

# SEROLOGICAL AND MOLECULAR IDENTIFICATION OF *Cucumber mosaic virus* (CMV) INFECTING BANANA CROPS IN ECUADOR

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## Abstract

*Cucumber mosaic virus* (CMV) is an important viral pathogen in banana plants (*Musa* spp.) in the world. CMV is an aphid transmitted plant virus belonging to the genus *Cucumovirus* and family *Bromoviridae*. Ecuador is one of the major banana export countries in the world. The infection of banana crops by CMV is a major problem in Ecuador 'Therefore', 'for this reason', in the present study, standardization of triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) and Reverse Transcription-PCR (RT-PCR) was carried out for the detection of CMV infected banana samples from the Ecuador. The CMV suspected banana samples (n=24) were collected in the major growing areas of Santa Elena, Los Rios, Santo Domingo and Guayaquil provinces from Ecuador. Among these collected banana samples, only four samples were confirmed as CMV infected by TAS-ELISA using the CMV specific antiserum. The RT-PCR was performed from CMV infected seven banana samples were DNA fragments of 500 bp were amplified by using the CMV-CP gene specific primers. Further, the standardized RT-PCR and TAS-ELISA can be used for the screening of CMV infected banana plant materials at any time during the growing seasons.

**Keywords:** Banana, *Cucumber mosaic virus*, TAS-ELISA, RT-PCR

## IDENTIFICACIÓN SEROLÓGICA Y MOLECULAR DE *Virus del Mosaico del Pepino* (CMV) INFECTANDO EL CULTIVO DE BANANO EN ECUADOR

### Resumen

El *Virus del Mosaico del Pepino* (CMV) es un importante patógeno viral en las plantas de banano (*Musa* spp.) en el mundo. CMV es un virus vegetal transmitido por un áfido perteneciente al género *Cucumovirus* de la familia *Bromoviridae*. Ecuador es uno de los mayores exportadores de banano en el mundo. La infección en los cultivos de banano debido a CMV es un problema importante en el Ecuador. Debido a ello, en el presente estudio se realizó la estandarización de un ensayo de inmunoabsorción ligado a una enzima – sandwich triple anticuerpo (TAS-ELISA) y PCR- transcripción reversa (RT-PCR) para la detección de muestras de banano de Ecuador, infectadas con CMV. Las muestras de banano sospechosas con CMV (n=24) fueron recolectadas de las principales áreas de producción de las provincias de Santa Elena, Los Ríos, Santo Domingo y Guayaquil en Ecuador. Entre las muestras recolectadas, solamente cuatro muestras fueron confirmadas con la infección de CMV utilizando el antisuero específico para CMV. Para la RT-PCR se utilizó siete muestras de banano infectadas con CMV, de las cuales se obtuvieron fragmentos de ADN de 500 pares de bases mediante amplificación utilizando primers CMV-CP de genes específicos. Adicionalmente, los protocolos RT-PCR y TAS-ELISA estandarizados pueden ser utilizados para la detección de material vegetal infectado con CMV en cualquier momento dentro de los estadios de crecimiento.

**Palabras Clave:** Banano, *Virus del Mosaico del Pepino*, TAS-ELISA, RT-PCR

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## I INTRODUCTION

Banana (*Genus Musa*) is one of the major food and horticultural crop and it is cultivated in more than 130 countries in tropical and subtropical regions of the world. It is a major staple food and income for millions of smallholder farmers across the many countries. In developing world's banana is the sixth most important global food crop after maize, rice, wheat, potato and cassava [1]. The world total production of banana was estimated at 107 million tons from 5.08 million ha in 2013 ([www.faostat/banana/2013](http://www.faostat/banana/2013)). In Ecuador the total production of banana was estimated at 5.99 million tons from 0.19 million ha ([www.faostat/banana/2013](http://www.faostat/banana/2013)) and also Ecuador is one of the major banana export country in the world [1].

