NAPPO
North American Plant Protection Organization
Organización Norteamericana de Protección a las Plantas

Pilot for harmonization of diagnostic protocols for seed pests focused on *Tomato brown rugose fruit virus* (ToBRFV)

Terms of Reference

NAPPO Seeds Expert Group

April 2022

Contents

Introduction

In 2019, the Mexican seed association (Asociación Mexicana de Semilleros A. C., AMSAC), submitted a new project proposal for NAPPO's consideration on "Recognition of diagnostic tests for seed pests among NAPPO member countries". The NAPPO Executive Committee (EC) and Advisory and Management Committee (AMC) reviewed the proposal and suggested a more targeted scope, and, in late October 2019, the EC endorsed the "Pilot for harmonization of diagnostic protocols for seed pests focused on *Tomato brown rugose fruit virus* (ToBRFV)". In March 2020, NAPPO virtually launched a regional seeds expert group (EG) to work on the pilot project. The entire project, including drafting a final report and recommendations is anticipated to span 24-30 months.

Membership

This EG has robust and active participation from seed health and diagnostic experts from all three NAPPO member countries and regional industry representatives. The members of the expert group and their affiliations are:

Country	Name	Organization
	Patricia McAllister	CFIA
	Pamela Ross	CFIA
	Huimin Xu (Ad hoc member)	CFIA
	Jennifer Nickerson (Ad hoc member)	CFIA
	José Manuel Cambrón Crisantos	SENASICA
	Jessica Berenice Valencia Luna	SENASICA
	Daniela Alejandra Bocanegra Flores	SENASICA
	Ángel Ramírez Suárez	SENASICA
	Beatriz Xoconostle Cazares	CINVESTAV
	Eduardo Garrido Ramírez	INIFAP
	Marlene Ortiz, **Vice-Chair** (Industry Contact)	AMSAC
	Mario Puente Raya (Industry Contact)	AMSAC
Country	**Name**	**Organization**
	Geoff Dennis	USDA, APHIS
	Vessela Mavrodieva	USDA, APHIS
	Kevin Ong	Texas A&M University
	Nancy Osterbauer	USDA, APHIS
	Ed Podleckis, **Chair**	USDA, APHIS
	Ric Dunkle (Industry Contact)	ASTA
	Samantha Thomas (Industry Contact)	Bayer Crop Science

Rationale/Objective

In endorsing the pilot, the EC used the following rationale:

- ToBRFV is a quarantine pest for all NAPPO member countries;

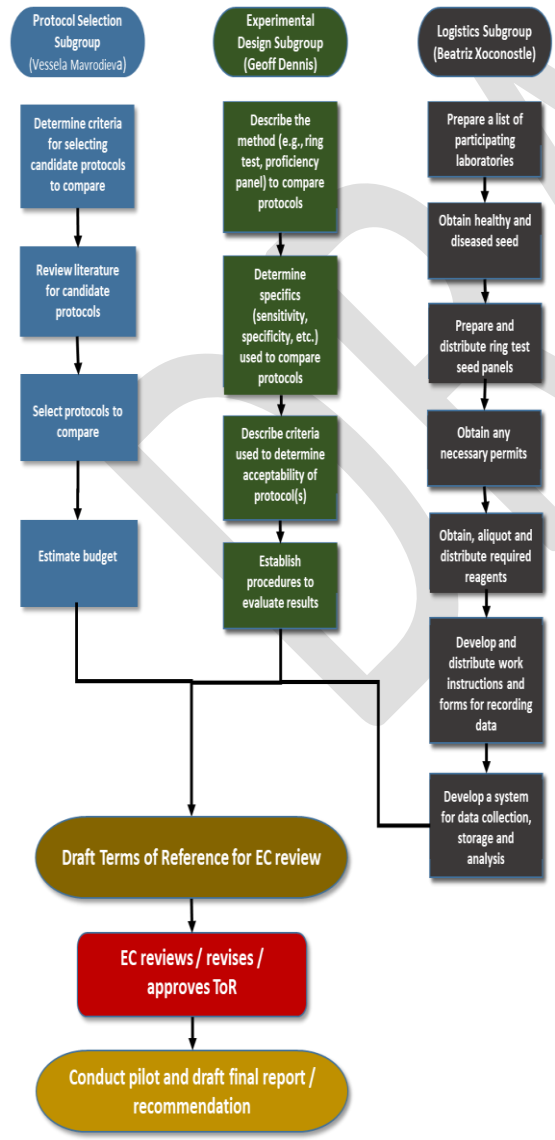
- ToBRFV is a pest of tomato and pepper plants for planting, fruit and seed;
- These commodities are heavily traded among the NAPPO member countries;
- The seed industry is very concerned about this pest; and,
- Harmonization of diagnostic protocols for ToBRFV would have immediate benefits for trade among NAPPO member countries by alleviating the delays and costs of conflicting seed testing results and unnecessary retesting of seed shipments.

