Grower Summary

On site detection of Tomato brown rugose fruit virus: validation of ToBRFV diagnostics (LAMP) for use by UK growers

PE 035

Final report

Project title:	**On site detection of Tomato brown rugose fruit virus: validation of ToBRFV diagnostics (LAMP) for use by UK growers**
Project number:	PE035
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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

Tomato brown rugose fruit virus is a tobamovirus of regulatory importance. LAMP approaches have been evaluated and validated for environmental swab sampling to detect the virus. This work has included comparative testing between LAMP and RT-qPCR, optimisation of extraction and sampling (swabbing), generation of validation data on sensitivity, specificity and matrix effects. These data indicate that LAMP will reliably detect ToBRFV from an RNA extraction to a dilution of 10^{-4}, compared to 10^{-6} for qPCR. The primers do not cross-react with either common tomato viruses or other members of the same genus. Initial testing has indicated some surfaces are challenging for the recovery of virus, likely due to their physical properties e.g. the porosity of concrete. Whilst there may be some applications for infield use, the use of swabs for inspector and grower testing cannot be supported due to the loss of sensitivity from non-extracted samples.

Background

Tomato brown rugose fruit virus has rapidly become the major virus of concern for the tomato industry. Surveillance for this virus involves sampling large numbers of plants, from a glasshouse which is time consuming for the inspector, costly, and only gives a ‘snapshot’ of the of a single point in the season. The current approach is to take leaf samples from 200 plants from a glasshouse of 10s if not 100s of thousands of plants. Whilst these numbers are statistically robust in line with ISPM 31, these levels of testing are set to give adequate levels of detection within the limits of inspector and laboratory resources. Work recently completed at Fera indicates that the timing and point of sampling on individual plants can have a strong influence on the reliability of detecting the virus (AHDB PE 034).

Loop-mediated isothermal amplification (LAMP) is a form of PCR testing which uses multiple primer sets to detect the presence of a target. However, unlike conventional PCR chemistry methods which require multiple cycles of rapid heating and cooling, the reaction in LAMP works at a single temperature. This means the equipment for running the tests can be much smaller than traditional PCR machines, making the equipment portable. Additionally, LAMP chemistry is less prone to the influence of inhibitors interfering with the diagnostic reaction, which means it can be used with crude sample preparations, and so may be amenable for onsite testing with low equipment resource requirements.

An alternative approach using onsite testing to focus on screening through environmental monitoring rather than through testing would have the potential advantage of reducing

resource inputs and allowing ongoing monitoring throughout the season. This environmental monitoring could be achieved by testing of critical points in the process, using LAMP diagnostics for testing swabs and irrigation water. This would potentially allow a more comprehensive approach throughout the season for less cost. However, where and how to best apply this technology has not been explored. LAMP for ToBRFV has already been initially evaluated for swabs and water samples but this requires validation to be completed for this application.

To support this, a scope of validation needed to be established. This involved mapping glasshouse processes to identify critical points, such as high traffic areas to develop sampling guidelines focused on areas where virus is likely to concentrate. Once this is established, LAMP is validated for inspection and grower support testing by comparing the applicability of the test for onsite and laboratory-based testing of plant and non-plant samples, such as comparing the influence of swab types and different surfaces. Additionally swab samples were collected during a visit to an active outbreak and tested to compare current laboratory methods and the application of LAMP for outbreak and post-outbreak monitoring. The main aims of this project are to establish if there are there alternative (improved) approaches for surveillance of a virus in a controlled environment rather than “snapshot testing” of small proportion of plants. The two key questions to be answered are:

- What are the ways which LAMP could be used to support inspectors and growers in surveying for the presence of ToBRFV?
- Does LAMP offer sufficient sensitivity and specificity to allow for onsite testing for the presence of ToBRFV?

Summary

The principal objectives of the project were:

1. Map glasshouse processes to identify key areas of virus concentration to fix a scope of validation
2. Validation of ToBRFV LAMP against current “gold standard” methods (real-time qPCR
    a. Establish sensitivity and specificity (inclusivity and exclusivity) characteristics for the test
    b. Determine any “matrix” effect from choice of swab or swabbed surface

To identify the key areas where an environmental monitoring process could focus sampling effort growers, inspectors and researchers attended an online workshop. The aim of the workshop was to identify the areas where virus residues were likely to accumulate within a glasshouse production system. To achieve this discussion was based around the general schematic process shown in Figure 1.

