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A rapid fluorescence-based real-time isothermal assay for the detection of *Cucurbit yellow stunting disorder virus* in squash and watermelon plants

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ABSTRACT

Cucurbit yellow stunting disorder virus (CYSDV) is a single-stranded positive-sense RNA virus that produces devastating disease in watermelon and squash. Foliar symptoms of CYSDV consist of interveinal yellowing, brittleness, and thickening of older leaves leading to reduced plant vigor. A rapid diagnostic method for CYSDV would facilitate early detection and implementation of best viral-based management practices. We developed a rapid isothermal reverse transcription-recombination polymerase amplification (exo RT-RPA) assay for the detection of CYSDV. The primers and a 6-fluorescein amidite (6-FAM) probe were developed to target the nucleocapsid gene. The real-time assay detected CYSDV at 2.5 pg purified total RNA extracted from CYSDV-infected leaf tissue and corresponded to 10 copies of the target molecule. The assay was specific and did not cross-react with other common cucurbit viruses found in Florida and Georgia. The performance of the exo RT-RPA was evaluated using crude extract from 21 cucurbit field samples and demonstrated that the exo RT-RPA is a rapid procedure, thus providing a promising novel alternative approach for the detection of CYSDV.

Cucurbit yellow stunting disorder virus (CYSDV), a member of the genus *Crinivirus*, family *Closteroviridae,* is a widespread and devastating viral pathogen infecting cucurbit crops [1–10]. CYSDV is transmitted in a semipersistent and non-circulative manner by the whitefly *Bemisia tabaci*. CYSDV produces symptoms that are most obvious in the older leaf tissue and consist of leaf interveinal yellowing, brittleness and thickening, and reduced plant vigor. The fruits of CYSDV-infected plants may also be impacted with yield and quality reductions as high as 60–80% [1,2]. Physiological, nutritional disorders, or pesticide toxicity are often mistaken for the symptoms of CYSDV [11], and therefore an effective and efficient technique for diagnosing CYSDV is required. A reliable diagnostic is also necessary to differentiate between symptoms induced by CYSDV and other criniviruses that can be very similar in appearance [11] and to detect CYSDV in non-symptomatic weedy species acting as a pathogen reservoir [12,13].

CYSDV is a relatively low titre, phloem-limited virus and therefore sensitive diagnostics are essential for identification. Several methods for the detection of CYSDV have been developed. These include enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR), real-time quantitative RT-PCR, and nucleic acid hybridization assays [14–18]. These methods, however, are time consuming to perform and require considerable experience to consistently and successfully perform the assays. RPA is an isothermal nucleic acid-based diagnostic assay that is rapid, sensitive, and easily performed in the field by a person with minimal laboratory skills [19]. RPA-based assays have been developed to detect other plant viruses in tissues in a highly sensitive and selective manner [20]. In this study, a rapid and reliable exo-RPA assay for real-time detection of CYSDV that uses crude-plant extract as template was developed.

\Templates for the nucleic acid assays consisted of 250 ng total RNA extracted from a leaf of an individual plant sample using a RNeasy Mini Kit (Qiagen Inc., Valencia, CA) or crude-plant leaf extract. Crude plant extract was prepared by macerating 0.01% (w/v) leaf tissue in extraction buffer consisting of 1X phosphate buffered saline, pH 7.4, containing 0.02% Tween-20 (PBS-T). A 1:10 dilution of the crude extract in extraction buffer was used in the RT-RPA reactions. The CYSDV specific primers and probe designed for exo-RT-PCR were based on the coat protein sequence (GenBank accession FJ492808.1) and were CYSDV2cpF 5′-ACATGGAGG

