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An Overview of the Sugarcane Mosaic Disease in South America

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ABSTRACT

Sugarcane mosaic, one of the most important viral diseases of sugarcane, is widely distributed in the world and its economic significance varies among regions. Economic losses depend on varietal susceptibility, virus strains, interaction with other diseases, vector population and environmental conditions. Although not a major problem in some countries, sugarcane mosaic has caused substantial yield losses in other countries (Argentina, Brazil) due to severe outbreaks. Numerous strains of *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV) are commonly associated with mosaic symptoms. Both viruses are members of the SCMV subgroup in the genus *Potyvirus* of the family *Potyviridae* and their genetic variability could be effectively assessed only through DNA sequence comparisons. The greater genetic variability of viruses associated with sugarcane mosaic needs to be taken into consideration in breeding and biotechnology programmes for resistance to mosaic. The most effective way to control sugarcane mosaic has been through the use of resistant cultivars, which requires a complete understanding of the genetic diversity of the pathogens as well as their interaction with cultivars; resistance breakdown can occur when new strains or viruses appear. However, the production of healthy and genetically pure seed cane could be an available tool to reduce the pathogenic load in sugarcane-growing areas. This could be achieved through hydro-heat-treatment followed by apical meristem *in vitro* culture and micropropagation. It is also relevant to implement extreme quarantine measures to prevent the entry of new pathogens or variants of the established ones through germplasm exchange.

Keywords: coat protein gene, genetic diversity, *Potyvirus*, SCMV, SrMV

Abbreviations: aa, amino acid; **AFLP**, amplified fragment length polymorphism; **BSA**, bulked segregant analysis; **CENICAÑA**, Centro de Investigaciones de Caña de Azúcar en Colombia; **CIMMYT**, Centro Internacional para el Mejoramiento de Maíz y Trigo; **CONICET**, Consejo Nacional de Investigaciones Científicas y Técnicas; **CP**, coat protein; **EEAOC**, Estación Esperimental Agroindustrial Obispo Colombres; **INSIBIO**, Instituto Superior de Investigaciones Biológicas; **JGMV**, *Johnsongrass mosaic virus*; **MAS**, marker-assisted selection; **MDMV**, *Maize dwarf mosaic virus*; **nt**, nucleotide; **PCR**, polymerase chain reaction; **PenMV**, *Pennisetum mosaic virus*; **QTL**, quantitative trait locus; **RFLP**, restriction fragment length polymorphism; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **SCSMV**, *Sugarcane streak mosaic virus*; **SCMV**, *Sugarcane mosaic virus*; **SCYLV**, *Sugarcane yellow leaf virus*; **SSR**, simple sequence repeat; **UNT**, Universidad Nacional de Tucumán; **ZeMV**, *Zea mosaic virus*

CONTENTS

DISEASE IMPACT IN SOUTH AMERICA

Of all the diseases affecting sugarcane, viral diseases have a particular interest due to the losses that they cause in susceptible cultivars, the inefficiency of chemical control and the scarcity of resistant plant material to all viruses and their strains (Ordosgoitti *et al*. 1982; Rea *et al*. 1994). Sugarcane mosaic, one of the most important viral diseases of sugarcane, caused by *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV), is widely distributed in the world (Koike and Gillaspie 1989) and its economic significance varies according to regions. SCMV is documented worldwide wherever corn and sugarcane are cultivated (Centro Internacional para el Mejoramiento de Maíz y Trigo, CIMMYT 2010). Although not a major problem in some countries, sugarcane mosaic has caused substantial yield losses in others due to severe outbreaks (Perera *et al*. 2009). Economic losses depend on varietal susceptibility, virus strains, its interaction with other diseases, vector population and environmental conditions (time period and sugarcane growing area involved) (Goodman 1999).

Between 1914 and 1916, a severe epidemic of this disease took place. In Argentina, mosaic is the most important viral disease of sugarcane and the disease caused losses up to 80% of the sugar production in Tucumán, located in the north of the country (Ahmed *et al*. 2007). This crisis was overcome thanks to the prompt intervention of Estación Experimental Agroindustrial Obispo Colombres (EEAOC),

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a local institution whose aim is to deal with issues related to agricultural production. The resistant materials from Java (Indonesia), which had been previously incorporated and tested, were the key to eradicate the disease as soon as it broke out. So, susceptible varieties were gradually replaced with resistant ones ('POJ' varieties) (Ploper *et al*. 2009). Currently, extreme quarantine measures have been implemented to prevent the entry of new pathogens or variants of the established ones (Ullivarri 2010). In Tucumán, Argentina, three varieties are planted in more than 85% of the sugarcane producing area: 'LCP 85-384' (44.3%), 'TUCCP 77-42' (22.8%) and 'CP 65-357' (18.4%). This fact turns the crop extremely vulnerable to disease attack. Thus, in this province, 65% of the variety 'CP 65-357' is affected by mosaic disease (Ramallo 2005).

In the mid-1920s, an epidemic threatened the sugar industry in both Brazil and the USA (Louisiana) (Koike and Gillaspie 1989). The experience of the previous decade in Argentina helped to solve the crisis; the procedure proposed by EEAOC in 1914 was used that time in Brazil and the USA, where the epidemic was controlled when susceptible varieties were replaced by resistant hybrids (Ploper *et al*. 2009). In Brazil, however, as the disease was supposedly eradicated, some susceptible varieties were planted again; as a result, new disease epidemic cycles occasionally took place (Gonçalvez *et al*. 2007b). In studies carried out in Brazil, between 1971 and 1972, SCMV was found in tolerant clones with 100% infection and it caused losses of 18%; in susceptible clones, however, with 25% infection, SCMV caused losses up to 75% (Matsuoka and Costa 1974).

In Venezuela, mosaic and ratoon stunting are the main concern from an economic point of view since losses in susceptible materials may exceed 20% (Nass *et al*. 1991; Mendez *et al*. 2005).

In Ecuador, the two major virus diseases of sugarcane are mosaic and yellow leaf, caused by *Sugarcane yellow leaf virus* (SCYLV). The incidence of SCYLV in commercial fields is high and the disease is widespread in the country. Mosaic is found in fewer locations but, when present, yield losses can reach 1.14 tons of cane/ha per 1% disease incidence (Garcés *et al*. 2006).

