Project Title: Cas12a-based rapid method for early detection of X-disease phytoplasma

Report Type: Final

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Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ 85,800
Total Project Request for Year 2 Funding: $ 89,232

Other related/associated funding sources: Awarded (Harper)
Funding Duration: 2022 - 2023
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Agency Name: USDA-ARS
Notes:

WTFRC Collaborative Costs: None

Budget 1

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Item	1/3/2023	1/3/2024
Salaries[1]	$20,925.00	$21,762.00
Benefits[1]	$7,421.00	$7,718.00
Wages		
Benefits		
RCA Room Rental		
Shipping[2]	$254.00	$316.00
Supplies[3]	$14,300.00	$14,940.00
Travel	$3,000.00	$3,000.00
Plot Fees		
Miscellaneous		
Total	$45,900.00	$47,736.00

Footnotes: 4% inflation for year 2. [1]Postdoc salary for 4.5 months and postdoc benefit rate at 35.5%.[2]Shipping materials to PSU. [3]Including budget for co-PI Scott Harper: $6000 for year 1 and $6240 for year 2.

Budget 2

Co PI 2: Dr. Yinong Yang
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Item	1/3/2023	1/3/2024
Salaries	$24,311.00	$25,283.00
Benefits	$8,752.00	$9,102.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$4,837.00	$5,111.00
Travel	$2,000.00	$2,000.00
Plot Fees		
Miscellaneous		
Total	$39,900.00	$41,496.00

Footnotes: 4% inflation for year 2.

Budget 3

Co-PI 3: Dr. Scott Harper
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Footnotes: Scott's budget is included in PI Zhao's budget 1.

Objectives:

1. To establish and optimize a Cas12a-based method for early and rapid detection of cherry X-disease phytoplasma;

2. To apply the Cas12a-based method for field sample diagnosis (cherry, insects, weeds);

3. To train and promote the Cas12a-based method for diagnosis of X-disease phytoplasma.

Significant Findings

- XDP-specific RPA primers and crRNAs have been identified and synthesized based on the DNA sequence alignment and analysis of the *SecY* genes from the phytoplasma 16SrIII subgroups (Figures 1-2).
- An XDP-specific primer pair has been selected and validated for its high efficiency of RPA.
- A specific crRNA was selected for the XDP Cas12a detection assay.
- A two-pot RPA/Cas12a assay has been successfully developed for highly sensitive and specific detection of different XDP strains (Figures 3-4).
- A simple protocol of the RPA/Cas12a assay has been established for rapid detection of XDP.
- The RPA/Cas12a method has been demonstrated to be capable of specific and rapid detection of XDP-infected samples from the field (Figures 5-6).
- Double blind assay results from Cas12a-based tests for more than 100 samples of sweet cherries, weeds and insects correlated well with the qPCR assay results (Figures 7-10).
- Developed a 5-min genomic DNA extraction method for the RPA/Cas12a assay (Figure 11).
- One-pot RPA/Cas12a assay based on the cap/spin approach was recently established to facilitate the field-deployable detection of XDP in the near future.

Methods:

Procedures and Methodology. Since the initiation of this project in January 2023, we have successfully established an RPA/Cas12a method for highly specific, supersensitive, and rapid detection of cherry X-disease phytoplasma (XDP).

The DNA sequences of *SecY* genes from the XDP strains and other 16SrIII subgroups were obtained and aligned using sequence analysis tools. RPA primers and crRNAs were designed, chemically synthesized, and experimentally tested in RPA and Cas12a assays, respectively. They were evaluated for their specificity, amplification or cleavage efficiency, compatibility, and reproducibility. In the subsequent two-pot assay, both RPA and Cas12a reactions were performed under the isothermal condition at 102ºF (39ºC) (see **Fig. 1**). The fluorescent signal released from the oligo reporter due to Cas12a trans-cleavage was detected and quantified by a fluorescence microplate reader using following setting: excitation 485/20; emission 530/25nm. The RPA/Cas12a assay for XDP detection can be completed within one hour.

