NAPPO
North American Plant Protection Organization
Organización Norteamericana de Protección a las Plantas
MEXICO - USA - CANADA

Pilot for the Harmonization of Diagnostic Protocols for Seed Pests Focused on Tomato brown rugose fruit virus (ToBRFV)

Final report prepared by the NAPPO Seeds Expert Group and presented to the NAPPO Executive Committee

May 2023

Contents

Executive Summary

- Five diagnostic protocols used by NAPPO member countries were selected for this pilot.
- Nine laboratories from the NAPPO region conducted the experiments.
- Data generated by the laboratories was used to evaluate the selected protocols.
- Based on ring test data analysis:
    - Protocols **A** (CDFA: T. Tian. Unpublished), **B** (ISHI-Veg/NSHS protocol), **C** (USDA-APHIS; Chanda *et al*., 2021), and **D** (USDA-APHIS; Dey *et. al*., 2021) produced comparable results and could be recommended for use as interchangeable diagnostic protocols for the detection of ToBRFV in tomato and pepper seeds. These protocols include two real-time PCR and two conventional PCR methodologies.
    - Furthermore, protocols **B**, **C**, and **D** *performed optimally* in relation to all assayed parameters and variables evaluated.
- Based on the results obtained, protocols **B**, **C** and **D** could be considered for use by the three NAPPO member country NPPOs for phytosanitary testing of seeds for the presence of ToBRFV.
- Having NAPPO member country NPPOs using optimal protocols would avoid retesting by exporting and importing countries thereby facilitating expeditious safe interregional trade of tomato and pepper seeds.
- The experimental design used in this pilot can serve as a model for future studies to evaluate additional diagnostic protocols for the detection of ToBRFV or protocols for other seed-transmitted regulated pests.

Introduction

In April of 2022, the NAPPO Executive Committee (EC) approved the Terms of Reference (ToR) for the NAPPO project entitled "*Pilot for harmonization of diagnostic protocols for seed pests focused on Tomato brown rugose fruit virus (ToBRFV)*". The ToR document provided information on the rationale and general objectives of the project, suggested project methodology, a list of potential participating laboratories, expected budget and general workplan, and provided a list of the subject matter experts (from government and industry) that formed the NAPPO Seeds Expert Group (EG). Approval of the ToR by the NAPPO EC was a requirement for the NAPPO Seeds EG to initiate the design, preparation, execution, and data analyses for this extremely high impact project.

This report provides a summary of project outcomes. It also includes a section on ***Lessons learned*** to consider for future projects on harmonization of diagnostic protocols for seed transmitted pests in our region, as well as a section on ***Conclusions and Recommendations*** based on project results.

Methodology and workplan

Experimental design

To avoid bias, protocols selected for virus detection and quantitation were labeled A, B, C, D and E before supplying all test materials to participating laboratories (See Table 1 below). The comparative analyses of the performance of the selected PCR-based diagnostic protocols (***test performance study***) used a ring test format. Three conventional PCR (cPCR) and two real-time PCR (RT-PCR) protocols were assayed. Experimental panels (ToBRFV analytical samples, positive and negative tomato and pepper seed samples, and controls) were also codified to minimize bias during the experimental phase.

Table 1. ToBRFV diagnostic protocols tested.

Code	Source	Type
A	California Department of Food and Agriculture (CDFA), T. Tian, unpublished protocol (protocol suggested by the Canadian Food Inspection Agency (CFIA)	cPCR
B	International Seed Health Initiative for Vegetable Crops / National Seed Health System (ISHI-Veg/NSHS)[1]	RT-PCR
C	United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS), Chanda *et al*., 2021	RT-PCR
D	USDA-APHIS, Dey *et al*., 2021	cPCR
E	Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), Rodríguez Mendoza *et al*., 2019.	cPCR

Three standard operating procedures (SOPs) were developed using the codes assigned to the protocols and samples (each given sample was labeled differently within each SOP). SOPs were designed so that all protocols but not all samples were tested by each participating laboratory. Samples were tested in duplicate and/or triplicate to capture variation resulting from sample

[1] Modified ISHI-Veg protocol validated through the National Seed Health System (NSHS)

amplification for each protocol in each laboratory. Variation between technicians in each participating laboratory was also evaluated.

Additional instructions were developed on how to enter the resulting data into the APHIS Laboratory Portal. Several virtual training sessions were scheduled by the Secretariat with participating laboratories before initiating the experimental phase of the project.