In Colombia, the highest incidence of mosaic was observed in the creole varieties until 1930, when these varieties were replaced by 'POJ 2878', resistant to the disease. However, the disease appeared again in 1974 with the establishment of the 'CP 57-603', a variety susceptible to the disease. In 1978, in the Cauca river valley, the incidence of infection in 'CP 57-603' commercial crops was 15% on average. In 1981, the mosaic incidence considerably increased and varied between 30 and 40%; in some cases, it increased up to 100% (Victoria *et al*. 1995; Centro de Investigaciones de Caña de Azúcar en Colombia, CENICAÑA 2005).

SYMPTOMS

Mosaic is identified primarily by its leaf symptoms. As with most sugarcane diseases, the symptoms may vary in intensity with the cane variety, growing conditions, and the strain(s) of the virus involved. Young, rapidly growing plants are more susceptible to infection than more mature or slower growing plants (Comstock and Lentini 2005). The plant growth can also be reduced, according to the virus strain involved, especially when the infection takes place in the early stages of development (Koike and Gillaspie 1989; Gonçalvez *et al*. 2007b).

The most distinctive symptom is a pattern of contrasting shades of green, often islands of normal green on a background of paler green or yellowish chlorotic areas on the leaf blade. Generally, the chlorotic areas are diffuse, but they may be sharply defined in some clones infected with certain virus strains. Chlorotic areas are most evident at the base of the leaf; these areas may also be present on the leaf sheath, but rarely on the stalk (Comstock and Lentini 2005). The infection may be accompanied by varying degrees of leaf reddening or necrosis, especially when symptoms are severe (Ramallo 1981).

CAUSAL AGENTS

SCMV and SrMV are commonly associated with mosaic symptoms. Both viruses are members of the SCMV subgroup in the genus *Potyvirus* of the family *Potyviridae* (Xiao *et al*. 1993). This family is the largest and economically most important group of plant viruses, with *Potyvirus* being its most significant genus (Brunt 1992). Four other viruses - *Maize dwarf mosaic virus* (MDMV), *Johnsongrass mosaic virus* (JGMV), *Pennisetum mosaic virus* (PenMV), and *Zea mosaic virus* (ZeMV) - are also included in the SCMV subgroup although they have never been isolated from sugarcane (Chatenet *et al*. 2005).

All members of the *Potyviridae* family have filamentous particles 650–900 nm or 500–600 and 200–300 nm in length and 11–13 nm in width, made up of about 2,000 units of a single structural coat protein (CP) surrounding a linear, single-stranded positive sense monopartite or bipartite RNA genome of 8,500–12,000 nucleotides (nt) with a poly (A) tail at the 3′-terminus and probably a genome-linked protein (VPg) at its 5′-terminus (Chen *et al*. 2001a). The genome or genome segments are translated into polyproteins which are subsequently processed by virus encoded proteases into functional proteins.

These viruses induce characteristic pinwheel or scroll-shaped inclusion bodies in the cytoplasm of the infected cells (Edwardson and Christie 1996; Gonçalvez *et al*. 2007b). These cylindrical inclusion bodies, which can be seen by electron microscopy, are formed by the virus encoded protein and can be considered as the unique phenotypic criterion for assigning viruses to the family (Ward and Shukla 1991).

In the SCMV group, complete nt sequences have been determined for MDMV (Kong and Steinbiss 1998), JGMV (Gough and Shukla 1993), SCMV (Fan *et al*. 2003) and SrMV (Yang and Mirkov 1997). There are also partial sequences, mostly from the 3′-terminal region, of several isolates of these viruses and particularly of SCMV (Frenkel *et al*. 1991; Xiao *et al*. 1993; Handley *et al*. 1996; Oertel *et al*. 1997; Suranto *et al*. 1998; Seifers *et al*. 2000; Perera *et al*. 2009).

In Argentina, a technique for the purification of the viral particle of SCMV has been optimized. This technique consists in grinding leaves of a diseased plant to extract the sap, which will be filtered, centrifuged and ultra-centrifuged. Thus, the clarification and concentration of viral particles will be achieved. Next, the concentrate is again centrifuged, this time in sucrose gradient, in order to gather the particles in only one stratum (Ramallo 1989).

Summers (1934, 1935) was the first to recognize the virus strains according to the different symptoms they produced in the 'CP 28-40' variety. Strains differ in their physical and chemical properties as well as in the symptoms they cause (Abbott and Tipett 1966). Also, the various strains differ in their host range, ability to cause infection and in the degree of injury they cause. Strains can be separated by distinctive symptoms shown on selected indicator clones and designated by letters (Comstock and Lentini 2005). The determined SCMV species included strains A, B, D, E, SC, Isis and Brisbane from sugarcane, BC from blue couch grass [*Digitaria scalarum* (Schweinf.) Chiov.], MDB (formerly MDMV-B) from corn, Bundaberg from wild sorghum and Sabi from sabi grass [*Urochloa mosambicensis* (Hack.) Dandy]. SrMV comprised strains H, I and M from sugarcane (Shukla *et al*. 1992, 1994). However, as sugarcane mosaic has been reported in more than 70 countries and, because the published strains have been described from only a few of these countries (Grisham 2000), the number of existing SCMV and SrMV strains is expected to be much greater. Moreover, numerous isolates or strains have not yet been investigated such as SCMV-C, F, G, K and L from the United States (Shukla *et al*. 1994) and

SCMV-N from India (Kondaiah and Nayudu 1985).

In Argentina, the sugarcane industry began in Tucumán 190 years ago and, since then, different mosaic symptoms have been described in infected plants. The causal agent was first identified by Bennet in 1941 as SCMV strain B. Two additional SCMV strains, A and F, and SrMV strain I, were detected in 1981 by biological assays (Ramallo 1981). This strain identification, based on symptom expression and serological methods, has proved to be inconsistent and unreliable. In 2005, the predominance of SCMV strain E in Tucumán was determined by RT-PCR-RFLP (reverse transcriptase – polymerase chain reaction - restriction fragment length polymorphism) (Fontana *et al*. 2005). More recently, using the same methodology, new RFLP profiles were obtained, indicating that potential new SCMV strains were present (Perera *et al*. 2007). In 2009, SCMV and SrMV were confirmed as the main causal agents of the mosaic disease in sugarcane-growing areas in Tucumán and the great genetic diversity found was only revealed by sequencing (Perera *et al*. 2009).