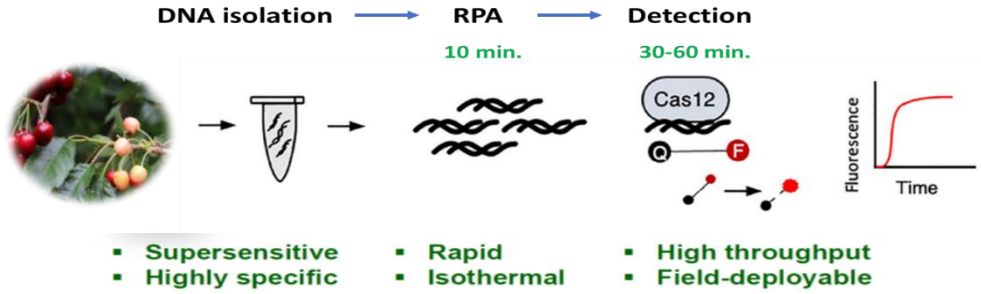


Fig. 1. Illustration of the RPA/Cas12a assay for XDP detection, which includes total DNA extraction, target DNA amplification by RPA and subsequent Cas12a detection with a fluorescence microplate reader based on trans-cleavage of oligonucleotide reporters.

Once successfully established our assay, we validated our assay specificity by using pure DNA fragment and DNA from infected cherry samples and determined the sensitivity of the assay by 10x

serial dilution of pure DNA samples and infected cherry DNA samples (see **Figs. 4-6**). To further confirm our method, double blind experiments were carried out using the Cas12a-based assay for more than 100 samples of sweet cherries, weeds and insects and compared with those of the qPCR assay results (**Figs. 7-10**) In addition, we adapted and optimized a 30-second rapid method to extract genomic DNAs from cherry plant samples based on cellulose dipsticks (**Fig. 11**).

Types and timing of anticipated results. The step-by-step protocol for the RPA/Cas12a-based diagnostic assay for cherry XDP was finalized, including RPA primer pairs, specific crRNA, and FAM reporter. We have also determined the detection limit and sensitivity of the assay. We optimized the times of RPA reaction, Cas12a detection procedure and signal detection by fluorescent microplate reader, which will significantly shorten the procedure and obtain results within an hour. The method was also verified by double blind assay and compared with qPCR results.

Potential problems or limitations. We did not encounter technical problems and limitations so far as the method is technically straightforward.

Results and Discussion:

Based on the DNA sequence alignment of *SecY* genes from the XDP strains and other 16SrIII subgroups, five RPA primer pairs were designed, synthesized, and experimentally tested for specificity, amplification efficiency and reproducibility. The best pair of primers (FP1/RP1) was selected for highly efficient and specific amplification of XDP (**Fig. 2**). Importantly, the FP1/RP1 primer pair did not amplify DNAs from cherry plant or other phytoplasmas such as Phytoplasma solani and Phytoplasma mali.

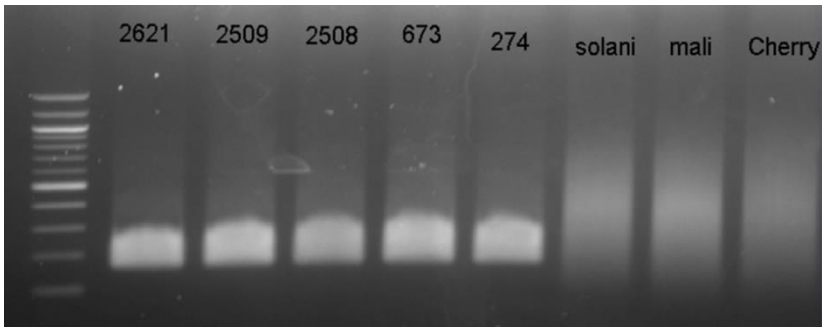


Fig. 2. Specificity and amplification efficiency of the FP1/RP1 primer pairs. Amplification of 185 bp fragment by the RPA assay only occurred with the XDP DNA samples (2621, 2509, 2508, 673, 274), but not with the DNA samples from Phytoplasma solani, Phytoplasma mali or cherry plant.