The *Cucumber mosaic virus* was identified in vegetables like cucumber, pepper, lettuce, spinach, lupin and red currant crops [2,3,4,5,6,7]; ornamental plants like gladiolus, lily, impatiens, begonia and honeysuckle [8,9,10,11,12] and also weed plants. [2,13] Until recently CMV isolates were detected using biological tests or serological methods, mainly ELISA [9, 12]. Previously, *Cucumber mosaic virus* was identified in different plants by molecular methods. [14,15,16]

Mosaic or infectious yellowing in banana is an important viral disease in banana caused by *Cucumber mosaic virus* (CMV). It has a wide host range, infecting more than 1200 plant species in tropical and subtropical regions in the world [17]. First report of CMV on banana in New South Wales (NSW) in 1929 and also recognized in most banana growing areas of the world [18, 19]. CMV is transmitted through planting material and non-persistently by several aphid species i.e., *A. gossypii*, *A. craccivora*, *R. maidis*, *R. prunifolium*, and *Myzus persicae* [20]. CMV isolates were grouped into two major subgroups (1 and 2) based on the serological and molecular characters [21]. Phylogenetic analysis of a number of CMV isolates has led to further subdivision of subgroup I into IA and IB [22]. One of the subgroup (1B) of *Cucumber mosaic virus* is limited to Asia, and other two subgroups (1A and 2) distributed worldwide. CMV is the type member of the genus *Cucumovirus* (family *Bromoviridae*), has 29 nm isometric and its genome consists of tripartite (RNA1, RNA2 RNA3), linear, positive sense ssRNA with a total length of 8621 nucleotides [23]. RNA 1, RNA 2 encode 1a, 2a proteins involved in virus replication [24], RNA 3a encodes movement protein (MP) [25] and 3b expressed from RNA 4 coat protein (CP) [26, 27]. The RNA viruses have a genetic variation is high due to the absence of proofreading ability of the RNA replicase.

The banana crop is infected by *Cucumber mosaic virus* and it is a major problem in Ecuador. To overcome this problem, TAS-ELISA kit protocol (Agdia, USA) was modified for the detection of CMV in banana. The *Cucumber mosaic virus* suspecting banana samples were collected from the major growing areas of Santa Elena, Los Ríos, Santo Domingo and Guayas states in Ecuador. In this present study, standardization of serological (TAS-ELISA) and molecular (RT-PCR) identification of CMV infecting banana crop in Ecuador.

## II MATERIALS AND METHODS

### Survey and sample collection

The CMV suspected banana plants (n=24), which showed leaf mosaic, yellow stripes in leaves, leaf distortion and stunting of plant (Fig. 1a & 1b) were collected from major growing areas of Santa Elena, Los Rios, Santo Domingo and Guayas states from Ecuador (Table 1).



**Figure 1a & 1b:** Symptoms associated with natural occurrence of *Cucumber mosaic virus* on banana: leaf mosaic, yellow stripes in leaves, leaf distortion and stunting of plant.

**Table 1:** Survey and collection of *Cucumber mosaic virus* (CMV) infecting banana samples were confirmed by TAS-ELISA and RT-PCR.

S.No	Place	Crop	Samples	TAS-ELISA	RT-PCR
1	Los Rios	Banana	6	1	2
2	Guayaguil	Banana	4	1	2
3	Santa Elena	Banana	4	0	1
4	Santa Domingo	Banana	10	2	2
<b>Total</b>			<b>24</b>	<b>4</b>	<b>7</b>

### Enzyme-linked immunosorbent assay (ELISA):

Triple antibody sandwich (TAS)-ELISA (Agdia, USA) kit method was modified and performed by following this protocol. The total procedure is completed within 3-4 hours as compared to kit protocol. The microtitre plates were coated with antiserum diluted in carbonate buffer (pH 9.6) according to the manufacturer's instructions. The IgG antibody was diluted in the carbonate buffer in the proportion of 1: 1000<sup>o</sup> (v/v); add 100 µl of antibody into plates and incubated for 60 min at 37°C. The leaf samples exhibiting mosaic, yellowing and healthy plant samples were ground in general extraction buffer with 0.5ml/l Tween-20 and 2% polyvinyl pyrrolidone, these

leaf extracts were used as antigens. The antibody coated plates were washed three times with PBS-T buffer. 100 µl of each sample was loaded into wells of ELISA plates and incubated at 37°C for 60 min. After incubation, the antigen coated plates were washed three times with PBS-T buffer. The antibody conjugated with alkaline phosphatase diluted in PBS-TPO buffer according to the manufacturer's instructions. Add 100 µl of conjugated with alkaline phosphatase and incubate at 37°C for 60 min. The plates were washed three times with PBS-T buffer. Absorbance at 405 nm (measured with an ELISA Reader; Biotek ELX 800, USA) was read 30 min after incubation with the substrate p-nitrophenyl phosphate (1 mg/ml, pH 9.8). A sample was considered positive if the absorbance was at least three times greater than that of the healthy control plant (negative control).