In Brazil, only SCMV produces mosaic symptoms in sugarcane but it does not cause major losses due the selection of resistant varieties and the roguing practice in commercial plantations. However, this virus infects other grasses, such as corn, *Zea mays* (Costa *et al*. 1971). The corn area planted in Brazil has increased, thus contributing to the mosaic incidence and the permanence of the inoculum source (Waquil *et al*. 1996). As a result, the possibility that new SCMV strains are spreading in the field increases. Recently, a new and more severe strain capable of infecting tolerant cultivars in the field was found (GenBank accesion number: AY819716). One of the most widely grown varieties in Brazil, 'RB72-454', considered resistant, showed mosaic symptoms (Goncalves *et al*. 2007b).

In Colombia, the strains of SCMV reported are A, B and D. A and B strains coexist in some varieties (Victoria *et al*. 1984).

The presence of SCMV strains, A, B, D, and H has been confirmed in Venezuela (Rea *et al*. 1994; Garrido and Uzcategui 2000) and strain B has been determined as the predominant one with the most severe damage on the crop (Ordosgoitti and Aponte 1986; Madriz 1992). A study revealed the existence of different susceptibility degrees to strain B in the germplasm bank and in the sugarcane breeding program parents in Venezuela (Rea *et al*. 1994). Also in Venezuela, the presence of SCMV strain D in an alternative host, St. Augustine grass (*Stenotaphrum secundatum*), has been reported for the first time (Ferreira 1990; Garrido *et al*. 1998). Also, recently, SCMV-MB was reported for the first time, infecting sugarcane in natural conditions in Venezuela, and possibly in the world; in the literature no other references associated with the identification of this strain in sugarcane, were found. The identification of this strain infecting sugarcane suggests the need of evaluating commercial and experimental materials currently grown in the country in order to select the genotypes with higher resistance. Thus, it is also of great interest to carry out studies to determine the distribution of this strain in the main sugarcane producing areas in Venezuela (Mendez *et al*. 2005). SCMV-MB had been reported before in Venezuela infecting corn in San Javier, Yaracuy (D'Lima and Garrido 1993) and sorghum in Maracay, state of Aragua (Garrido 2000).

The strain prevalence has shifted in time in several sugarcane growing areas, probably as a consequence of changes in cultivar adoption as it was reported in Louisiana (USA) (Summers *et al*. 1948; Koike and Gillaspie 1989; Grisham and Pan 2007) and Tucumán (Fontana *et al*. 2005; Perera *et al*. 2009).

Another virus, *Sugarcane streak mosaic virus* (SCSMV), was identified (Hall *et al*. 1998) and is the major cause of mosaic symptoms in commercial sugarcane cultivars in several Asian countries (Chatenet *et al*. 2005). This virus could belong to an undescribed new genus within the *Potyviridae* family (Hema *et al*. 2002; Adams *et al*. 2005) and can infect sugarcane simultaneously with SCMV (Chatenet *et al*. 2005). In India, SCSMV and SCMV are found to cause mosaic together or separately and among the two viruses, the former was more frequently detected in sugarcane varieties (Viswanathan *et al*. 2007; Viswanathan and Karuppaiah 2010). Detailed characterization of SCSMV genome in India established this virus in the new genus *Susmovirus* (Viswanathan *et al*. 2008) and further studies by Xu *et al*. (2010) also confirmed its genus status. This virus was recently found in a germplasm collection in Colombia (Cardona *et al*. 2006), a fact which indicates the importance of establishing a standard diagnostic protocol for SCSMV detection in quarantine stages. In Argentina, SCSMV was not detected although its molecular detection was optimized (Perera *et al*. 2009).

SPREAD AND TRANSMISSION OF THE DISEASE

There are three main modes for SCMV to spread: (1) by aphid vectors, (2) by infected seed cane and (3) by mechanical inoculation. Only aphid vectors and infected seed cane are relevant in the field whereas mechanical transmission is only significant in greenhouse and laboratory research (Comstock and Lentini 2005).

Natural infections of SCMV have been reported on a number of cultivated and wild grass species. Corn and sorghum, if planted next to sugarcane, may serve as an infection source as it happened in Brazil (Gonçalvez *et al*. 2007b). The importance of transmission of the disease from alternative hosts is yet to be studied (Comstock and Lentini 2005).

There are at least 12 species of aphids that can transmit SCMV from diseased to healthy plants: *Acyrfhosiphon pisum* (Harris), *Uroleucon ambrosiae* (Thomas), *Hyperomyzus lactucae* (L.) (Abbott and Charpentier 1963), *Aphis nerii* Boyer de Fonscolombe, *Carolinaia cyperi* Ainslie (Tate and Vandenberg 1930), *Hysteroneura setariae* (Thomas) (Ingram and Summers 1936), *Aphis fabae* Scopoli, *Myzus persicae* (Sultzer), *Rhopalosiphum rufiabdominalis* (Sasaki), *Lipaphis erysinii* (Kaltenbach), *Melanaphis sacchari* (Zehntner) (Bhargava *et al.* 1971) and *Schizaphis graminum* (Rondani) (Ingram and Summers 1936).

Brandes (1920) demonstrated that the corn leaf aphid *Rhopalosiphum maidis* could transmit mosaic from diseased to healthy sugarcane. This insect remained the only known vector until Ingram and Summers (1935) determined that the rusty plum aphid *Hysteroneura setariae* and the green bug *Schizaphis graminum* could also transmit the disease in sugarcane. The aphids transmit these viruses in a non-persistent manner, i.e., viruses do not reproduce in the aphid. The acquisition periods frequently range from a few seconds to a few minutes, while the retention process can take some hours (Shukla *et al*. 1994). Aphids (both adults and nymphs) transmit the virus during feeding and a latent period is not required for transmission to new host plants. Aphids do not retain the virus after molting. The virus overwinters in alternate hosts (CIMMYT 2010). The spread of mosaic is most rapid when vector populations are high, susceptible sugarcane varieties are present, and SCMV-infected plants are plentiful.

