Doi: https://doi.org/10.17584/rcch.2024v18i3.17841

Occurrence and molecular characterization of cucumber mosaic virus (CMV) in plantain orchards in Caldas and Risaralda departments of Colombia

Presencia y caracterización molecular del virus del mosaico del pepino (CMV) en cultivos de plátano de los departamentos de Caldas y Risaralda en Colombia

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Chlorotic streaking and mosaic in plantain leaf.

Photo: M. Betancourt

ABSTRACT

Cucumber mosaic virus (CMV) affects plantain (*Musa* AAB) orchards in most producing regions across the globe. This study aimed to determine the occurrence and characterize the CMV affecting plantain orchards in the departments of Caldas and Risaralda, Colombia. Leaf tissue samples were collected from six municipalities, then screened for CMV diagnosis using the enzyme-linked immunosorbent assay (ELISA). The samples with a positive reaction were confirmed by reverse-transcription polymerase chain reaction (RT-PCR). The results confirmed the presence of CMV, with an incidence of 2.8% in the study region. For the molecular characterization of the virus, a partial region of the RNA-dependent RNA polymerase (RdRp) gene was amplified and sequenced. The sequence analysis showed that the isolates shared nucleotide identities greater than 97.7% with GenBank accession MG696855.1, corresponding to a CMV isolate obtained from lulo (*Solanum quitoense*) in Colombia. Phylogenetic analysis grouped the sequences generated in this study with reference sequences from subgroup IA. Our results contribute to the understanding and updating of CMV population dynamics in this important plantain production region.

Additional key words: CMV; ELISA; molecular detection; *Musa* AAB.

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RESUMEN

El virus del mosaico del pepino (CMV) afecta los huertos de plátano (*Musa* AAB) en la mayoría de las regiones productoras del mundo. El objetivo de este estudio fue determinar la presencia y caracterizar el CMV que afecta huertos de plátano en los departamentos de Caldas y Risaralda, Colombia. Se recolectaron muestras de tejido foliar de seis municipios, luego se analizaron para el diagnóstico de CMV mediante el ensayo inmunoabsorbente ligado a enzimas (ELISA). Las muestras con una reacción positiva se confirmaron mediante la reacción en cadena de la polimerasa con transcripción inversa (RT-PCR). Los resultados confirmaron la presencia de CMV, con una incidencia de 2,8% en la región estudiada. Para la caracterización molecular del virus, se amplificó y secuenció una región parcial del gen de la ARN polimerasa dependiente de ARN (RdRp). El análisis de secuencia mostró que los aislamientos compartían identidades de nucleótidos superiores al 97,7% con la accesión GenBank MG696855.1, correspondiente a un aislado de CMV obtenido de lulo (*Solanum quitoense*) en Colombia. El análisis filogenético agrupó las secuencias generadas en este estudio con secuencias de referencia al subgrupo IA. Nuestros resultados contribuyen a la comprensión y actualización de la dinámica poblacional del CMV en esta importante región productora de plátano.

Palabras clave adicionales: CMV; ELISA; detección molecular; *Musa* AAB.

Received: 02-07-2024 Accepted: 12-08-2024 Published: 10-09-2024

INTRODUCTION

Colombia reported in 2022 approximately 434,723 ha under cultivation and a plantain production of 4,460,748 t (MinAgricultura, 2024), with 'Dominico Hartón' being the most widely commercially grown cultivar (Catellanos and Lucas, 2011). Viral diseases are significant phytosanitary constraints affecting the crop. Among them, banana mosaic disease (BMD), caused by cucumber mosaic virus (CMV) (Stover, 1972), has been a prominent global concern. CMV exhibits a broad geographical distribution and infects numerous hosts, encompassing over 1,300 species distributed across more than 100 families of monocotyledons and dicotyledons, including fruits, vegetables, and forages (Joshi *et al*., 2023b). CMV is transmitted through seeds, tools, and aphids species in a non-persistent manner (Khaled-Gasmi *et al*., 2023).

CMV has been documented in all major *Musa* sp. production regions in several countries, such as Argentina (Cabrera *et al*., 2018), Ecuador (Buitrón-Bustamante and Morillo-Velastegui, 2017), Ethiopia (Kebede and Majumder, 2021), India (Joshi *et al*., 2023a), and Turkey (Fidan and Koç, 2019). In Colombia, CMV has been identified in the departments of Valle del Cauca, Risaralda, Quindio, and Caldas, affecting both plantain and banana crops (Belalcázar *et al*., 1996; López-Cardona *et al*., 2014).

The symptoms induced by CMV are variable, and their expression level depends on the virus strain, the species, and the host plant (Joshi *et al*., 2023b). In plantains and bananas, the most characteristic symptom is leaf mosaic, but in advanced stages of the disease, symptoms can lead to pseudostem rot, flag leaf necrosis, stunted growth, and eventual plant collapse (López-Cardona *et al*., 2014; Manzo-Sánchez *et al*., 2014).

