ENHANCING POTATO VIRUS X DIAGNOSIS: CHELEX™ 100 RESIN RNA EXTRACTION METHOD EVALUATION

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Abstract

Potato Virus X (PVX) is a prevalent pathogen affecting potato crops, leading to substantial yield losses. This study investigates the efficacy of Chelex™ 100 resin RNA extraction method for PVX detection, through RT-qPCR analysis. We evaluate RNA yield, sensitivity, and detection limits of this method compared with Phenol-chloroform RNA extraction protocol. Our results demonstrate that Chelex™ 100 resin extraction yields superior RNA concentrations and exhibits greater sensitivity compared to ELISA immunoassay diagnostic test. RT-qPCR successfully detects PVX RNA in dilutions up to 1:100 using Chelex™ 100 resin extraction. These findings highlight the potential of Chelex™ 100 resin extraction coupled with RT-qPCR as a rapid and reliable method for PVX detection.
Keywords: PVX, Chelex, Molecular Diagnostics, Plant Virus.

Introduction

Potato (*Solanum tuberosum* L.) is one of Colombia's most important crops, with a cultivated area spanning approximately 113,000 hectares and yielding an average of 22.78 tons per hectare (FEDEPAPA, 2023). However, the presence of numerous viral pathogens, particularly Potato virus Y (PVY), Potato leafroll virus (PLRV), Potato yellow vein virus (PYVV), and Potato virus X (PVX), is associated with a negative impact on crop production (Campos & Ortiz, 2020; Guzmán et al., 2010; Kumar et al., 2022). Among them, PVX is one of the most widespread and economically damaging. It is transmitted through various means, including pollen, true seeds, contaminated farming equipment, and direct contact between healthy and infected foliage or roots (Kreuze et al., 2020; Verchot, 2022).

Incidence studies in Colombia have shown that yields can decrease by 3% to 50%, depending on the viral load (García et al., 2023; Sierra et al., 2021). This reduction is attributed to the successive planting of contaminated seeds, climatic conditions, and the response of different potato varieties to viral infection. The impact of PVX on crops generates the need of early and accurate diagnosis for effective disease management and mitigation of economic losses (Devi et al., 2024). Traditional methods for the detection of PVX, such as enzyme-linked immunosorbent assay (ELISA), have been the standard for diagnosis for decades (Boonham et al., 2014; Boston & Halİloğlu, 2011; Qamar et al., 2016; Raigond et al., 2022). While ELISA offers established protocols and reliable results, it is slow and laborious. Furthermore, for this reason, there is a need to develop alternative diagnostic methods that can offer greater

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sensitivity, specificity, speed, and practicality in the context of PVX detection (Hema & Konakalla, 2021; Khurana & Marwal, 2016). Given these requirements, the diagnosis of PVX has increasingly involved qPCR detection of nucleic acids.

Developing more effective diagnostic tools for PVX relies on a thorough understanding of its molecular characteristics. PVX belongs to the genus *Potexvirus* within the family *Alphaflexiviridae*, and is characterized by a genome composed of a single positive-sense RNA strand approximately 6.4 kilobases in length (Grinzato et al., 2020). The PVX genome encodes at least five open reading frames (ORFs), including the viral replicase (RdRp), movement proteins located in the Triple gene block (Tgb), and the viral capsid protein (CP). A methylguanosine cap is situated at the 5' end of the genome, while the 3' end is polyadenylated (Verchot, 2022). To enhance the sensitivity and specificity of diagnostic assays, previous research has focused on targeting for quantitative reverse transcription polymerase chain reaction (RT-qPCR) various regions of the PVX genome such as the capsid region, the RdRp gene, and other conserved sequences (Abbas & Hameed, 2012; Kumar et al., 2021; Ruíz et al., 2016).

