# EVALUATION OF DNA EXTRACTION METHODS FROM DRIED AND FROZEN TOMATO LEAVES FOR DETECTING BEGOMOVIRUSES BY PCR

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*SUMMARY*

*Six DNA extraction protocols for dried and frozen tomato [*Solanum lycopersicum *(Mill)] leaves were evaluated to detect begomoviruses using polymerase chain reaction (PCR). The extraction method comparisons were based on DNA quantity and quality, extraction cost, and processing time. Four samples of dried and frozen tomato leaves, collected in the Venezuelan states of Zulia, Mérida and Táchira, were evaluated. The DNA concentration and the ratios of 260 and 280µm absorbances were measured using spectrophotometry. Begomovirus detection was performed using degenerate primers for viral components A and B (DNA-A and DNA-B), and PCR band intensity was recorded using a visual scale. The SDS method produced greater DNA quantity and quality, and it is faster and less expensive than the other extraction protocols tested. Begomovirus DNA-B was more consistent in amplification than DNA-A, suggesting higher variability of the A component. Resuspended DNA pellets minimized the effects of inhibitors on the amplification because of the high sensitivity of the PCR technique, which also allowed begomovirus detection even when using small amounts of dried and frozen leaf tissue. The DNA isolated by the SDS method provides a simple and economical option for begomovirus screening in tomato using PCR, and could be used for large-scale epidemiological studies.*

## Introduction

Tomato [*Solanum lycopersicum* (Mill)] is a vegetable extensively distributed worldwide. It is also great importance in human consumption (Hernández *et al*., 2008), having a high production volume of 129,942,416t, and a yield of 27,980kg·ha$^{-1}$ (FAOSTATS, 2009). Although its cultivation is often highly profitable, it also has high risks due to complex problems in the management of diseases including viruses, especially begomoviruses, which are common pathogens in several regions of Venezuela (Debrot *et al*., 1963; Nava, 1999; Nava *et al*., 2006), as well as in subtropical and tropical zones of other countries (Chowda *et al*., 2005; Moriones and Navas-Castillos, 2000; Polston and Anderson, 1997).

Begomoviruses are transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Fauquet *et al*., 2006), and possess one or two molecules of circular single-stranded DNA (called DNA-A and DNA-B) protected by a capsid of twin quasi-icosahedric particles (Fontenelle *et al*., 2007). The viral components have been identified in plant tissues and in their vector using cytological, serological, and hybridization tests (Mason *et al*., 2008). Molecular diagnosis using the polymerase chain reaction (PCR) has high sensitivity and precision because it amplifies DNA segments from small fresh, dry or frozen quantities of tissue (Royce and McLaughlin, 1997), and has been successfully adopted for the detection of begomoviruses (Ghosh *et al*., 2009).

In plants, however, there is no universally applicable protocol to extract high quality DNA. Tomato plants have polyphenols and tannins that participate in the degradation of DNA during extraction (Varma *et al*., 2007). The steps that govern an extraction method begin with the rupture of tissues and cell walls using a variety of buffers, followed by membrane disruption using detergents such as sodium dodecyl sulfate (SDS) and Triton®. To eliminate protein contaminants, reagents such as proteinase K, ethylenediaminetetraacetic acid (EDTA), chloroform:isoamyl and mercaptoethanol are used. Cleaning phenol residues requires mercaptoethanol, SDS, PVP (polyvinylpyrrolidone), sodium sulfite, or cesium chloride (CsCl). Carbohydrates are reduced using cetyl trimethyl ammonium bromide (CTAB), NaCl, or KCl. Finally, isopropanol and ethanol are used to precipitate DNA (Rocha, 2002).

Measurement of DNA concentration has been carried out using high performance liquid chromatography (Diadema *et al*., 2003), absorption and fluorescence emission spectra at 458nm with the dye Hoechst 33258, fluorescence induction by UV light using intercalation of ethidium bromide, and spectrophotometric analysis of absorbance readings at 260nm ($A_{260}$) and 280nm ($A_{280}$). Due to its rapidity, simplicity and negligible ef-

**KEYWORDS / CTAB / DNA Isolation / Geminiviridae / SDS /**

Received: 04/21/2010. Modified: 09/29/2010. Accepted: 10/11/2010.