In addition, three crRNAs were designed, chemically synthesized, and tested for specificity, cleavage efficiency and compatibility with RPA. One specific crRNA was selected for the XDP Cas12a detection assay.

After the establishment of individual RPA and Cas12a detection assay, a two-pot RPA/Cas12a assay was successfully developed in Co-PI Dr. Yang's lab at Penn State for highly sensitive and specific detection of XDP DNA. Preliminary data showed that the RPA/Cas12a assay can detect XDP DNA at the sensitivity level of at least 100 aM (**Fig. 3**). Subsequently, we further optimized the assay reagents and conditions and achieved the attomolar detection sensitivity (~1 copy per microliter) in both Yang and Zhao labs (**Fig. 4**).

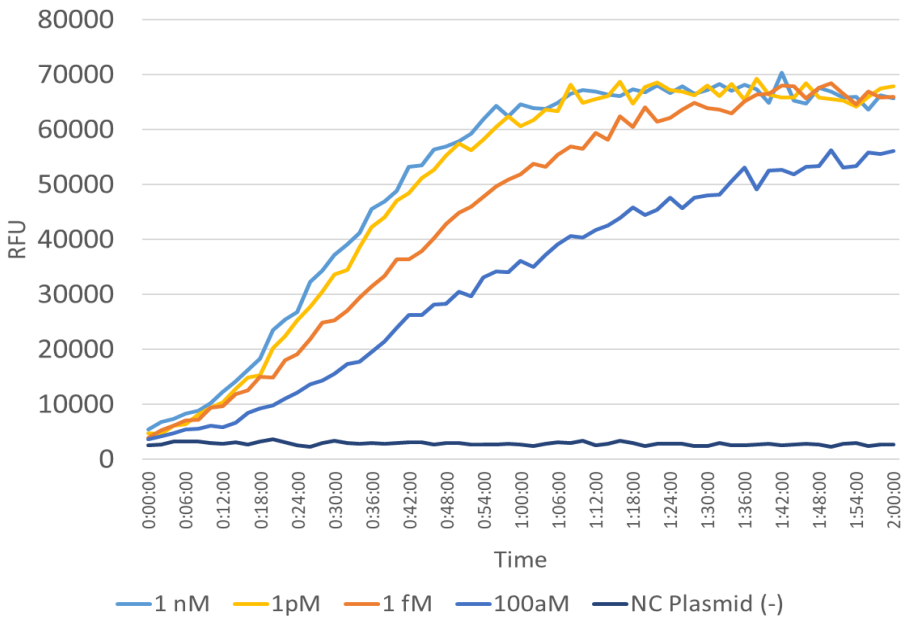
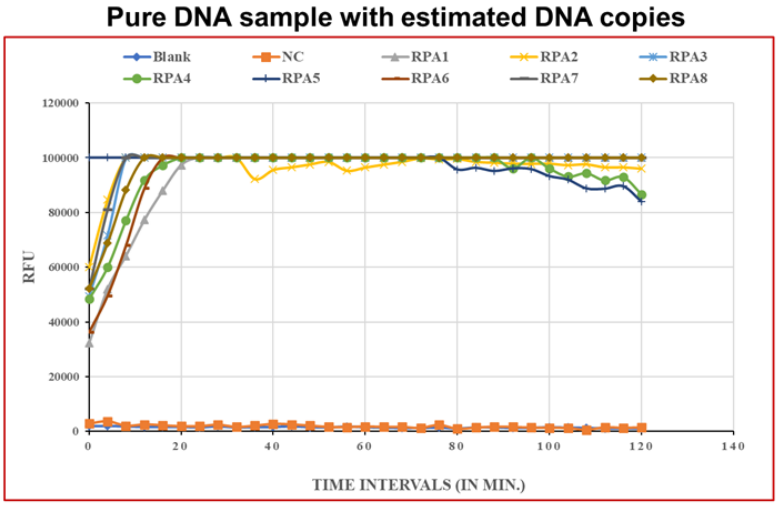