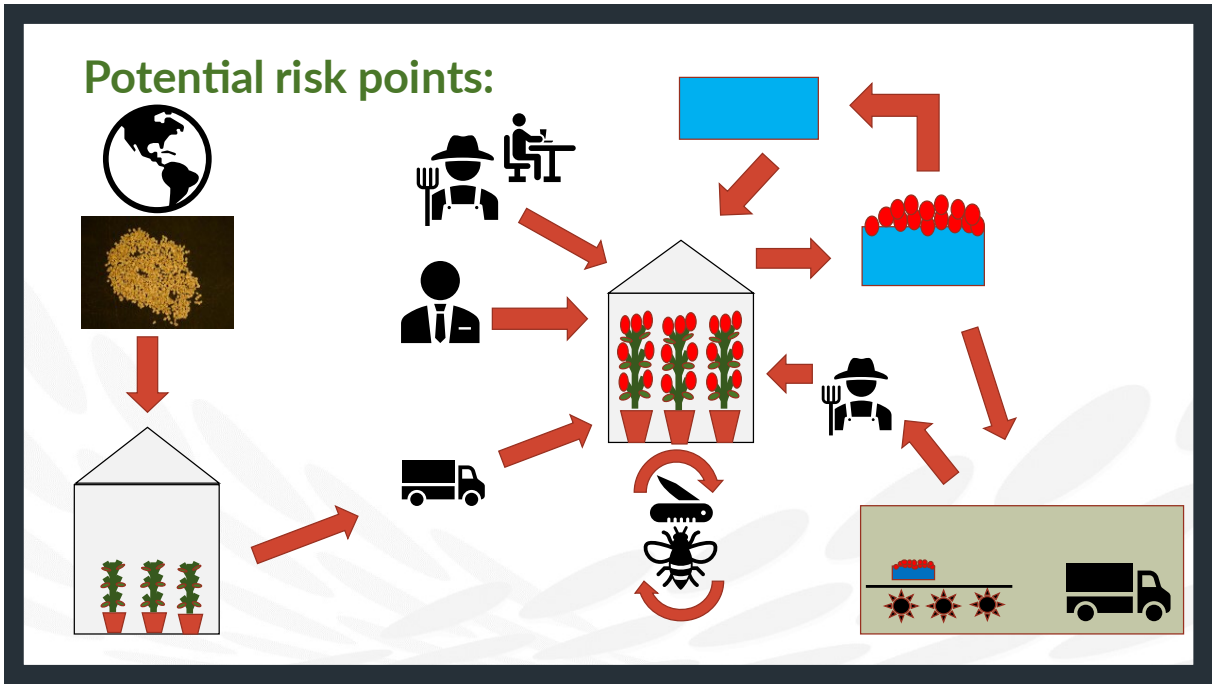

Figure 1. Potential risk points in the tomato production chain showing (left to right) global seed introductions into propagation, transport, inputs into the production system including staff, management, glasshouse practices (cutting, pollination), and via the packhouse.

Discussions based around this schematic helped to identify the key areas where process and diagnostic controls were currently in place (e.g. seed testing and checking propagation plants prior to despatch), and also areas where no controls were currently in place but would be potential points of concentration of virus residues Figure 2. These were considered to be key areas for where environmental swabbing could be used for monitoring for the presence of ToBRFV. These included surfaces in the glasshouse including both fixed and mobile equipment, PPE and peripheral equipment such as computer terminals. Other areas which were identified were areas outside the main glasshouse, such as the in the canteen, onsite accommodation and other surfaces such as mobile phones and surfaces in cars where staff (especially managers) may move between glasshouse sites. It was agreed that for the main validation work the surfaces used for previous survival and disinfection work would give a

good representative cross section of surfaces, namely: glass, stainless steel, aluminium, hard plastic (picking crate), soft plastic (polythene sheeting), and disposable gloves (nitrile type).