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## EVALUACIÓN DE MÉTODOS DE EXTRACCIÓN DE ADN DE HOJAS DE TOMATE SECAS Y CONGELADAS PARA DETECCIÓN DE BEGOMOVIRUS POR PCR

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*RESUMEN*

*Seis protocolos de extracción de ADN para hojas de tomate [*Solanum lycopersicum *(Mill)] secas y congeladas fueron evaluados para la determinación de begomovirus utilizando la reacción en cadena de polimerasa (PCR). La comparación de los métodos de extracción se basó en la cantidad y calidad del ADN, costo de extracción y tiempo de procesamiento. Fueron evaluadas cuatro muestras de hojas de tomate secas y congeladas, recolectadas en los estados venezolanos de Zulia, Mérida y Táchira. La concentración de ADN y la relación de absorbancias a 260 y 280nm fueron determinadas por espectrofotometría. La detección de begomovirus se llevó a cabo utilizando cebadores degenerados para los componentes virales A y B (ADN-A y ADN-B), y la intensidad de las bandas de PCR se registró usando una escala visual. El método SDS produjo mayor cantidad y calidad de ADN, y es más económico y rápido que los otros protocolos de extracción ensayados. El ADN-B de begomovirus fue más consistente en la amplificación que el ADN-A, sugiriendo una mayor variabilidad del componente A. Los pellets de ADN resuspendidos minimizaron los efectos de inhibidores en la amplificación debido a la alta sensibilidad de la técnica de PCR, lo que también permitió la detección de begomovirus, incluso utilizando pequeñas cantidades de tejido de hojas secas y congeladas. El ADN aislado por el método SDS provee una opción sencilla y económica para la detección de begomovirus en tomate usando PCR, y podría ser empleado para estudios epidemiológicos de gran escala.*

## AVALIAÇÃO DE MÉTODOS DE EXTRAÇÃO DE DNA DE FOLHAS DE TOMATE SECAS E CONGELADAS PARA DETECÇÃO DE BEGOMOVIRUS POR PCR

Carla Méndez, Alfredo Faría, Francisco Osorio-Acosta e Alba Nava

*RESUMO*

*Seis protocolos de extração de DNA para folhas de tomate [*Solanum lycopersicum *(Mill)] secas e congeladas foram avaliadas para a determinação de begomovirus utilizando a reação em cadeia de polimerase (PCR). A comparação dos métodos de extração se baseou na quantidade e qualidade do DNA, custo de extração e tempo de processamento. Foram avaliadas quatro amostras de folhas de tomate, secas e congeladas, colhidas nos estados venezuelanos de Zulia, Mérida e Táchira. A concentração de ADN e a relação de absorvâsncias a 260 e 280nm foram determinadas por espectrofotometría. A detecção de begomovirus foi realizada utilizando cevadores degenerados para os componentes virais A e B (DNA-A e DNA-B), e a intensidade das bandas de PCR se registrou usando uma escala visual. O método SDS produziu maior quantidade e qualidade de DNA, e é mais econômico e rápido que os outros protocolos de extração ensaiados. O DNA-B de begomovirus foi mais consistente na amplificação que o DNA-A, sugerindo uma maior variabilidade do componente A. Os pellets de DNA resuspensos minimizaram os efeitos de inibidores na amplificação devido a alta sensibilidade da técnica de PCR, o que também permitiu a detecção de begomovirus, inclusive utilizando pequenas nos tecidos de folhas secas e congeladas. O ADN isolado pelo método SDS provee uma opção simples e econômica para a detecção de begomovirus em tomate usando PCR, e poderia ser empregado para estudos epidemiológicos de grande escala.*