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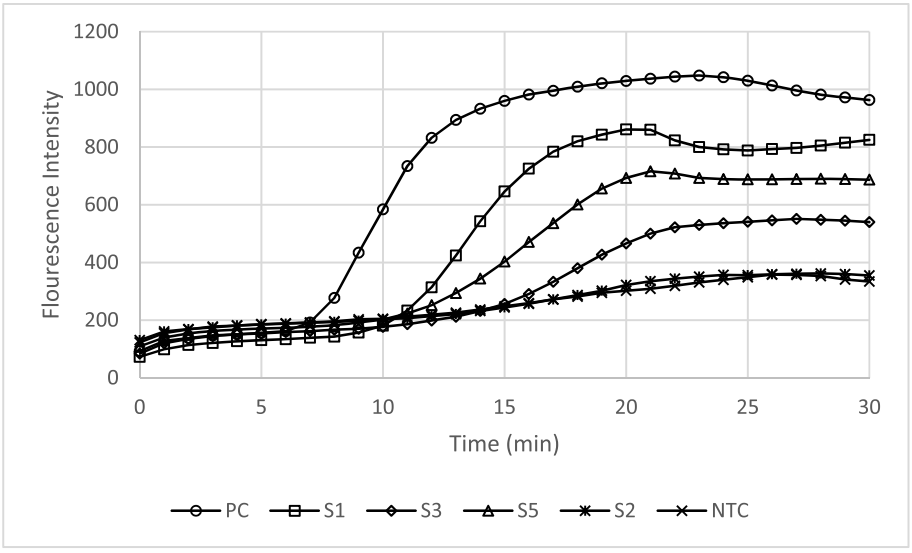


Fig. 1. Optimized cucurbit yellow stunting disorder virus (CYSDV) exo RT-RPA reaction using purified total RNA as template. S1, S3, and S5 were samples from CYSDV symptomatic watermelon plants. S2 was a sample from an asymptomatic watermelon plant. The positive (PC) and negative control (NC) consisted of 250 ng purified total RNA from leaves of a CYSDV-infected or non-infected watermelon plant, respectively. Non-template control (NTC) was sterile distilled water (SDW), which was the RNA elution buffer.

ACGGCTGACTTTATCAATTATG-3'; CYSDVcpR 5′-CAACAGATTGGCTGCC AAATCGTCTTCGGA-3'; and CYSDVcp1 5′-TTGAAGGCAACAGGAAAGGT CACTGTTGACCA[FAMdT]C[A-THF]C[BHQdT]TCAAGCGAAAC ATG[SPC03]-3′, where FAM is 6-carboxy-fluorescein, THF is tetrahydrofuran, BHQ is the black hole quencher, and SPC03 is 3′ spacer.

The exo-RT-RPA assays were performed at 40 °C for 30 min using an AmpliFire isothermal fluorometer and the Amplify RP XRT Discovery Kit (Agdia Inc. Elkhart, IN). Each 25 μL reaction contained a lyophilized pellet, 14.75 μL rehydration buffer, 0.42 μM of each primer, 0.12 μM of probe, 100 units of Superscript II reverse transcriptase enzyme (Thermo Fisher Scientific Inc., Waltham, MA), nuclease-free water, and template consisting of 50 ng total RNA or 0.5 μL crude extract. A final concentration of 14 mM magnesium acetate was added to each reaction and the sample briefly mixed, centrifuged and incubated as described above. Examples of exo RT-RPA reactions are presented in Figs. 1 and 2.

Detection sensitivity of the exo RT-RPA was evaluated using a 10-fold serial dilution series between 10 pg/uL and 1 fg/uL of purified total RNA from leaf tissue of a CYSDV-infected watermelon plant. Dilutions were made with 10 ng/uL of purified total RNA extracted from asymptomatic leaves of a watermelon plant. Purified total RNA rather than sterile distilled water (SDW) was used as the dilution medium in the sensitivity assays to avoid overestimation of sensitivity associated with SDW. Results show that the sensitivity of detection for CYSDV using exo RT-RPA was 100 fg/uL (2.5 pg RNA) purified total RNA from watermelon leaves infected with CYSDV (Fig. 3). Another RT-RPA recently developed for watermelon reported a similar detection limit of 1.0 pg purified total RNA for *Cucumber green mottle mosaic virus* (CGMMV) [21].

