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Research Final Report #140
Disease Management
Development of a grower friendly detection method for viruses infecting ornamentals

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1. Background

Tobacco mosaic virus (TMV), *Hosta virus X* (HVX), *Cucumber Mosaic Virus* (CMV), *Tomato spotted wilt virus* (TSWV), and *Impatiens necrotic spot virus* (INSV) are major pathogenic viruses infecting ornamental and nursery plants and causes significant losses impacting growers and the ornamental industry. These five viruses exist in complex epidemiological environments artificially created, and share common areas with a variety of ornamental plants during propagation and different insect vectors species that may feed on these hosts. As a consequence, these viruses are inadvertently spreading in propagative materials of ornamental plants and are being disseminated intensively.

The economic impact of plant viruses on ornamental crops is high because symptoms frequently do not develop during production stages and may not be identified until the plants reach the retailer or customer (Sadof and Sclar 2002, Malcolm, 2006, Hayward, 2008). This can result in limitations on the exportation of a number of plant ornamental products. Frequently, nursery growers ship seemingly healthy plants, which are later discarded or set on hold or rejected by retailers or government regulatory agencies. Moreover, sampling and shipping ornamental samples to diagnostics laboratories, and the timely return of accurate results so that growers can make proper disease management decisions, are challenging tasks. Novel user-friendly instruments and virus detection protocols can facilitate the improved ability to produce virus-free nursery stock. These tools can be used by both growers who require ability to make timely disease management decisions in farms and scientists, such as breeders and plant pathologists, to implement such methods in plant breeding programs and development of new integrated pest management (IPM) guidelines. We hypothesize that combining such technological tools allows prototyping a farm friendly kit and methods.

The objectives of this project were: **(1)** To develop five Helicase Dependent Amplification (HDA) assays for grower-friendly detection and identification of ornamental plant viruses, including the following: *Impatiens necrotic spot virus* (INSV)*, Tomato spotted wilt virus* (TSWV)*, Cucumber mosaic virus* (CMV)*, Tobacco mosaic virus* (TMV)*,* and *Hosta virus X (*HVX). **(2)** To develop a 'HDA Grower Kit' and validate a novel Elution-Independent Collection Device (EICD) designed for rapid collection of microorganisms and recovery of nucleic acids to include a multi-target, non-infectious, clonable artificial positive control. **(3)** To develop a Multiplex RT-PCR for the simultaneous detection of these five viruses on ornamentals to be applied by trained diagnosticians if confirmation is required by a nursery. **4)** To transfer both

HDA and Multiplex RT-PCR technologies nation-wide through outreach including a 'hands on' workshop introducing guidelines for standard operational procedures, and cost analysis.

2. Materials

2.1 Extractions using Elution Independent Collection Devices

Rather than using conventional nucleic acids (DNA/RNA) extraction kits commercially available, we will use a novel Elution-Independent Collection Device (EICD). EICD was conceived to allow a rapid (3 min.) sample collection-preparation of microorganisms, nucleic acid recovery and long-term room temperature storage, and requires far less time than the 10-30 minutes required using commercially available kits (1). EICD proved to streamline sample processing for PCR based assays or ELISA. EICD is easy-to-operate, collects fluid specimens by contact and lateral flow filtration. The Office of Intellectual Property of Oklahoma State University is actively seeking for potential licensees for EICD (US patent 9423398 B2). EICD uses a soluble matrix to collect fluids by contact and lateral flow. Subsequently, minute pieces (1.2 mm diameter) of the central soluble element of EICD will be dissolved directly in commercial RT-PCR and RT-HDA mixtures without an intermediate elution step, thereby streamlining PCR and HDA based assays. Samples of the five virus studied were collected with EICD and tested by both RT-PCR and RT-HDA

2.2 Reverse Transcription Helicase Dependent Amplification (HDA)

Primer pairs for Reverse Transcription-Helicase Dependent Amplification (RT-HDA) were designed to amplify regions from a conserved segment of the capsid protein gene of HVX and CMV; nucleocapsid protein gene of TSWV and INSV, and movement and capsid protein gene of TMV. The primers were identified as CMV 1F/1R, INSV 1F/1R, HVX 1F/1R, TSWV 1F/1R, and TMV9F/9R amplify diagnostic products of 78, 79, 118, 107, and 110 bp, respectively (Table 1). The oligonucleotide primer sequences were designed using the Web-interface applications Primer3 (Rozen & Skaletsky, 2000), following described specifications for HDA (Goldmeyer et al. 2007).

The primers were examined *in silico* (with software assistance) for their thermodynamics, internal structures, and self-dimer formation using mFold (Zuker et al., 2003) following described parameters (Arif and Ochoa-Corona, 2013). The specificity of each primer was subsequently confirmed *in silico* using BLASTn (Altschul et al., 1990) and direct sequencing of the obtained products.

Lypholized infected sap from Agdia Inc. (Elkhart, IN) was used as the reference positive control for targeted TMV, HVX, CMV, TSWV and INSV. Additionally a multi-target, non-infectious, clonable, artificial positive control (APC) was also constructed as previously described by Caasi et al. (2013). The expected product of each primer set using the artificial positive control is of 80 bp for primer sets CMV1F/1R, INSV 1F/1R, and HVX 1F/1R, and of 81 bp for TSWV 1F/1R and TMV9F/9R (Fig.1). The RT-HDA protocol was also adapted to perform in single step format (reverse transcription and HAD reaction in a single step and tube) using crude sap from plant directly as the source of viral RNA, called one step RT-HDA .

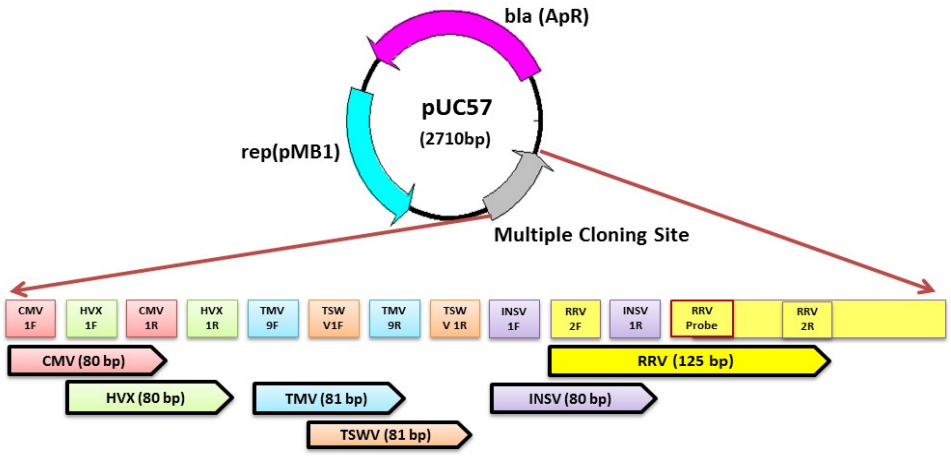

Figure 1. Multi-target APC carrying multiple custom synthetic APC inserts, not to scale. The insert is made by custom synthesized tandems of forward and reverse complement priming sequences ligated into the multiple cloning site of pUC57 to make a circular and clonable, non-infectious construct. Each PCR product amplified from the APC construct has a unique identifiable nucleotide sequence useful for further PCR-product verification and quality control. A *Rose rosette virus* (RRV) positive control that generates a product of 125 was alsoincluded.