CMV belongs to the *Cucumovirus* genus, *Bromoviridae* family (Joshi *et al*., 2023b). It is composed of a tripartite genome of positive-sense, single-stranded RNA (RNA 1, RNA 2, and RNA 3) and an additional subgenomic RNA (RNA 4) derived from RNA 3. RNA 1 and RNA 2 encode proteins 1a and 2a, respectively, which participate in virus replication (Hayes and Buck, 1990). RNA 2 also encodes protein 2b, which functions as a post-transcriptional gene silencing suppressor and is involved in symptom induction in the host plant (Palukaitis and García-Arenal, 2003). RNA 3 encodes protein 3a, which is involved in cell-to-cell virus movement (Khan *et al*., 2012), and protein 3b, the capsid protein CP (Mochizuki and Ohki, 2012). The subgenomic RNAs (RNA 4 and RNA 4A) also encode CP (Jacquemond, 2012).

Based on phylogenetic analysis, CMV was first categorized into two subgroups, I (A and B) and II

(Anderson *et al*., 1995; Roossinck *et al*., 1999). However, a complete genome analysis of CMV strains showed that some strains did not cluster into subgroups I and II, leading to their classification into a new subgroup called subgroup III (Liu *et al*., 2009). Strains of the virus that do not cluster within the three subgroups have also been found, representing recombinant variants of the virus (Thompson *et al*., 2015). Subgroups IA and II are distributed globally, and subgroup IB, originally from Asia (Roossinck, 2001), has also been reported in other regions, including Nigeria (Apalowo *et al*., 2022), the United States (Nouri *et al*., 2014), and Ecuador (Ganchozo-Mendoza *et al*., 2024). Subgroup III has been documented in China (Liu *et al*., 2009). CMV strains belonging to subgroup I are regarded as more virulent than those in subgroup II (Li *et al*., 2020).

CMV has held a significant position among the viruses affecting plantain orchards in Colombia due to the associated yield losses. In the studied region, losses exceeding 50% have been reported in 'Dominico Hartón' (López-Cardona *et al*., 2014). However, there is limited and outdated information available regarding the virus's incidence and genetic characteristics. Therefore, this study aims to determine the occurrence of CMV in 'Dominico Hartón' orchards in the departments of Caldas and Risaralda and to characterize the virus at the molecular level, contributiing to disease prevention and the implementation of integrated management strategies.

MATERIALS AND METHODS

Location

Six plantain farms were selected across six municipalities: Belalcazar, Risaralda, Chinchina in Caldas department, and Marsella, Belen de Umbria, Apia in Risaralda department, Colombia (Fig. 1). These farms were characterized by the presence of foliar symptoms associated with viral diseases in a survey conducted from June to October 2020. In each farm,