Fig. 3. The initial sensitivity test of the RPA/Cas12a assay for XDP DNA detection. A dilution series (1 nM, 1 pM, 1 fM, 100 aM) of plasmid DNA containing the *SecY* gene target region were used along with negative control plasmid. XDP DNA at 100 aM level could be readily detected by fluorescence microplate reader within 12 minutes of Cas12a reaction.



Sample	Copy no.
RPA1	1
RPA2	10
RPA3	100
RPA4	1000
RPA5	10000
RPA6	100000
RPA7	1000000
RPA8	10000000

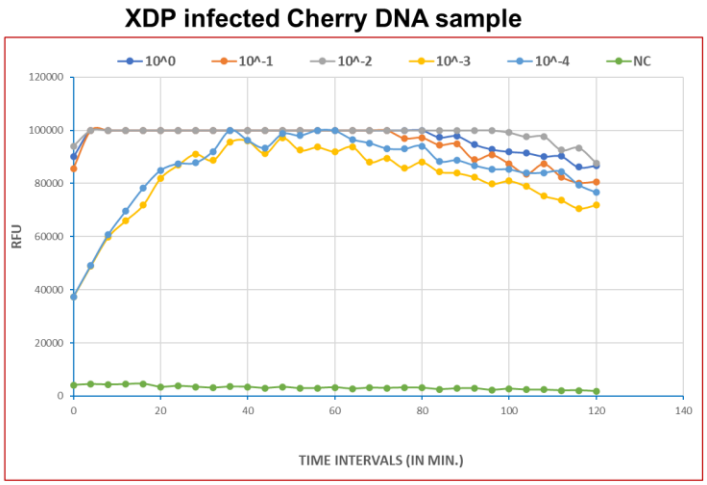
NC: Negative control (non-infected sample); **Blank:** Water.

The max fluorescence reading is 100000 RFU.

Fig. 4. Detection of XDP DNA by the RPA/Cas12a assay at the single copy sensitivity. The XDP *SecY* gene fragment was amplified by PCR, quantified for DNA concentration and used to generate serial dilutions for the detection sensitivity test.

To validate the RPA/Cas12a assay for XDP detection of field samples, we used the assay protocol provided by Co-PI Dr. Yang to detect XDP at Washington State University in Prosser. We were able to successfully replicate and validate the RPA/Cas12a assay for XDP detection (**Figs. 4-6**). We could detect one to two copies of DNA by the RPA/Cas12a assay within 10 min using purified DNA and cherry DNA samples, respectively (**Figs. 4-5**). Furthermore, the two-pot RPA/Cas12a assay was

demonstrated to be capable of specific and rapid detection of XDP-infected samples from the field with various concentration of XDP, ranging from 300 copies to 1 million copies of DNA (with Ct values 18 to 31) (**Fig. 6**). These results showed a very higher sensitivity of RPA/Cas12a assay for XDP detection in the field samples. Double blind experiment results from Cas12a-based assay for more than 100 samples of sweet cherries, weeds and insects correlated well with the qPCR assay results (**Figures 7-10**). These results suggest that this method is capable of detecting XDP at low titer and applicable to various samples from the field.