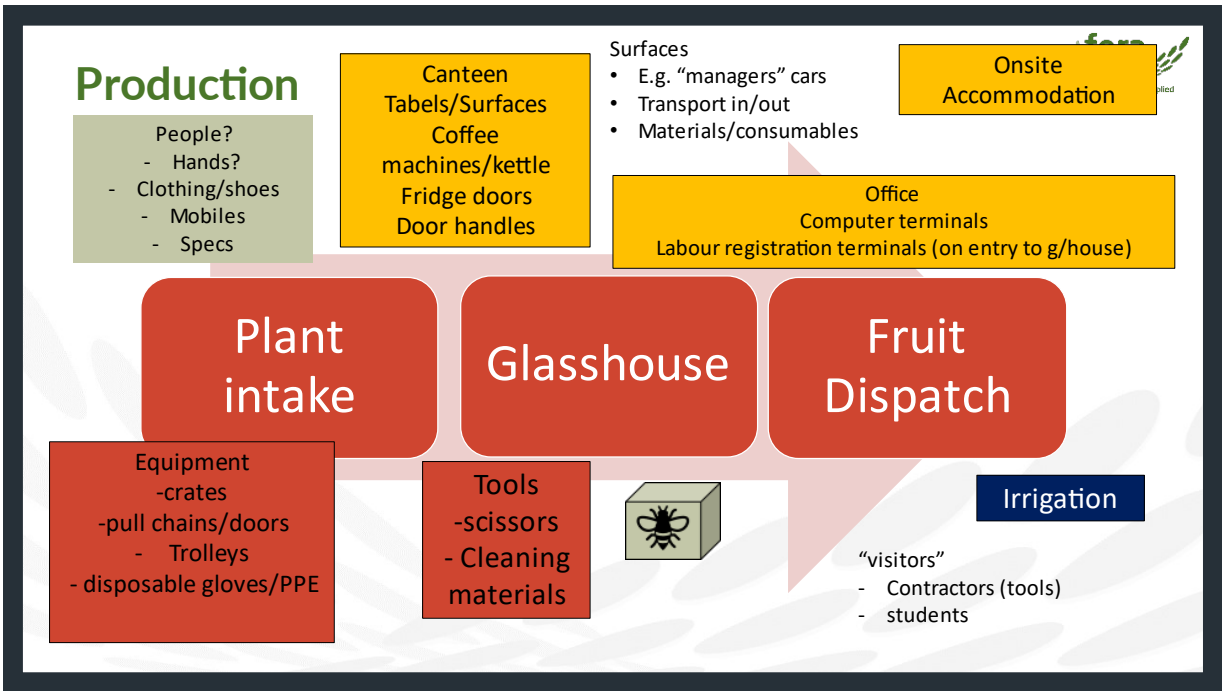

Figure 2 Areas for potential accumulation of environmental residues of ToBRFV identified during the stakeholder's workshop.

The current "gold standard" method, real-time RT-PCR, cannot be reliably used on samples using crude extractions, and requires RNA to be extracted from samples prior to testing. One consequence of this is that key aspects of test performance, such as analytical and diagnostic sensitivity should be compared with the current method to determine the relative limits of detection. For this reason, all validation was done in two stages to give a proxy measure of relative sensitivity. Initially the LAMP method was compared directly with existing real-time RT-PCR methods used in the laboratory on the same RNA extracts. Subsequently LAMP was trialled on crude preparations using leaf or swab samples diluted in a polyethylene glycol buffer. In this report real-time RT-PCR results are presented as the cycle threshold (Ct) which is the number of PCR heating cycles to register a positive result. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta). The Ta measurement is specific to the target product of the LAMP reaction and serves as a secondary quality check on the test result.

A comparison of detection sensitivity between LAMP using and RNA extract (Figure 3) indicated that the limit of detection (LoD) was around 1 part in 1,000,000 ($1x10^{-6}$). Crude extractions only gave a LoD around 1 part in 10,000 ($1x10^{-4}$), a hundred fold less than extracted RNA. However, the impact on the time taken to register the sample as a positive

reaction was impacted taking more than 10 minutes longer for the equivalent detection at $1x10^{-4}$. Due to the mechanics of the LAMP process, which require fluorescence to be emitted during the reaction, the stronger preparations (neat and a 1:10 dilution) could not be read by the machine, registering a "negative" result.