2.3 Inclusivity and exclusivity assays. These two assays were performed to determine the specificity of the primers. The inclusivity panel consisted of the positive control of the target viruses including *Tobacco mosaic virus* (TMV), *Hosta virus X* (HVX), *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), and *Impatiens necrotic spot virus* (INSV) (Agdia, Inc, Elkhart, IN). The exclusivity panel consisted of eleven related (near-neighbor) virus positive controls (Agdia) which include: *Cucumber green mottle mosaic virus* (CGMMV), *Kyuri green mottle mosaic virus* (KGMMV), *Odontoglossum ringspot virus* (ORSV), *Potato virus X* (PVX), *Pepino mosaic virus* (PepMV), *Potato aucuba mosaic virus* (PAMV), *Iris yellow spot virus* (IYSV), *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV), *Tomato aspermy virus* (TAV), and *Peanut stunt virus* (PSV).

2.4 Sensitivity assays. These assays were performed using the plasmid DNA and infected host tissue to determine the detection limit of the each primer set. RT-HDA sensitivity assays were assessed and validated performing the assays in a thermal cycler, and water bath incubators (Fig.2). The sensitivity of the RT-HDA and Reverse Transcription-PCR (RT-PCR) were compared. RT-PCR was considered the reference method.

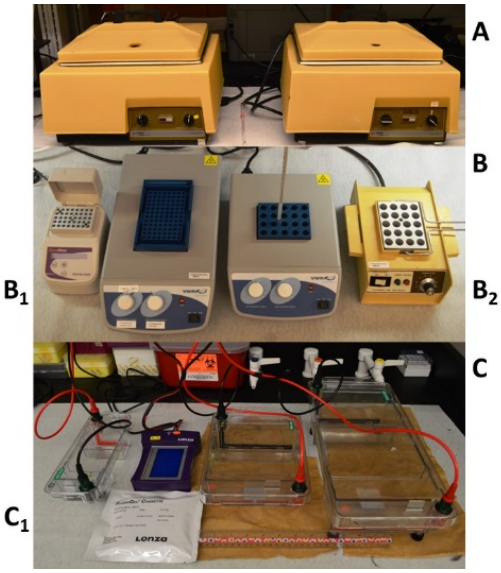

Figure 2. Different equipment tested during HDA experimentation. A) Standard water baths. B) Different types of dry bath tested, B1 allows loading reaction tubes in PCR like blocks and B2 have wider-loose wheels that were filled with water or mineral oil. Best results were obtained with B1 (GeneMate mini dry bath from Bioexpress, Cat. No. D-2260) and standard water baths. C) Electrophoresis equipment tested showing the fast precasted system (C_1) from Lonza and standard electrophoresis. Other similar and competitive equipment are available in the market. C_1 showed to be the most friendly to use for non-skilled operators.

2.5 HDA with dried reagents seeking simplification of the assay. This method was tested using primers specific for CMV and HVX. The aim of the approach was developing ready to use assay tubes for direct use on farms. The experiment explored developing HDA assay reaction tubes containing all reagents dehydrated ready for reconstitution with water for easy and rapid use in point of care or nurseries. The components of this dry reaction format were prepared in the lab by drying out the reaction components in a vacuum DNA concentrator. The tubes were prepared for use after 24 h, 7 days, 14 and 30 days. All tubes were left at room temperature once the pellet was formed. Once the tubes were ready infected plant samples were crushed using a pestle in ready to use samples pouches containing extraction buffer (Agdia, Inc. Elkhart, IN) as shown in figure 3.

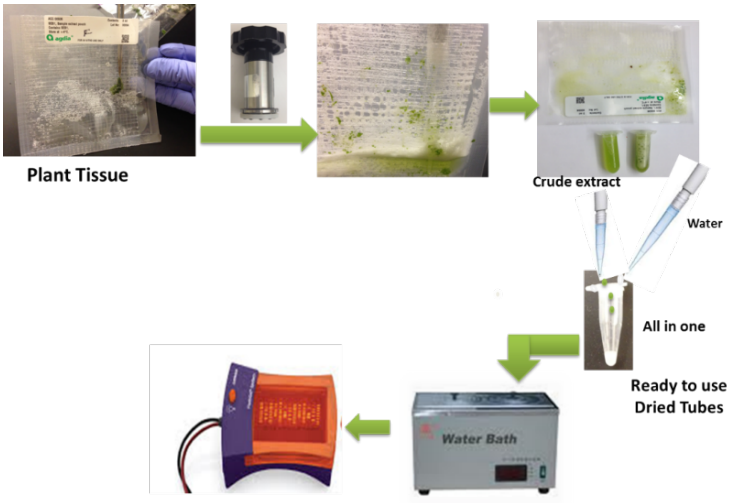

Figure 3: Representation of rapid and direct sample collection for RT-HDA for field detection of a targeted virus from infected plant samples.

2.6 Reverse Transcription Multiplex PCR (RT-mPCR)

A RT-PCR for the simultaneous detection of the selected five viruses on ornamentals was developed for use of trained diagnosticians if confirmation is required by a nursery. Primer pairs for Reverse Transcription multiplex PCR (RT-PCR) were designed to amplify targeted gene sequences of each virus that were aligned using CLUSTALX (Larkin et al., 2007) and examined for conserved regions. PCR primers specific for detection of HVX and CMV were designed using the consensus sequences of aligned capsid protein (CP) genes. The primer pairs for TSWV and INSV were designed for targeting the consensus sequences of nucleocapsid protein (NP) genes. Primer TMV10F was designed for targeting the movement protein (MP) gene of TMV while TMV10R was designed for using the CP gene. Oligonucleotide primers sequences were designed using the Web-interface applications Primer3 (Rozen & Skaletsky, 2000), and were

subsequently examined *in silico* for their thermodynamics, internal structures, and self-dimer formation using mFold (Zuker et al., 2003) following described parameters (Arif and Ochoa-Corona, 2013).
The inclusivity panel consisted of positive control from Agdia (Agdia, Inc, Elkhart, IN) which include TMV, HVX, CMV), TSWV, and INSV, and eleven near-neighbor virus positive controls (exclusivity panel), CGMMV, KGMMV, ORSV, PVX, PepMV, PAMV, IYSV, GRSV, TCSV, TAV, and PSV.
The specificity of each primer was confirmed *in silico* using BLASTn (Altschul et al., 1990).
Single virus RT-PCR and RT-mPCR assays were validated using reference positive controls, and sensitivity assays. These two methods were also assessed before screening field samples. Forty-six infected ornamental plant samples consisting of 25 different plant species were collected from five states: Indiana, Illinois, Pennsylvania, Oklahoma, and Texas (Annex 1), and were screened using this assay for the five selected plant viruses.
In any case the *nad5* plant gene was targeted as internal control to avoid false negatives.