### Isolation of RNA

Approximately 100 mg of the virus suspected leaf samples were taken for isolation of total RNA. The leaf material was ground into fine powder in a mortar using pestle with liquid nitrogen. The fine powder was transferred into a sterile eppendorf tube; add 0.5 ml of plant RNA reagent and then incubated at room temperature for 5 min for dissociation of nucleoproteins. Next, 0.1 ml of 5M sodium chloride mix were added and mixed thoroughly by inversion and 0.3 ml of chloroform was added and shaken vigorously for 15 seconds and allowed to stand for 2-5 min at room temperature. The resulting mixture was centrifuged at 12,000 x g for 10 min at 4 °C. Centrifugation separates the mixture into 3 phases: a lower red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The upper separated aqueous phase was transferred to a fresh tube and equal volume of isopropanol was added. The mixture was shaken vigorously and incubated at room temperature for 10 min and centrifuged at 12000 x g for 10 min at 4°C to pellet the RNA. The RNA pellet was then washed with 1 ml of 75% ethanol by centrifugation at 7500 x g for 1 min. The RNA pellet was briefly dried and re-suspended in 50 µl of RNase-free DEPC treated water.

### Reverse Transcription Polymerase chain reaction (RT-PCR):

#### cDNA synthesis:

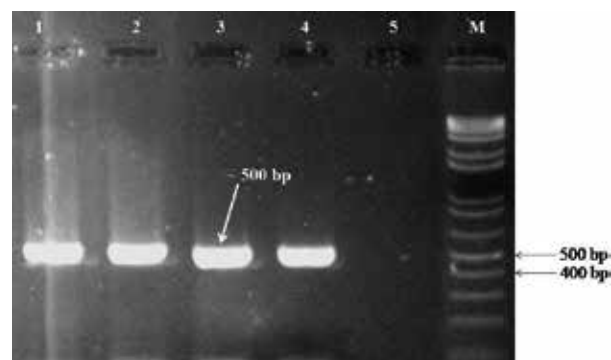
Reverse transcription reactions were done using SuperScript III Reverse Transcriptase (Invitrogen, USA) with random hexamer primers following the manufacturer's instructions. In the first step of cDNA synthesis, 5 µl total RNA, 1 µl (50ng/µl) random hexamer primers, 1 µl 10mM dNTP's and 3 µl

nuclease-free water were mixed and denatured at 65°C for 5 min and then kept on ice. To this mixture, 4 µl 5× first strand buffer, 1 µl (40 U/µl) RNasin® Plus RNase Inhibitor, 2 µl (0.1 M) DTT and then kept at 37°C for 2 min and then 1.0µl (200U/µl) MLV- reverse transcriptase and 2 µl nuclease-free water was added. These samples were kept in a thermocycler at 25°C for 15 min, 37°C for 50 min, 70°C for 15 min and 4°C for 1 min. The synthesized cDNA was stored at -20°C. The genome sense primer, CMV-F TATGATAAGAAGCTTGTTCGCG and antisense primer CMV-R 5'- GCCGTAAGCTGGATGGACAA-3' were used to amplify the complete CP gene of CMV [28].

The reverse transcribed cDNA (crude cDNA, 2 µl) was then subjected to PCR in a 25 µl reaction volume involving 2.5 µl of 10 x PCR buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 10 pmol of CMV-forward and CMV-reverse primers and 1U of Taq DNA polymerase and the PCR mix was subjected to thermal cycling conditions of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 2 min with a final extension of 72°C for 10 min in a thermal cycler (Bio-Rad, USA). Amplified products were resolved following electrophoresis through a 1% agarose gel containing SYBR® Safe (Invitrogen, USA).