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fects on sample integrity, spectroscopic absorption has been widely used as the preferred method for DNA and RNA quantification (Sambrook and Russell, 2001). The proportion of absorbances ($A_{260}$:$A_{280}$) provides an estimate of the purity of the nucleic acids. Pure preparations of DNA and RNA have $A_{260}$:$A_{280}$ values of ~1.8 and ~2.0, respectively (Sambrook and Russell, 2001; Diadema *et al*., 2003; Puchooa, 2004; Moyo *et al*., 2008). The objective of the present study was to evaluate six DNA extraction methods on samples from dried and frozen tomato leaves for the detection of begomoviruses using PCR.

### Materials and Methods

*Samples*

Tomato [*Solanum lycopersicum* (Mill)] leaf samples, collected from four production zones in Venezuela, were dried at room temperature between sheets of paper for about 2-3 weeks and then frozen at -20°C until use. Sample 71-v, in which leaf curl symptoms were present, was from Las Camelias farm, located in Caña Brava, Páez municipality, Zulia State. Sample 57-v had mottling and deformation of leaves (Nava *et al*., 1998) and was collected from La Mesa farm, in Los Estanques, Sucre municipality, Mérida state. Sample 354-v showed severe yellow mosaic, and was collected in San Rafael, Cárdenas municipality, Táchira state. The last sample, 385-v, exhibited yellow mosaic, leaf curl and severe cupshaped leaves (Faría and Nava, 2009), and was from Las tablas farm, located in Vía Monte Carmelo, Andrés Bello municipality, Táchira state. Three replicate samples of 3mg each were placed in individual 1.5ml Eppendorf tubes for further DNA extraction.

*DNA extraction*

Six different extraction protocols were compared. DNA was extracted from leaves showing viral symptoms using the following methods: CTAB (Doyle and Doyle, 1987), SDS (Gilbertson *et al*., 1991), 1X CTAB/ 10% CTAB (Rogers and Bendich, 1985), KCl/ $Na_2SO_3$ (Baranwal *et al*., 2003), Gem-CTAB (Rouhibakhsh *et al*., 2008), and 2X CTAB/ RNAse/ CsCl (Rath *et al*., 1998), with some modifications. For all of the protocols, tissue homogenization was carried out using a pellet pestle (Thomas Scientific, Swedesboro, USA) and drill-mounted Eppendorf tube, and DNA pellets were dissolved in 50µl of TE-8 1X buffer. RNAse A (20µg) was added to the protocol; KCl/$Na_2SO_3$,

Gem-CTAB, and 2X CTAB/ RNAse/ CsCl. Modifications for the 1X CTAB/ 10% CTAB method were the utilization of 170µl of 1X CTAB extraction buffer, 170µl of 10% CTAB, 200µl of CTAB precipitation buffer, 100µl of saline TE, and 200µl of 80% ethanol to wash the pellet. The DNA was resuspended in 50µl of 0.1X TE, omitting the use of yeast RNAt. For the $KCl/Na_2SO_3$ method, 500µl of extraction buffer were used, the incubation period at -80ºC was of 30min, and the pellet was washed with 200µl of 70% ethanol. The Gem-CTAB protocol utilized 500µl of Gem-CTAB buffer. For the 2XCTAB/RNAse/ CsCl method, contaminants were eliminated using chloroform:isoamyl alcohol (24:1) without phenol. The DNA concentration ([DNA]) was measured in $ng \cdot \mu l^{-1}$, and the quality measured through the ratio $A_{260}$:$A_{280}$ (Sambrook and Russell, 2001) using a spectrophotometer (WPA, Biowave, Biochrome). All readings were carried out in triplicate.