However, for RT-qPCR detection to be effective, it is imperative to develop a simplified nucleic acid extraction method designed for PVX detection. Traditional extraction techniques, such as commercial kits or the Phenol-chloroform extraction method, pose inherent challenges, including complexity and reliance on hazardous chemicals, making their widespread adoption difficult, especially in resource-limited settings (Bhat & Rao, 2020). Therefore, this study focuses on exploring the efficacy of Chelex™ 100 resin extraction, a simpler and safer alternative (Shetty, 2020; Shiaw et al., 2010), to isolate high-quality nucleic acids from PVX-infected potato samples. Integrating Chelex™ 100 resin extraction with PCR-based diagnostic assays offers a rapid, reliable, and easy-to-use approach for PVX detection.

Materials and Methods

Plant material and virus isolates

For the diagnosis of PVX, symptomatic *Solanum tuberosum* plants were collected from a pool of *in vitro* specimens originated from crops located in Ventaquemada, Boyacá, Colombia. The plants were screened for PVX using double-antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) with polyclonal antibodies from Agdia (Indiana, USA), following the manufacturer's protocol. Ten potato plants confirmed as PVX positive by ELISA were used for RNA extraction and molecular analysis by RT-qPCR.

RNA extraction

In this study, two distinct RNA extraction protocols were employed to assess their efficacy in isolating viral RNA from potato *in vitro* plants. The first method utilized the Phenol-chloroform technique (method I), a traditional approach widely used in RNA extraction, serving as a benchmark against which the effectiveness of the second method was compared. The method II employed Chelex™ 100 resin. This choice was made due to the known advantages of Chelex™ 100, including its rapid protocol and ease of use (Gautam, 2022; Seufi & Galal, 2020). The starting material for RNA extraction in both methods was represented by 50 mg of infected potato plant tissue, ground into a fine powder inside a 2 mL tube by using liquid nitrogen and a thin metal rod.

Phenol-chloroform method

Total RNA was extracted from samples using method I, modified from the protocol described by Kingston, 2010. The ground plant material was resuspended in 1 ml of lysis buffer (pH 8.2), containing 1% Sodium Dodecyl Sulfate (SDS), 0.18 M Tris, 4.5 mM EDTA, and 0.09 M

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Lithium Chloride. Then, 300 µL of phenol equilibrated with TLE solution (0.2 M Tris, 0.1 M Lithium Chloride, 5 mM EDTA) were added. The mixture was vortexed for 2 minutes, followed by the addition of 600 µL of chloroform, and incubated at 50°C for 20 minutes. Post-incubation, the tubes were centrifuged at 13,000 rpm for 20 minutes at 4°C. The clarified aqueous phase was extracted twice with 600 µL of phenol equilibrated with TLE solution and 600 µL of chloroform. RNA precipitation was achieved by adding a 1:10 ratio of 3 M Sodium Acetate to the sample volume, an equal volume of isopropanol, followed by 12 to 16 hours incubation at -20°C. Subsequently, the tubes were centrifuged at 13,000 rpm for 20 minutes at 4°C. The resulting pellet was washed twice with 70% ethanol, air-dried, and resuspended in 50 µL of RNase-free water.

Chelex™ 100 resin method

The method II was modified from Dreskin et al., 2022, where 50 mg of potato plant ground tissue was homogenized in a 0.5 mL microtube containing 120 µl solution of 6% Chelex™ 100 resin. The mixture was vortexed for 1 min, incubated at 95 °C for 10 min and then refrigerated for 3 min at -20 °C. After, the samples were centrifuged at 13,000 rpm for 1 minute at 4 °C. Approximately 20 µl of the supernatant containing the RNA was transferred to a clean 0.5 mL microtube (Fig 1).

RNA Quantification and Storage

The RNA samples were quantified using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA concentration measurements were repeated three times for each sample. Subsequently, the samples were stored at -80°C for further RT-qPCR analysis.