The importance of the different species of vectors depends not only on their effectiveness in transmitting the virus, but also on their numbers, which are influenced by numerous environmental factors and by aspects of their behaviour (Raccah 1983). To determine the potential for virus transmission by naturally occurring vectors in a field, vectors must be trapped in the field and then tested for infectivity (Harborne 1988).

In Ecuador, SCMV is transmitted by the corn leaf aphid, *Rhopalosiphum maidis* Fitsh, but not by the yellow sugarcane aphids, *Sipha flava* Forbis, or *P. sacchariciida*, also present in the area. In experiments made in this country, efficient transmission of SCMV by *R. maidis* was obtained with 2 hr for virus ingestion from the host plant, 0.5 hr for insect fasting period and 0.5 hr for the inoculation access period (Garcés *et al.* 2006).

Also in Venezuela, the high incidence of mosaic has a

high correlation with the presence of the aphid *R. maidis*. This aphid was mainly responsible of the disease transmission; however, *Sipha flava* and *Melanaphis sacchari* are also present (Figueredo *et al*. 2004).

In Brazil, the main vector for the SCMV transmission is also the aphid *R. maidis* (Gonçalves *et al*. 2007a).

The main vector registered of SCMV in the Cauca river valley (Colombia) is the aphid *R. maidis*, which has sorghum and corn as their primary source of inoculum. In 1992, another aphid, *Hysteroneura setariae* Thos was also found in the area but their transmission efficiency is lower than that of *R. maidis* (Centro de Investigación de la Caña de Azúcar de Colombia, CENICAÑA 2010).

DETECTION

Before 1997, the only reported method of distinguishing between different SCMV and SrMV strains was to inoculate differential hosts with sap extracted from infected plants and observe if the plants developed characteristic symptoms of the different virus strains. However, the use of host differentials is time-consuming and labour-intensive and more importantly, it does not reveal the range of viral diversity. Additionally, reliable studies require the use of a set of standard differential hosts and previously described viral strains. Serological-based assays (Koike and Gillaspie 1989), such as DAS-ELISA tests, using polyclonal antibody raised against SCMV (Shukla *et al*. 1992) and RT-PCR protocols (Smith and Van de Velde 1994) are currently available to identify SCMV and SrMV.

In 1997, Yang and Mirkov (1997) were the first to report the development of an RT-PCR-based RFLP analysis protocol to distinguish between SCMV and SrMV as well as between strains within each virus. A pair of RT-PCR primers that amplified a fragment of the CP gene was used to detect SCMV, and a second pair to detect SrMV. The RT-PCR products were then subjected to an RFLP analysis to differentiate individual strains. The CP is the best characterized of all the gene products and consists of the highly variable surface-exposed amino-(N)-terminus, a highly conserved core region, and a surface-exposed carboxyl-C-terminus (Shukla *et al*. 1988). The N-terminal part of the CP is the most variable region in the virus that it is unique to each viral type and, thus is the region where most strain variation occurs (Goodman *et al*. 1998). It contains the major virus-specific antigenic determinants, whereas the core protein is highly conserved among various *Potyvirus* spp. (Shukla *et al*. 1988; Shukla and Ward 1989).

On the other hand, virus diversity studies may require the characterization of a large number of isolates, which may be impractical if standard sample preparation and processing methodology are using. A simple protocol that yields good quality sequence information for the CP gene of viruses causing sugarcane mosaic (SrMV and SCMV) was developed by Gómez *et al*. (2009). This protocol requires neither viral RNA purification nor cloning of RT-PCR products. It was designed for rapid processing of samples in large scale molecular epidemiology and evolutionary sugarcane studies on virus populations. It was tested in a mosaic virus diversity study from sugarcane infected samples in the field. A total of 522 symptomatic leaf samples belonging to 106 different genotypes collected from 111 sampling sites throughout the sugarcane growing area of Argentina (provinces of Jujuy, Salta, Tucumán and Santa Fe) and neighbouring countries (Bolivia, Uruguay and Paraguay) were extracted and analyzed by RT-PCR using the optimized protocol described in this study. A total of 489 samples were positive for SCMV (94%), and 12 for SrMV (2.3%), with only 2 samples showing co-infection (0.4%). Twenty-three symptomatic samples (4.4%) tested negative for both viruses and it may be attributed to a series of factors which include sequence polymorphisms in primer binding sites, presence of RT-PCR inhibitors, symptom misidentification, low virus titer or a different causal agent (Gómez *et al*. 2009).

GENETIC DIVERSITY

The availability of the RT-PCR-based RFLP protocol provided a practical and efficient method to identify and differentiate virus strains causing mosaic (Yang and Mirkov 1997).

A genetic diversity study of the viruses associated with sugarcane mosaic disease was performed in Tucumán, Argentina (Perera *et al*. 2009). In order to investigate the whole viral genetic diversity, authors did not restrict the sampling to commercially grown cultivars; instead, they collected samples from sugarcane-breeding field trials that included advanced promising cultivars of the local breeding program. Samples from provinces of Salta and Jujuy were included and considered as a different geographical and agroecological group. Fifty-two samples (59.8%) were found to be infected by both SCMV and SrMV, whereas 32 samples (36.8%) were infected only by SCMV and 3 samples (3.4%) only by SrMV. Co-infection between SCMV and SrMV was found only in samples from Tucumán. Samples from Salta and Jujuy were only infected by SCMV. Koike and Gillaspie (1989) suggested that mixtures of strains might become unstable, resulting in one strain becoming dominant. Although joint infection by related viruses is unusual, it does seem to occur in some vegetatively propagated crops (Chen *et al*. 2001b). In this respect, although there have been many studies in which specific primers for SCMV and SrMV were used jointly, there have been only two reports of the coexistence of both viruses in sugarcane (Chen *et al*. 2002; Gómez *et al*. 2009). The high co-infection (68.4%) in Tucumán found by Perera *et al*. (2009) may be the consequence of the use of different sugarcane genotypes, the effect of agroecological conditions, and/or the incidence of vector populations compared with other sugarcane growing areas in Argentina and the world. The differentiation of virus strain in that study was performed as previously described (Yang and Mirkov 1997). Nine different RFLP profiles produced by the digestion with restriction enzymes *Taq*I and *Hinf*I of the PCR products of the CP gene for SCMV were found. RFLP profiles of 41% of the SCMV-positive samples coincided with strain E, whereas the other eight profiles showed complex patterns of polymorphisms that did not totally match with other known strains of SCMV. So, 59% of the samples produced banding patterns that did not match with those for known strains. Consequently, a single mutation is sufficient for an isolate to lose a restriction site and hamper typing by the RFLP-RT-PCR method (Marie-Jeanne *et al*. 2000). The RFLP analysis of the SrMV-specific PCR products with *Hga*I indicated the existence of three known SrMV strains: H, I, and M. Strains M and I were found in 68 and 14% of the samples, respectively, whereas strain H was found in association with strain M in only 18% of the samples. Nevertheless, no association between the kind of RFLP profiles of SCMV and SrMV was detected, indicating that there was no relationship between the SCMV and SrMV strains found in co-infected samples. The RT-PCR fragments belonging to each RFLP profile were purified, cloned into a vector, and sequenced. The CP-encoding region was aligned and differences were found through the entire sequence of SCMV and SrMV. No gaps were detected within each group of sequences. The cloned fragments of SCMV contained 900 nt and encoded 300 amino acids (aa); for SrMV, they contained 871 nt and encoded 290 aa. The nt sequence identity ranged from 95.89 to 99.88% within the SCMV group. When pairwise comparisons of the nt sequences were performed, all the SCMV sequences, even those classified as SCMV strain E, had a higher nt identity with SCMV strain E (95.66 to 97.07%) than with the other strains (A, B, and D) reported by Yang and Mirkov (1997). Currently, other than SCMV strain E, which predominance was determined by an RT-PCR based RFLP technique in2005 (Fontana *et al*. 2005), the major strain identified by RT-PCR-based RFLP in Argentina region belongs to an unknown profile that did not match any known strains. These