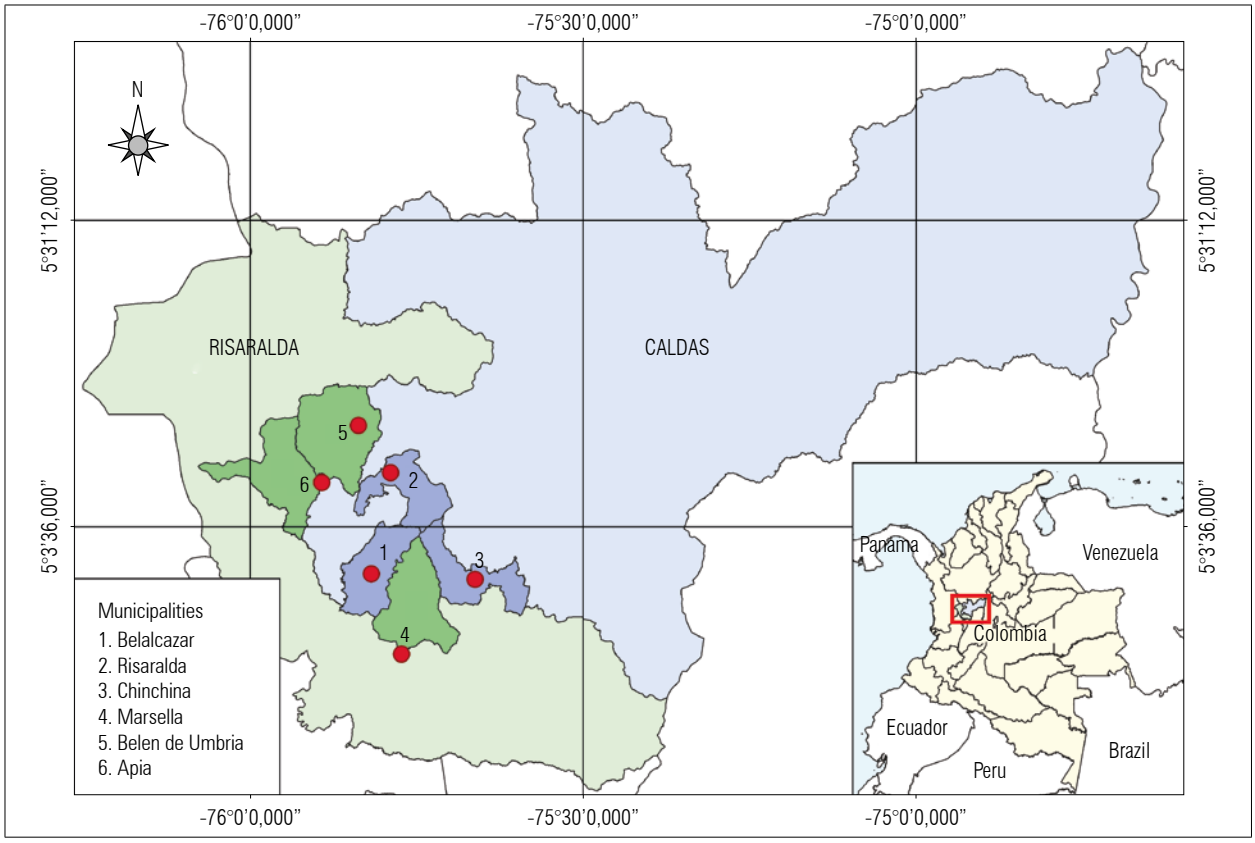

Figure 1. Geographical location of the selected farms for sample collection. The red dots indicate the six farms selected in the municipalities of the departments of Caldas and Risaralda, Colombia. Source: K. Rodríguez and L.-N. Martínez.

a zigzag random survey was conducted within the cultivated area (Gauhl *et al*., 1999).

Plant material collection

Symptoms associated with viral diseases were recorded, and 30 leaf tissue samples were randomly collected from each farm, including both symptomatic (chlorotic streaking and mosaic) and asymptomatic plants, for a total of 180 samples. This collection was conducted under a permit issued by the Autoridad Nacional de Licencias Ambientales - ANLA (Resolution N°. 1466, December 3, 2014). The samples consisted of the central region of the complete foliar surface, including the central vein of the third-youngest leaf of each plant (López-Cardona *et al*., 2014; Mateus-Cagua and Rodríguez-Yzquierdo, 2019). Each sample was placed in a paper bag and then in a resealable plastic bag (Ziplock®), properly labeled, and stored at 4°C. The samples were processed and preserved at the molecular biology laboratory of the Corporación Colombiana de Investigación Agropecuaria – AGROSAVIA, Palmira research station.

Serological CMV detection

The collected plantain leaf tissue samples (n=180) were analyzed for CMV detection using the ELISA assay by Agdia®, following the manufacturer's recommendations. The analysis of the samples was carried out in duplicate. As a blank control 1X extraction buffer (GEB) was included, while healthy plant tissue was used as a negative biological control. Additionally, infected plant tissue with CMV, commercially obtained from Agdia®, was used as a positive control. Colorimetric results were quantified at 30, 60, and 120 min after incubation at an absorbance of 405 nm using an Epoch model - microplate spectrophotometer (Bio Tek®). Results were considered positive when the optical density value exceeded the average of the negative control plus three times the standard deviation of the set of negative samples. The results obtained with the ELISA technique for each of the evaluated samples were recorded as either positive or negative. Based on this data, the disease incidence in the study region was determined using the following formula: Disease incidence (%) = (Number of infected plants / Total number of analyzed plants) * 100 (El Gamal *et al*., 2022).

Molecular CMV detection

The samples that showed a positive reaction in the ELISA test were used for the molecular characterization of the virus. RNA extraction from leaf tissue samples was performed using the CTAB protocol described by Chang *et al*. (1993). Complementary DNA (cDNA) was synthesized following the instructions provided in the Verso cDNA Synthesis kit (ThermoScientific®) and stored at -20°C. The quality of the cDNA was verified by amplifying a region of the 18S ribosomal RNA (rRNA) using the primer pair 18SF 5' GAGAAACGGCTACCACATCCA 3' and 18SR 5' CGTGCCATCCCAAAGTCCAAC 3', following the conditions reported by Du *et al*. (2006). For each reaction, 1X My Taq Red Mix 2X, 0.2 μM of each primer, and 1 μL of cDNA were added for master mix preparation in a final volume of 12.5 μL. The amplification program consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of an initial step at 95°C for 30 s, 52°C for 60 s, 72°C for 60 s, and a final elongation at 72°C for 10 min.