To evaluate the specificity of the CYSDV exo RT-RPA, the primers and probe were tested against other common cucurbit infecting viruses

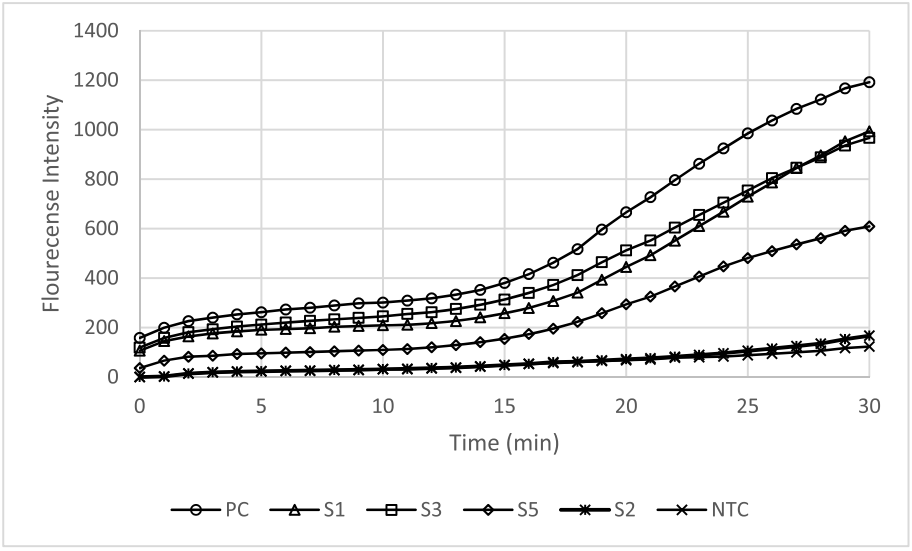


Fig. 2. Optimized cucurbit yellow stunting disorder virus (CYSDV) exo RT-RPA reaction using crude plant extract as template. S1, S3, and S5 were samples taken from CYSDV symptomatic watermelon plants. S2 was a sample from an asymptomatic watermelon plant. The positive (PC) and negative control (NC) contained crude plant extract with the former spiked with 250 ng purified total RNA from leaves of a CYSDV-infected watermelon plant. Non-template control (NTC) was PSB-T RPA elution buffer.

