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Comparison of Two Diagnostic Methods for Evaluation of *Sugarcane yellow leaf virus* Concentration in Brazilian Sugarcane Cultivars

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ABSTRACT

Sugarcane yellow leaf virus (ScYLV) is one of the main virus diseases infecting sugarcane (*Saccharum* sp.) in major sugarcane-producing areas around the world. The virus belongs to the *Luteoviridae* family and is transmitted by different aphid species. This work was carried out to evaluate ScYLV concentration in different tissues of infected plants (with or without symptoms), and to compare a serological (DAS-ELISA) and a molecular (RT-PCR) method of detection. Both tests were highly specific and their sensitivity was very similar. Both methods of detection revealed the presence of ScYLV in asymptomatic and symptomatic sugarcane plants, suggesting different levels of tolerance or resistance. To better understand some aspects of virus distribution, virus concentration was also evaluated in younger and older leaves and in two parts of the leaf (the midrib and the leaf blade). Virus concentration was significantly higher in the younger leaves compared to the more mature leaves, and there was a significant increase in virus concentration in the midribs. There was no relationship between virus concentration in infected plants and intensity of symptoms. These results provide information on the most appropriate method for routine ScYLV detection and identify the best plant tissue to be used for a reliable diagnosis.

Keywords: aphids, DAS-ELISA, luteovirus, PCR, ScYLV, virus concentration
Abbreviations: **BWYV**, *Beet western yellows virus*; **BYDV**, *Barley yellow dwarf virus*; **DAS-ELISA**, double antibody sandwich-enzyme linked immunosorbent assay; **NASBA**, nucleic acid sequence-based amplification; **PNPP**, *p*-nitrophenyl phosphate; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **ScYLV**, *Sugarcane yellow leaf virus*; **SDV**, *Soybean dwarf virus*; **TBIA**, tissue blot immunoassay

INTRODUCTION

The dissemination of plant virus diseases either by their natural vectors or through the propagation of infected material, causes serious economic losses every year throughout the world (Gergerich and Dolja 2006). Sugarcane (*Saccharum* sp.), as most other crops that are vegetatively propagated, perpetuates pathogens accumulated during successive propagations, causing great crop losses and affecting the survival of plants (Gergerich and Dolja 2006). *Sugarcane yellow leaf virus* (ScYLV) is a viral disease affecting sugarcane and has been identified in major sugarcane-producing areas of the world (Rassaby *et al.* 2004; Abu Ahmad *et al.* 2006; Viswanathan *et al.* 2008). Production of sucrose is significantly reduced in infected plants and the virus is responsible for losses in productivity up to 50% in some sugarcane cultivars (Vega *et al.* 1997; Rassaby *et al.* 2004; Lakshmanan *et al.* 2005).

The virus belongs to the *Luteoviridae* family (Smith *et al.* 2000) and is transmitted in a semi-persistent way by the aphid species *Sipha flava* (Lopes *et al.* 1997), *Melanaphis sacchari* and *Rophalosiphum maidis* (Scagliusi and Lockhart 2000), *R. rufiabdominalis* (Schenck and Lehrer 2000) and *Ceratovacuna lanigera* (Li *et al.* 2008), although *M. sacchari* seems to be the only important vector for field spread of the disease (Lehrer *et al.* 2007). Typical symptoms in susceptible plants are pronounced yellowing of the midribs followed by leaf necrosis and occasionally red coloration of the adaxial surface. Symptoms also include shortening of terminal internodes, yellowing of leaves, and sucrose accumulation in the midribs (Vega *et al.* 1997; Gonçalves *et al.* 2005). Some sugarcane cultivars infected with ScYLV do not show any disease symptoms, increasing the risk of propagating virus-infected material (Korimbocus *et al.* 2002). Symptoms can also vary according to abiotic factors such as soil conditions and temperatures (Vega *et al.* 1997; Scagliusi and Lockhart 2000).

As a general rule, polyclonal-antibody based enzyme immunoassays are usually used for routine plant virus detection, but alternative methods may be necessary when increased sensitivity or specificity is required (Torrance 1992; Gonçalves *et al.* 2002; Korimbocus *et al.* 2002). ScYLV detection has been made, most of the times, through serological analyses, using DAS-ELISA (Scagliusi and Lockhart 2000; Chatenet *et al.* 2001; Viswanathan and Balamuralikrishnan 2004) and TBIA (Schenck *et al.* 1997; Fitch *et al.* 2001; Lehrer and Komor 2008). However, since virus concentration can vary within plant tissues and can be influenced by plant age and between different cultivars, more sensitive detection methods are sometimes necessary to improve reliability. After the polymerase chain reaction (PCR) was developed, the technique was soon applied to plant virus detection (Wong 2002). The advantages of PCR as a diagnostic tool include speed, versatility and exceptional sensitivity. The method is reportedly 10^2-10^5 times more sensitive than ELISA (Parakh *et al.* 1995; Spiegel *et al.* 1996; Nassuth *et al.* 2000). However, there is no single universal test, and diagnostic assays must be developed and optimized for each pathogen. PCR has some limitations when used for large-scale routine testing, being more expensive and labour intensive than ELISA (Figueira *et al.* 1997). The challenge with all types of tests is to make them