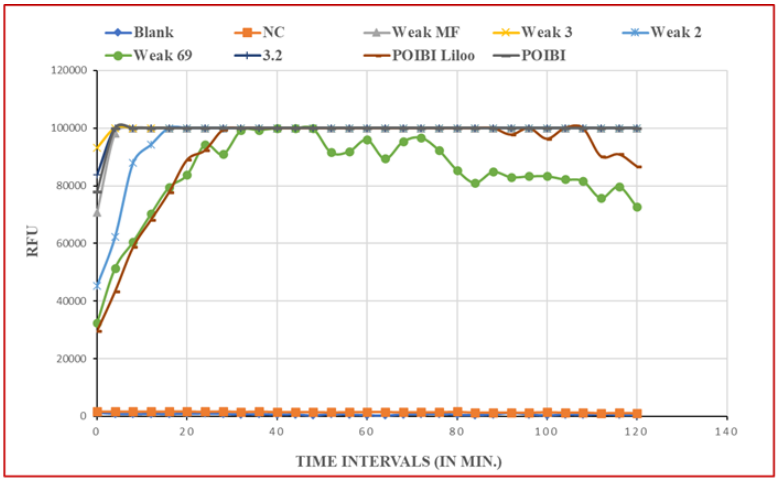


Template	Copy no.
10^{0}	20000
10^{-1}	2000
10^{-2}	200
10^{-3}	20
10^{-4}	2

NC: Negative control (non-infected sample)

The max fluorescence reading is 100000 RFU.

Fig. 5. Detection of XDP by RPA/Cas12a in a DNA extract from an infected cherry tree.



Cherry samples
i) Weak MF
ii) Weak 3
iii) Weak 2
iv) Weak 69
v) 3.2

Other phytoplasma

i) POIBI Liloo
ii) POIBI

NC: Negative control (non-infected sample); **Blank:** Water.

The max fluorescence reading is 100000 RFU.

Fig. 6. Detection of XDP from infected cherry samples and other phytoplasmas.

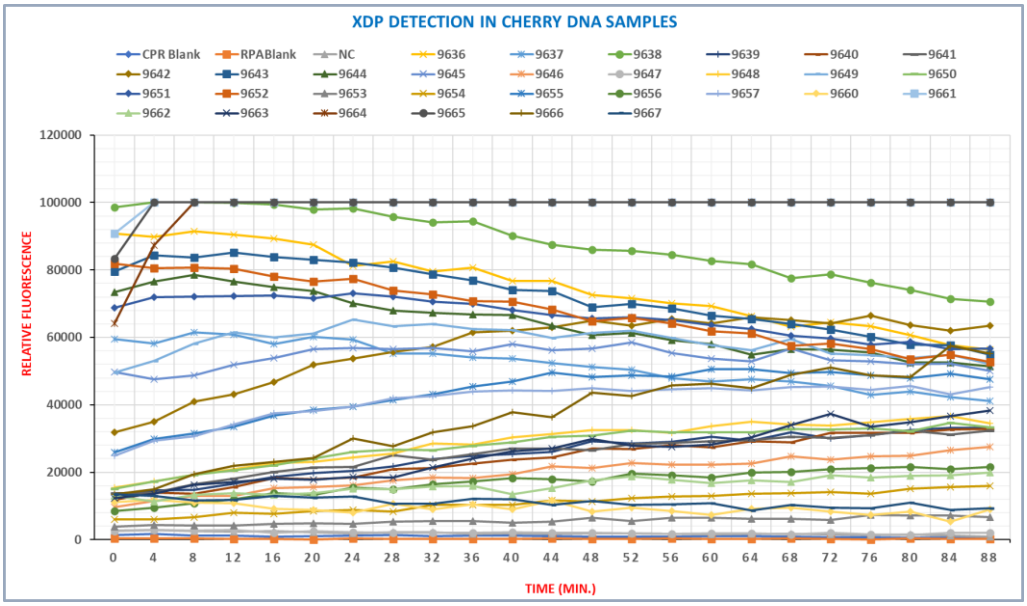


Fig. 7. Double blind tests of 30 cherry samples (all positive) with Ct values ranging from 21 to 39.

