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Unraveling complex viral infections in cassava (*Manihot esculenta* Crantz) from Colombia

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ABSTRACT

In the Americas, different disease symptoms have been reported in cassava including leaf mosaics, vein clearings, mottles, ring spots, leaf distortions and undeveloped and deformed storage roots. Some viruses have been identified and associated with these symptoms while others have been reported in symptomless plants or latent infections. We observed that reoviruses associated with severe root symptoms (RS) of Cassava Frogskin Disease (CFSD) are not associated with leaf symptoms (LS) observed in the cassava indicator plant 'Secundina'. Neither were these LS associated with the previously characterized *Cassava common mosaic virus*, *Cassava virus X*, *Cassava vein mosaic virus* or phytoplasma, suggesting the presence of additional pathogens. In order to explain LS observed in cassava we used a combination of biological, serological and molecular tests. Here, we report three newly described viruses belonging to the families *Secoviridae*, *Alphaflexiviridae* and *Luteoviridae* found in cassava plants showing severe RS associated with CFSD. All tested plants were infected by a mix of viruses that induced distinct LS in 'Secundina'. Out of the three newly described viruses, a member of family *Secoviridae* could experimentally induce LS in single infection. Our results confirm the common occurrence of complex viral infections in cassava field-collected since the 1980s.

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1. Introduction

Cassava (*Manihot esculenta* Crantz) is native to the southern border of the Amazon basin where it has been eaten for centuries by indigenous people (Olsen and Schaal, 1999). It was then spread and cultivated in Latin America, West India, Africa and Asia increasing worldwide production to about 250 million tons per year (FAOSTAT, 2011). Colombia is the third largest producer of cassava in Latin America and has increased its production by ~30% over the last 4 years (FAOSTAT, 2011). Cassava is an important source of calories for human consumption and has played an important role in subsistence agriculture and food security because it requires few inputs and tolerates dry weather conditions (FAO, 2013). However, cassava is vegetatively propagated and thus infections tend to accumulate over different crop cycles with a cumulative negative effect on the quality of cassava planting material and plant yield potential (Calvert et al., 2012).

Virus infections have a devastating effect on cassava yield in Africa (Legg et al., 2006; Mbanzibwa et al., 2011), and there is evidence for the occurrence of several viral diseases affecting cassava in the Americas which cause significant yield losses (Calvert et al., 2012). Cassava Vein Mosaic Disease (CVMD) and Cassava Common Mosaic Disease (CCMD) were the first two cassava diseases reported in South America (Costa, 1940; Silva et al., 1963). Affected plants show characteristic leaf symptoms (LS) that have been associated with *Cassava vein mosaic virus* (CsVMV; Family *Caulimoviridae*, Genus *Cavemovirus*) and *Cassava common mosaic virus* (CsCMV; Family *Alphaflexiviridae*, Genus *Potexvirus*), respectively. CVMD has been reported only in Brazil (Calvert et al., 1995) and little research has been published on the epidemiology and control of its associated virus. CCMD has been reported in Brazil, Colombia, Paraguay, Africa and Asia (Silberrschmid, 1938; Costa and Kitajima, 1972; Chen et al., 1981; Aiton et al., 1988), and although the disease has been considered a minor problem in the Americas, prolonged cold periods can cause severe LS and high yield losses (Costa and Kitajima, 1972). Purification of CsCMV (Fig. 1A) and genome sequencing allowed its classification as a member of genus *Potexvirus* (Kitajima et al., 1965; Calvert et al., 1996). ELISA tests using a polyclonal antiserum against a Brazilian isolate showed no serological relation between CsCMV and *Cassava virus X* (CsVX), another putative *Potexvirus* member associated with symptomless infections in cassava (Fig. 1B) (Harrison et al., 1986). Distinct strains of CsCMV have different serological and biological properties (Elliot and Zettler, 1987; Marys and Izaguirre-Mayoral, 1995) compared to

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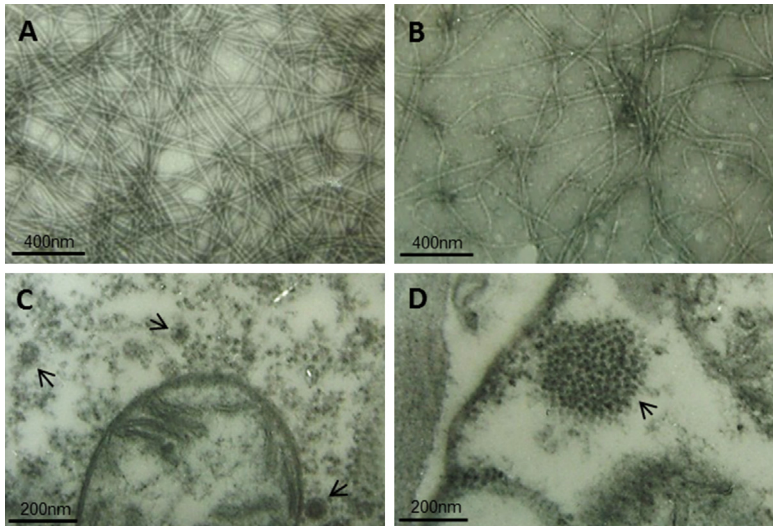