*PCR*

Two sets of primers were used for each viral component. The pair PAR1c496 and PAL1v1978 amplifies 100bp of the A component, and the pair PVL1v2040 and PCRc154 amplifies ~700bp of the B component (Rojas *et al*., 1993). PCR was carried out in a Mastercycler EP gradient S (Eppendorf, Hamburg, Germany) thermocycler. Amplification was performed in a volume of 25µl in each PCR tube. The reaction mixture contained 1X green GoTaq® flexi buffer, 2.5mM $MgCl_2$, 0.4mM dNTPs, 0.2µM of each primer, 0.15U/µl GoTaq® DNA Polymerase, 0.75µl of genomic DNA, and RNAse free water up to 25µl. The PCR mixture was subjected to an initial cycle of denaturalization for 2min at 94ºC, 35 cycles for 30s at 94ºC, 35s at 55ºC, 1.15min at 72ºC, with increments of 3s, and a final extension for 7min at 72ºC (Rojas *et al*., 1993). The DNA of the positive and negative controls for PCR was extracted using the SDS method. Samples 451-v and 450-v were used as positive controls to detect DNA-A and DNA-B, respectively (Nava and Faría, unpublished data); a sample from healthy tomato grown under laboratory conditions was used as a negative control, and pure water (Promega, Madison, USA) was used as an absolute negative control. To verify the expected sizes of the PCR products, a 100bp DNA ladder was used (100bp DNA ladder, Promega, Madison, USA). The PCR products were run on 1.5% (p/v) agarose gels in TBE (0.045M Tris-borate, 0.001M EDTA, pH 8.0) for 10min at 100V, 15min at 75V, and 30min at 50V. Gels were stained with ethidium bromide at $0.0015mg \cdot ml^{-1}$ (Faría and Nava, 2009). The photographs were taken in a UV transilluminator (UVP-Bio-Imagin System, Epichemi 3 Darkroom, USA).

*Statistical analysis*

A split plot in a completely randomized design was used, where the main plot was the samples and the secondary plot was the DNA extraction methods. Statistical analyses were carried out using Statistix 8.0 (Tallahassee, Florida, USA), analysis of variance was performed on transformed values of [DNA] (ln[DNA]) and the ratio $A_{260}$:$A_{280}$ ($Arctg\sqrt{(A_{260}:A_{280})}$), mean comparisons were performed using Tukey's test. The intensity of the expected PCR bands was measured on a visual scale (0-7), where 0 was absence of the band and 7 was the greatest intensity observed, and the values were analyzed using a nonparametric Kruskal-Wallis test. The selection of the best DNA extraction method was based on the quantity and quality of DNA, the unitary cost and the extraction time required to process 12 samples for each method.

TABLE I
DNA CONCENTRATION AND $A_{260}$:$A_{280}$ RATIO OBTAINED FROM FOUR SAMPLES OF DRIED AND FROZEN TOMATO (*Solanum lycopersicum*) LEAVES SUBJECTED TO SIX METHODS OF DNA EXTRACTION

| Extraction methods | [DNA] ($ng \cdot \mu l^{-1}$) | $A_{260}$:$A_{280}$ ratio |
|---|---|---|
| CTAB | 1,428.8 a | 1.3555 a |
| SDS | 1,347.4 a | 1.2618 abc |
| 2X CTAB/ RNAse/ CsCl | 578.5 b | 1.1584 bc |
| KCL/ Sodium sulfite | 567.2 b | 1.2797 abc |
| Gem-CTAB | 449.1 b | 1.3577 ab |
| 1X CTAB/ 10% CTAB | 470.1 b | 1.1369 c |
| Mean (X) | 806.93 | 1.2583 |

Within each column, mean values accompanied by the same letter are statistically similar using Tukey's test with $\alpha$=5%.

