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Virus Diseases of Sugarcane. A Constant Challenge to Sugarcane Breeding in Brazil

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ABSTRACT

Sugarcane mosaic virus (SCMV) and *Sugarcane yellow leaf virus* (SCYLV) are the two main viruses infecting sugarcane in Brazil. The authors analyze the emergence and current status of these pathogens and what has being done to minimize losses and prevent new outbreaks in sugarcane. It was shown that SCMV is the only causal agent of mosaic in sugarcane in the country. Most sugarcane cultivars are believed to be tolerant or intermediate resistant to mosaic, although new cases have been reported in the field. One recently characterized SCMV isolate comprises a novel severe strain capable of infecting cultivars previously considered to be resistant. SCYLV, the causal agent of sugarcane yellow leaf, is widely distributed in Brazil and other sugarcane producing countries causing significant yield losses. The virus became widespread in the field and in parental clones used in sugarcane breeding programmes. Sensitive and reliable detection methods for SCYLV were developed and have been routinely applied for diagnosis while screening for resistance, virus elimination for germplasm exchange, and production of virus-free seed cane. Screening for resistance to sugarcane mosaic and sugarcane yellow leaf is considered a primary and essential step in sugarcane breeding programmes in Brazil.

Keywords: sugarcane mosaic, *Sugarcane mosaic virus*, *Potyvirus*, *Sugarcane yellow leaf virus*, sugarcane yellow leaf disease, *Polerovirus*, *Luteoviridae*

Abbreviations: CP, coat protein; **DAS-ELISA**, double antibody sandwich-enzyme linked immunosorbent assay; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **SCMV**, *Sugarcane mosaic virus*; **SCYLV**, *Sugarcane yellow leaf virus*; **TBIA**, tissue blot immunoassay; **YLD**, yellow leaf disease; **YLS**, yellow leaf syndrome

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INTRODUCTION

Sugarcane presents more than 200 diseases caused by fungi, viruses, bacteria, phytoplasmas, and nematodes which figure as one of the main factors contributing to sugarcane yield losses worldwide. Historically and potentially, viruses are amongst the world's most important sugarcane diseases causing remarkable epidemics and losses of major proportions. As a consequence sugarcane viruses always must be taken into account in breeding programmes worldwide where selection and elimination of susceptible clones following resistance tests have become a routine procedure.

The two main viruses infecting sugarcane in Brazil are *Sugarcane mosaic virus* (SCMV) and *Sugarcane yellow leaf virus* (SCYLV). There is only one report of *Sugarcane bacilliform virus* (SCBV) (Vega and Sordi 1991) in quarantine material, and no other sugarcane infecting virus was reported in the country so far.

Mosaic is one of the most widespread virus diseases affecting sugarcane, maize, sorghum and other Poaceous plants worldwide. Sugarcane mosaic was one of the first plant epidemics reported in the world in the beginning of the 20th century. The disease was responsible for drastic epidemics in sugarcane in Argentina, Brazil, Cuba, Puerto Rico, and USA in the beginning of the 20th century, accounting for the near collapse of the sugarcane industry (Abbott 1961; Yang and Mirkov 1997). These outbreaks were historically remarkable leading to the introduction of interspecific *Saccharum* hybrids imported from Java, in order to control the rapid spread of the disease in the noble canes (*Saccharum officinarum* L.) grown at the time in these countries (Koike and Gillaspie 1989). In Brazil, mosaic was

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introduced by mean of contaminated seed cane, probably from Argentina, causing drastic economic losses in the 1920-1930 decade. With the supposed eradication of the disease by the use of resistant hybrids, susceptible noble canes were replanted and new disease cycles took place in the mid-1930s. The problem was circumvented with the establishment of sugarcane breeding programs in the country, in combination with some field practical approaches. However as will be discussed, mosaic remains as a major disease to be considered for selection and development of new cultivars.

Yellow leaf of sugarcane, caused by SCYLV, has been reported in most sugarcane producing areas worldwide. For this reason it has been the most studied sugarcane disease since its appearance in the beginning of the 1990's, with dozen of papers dealing with different aspects of the virus and its effects in sugarcane published in the last years. The virus was responsible for drastic economic losses in southeast Brazil in the beginning of the 1990's and remains a major concern for sugarcane breeders.

Sugarcane yellow leaf syndrome (YLS), as the disease was primarily referred to, was first observed in Hawaii in 1988 and in Brazil in 1990 (Vega *et al.* 1997; Schenck 2001). The term YLS also was used in order to consider a similar disease caused by a phytoplasma in certain areas of the world (Moonan and Mirkov 2002; Arocha *et al*. 2005). In the last years the term yellow leaf of sugarcane has been employed to name the viral form of the disease (Abu Ahmad *et al*. 2007; Yan *et al.* 2009). Presently, SCYLV is endemic in all growing sugarcane areas in Brazil, but its damage is frequently ignored by growers in reason of the use of tolerant varieties and the absence of visual symptoms until the last stages of the cane cycle.

