Final report on a research grant proposal to: American Bramble Growers Research Foundation (NABGRF)

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Project Title: Adoption of a standardized virus diagnostic protocol for blackberry and raspberry in Mexico and later validation through a field survey

Project Duration: 03/01/2023 to 12/31/2023

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Objectives:

1. Obtain and standardize individual RT-PCR assays for raspberry and blackberry viruses.
2. Empirically test and validate the new assays using positive controls.
3. Screen select raspberry and blackberry fields for viruses in Mexico.
4. Incorporate new genomic data into a more complete characterization of genetic variation across *Rubus* viruses.
5. Disseminate research progress and results.

Accomplishments:

Objective 1:
Overall, ten different reverse transcription-PCR (RT-PCR) assays using the primers listed on Table 1 and corresponding protocols were obtained from the Arkansas Clean Plant Center (ACPC, University of Arkansas System). The ACPC is part of the National Clean Plant Network (NCPN) in the USA; consequently, the obtained assays have previously been validated in multiple occasions by different NCPN centers. Subsequently, RT-PCR assays were optimized under the local conditions at the Bioengineering Center in Tecnologico de Monterrey (Mexico) to ensure that each *Rubus* virus is efficiently amplified. Hence, adjustments to these assays mainly involved a change in the annealing temperature of the primers. Given the genetic diversity known to exist for some of the studied viruses, RT-PCR assays may involve more than one set of primers.

Table 1. Standardized assays for detection of pathogens infecting raspberry and blackberry.

Virus Assay	Primer	Sequence	Annealing T°
RBDV	RBDV-1 F	F: AGATCCATGACGGATGT	55
	RBDV-1 R	R: AGATATGCCCACTAGCAG	
	RBDV 2 F	F: AAAGACKYSCAGAAATCCGTTA	
	RBDV 2 R	R: TGWAWARGAAGTTDGCCCATTT	
RLMV	RLMV-CPH F	F: CGAAACTTYTACGGGGAAC	55

	RLMV-CPH R	R: CCTTTGAAYTCTTTAACATCGT	
BRNV	BRNV C2 F	F: TGTTACCCGGGAGAATGGAAGTTGC	55
	BRNV C2 R	R: ATCGAGGACCTCACCCTATCTGAGC	
	BRNV-2 F	F: CAATGTCTTGGAAGCCAC	
	BRNV-2 R	R: AGCATGGTTCGTCATCTG	
TomRSV	TomRSV F	F: CCGTTAGCAGCTTCCAAAAG	56
	TomRSV R	R: GTCCTCATGGAACCTTTCTC	
RYNV	RYNV-6 F	F: CGTGATAACGGCTTGGTTTT	56
	RYNV-6 R	R: CGTAAGCGCAGATTTCTTCC	
	RYNV-3 F	F: TGCTGACATCACACCAACAC	
	RYNV-3 R	R: AGCCTTCAAACTCTTCTCCC	
SLRSV	SLRSV F	F: CCTCTCCAACC-TGCTAGACT	56
	SLRSV R	R: AAGCGCATGAAGGTGTAACT	
SNSV	SNSV MPbeg F	F: GGGATCGATTGGTTAGGACCGTCAT	55
	SNSV MPbeg R	R: CAGTGTTTACGGCTGCGAAG	
	SNSV CPbeg F	F: GAGTATTTCTGTAGTGAATTCTTGGA	
	SNSV CPbeg R	R: ACACCACCATTGCGCATACATCTC	
	SNSV CPbend F	F: ATTATTCTTAATGTGAGGCAACTCG	
	SNSV CPbend R	R: CAGTGTTTACGGCTGCGAAG	
CRLV	CRLV_O F	F: GCCAGTTTCTCCAGTGAACC	60
	CRLV_O R	R: CAGTTGAACGGATTTAACC	
TBRV	TBRV_CP F	F: GCCTGTCTCTCTCGCAATG	60
	TBRV_CP R	R: AAGGAGCCAAACTGAAATGT	
TRSV	TRSV F	F: ATTGGGGTGCTTACTGGCAAGG	60
	TRSV R	R: GGGAGAAAGCTCATTTACACAAGC	

Objective 2:
All the virus assays included in Table 1 were challenged using infected material and healthy plants (virus free controls). The positive controls were obtained from the ACPC via an import permit, and they are used as routine controls in the testing process at the ACPC. These positive controls were RNA extracts from diseased plants and/or virus-mimicking positive controls (ViMAPCs). The ViMAPCs are a novel technology developed by the University of Arkansas System, which avoids the need of a natural infection by the targeted virus. As a result of this initial validation, these assays specifically and efficiently detected the different studied pathogens, generating clean amplicons with the corresponding size. On the other hand, healthy plants tested negative during the screening using the RT-PCR assays.