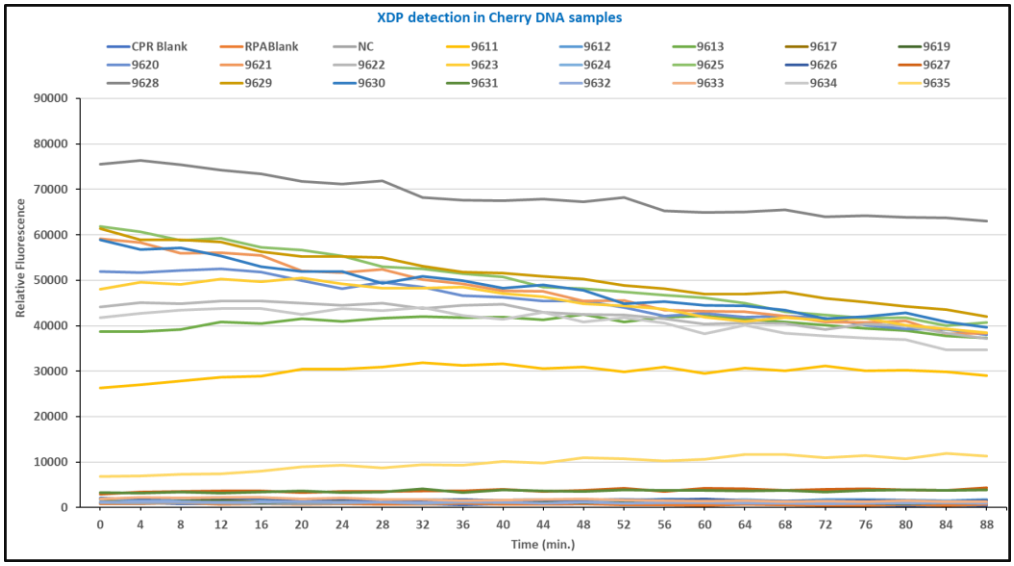


Fig. 8. Double blind tests of 21 cherry samples. 14 samples tested positive with CT values ranging from 21 to 36 and 7 samples tested negative.

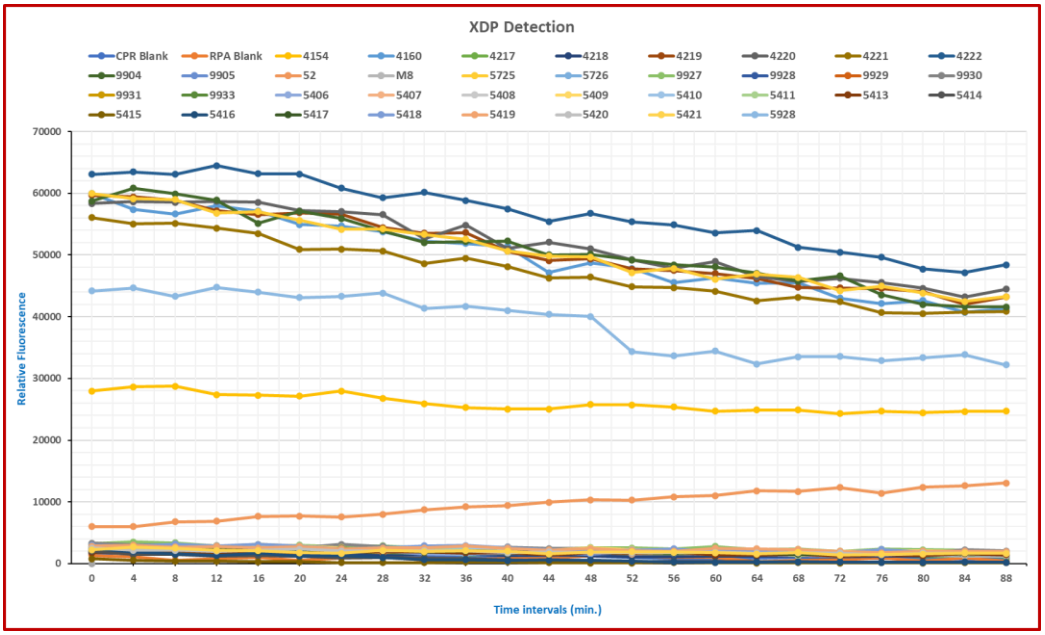


Fig. 9. Double blind tests of 36 samples from weed, leafhopper, cherry and celery. 10 samples tested positive with Ct values ranging from 26 to 38.