SUGARCANE MOSAIC VIRUS

Characterization of strains in Brazil

The *Sugarcane mosaic virus* complex, in the genus *Potyvirus*, family *Potyviridae*, consists of seven viruses that infect different species of poaceous, and includes SCMV, *Sorghum mosaic virus* (SrMV), *Sugarcane streak mosaic virus* (SCSMV), *Johnsongrass mosaic virus* (JGMV), *Maize dwarf mosaic virus* (MDMV), *Pennisetum mosaic virus* (PenMV) and *Zea mosaic virus* (ZeMV). Although belonging to the SCMV subgroup, the last four viruses have never been isolated from sugarcane (Chatenet *et al.* 2005), indicating that only SCMV, SrMV and SCSMV can naturally infect this crop. On the other hand mixed infections of SCMV and SrMV, or SCMV and SCSMV have been described in Argentina (Perera *et al.* 2009) and some Asian countries (Chatenet *et al.* 2005), respectively. It is important to note that despite SrMV was described infecting sugarcane in the province of Tucumán, north of Argentina, close to the Brazilian border, this virus was never reported in sugarcane or maize crops in Brazil so far.

In fact, until the middle of 2000s there was a lack of information regarding the occurrence of the species of the *Sugarcane mosaic virus* subgroup and the genetic variability of SCMV in Brazil. Mostly results were based exclusively in symptoms and Transmission Electron Microscopy (TEM) examinations, with reports of SCMV and MDMV. More recently, in surveys performed from 2003 to 2007 in the south, southeast and central Brazil in sugarcane, maize and sorghum crops, several virus isolates were investigated by mean of biological tests in indicator plants, ELISA, RT-PCR, and sequence analysis (Gonçalves *et al.* 2004, 2007a, 2007b; Gonçalves 2010). It was found that the unique potyvirus causing mosaic symptoms in these crops in Brazil was SCMV, contrasting previous reports of MDMV infecting maize (Gonçalves *et al*. 2011). In Brazil a peculiar epidemiological character of the mosaic disease is the proximity of sugarcane and maize crops in some regions of the country, what may contribute to increase the dissemination of SCMV. In addition, in the last 12 years, maize has been grown during the whole year with new cultivars adapted to the colder and drier season (winter maize). These conditions increase the source of inoculum of SCMV and the survival of its main aphid vector *Rhopalosiphum maidis* in these crops.

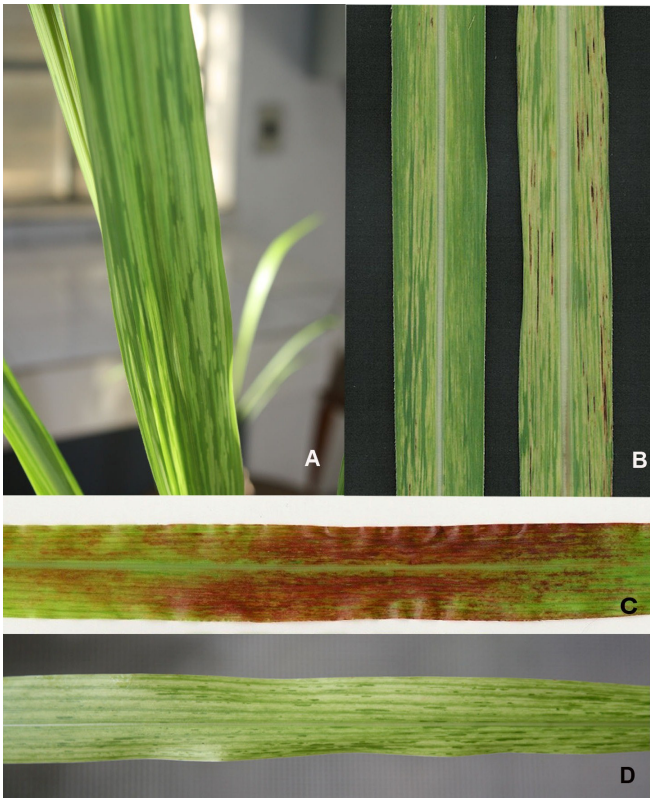

Fig. 1 (**A**) Sugarcane plant from commercial fields infected with SCMV strain RIB-1, showing severe mosaic symptoms; (**B**) Sugarcane leaves from plants naturally infected with SCMV strain RIB-1, showing severe mosaic and partial necrosis along the leaf blade; (**C**) Detail of reddening and necrosis symptoms in sorghum 'Rio' (*Sorghum bicolor* 'Rio') leave from plant inoculated with SCMV strain RIB-1; (**D**) Detail of mosaic symptom in sorghum 'Rio' leave from plant inoculated with SCMV strain RIB-1.

During the surveys, the SCMV isolate so-called Rib-1, described by Gonçalves *et al.* (2007a) was found causing mosaic outbreaks in sugarcane in São Paulo state, the main sugarcane and ethanol producing area in Brazil. This isolate induced severe symptoms in sugarcane and some host plants (**Figs. 1A-1D**), and was found infecting sugarcane commercial varieties and clones considered to be resistant to mosaic. Biological indexing with indicator plants, TEM examinations, and serological tests with antisera for species of the SCMV subgroup revealed distinct features of this isolate. Total RNA was extracted from infected plants and submitted to RT-PCR amplification with specific primers covering the capsid protein gene (CP) and C-terminal end of the Nib coding regions of SCMV subgroup. After multiple sequence alignments and phylogenetic profile analysis of the CP gene (GenBank accession number AY819716), SCMV-RIB-1 was considered a new SCMV strain, clustering with other two SMCV Brazilian isolates from sugarcane, JAU-1 (GenBank AY819717) and PIR-2 (GenBank AY819718) and with three Australian strains (**Fig. 2**). The higher nucleotide identity of SCMV sequences with Australian isolates also has been reported by Perera *et al.* (2009) in Argentina, indicating that SCMV in these two countries probably had a common origin.