Objective 3:
To further test the efficiency of the new assays, during the spring and summer of 2023, a total of 30 samples were obtained from raspberry and blackberry populations with a historically high incidence of pathogens or with virus-like symptoms (Figure 1). These *Rubus* populations included commercial fields located in Jalisco, Michoacan and Guanajuato (Table 2).

Figure 1. Blackberry and raspberry plants collected during the field survey.

Table 2. Raspberry and blackberry samples from commercial fields and analyzed by RT-PCR.

Origin	Plant	Sample ID
Michoacan (Zamora)	Blackberry	BK-1
		BK-2
		BK-3
		BK-4
		BK-5
		BK-6
		BK-7
		BK-8
		BK-9
Jalisco (San Antonio Matute)		BKS-1
Jalisco (Jocotepec)		BKD-1
Michoacan (Patzcuaro)		BKP-1
		BKP-2
Jalisco (Jocotepec)	Raspberry	RAD-1
		RAD-2
		RAD-3
		RAP-1
		RAP-2
		RAP-3
Jalisco		RA-1
		RA-2
Guanajuato		RAG-1
		RAG-2
		RAG-3
		RAG-4
		RAG-5
		RAG-6
		RAG-7
		RAG-8
		RAG-9

As a result, five *Rubus* samples tested positive for black raspberry necrosis virus (BRNV), strawberry necrotic shock virus (SNSV), cherry leaf roll virus (CLRV), tomato ringspot virus (ToRSV) and Rubus yellow net virus (RYNV) by the RT-PCR assays. Subsequently, virus infection was confirmed by Sanger sequencing. The identification of the previous mentioned viruses represents the first detection of RYNV, BRNV, SNSV, CLRV and ToRSV infecting *Rubus* plants in Mexico. These results provide updated information on virus occurrence in berry crops in Mexico and help growers in their disease management decisions.

Objective 4:
As part of this research project, genetic diversity of *Rubus* viruses identified in Mexican commercial fields was investigated by Sanger sequencing. Consequently, 5 partial genome sequences of RYNV, BRNV, SNSV, CLRV and ToRSV were obtained and deposited in the

GenBank database. The nucleotide sequence of these isolates exhibited considerable homology with known variants previously characterized from the USA. The addition of new genetic data results in a more complete description of genetic variation across the studied pathogens.

Objective 5:
Results of this study have been presented during growers' meetings organized by the Tecnologico de Monterrey. A press release will be shared with local growers and stakeholders; likewise, a peer-reviewed scientific article is being prepared and will be submitted to the Plant Disease journal by the end of March 2014. A poster will be presented during the 2024 American Phytopathological Society (APS) annual meeting. Lastly, the novel detection tools (RT-PCR assays) are being shared with diagnostic labs, public and private, and plant pathologists involved in the berry industry in Mexico.

Summary:
The most efficient way of controlling viruses in raspberry and blackberry involves using pathogen exclusion strategies aided by early detection. The main goal of this project was to harmonize the *Rubus* virus testing conducted in Mexico with USA's protocols. Thus, the here-adopted testing process will be made available to local diagnostic labs aiming an effective control of raspberry and blackberry viruses in Mexico and beyond. This work is suitable as there has been a significant increase in movement of plant material inside and outside the country, highlighting the necessity for more robust pathogen detection analyses. Additionally, RT-PCR assays were used to screen select raspberry and blackberry populations for targeted viruses. As a result, five *Rubus* plants collected in Michoacan, Jalisco and Guanajuato were determined positive for RYNV, BRNV, SNSV, CLRV and ToRSV, such plants exhibited different virus-like symptoms. This is the first report of RYNV, BRNV, SNSV, CLRV and ToRSV infecting *Rubus* in Mexico, which expands the known virus population (virome). The next logical step should be implementing procedures to prevent outbreaks of virus diseases in berry crops in Mexico.