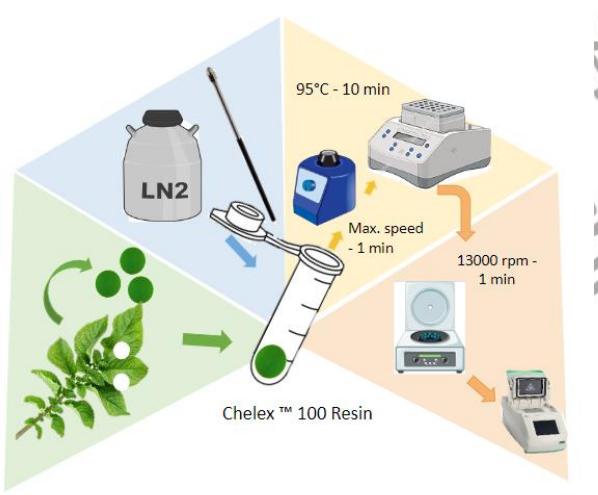

Figure 1. Diagram of rapid protocol applied for RNA extraction using Chelex™ 100 Resin on PVX infected potato plants.

RT-qPCR assay

The RT-qPCR was performed in single-tube reactions with a total volume of 10 µL. Each contained 2 µL of the RNA template, 5 µL of (2x) qScript XLT One-Step RT-qPCR ToughMix (Quantabio, Germany), 0.25 µL of each primer (10 µM), 1.5 µL of BSA (20 mg/mL), 0.5 µL of DMSO and 0.5 µL ddH2O for a final volume of 10 µL. The extracted RNA was normalized to 100 ng/µL, and 1:10, 1:20, 1:50, and 1:100 dilution series were prepared to evaluate the sensitivity of the test. The primers PVX-F (5'-TGGAAGGACATGAARGTGC-3') and PVX-R (5'-CGAATTTGTGCTCAGGCTTG-3') were used to amplify a 282 bp fragment from the capsid protein (ORF5) gene (Yan, 2021). Real-time PCR was conducted on a CFX96 Real-

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Time System (Bio-Rad, USA), with the following thermal cycling conditions: 55 °C for 10 min, 95 °C for 3 min, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The PCR products were analyzed by gel electrophoresis on a 3% agarose gel. Products were visualized using a Bio-Rad ChemiDoc XRS Gel Photo Documentation System (Bio-Rad, USA) and compared to the HyperLadder 50 bp DNA ladder (Bioline, USA).

Results and Discussion

RNA Quantity Assessment

This study focused on streamlining the RNA extraction process for PVX detection, thereby enabling a more efficient process achieving optimal sensitivity using quantitative reverse transcription polymerase chain reaction. We evaluated two distinct extraction methods. The method I, is a widely described standard protocol known for its efficiency albeit labor-intensive nature and safety concerns (Toni et al., 2018). The method II uses the Chelex™ 100 resin recognized for its ability to preserve nucleic acid integrity while offering expedited processing (Gautam, 2022; Sajib et al., 2017; Walsh et al., 1991).

Samples subjected to extraction method I exhibited RNA concentrations between 100 to 140 ng/µL, whereas those processed using method II displayed concentrations exceeding 500 ng/µL (Table 1). This difference highlights the superior RNA yield achieved with method II, exceeding the minimum concentration threshold for PCR development, which is approximately 10 ng/µL (Lorenz, 2012).

Chelex™ resin is a styrene-divinylbenzene copolymer, which has been reported as a chelating agent of divalent cations like magnesium and calcium (Gautam, 2022). The chelation mechanisms inhibit nuclease activity, ensuring the preservation of nucleic acids during the extraction process in complex samples and facilitating the release of nucleic acids from cells or tissues (Singh et al., 2018). The polar resin binds polar cellular components while DNA and RNA remain in the water solution above Chelex™ (Panda et al., 2019). Moreover, the method utilizes thermal denaturation to extract nucleic acids from plant tissues. Raising the temperature of the sample promotes the binding of the resin to the ions and facilitates the release of the nucleic acids from the cells (Guan et al., 2021; Lim et al., 2022).