The immediate objective of this project is to systematically evaluate selected molecular diagnostic (RT-PCR) assays currently employed by NAPPO member countries and major trading partners. Based on this evaluation, the expert group will develop a recommendation for a protocol OR protocols for phytosanitary testing of tomato and pepper seeds by NAPPO member countries. A longer-term goal of the study is to develop a NAPPO standard procedure to evaluate seed diagnostic protocols that could be applied to future diagnostics developed for other seed pathogens.

Methodology / Plan of Work

The first task of the EG was to develop this Terms of Reference (ToR) document, or a plan of work, for the pilot. The ToR outlines the project tasks and experimental design developed by the EG. Once the ToR document is approved, the EG initiates the pilot. Upon completion of the laboratory testing phase of the pilot, the EG evaluates and documents the pilot results, including lessons learned and recommendations. Depending on results, the EG will develop a project proposal for consideration by NAPPO that has broader and more strategic objectives concerning the harmonization of diagnostic protocols for seed pests. In developing the ToR, the Seeds EG has outlined a plan of work (**Figure 1**) that is grouped into three core functions: protocol selection, experimental design, and logistics. Three subgroups were created to determine and complete the required tasks outlined below.

Figure 1. Seed EG ToBRFV pilot project workflow



Selection of Protocols

Subgroup 1 focused on identifying and selecting candidate RT-PCR protocols. Seventeen protocols were initially identified from literature reports or through their use by private and government laboratories around the world. Five protocols were selected for evaluation in the pilot (**Table 1**). The five selected protocols were chosen based on several factors:

- Their use by one or more of the NAPPO member country official diagnostic laboratories;
- The type of PCR protocol (i.e., conventional RT-PCR versus real-time RT-PCR);
- The target ToBRFV sequence (e.g., encoding the coat protein, movement protein or, RNA-dependent RNA polymerase);
- The protocol has been validated.

Three conventional RT-PCR protocols used by the APHIS Plant Pathogen Confirmatory Diagnostics Laboratory (PPCDL), SENASICA and CFIA were selected. Additionally, two real-time RT-PCR protocols, one adapted by the APHIS PPCDL, and one developed by the International Seed Federation's (ISF) International Seed Health Initiative for Vegetable Crops (ISHI-Veg) and validated by the U.S. National Seed Health System (NSHS) were selected.

Table 1. **Summary of selected PCR Protocols**

Source	Primer	Target Region	Reference
Conventional RT-PCR			
CFIA	TBRFV-MF1 TBRFV-MR1	MP[1]	T. Tian, CDFA unpublished
SENASICA	ToBRFV-F* ToBRFV-R*	RdRp[2]	Rodriguez-Mendoza *et al*. 2019. *Mexican Journal of Phytopathology*, 37(2):345-356.
USDA-APHIS	FL782 FL783	CP[3]	Dey *et al*. 2021. *New Disease Reports*, 44, e12028.https://doi.org/10.1002/ndr2.12028
Real-time RT-PCR			
USDA-APHIS	KL 18-59 ToBRFV-F KL 18-60 ToBRFV-P1 KL 18-61 ToBRFV-R1	MP	Chanda *et al*. 2021. *Plant Disease*, 105: 3643-3652.
ISHI-Veg / NSHS	CaTA28Fw CaTa28Pr CaTa28Rv	MP	ISF ISHI-Veg. 2020. https://worldseed.org/wp-content/uploads/2020/11/Tomato-ToBRFV_2020v1.5.pdf?_ga=2.66722734.1075065090.1644270496-1579089872.1623958967
	CSP1325Fw CSP1325Pr CSP1325Rv	CP	

[1]**MP**- Movement protein
[2]**RdRp**- RNA-dependent RNA polymerase
[3]**CP**- Coat protein

Experimental Design

Subgroup 2 was tasked with developing the experimental design for the pilot project. Among the design elements determined were the format (i.e., a ring test), evaluation metrics (e.g., sensitivity, specificity, precision), composition of test panels, number of replicates and number of participants required for statistically valid results. A ring test format was selected and designed in accordance with internationally accepted principles for diagnostic methods validation. An important topic of discussion early in the development of the ring test format was whether to dictate a single nucleic acid extraction procedure for all participating laboratories to use. In the end, the EG consensus was for each laboratory to use the extraction procedures they normally employ. The decision was intended to simplify logistics in organizing the ring test and examine a process that more closely reflects how the diagnostic would likely be used after the pilot is concluded. The components of the ring test seed panels are summarized in **Table 2**.