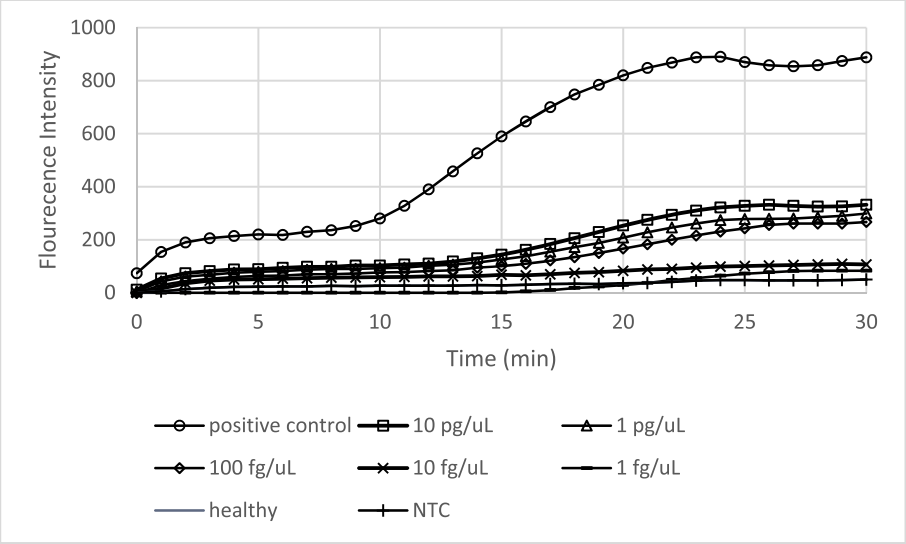


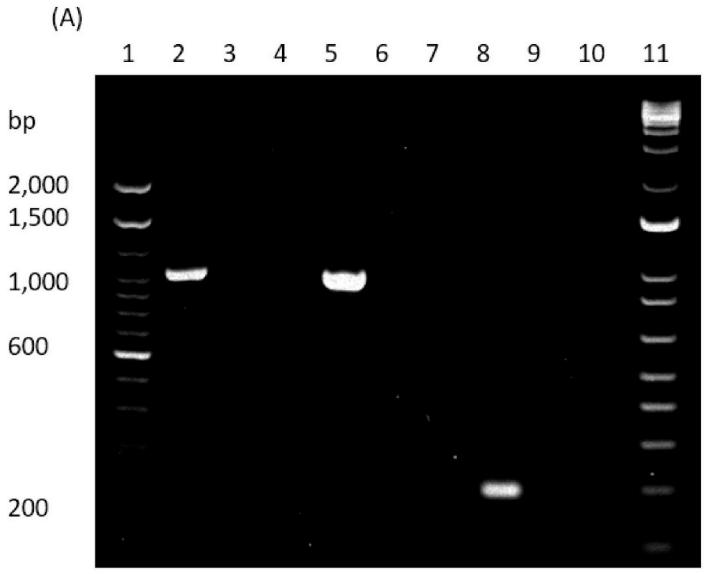
Fig. 3. Sensitivity of exo RT-RPA using purified total RNA from cucurbit yellow stunting disorder virus (CYSDV)-infected watermelon leaves. The positive control (PC) sample contained 250 ng total RNA from leaves of a CYSDV-infected watermelon. The healthy sample contained of 250 ng total RNA extracted from leaves of an asymptomatic watermelon plant. Non-template control (NTC) was sterile distilled water (SDW).

in Florida and Georgia including *Cucurbit leaf crumple virus* (CuLCrV), *Squash vein yellowing virus* (SqVYV), and *Papaya ringspot virus* (PRSV). These viruses were selected because they are the most common ones affecting cucurbits in Florida and they are often in mixed infections with CYSDV [8,17]. Fresh samples of CuLCrV, SqVYV, or PRSV affecting watermelon or squash were collected from commercial grower fields and research plots in Florida. Purified total DNA extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) or total RNA extracted as described above was used as template for all specificity assays. PCR or RT-PCR followed by PCR clean-up, and sequencing was used to confirm CuLCrV or SqVYV in infected tissues [22,23]. Custom primers PRSVfor2 5′- TCACTTTAGTAACGCGGCAGAAGCATACAT -3′ and PRSVrev2 5′- TTACTAACACTGCCGTCCATACCAAACATT -3′ were used to amplify a 180 bp product that was also sequenced to confirm PRSV infection. Sterile distilled water (SDW) and leaves from asymptomatic squash or watermelon was used as the non-template (NTC) and negative controls, respectively. Results show that pathogen specific PCR or RT-PCR detected CuLCrV, SqVYV, or PRSV in symptomatic test samples, however, the same nucleic acids tested negative for CYSDV in exo RT-RPA (Fig. 4). The amount of fluorescence generated from the exo RT-RPA with CYSDV infected tissue was much greater than tissue infected with CuLCrV, SqVYV, PRSV, NTC, and asymptomatic plant tissue. This finding confirms that the exo RT-RPA developed in this study is highly specific for CYSDV.