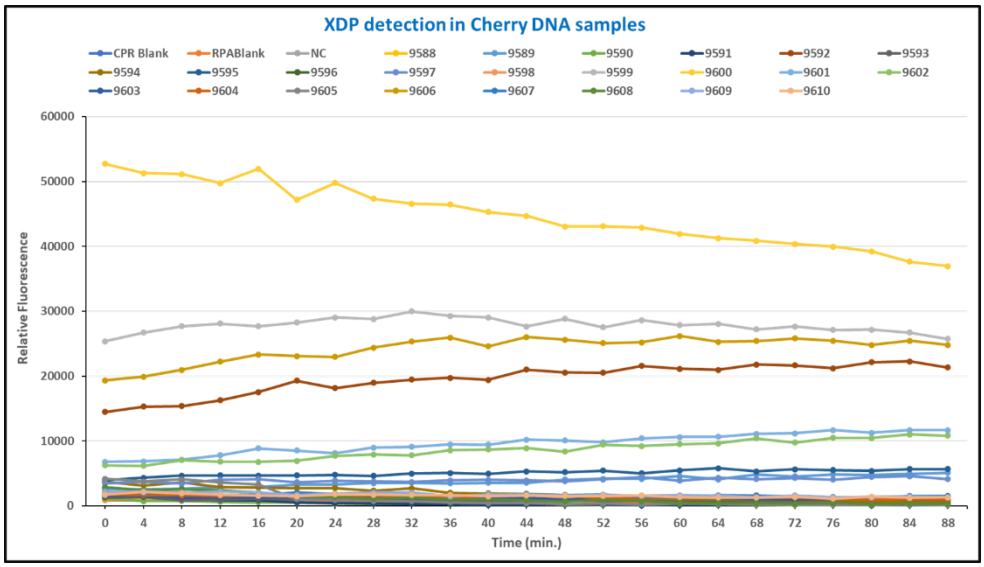


Fig. 10. Double blind tests of 23 cherry samples. Only 9 samples tested positive with CT values ranging from 31 to 39.

In addition, we adapted and optimized a 30-second rapid genomic DNA extraction method based on cellulose dipsticks (**Fig. 11**). This method comprises the grinding of 1-cm cherry tissues, including leaf, stem, bark, fruit, and flower in DNA extraction buffer. The cellulose dipstick dipped 5 times each in DNA extraction buffer and washing buffer. The purified DNA on the cellulose dipstick was then eluted in the dH_2O or RPA reaction solution for amplification. This DNA extraction method is simple and can rapidly extract DNAs from various plant tissues.