The information presented above on the occurrence of new SCMV genotypes infecting sugarcane and maize in Brazil reinforces the need of employment of effective measures to control mosaic. The use of healthy seed cane, including the practice of roughing in nurseries in order to provide farmers with healthy propagation material, and cons-

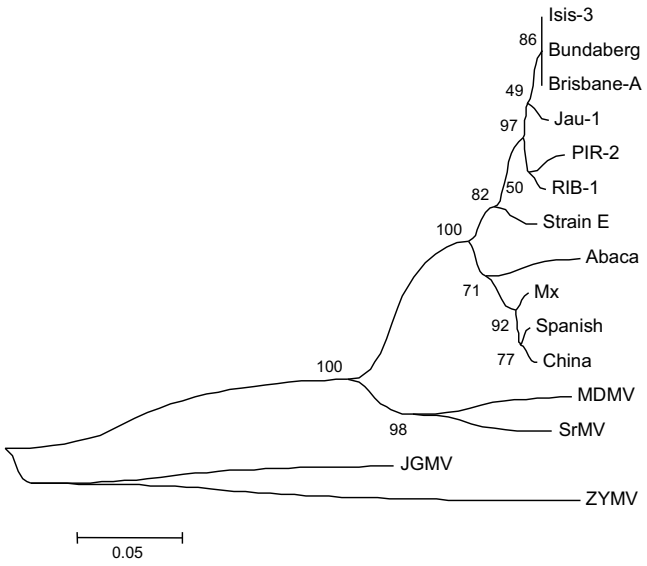

Fig. 2 Phylogenetic tree constructed by neighbor-joining analyses of the amino acid deduced capsid protein nucleotide sequences of SCMV isolates and other related *Potyviridae* species. The percentage of bootstrap replicas (2000 repetitions) observed for each branch is indicated. Abbreviations and respective GenBank accession numbers: SCMV RIB-1 (AY819716); SCMV JAU-1 (AY819717), SCMV PIR-2 (AY819718); SCMV Mx (AAO41684); SCMV Bundaberg (AAC17482); SCMV Isis-3 (AF006728); SCMV Abaca (AY222743); SCMV Spanish (CAC38364); SMCV Strain E (AAX35329); SCMV Brisbane Strain A (CAC81986); SCMV China (CAC82225); MDMV (CAA04929); SrMV (CAC84434); JGMV (NP619668); ZYMV (out-group) (CAC87636) (Gonçalves *et al*. 2007a).

Fig. 3 Details of SCYLV-infected sugarcane leaves. (**A**) Abaxial surface showing yellowing of the midrib along with partially bleached and dried leaf blade; (**B**) Adaxial surface showing reddening of the midrib along with partially bleached and dried leaf blade; (**C**) General view of a sugarcane field planted with variety SP71-6163, showing severe yellowing and necrosis caused by SCYLV infection, beside asymptomatic tolerant varieties (photo **3C** by Dr. Alvaro Sanguino).

tant monitoring of commercial fields remain as basic measures to be taken. However the main concern regarding mosaic in sugarcane is the application of the recent knowledge on genetic variability of SCMV by sugarcane pathologists and breeders, in order to develop durable resistance to the pathogen. A recent approach for such challenge, fastening the conventional breeding techniques is the use of marker assisted selection, as will be discussed forward. The genetic engineered virus resistance in sugarcane is another tool that offers a promising alternative for rapid development of SCMV resistant and high productive cultivars (Cheavegatti-Gianotto *et al.* 2011).

SUGARCANE YELLOW LEAF VIRUS

Biological and molecular characterization

Symptoms of SCYLV infection in susceptible varieties like SP71-6163 and IAC89-2135 are characterized by intense yellowing of the midrib on the abaxial surface of mature leaves (**Fig. 3A**). Older leaves show a red coloration of the midrib on the adaxial surface (**Fig. 3B**). Afterwards the leaf blade becomes yellow, dry and bleached, proceeding from the tip toward the base of the leaf, and tissue necrosis can eventually take place (**Fig. 3C**) (Vega *et al.* 1997; Gonçalves *et al.* 2005). Frequently, there is also reduction in growth resembling that of drought stress (Vasconcelos *et al.* 2009).

The first studies linking the disease to a possible viral cause were reported by Vega *et al.* (1997). These authors found virus particles in the phloem of affected leaves and detected cross reactions in the affected vascular tissues with an antiserum for a related luteovirus by Tissue Blot Immunoassay (TBIA). Subsequently, analysis of purified particles and viral RNA sequences revealed that the virus belongs to the genus *Polerovirus*, family *Luteoviridae*, and probably originated from recombination events between other species in this family (Maia *et al.* 2000; Moonan *et al.* 2000). The virus was found to be transmitted by several aphid species including *Melanaphys sacchari*, *R. maidis* and *Sypha flava* (Lopes *et al.* 1997; Scagliusi and Lockhart 2000), although only *M. sacchari* is considered important for field spread (Lehrer *et al.* 2007a). By the end of 1990's, when diagnostic tests for SCYLV were available, the disease had been reported in mostly principal sugarcane-growing countries worldwide. In Brazil the cultivar SP71-6163, extensively grown in the state of São Paulo when the disease first appeared in the country, reached yield losses of as high as 50% in the mid-1990s (Vega *et al.* 1997).