Table 1. Total RNA concentration obtained from 50 mg of plant tissue, using extraction method I and method II. Each value represents the average of the replicates of each sample and the standard deviation.

Sample No.	Average Total RNA Concentration ± S.D. (ng/µL)	
	Phenol-chloroform (method I)	Chelex™ 100 resin (method II)
1	115 ± 3.7	532 ± 7.8
2	113 ± 4.5	550 ± 5.7
3	118 ± 2.8	524 ± 6.9
4	125 ± 4.9	567 ± 8.2
5	132 ± 4.1	511 ± 6.3
6	138 ± 3.8	546 ± 4.6
7	114 ± 5.3	520 ± 9.1
8	107 ± 5.1	563 ± 7.6
9	121 ± 6.0	507 ± 5.4
10	137 ± 4.7	578 ± 9.5

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Our results demonstrate that method II is simpler and faster when it is compared with method I. Additionally, it does not require the use of organic solvents, making it safer and easier to handle. However, Chelex™-resin is generally less effective at removing contaminants and may yield lower purity of RNA than method I.

RT-qPCR for RNA Detection

The RT-qPCR amplification targeted the capsid protein (ORF5) gene of PVX. The size of the fragment obtained was 282 base pairs (Fig. 2), consistent with the expected result. The bands obtained from the amplification of the RNA extracted with method II were thicker and more pronounced compared to the bands obtained from RNA extracted with method I. This observation could suggest a more effective RNA extraction using the method II, resulting in visibly stronger bands indicating successful amplification of the ORF5 gene. This band intensity can be attributed to the efficiency of the Chelex™ 100 resin as a chelating agent, which allows preserving the integrity of the nucleic acid during the extraction process and its subsequent analysis.