3. Results and discussion

3.1 Sample collection and the Elution Independent Collection Device

Data from a previous project and this project had shown that diagnostic targets of eleven different viruses, fifteen bacteria, one fungus, one insect and one plant gene (used as internal control) were successfully amplified in one-step RT-PCR assays without intermediate RNA extraction using commercial kits. Identical results were obtained with the five studied viruses. EICD prototypes were effective for use with sap from infected plants. EICD is a simple rapid sampling choice for plant health biosecurity, health, veterinary, forensics, and food quality applications. Results obtained with the five studied viruses indicate the EICD can be used for collection and storage at room temperature of infected plant sap and therefore can be part of the kit (Figure 12).

3.2 Reverse Transcription-Helicase Dependent Amplification

New designed primer sequences for five independent, one-step, single tube, reverse transcription helicase dependent amplification (RT-HDA) reactions are provided in Table 1.

Virus	Primer code	Primer Sequence (5-3')	RT-HDA product size (bp)
CMV	CMV1F	CGCGCATTCAAATTCGAGTTAATCCTT	78
	CMV1R	AGGCAGGAACTTTACGGACTGTCACC	
INSV	INSV1F	TGGAATGATAAAGGACAATGGATCTGC	79
	INSV1R	ATATGAGGCAATCAGAGGGTGACTTGG	
TSWV	TSWV1F	TCAGGCTTGTAGAGGAAACTGGGAATT	107
	TSWV1R	TGCATCATCAAGAGGTAATCCATAGGC	
TMV	TMV9F	CGGTGGCCATAAGGAGCGCTATAAATA	110
	TMV9R	AGAGGTCCAAACCAAACCAGAAGAGCT	
HVX	HVX1F	CGATGGGCTACAAAGAAAACACGAAGT	118
	HVX1R	AGCTCCTCCTCTGTTGGTTGTCTGATT	

Table 1. Primers sequences to specifically detect and discriminate CMV, INSV, TSWV, TMV and HVX by RT-HDA.

All primers were found to be specific for each of the targeted plant viruses and no cross-reactivity was observed against eleven near-neighbor non-target viruses (exclusivity panel). Sensitivity of RT-HDA using plasmid

DNA is 1 fg for TMV, HVX and CMV and 10 fg for TSWV and INSV (Fig. 4).

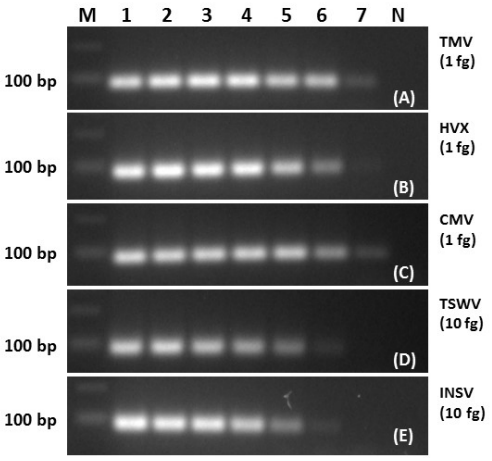

Figure 4. RT-PCR Sensitivity Assays using ten fold serially diluted plasmid DNA (Artificial positive control) from 1 ng to 1 fg. Primers used were: (A) TMV 9F/9R primers, (B) TSWV 1F/1R primers, (C) HVX 1F/1R primers, (D) CMV 1F/1R primers, and (E) INSV 1F/1R primers (Table 1). Lane M, 100 bp ladder, , Lane 1- 1ng, Lane 2-100 pg, Lane 3-10 pg, Lane 4-1 pg, Lane 5-100 fg, Lane 6-10 fg, Lane 7-1fg, Lane N-non-template control (NTC, water).

3.2.1 RT-HAD Thermal cycler vs. water bath.

RT-HDA was tested in a thermal cycler (Eppendorf) and water bath (Thermo scientific) respectively using cDNA made from RNA from reference positive control infected sap. The sensitivity of RT-PCR was 1 fg for all five viruses (Fig.4 A1-E1). The sensitivity of RT-HDA is 1 fg for TMV, TSWV and HVX (Fig.4. A2-C2), and 10 fg for CMV and INSV (Fig.4. D2-E2). Differences in sensitivity performing RT-HDA in thermal cycler or water bath using the same primer set (Table 1) is minimal (10 fg), and only detectable with CMV and INSV primer sets (Fig.5. D1-D2 and E1-E2).

3.2.2 RT-HAD vs. RT-PCR.

RT-PCR was used as reference method, and was compared with RT-HDA using the primers developed for RT-HDA (Table 1), Figure 6). RT-PCR and RT-HDA were performed with ten fold serially diluted cDNA obtained from infected sap reference positive control. The RT-PCR limit of detection or sensitivity varied within a range of 1-10 fg and 1-100 fg for RT-HDA. In general, it is evident that RT-PCR allows brilliant amplified products and ten-fold average lower sensitivity compared to RT-HDA. The variation between these two methods is be due to differences in the thermodynamics of the two different reaction and differences in the polymerases used. RT-PCR depends on the thermal cycler temperature to unwind the strands of DNA, whereas RT-HAD uses a helicase mix to unwind the DNA strands for the polymerase extends the chain synthesis similarly as in PCR. However, even if less brilliant products the reaction is still clearly visible.