The alterations in sugarcane photosynthetic apparatus, plant metabolism and ultrastructure of vascular system caused by SCYLV infection have been extensively investigated. Once the luteovirus infection is restricted to the phloem, the characteristic yellowing and necrotic symptoms derive primarily from sieve elements and companion cells obstruction (Esau 1957), besides deterioration of mesophyll cells and chloroplast structure (Gonçalves *et al*. 2005; Yan *et al*. 2009). SCYLV-infected asymptomatic plants showed CO_2 assimilation rates and stomatal conductance 10-30% lower than healthy plants, and water relations resembling those of salinity- and drought-stressed plants (Lehrer and Komor 2009). On the other hand, when compared to healthy plants, symptomatic infected plants showed a reduction in the potential quantum efficiency for photochemistry of photosystem (PSII) and reduction in the CO_2 net exchange rates. In addition, reductions were found in the contents of photosynthetic leaf pigments and in the chlorophyll *a*/*b* ratio (Gonçalves *et al.* 2005) along with ultrastructural changes in Krans cell chloroplasts (Yan *et al*. 2009). Carbohydrate content in the leaves also was increased as a secondary effect of the SCYLV infection, suggesting a reduction of assimilate export in infected plants (Fontaniella *et al*. 2003; Gonçalves *et al.* 2005; Lehrer *et al*. 2007b).

Development of diagnostic tools

The high economic losses, and the rapid spread of sugarcane yellow leaf disease in several sugarcane growing countries during the 1990's brought special attention for the development of effective and reliable diagnostic methods for the virus. Once sugarcane is propagated vegetatively and prone to viral dissemination via seed cane, SCYLV screening plays an essential role for controlling the disease

during seed cane production and germplasm exchange. Scagliusi and Lockhart (2000) developed a polyclonal antiserum raised against SCYLV that was largely used to routine detection by TBIA and DAS-ELISA. However the production of antiserum for SCYLV is time and labor consuming because of the difficulties in purifying the virus in large concentrations from infected sugarcane. Monoclonal antibodies to SCYLV were also produced using a recombinant readthrough protein (Korimbocus *et al.* 2002); however, limitations were found in applying these antibodies in routine diagnostic tests.

Once the initial evidence suggested the association of a luteovirus with YLS, degenerated RT-PCR primers based on conserved nucleotide sequences of other members of *Luteoviridae* were developed, and successfully amplified a SCYLV specific product (Irey *et al.* 1997). Based on the sequence of this first RT-PCR product, more specific primers were developed and applied for SCYLV detection (Comstock *et al*. 1998; Gonçalves *et al*. 2002). In the following years other RT-PCR primers and new PCR-based techniques were developed to improve SCYLV detection fastness and sensitivity. Real time detection of the virus using isothermal nucleic acid sequence-based amplification (NASBA) combined with molecular beacons probes, and RT-PCR with Taqman were developed, enabling detection of as low as 10 fg of purified virus (Gonçalves *et al*. 2002; Korimbocus *et al.* 2002). One step multiplex RT-PCR assay to detect simultaneously SCYLV and other sugarcane infecting viruses, like SCMV, SrMV, and SCSMV, was recently developed and can reduce even more cost and labour for routine diagnostic, besides facilitate sugarcane virus testing in germplasm exchange and epidemiological studies of these viruses (Xie *et al.* 2009). Due to the yellow leaf dissemination to other sugarcane growing areas, and the occurrence of asymptomatic plants, quantitative real-time RT-PCR assays have been largely employed in sugarcane breeding programs to detect SCYLV in cultivars thought to be virus free, when using serology based tests, as TBIA (Viswanathan *et al.* 2009; Zhu *et al.* 2010a; Gonçalves *et al*. unpublished data). Asymptomatic SCYLV infected plants also have been diagnosed by high-resolution hyperspectral remote sensing (Grisham *et al*. 2010). These authors postulate that to detect remotely SCYLV infections in the field, without a laboratory based diagnostic technique may provide an efficient method to prevent the growers the cultivation and propagation of virus infected seed cane.

Importance in Brazil in the last decade

The losses caused by SCYLV in the Brazilian sugarcane commercial varieties currently planted are poorly known, but the virus became endemic in the main sugarcane-growing areas of the country (Gonçalves 2008) making the development of resistant cultivars to yellow leaf essential in sugarcane breeding programs. The main reason for the lack of studies to determine the potential impact of yellow leaf in commercial areas is the absence of visual symptoms in most of the sugarcane varieties. In fact, yellow leaf symptom expression may show a fluctuation that depends on the developmental stage of the infected plants, and usually begins around 200 days of growth, disappears at 400 days and reappears around 500 and 600 days (Lehrer and Komor 2008). On the other hand, Grisham *et al*. (2010) reported that rare observations of SCYLV symptoms were made in cane younger than 9-months-old in Louisiana, where sugarcane is 6-9 months of growth when harvested. Despite most of the sugarcane planted in Brazil have been asymptomatic in commercial fields, several samples taken from different growing regions from 2005 to 2010 tested positive for SCYLV by RT-PCR (unpublished data). This tolerance or intermediate resistance is a result of breeding selection to sugarcane yellow leaf disease in the last 10 years in the country.