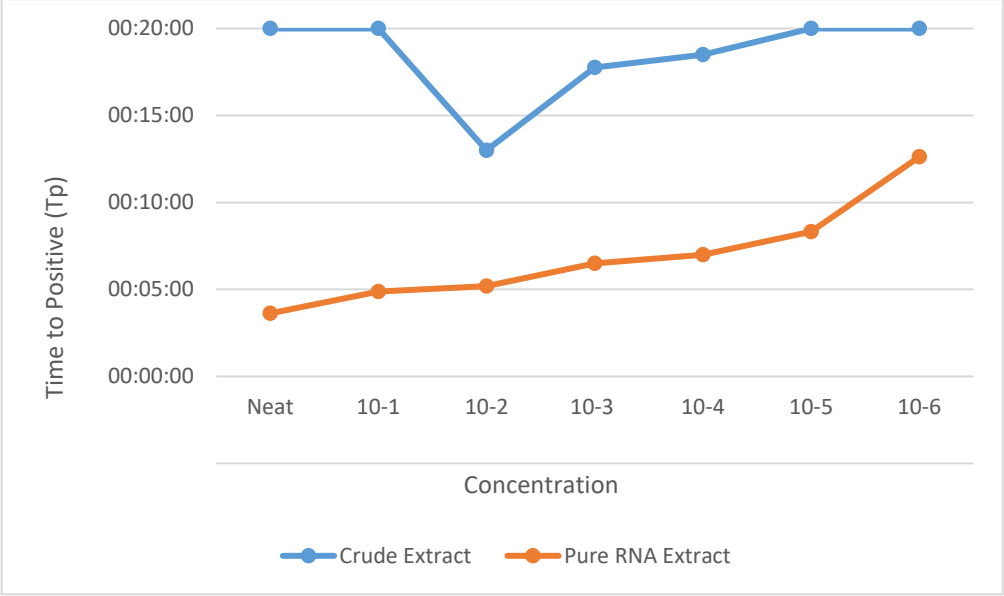

Figure 3 Comparison of detection of ToBRFV using Isothermal Amplification by 10-fold serial dilution. Each increment represents a further 1:10 dilution of samples plotted against the "time to detection".

Ensuring a test detects the desired target, but does not detect non-targets is termed the specificity. When designing molecular primers sets (tests) such as for PCR, real-time PCR or LAMP, allowances are made for variations in the target genomes using a broad range of isolate genetic sequences as the basis for the design, during this phase a computer based "*in silico*" check is also carried out to ensure that the primers should not cross react with any other known virus sequences. As a confirmation of this a laboratory based specificity study was also carried out, this included regulated and non-regulated viruses and viroids which are either commonly tested for by laboratories (e.g. potato spindle tuber viroid, PSTVd), or which are commonly found in glasshouse samples (e.g. pepino mosaic virus, PepMV) or other viruses closely related to ToBRFV .

With the exception of one PepMV isolate, the assay detected all target isolates, and no non-target isolates, and performed as expected. In one case, an isolate of PepMV-Ch2 a positive result was obtained. This was further investigated with alternate test methods, and other isolates of this virus strain were also tested to ensure no cross reaction. The ToBRFV assay

did not detect ToBRFV from any other isolate. However, further testing of the initial positive isolate confirmed this result was the consequence of the isolate being contaminated with ToBRFV, likely the result of a co-infection in the glasshouse.

Table 1 Specificity testing of LAMP assay for ToBRFV showing negative reations with non-target viruses and viroids, and detection of ToBRFV test and controls. [(1)] PepMV sample tested positive, later shown to be cross-contamination of the sample. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta).