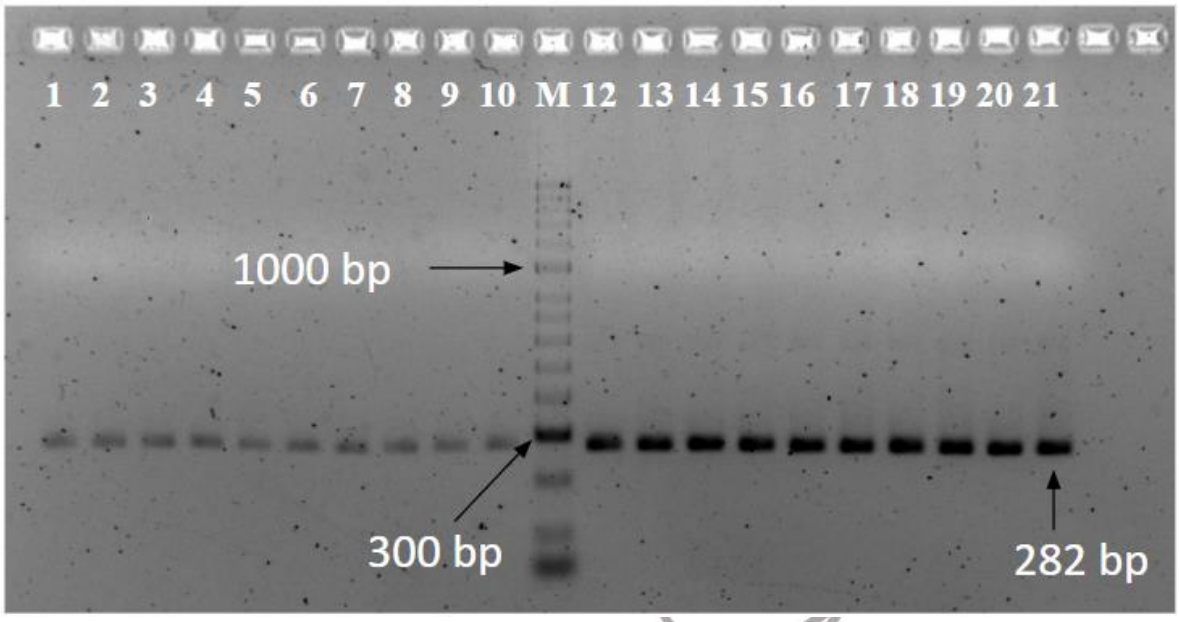

Figure 2. Agarose gel electrophoresis of amplimers of the capsid protein (ORF5) gene from PVX-infected potato plants, obtained using RNA extracted with the method I, lanes 1-10 and the method II, lanes 12-21. Lane M shows the molecular marker: Hyperladder 50 bp.

Detection sensitivity comparison for PVX

For sensitivity evaluation, the RNA extracted with method II from PVX-infected tissue was diluted at ratios of 1:10, 1:20, 1:50, and 1:100, and then analyzed using RT-qPCR. Results demonstrated that RT-qPCR can detect the 1:10, 1:20, 1:50 dilutions between 25 to 26 Ct values with a standard deviation of ± 2.1, indicating robust sensitivity within this dilution range. However, the fluorescence level decreased at the 1:100 dilution (Fig. 3-A), but PVX RNA was still detectable, indicating a reduction in the sensitivity. The analysis of the denaturation curves allowed identification of a single melting temperature (Tm) value among the analyzed samples: Tm =79.5±1°C, indicating the occurrence of a single variant of this virus in the potato plants analyzed (Fig. 3-B).

The RT-qPCR analysis was also conducted with dilutions derived from nucleic acids extracted from method I to compare the sensitivity in detecting the PVX gene in the RNA obtained from both evaluated extraction methods (data not shown). The amplification results of the dilutions

were confirmed by gel electrophoresis, where PCR products of 282 base pairs were obtained for each dilution of each extraction method, as depicted in Figure 4.

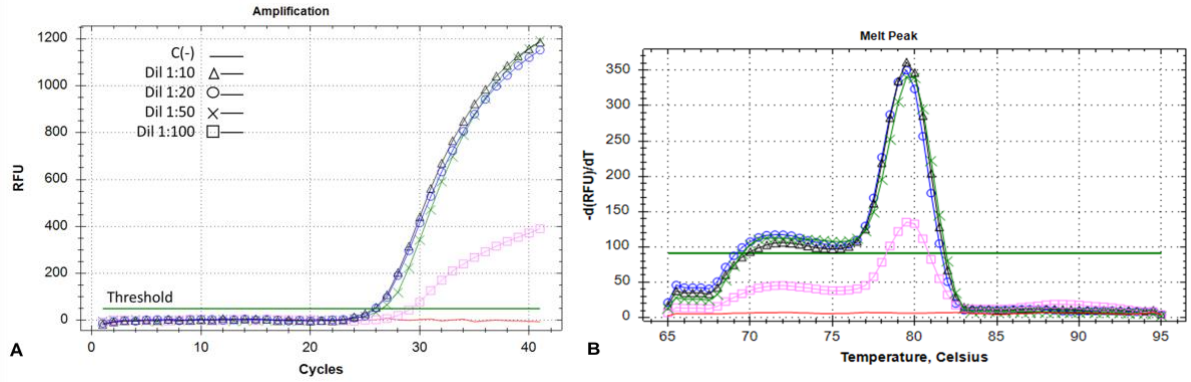

Figure 3. (A) Amplification curves by RT-qPCR for dilutions 1:10, 1:20, 1:50 and 1:100 using the SYBR Green I system and the primers for the detection of PVX in infected potato tissues. (B) Profiles of the denaturation curves of PVX-specific amplicons obtained by RT-qPCR in the different dilutions.