## Results and Discussion

*DNA quantity*

The greatest concentration of DNA was observed with the CTAB ($1,428.8ng \cdot \mu l^{-1}$) and SDS ($1,347.4ng \cdot \mu l^{-1}$) methods (Table I), achieving a sufficient nucleic acid amount from small quantities (3mg) of dried and frozen tissue of tomato leaves. Generally, these values were greater than those obtained by the original authors of each method (Rath *et al*., 1998; Baranwal *et al*., 2003; Rouhibakhsh *et al*., 2008). Nevertheless, the quantity of DNA is relative (Rocha, 2002); its final concentration is influenced by the species, the genome size, the ploidy, the size of the cells at different phenological phases of the plant, the amount and type of tissue (Rogers and Bendich, 1985), the proportion between high quantities of tissue and volume of the extraction buffer used (Moyo *et al*., 2008), and the effect of some substances in the extraction buffers that maintain the stability of the pure DNA (Rogers and Bendich, 1985; Anchordoquy and Molina, 2007).

*DNA quality*

The $A_{260}$:$A_{280}$ ratio for the six evaluated methods was <1.5 (Table I), revealing the presence of contaminants in the preparation, such as proteins and phenols (Stamm, 2006). A greater ratio of $A_{260}$:$A_{280}$ (1.35) was obtained by using the CTAB method, but was smaller than that reported initially for the 2X CTAB/ RNAse/ CsCl method (Rath *et al*., 1998). However, the great majority of studies on begomovirus detection have used the intensity of the PCR product bands as indirect criteria for measuring DNA quality (Gilbertson *et al*., 1991; Baranwal *et al*., 2003; Rouhibakhsh *et al*., 2008), although this does not definitively indicate the degree of contamination by different compounds.

*Detection of viral components (PCR)*

The six methods of DNA extraction evaluated influenced the intensity of the bands of both components of begomovirus (Table I). The greater intensity of both components was observed when the DNA was obtained using the CTAB and SDS methods (Figures 1 and 2). Also, greater detection of component B was observed (Figure 2), due to a greater consistency of segment amplification. Similar data have been reported in tomato where begomoviruses were visualized using degenerate primers (Faría and Nava, 2009), and in jute using specific primers (Ghosh *et al*., 2009). The intensity of the PCR bands shows that an ef-

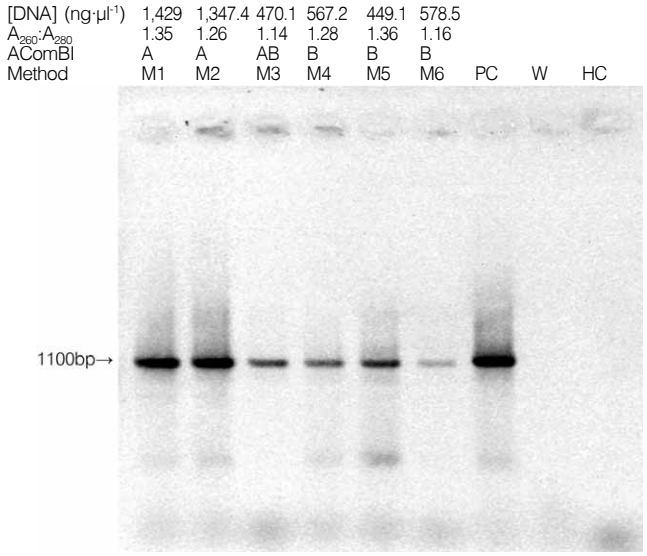


Figure 1. DNA quantity and quality ($A_{260}$:$A_{280}$), A component band intensity (AComBI) obtained using six methods of DNA extraction from samples of dried and frozen tomato leaves. M1: CTAB, M2: SDS, M3: 1X CTAB/ 10% CTAB, M4: KCl/ $Na_2SO_3$, M5: Gem-CTAB, M6: 2X CTAB/ RNAase/ CsCl, PC: positive control, HC: healthy control, W: water. Values with the same letter are statistically similar using Kruskal-Wallis test and $\alpha$=5%.