The Agronomic Institute of Campinas (IAC) sugarcane breeding program in São Paulo, Brazil, works applying a phenotypical clone selection strategy that takes 6 to 7 years for the regional phase assessments, and 10 to 12 years in total to release a genotype as a commercial variety (Landell *et al.* 2005). During the several selection stages, resistance to sugarcane yellow leaf is one of the main traits considered. In the last years typical symptoms of SCYLV infection have been frequently observed in seedlings and even during the more advanced stages of selection for breeding, being responsible for discard of promising clones. Similarly, Comstock and Miller (2003) reported that in the CP-cultivar breeding program at Canal Point, Florida, USA, the incidence of samples with SCYLV infection generally increased from the first to the last stage of selection.

Molecular breeding techniques for resistance to sugarcane infecting viruses, with emphasis to SCYLV have been extensively studied and discussed in the last years (Smith and Harding 2001; Gilbert *et al.* 2009; Glynn *et al.* 2010; Zhu *et al.* 2010b; Cheavegatti-Gianotto *et al.* 2011). It is usually emphasized that marker assisted selection applied for resistance to SCYLV can speed up, and greatly enhance the process for the development of resistant cultivars, as will be discussed next.

SCREENING FOR NATURAL RESISTANCE: BREEDING AND BIOTECHNOLOGY

Breeding for mosaic and yellow leaf resistance

Undoubtedly the adoption of resistant varieties is the most practical and efficient means of controlling sugarcane diseases and this is also true for sugarcane viruses such as SCMV and SCYLV.

Screening for viral resistance is by far an important step in any sugarcane breeding program, once neglected viral diseases can cause considerable losses in susceptible varieties. Yield losses ranging from 11 up to 50% have been reported in susceptible varieties under severe infection with mosaic (Singh *et al.* 2003, 2005), from 40 up to 50% with SCYLV (Vega *et al.* 1997) or even 10 up to 30% in SCYLV infected asymptomatic plants (Lehrer and Komor 2009; Lehrer *et al.* 2010). In fact, SCMV infection may lead to reductions in growth parameters such as cane diameter, cane weight and number of internodes as also reduction in brix, sucrose contents, purity, and commercial cane sugar (Viswanathan and Balamuralikrishnan 2005). SCYLV infection alters sugar metabolism (Gonçalves *et al.* 2007a; Lehrer *et al.* 2010) and root system development (Vasconcelos *et al.* 2009) conducting to the yield losses.

Most of the sugarcane breeding programs conduct SCMV and SCYLV resistance evaluation through field trials under suitable environmental conditions for virus incidence at sites of high infection, usually during the early stages of selection and on the initial plant development. In fact, plant age has a definite impact on SCMV infection and the progress of infection decline with ageing (Balamuralikrishnan *et al.* 2003). Field trials evaluation, however, depends on the development of the visual symptoms which are not a mandatory expression of the viral pathology (Huckett and Botha 1996), and also of the virus strain that prevails at the experimental site. SCMV has several strains infecting sugarcane (Gonçalves *et al.* 2007a, 2007b; Pereira *et al.* 2009; Gonçalves *et al.* 2011) which implies that the response of a sugarcane clone or variety may vary according to the virus strain.

Screening for virus resistance also has been conducted under greenhouse conditions with artificial inoculation of a known strain (Schenck and Lehrer 2000; Galdeano *et al.* 2007). Different methods were developed and evaluated for their efficiency in SCMV artificial inoculation (Srisink *et al.* 1994; Gemechu *et al.* 2004; Chaves-Bedoya *et al.* 2011). These methods combined with molecular or serological virus diagnosis tests (Huckett and Botha 1996; Balamuralikrishnan *et al.* 2004; Galdeano *et al.* 2007) contribute to resistance screening even in symptomless varieties to confirm the presence of the virus and strain identification.

Concerning SCYLV, the TBIA, double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), and RT-PCR have been successfully used to detect the presence of the virus in clones and varieties and especially in sugarcane quarantine (Chatenet *et al.* 2001; Viswanathan *et al.* 2009).

Under greenhouse conditions, screening for sources of resistance to SCMV strain H was earlier described by Grisham *et al.* (1992) in a wide range of sugarcane relatives and in *Saccharum* inter-specific hybrids. Among the genus and species evaluated, *Erianthus*, *S. spontaneum*, *S. barberi* and *S. sinense* were the most resistant while *S. robustum* was the most susceptible. *S. officinarum* and inter-specific hybrids were classified as intermediate resistant.

Regarding to SCYLV, Comstock *et al.* (2001) investigated the incidence of the virus in sugarcane clones from the germplasm collection from the Florida Sugarcane Experimental Station. According to these authors, clones free of SCYLV infection may be sources of resistance to be used in breeding programs to develop resistant cultivars. The incidence of SCYLV was higher in *S. officinarum* accessions (75.8%) in contrast to *S. spontaneum* (7.0%). The incidence of SCYLV in *S. robustum*, *S. sinense* and *S. barberi* was 62.5, 46.2 and 13.6%, respectively. In addition, *S. spontaneum* was pointed out as the most resistant and *S. officinarum* as the most susceptible. Similar results were reported by Komor (2011) in a survey for SCYLV infection in sugarcane clones and wild relatives of sugarcane tested by TBIA, at the Hawaii Agriculture Research Center. Sixty to seventy per cent of the sugarcane hybrids, noble canes (*S. officinarum*) and *S. sinense* were infected by SCYLV while only 27% of *S. spontaneum* clones and 10% of the *S. robustum* and the *Erianthus* were infected with the virus. It is important to consider that differences in pathogenicity occurs within SCYLV genotypes, and capacity of the virus to multiply in sugarcane plants may also vary according to the virus isolate or strain (Moonan and Mirkov 2002; Abu Ahmad *et al*. 2007; Viswanathan *et al.* 2008; Wang and Zhou 2010).