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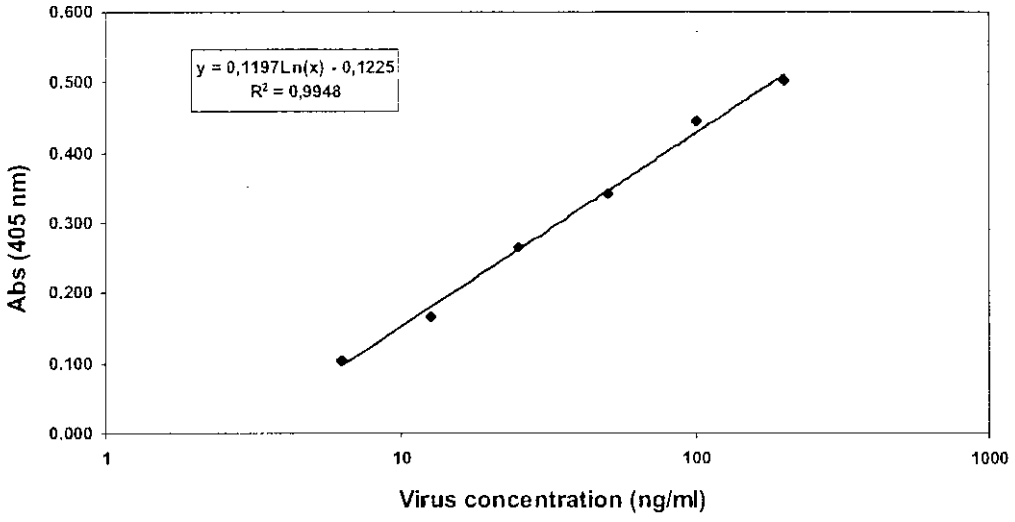

Fig. 1 Regression line obtained using six different purified virus concentrations in DAS-ELISA tests.

faster, less labour intensive, and if possible, relatively cheaper without losing reliability.

In order to identify the most appropriate method for ScYLV detection, considering costs, sensitivity and specificity, two diagnostic methods, DAS-ELISA and RT-PCR were assessed. The influence of different sugarcane cultivars and different tissue sections, as well as the relationship between virus concentration and intensity of the symptoms on the plants were investigated.

MATERIALS AND METHODS

Plant material

A range of Brazilian sugarcane cultivars (SP 70-1143, SP 71-6163, SP 80-1816, SP 80-1842, SP 80-165, SP 80-3280, SP 81-3250, SP, 84-2025, SP 85-3877, SP 86-155, SP 87-344 and SP 87-396), bred at the Sugarcane Technology Center (São Paulo, Brazil), were grown from stalks of symptomatic ScYLV infected plants, in pots in a greenhouse under natural sunlight. Infection by ScYLV was confirmed by DAS-ELISA using a specific antiserum raised against the virus (Scagliusi and Lockhart 2000). Plants of the same cultivar developed from meristem tip culture and grown in an insect-proof greenhouse were also used for this work. Virus concentration was evaluated in younger and older leaves (+1 and +4 leaves) of the same sugarcane plant, and in two parts of the same leaf (separate samples of the midrib and the leaf blade). Healthy seedlings were used as negative controls.

Serological analyses

DAS-ELISA test was used according to the procedure described by Clark and Adams (1977), with some modifications. Multiwell ELISA plates (Corning Costar 9018) were coated with 100 µL ScYLV antiserum (AS-ScYLV) raised against purified virus (Scagliusi and Lockhart 2000), diluted 1:1,000 in carbonate buffer (Merck), pH 9.6. The test samples were prepared by grinding 1.0 g of leaf tissue with liquid nitrogen in 100 mM $NaKPO_4$ (Sigma), pH 6.0, 1% Na_2SO_3 (Sigma), and 0.05% Tween-20 (Bio-Rad). The extracts were centrifuged at 12,000 × *g* for 15 min and 100 µL of the supernatant was collected for the assay. Tissue samples from healthy sugarcane seedlings were used as negative controls.

Alkaline phosphatase immunoglobulin conjugate (IgG-AP) was diluted to 1:1,000 in 20 mM Tris/HCl (Bio-Rad), pH 7.4, containing 150 mM NaCl (Sigma), 0.05% Tween-20 (Bio-Rad), 0.2% BSA (Bio-Rad) and 2% skimmed powdered milk (Bio-Rad). At each stage, plates were incubated for 2 hrs at room temperature (21°C) or overnight (4°C). The *p*-nitrophenyl phosphate (PNPP - Sigma) solution (0.5 mg/mL) was added to the plates and final absorbance was read at 405 nm in an ELISA plate reader (BioRad), beginning after 1 hr of incubation. Samples were considered positive when absorbance values were 2 times higher (Sutula *et al.* 1986; Khentry *et al.* 2006) than the negative controls (healthy seedlings). Different concentrations of purified virus samples (6.25, 12.5, 25, 50, 100 and 200 ng/mL) were included in the assays and they were used as a standard to determine virus concentration in the test samples (regression line, **Fig. 1**). Purification method was used according to the procedure described by Scagliusi and Lockhart (2000).