Fig. 1. Photographs of viral particles or virus-like particles derived from cassava plants infected with CsCMV and CsVX or affected by CFSD, CCMD and 'Cassava Caribbean mosaic disease', observed by transmission electron microscopy. (A) CsCMV purified particles from a cassava plant displaying CCMD symptoms (image from 1990), (B) CsVX purified particles (image from 1990) from plants affected by CFSD, (C) Leaf section of a parenchyma cell derived from a cassava plant affected by 'Cassava Caribbean mosaic disease' and CFSD (FSD80) showing spherical-like particles of around 70 nm in diameter, (D) or spherical-like particles of around 25 nm (image from 1990). Size bars are shown for each picture.

the Brazilian isolate used as a reference (Nolt et al., 1991) suggesting greater variability among potexviruses in cassava. Indeed a putative potexvirus named *Cassava Colombian symptomless virus* (CsCSLV) was reported in cassava (Lennon et al., 1986) but not associated with LS. To our knowledge there is no original sample, antisera or sequence information available to identify this virus.

In 1971 in the Department of Cauca, Colombia, a disease that affected cassava storage roots caused yield losses up to 89% (Pineda et al., 1983). Due to the type of root symptoms (RS) observed, the disease was named Cassava Frogskin Disease (CFSD) (Fig. 2E–H). Mild symptoms include the enlargement of the corky layer to form raised lip-shaped fissures in the roots (Fig. 2E), but the disease is symptomless in leaves of most cassava landraces. Severely affected roots present constriction zones and failure of the storage root to accumulate starch (Fig. 2F–H). CFSD has been associated with diverse RS reported in Brazil, Venezuela, Costa Rica, Panama and Peru, and it is the major constraint to cassava production in Latin America (Chaparro-Martinez and Trujillo-Pinto, 2001; Calvert and Thresh, 2002; Calvert et al., 2012). Different pathogens have been associated with CFSD; initial studies aiming to identify the causal agent of the disease detected particles similar to those of members of genus *Closterovirus* and mycoplasma-like structures (Pineda et al., 1980). In 1981, a disease similar to CFSD but with additional severe LS was described in the Caribbean coastal region of Colombia (Department of Magdalena) affecting the local cassava landrace 'Secundina' (COL2063). The associated LS were characterized by bright yellow mosaics (Fig. 2C) and the disease was named 'Caribbean mosaic' (Pineda et al., 1982). Later studies performed by Nolt et al. (1992) suggested that 'Caribbean mosaic' and CFSD were the same disease, and the mosaic symptoms induced in 'Secundina' upon grafting were then recommended for indexing of CFSD (Nolt et al., 1992). More detailed microscopy analysis of plants displaying typical LS of 'Caribbean mosaic' revealed the presence of elongated (*not shown*) as well as spherical virus-like particles (Fig. 1C and D) suggesting mixed virus infections. In 1982, CsVX was first isolated from a symptomless cassava plant (Angel et al., 1987) and then detected by ELISA in cassava plants affected by CFSD (Nolt et al., 1992). Thus, over the years, different viruses have been detected in plants showing RS associated with CFSD. More recently, the analysis of double-stranded RNA (dsRNAs) and total DNA from cassava roots allowed the identification and partial characterization of *Cassava frogskin associated virus* (CsFSaV; Family *Reovirirdae*, Genus unassigned) (Calvert et al., 2008) and a phytoplasma (Alvarez et al., 2009), both detected in plants displaying RS of CFSD.

Additional evidence of mixed infections in cassava comes from recent studies: (1) the work by Calvert et al. (2008) first reported CsFSaV association to CFSD, but also indicated the presence of a second type of isometric particles in CFSD-affected plants. (2) A recent report on a phytoplasma associated with CFSD where it is shown that in single-infection, the phytoplasma does not induce LS or RS on cassava indicator plants (Alvarez et al., 2009). (3) Preliminary observations during virus indexing showing that plants infected with CsFSaV induce different kinds of LS in the indicator plant 'Secundina', suggesting the presence of additional uncharacterized viruses or viral strains. Based on these observations we have used serological, biological and molecular methods to identify additional viruses in cassava plants showing varying degrees of LS. We thus unraveled and confirmed suspected disease-associated mixed virus infections in cassava and reported the presence of three novel viruses belonging to the families *Alphaflexiviridae*, *Secoviridae* and *Luteoviridae* in plants collected since the 1980s in different regions of Colombia. The results indicate that mixed virus infections in cassava are more common and widespread than previously known and that they are associated with a variety of LS in 'Secundina'.

2. Materials and methods

2.1. Virus isolates and plant material

Plants from the *in vitro* cassava germplasm collection maintained at the International Center of Tropical Agriculture (CIAT), Colombia (Table 1, upper half) were propagated in a humid chamber for 3 weeks before being transferred to a greenhouse at CIAT as indicated below. Stem cuttings of different cassava varieties, displaying CFSD root symptoms, were collected between 1980 and 2012 from various regions of Colombia including farms and experimental fields (Table 1, lower half). Plants were grown and maintained at $28 \pm 5\,^{\circ}C$ and 70–80% relative humidity in a quarantine insect-proof greenhouse at CIAT, in 25 cm × 25 cm plastic pots

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