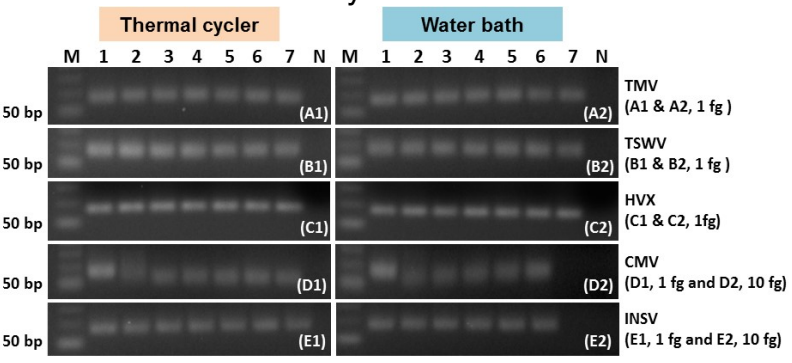

Figure 5. RT-HDA Sensitivity Assays. Comparison of RT-HDA using thermal cycler (left) and water bath (right) with (A) TMV 9F/9R primers, (B) TSWV 1F/1R primers, (C) HVX 1F/1R primers, (D) CMV 1F/1R primers, and (E) INSV 1F/1R primers. Lane M, 50 bp ladder. Lanes 1-7 serially diluted plasmid DNA from 1 ng to 1 fg, Lane N, non-template control (NTC; water

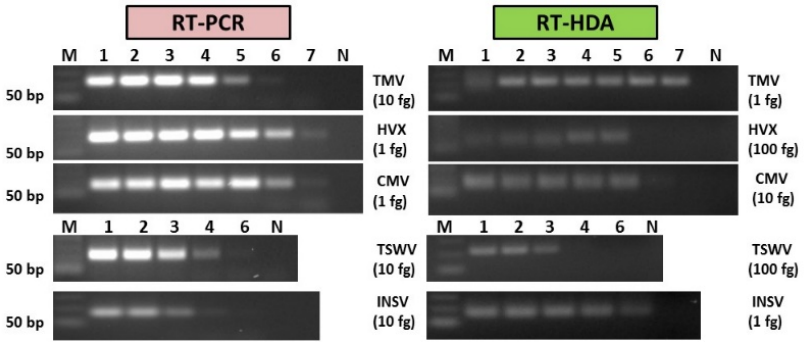

Figure 6. Comparison of sensitivity between RT-PCR and RT-HDA from serially diluted cDNA. Viral targets were amplified using primers TMV 9F/9R, HVX 1F/1R, CMV 1F/1R, TSWV 1F/1R, and INSV 1F/1R (Table 1). The limit of detection of each primer set is indicated at the right of each gel picture. Lane M, 50 bp ladder. Lanes 1-7 are serially diluted infected host cDNA from 1 ng to 1 fg, Lane N, non-template control (NTC; water).

3.3 One step RT-HDA (Reverse transcription HDA)

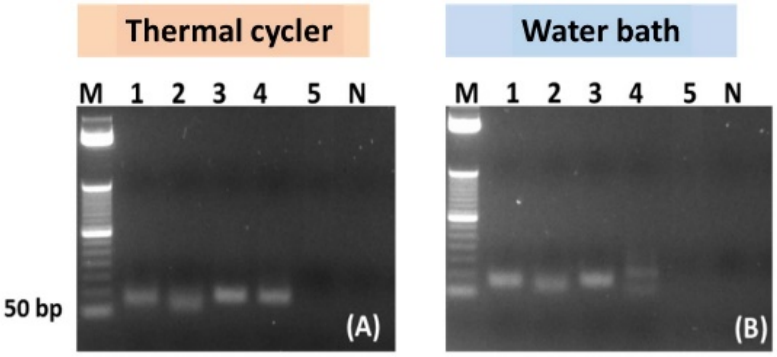

Figure 7. One step RT-HDA using plant sap (directly) with HDA primers shown in Table 1. The same experiment was performed in both a thermal cycler (left) and in a water bath (right). Lane M, 50 bp ladder. Products are from crude extract of infected plant sample. Lane 1 is CMV, lane 2 is HVX, lane 3 is INSV, lane 4 is TMV, lane 5 is TSWV, Lane N is non-template control (NTC; water).

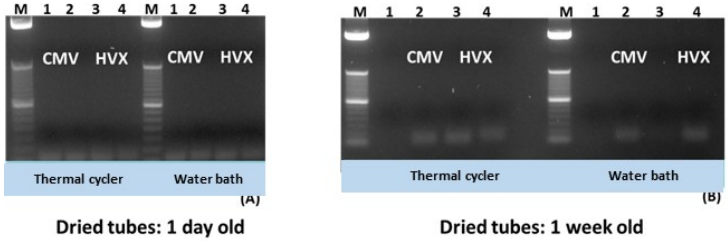

Figure 8. One step RT-HDA using dried tubes using HDA primers shown in Table 1. Reactions on the left were performed in thermal cyler and on the right with a water bath. (A) Dried HDA tubes after 24 hours. Lane M, 100 bp ladder, lane 1,2 crude extract of plant sample infected with CMV, lane 3,4 crude extract of plant sample infected with HVX. (B) Dried HDA tubes after a week. Lane M, 50 bp ladder, lane 1,2 crude extract of sample infected with CMV, lane 3, 4 crude extract of sample infected with HVX .

3.4 Reverse Transcription Multiplex PCR

New designed primer sequences for single tube, two-steps, reverse transcription multiplex endpoint PCR (RT-mPCR_are provided in Table 2.

Virus	Primer code	Primer Sequence (5-3′)	RT-mPCR product size (bp)
TMV	TMV10F	GGGTTTCTGTCCGCTTTCTCT	493
	TMV10R	CGACAGTTCGAGCTTGTTGTG	
HVX	HVX2F	TCATTAGCACAAGGCTCCCAAG	362
	HVX2R	TGAGGATGGAGTCGAAGGTGT	
CMV	CMV2F	GAGTCTTGTCGCAGCAGCTTT	249
	CMV2R	TCGGCAAAGGATTAACTCGAA	
TSWV	TSWV2F	ATTGCTTCCCACCCTTTGATT	181
	TSWV2R	GGTCAATCCCGAGGTCTTTGT	
INSV	INSV4F	CCGAAGACTTGATGCAATGGT	112
	INSV4R	TGAGGCAATCAGAGGGTGACT	

Table 2. Primers sequences for single, multiplex, and specific detection and discrimination TMV, HVX, CMV, TSWV, and INSV, by RT-mPCR. Note the different size of the products respect table 1.