CMV detection was performed by amplifying a partial region of the RNA-dependent RNA polymerase gene using the primer pair CMVF 5' TAACCTCCCAGTTCTCACCGT 3' and CMVR 5' CCATCACCTTAGCTTCCATGT 3', following the conditions reported by Grieco *et al*. (2000). For each reaction, 1X My Taq Red Mix 2X, 0.2 μM of each primer and 1 μL of cDNA were added for master mix preparation in a final volume of 12.5 μL. The amplification program consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of an initial step at 95°C for 45 s, 52°C for 30 s, 72°C for 60 s, and a final elongation at 72°C for 10 min. The amplification process was carried out using an Agilent Technologies SureCycler 8800 thermocycler. The amplified products were visualized on a 1.6% agarose gel, dyed with GelRed (Biotium) using the gel documentation system Enduro GDS (Labnet).

Nucleotide identity and phylogenetic analysis

Purification of the amplified PCR products was done using the Qiagen® QIAquick PCR & Gel Cleanup kit (Qiagen, Germany) and sent for amplicon direct sequencing to Macrogen, Seoul. Sequences were cleaned, assembled, and aligned using Geneious Prime® 2021.0.3 software. The nucleotide identity

was determined by homology with sequences in the NCBI (National Center for Biotechnology Information) using the BLASTN (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed in August 2023). In order to determine the clustering of CMV isolates affecting plantain orchard in the departments of Caldas and Risaralda, a phylogenetic analysis was performed using the sequences generated in this study. A group of twenty reference sequences of a partial region of the RNA-dependent RNA polymerase gene, representing subgroups I (A and B), II, and III from different crops and reported in various parts of the world, were retrieved from GenBank. An isolate of peanut stunt virus (accession NC_002039.1) was used as an outgroup. Sequence alignment was conducted using the ClustalW program in MEGA6 version 11.0.11 (Tamura *et al*., 2021). A consensus alignment of 485 positions was used to infer the phylogenetic relationships of the CMV isolates using the maximum likelihood method, with the T92+G substitution model best fitting the data, and bootstrap values were calculated using 1,000 random subsamples.

RESULTS AND DISCUSSION

Plant material collection

From the group of samples collected, 25 out of 180 (13.9%) exhibited symptoms associated with viral diseases. In the department of Caldas, a higher proportion of symptomatic plants was observed (16.6%), with a distribution of 8.9% in the municipality of Chinchina, followed by Belalcazar and Risaralda with 4.4 and 3.3%, respectively. In the department of Risaralda, 11.1% of plants exhibited viral symptoms, distributed across the municipalities of Marsella (5.6%), Belen de Umbria (3.3%), and Apia (2.2%).

Similar results were found by Alarcón *et al*. (2005), who observed symptoms indicative of viral diseases in various plantain and banana cultivars established in an experimental farm in the municipality of Palestina (Caldas) with incidences ranging from 2 to 24%. These findings are consistent with those reported by Belalcázar *et al*. (1996), who observed an incidence of 24% for CMV in Gros Michel established in the municipality of Caicedonia (Valle del Cauca).

Regarding the characteristics of symptoms, these primarily appeared on the leaf surface, with chlorotic streaks (14/25) and general chlorosis of the lamina (7/25) being the most characteristic symptoms, while mosaic was observed less frequently (4/25) (Fig. 2A, B). During sample collection, some plants displaying symptoms such as flag leaf necrosis and pseudostem splitting were also observed (Fig. 2C, D). In some cases, phyllotaxis alteration was noted as well (Fig. 2E). This general symptom description has been previously reported in plantain and banana crops associated with viral diseases (López-Cardona *et al*., 2014; Selvarajan, 2015; Bhat *et al*., 2016; James *et al*., 2021).

Serological CMV detection

Out of the 180 analyzed samples, five showed a positive reaction to CMV. Specifically, four of these positive samples originated from the department of Caldas, and one was from the department of Risaralda.

The average absorbance values for the samples exhibiting a positive reaction to the virus ranged from 2.965 to 3.800 nm. The positive control displayed an average absorbance of 3.632 nm, while the negative control exhibited 0.142 nm, and the blank control registered 0.128 nm, thus demonstrating the reliability of the test (Koua *et al*., 2020). Based on the results, the incidence of CMV in the study region was 2.8%, with a distribution per department of 4.4% (4/90) in Caldas and 1.1% (1/90) in Risaralda.

The use of the ELISA technique for the detection and diagnosis of CMV in plantains has recognized advantages as it provides a reliable diagnosis due to its high degree of sensitivity, specificity, and reproducibility (Vidal *et al*., 2012). Furthermore, it can be used as a screening test, allowing for the analysis of samples on a large scale, from symptomatic and asymptomatic plants, where the virus can be present in early infection stages and at low concentrations (Koua *et al*., 2020). ELISA has been widely used for CMV detection with satisfactory results in *Musa* sp. (López-Cardona *et al*., 2014; Cabrera *et al*., 2018; Viswanath *et al*., 2021).