Reagents and experimental panels

Experimental panels (= samples to be tested) were prepared and labeled using the codified information based on the SOP assigned to each participating laboratory. Import permits were obtained to ship the reagents and experimental panels from the laboratory in the United States that assembled the materials, to participating laboratories in Canada, Mexico, and the United States.

Prior to initiating the experimental phase, participating laboratories were required to complete a pre-test to ensure that the reagents worked with the RNA extracted by each laboratory. This was essential as each laboratory used their own RNA extraction protocol.

Results

The participating laboratories conducted the testing and collected data. Eight laboratories contributed complete datasets for all real-time RT-PCR parameters evaluated. Data from seven laboratories determined a universal sample cutoff of 34 cycles using qRT-PCR. The following parameters were evaluated for all protocols tested:

Limit of detection (LOD)

Defined as the percentage of samples containing detectable target (ToBRFV) at a given concentration. Values ranged from 0% for negative samples (no virus present) to 100% (all samples containing virus are detectable at the specified concentration). LOD results for all protocols are shown in Figure 1. Protocols B, C and D yielded the lowest LOD. Protocols B and C had an LOD of **50%** when the diagnostic sample was diluted 1/1000, while Protocol D had an LOD of **79%.**

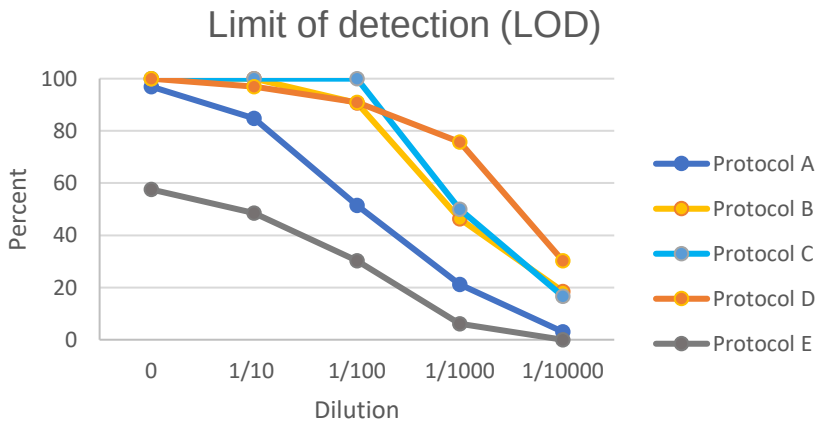

Figure 1. Limit of detection (LOD) for all protocols tested (A-E) in undiluted to 1/10000 diluted RNA samples extracted from ToBRFV-infested tomato seeds.

Specificity

Defined as the assay's non-specific or cross-reacting amplification when testing negative material. To test for specificity, three sample types were used:

- A cross-reacting analytical sample created using a synthetic template of tomato mottle mosaic virus (ToMMV) closely related to ToBRFV. This sample was tested at high and low virus concentrations.
- Healthy tomato seeds (not infected with ToBRFV).
- Healthy pepper seeds (not infected with ToBRFV).

All protocols yielded comparable specificity levels regardless of potential cross-contaminating pathogen titer or host matrix. A significant difference in specificity was found only in healthy pepper seed samples with Protocol B. Determining the source of this difference was beyond the scope of the study (it could be related to the host matrix or the possible presence of a pathogen other than ToMMV or ToBRFV) (Table 2).

Table 2. Specificity test results for protocols A-E using ToMMV analytical samples and healthy tomato or pepper seed controls. Results are percent negative ± the 95% confidence interval.

	Conventional PCR			**Quantitative real time-PCR**	
Samples used to test specificity	**A**	**D**	**E**	**B- Coat Protein**	**C**
Cross reacting analytical sample at **High** ToMMV virus concentration. In-vitro transcript in molecular grade water.	83.3±13.9	100.0±3.9	100.0±3.9	100.0±1.6	92.6±4.1
Cross reacting analytical sample at **Low** ToMMV virus concentration. In-vitro transcript in molecular grade water.	77.8±10.3	94.4±11.1	100.0±5.1	100.0±1.6	91.7±3.8
Healthy tomato seeds.	100.0±4.6	95.8±11.9	100.0±4.6	100.0±1.8	100.0±2.8
Healthy pepper seeds.	96.3±11.3	100.0±4.4	100.0±4.6	77.8±3.6	100.0±3.5

Sensitivity

Sensitivity is defined as a measurement of true positives over the range of samples tested (percent detection of ToBRFV positive material). Protocols B, C and D performed optimally, having a higher percentage of sensitivity compared to protocols A and E (Figure 2).