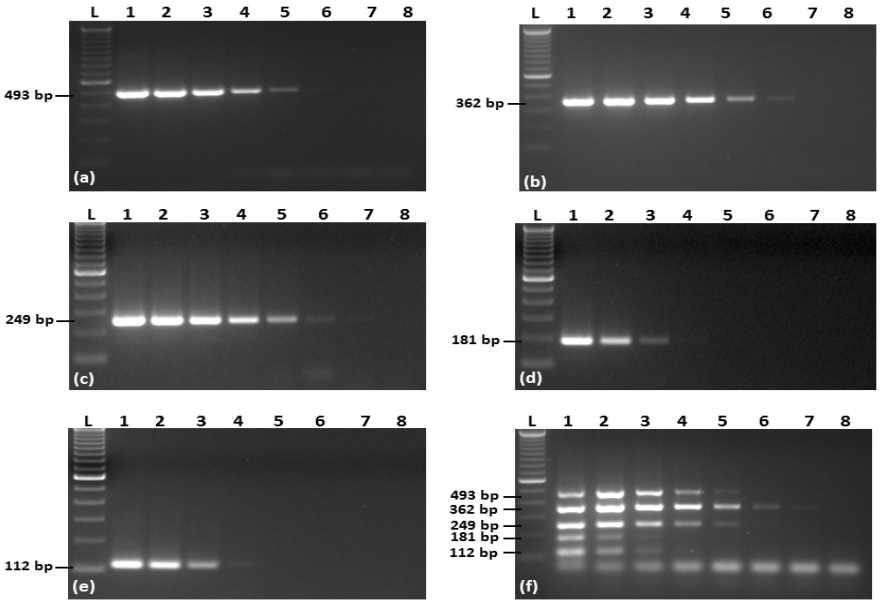

Figure 9. Specificity of multiplex RT-PCR using a mixture of five primer pairs TMV10F/10R (493bp), HVX2F/2R (362 bp), CMV2F/2R (249bp), TSWV2F/2R (181bp) and INSV4F/4R (112 bp) (Table 2). Lane L: 100 bp ladder; lane1: mixture of cDNAs to include *Tobacco mosaic virus* (TMV), *Hosta virus X* (HVX), *Cucumber Mosaic Virus* (CMV), *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus*(INSV) from reference positive control infected sap. NTC (non-template control, water). The sensitivity of this method range between 100 fg to 10 pg. TMV 1 fg, HVX 10 fg, 10 pg CMV, TSWV and INSV.

Figure 10. Sensitivity assays of single (a-to e) and multiplex (f) RT-PCR performed using 10-fold serial dilution of viral cDNA from targeted reference positive control infected sap. (a) Single RT-PCR using primer set TMV2F/2R from TMV cDNA, (b) Single RT-PCR using primer set HVX2F/2R from HVX cDNA, (c) Single RT-PCR using primer set CMV2F/2R from CMV cDNA, (d) Single RT-PCR using primer set TSWV2F/2R from TSWV cDNA, (e) Single RT-PCR using primer set INSV4F/4R from INSV cDNA. Figures: a-e, lane L: 100 bp ladder; lane 1-7: 10-fold serial dilution of cDNA from 1 ng to 1 fg per reaction; lane 8: NTC (non-template control, water). (f) Multiplex RT-PCR using 10-fold serial dilution of a mixture of cDNAs to include TMV, HVX, CMV, TSWV, and INSV and a mixture of five primer sets (Table 2). Lane L: 100 bp ladder; lane 1-7: 10-fold serial dilution of cDNAs (TMV, HVX, CMV, TSWV, INSV) starting from 1 ng to 1 fg per reaction; lane 8: NTC (non-template control, water).

3.5 Field validation of RT-mPCR

Among the 45 field samples tested, 30 were found to be infected Annex 1. Nine samples were found infected with more than one virus and seven samples were found co-infected with two

different viruses. One sample (Lobelia) was found to be co-infected with four (TMV, CMV, TSWV and INSV) and Petunia was co-infected with three viruses (TMV, TSWV and INSV). None of the samples tested positive for
all five viruses simultaneously.

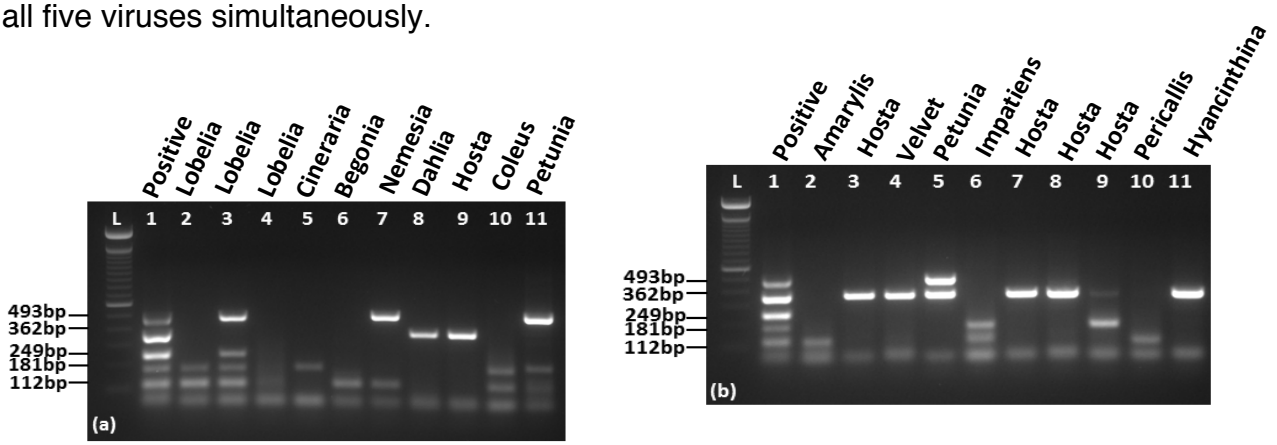

Figure 11. Simultaneous detection of naturally occurring virus infection in nursery samples using multiplex RT-PCR. (a) Lane L: 100 bp ladder, lane 1-11: Multiplex positive control, PL1, PL2, PL3, PC1, PB1, PN1, PD1, PH1, PCA1, PP1, respectively; (b) Lane L: 100 bp ladder, lane 1-11: lane 1 is a multiplex positive control, lanes 2-11 correspond to samples A20, H27, V27, P27, P622, H12, H13, HF2, P68, and HY2, in Annex 1 respectively.

4. Cost Analysis

4.1 Cost of methods

Method	[1]Extraction cost ($)	[2]EICD	[3]Cost per virus and pool of five viruses ($)	[4]Total cost* ($)
Endpoint RT-PCR	10.5	To be determined by the manufacturer	0.35 per virus, (0.35x5)= 1.75	12.25
RT-mPCR	10.5	To be determined by the manufacturer	0.35 per 5 viruses = 0.35	10.85
RT-HDA	10.5	To be determined by the manufacturer	2.68 per virus, (2.68x5)= 13.4	23.9
RT-HDA kit	Leaf tissue directly macerated in the pouch	To be determined by the manufacturer	6.38 per virus (6.38X5)=$31.9 Thermoscript RT per virus = $2.17 (5X2.17)=$10.85	58.74

			Trehalose per virus= $ 0.03. (5X0.03)=$0.15 Lonza precast gels=$15.84	
RT-qPCR (SYBR Green)	10.5	To be determined by the manufacturer	0.93 per virus, (0.93x5)= 4.65	15.15