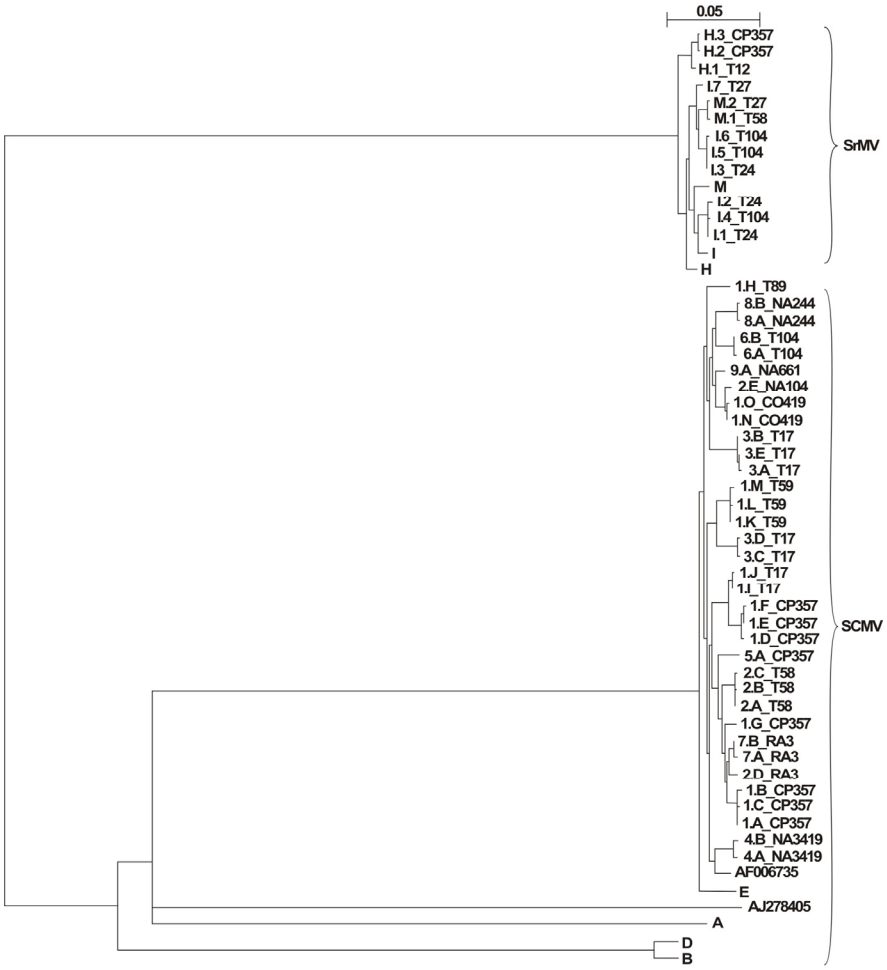

Fig. 1 Phylogenetic tree obtained with Clustal X (Thompson *et al*. 1997) from *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV) multiple alignment of the nucleotide sequence of the coat protein gene-amplified fragment. Abbreviations and accession number in the GenBank of known strain sequences: M (U57360), H (U57358), I (U57359), A (U57354), B (U57355), D (U57356), and E (U57357). SCMV isolates were named with a number equivalent to the restriction fragment length polymorphism (RFLP) profiles (1 to 9) and a letter corresponding to the different isolates belonging to each profile. SrMV isolates were designated with a letter corresponding to the three RFLP profiles (M, H, and I) and a number corresponding to the different isolates belonging to each profile. Local sugarcane genotype identities are assigned by the Sugarcane Breeding Program at Estación Experimental Agroindustrial Obispo Colombres and at Chacra Experimental Colonia Santa Rosa. Genotype abbreviations were used for the purpose of this phylogenetic analysis. Reprinted from **Perera MF, Filippone MP, Ramallo J, Cuenya MI, García ML, Ploper LD, Castagnaro AP** (2009) Genetic diversity among viruses associated with sugarcane mosaic disease in Tucumán, Argentina. *Phytopathology* **99 (1)**, 38-49, with kind permission of The American Phytopathological Society.