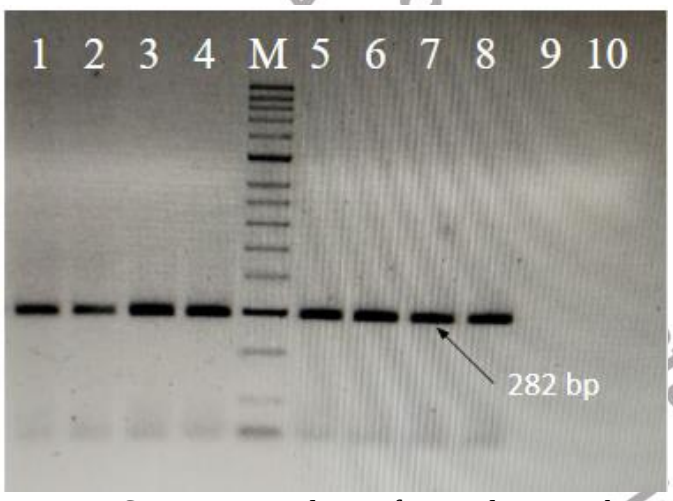

Figure 4. Sensitivity analysis of PVX detection by RT-qPCR from RNA isolated using Method I (lanes 1-4) and Method II (lanes 5-8). Lanes 1 and 5 show the 1:10 dilution, lanes 2 and 6 the 1:20 dilution, lanes 3 and 7 the 1:50 dilution, and lanes 4 and 8 the 1:100 dilution for each method. Lane M represents the Hyperladder 50 bp molecular marker, and lanes 9-10 are negative controls.

From a previous study, data on sensitivity limits were obtained from the conventional immunoassay ELISA used for the diagnosis of PVX (Yan, 2021), with the objective of comparing the detection limits with the RT-qPCR technique in different dilution ranges (Table 2). The sensitivity results showed that RT-qPCR, from both RNA extraction methods, demonstrated higher sensitivity for PVX detection compared to reported ELISA assays, detecting up to a 1:100 dilution, whereas ELISA was limited to detect up to a 1:20 dilution.

Table 2. Comparison of the detection sensitivity of ELISA (Yan, 2021) and RT-qPCR for PVX with both extraction methods applied. "+" indicates the positive detection of the virus and "-" indicates a negative result for the detection.

Dilutions	ELISA	RT-qPCR

		Phenol-chloroform (method I)	Chelex™ 100 resin (method II)
1:10	+	+	+
1:20	+	+	+
1:50	-	+	+
1:100	-	+	+

Furthermore, the RT-qPCR technique offers advantages in terms of sensitivity and speediness of results. RT-qPCR exponentially amplifies specific nucleic acid sequences within a short timeframe, facilitating the detection of even small quantities of viral RNA; this high sensitivity is particularly crucial in the context of crop infectious diseases, where early detection is desirable for containment and mitigation efforts. (Jeong et al., 2014).
Additionally, ELISA assays could incur higher costs and are less efficient compared to PCR-based methods (Boonham et al., 2014). ELISA procedures typically require approximately 8 hours to yield results, while Chelex™ RNA extraction coupled with RT-qPCR provides more precise results in just 3 hours, significantly reducing time and labor costs. In addition, ELISA immunoassay may include the risk of false negative results, since it relies on antibody-antigen interactions, which may not be as sensitive in detecting low viral concentrations. This is particularly evident when testing is conducted during the early stages of infection when viral antigen concentrations are low (Cassedy et al., 2021), compromising the accuracy of the diagnosis.

Conclusion

The study establishes the efficacy of the Chelex™ 100 resin RNA extraction method in optimizing the extraction process for PVX detection via RT-qPCR. While the Phenol-chloroform method remains reliable, the Chelex™ 100 resin method offers expedited processing, higher RNA yield, and comparable sensitivity. These findings not only contribute to the optimization of PVX diagnostic protocols but also underscore the importance of RNA extraction methodologies in molecular biology research.

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