Seed health test flow for detection of Tobamoviruses and ToBRFV

**Seed health test flow for detection of tobamoviruses and ToBRFV**






tomato and capsicum is in use by the seed industry for many years, and found to be fit for purpose as it detects all *Tobamoviruses*, including ToBRFV. It does not, however, provide information about the specific *Tobamovirus(es)* that caused local lesions on the leaves of indicator plants. This is no problem for seed trade as a negative test, i.e. no lesions on leaves of the indicator plants, indicates no infectious virus present in the seed lot and that it can be traded safely.

Several countries, such as Mexico, Turkey, South Korea and Australia, have put in place import restrictions on tomato, capsicum and eggplant seed to contain or prevent the spread of ToBRFV through infected seeds. These import requirements vary from country to country with some accepting results obtained with the local lesion assay with ELISA as a pre-screen, but others require ToBRFV-specific PCR tests.

To facilitate trade, a ToBRFV-specific PCR has now been added to ISHI-Veg's current method. It can be used to identify ToBRFV after a positive ELISA or to detect ToBRFV by running the PCR test directly on a seed extract. For positive results from the ELISA and TaqMan tests, the local lesion assay should be run to confirm viability and pathogenicity of tobamovirus in the seed lot. The final conclusion on the status of the seed lot is based on the result of the local lesion assay (see the flow chart).

The advantage of the TaqMan PCR is that it provides an immediate conclusion on the identity of the virus present. In addition the test is a 'one-tube assay' which reduces not only labour costs but also the risk of cross contamination. Cross contamination is of serious concern in molecular tests when run using a multi-tube system; PCR tests are very sensitive and are able to react positively to miniscule aerosol particles containing ToBRFV present in the lab. Such false positive results may lead to rejected seed lots even though no infectious virus is present.

TaqMan PCR also allows multiplex assays to be developed for simultaneously detecting viroids and other viruses, like specific *Tobamoviruses* and Pepino Mosaic Virus. ISHI-veg is already engaged in such a project.

**Table 2.** Specificity of ToBRFV TaqMan PCR in combination with different PEC tested on several plant species (*source: ISHI-Veg members*)

| Virus tested | Specific-PCR | | PEC-PCR | |
|---|---|---|---|---|
| | CSP1325 | CaTa28 | BacV / DLVd / SqMV with *Tobamovirus* | BacV / DLVd / SqMV with PEC spike |
| ToBRFV | Detected | Detected | No cross reaction | PEC detected |
| ToMMV | Not detected | Not detected | No cross reaction | PEC detected |
| TMV | Not detected | Not detected | No cross reaction | PEC detected |
| BePMV | Not detected | Not detected | No cross reaction | PEC detected |
| TMGMV | Not detected | Not detected | No cross reaction | PEC detected |
| ToMV | Not detected | Not detected | No cross reaction | PEC detected |
| PMMoV | Not detected | Not detected | No cross reaction | PEC detected |
| PaMMV | Not detected | Not detected | No cross reaction | PEC detected |
| TSAMV | Not detected | Not detected | No cross reaction | PEC detected |
| CGMMV | Not detected | Not detected | No cross reaction | PEC detected |
| TSWV | Not detected | Not detected | No cross reaction | PEC detected |
| PepMV | Not detected | Not detected | No cross reaction | PEC detected |
| Negative Control tomato | Not detected | Not detected | Not applicable | PEC detected |
| Neg. Control capsicum | Not detected | Not detected | Not applicable | PEC detected |
| Neg. Control N. glutinosa | Not detected | Not detected | Not applicable | PEC detected |
| Neg. Control N. bentamiana | Not detected | Not detected | Not applicable | PEC detected |

## ToBRFV TaqMan PCR test

Primers are an important feature of PCR assays for identification and detection, as they are designed on specific genetic targets. ToBRFV-specific primer sequences developed by ISHI-veg are presented in Table 1. Within *Tobamoviruses* there is genetic variability [8] that may cause false negative results.

Photo 2a and 2b: ToBRFV infection of tomato: Mild mosaic on leaves and uneven ripening and necrotic lesions on fruit (Courtesy of David Levy, Hazera seed, Ltd)



Before ToBRFV-specific primers can be used in routine testing, it is essential to verify that they give reliable and consistent results when detecting ToBRFV. They should give a positive reaction with the target pathogen but not with, for example, closely related non-pathogenic organisms, in other words the test should not give false positive results. False negative results are even more worrisome as infected or contaminated seed may potentially cause a disease outbreak. To minimize the risk of false negative results during testing, pathogen isolates collected from different geographical regions, time periods and hosts are tested to verify if primers detect the complete range of known target isolates. Forty-five different virus strains tested showed no cross reactivity and ToBRFV strains were always detected. The specificity of the ToBRFV primers was thus demonstrated (see Table 2)


Photo 3a and 3b: Lesions on a tobacco leaf inoculated with ToBRFV-infected tomato leaf (left) and Tobamovirus-infected seed extract (right) (Courtesy of Gerbert Hiddink, Enza Zaden)

## Validation

As leaf and seed components are known to interfere with PCR reactions or degrade target material, the assay was tested on tomato, capsicum and tobacco leaves and on seeds of tomato and capsicum. Leaves of ToBRFV-infected tomato and tobacco plants tested positive and non-infected leaf material gave a negative result. For capsicum no infected leaf was available but in tests on non-infected leaves a negative result was obtained.