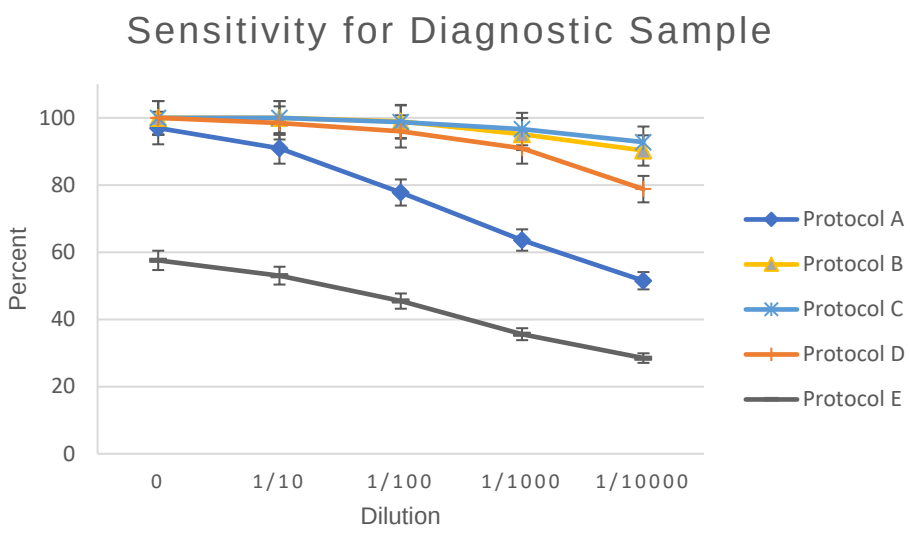

Figure 2. Sensitivity results for protocols A-E for diagnostic samples (ToBRFV infested tomato seeds). Sensitivity percentages are for undiluted down to 1/10000 diluted RNA samples extracted from ToBRFV-infected tomato seeds.

Precision

Precision is the assessment of assay variability between tests. Precision was evaluated only for the two RT-PCR protocols (B and C). Three categories were evaluated:

- Repeatability - to assess replicates in the same test.
- Intermediate precision - to assess replicates between technicians in the same lab.
- Reproducibility – combined replicates of all laboratories.

Trends for the above categories were as expected. Percent variability was evaluated by percent coefficient of variation (standard deviation divided by the average times 100; CV). Repeatability was less than 2% CV, intermediate precision ranged between 3.55 to 4.38% CV, and reproducibility was below 9%. Results are within generally accepted limits (source 1, source 2, source 3, source 4) and indicate good assay precision (Table 3).

Table 3. Parameters to determine assay precision for each target of protocols B and C.

	Variables to determine assay precision (values in %)		
Protocol	Repeatability	Intermediate precision	Reproducibility
B- Movement protein gene	1.65	3.55	8.57
B- Coat protein gene	1.53	3.73	8.58
B- Plant nad5	1.43	4.38	5.11
C- Movement protein gene	1.85	4.01	8.68
C-Plant nad5	1.67	3.73	7.73

Linearity, accuracy, and sample cutoff

Linearity was determined using a five-point calibration curve for each lab. At least three concentration points were needed to establish a curve and obtain a coefficient of determination

(r^2), which measures how linear the points are on the curve (Table 4). A perfect curve fit (r^2=1.0) suggests little to no variability among serial dilutions. Values from seven laboratories had r^2>0.99, suggesting a good fit for a laboratory's five-point calibrator curve. The calibration curves were used to determine the **accuracy** and the **sample cutoff** for protocols B and C (Table 4).

Accuracy of a real-time PCR curve is the slope of the calibration curve. Perfect accuracy, or 100%, is rooted in a slope of -3.32. The slope represents the change of cycle value as concentration decreases, or -3.32 cycles (ΔCq), per calibration curve point. As the slope ΔCq increases or decreases from this value, accuracy drops. Acceptable accuracy falls within a range of ± 0.3, from -3.02 to -3.62. The seven laboratories assessed produced a slope within the expected range.

Sample cutoff was determined by subtracting 3.32 from a predicted assay cutoff (predicted end-of-measurement cycle value) for each of the seven laboratories assessed and subtracting the generated slope for each laboratory from the assay cutoff. The *sample cutoff* value determined whether a sample value, when evaluated against the calibrator curve, was positive or negative.

In summary, calibrator curves for the seven laboratories assessed could be applied to accurately make positive or negative sample determinations for protocols B and C.

