Ring test provides the basis for harmonization of ToBRFV diagnostic protocols for seeds in the NAPPO region

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ABSTRACT: Global seed trade is subjected to various national, regional and international regulations to prevent the spread of harmful seed-borne and seed-transmitted pathogens including freedom from pest certification in seed lots. The use of different pest detection protocols by trading partners can produce different test results that may require additional analysis resulting in trade delays. Establishing comparability of the protocols used by trade partners can harmonize test results, thus benefiting trade. NAPPO conducted a pilot project on harmonizing diagnostic protocols for a seed-transmitted virus, *Tomato brown rugose fruit virus* (ToBRFV), an emerging pathogen that has hampered tomato and pepper world-wide seed production and trade. The project partnered academia, industry, trade organizations and national plant protection organizations (NPPOs). Five RT-PCR protocols (three end-point and two real-time) were selected for comparability studies via a ring test consisting of analytical, diagnostic and calibrator samples. Nine laboratories from NAPPO countries participated in the ring test, generating 3,680 data points. Four out of five methods were found transferrable between the laboratories, and three of those demonstrated optimal performance for accurate, reproducible and user-friendly detection.

RATIONALE: ToBRFV is a seed transmitted tobamovirus under regulation in Canada, USA and Mexico. Solanaceous seeds are traded heavily among the NAPPO countries, thus ToBRFV is a significant concern for the seed industry and seed trade.

OBJECTIVE: To harmonize diagnostic protocols for ToBRFV testing in seeds in the NAPPO region. Expected benefits include alleviating delays and reducing retest costs, thus facilitating trade and increasing its predictability.

PROTOCOL SELECTION

- Criteria for protocol selection:
  - Molecular assays for ToBRFV
  - Validated data available
  - Used by NAPPO countries or trading partners
- Direct seed testing only
- Specific nucleic acid extraction protocols not prescribed

Table 1. RT-PCR diagnostic protocols A-E selected for the NAPPO ring-test

Protocol ID	Target	RT-PCR Type	Steps	Internal Control	Reference
A	MP	End-point	1-step	NO	T. Tian, CDFA, USA, unpublished
B	MP & CP	Real-time	1-step	YES	ISF ISHI-Veg. 2020. https://worldseed.org/wp-content/uploads/2020/11/Tomato-ToBRFV_2020v1.5.pdf?_ga=2.66722734.1075065090.1644270496-1579089872.1623958967
C	MP	Real-time	1-step	YES	Chanda *et al*. 2021. *Plant Disease*, 105: 3643-3652.
D	CP	End-point	1-step	NO	Dey *et al*. 2021. *New Disease Reports*, 44, e12028.https://doi.org/10.1002/ndr2.12028
E	RdRp	End-point	2-step	YES (2nd reaction)	Rodriguez-Mendoza *et al*. 2019. *Mexican Journal of Phytopathology*, 37(2):345-356.

RdRp: RNA dependent RNA polymerase; MP: Movement Protein; CP: Coat Protein

RING TEST MATERIAL

Table 2. Ring test samples description

ID	Material	Target	Host matrix	Status	Observations	Extraction	Tests
A	Transcript (target mix)	ToBRFV	NO	Unknown	Analytical LOD	No	5 dilutions prepared by the participants
B	Seed tomato (2 samples)	ToBRFV	YES	Unknown	Diagnostic LOD Specificity, Intermediate Precision	YES	2 diagnosticians, 1 sample each, 5 dilutions from each and test.
C	Transcript (target mix)	ToMMV	NO	Unknown	Analytical Specificity	No	2 dilutions ready to test
D	Seed tomato	no	YES	Unknown	Diagnostic Specificity	Yes	Extract and test
E	Seed pepper	no	YES	Unknown	Diagnostic Specificity	Yes	Extract and test
PPC	Seed tomato	ToBRFV	YES	Known	Positive Process Control	Yes	Extract and test
NPC	Seed pepper	no	YES	Known	Negative Process Control	Yes	Extract and test
NPC	Seed pepper	no	YES	Known	Negative Process Control	Yes	Extract and test
Cal	Transcript (target mix)	ToBRFV	NO	Calibrator	QC and data validity	No	5 dilutions ready to test

RING TEST SCHEME

- Parameters evaluated: LOD, sensitivity, specificity and precision
- Nine laboratories participated in the ring test.
- Each lab tested all 5 protocols (A-E)
- A set of 2 known seed samples provided for a pre-test
- Samples tested in triplicate to evaluate repeatability
- Single-source RT-PCR reagents to minimize lab-to-lab variability
- Three SOPs with detailed experimental design descriptions