To test suitability of the PCR test on seeds, tests were carried out in multiple labs. Tomato and capsicum seed extracts were subjected to RNA extraction on the ELISA extract (250 seeds). ToBRFV was detected in ToBRFV infected tomato seeds. As no ToBRFV-infected capsicum seeds were available, infected tomato leaf material was added to healthy seed extracts. The PCR test was as sensitive as the ELISA on all the positive seed extracts showing suitability of the PCR assay for identification and detection of ToBRFV.

The local lesion assay is sufficiently sensitive to

To prevent that, ISHI-veg always aims to have at least two primer sets based on different areas of the pathogen genome in molecular assays.

Another essential component in PCR assays on seed extract is the ability to verify proper execution of the test. This is done by adding a known amount of another virus to the seed extract, the so-called Positive Extraction Control (PEC). The use of a PEC is essential and the user is free to choose any PEC provided it is shown that the one selected does not influence test results. ISHI-veg used a sequence from *Bacopa chlorosis virus* (BaCV) but other PECs such as *Squash mosaic virus* (SqMV) and *Dahlia latent viroid* (DLVd) have also been used and are compatible with the ToBRFV-specific primers.

**Table 3.** ToBRFV TaqMan PCR assay on a sample of one ToBRFV infected seed in 999 healthy tomato seed (*source: Naktuinbouw*)

| Sample and controls | Triplex ToBRVF + BaCV PCR | | |
|---|---|---|---|
| | ToBRFV-CSP1325 | ToBRFV-CaTa28 | BaCV |
| 1 | Detected | Detected | Detected |
| 2 | Detected | Detected | Detected |
| 3 | Detected | Detected | Detected |
| 4 | Detected | Detected | Detected |
| 5 | Detected | Detected | Detected |
| 6 | Detected | Detected | Detected |
| 7 | Detected | Detected | Detected |
| 8 | Detected | Detected | Detected |
| Positive Isolation Control | Detected | Detected | Detected |
| Negative Control Seed | Not detected | Not detected | Detected |
| Buffer control | Not detected | Not detected | Detected |
| Negative Process Control | Not detected | Not detected | Not detected |
| Positive Amplification Control | Detected | Detected | Detected |



prevent disease outbreaks from infected seeds in the past two to three decades. The ToBRFV TaqMan PCR has been developed to be more sensitive than the local lesion assay to exclude any false negative test results from the TaqMan PCR assay. This is in line with ISHI-Veg's technical paper on real-time PCR pre-screening (see 9).

There are studies ongoing on the sensitivity of the PCR to enable a bigger subsamples size, such as 1,000 seeds instead of 250 (see Table 3).

## Summary

A new *Tobamovirus*, ToBRFV, has recently become a threat to the tomato industry, as it overcomes the Tm-2² resistance in tomato. *Tobamoviruses* have been a burden for growers and seed industry alike for decades. Easy mechanical transmission of the virus is a threat to modern fresh produce farming. Good and appropriate management of the disease has contributed greatly to current levels of production. The seed industry has had a leading role in the management of these *Tobamoviruses* through the development of resistant varieties and seed health testing.

The fact that there have been no outbreaks of *Tobamoviruses* in tomato, capsicum and eggplant linked to a failure of the seed health test, shows that the current method is fit for purpose and disease management strategies are effective. Like other *Tobamoviruses* ToBRFV can also be managed by carrying out the correct seed health tests and implementing strict hygiene management.

ISHI-Veg aims to secure the delivery of sufficiently healthy seed to customers by developing methods for seed health testing that are internationally recognized as reference methods and accepted as industry standards. The current test for Tobamoviruses, a local lesion assay in combination with pre-screen ELISA on 3000 seeds, is able to detect the new Tobamovirus ToBRFV.

To facilitate the international movement of seed and help companies comply with import requirements for ToBRFV, ISHI-Veg has developed a new method for specifically detecting ToBRFV. This TaqMan assay subjected to a seed extract can be used either to detect seed lots for ToBRFV or to identify ToBRFV after a positive ELISA.

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