### Primer testing

The CMV primer set, were initially tested against 4 different infected plants that provided template for sequencing (Fig. 2). To obtain a positive control, PCR product were sent to MacroGen Inc. (Seoul, South Korea) for sequencing. Thus, the isolated number three (assigned as KP877623 NCBI accession number) was used as a positive control (Fig 2). After initial screening, the primers were tested with the 24 samples. To determine their reliability. Specificity was determined by using cDNA of non-target Banana streak virus and one cDNA of an apparently healthy plant.



**Figure 2:** Agarose gel electrophoresis of RT-PCR products. Lane M- 1Kb DNA ladder; Lane 1, 2, 3, 4- CMV infected banana samples; Lane 5-Healthy banana sample. Sample N° 3 sequenced and assigned as KP877623- NCBI accession number.

### III RESULTS AND DISCUSSION

The presence of CMV was detected in 7 of the 24 banana plants from different places of the Santa Elena, Los Ríos, Santo Domingo and Guayas States from Ecuador. The symptoms of CMV infected Banana plants showing leaf mosaic or chlorosis, yellow line stripes, ring spots in the affected leaf lamina (Fig. 1a & 1b). Deformation and curling of leaves are observed in some of the banana plants. The Rosette appearance of leaf arrangement and conspicuous inter-veinal chlorosis are also symptoms of the disease. Depending on the virus strain and temperature, the symptoms are changing. When the temperature falls below 24°C severe symptoms were observed. Necrosis of emerging cigar leaves leading to varying degree of necrosis in the unfurled leaf lamina. Affected plants are stunted and throw small bunches with malformed fingers. Sometimes plants may die if the severe strain of the virus affects the plant. The collected samples were initially screened by TAS-ELISA method (Agdia, USA against the nucleocapsid protein of CMV (A405 = 2.40). Among these (n=24) collected banana samples, only four samples were confirmed as CMV infected by double antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) by using the CMV specific antiserum, whereas seven samples were confirmed by RT-PCR. In RT-PCR, a single band of the expected size (~500 bp) (Fig. 2) corresponding to the coat protein gene of CMV was observed when total RNA extracted from infected tissue was used. Optimization of annealing temperature is an essential and critical parameter for amplification of the target gene. The PCR conditions were further validated by amplification with healthy sample, a sample with similar symptomatology having Banana Streak virus (BSV) and water sample control. The PCR results under UV-transilluminator revealed that the infected banana samples gave a single band of CMV-CP gene and there was no band in the healthy, BSV plant and water samples. It's concluded that, the selective primer pair of CMV with optimized PCR conditions can be used further for the detection and diagnosis of CMV by RT-PCR. For further studies all the amplified RT-PCR products were sent for sequencing to identify the sub group of CMV in Ecuador. Niblett [19] observed the CMV infected plants showing yellow mosaic and stripes on leaves, leaf distortion along with stunting of banana plants. Magee [29] described the high disease incidence rate is high and with a reduction of yield in Australia due to the *Cucumber mosaic virus*. Hu [30] observed sometimes no apparent or obvious symptoms in banana infected plants. Zitter [17] described the CMV infecting different host plants including monocots, dicots, herbaceous and woody species. In recent, CMV infected other plant hosts are

*Agave* [31], *Cynara scolymus* [32] and *Zea mays* [33]. Recombination and re-assortment play an important role in CMV evolution and infect various new host plants [34]. Cucurbitaceous plants are not cultivated along with banana crop; the CMV is easily spread to banana plants. The banana growers must be aware of external symptoms of mosaic disease and must destroy such banana plants and noticed without further spread. This RT-PCR method is significantly improved monitoring and forecasting of banana mosaic epidemics. Further studies will use the RT-PCR method for the characterization of CMV infected banana plants during the growing seasons in Ecuador.

### IV CONCLUSION

TAS-ELISA has been used to detect CMV in banana, but gave fewer results when compared to RT-PCR. These results showed that RT-PCR is more sensitive than TAS-ELISA. The RT-PCR method was standardized and successfully amplified the coat protein gene of CMV for detection. This method is important and development of diagnosing the infection of CMV in banana crops, it is very useful for the banana growers in Ecuador.

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