Another key decision in the experimental design was each participating laboratory would test all five of the protocols selected for the ring test. Having every participating laboratory test all five selected protocols would minimize unexplained variability in the results.

To ensure the statistical reliability of the ring test results, the EG considered guidelines from two international standards that at least eight laboratories needed to participate to in the ring test.

Table 2. **Composition of ToBRFV ring test seed panels**

Sample ID	Sample Name	Description	Comment
A	**ToBRFV Analytical Sample**	ToBRFV *in vitro* transcripts[1] in molecular grade water	Analytical sample created from ToBRFV transcripts to observe sensitivity and limit of detection. Sample A is serially diluted 1:10 before testing to form a standard curve.
B	**Positive Tomato Seed**	ToBRFV seed sample in relatively high concentration	Sample B is serially diluted 1:10 post-RNA extraction to form a standard curve and characterize limit of detection.
C	**Cross-reacting Analytical Sample**	Non-target virus *in vitro* transcripts[1] or total RNA	Analytical sample created from a healthy sample with ToMV[2] or ToMMV[3] introduced to observe specificity.
D	**Negative Tomato Seed**	ToBRFV-free seed sample	
E	**Negative Pepper Seed**	ToBRFV-free seed sample	
PPC	**Positive Process Control**	ToBRFV positive seed	Used to confirm successful sample processing
NPC-T	**Negative Process Control-Tomato**	ToBRFV-free seed sample	Used to validate that contamination or spurious amplification is not arising from something in the sample matrix or arising during the extraction process.
NPC-P	**Negative Process Control-Pepper**	ToBRFV-free seed sample	Used to validate that contamination or spurious amplification is not arising from something in the sample matrix or arising during the extraction process.
Calibrator	**Purified RNA**	ToBRFV *in-vitro* transcript[1] in buffer solution in molecular grade water in five serial dilutions	Used to characterize linearity for real-time PCR assays.
NTC		molecular grade water	Omits any RNA template from the reaction; serves as a general control for extraneous nucleic acid contamination.

[1] *in vitro* transcript is a mix of transcripts from the three diagnostic target regions
[2] *Tomato mosaic virus*
[3] *Tomato mottle mosaic virus*

Nine laboratories, two from Canada, three from Mexico and four from the United States have agreed to participate. Each of the participating laboratories is authorized or accredited by their respective NPPO to conduct diagnostic testing. The participating laboratories are listed below in **Table 3**.

Table 3. **Participating Laboratories**

	Name	Organization	Location	Type
	United States			
1	Plant Pathogen Confirmatory Diagnostics Laboratory (PPCDL)	USDA	Laurel, Maryland	Government
2	Seed Science Center	Iowa State University	Ames, Iowa	Academia
3	California Seed and Plant Laboratory	CSP Labs	Pleasant Grove, California	Private
4	UF/IFAS Plant Diagnostic Center	University of Florida	Gainesville, Florida	Academia
	CANADA			
5	CFIA Charlottetown Laboratory	CFIA	Charlottetown, Prince Edward Island	Government
6	CFIA Fallowfield Laboratory	CFIA	Nepean, Ontario	Government
	MEXICO			
8	Laboratorio de Virología CNRF/SENASICA	SENASICA	Tecámac, State of Mexico	Government
9	Laboratorio de Biología Molecular y Genómica Funcional	CIAD	Culiacán, Sinaloa	Academia
10	Laboratorio de diagnóstico Integral Fitosanitario (LADIFIT)	LADIFIT	Montecillo, State of Mexico	Academia

Logistics

Subgroup 3 addressed multiple issues of logistics for implementing the ToBRFV ring test. These include obtaining ToBRFV-free and infected seeds for the test panels, preparation of the test panels, obtaining and aliquoting reagents, randomization of the test panels, arranging necessary permits, and determining how test panels and reagents would be distributed and by whom. Since the participating laboratories are not necessarily familiar with all five PCR protocols evaluated, and to ensure consistency in execution of the protocols, detailed work instructions for each of the five protocols were written and translated for distribution to each laboratory. The EG further agreed to provide sufficient test material and reagents for participating laboratories to conduct "practice" tests. A system for data collection, storage and analysis was identified and electronic forms for recording data were developed.