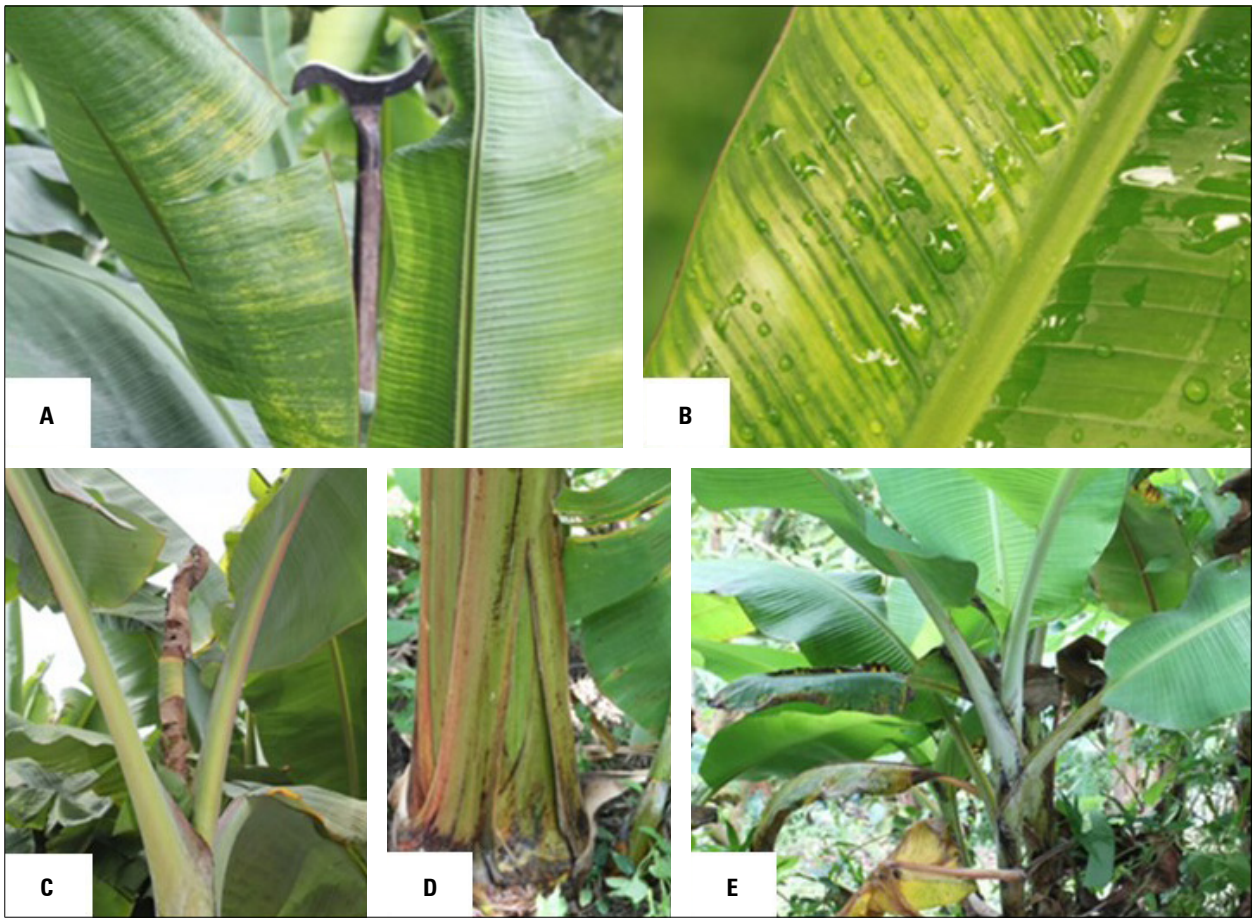

Figure 2. Symptoms of viral infections in 'Dominico Hartón' cultivar. A) chlorotic streaking, B) mosaic, C) flag leaf necrosis, D) pseudostem splitting, E) plant phyllotaxis alteration. Source: M. Betancourt.

Molecular CMV detection

For the molecular characterization, the RNA extracted from the CMV positive samples tested by ELISA yielded concentrations ranging from 555.1 to 2,916 ng μL^{-1}, with absorbance 260/280 and 260/230 ratios ranging between 2.09-2.23 and 2.09-2.37, respectively. The amplification of the 18S rRNA region resulted in a fragment of approximately 250 bp in all analyzed samples (Fig. 3A), consistent with the size reported by Du *et al*. (2006), indicating proper cDNA synthesis. The RT-PCR results confirmed the presence of CMV, the expected fragment of approximately 513 bp (Grieco *et al*., 2000) was amplified in each of the samples (Fig. 3B).