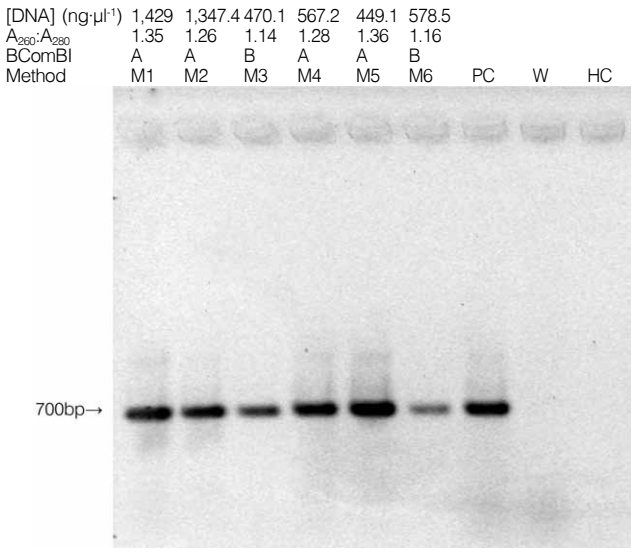


Figure 2. DNA quantity and quality ($A_{260}$:$A_{280}$), B component band intensity (BComBI) obtained using six methods of DNA extraction from samples of dried and frozen tomato leaves. M1: CTAB, M2: SDS, M3: 1X CTAB/ 10% CTAB, M4: KCl/ $Na_2SO_3$, M5: Gem-CTAB, M6: 2X CTAB/ RNAse/ CsCl, PC: positive control, HC: healthy control, W: water. Values with the same letter are statistically similar using Kruskal-Wallis test and $\alpha$=5%. Values in bold indicate a compensation effect.

ficient amplification depends on the primer sequences (Nagata *et al*., 2004) and the quality of the DNA, which is affected by the presence of inhibitors in the extract (Dalmon *et al*., 2000). In this study, a compensatory effect was observed between DNA concentration and the $A_{260}$:$A_{280}$ ratio in relation to intensity of the PCR product bands for component B (Figure 2; Ghosh *et al*., 2009). The Gem-CTAB and KCl/$Na_2SO_3$ methods provided low DNA concentrations; however, its higedquality increased the intensity of the PCR bands to allow the detection of the begomovirus B component.

*Time and cost*

The SDS method was performed in a shorter time (2h, 9min) and was less expensive (USD1.46; Table II), while no harmful reagents such as chloroform (hazardous), CTAB or liquid nitrogen (costly) were used. A summary of the results obtained regarding time and cost to process the samples is shown in Table II. Although the weighing and homogenization of samples are time consuming as a component of plant DNA extraction procedure (Csaikl *et al*., 1998), in this work we did not include this time, given that such activities were equal for all the methods tested.

TABLE II
TIME AND COST FOR EACH METHOD OF DNA EXTRACTION USED ON FOUR SAMPLES OF DRIED AND FROZEN TOMATO (*Solanum lycopersicum*) LEAVES

| Method | Time (hours:min)‡ | Cost of extraction (chemicals + supplies)§ |
|---|---|---|
| CTAB | 03:18 | 1.50 |
| SDS | 02:09 | 1.46 |
| 1X CTAB/ 10% CTAB | 02:15 | 1.55 |
| Gem-CTAB | 03:16 | 2.92 |
| KCL/ sodium sulfite | 03:43 | 2.79 |
| 2X CTAB/ RNAse/ CsCl | 05:49* | 3.62 |

* Includes an overnight step addition to the 5:49h.
§ Cost in USD on 07/17/2009, from Sigma-Aldrich online.
‡ Time required to process 12 samples.

## Conclusions

The SDS protocol produced a greater quality and quantity of DNA for the detection of begomoviruses using degenerate primers in PCR, and it was also the fastest and the least expensive method among the six DNA extraction protocols evaluated on dried and frozen tomato leaves. The presence of begomoviruses was detected by PCR using degenerate primers even though a low concentration of DNA was obtained in most cases, probably due to a compensatory effect from high quality of DNA. The reduced detection of begomovirus A component suggests variability among the samples and, consequently, low hybridization with the degenerate primers. Small amounts of dried and frozen tomato leaf tissue are sufficient to detect begomoviruses given the high sensitivity of PCR.

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