Group	Target	Extracted RNA		
		Tp	Ta	Result
Viroids	PSTVd	/	/	Neg
	CLVd	/	/	Neg
	PCFVd	/	/	Neg
Tomato infecting viruses	PepMV Ch1	/	/	Neg
	PepMV Ch2	8:00	84.89	Contamination[1]
	PepMV Ch2 (isolate 2)	/	/	Neg
	PepMV EU	/	/	Neg
	STV	/	/	Neg
	TSWV	/	/	Neg
	TYLCV	/	/	Neg
	PVX	/	/	Neg
	PVY	/	/	Neg
Tobamoviruses	TMV	/	/	Neg
	ToMV	/	/	Neg
	PMMoV	/	/	Neg
	ToBRFV	3:00	84.96	Pos
	ToMMV	/	/	Neg
	UTobV1	/	/	Neg
Controls	No Template	/	/	Neg
	H2O 1	/	/	Neg
	H2O 2	/	/	Neg
	H2O 3	/	/	Neg

	Pos 1	5:45	84.97	Pos
	Pos 2	5:30	84.93	Pos
	Pos 3	5:45	84.93	Pos

Other aspects of test performance characteristics were investigated including the use of different swab types, and the influence of swabbing different surfaces Figure 4. The surfaces used were the same standard range of surfaces investigated in previous projects on survival and disinfection of ToBRFV (PE033/a). Swabs of sap contamination from inert surfaces, such as glass, steel and aluminium gave robust and rapid detection of ToBRFV. Similarly swabbing nitrile disposable gloves gave similar rapid detection of the virus. Plastics such as polythene sheeting and hard plastic (picking tray) may influence the detection of virus from a swab sample, possibly due to electrostatic charge in the plastic interacting with the virus or sap. Within the experimental work here, infected sap inoculated onto concrete could not be detected using a swab sample.

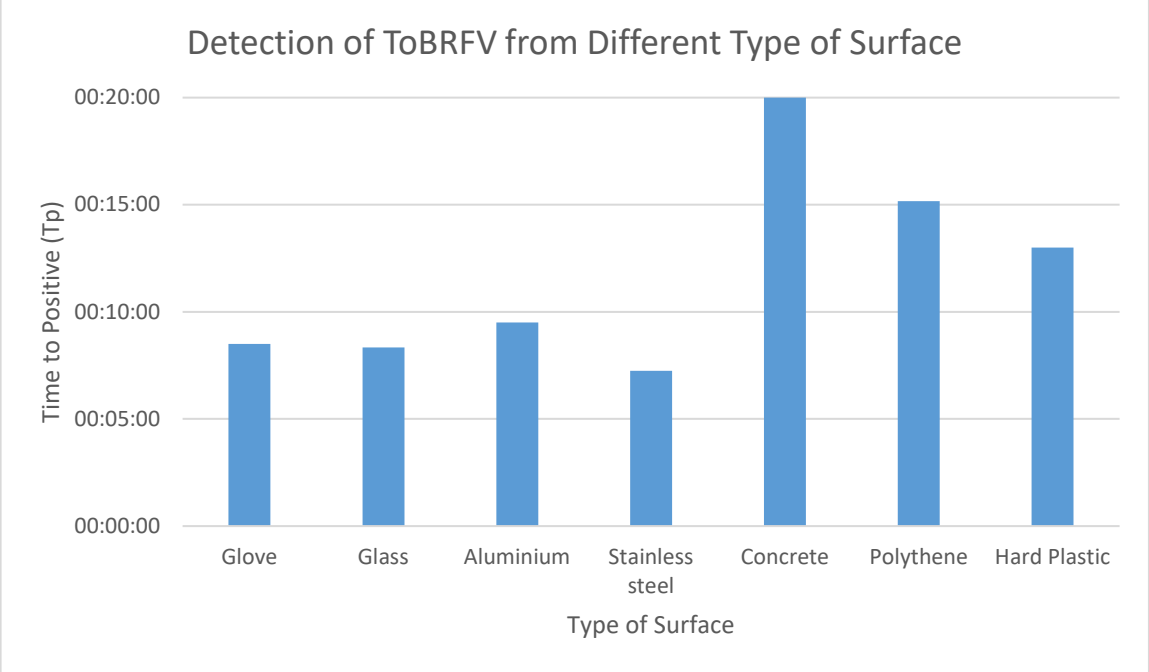

Figure 4 Detection from different surface types showing time to detection

Following validation work to define the performance characteristics of the test, and to optimise the sampling process, the swab sample method was trialled in two glasshouse scenarios. One of these was as part of the statutory plant health action following a recurrent outbreak in the UK, the second scenario was swabbing the glasshouse cubicle at Fera which has been

used for containing previous work conducted on ToBRFV including work reported in PE 033/a and PE 034 (Table 2). This work was crucial to understanding the limits of detection of the investigated methods with comparison to the current standard method.