Nucleotide identity and phylogenetic analysis

The analysis of the sequences obtained in this study revealed nucleotide identities ranging from 97.7 to 98.7% with the GenBank accession MG696855.1, corresponding to a CMV strain obtained from lulo (*S. quitoense*) in Colombia. The five consensus sequences generated in this study were deposited in GenBank with accession numbers OR588863, OR588864, OR588865, OR588866, and OR588867. In the phylogenetic tree, three well-defined groups were formed among the 20 sequences retrieved from GenBank, consisting of subgroups I (A and B), II, and III. The analysis revealed that the four CMV strains obtained in Caldas and the CMV strain from Risaralda exhibited closer phylogenetic relationships with accessions from subgroup IA (Fig. 4). This result is consistent with what Roossinck (2001) reported, where subgroup IA is described as having a worldwide distribution, including previous reports in the Americas (Rivera-Toro *et al*., 2020; Bello *et al*., 2023).

In this study, we report a low incidence of CMV in farms cultivating 'Dominico Hartón' in the Caldas

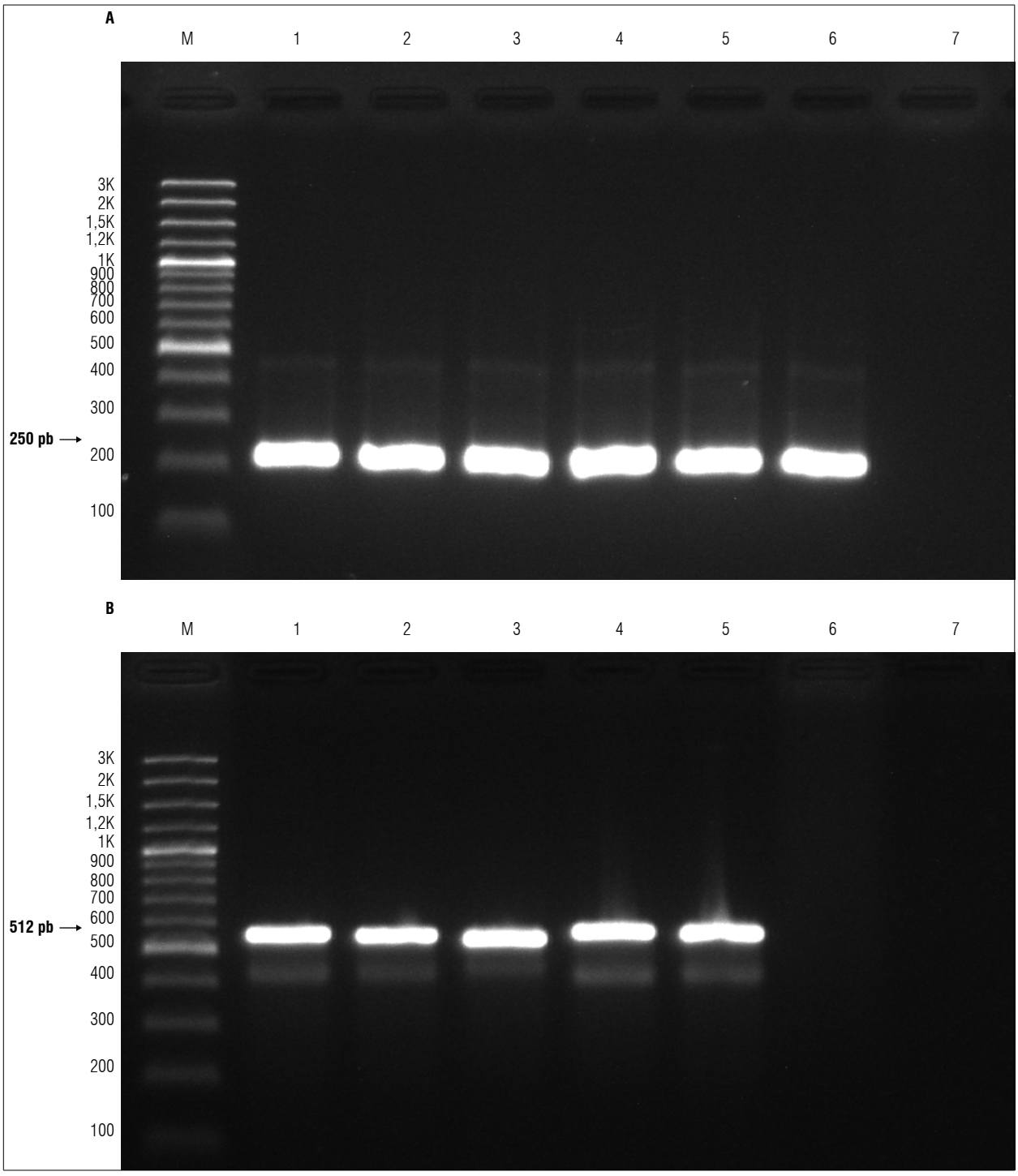

Figure 3. Agarose gel electrophoresis of RT-PCR amplified products from *Musa* sp. A) 18S RNAr region and B) RNA2 CMV region using CMVF/CMVR primers. M: Molecular weight marker of 100 bp. Lines 1, 2, 3, 4 and 5 correspond to PB001, PR058, PP065, PP067 samples from Caldas and PR157 sample from Risaralda, respectively. 6: Biological negative control. 7: no-template control.