To determine the reliability of exo RT-RPA in the detection of CYSDV, leaf tissue from 21 symptomatic and asymptomatic watermelon and squash samples were collected from different fields across Florida and analyzed using exo RT-RPA and RT-PCR. Experiments were replicated three times with purified total RNA and crude leaf extract as template. RT-PCR was completed using Superscript III One Step RT-PCR system with Plantinum Taq DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA). The RT-PCR primers included those previously described including CYScp1F/CYScp1R and CYShspF/CYShspR [8] or the RT-RPA primers designed in this study with an annealing temperature of 63 °C and expected amplicon size of 249bp. The exo RT-RPA produced similar reaction types as RT-PCR in 20 out of the 21 leaf samples tested (Table 1). The sample showing disagreement produced a positive reaction for exo RT-RPA and a negative reaction for RT-PCR and was direct sequenced using procedures described previously [24]. The sequencing results for the exo RT-RPA product showed 98% identity to the expected CYSDV sequence (GenBank accession FJ492808.1). Further testing is required to determine if the CYSDV exo RT-RPA described in this study is consistently more sensitive than RT-PCR using primers described by Ref. [8] in detecting CYSDV in field samples.

The exo RT-RPA and RT-PCR identified CYSDV in 10 and 9 out of 13 symptomatic squash and watermelon plants, respectively (Table 1). These results highlight the necessity of having a reliable nucleic acid diagnostic for CYSDV and that symptoms alone are not definitive at identifying viruses that infect cucurbits. Of the asymptomatic samples, 7 of the 8 plants tested negative for CYSDV; however, one asymptomatic plant tested positive for CYSDV in both exo RT-RPA and RT-PCR. These results suggest that the exo RT-RPA would be useful in the early detection of CYSDV in asymptomatic tissue.

The ability to use crude plant extract as template in the exo RT-RPA greatly reduced the amount of time required to complete the diagnostic exo RT-RPA assay. RT-PCR cannot be performed on crude extract and therefore PCR requires an additional hour more than RPA to extract nucleic acid. Additional time required for the exo RT-RPA was further reduced by adding Moloney murine leukemia virus (MMLV) reverse transcriptase directly to the RPA reaction to simultaneously synthesize cDNA at 40 °C while carrying out the isothermal RPA reaction. The RT-PCR required separation of the cDNA and DNA amplification steps, adding another 15 min to the assay. The RT-PCR DNA amplification process including, denaturing, annealing, and extension took an additional 60 min beyond isothermal incubation of exo-RT RPA. Moreover, the exo RT-RPA used a florescent probe to examine the product and this step eliminated another 45 min required for visualizing the RT-PCR results using agarose gel electrophoresis. Others have reduced the agarose gel electrophoresis step of RT-PCR by introducing a fluorescent probe for the detection of CYSDV [25]. In this study, the entire exo RT-RPA assay using crude extract as template took 40 min which is much less time than the 240 min required for RT-PCR in detecting CYSDV in