changes in strain identity could be explained by changes in the sugarcane cultivars used in the region (Koike and Gillaspie 1989; Grisham 2000), where new strains appeared when new cultivars were grown. In order to obtain genetic variability in agronomical traits, the EEAOC breeding program is constantly importing foreign germplasm, mainly from Louisiana, that, after quarantine, is incorporating into the crossing schedule. However, all SCMV sequences shared a higher nt identity with Australian strains AF006735 and AF278405 (96.60 to 97.89% and 99.00 to 99.90%, respectively) than those from the United States. Nucleotide sequence identities (and aa similarities) have been widely used for *Potyvirus* taxonomic purposes (Shukla and Ward 1989; Rybicki and Shukla 1992), taking into consideration that all CP gene nt identity percentages vary between 40 and 70% for different potyviruses and are above 90% for different strains of the same virus (Frenkel *et al*. 1989). Within the SrMV group, the nt sequence identity ranged from 97.36 to 99.88%. When pairwise comparisons were performed, the sequences of the SrMV strains predicted as H, M, and I by RFLP analysis did not yield the highest expected nt identity with the sequences of the corresponding strains reported by Yang and Mirkov (1997). So, isolate characterization may be best achieved by analyzing sequence data directly (Gómez *et al*. 2009), and sequence data for the CP gene of the *Potyviridae* family has been shown to be suitable for phylogenetic studies (Adams *et al*. 2005). These results question the RFLP method to discriminate strains. Not only does this technique fail to detect the entire range of genetic diversity of the viruses but it also might mask differences. Goodman (1999) found that the results obtained using the simple RFLP technique for SCMV strain identification were not in complete agreement with those obtained using sequence comparisons of the CP gene fragments. Today, DNA sequence data are only one of the sources of information used in virus classification. However, this source is becoming increasingly important, with the CP region being highly discriminatory for diagnostic and taxonomic studies if only a subportion of the genome is to be sequenced (Adams *et al*. 2005). Viral strain identification at the genomic level would provide valuable information for the development of appropriate *in vitro* diagnostic tests as well as for determining mechanisms for increased disease resistance (Goodman 1999).

Also, in the Perera *et al*. (2009) study, a phylogenetic tree was constructed based on the nt sequence alignment of the core region of the CP gene from the 35 SCMV and 12 SrMV different sequences obtained, where nine sequences of the CP gene from known viruses (obtained from GenBank) were included for comparisons (**Fig. 1**). As expected, the SCMV and SrMV isolates were clustered in independent branches. No correlation was observed between

the SCMV groups and the geographical origin of the SCMV isolates. Nevertheless, the isolates from Salta (1.N, 1.O, 2.E, 8.A, 8.B, and 9.A) and Jujuy (4.A and 4.B) belonged to different branches. A correlation between host genotype and the sequence of the SCMV CP gene has been reported (Xiao *et al*. 1993), indicating that infected hosts may have exerted a selection pressure for virus evolution. A weak correlation among viruses isolated from the same sugarcane genotypes, especially for SCMV was found. This may be due to the fact that different sugarcane genotypes were sampled in the three regions and, as Espejel *et al*. (2006) and Gemechu *et al*. (2006) have reported SCMV distribution seems to be more related to host than to geographical origin.

Since, as in the previous study by Perera *et al*. (2009), it was not possible to establish clearly if the host sugarcane genotype or the geographical origin of the isolate differentially affect the virus evolution, samples from four sugarcane genotypes with mosaic symptoms were collected in five localities of Tucumán, by the same research group. SCMV was detected in 70% of the samples and SrMV in 94% of the samples. Again, a high coinfection rate between both viruses (64%) was found. Sequences obtained from SCMV and SrMV grouped separately and the distribution of the isolates indicates no clear association between viral isolates and sugarcane genotypes or geographical origin (unpublished data). In fact, data obtained by Goodman (1999) indicate clearly that no association exists between SCMV strain prevalence and specific cultivars or regions.

A greater genetic variability in the Tucumán region was found by Perera *et al*. (2009) compared with that determined by Handley *et al*. (1998) for Australia, the United States, and South Africa, where similar values of variability among SCMV isolates were found. In addition, this genetic variability in the nt sequences of SCMV (0.12 to 4.11%) and SrMV (0.12 to 2.64%) in sugarcane should be taken into consideration in the local breeding program for resistance to mosaic disease.

Another relevant result is that in Tucumán, the commercial cultivar 'CP 65-357', released in 1989 and currently planted in 18% of the sugarcane production area (Ahmed *et al*. 2007), was infected by several virus genotypes (different RFLP-RT-PCR profiles and different CP sequences) (Perera *et al*. 2009). This confirms the high susceptibility to mosaic of this important cultivar, which was the most widely planted cultivar between 1994 and 2002, when it occupied 34% of the production area in Tucumán. Also, SrMV strain H was found in this cultivar by RT-PCR-based RFLP, whereas Grisham and Pan (2007) reported that, in 2003 in Louisiana (USA), 'CP 65-357' was infected with SrMV strain I. This was in contrast to what had been found in earlier surveys, with SrMV strain H being the most commonly recorded one (Grisham and Pan 2007).

In another study carried out in Brazil, the percentage of nt identity of CP gene sequence between a new isolate (AY819716) and other isolates from Brazil and other world regions deposited in the GenBank was 96-97 and 92-96%, respectively. This new isolate, more severe, differs in the N-terminal region, however, in the phylogenetic analysis it is grouped with other isolates less severe, both Brazilian and Australian (Gonçalves *et al*. 2007b). The N-terminal region of the CP sequence showed the highest variability among *Potyviridae* members, and often determines the distinction of strains and species within the family (Shukla *et al*. 1994). One possible explanation for this diversity is the duplication of short peptide motifs, as it was reported for SCMV and SrMV strains (Frenkel *et al*. 1991). The differences in pathogenicity may be associated with other genome regions of the *Potyviridae*, such as P1 and HC-Pro proteins (Revers *et al*. 1999).