Table 2 Swab results from the experimental glasshouse at Fera, showing a direct comparison of rates of detection with real-time RT-PCR, LAMP on extracted RNA and LAMP on a crude (PEG) extract. Real-time RT-PCR results are presented as the cycle threshold (Ct) which is the number of PCR heating cycles to register a positive result. LAMP results are presented as the time to positive amplification (Tp) and the annealing temperature of the LAMP product (Ta).

	Real-time RT-PCR				
	RNA extract	LAMP			
		RNA Extract		Crude (PEG)	
Sample type	Avg Ct	Tp	Ta	Tp	Ta
window 1	28.43	00:10:45	84.51	/	/
window 2	28.27	00:11:15	84.52	/	/
window 3	29.11	00:11:15	84.86	/	/
Bench edging - face out	34.37	/	/	/	/
Bench edging - face in	32.24	/	/	/	/
ladder	25.93	00:08:30	84.66	00:10:45	84.61
wall 1	34.88	/	/	/	/
wall 2	28.15	00:12:15	84.72	/	/
floor	26.96	/	/	00:04:45	84.46
plant pot 1	26.93	00:06:30	84.77	/	/
plant pot2	26.77	00:06:45	84.58	/	/
plant pot black tray	21.08	00:05:45	84.66	00:14:45	84.61
Stand - leg	32.25	/	/	/	/
Stand - middle bar	29.47	00:18:15	84.44	/	/
Stand - grid panel	18.89	00:05:30	/	00:06:30	84.26
Glove	36.70	/	/	/	/
Tyvek sleeve	36.60	/	/	/	/
Plastic apron	31.06	00:16:15	84.79	/	/
H2O	40.00	-	-	-	-
ToBRFV + (avg)	22.98	00:06:00	85.3	00:06:23	85.15

This testing indicated that the comparative limit of detection of the LAMP when testing extracted RNA is equivalent of a real-time PCR result of 30-31Ct (Table 2). This means that

this testing is approximately the same sensitivity as conventional PCR methods for ToBRFV testing. However, this comparative lack of sensitivity meant that the LAMP method only detected 60% of the swab samples which would have been considered positive by real-time RT-PCR. Testing crude extractions did detect the presence of ToBRFV in some samples, however this was poor by comparison to RNA extraction LAMP and real-time RT-PCR, only detecting around 25% of the samples that the current standard method would have detected.

The results presented here indicate that LAMP testing does have potential for screening testing for ToBRFV, and when used on extracted sample RNA gives comparable levels of detection to conventional PCR methods. However, the loss of sensitivity incurred when using the approach on crude preparations from swabs means that this approach cannot be currently recommended as an infield test. The testing carried out in glasshouses as part of this project indicates the high risk of contamination following an outbreak of ToBRFV with environmental residues of the virus being present in areas where there had been no direct contact with the virus. Work is ongoing at Fera and with international partners to better understand the sources of, and risks associated with, these environmental residues.

Financial Benefits

There are no direct financial benefits to growers from this work, however, these data have validated the use of swab testing by both LAMP and real-time RT-PCR. These results indicate that laboratory based testing of environmental swabs could provide a way of monitoring glasshouses for the presence of ToBRFV allowing early intervention in an outbreak. However, due to the persistence of the virus, swab testing by molecular diagnostic methods does not provide an effective way to demonstrate freedom from the virus.

Action Points

- Swab testing with laboratory based testing can be used to monitor for the presence of ToBRFV
- In the event of a ToBRFV outbreak the virus can rapidly contaminate surfaces and these may form a source for future carry over infections
- LAMP testing provides another potential diagnostic method in the battle against ToBRFV, and could be used in conjunction with RNA extraction procedures, however cannot be recommended for testing crude sample preparations at this time.