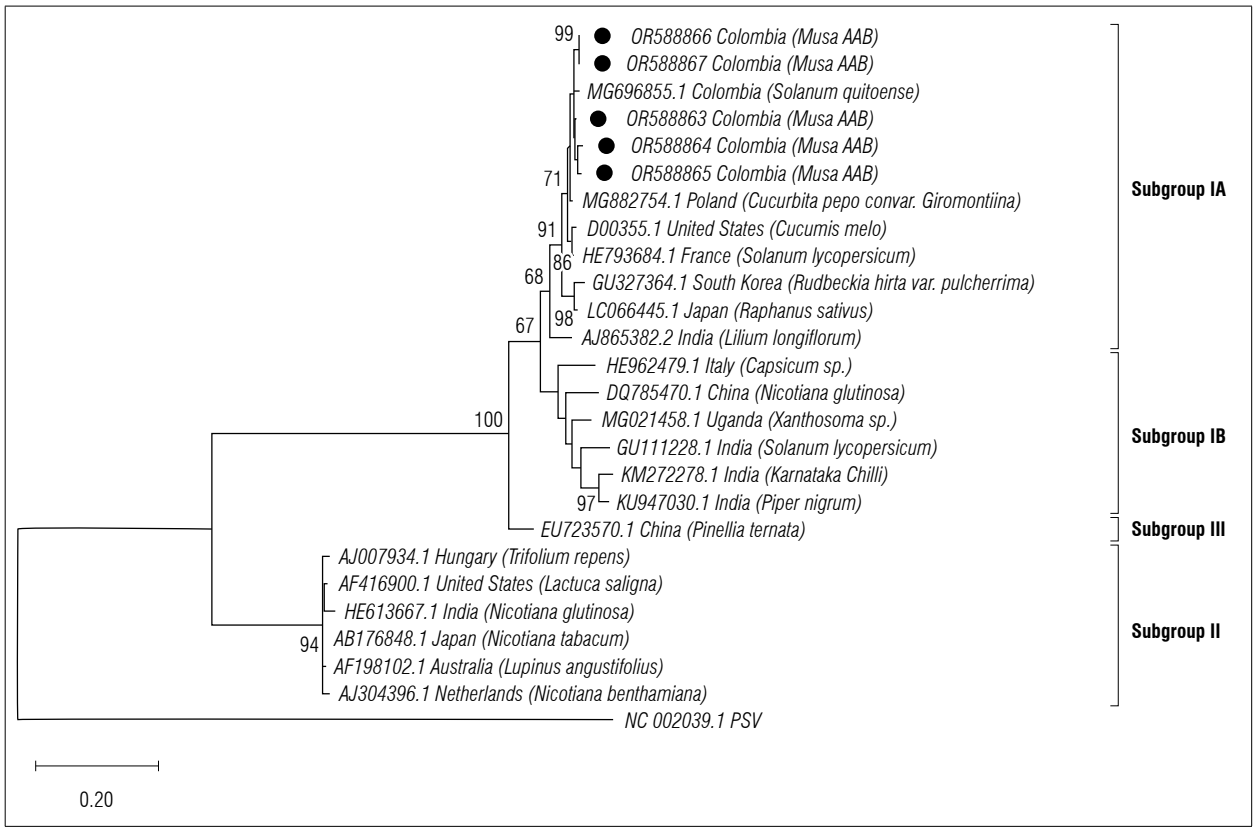

Figure 4. Phylogenetic tree generated from the alignment of 485 positions of a partial region of the RNA-dependent RNA polymerase (RdRp) gene of CMV. The analysis illustrates the relationship between CMV strains detected in 'Dominico Hartón' in Colombia, indicated by black dots, and those retrieved from GenBank.

and Risaralda departments. Conversely, previous studies have reported higher CMV incidences in this cultivar within the region. Initially, Reichel *et al*. (1996b) determined a virus incidence of 31% in the Quindío department, affecting 'Dominico Hartón' orchards based on serological tests. Later, Alarcón *et al*. (2005) reported a 15% incidence of CMV in 'Dominico Hartón' plants in experimental plots as well as in the *Musa* sp. collection established at the Montelindo farm of the Universidad de Caldas, in the municipality of Palestina (Caldas). Similarly, a study conducted by López-Cardona *et al*. (2014) found CMV incidences below 14% in 'Dominico Hartón' orchards in the departments of Quindío (12%), Caldas (12%), and Risaralda (14%).