In order to perform a diversity analysis from South America, nt sequences of SCMV and SrMV were searched in GenBank, DataBank of Japan (DDBJ) and European Molecular Biology Laboratory (EMBL) databases. Two-hundred seventy six (276) CP gene sequences for SCMV, out of which only 35 are from Argentina and 16 from Brazil,

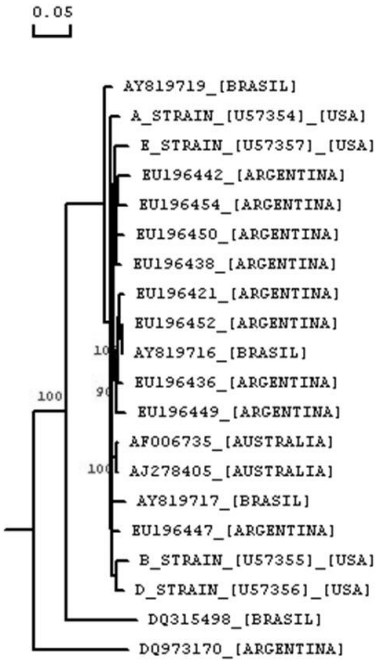

Fig. 2 Phylogenetic tree obtained with DNAMAN from *Sugarcane mosaic virus* (SCMV) multiple alignment of the nucleotide sequence of the coat protein gene from different Brazilian and Argentinian isolates, obtained from GenBank.

were found. To SrMV, 55 CP gene sequences, out of which 12 belong to Argentina, were found. The CP encoding regions (amplicon of 900 nt including SCMV F4/R3 primer sequences) of some SCMV sequences from GenBank, were aligned and their phylogeny determined by DNAMAN Version 7 (2009) using the maximum likelihood option with a bootstrap analysis of 10,000 random replications (**Fig. 2**). Gaps within this group of sequences were detected only in the first 300 nt. Brazilian and Argentinean isolates clustered together, so no correlation was observed between the SCMV groups and the geographical origin of the SCMV isolates, as Perera *et al*. (1999) reported. They also clustered with isolates from Australia. Also, AY819716, a more severe isolate from Brazil (Gonçalves *et al*. 2007b) is grouped with the other isolates from Brazil and Argentina. However, Alegria *et al*. (2003) found a strong correlation between the phylogenetic groups and the geographical origin of the SCMV isolates. Taking into account the similarity between the SrMV sequences from Argentina and the USA (**Fig. 1**), and the absence of SrMV in Brazil (Gonçalves *et al*. 2004), it is suggested that these introductions were mainly from the United States. For that reason, the SrMV diagnostic technique was urgently implemented in Argentina in order to avoid SrMV introduction, as seems to have happened in the past.

On the other hand when SCMV sequences were compared with USA sequences, the most of the Brazilian and Argentinean sequences had a higher nt identity with SCMV strain E than with the other strains (A, B, and D) reported by Yang and Mirkov (1997). Three isolates, clustered separately from the rest (AY819719, DQ973170 and DQ315498). AY819719 were isolated from sugarcane in Brazil, instead of DQ973170 from Argentina and DQ315498 from Brazil that they were recovered from corn (Souza *et al*. 2005; Lenardon and Giolitti 2006). So, infected hosts may have exerted a selection pressure for virus evolution (Xiao *et al*. 1993). Sugarcane and corn isolates of SCMV were clearly differentiated and formed two groups as Alegria *et al*. (2003) found. Their results also suggested that the sugarcane and corn groups derived from a common ancestor and diverged into two groups according to their respective host.

Unlike Argentinean SCMV, which may have come from Brazil or Australia, Brazilian SCMV comes from Australia;

Brazil imports germplasm for its breeding programs from Australia. Results indicate that in Argentina sugarcane quarantine of materials coming from the USA is effective at preventing the spread of SCMV; detection has been optimized and is routinely carried out (Ramallo *et al*. 2000). However, several materials have been introduced in Argentina from Brazil, Australia and other countries without a quarantine stage (Cuenya pers. comm.). Also serological tests used before 2005, when molecular detection of pathogens has been implemented, may have failed. The results also reinforce the importance of proper implementation of quarantine and diagnostic protocols for germplasm exchange to prevent the introduction of new pathogens or new strains into sugarcane-growing locations (Croft *et al*. 1996).

PREVENTION AND CONTROL

The more effective way to control sugarcane mosaic has been the use of resistant cultivars (Xia *et al*. 1999) and by planting healthy seed cane (Cronje 2001). Periodic surveys of SCMV strains are necessary so that all clones may be tested against prevalent strains (Comstock and Lentini 2005). This requires a complete understanding of the genetic diversity of the pathogens as well as their interaction with cultivars because resistance breakdown can occur when new strains or viruses appear (Grisham and Pan 2007). For that reason, it is relevant to carry out genetic diversity studies in the different sugarcane growing areas. Currently, mosaic disease has been controlled in many countries by using resistant varieties, although plants with symptoms are frequently observed during the evaluation of clones in the breeding programs and occasionally in nurseries and commercial plantations (Gonçalves *et al*. 2007b). In addition, careful planning of crop management practices, including planting and harvesting seasons, are used for disease control in order to avoid having young canes during peak aphid activity periods (Cronje 2001).

Breeding for resistance has proven to be difficult due to the complexity of the sugarcane genome (Handley *et al*. 1998), and resistance to mosaic remains a major selection criterion in the breeding programs. As a consequence, susceptibility to SCMV still restrains the cultivation of several elite sugarcane cultivars (Lomonosoff 1995). Also, it is estimated that mosaic has been responsible for the elimination of at least 40% of sugarcane germplasm in breeding programs (Huckett and Botta 1996). Sugarcane breeding programs use a collection of germplasm, which is likely to include basic germplasm as well as hybrids. The success of these programs may depend on the program's ability to source and import new germplasm and on its skill in using that germplasm effectively. Importers habitually quarantine these clones for 1-2 years before planting them in the field, and when diseases are discovered, the plants are destroyed. This prevents disease spreading (Hogarth and Berding 2005). Quarantine of clones has been greatly enhanced by the development of biotechnological pathology screens (James *et al*. 2004). Many organisations now subject clones to a range of pathology screens before export and after import. These procedures have minimised the risk of disease movement among countries, and represent a major step forward in improving the safety of clonal exchange. For example, the EEAOC has a 2-year phytosanitary quarantine for the materials incorporated mainly from Louisiana (USA) at Chacra Experimental Agrícola Santa Rosa in Salta, Argentina. Observations of symptoms and serological tests to detect pathogens were used in these materials; however, since 2005 molecular diagnoses have been implemented. PCR protocols to detect two bacterial diseases, ratoon stunting (*Leifsonia xyli* sp. *xyli*) and leaf scald (*Xanthomonas albilineans*), and RT-PCR protocols to detect SCMV, SrMV, SCSMV and ScYLV were optimized and are routinely applied (Filippone *et al*. 2010).