[1]The RNeasy plant minikit (Qiagen) for RNA extraction, costs about $6.50, and synthesis of 10 µl of cDNA from the RNA cost about $4. Therefore, a total of about $10.50 per sample.
[2]The cost of the Elution independent collection device (EICD) is yet to be determined by the manufacturer.
[3] Only Includes the cost of reagents used for amplification, and may vary among manufacturers.
[4]Total cost do not include labor charges, storage, consumables and indirect costs.
Grower

4.2 Cost of Standard essential lab vs. HDA Grower Kit

Standard essential lab kit equipment for laboratories	**Estimated Cost ($) (prices may vary among distributors)**	**HDA Grower Kit**	**Estimated Cost ($) (prices may vary among distributors)**
Dry block (for cDNA)	$600	GeneMate minidry bath with PCR tube block	$400
Thermal cycler	$2000-8000	LONZA FlashGel® Dock	$440
Gel electhrophoresis tank	$600	LONZA Flasgel DNA cassette	$110
Gel electrophoresis power supply	$400	Gel electrophoresis power supply	$400
Two micro Pipettes for liquid handling (100-1000 μL and 20-2000μL)	$400	Two micro Pipettes for liquid handling (100-1000 μL and 20-2000μL)	$400
		Fixed volume micropipettes, or alternatively regular pipettes as listed below	$100

Positive control. Infected tissue may require USDA permit or accreditation.	$15-30	Artificial positive control Not infectious, no permit required (generated during this research)	To be determined by the manufacturer
Total capital investment	$4,000-10,000	**Total capital investment**	$1,450-1,750

4.3 Technology transfer.

Both RT-HDA and RTmPCR protocols were transferred to the Oklahoma State University, Plant Disease and Insect Diagnostic Laboratory (PDIDL) including 'hands on' and protocols.

5. Conclusions

Two detection-identification methods were developed for TMV, HVX, CMV, TSWV and INSV.

The first method amplifies the targeted viruses by HDA at a fixed temperature (isothermal) and the second is a multiplex endpoint RT-PCR (standard multiplex). The HDA protocols are for health assessment of propagative plant material of propagation blocks at nursery level. The multiplex RT-PCR was developed for detection and/or confirmation of the same five viruses in samples sent to plant clinics/laboratories by nurseries implementing HAD and seeking confirmation. The use of a sample device (EICD) is applicable to both of the methods and by nurseries and subsequently by the diagnostic lab (after shipping) ensures repeatability of these two methods targeting without plant variability the same specimen (Fig. 13).

Moreover, HDA reactions occurs at constant temperature, which allows the use of an alternative source of heat such as a dry block or a water bath in substitution of the thermal cycler, reducing capital expenditure and training of personnel because dry blocks or water baths are easy to use and to implement if compared to thermal cyclers and standard multiplex RT-PCR. This study proposes a prototype of grower friendly virus-diagnostic kit and protocols for five independent, one-step, single tube, reverse transcription helicase dependent amplification (RT-HDA) reactions (Fig. 12). HDA amplifies DNA products no larger than 120 base pairs (bp). This limitation did not allowed developing a multiplex HDA since HDA products are distinguishable by visual means when differing by 40-50 bp only. The technology transfer and hand-on training for non-specialized nursery personnel is still required, however, is not intensively demanding.

The second method is an endpoint (standard) two steps reverse transcription multiplex PCR (RTmPCR) that uses a second set of five primers designed for either simultaneous detection or specific discrimination of the five-targeted viruses or alternatively one virus at the time in single tube reactions. This RTmPCR method performed well during a survey of 25 different host species.

This method was developed for use in diagnostics networks and routine diagnostics in laboratories equipped with thermal cyclers and specialized personnel. The method has

additional applications in disease management, routine screening of ornamentals and nursery plants during breeding or biosecurity, microbial forensics.

Both of these methods RT-HAD and RTmPCR are sensitive, specific and able to detect mixed infections of the targeted five plant viruses.

The capital investment of nurseries seeking stepping up over self-diagnostics capabilities is low if implementing HDA or an alternative isothermal methods, if compared to standard molecular RT-PCR (Section 4.2, Cost of standard essential lab vs. HDA grower kit). The race for developing rapid and friendly to use point of care methods by non-skilled personnel is searching attentively for new accurate regents and technologies with detail, and therefore new technological additions in this field are expected in the new future.

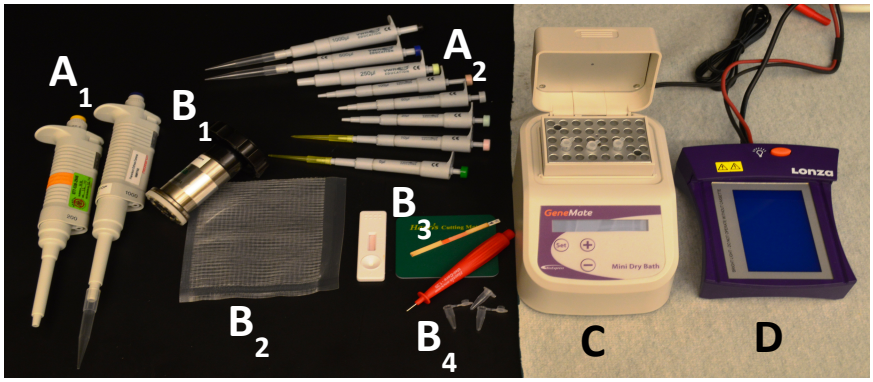

Figure 12. Grower friendly kit for HDA. The kit may use either (A_1) regular pipettes or alternatively (A_2) fixed volume micropipettes for small volume handling of liquids. Plant tissue samples are macerated with a tissue homogenaizer (B_1) in mesh plastic sample bags (B_2) from where 0.5 ml is transferred to an EICD (B_3). A 1.2 mm diameter or very small section of the EICBD membrane is excised with a Harris pouncher (or alternative brand) on a clean vinyl mat and transferred into a PCR tube loaded with the RT-PCR mix (B_4). (C) HDA reactions are incubated in a GenMate dry-bath and (D) electrophoresis and visualization of the HDA products is performed in a fast pre-casted system from Lonza. Other similar and competitive Brands are available in the market. Mesh plastic sample bags preloaded with buffer are commercially available.

6. Impact to the industry

This research project explored the development of an isothermal HDA detection method to detect five plant viruses within a perspective of a broad plant virus detection platform. The platform will function integrating ornamental growers that collect own samples and self-perform HDA tests at the nursery to assist timely integrated pest management decision-making and/or pathogen-free certification purposes. If confirmation is desired, EIDCs will be mailed to the diagnostic laboratory, where further RT-PCR testing allows confirmation that will be communicated back to nursery personnel. Shipping of EIDCs guarantees an exact confirmation from the original source tested at the farm, and allows samples can be stored for further testing or monitoring purposes at room temperature for a year.