Resistance to SCYLV also has been done exploring possible sources of host plant resistance to the sugarcane aphid *M. sacchari* (Zehntner), the main vector of SCYLV (Gonçalves *et al.* 2002; Fartek *et al.* 2008; Akbar *et al.* 2010). In this approach, the effect of the variety on the insect populations was evaluated in terms of the impact on the reproductive cycle of the insect vector (antibiosis) and in relation to its repellent effect or non-preference (antixenosis). In greenhouse studies conducted to categorize five commercial sugarcane varieties (L 97-128, CP 85-384, HoCP 96-540, Ho 95-988 and HoCP 91-555) in relation to antixenotic or antibiotic effects to *M. sacchari* and *S. flava*, differences in the duration of the reproductive period and fecundity were revealed among the varieties for both aphid species, allowing to rank them in a scale from the most to the least susceptible (Akbar *et al.* 2010).

However, according to Comstock and Miller (2003), the inoculation of a high number of plants using insectary aphids limits the number of clones that can be evaluated for sugarcane yellow leaf. On the other hand, it's highlighted that resistance evaluation based on natural infection by aphids requires several years to assure adequate exposure of plants, as the spread of SCYLV is not fast enough to allow an efficient screening of populations for the resistance incorporation into a cultivar development program.

Although resistance and susceptibility status of some varieties have been investigated for mosaic and yellow leaf in sugarcane breeding programs, there is a lack of information on the inheritance and genetics of the disease. Probably this is due to the complexity of the sugarcane genome allied to the fact that most of the sugarcane traits are multigenic, multi-allelic and quantitatively inherited (Hoarau *et al.* 2007). For instance in maize, that is taxonomically related to sugarcane, resistance to SCMV has been assigned to two major dominant genes (or regions) Scmv1 and Scmv2 (Melchinger *et al.* 1998) both essential for the expression of the resistance to SCMV (Xing *et al.* 2006).

In the case of SCYLV, individual plants derived from crosses between Hawaiian cultivars with different responses to the virus were inoculated with viruliferous aphids and subsequently tested by TBIA for SCYLV infection. The results showed that the progeny of a susceptible cultivar was mostly susceptible, and that from a resistant cultivar was mostly resistant. Pedigree analysis of susceptible and resistant cultivars reveled that parental crosses of a resistant female with a susceptible or an unknown male resulted in 90% and 10% of resistant and susceptible progeny cultivars, respectively, while in the case of a susceptible female, 75% of progeny cultivars were susceptible. Moreover, controlled cross between a SCYLV resistant *S. robustum* (cv. 'Mol 5829') and a SCYLV susceptible *S. officinarum* (cv. 'LA Purple') showed that 85% of the progeny was free from SCYLV infection in contrast with 15% of the progeny infected, suggesting that resistance to SCYLV is a dominant trait (Komor 2011).

Besides the conventional breeding, an alternative approach for developing mosaic and yellow leaf resistant cultivars has been the use of biotechnology, such as genetic transformation, once promising clones or even high productive varieties are eliminated due to virus susceptibility. This probably occurs because of the high genetic complexity and low fertility of sugarcane, hampering traditional breeding efforts what makes sugarcane a prime candidate for improvement through genetic engineering (Ingelbrecht *et al.* 1999). In addition, transgenic sugarcane plants having stable inheritance of the transgenes could be successfully used as parents in a breeding program (Butterfield *et al.* 2002).

Post-transcriptional gene silencing was applied to introduce resistance to mosaic in high sucrose yield but susceptible cultivars using the viral coat protein gene (CP) isolated from a SCMV strain prevalent in the South Africa Midlands. Basically, in this approach gene silencing is induced by the transcription of a viral gene introduced in the genome of the host plant resulting in the degradation of the corresponding gene during a putative virus infection (Sooknandan *et al.* 2003). Gilbert *et al.* (2005) used biolistic transformation with an untranslatable form of the SCMV strain E CP gene (Ubi-eut) to confer resistance to SCMV.

Introduction of resistance to SCYLV through genetic transformation has a major impact in breeding programs having a lack of parents that can be used as a source to incorporate resistance by traditional breeding techniques (Butterfield *et al*. 2002). Genetic transformation of the 'CP92-1666' cultivar, through an untranslatable SCYLV CP correspondent DNA fragment in the antisense orientation, led to the production of two transgenic clones with low yield potential but high levels of resistance to SCYLV. Nevertheless, these transgenic clones are being used as parents in crosses to combine SCYLV resistance with agronomic characteristics of high-yielding germplasm (Gilbert *et al.* 2009; Glynn *et al.* 2010). Moreover, SCYLV-resistant transgenic sugarcane derived from a susceptible cultivar (H62-4671) was transformed with an untranslatable fragment of the SCYLV CP gene. The transformed lines showed lower virus titer compared to the non-transformed, susceptible parent (Zhu *et al.* 2010b).