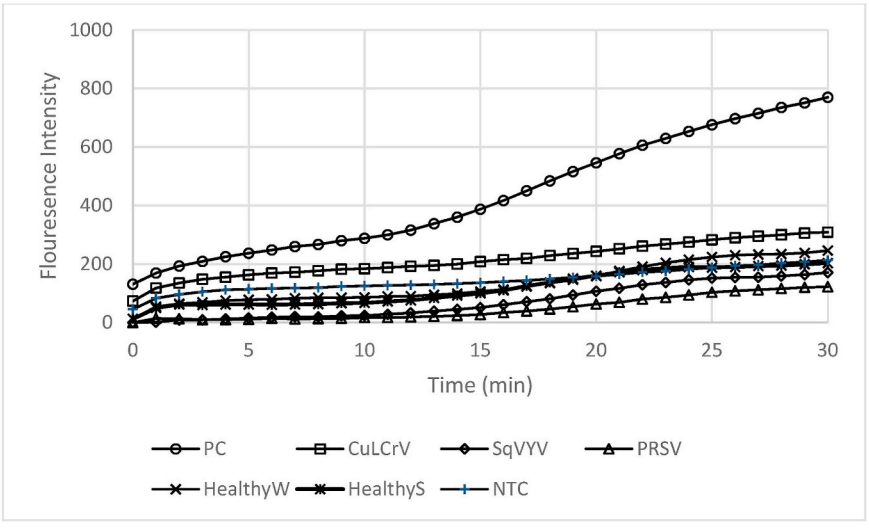


Fig. 4. Specificity of the cucurbit yellow stunting disorder virus (CYSDV) exo RT-RPA. (A) PCR or RT PCR for cucurbit leaf crumple virus (CuLCrV) (L2-4), squash vein yellowing virus (SqVYV) (L5-7), or papaya ringspot virus (PRSV) (L8-10). L1: Invitrogen 100 bp DNA ladder. L2, 5 and 8: DNA/RNA from a symptomatic leaf. L3, 6, 9: DNA/RNA from an asymptomatic leaf. L4, 7, and 10: NTC. L11: Invitrogen TrackIt 1 kb Plus DNA Ladder. (B) CYSDV exo RT-RPA using nucleic acid samples positive for CuLCrV, SqVYV, and CYSDV. The positive control (PC) included 250 ng purified total RNA from leaves of a symptomatic watermelon and the negative control (NC) included the same amount of RNA from asymptomatic watermelon (healthyW) and squash (healthyS) leaf tissue. The non-template control (NTC) was sterile distilled water (SDW).

squash and watermelon tissue while providing similar levels of sensitivity.

CYSDV is a serious disease of squash, watermelon and melons because it can have a devastating impact on foliage and fruits. A reliable diagnostic for CYSDV is essential because symptoms are confused with those of other cucurbit viruses or abiotic disease. Here, we report on the development of a rapid, sensitive, and specific exo RT-RPA that can be used to identify CYSDV in watermelon and squash field samples. The development of the exo RT-RPA enables future studies to explore possibilities of early detection of CYSDV and the movement of CYSDV in plants, as influenced by the whitefly vector, in characterizing epidemiology and development of best management practices for the disease.

Declaration of competing interests

The authors declare they have no conflicts of interest.

CRediT authorship contribution statement

Melanie L. Kalischuk: Conceptualization, Investigation, Writing - original draft. **Pamela D. Roberts:** Conceptualization, Writing - review & editing, Funding acquisition. **Mathews L. Paret:** Conceptualization, Writing - review & editing, Resources, Funding acquisition.

Table 1
Comparative assay of 21 field samples of watermelon and squash in 2018 and 2019 by RT-PCR and exo RT-PCR. Two different primer sets were used for PCR: CYScp1F/CYScp1R and CYShspF/CYShspR (Set A primers) and CYSDV2cpF/CYSDVcpR (Set B primers). Exo RT-PCR was done using Set B primers.

Collection date	Host	Symptomatic/ asymptomatic	RT-PCR[a]		Exo RT-PCR[b]
			Set A primers	Set B primers	Set B primers
10/05/18	watermelon	symptomatic	+		+
10/05/18	watermelon	asymptomatic	–		–
25/10/18	watermelon	symptomatic	+		+
25/10/18	watermelon	asymptomatic	–		–
07/11/18	watermelon	symptomatic	+		+
07/11/18	watermelon	asymptomatic	–		–
01/04/19	watermelon	symptomatic	+		+
01/04/19	watermelon	symptomatic	–		+
01/04/19	watermelon	symptomatic	–		–
01/04/19	watermelon	asymptomatic	–		–
11/04/19	squash	symptomatic	+		+
11/04/19	squash	asymptomatic	–		–
30/04/19	watermelon	symptomatic	+		+
30/04/19	watermelon	asymptomatic	–		–
30/04/19	squash	symptomatic	+		+
30/04/19	squash	asymptomatic	–		–
05/11/19	watermelon	symptomatic	+	+	+
05/11/19	watermelon	symptomatic	+	+	+
05/11/19	watermelon	asymptomatic	+	+	+
05/11/19	watermelon	symptomatic	–	–	–
05/11/19	watermelon	symptomatic	–	–	–

[a] Purified total RNA as template.
[b] Crude extract and purified total RNA as templates.

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