The envisioned approach is possible for ornamental growers seeking speeding up sanitary decision making at their nurseries incorporating low cost molecular diagnostics and keeping partnership with local plant diagnostic laboratories. The development of local or regional diagnostic platforms combining the developed technologies will facilitate improved disease management resulting in a higher quality product offered. This will also improve competitiveness of the industry, nationally and internationally.

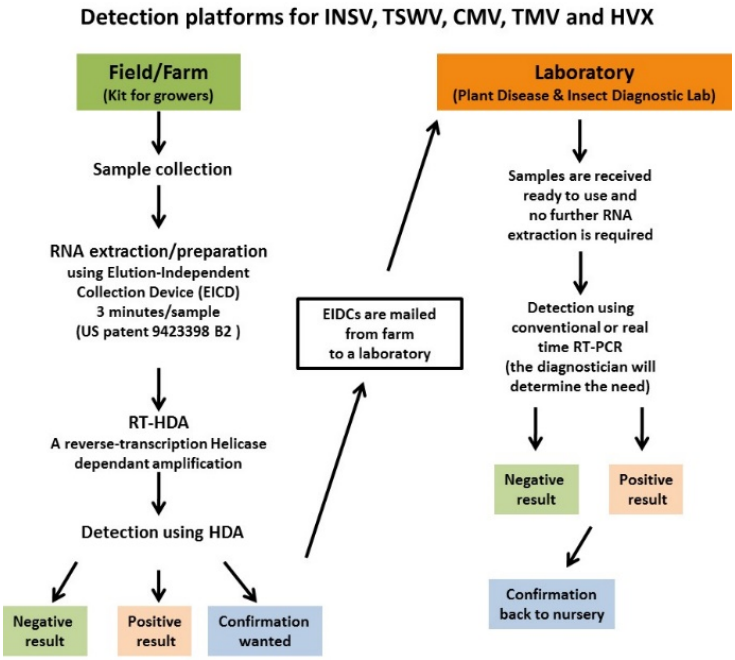

Figure. 13. Flow chart showing the interaction between ornamental growers and a plant disease diagnostic laboratory.

Annex.1

Sample	Host	Location	Multiplex RT-PCR output				
			TMV	HVX	CMV	TSWV	INSV
PL1	Lobelia	Pennsylvania	-	-	-	+	+
PL2	Lobelia	Pennsylvania	+	-	+	+	+
PL3	Lobelia	Pennsylvania	-	-	-	-	+
PC1	Cineraria	Pennsylvania	-	-	-	+	-
PM1	Mandevilla	Pennsylvania	-	-	-	-	-
PT1	Tradescantia	Pennsylvania	-	-	-	-	-
PS1	Snapdragon	Pennsylvania	-	-	-	+	+
PB1	Begonia	Pennsylvania	-	-	-	-	+
PGI1	Garden impatiens	Pennsylvania	-	-	-	-	+
PN1	Nemesia	Pennsylvania	+	-	-	-	+
PD1	Dahlia	Pennsylvania	-	+	-	-	-
PL4	Lysimachia	Pennsylvania	-	-	-	-	-
PH1	Hosta	Pennsylvania	-	+	-	-	-
PH2	Hosta	Pennsylvania	-	+	-	-	-
PCG1	Coleus	Pennsylvania	-	-	-	-	-
PCA1	Coleus	Pennsylvania	-	-	-	+	+

PCB1	Coleus	Pennsylvania	-	-	-	-	-
PCAL1	Calibrachoa	Pennsylvania	-	+	-	-	-
PP1	Petunia	Pennsylvania	+	-	-	+	+
A20	Amarylis	Indiana	-	-	-	-	+
A27	Anemone	Indiana	-	-	-	-	-
H27	Hosta	Indiana	-	+	-	-	-
V27	Velvet plant	Indiana	-	+	-	-	-
TL2	Toad lily	Indiana	-	-	-	-	-
P27	Petunia	Indiana	+	+	-	-	-
P20	Pepper	Indiana	-	-	-	-	-
P622	Impatiens	Oklahoma	-	-	-	+	+
CR1	Coleus Rustic orange	Oklahoma	-	-	-	-	-
C719	Coleus	Oklahoma	-	-	-	-	-
H12	Hosta	Oklahoma	-	+	-	-	-
H13	Hosta	Oklahoma	-	+	-	-	-
T5	Tomato	Oklahoma	-	-	-	-	-
SF1	Straw Flower	Oklahoma	-	-	-	-	-
HH1	Hosta	Oklahoma	-	-	-	-	-
HPF1	Hosta	Oklahoma	-	+	-	-	-
HF2	Hosta	Oklahoma	-	+	-	+	-
HF1	Hosta	Oklahoma	-	+	-	-	-
HP1	Hosta	Oklahoma	-	+	-	-	-
HP2	Hosta	Oklahoma	-	+	-	-	-
HF7	Hosta	Oklahoma	-	+	-	-	-
H18	Hosta	Oklahoma	-	+	-	-	-
HY2	Hyancinthina	Oklahoma	-	+	-	-	-
HY1	Hyancinthina	Oklahoma	-	-	-	-	-
P68	Pericallis	Illinois	-	-	-	-	+
Z947	Zinnia	Texas	-	-	-	-	-
GD955	Gerbera daisy	Texas	-	-	-	-	-

Annex 2. Assay Protocols

HAD Protocol

The HDA reaction was carried out in a 25 µl reaction volumes using IsoAmp® III Universal tHDA Kit (Biohelix Corporation, Beverly, MA) as per the manufacturer's instructions. A two-step protocol was followed consisting of two separate reaction mixes, A and B. Mix A consisted of 1.25 µl of 10X annealing buffer II, 0.5 µl (5 µM) of each forward and reverse primer, 1 µl of template DNA and 9.25 µl nuclease free water. Mix B consisted of 1.25 µl of 10X annealing buffer II, 1 µl $MgSO_4$ (100 mM), 2 µl NaCl (500 mM), 1.75 µl IsoAmp dNTP solution, 1 µl IsoAmp III Enzyme mix and 5.5 µl nuclease free water. Two sets of reactions were prepared. Each mix was mixed by pipetting followed by brief centrifugation and was placed on ice. The mix A was incubated at 95 °C for 2 min and then placed promptly on ice. The 12.5 µl of mix B was added to mix A, mixed by pipetting, followed by brief centrifugation, the mix was overlaid with immersion oil, and the mixture was incubated at 65 °C for 120 min using a thermal cycler and another set simultaneously in the water bath. At the end of the reaction the enzyme was deactivated by incubating the mixture at 95 °C for 3 min. The negative control (non-template control; water) was included in each sensitivity assay. The amplified product was loaded on a 2 % agarose gel in 1X TAE buffer.