Table 4. Linearity and accuracy of the results of seven participating laboratories. Good linearity is indicated with an r^2 value greater than 0.98. Accuracy (calibration curve slope) is expected in the range of -3.32±0.3

Laboratory	Linearity (r^2)	Accuracy (ΔC_q)
1	0.9957	-3.18
2	0.9995	-3.38
3	0.9983	-3.38
4	1.0000	-3.33
5	0.9964	-3.37
6	0.9967	-3.48
7	0.9986	-3.27

Likelihood ratios

Positive (PLR) and negative (NLR) likelihood ratios are used to demonstrate that each test works properly. PLR and NLR ratios consist of all valid sample data points (e.g., Protocol A PLR and NLR ratios reflect valid data generated by all laboratories). The closer these values are to 1 the more difficulty the test has in distinguishing positive and negative samples. A PLR above 5 and an NLR below 0.5 confirm some capacity for distinguishing positive from negative samples (Table 5).

Table 5. Likelihood ratios for protocols A-E. Protocol B was tested for the movement (MP) and coat (CP) protein regions. Values were estimated for ToBRFV infected seed, healthy tomato seeds, and healthy pepper seed samples.

Protocol	Positive likelihood ratio (PLR)	Negative likelihood ratio (NLR)
A	28	0.49
B (Target 1 and 2)	17	0.08
C	29	0.07
D	38	0.22
E	6(*)	0.72(*)

(*): As stated above, a low PLR (around or below 5) and a high NLR (above 0.5) suggest difficulties for the protocol to produce comparable results when used by different laboratories.

The confidence level in the results when one sample was tested with all five protocols is shown in Table 6. For example, there is a 91% level of confidence that the assessment of the analytical sample (first row in table 6) has a 97.2% or greater chance of being positive for ToBRFV.

Table 6. Verification of sample matrix consistency.

Sample	Dilution	Confidence level (CL)(%)	Probability of sample identified accurately
ToBRFV in vitro transcript in molecular grade water	Undiluted	91	97.2%
	1/10	91	94.2%
	1/100	91	91.5%
	1/1,000	91	88.8%
	1/10,000	91	82.7%
Non-target virus *in vitro* transcript	High	91	89.6%
Non-target virus *in vitro* transcript	Low	91	85.7%
Tomato seeds samples infected with ToBRFV	Undiluted	93	85.6%
	1/10	93	83.8%
	1/100	93	79.2%
	1/1,000	93	72.4%
	1/10,000	93	65.5%
Healthy tomato seeds		91	92.8%
Healthy pepper seeds		91	91.8%

Conclusions and recommendations

The most relevant conclusions and recommendations are listed in the Executive Summary of this report. Additional conclusions and recommendations include the following:

- The internal control plant mitochondrial gene target nad5 employed was successfully amplified in all tested protocols (two real-time PCR and one conventional PCR).
- Consideration should be given to possibly inviting other countries outside the NAPPO region to participate in future ring test studies, particularly focused on NAPPO trading partners.

Next steps

As was stated in the approved Terms of Reference, with the completion of the ring test and the approval of this report by the NAPPO Executive Committee, the NAPPO Seeds EG will assemble a small subgroup of subject matter experts to draft a manuscript for publication in a scientific journal. Publication of the manuscript will conclude the project and the NAPPO Seeds EG small group will be disbanded.

Lessons learned

This has been the most complex NAPPO project since the Secretariat relocated to Raleigh, NC. It was directed by one of the largest NAPPO Expert Groups. This brought with it challenges for the NAPPO Secretariat in terms of efficiently managing the EG, meeting project timelines, and issues associated with decision-making and project logistics. Below we outline some key points

that could be taken into consideration for future projects of a similar nature, including those aimed at evaluating other pathogens/diagnostic systems:

- A smaller EG with good representation from the NPPOs and industry would improve management, coordination, and decision-making.
- Sub-groups with specific tasks proved to be an effective way to advance the work of this EG. However, rather than having subgroups working linearly, we suggest having subgroups working concurrently on some tasks to save time.
- Whenever possible, important decisions should be made through a voting system.
- *Ad-hoc* members assigned to specific tasks proved to be a valuable and effective strategy to accomplish important project objectives.
- It proved valuable to develop specific guidelines for document and data sharing from the outset of the project. This should include the roles and responsibilities of the parties involved.
- Responsibility for preparation of project related documentation should be the responsibility of future Seeds EGs.
- Face-to-face meetings did not happen due to COVID 19. However, we suggest including face-to-face meetings in future projects. A face-to-face meeting would prove especially valuable in the first 3-4 months of the project to expedite role assignments and planning.