Infected seed. ToBRFV infected tomato seed was donated by industry to California Seed and Plant Labs (CSPL), an ISO 17025 and National Seed Health System accredited testing laboratory, to be used as reference material in research and diagnostic testing. CSPL, in turn, provided a portion of this seed to the NAPPO project. The infected seed, procured by CSPL from multiple producers, was mixed to create

the positive reference material. This positive reference seed mixture was then sent to PPCDL, where it was further mixed with commercially available ToBRFV-free tomato seed to create ToBRFV-positive seed samples used to evaluate the performance of the selected PCR protocols in the ToBRFV ring test.

Analytical samples (RNA transcripts). A mix of *in vitro* transcripts from the three diagnostic target regions of ToBRFV (coat protein, movement protein, RNA dependent RNA polymerase) were created in the Biotechnology Department at the Centro de Investigación y de Estudios Avanzados (CINVESTAV). The transcripts were sent to PPCDL to be evaluated and increased in concentration. The transcript samples are used to determine the precision, sensitivity, and limit of detection of the assays. Transcripts for the "calibrator" and cross-reaction samples were developed at PPCDL.

Panel components testing. The components of the ring test panel, including reagents, were tested using each of the RT-PCR protocols selected for evaluation to ensure compatibility with the different PCR protocols. The testing was conducted at PPCDL.

Assembly of the ring test panel packages. Discussions by the EG arrived at a consensus that to ensure consistency and reduce variability as well as increase efficiency, reagents for the ring test would be purchased and assembled by a single facility. It was agreed that PPCDL would work with the NAPPO Secretariat to purchase required reagents. These reagents include PCR primers and probes, buffers, nucleotides, enzymes, reaction mixes, etc. All reagents for the participants have been received, aliquoted and individual packages for the participating laboratories were prepared by staff at PPCDL. As noted earlier, PPCDL also obtained the required seed samples and prepared RNA transcripts, including those provided by CINVESTAV. Each of the five PCR protocols being evaluated requires a specific combination of reagents. All the components have been blinded to reduce potential bias. Each laboratory will receive sufficient reagents and samples to conduct a set of "practice" reactions in addition to the actual ring test.

Panel distribution. To avoid any potential for bias, ensure consistency, reduce variability, and increase efficiency, a different facility (Texas A&M University) that has participated in the pilot project development but will **not** be analyzing ring test samples will assemble randomized, blinded panels and distribute them together with reagent packs to participating laboratories. The actual delivery method for distributing the ring test panel and reagent packages is still being finalized, but the current proposal is to deliver them to U.S. and Canadian laboratories by express carrier and hand carry them to Mexico City for subsequent transport to participating Mexican laboratories.

***Detailed work instructions*.** A file was compiled containing the technical description of each of the five RT-PCR protocols to be evaluated in the ring test and suggested RNA extraction methods. The document was prepared in English and Spanish, detailing the work instructions to conduct conventional RT-PCR, real time RT-PCR assays, and interpreting and reporting results. The document includes a list of references and recommendations for each of the evaluated RT-PCR protocols.

Reporting

The EG agreed that there would be a central repository for collecting and analyzing the ring test data. The APHIS Laboratory Portal (ALP) was chosen as that repository. The ALP is an online service administered by the National Animal Health Laboratory Network (NAHLN; USDA-APHIS-Veterinary Services) that collates massive quantities of data with little manipulation, two traits that will help inform international partners promptly. The USDA-APHIS National Plant Protection Laboratory Accreditation Program utilizes Proficiency Testing modules within the ALP to capture data generated during proficiency testing events. Participating laboratories will need to register with the ALP. Instructions for registering and using the ALP are being drafted and a virtual training session is being planned.

The ring test data will be used to compare the five selected PCR protocols for the following validation criteria:

Limit of detection (LOD)	A validation category that accounts for an assay's percent detection as the material is serially diluted.
Linearity	A validation category that accounts for an assay's amplification efficiency (accuracy), standard error (precision), limit of quantification, and estimated cutoff (sensitivity).
Precision	A validation category that accounts for an assay's variability between tests.
Sensitivity	A validation category that accounts for an assay's percent detection of positive material. This is a subset of selectivity, a validation category that accounts for an assay's global accuracy (total false reactions).
Specificity	A validation category that accounts for an assay's non-specific or cross-reacting amplification in negative material. This is also a subset of selectivity.

The results of the ring test will be compiled in a report and presented to the NAPPO EC. With EC approval, the EG intends to publish the results of the ring test as a peer-reviewed journal article to contribute to the public knowledge on ToBRFV diagnostics.