LOGISTICS

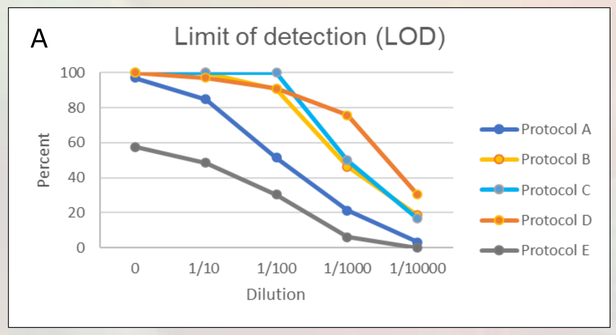
- Import Permits obtained for shipping the ring test panels
- Three blind randomized panels (3 SOPs) were assembled
- Panels' temperature was tracked during delivery
- Data were collected through the APHIS Laboratory Portal
- Training provided to participating laboratories for data entry

PRODUCTION of panel samples and reagents

- Three laboratories cooperated to produce and verify ring test materials
- Produced 382 seed and 120 composite transcript samples with desired target titer
- Characterized and verified
  - Samples homogeneity with each of the five protocols
  - Samples' stability: over time and during transport
- Assembled and validated forty-seven (47) reagent packs for protocols A-E

RING TEST RESULTS ANALYSIS

Fig. 1: LOD (A) and sensitivity (B) for protocols A-E in five 10X serial dilutions of RNA extracted from sample B (ToBRFV infected seed).



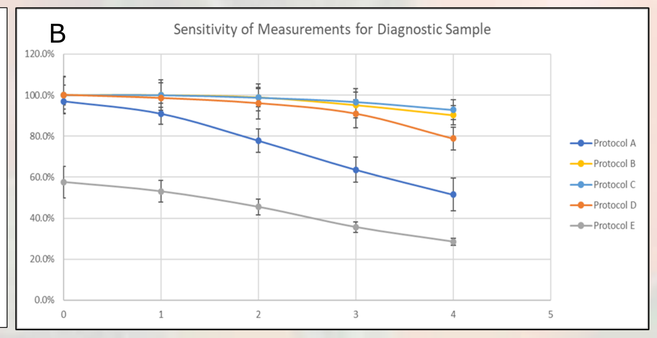


Table 3. Specificity test results for protocols A-E. Results are percent negative ± the 95% confidence interval

Samples used to test Specificity	End-point RT-PCR Protocols			Real-time RT-PCR Protocols	
	A	**D**	**E**	**B** (CP target)	**C**
Sample C (ToMMV) High target concentration	83.3±13.9	100.0±3.9	100.0±3.9	100.0±1.6	92.6±4.1
Sample C (ToMMV) Low target concentration	77.8±10.3	94.4±11.1	100.0±5.1	100.0±1.6	91.7±3.8
Sample D (ToBRFV free)	100.0±4.6	95.8±11.9	100.0±4.6	100.0±1.8	100.0±2.8
Sample E (ToBRFV free)	96.3±11.3	100.0±4.4	100.0±4.6	77.8±3.6	100.0±3.5

Real-time Protocol	Rep	Int Pre	Rpo
Protocol B MP	1.7%	3.6%	8.6%
Protocol B CP	1.5%	3.7%	8.6%
Protocol B Nad5	1.4%	4.4%	5.1%
Protocol C MP	1.9%	4.0%	8.7%
Protocol C Nad5	1.7%	3.7%	7.7%

Table 4. Precision for each target of real-time RT-PCR protocols (B&C) calculated as the coefficient of variation (Standard Deviation/100)
Rep: repeatability of replicates of the same test (run)
Int Pre: intermediate precision: between two diagnosticians in the same lab (sample B)
Rpo: reproducibility of combined replicates for all laboratories.
Nad5: RNA internal control gene

CONCLUSION

- Protocols **A** (T. Tian. Unpublished), **B** (ISHI-Veg/NSHS protocol), **C** (Chanda et al., 2021), and **D** (Dey et. al., 2021) produced comparable results.
- Protocols **B**, **C**, and **D** performed optimally in relation to all assayed parameters and variables evaluated.
- 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.
- Scan the QR code for a full report.

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NAPPO Project Expert Group

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