Another approach that can contribute to enhance the development of virus resistant varieties is the use of molecular markers tightly linked to resistant genes. An association mapping population was used to identify markers linked to sugarcane yellow leaf disease and also for resistance to its vector (*M. sacchari*) under natural infestation. Significant marker-trait associations involving Amplified fragment length polymorphism (AFLP) and DArT (Diversity Array Technology) derived markers were detected either for SCYLV or aphid resistance (Fartek *et al*. 2011) opening possibilities for marker assisted selection for SCYL disease.

Virus elimination from seed cane and germplasm

Sugarcane is a vegetatively propagated and semi-perennial crop, meaning that the same plant will be harvested and regrown up to seven years, until it is replaced for another variety. During this time, there is a huge possibility that susceptible genotypes will accumulate viruses. It is well known that viruses can cause several damages to sugarcane growth and yield, since they use the plant cellular machinery for its replication. Besides their effect on sugarcane productivity, viruses alter the plant metabolism and usually increase their concentration along the vegetative propagation cycles. Therefore, the use of healthy seed cane is crucial to achieve a good productivity along the cultivation cycles.

The damage from viruses on sugarcane crop is dependent on the virus species and strain, the sugarcane genotype, as well as the virus concentration in the tissues, and its spread within plants. Many sugarcane genotypes decline on its performance as a result of the increase in virus accumulation, and for this reason, these genotypes must be replaced. The dissemination of viruses can be reduced if seed cane nurseries are established in order to certify that all newly propagated material is virus-free.

Elimination of plant viruses of infected clones is possible through a combination of different techniques, including chemotherapy (Balamuralikrishnan *et al.* 2002), thermotherapy, and tissue culture.

The removal of viruses by tissue culture has been reported to lead to an important yield increase in vegetative propagated crops, including sugarcane. On the other hand, thermotherapy has been a well-documented approach for the inactivation of the virus in the apical meristem, since the hot appears to reduce the movement of viral RNA particles into the apical meristem by inhibiting viral synthesis (Wang *et al.* 2007).

The most efficient method used for sugarcane virus elimination is meristem tip culture, associated or not to thermotherapy. This procedure takes into account that viruses may fail to invade the meristematic region. Four hypothesis are proposed to explain why apical meristem of infected plants are generally either free or carry a very low concentration of viruses. The first is based on the high metabolic activity in the meristematic region that inhibits the virus replication. The second is concerning with the lack of vascular system at meristematic tissues. Some viruses like SCYLV and the phytoplasma SCYP (Sugarcane Yellow Phytoplasma) spread through the vascular system, and as the meristem is not vascularized, viruses cannot invade the meristematic tissue. Another hypothesis is concerning to high endogenous and exogenous auxin levels, that seem to inhibit virus replication (van Loon 1979). Recently, a new hypothesis has been proposed that meristems tips escape from virus infection by RNA silencing, a mechanism by which virus RNAs are targeted and inactivated by antisense RNA fragments (Foster *et al*. 2002). Several evidences have supported the hypothesis of the existence of a RNA-surveillance mechanism, controlled by posttranscriptional gene silencing (PTGS) at the shoot apex, which acts to allow the selective entry of RNA in reproductive structures, protecting them from virus invasion (Moore *et al.* 2001; Foster *et al.* 2002). PTGS is characterized by the cytoplasmic degradation of RNA in a sequence specific manner and the presence of small 21 to 25 nucleotide fragments of the targeted sequence (Waterhouse *et al.* 1999). This precisely target degradation is probably the consequence of a natural defense system aiming to protect shoot apex against foreign RNA (Ratcliff *et al.* 1997).

From the exposed above, since in a shoot apical meristem many cells are virus-free, it is possible to dissect out a non-infected region and manipulate this explant *in vitro* to produce virus-free plants (Kane 2005). As only the meristematic dome (part of the shoot apex immediately distal of the first leaf primordia) and the immediate covering (first leaf primordia) are usually virus-free, the size of the meristem excised is critical (Ramgareeb *et al*. 2010). Several authors have proposed a meristem size ranging from 0.2 to 1.5 mm in length for virus elimination in sugarcane (Chatenet *et al.* 2001; Parmessur *et al.* 2002; Zhang *et al.* 2006). Ramgareeb *et al.* (2010) used a combination of thermotherapy by hot water treatment of SCMV and SCYLV infected sugarcane nodes at 50°C for 40 min followed by the germination of vegetative buds at 40°C during 6-8 weeks. Meristem tips ranging from 0.5 to 2 mm in size were introduced *in vitro*, resulting in sugarcane virus-free plantlets. However, the size of the meristems used as sources for *in vitro* explants affected shoot development. The same authors found that when apical meristems from field sugarcane plants were used as explant sources, only 46% of the 0.5 to 1 mm meristems with first or second leaf primordial were able to develop shoots, when 79% of 1-2 mm meristems with first to third leaf primordia developed shoots. On the other hand, when meristems from node shoots were used 54% of the explants with 0.5 to 1 mm developed shoots, and 100% of the explants with 1-2 mm developed shoots. Very short meristems are sensitive to desiccation, phenolic compounds and damage during dissections, factors that could negatively contribute to shoot development (Balamuralikrishnan *et al.* 2002). However, viruses may be present in meristems larger than 1 mm (Victoria *et al.* 1999). Ramgareeb *et al.* (2010) verified that hot water treatment had no significant effect on the node germination.