When prepared for multiple samples the steps preparing the two mixes is simplified, reagents comes with colored caps and is easy to follow. Throughput pipetting can substitute centrifugation steps.

One step RT-HDA (Reverse transcription HDA) to be used at the nursery.

This protocol can be directly performed using the RNA from plant sap. The RT-HDA reaction was carried out in 50 µl reaction volumes using IsoAmp® III Universal tHDA Kit (Biohelix Corporation, Beverly, MA) components. The 50 µl reaction consisted of 5 µl of 10X annealing buffer II, 1.75 µl $MgSO_4$ (100 mM), 4 µl NaCl (500 mM), 3.5 µl IsoAmp dNTP solution, 1 µl (5 µM) of each forward and reverse primer, 3.5 µl IsoAmp III Enzyme mix, 0.5 µl of Thermoscript RT (2.1 U/ µl, Invitrogen, cat#12236-022), 4 µl of RNA (Plant sap directly) and 25.75 µl of nuclease free water. The reaction mix is briefly vortexed or pipetted followed by brief centrifugation and overlayof the reaction with 50 µl of mineral oil. Incubation of the reaction was at 65 °C for 120 min using a thermal cycler or a water bath. At the end of the reaction the enzyme was deactivated by incubating the mixture at 95 °C for 3 min. The amplified product was loaded on a 2 % agarose gel in 1X TAE buffer or 10 µl of the amplified product was loaded on a precasted Lonza gel.

HDA with dried reagents seeking simplification for the operator

Sap aliquotes were transferred to a 2 ml eppendorf tubes and allow to decant for 1 min, then 4 µl of supernatant were taken. The HDA dried reaction was reconstituted to 50 µl and the mixture mixed by tapping with finger tips to dissolve the pellet. The HDA tubes were overlaid with the 50 µl of mineral oil and set in water bath at 65 °C for 120 min. the temperature was raised at 95 °C for 3 min at the end of the reaction . The amplified product was loaded in a 2 % agarose gel in 1X TAE buffer. The same procedure was performed with dried tubes after 1 week, 2 week and 1 month.

This experiment explored the development of a ready to use format for field detection of the pathogen, which is shown in Fig.3.

RT-PCR/PCR protocol

The RT-PCR/PCR assays were performed in a 20 µl reaction volumes consisting of 10 µl GoTaq Green Master Mix (Promega), 1 µl of each forward and reverse primer (5 µM), 1 µl of DNA/cDNA template (DNA in case of Artificial positive control; cDNA in case of infected positive control), and 7 µl nuclease free water (Ambion, Austin, TX). PCR was performed in an Eppendorf thermal cycler (Eppendorf, Hauppauge, NY) with the cycling parameters as follows: initial denaturation of 94ºC for 2 min followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 62ºC for 30 s, extension 72ºC for 60 s, and final extension at 72ºC for 3 min. A negative (non-template control; water) controls was also included in each assay. The amplified PCR product was separated by electrophoresis using 2% agarose gel in 1X TAE buffer.

RTmPCR cycling conditions.

Multiplex RT-PCR was performed to detect all five viruses in a single reaction. Multiplex RT-PCR assays were performed in 50 µl reactions composed of 25 µl of GoTaq Green Master Mix (2X GoTaq Green reaction buffer, 400 mM of each of four deoxynucleoside triphosphates, and 3 mM $MgCl_2$), 3.5 µl $MgCl_2$ (25 mM), 5 µl primer solution (containing all five primer sets; each primer at 2 µM concentration) and 3 µl cDNA (synthesized from plant sample or positive control). PCR cycling conditions were: initial denaturation at 95ºC for 4 min followed by 35 cycles at 94ºC for 30 s, 62ºC for 90 s, 72ºC for 45 s and a final extension at 72ºC for 4 min. A volume of 20 *µ*l of amplified multiplex PCR product was electrophoresed in a 2% agarose gel in 1X TAE buffer at 100V for 90 min.

Inclusivity and exclusivity assays were performed using the positive control previously described to determine the specificity of the primers.

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Annex 4. Publications.

This project generated two refereed scientific publications:

1. Shefali Dobhal, Jennifer D Olson; Mohammad Arif, Johnny A Garcia Suarez; Francisco M Ochoa-Corona. A simplified strategy for sensitive detection of Rose rosette virus compatible with three RT-PCR chemistries. Journal of Virological Methods. 232 (2016) 47–56.
2. Shefali Dobhal, Mohammad Arif, Jen Olsen, Abby Mendoza-Yerbafría, Stefanny Aguilar-Moreno, Marcos Perez-Garcia and Francisco M. Ochoa-Corona. Sensitive detection and discrimination method for studying multiple infections of five major plant viruses infecting ornamental plants in nursery environments. Ann Appl Biol. 166:286-296; DOI: 10.1111/aab.12182.

Annex 5. Presentation in meetings.

Attendance to two national meetings is reported.

American Phytopathological Society (APS) 2013

- Arif, M., A. Mendoza Yerbafria, G. S. Aguilar Moreno, M. Perez Garcia, J. Olson, D. Smith, F. M. Ochoa-Corona. 2013. Development of end-point multiplex RT-PCR and helicase dependent amplification for detection of CMV, HVX, INSV, TMV, and TSWV. Phytopathology 103(Suppl. 2):S2.9.
- S. Dobhal , M. Arif , J. Olson , A. Mendoza-Yerbafría , S. Aguilar-Moreno , M. Perez-Garcia , F. M. Ochoa-Corona. 2014. Screening for plant viruses including TMV, HVX, CMV, TSWV and INSV in ornamental and nursery crops from four states of the United States. Phytopathology 104(Suppl. 3):S3.34

American Phytopathological Society (APS) 2014

- S. Dobhal , M. Arif , J. Olson , F. M. Ochoa-Corona. Comparison of end point RT-PCR and RT-HDA using primer pairs for five viruses frequently infecting ornamental and nursery crops. Phytopathology 104(Suppl. 3):S3.34

Annex 6. Undergraduate student involvement.

Four undergraduate students participated received training and participated in this research. All were included as authors in the published articles. These students joined Oklahoma State University through an international-exchange program. Three students were affiliated Universidad Autónoma de Chapingo, Agroindustrial Engineering, Texcoco, Mexico, and one

from The University of the Army of Ecuador (ESPE), Quito Ecuador. Their travel and maintenance was funded by the Universidad Autónoma de Chapingo and ESPE respectively. The four students are:
Marcos Perez Garcia, Stefanny Aguilar Moreno, Abigail Mendoza Yerbafría and Johnny Suarez Garcia.

Annex 7. Conflict of interest.
The mention of trade names or commercial products in this project, derived publications and presentations in scientific meetings does not imply recommendation or endorsement by the authors and Oklahoma State University who declare no conflict of interest.