Although changes in factors such as climatic and agroecological conditions in the production areas, management practices, planting systems (monoculture, polyculture), and the quality of planting material could affect the dynamics of the virus population, we were unable to establish an association with those factors under the conditions of our study. Samples were collected during a survey conducted from June to October 2020. During this period, the climatic data did not indicate significant changes, with temperature and relative humidity ranges between 16-18°C and 85-87%, respectively, and a higher precipitation level (148 mm) recorded during plant collection in July in the municipalities of Chinchina and Marsella (Caldas). Additionally, the explored farms employed both monoculture and polyculture production systems, the latter primarily associated with coffee plantations, and vegetative propagation was carried out using their own plant material.

It has been reported that changes in climatic conditions can influence the multiplication of vector insect populations, virus dispersion, and transmission (Pérez-Vicente and Porras, 2015), but in our study, the presence of aphids in the fields was not observed. Therefore, to test the hypothesis of a reduction in

CMV incidence in this region over time, additional studies on CMV population dynamics and its vectors in the region will be required.

On the other hand, the results of this research showed that the presence of CMV was associated with both symptomatic and asymptomatic samples. Four out of the five samples with a positive reaction to CMV exhibited viral symptoms such as chlorotic mosaic and leaf streaking, which are typical symptoms of CMV infections (Koua *et al*., 2020, Bae *et al*., 2021). However, one sample was asymptomatic. Similar results have been reported in studies conducted by Buitrón-Bustamante and Morillo-Velastegui (2017), who identified CMV in plants without symptoms associated with viral infections, emphasizing the need to monitor nursery plants to ensure virus-free planting material and prevent the movement of contaminated plants to disease-free areas.

In contrast, considering the random nature of the sample collection, some plants exihibiting viral symptoms were found not to be associated with CMV. These plants could potentially be infected with other viruses reported in *Musa* sp. in the country, such as banana streak viruses (BSVs) (Reichel *et al*., 1996b), banana mild mosaic virus (BanMMV) (Reichel *et al*., 2003), and banana streak Obino l'Ewai virus (BSOLV) (López-Cardona *et al*., 2014). A previous study conducted in the region reported single infections of BSOLV affecting 'Dominico Hartón' orchards with an incidence of 12% (López-Cardona *et al*., 2014), and mixed infections of BSV/CMV (60%) and BSOLV/CMV (<1%) (Reichel *et al*., 1996b; López-Cardona *et al*., 2014).

Additionally, we report the presence in the study region of CMV strains classified in subgroup IA, contributing to the understanding of the virus in the region. CMV strains classified in subgroup IA have been identified in *Musa* sp. in different parts of the world (Cabrera *et al*., 2018; Koua *et al*., 2020), and it has been highlighted that strains from subgroup I induce more severe disease in hosts (Li *et al*., 2020). Although in Colombia there is insufficient phylogenetic information about CMV infecting *Musa* sp., a preliminary analysis yielded similar results. In this study, the characterization of the capsid protein of two CMV isolates obtained from 'Dominico Hartón' plantains and Gros Michel bananas revealed a high degree of conservation with members of subgroup I (Reichel *et al*., 1996a), later reclassified as subgroup IA by Rivera-Toro *et al*. (2020).

Taking everything into account, it is necessary to expand this research within the region and in other plantain-producing areas of Colombia to confirm the dynamics of CMV populations. Additionally, it is important to determine whether the findings of this study reflect a broader trend influenced by management practices, the use of healthy planting material, agroecological factors, or the presence of more widely distributed viral species.

CONCLUSION

CMV was detected at a low incidence in plantain orchards cultivated with 'Dominico Hartón' in Caldas and Risaralda departments, Colombia. The CMV sequences generated in this study clustered with subgroup IA GenBank reference sequences. This study contributes to updating the CMV status in an important plantain production region for the country and highlights the need for complementary studies to determine the prevalence, incidence, and distribution of this and other viruses affecting the crop. Such information is critical for developing effective control measures to minimize losses caused by viral diseases in the crop at the national level.

Acknowledgments

The authors would like to thank the plantain producers in the departments of Caldas and Risaralda, as well as the team at the La Selva research station, Agrosavia, located in Manizales, for their assistance in selecting farms and collecting plant material, and to K. Rodriguez for sequence editing and analysis. They also express gratitude to the *Ministerio de Agricultura y Desarrollo Rural* of Colombia for funding this research. This study is part of the project "Technological alternatives for the production and processing of plantain crops, ID 1000734".

Conflict of interest: No potential conflict of interest was reported by the authors. The funders had no role in the design of the study; data analysis, writing of the manuscript, or the decision to publish the results.

Author's contributions: D.M. Rodríguez-Mora: methodology, research, formal analysis, writing – original draft, writing – review and editing. L.N. Martínez-Caballero: methodology, research, formal analysis, writing – original draft, writing – review

and editing. M. Betancourt: methodology, resources, funding acquisition, project administration I. Moreno: conceptualization, methodology, research, formal analysis and interpretation, supervision, writing – original draft, writing – review – editing and translation.

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