Transfer of meristematic dome, with one or two leaf primordia has been proven to be efficient to obtain virus-free sugarcane plants. Several authors have reported success in the elimination of the main sugarcane viruses, i.e. SCMV (Leu 1972; Balamuralikrishnan *et al.* 2002; Zhang *et al.* 2006; Ramgareeb *et al.* 2010), SCYLV (Parmessur and Dookun 2000; Chatenet *et al.* 2001; Fitch *et al.* 2001; Pamessur *et al.* 2002; Ramgareeb *et al.* 2010) and *Fiji disease virus*, FDV (Wagib *et al.* 1995).

Parmessur *et al.* (2002) reported successful SCYLV and/or SCYP elimination from exotic sugarcane genotypes in quarantine. From 30 genotypes previously contaminated with one or both pathogens, 19 were completely virus-free after tissue culture. An important finding was concerning to the source of the explant (leaf roll, axillary buds, and meristem tips) used for *in vitro* culture. Those originated from callus exhibited 100% success after only one subculture when compared with meristem tips and axillary buds. Similar results were obtained with SCYLV by Pillay *et al.* (2003).

As not all meristem tips established are guaranteed to be virus-free, it is necessary to emphasize the need for sensitive diagnostic tools for virus indexing (Parmessur *et al.* 2002). According to these authors, the success in virus elimination from infected plants by tissue culture using callus from leaf roll is due the uneven distribution of the virus in the different tissues of the leaf. Furthermore, SCYLV is a virus restricted to the phloem, whereas somatic embryos have been found to arise mainly from nonvascular tissue (Guiderdoni and Demarly 1998). Therefore, plantlets derived from callus culture are more likely to be derived from virus-free cells, and hence be free from SCYLV. Fitch *et al.* (2001) also reported that it was possible to eliminate 100% of both SCYLV and SCYP when using callus culture from leaf roll explants, since both pathogens are limited to the phloem, and a lack of connection between somatic embryos and phloematic tissue certainly contribute to limit the movement of the virus. However, the possibility to the appearance of somaclonal variants or off-types when plants are originated from callus culture should be taken into account.

An important aspect regarding to sugarcane viruses indexation is concerning to the pathogen detection methods employed, since they vary in their sensibility and consequently in their efficiency. Several methods have been proposed for screening sugarcane for the presence of sugarcane viruses, including serological imunoassays (ELISA and TBIA) (Scagliusi *et al*. 1997; Schenck *et al.* 1997;

Comstock *et al.* 1998; Scagliusi and Lockhart 2000), reverse transcriptase polymerase chain reaction (RT-PCR) (Xie *et al*. 2009), real time PCR reactions like AmpliDet RNA (Gonçalves *et al.* 2002) and the fluorescent probe TaqMan (Korimbocus *et al.* 2002). The choice for the method to be used depends on the purposes and the infrastructure available.

Within this context, due to the impact of the SCMV to the sugar cane crop in Brazil, our group has been using the meristem tip culture associated with thermotherapy at 52°C for 30 min in order to establish nurseries free of the pathogen, as well as for purposes of germplasm exchange. Our experience has shown that the higher temperature also allows the elimination of others systemic pathogens, like *Leifsonia xyli* subsp. *xyli* and *Xanthomonas albilineans*, bacterial agents of "ratoon stunting disease" and "leaf scald disease", respectively. Meristem tips ranging from 1-1.5 mm in size, have been introduced *in vitro,* and the plantlets checked for the presence of SCMV and SCYLV by RT-PCR, according to the procedure described by Gonçalves *et al.* (2002, 2005). The efficiency in virus elimination has been from 70 up to 100 percent, depending on the genotype and its initial virus titer. Other possible reasons for the success or failure in virus elimination have been addressed in this chapter, reinforcing the need of a sensitive, reliable and robust diagnosis system to index the plantlets.

CONCLUSION

The information presented here from different aspects on biological and molecular characterization, diagnostic tools, phylogeny, breeding for resistance, and virus elimination give us a general overview on the situation of SCMV and SCYLV in Brazil. Some main points can be ruled out from the data presented here:

- Biological and molecular data confirm that SCMV is the only *Potyviridae* species infecting sugarcane in Brazil to date;
- The sequence data of Brazilian and Argentinean SCMV isolates suggests that they probably had a common origin;
- Great concern should be given to avoid the introduction in the country of SrMV and SCSMV, other two important potyviruses infecting sugarcane;
- The incidence of SCYLV in sugarcane varieties currently planted in Brazil is underestimated;
- Additional studies are necessary to evaluate the current impact of SCYLV in yield of Brazilian sugarcane varieties;
- In face of the new sugarcane and ethanol scenario in the country, the use of molecular breeding for developing resistance to SCMV and SCYLV is essential for enhancing the fastness and performance of Brazilian sugarcane breeding programmes;
- Efficient elimination of SCMV and SCYLV for germplasm exchange and seed cane production depends on the use of sensitive and robust RT-PCR-based diagnostic tools.

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