The genetic complexity of sugarcane renders traditional breeding laborious and makes it a prime candidate for improvement through genetic engineering. Transgenic sugarcane plants have been obtained via particle gun bombardment of embryogenic callus (Bower and Birch 1992; Gallo-Meagher and Irvine 1996), via electroporation of cells derived from embryogenic callus (Arencibia *et al*. 1995) and by *Agrobacterium tumefaciens* transformation (Arenciabia *et al*. 1998). Therefore, introducing specific genetic improvements, such as virus resistance, directly into elite sugarcane varieties is a realistic goal. Several strategies have been used to engineer virus resistance in plants (Baulcombe 1996). In CP and movement protein-mediated protection, a transgene derived homolog of a viral protein is expressed in plants, which interferes with or prevents various stages of the viral life cycle, resulting in attenuated disease symptoms or resistance (Ingelbrecht *et al*. 1999). Ingelbrecht *et al*. (1999) showed that transgenic mosaic virus resistance in sugarcane is based on posttranscriptional gene silencing. Recently the Australian sugar industry in collaboration with the University of Queensland and American scientists announced the beginning of the field trials of transgenic sugarcane resistant to mosaic virus. These plants were successfully tested; their sucrose yield was significantly higher and the disease incidence lower than non transgenic plants (ArgenBio 2005). Also Joyce *et al*. (1998) have selected, propagated and challenged with SCMV plants containing the CP transgene in greenhouse trials in Australia.

On the other hand, in corn, the major genes Scmv1 on chromosome 6 and Scmv2 on chromosome 3, conferring resistance against SCMV have been identified by quantitative trait loci (QTLs) (Kuntze *et al*. 1997) and bulked segregant analyses (BSA) (Xu *et al*. 1999). Also, both chromosome regions were further enriched for SSR (simple sequence repeat) and AFLP (amplified fragment length polymorphism) markers by targeted BSA in order to identify and map only markers closely linked to either Scmv1 or Scmv2. However, the question of whether (1) the Scmv1 region contains only one or more resistance genes against SCMV, and (2) the Scmv1 and the Scmv2 regions each harbour only a single locus or clusters of resistance loci against different viruses and other pathogens, can only be solved by cloning of these genes. Cloning of the Scmv1 region has been complicated because of the putative presence of two resistance genes in this region and the resulting difficulties in mapping the markers closely linked to one of the two resistance genes in that target region. Identification of recombinants between both QTLs is necessary to analyze them independently. In contrast, markers identified for the Scmv2 region seems to be suitable for marker-assisted selection (MAS) and map-based cloning (Dussle *et al*. 2003). No references were found in the literature about resistant genes to SCMV in sugarcane. However, by linkage disequilibrium mapping of commercial sugarcane germplasm, several AFLP markers were associated with the mosaic resistance trait (Butterfield and D´Hont 2006).

Management practices targeting insect vectors and control methods aimed at eradication have not been quite effective. For example, applications of insecticides have thus far failed to prevent the aphid vectors of SCMV from spreading the virus. Also, the practice of roguing, i.e., digging out and destroying diseased plants, is generally not considered feasible if the infection level exceeds 5% (Comstock and Lentini 2005). The removal of diseased plants is an invalid economic practice in commercial fields due to labour requirements, but it is highly recommended in propagation nurseries (Gonçalves *et al*. 2007b). In Colombia, also herbicides such as glyphosate are used for the destruction of these plants (Victoria *et al*. 1995).

Heat treatment of cuttings to control mosaic is partially effective but it is only practical in quarantine situations, and in some cases, sugarcane plants have recovered. However, these plants remain susceptible to reinfection by the same strain or other strains (Comstock and Lentini 2005).

As it was aforementioned, the most effective way to control sugarcane mosaic has been the use of resistant cultivars. For that reason, the breeding program of the EEAOC

releases resistant varieties; however, at the same time, the institution carries out a strategy to the sanitation of materials. Taking into account that infected seed cane is relevant as a way of spread and transmission of the disease in the field, this approach could gradually reduce the pathogenic load of the sugarcane growing areas in Tucumán. Since 2001, the EEAOC, has been working on a "Vitroplantas" project. On average, 55,000 sugarcane seedlings of the main sugarcane varieties are produced annually through *in vitro* meristem culture in the lab stage. These seedlings first undergo a rustification process and then, three more stages of conventional propagation (in Basic, Registered and Certified Nurseries) before being distributed among sugarcane growers. This project is supposed to guarantee seedling phytosanitary quality and genetic purity. The sanitation of the plant material is achieved through *in vitro* culture of apical meristem from donor plants hydro-heat-treated previously and held under natural light conditions in greenhouse with anti-aphid screen only 3 years. The micropropagation technique is widely used for the elimination of systemic diseases, especially the viral ones. Also, both meristem donor plants and micropropagated seedlings are evaluated by molecular diagnosis, a sensitive, rapid and reproducible choice. At the EEAOC, PCR protocols to detect two bacterial diseases, ratoon stunting and leaf scald and RT-PCR protocols to detect SCMV and SrMV, causing sugarcane mosaic disease are routinely applied (Paz *et al*. 2008). So, the systemic disease incidence significantly decreased in the field by using the *in vitro* culture, the micropropagation technique and the molecular diagnosis (Filippone *et al*. 2010). On the other hand, the plant tissue *in vitro* culture can produce somaclonal variation, which consists of genetic modifications in cultured cells and tissues (Larkin and Scowcroft 1981). So, a molecular methodology based on molecular markers to quantify and detect somaclonal variation in the project propagation scheme is routinely applied as a complement of the phenotypic evaluation in the field. In cases where this variation does occur, it is possible to detect it before releasing the material thus propagated (Sepúlveda Tusek *et al*. 2008; Perera *et al*. 2010). Regarding productivity, efficiency and safety, propagated plants from meristems are quite advantageous; in effect, in the short term, old and/or infected materials will be replaced by these healthy materials of high yield potential. This state-of-the-art technology, which is widely spread in sugarcane growing countries, has been incorporated by the EEAOC to obtain seedlings with phytosanitary quality and genetic purity, so as to offer them to the local growers.

For the aforementioned reasons, the great genetic variability of the causal agents of sugarcane mosaic disease should be taken into consideration in breeding programmes for resistance to mosaic and biotechnology strategies for disease management in the different sugarcane growing areas. Also, quarantine measures should be extreme in order to avoid the introduction of new pathogens during the germplasm exchange.

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