Budget

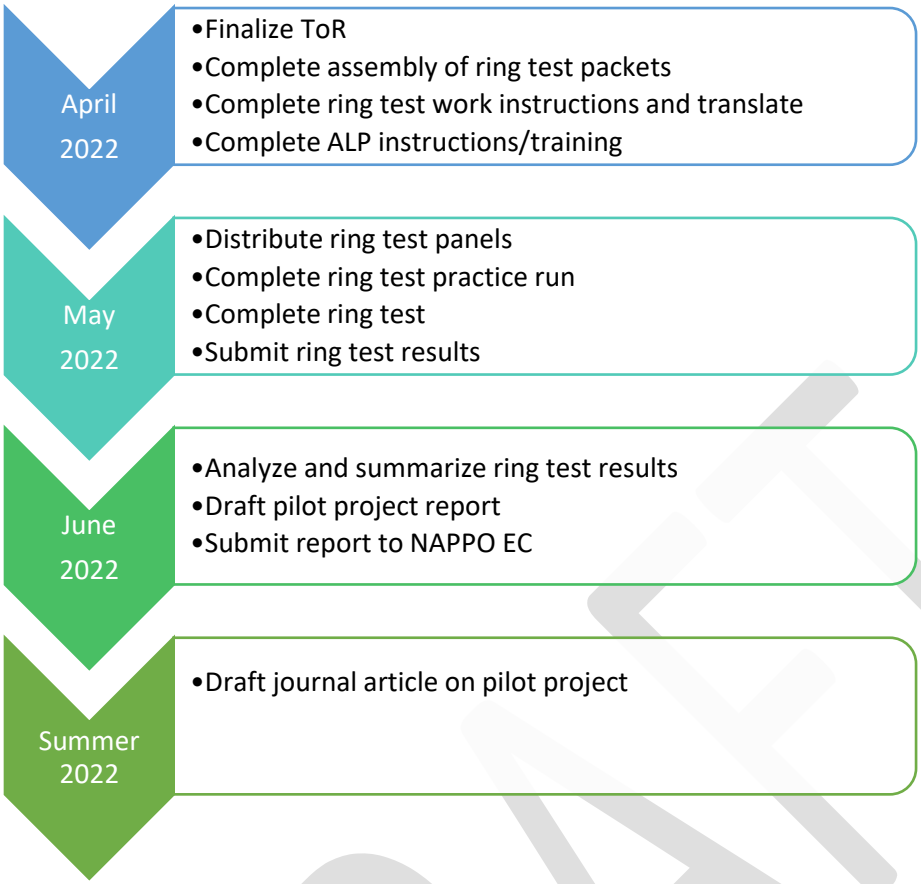
The protocol subgroup also developed an estimate of the costs (excluding labor) for completing the ring test to evaluate the five protocols (**Table 4**). To offset some of the cost to NAPPO, the American Seed Trade Association (ASTA) and the Asociación Mexicana de Semilleros (AMSAC) agreed to contribute $US 15,000.00 and $US 5,000.00, respectively, to the project.

Table 4. **Estimated Budget -** please note that this initial estimate was for 10 laboratories**.**

NAPPO ToBRFV ring test cost estimate				
Cost for participating laboratories (10 labs)				
Extraction and RT-PCR cost	sample #	cost/sample	total samples 10 labs	Cost for 10 labs
CFIA/CDFA MP cRT-PCR	1	$ 8.00	565	$ 5,650.00
SENASICA RdRp cRT-PCR	1	$ 25.00	540	$ 13,500.00
cRT-PCR PPQ (cost sharing)			510	$ 3,982.20
real-time RT-PCR PPQ (cost sharing)			700	$ 7,309.19
ISHI-Veg qRT-PCR protocols cost similar to PPQ			700	$ 7,309.19
15 extractions average per lab	n/a	$ 484.00	n/a	$ 4,840.00
				$ 42,590.58
Cost for ring test panel production and validation				
analytical material preparation				$ 5,500.00
extraction kits and supplies				$ 5,600.00
RT-PCR kits, primer&probes and supplies				$ 12,500.00
shipping cost estimate				$ 3,200.00
				$ 26,800.00
10% miscellaneous & incidentals				**$ 6,939.00**
Total cost				**$ 76,329.58**

Arrangements were made for the laboratories to develop their "shopping lists" then work through the NAPPO Secretariat to make necessary purchases.

Timeline



Conclusion

After a slower than anticipated start to the pilot project prompted by lengthy, but necessary, discussions around operational details and membership changes in the EG, the group has coalesced well and reached consensus on all important points of the pilot. Almost all preparatory work has been concluded and the EG is poised to bring the pilot project to a successful conclusion in the upcoming two to three months.