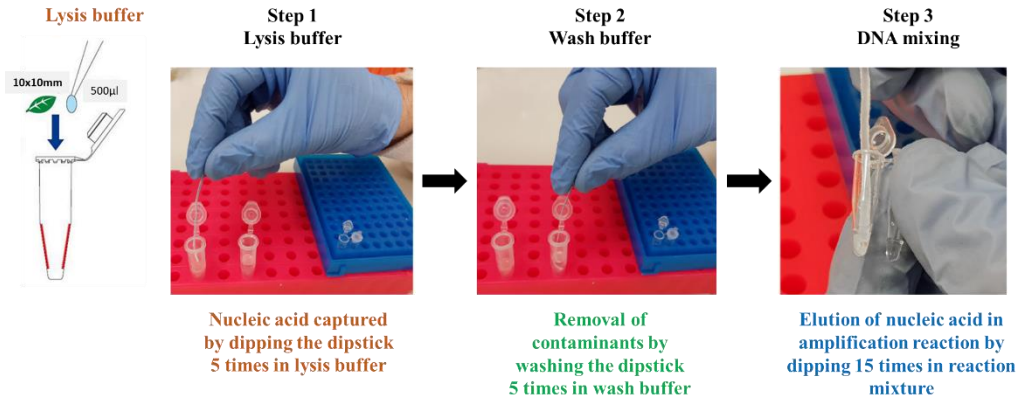


Fig. 11. Rapid extraction of genomic DNAs from the cherry tree samples.

Significance to the industry, and potential economic benefits. The cherry industry has been in an uncharted territory due to the cherry X-disease, which causes significant economic losses to growers and nurseries in the past several years. One major challenge in effectively managing the X-disease phytoplasma (XDP) is to find effective methods of early disease detection for non-fruit-bearing trees and for non-symptomatic trees/rootstocks in both orchards and nurseries. However, early detection of XDP is particularly challenging due to unculturable nature, low titer, and uneven distribution of the pathogens within infected plants as well as the ever changing genotypes of the pathogen. The RPA/Cas12a assay for XDP detection developed in the current study specifically addressed the industry's need by providing an early detection platform with the following advantages: a) highly sensitive, detecting as low as one to two copies of DNA within one microliter sample; b) highly specific, detecting the XDP target DNA with no cross reactivity; c) very rapid and have results within an hour; and d) can detect all current known genotypes of the pathogen. On the other hand, current laboratory-based testing by real-time PCR is the industry's standard for diagnosing an XDP infection not expressing symptoms, which could cost $60 per sample. The method we currently developed costs much less than that, which will provide the industry with an inexpensive alternative method for XDP diagnosis. Therefore, the significant and potential economic benefits to the cherry industry of the current study are multifaced. It not only provides the industry with much-needed tool for fast, supersensitive, and accurate diagnosis, but also saves time and money for both growers and nurseries.

Executive Summary

Project Title: Cas12a-based rapid method for early detection of X-disease phytoplasma

Key words: X-disease Phytoplasma, Diagnosis, Detection, CRSPR-Cas12a, Cherry

Abstract:

The cherry X-disease is at epidemic levels in the Pacific Northwest and caused significant economic losses in the past several years. To effectively manage the X-disease phytoplasma (XDP), it is critical that infected trees be identified as rapidly as possible, especially before symptoms appear. Recently, the clustered regularly interspersed short palindromic repeats/CRISPA-associated (CRISPR-Cas) system has been used as an extremely sensitive and rapid diagnostic tool for pathogen detection besides its common applications in genome editing. In this study, we have developed the recombinase polymerase amplification (RPA)/Cas12a diagnostic assay and a quick DNA extraction method to facilitate the rapid and super-sensitive detection of XDP. Based on the DNA sequence alignment and analysis of the *SecY* genes from the phytoplasma 16SrIII subgroups, XDP-specific RPA primers and crRNAs have been identified and validated for its high efficiency of RPA and for the XDP Cas12a detection assay. Thus, a two-pot RPA/Cas12a assay has been successfully developed for highly sensitive and specific detection of different XDP strains and demonstrated to be capable of specific and rapid detection of XDP-infected samples from the field. Double blind assay results from Cas12a-based tests for more than 100 samples of sweet cherries, weeds and insects correlated well with the current industry standard qPCR assay results. In addition, we also developed a 5-min genomic DNA extraction method for the RPA/Cas12a assay and established one-pot RPA/Cas12a assay based on the cap/spin approach, which will facilitate the field-deployable detection of XDP in the near future. In summary, the highly sensitive, specific, and rapid RPA/Cas12a assay for XDP detection not only provides the industry a much-needed platform for fast, supersensitive, and accurate diagnosis, but also saves time and money for both growers and nurseries.