RT-PCR (Reverse Transcriptase PCR)

The PCR method was performed according to the procedure described by Gonçalves *et al.* (2002). The primer sequences were: sense primer Forward - SCF (P1) (position 3660-3882) GCT.AAC.CGC.TCA.CGA.AGG.AAT.GT; anti-sense primer Reverse - SCR (P2) (position 4091-4109) GAA.GGG.GGC.CGG.GAA.GAC.T (Pharmacia Biotech, The Netherlands).

For total RNA extraction, sugarcane leaf samples were triturated with liquid nitrogen and 500 µL of the leaf powder was diluted in 1.0 mL Trizol (Total RNA Isolation Reagent - Gibco). 200 µL chloroform (Sigma) was added to the mixture and centrifuged at 12,000 × *g* for 15 min. After centrifuging, the aqueous phase was removed, mixed with equal volume of isopropanol (Sigma). The sample was centrifuged again at 12,000 × *g* for 5 min. The pellet containing RNA was washed with 75% ethanol (Sigma) and diluted in 50 µL of DEPC (Sigma) sterile treated water.

For first strand DNA synthesis (cDNA), samples were incubated at 95°C for 5 min followed by 40°C for 10 min. The reaction mix consisted of 1.0 µL reverse primer antisense (SCR), 3.0 µL purified RNA and 4.0 µL of a mixture containing 5X Strand Buffer (Gibco), 1.0 µL RNA-Guard (Gibco), 1.0 µL of 0.1 M DTT, 1.0 µL of 10 mM dNTP and 200 U Superscript II (Reverse Transcriptase - Gibco). The samples were incubated for 10 min at room temperature (21°C) followed by 1 hr at 37°C.

The PCR reaction mix consisted of 5.0 µL of 10X PCR Buffer (Gibco), 1.5 µL of 50 mM $MgCl_2$ (Gibco), 1.0 µL of 10 mM dNTP (Gibco), 2.5 µL reverse primer antisense (SCR), 2.5 µL forward primer (SCF), 0.5 µL *Taq*-polymerase enzyme (Amplitaq Gold - Perkin Elmer), 34 µL H_2O and 3.0 µL cDNA strand. Amplification cycles were in a Perkin Elmer Gene Amp, PCR System 2400, initially for 10 min at 94°C and 40 cycles of 30 sec at 94°C, 1 min at 60°C and 2 min at 72°C. Following PCR, the products were analyzed by electrophoresis on a 1% agarose gel (Gibco) and stained with ethidium bromide (Sigma). A 1 kb ladder (Gibco) was used as a standard molecular weight marker.

Statistical analyses

Experimental design was completely randomized with four replications. All statistical analyses were done using Agrobase 999 (Agromix Software, Inc. 1999) and Microsoft™ Excel® software.

RESULTS

ScYLV quantification – DAS-ELISA versus RT-PCR

Purified virus samples at various known concentrations were used to evaluate the suitability of DAS-ELISA, as a method for quantifying ScYLV in sugarcane tissues. Absorbance values showed a high correlation level with the logarithm of virus concentration, resulting in a regression line where $R^2 = 0.994$ (**Fig. 1**).

Our results showed that the DAS-ELISA method was able to detect up to 4.0 ng of virus/mL or 400 pg of virus per each ELISA plate well (using 100 µL per well). When these same samples were tested using RT-PCR, it was possible to detect lower virus concentrations, suggesting higher sensitivity. **Table 1** shows one example of a negative result for DAS-ELISA that was positive for RT-PCR. In this specific case, the tested plant (SP 81-3250) was originated from tissue culture, where attempts to eliminate the virus were made through meristem tip culture. The negative result obtained by DAS-ELISA was therefore probably due to the fact that when the plants were tested, they were very young (plants with leaves that were not fully developed, classified as -1 and 0 leaves), and virus concentration was extremely low (**Fig. 2**). However, when both techniques were compared and used for virus detection in adult plants, their specificity and sensitivity were comparable. For this reason, DAS-ELISA was used to study virus concentration in different sugarcane tissues and varieties, improving sample throughput and reducing costs.

Table 1 Comparison of sensitivity between DAS-ELISA and RT-PCR results.

Varieties	DAS-ELISA	RT-PCR
Healthy Sugarcane Seedlings	-	-
SP 81-3250 (meristem)	-	+
SP 70-1143 (meristem)	+	+
SP 71-6163 (meristem)	+	+
SP 80-1842	+	+
SP 80-1816	+	+
SP 86-155	+	+
SP 85-3877	-	-
SP 87-396	-	-

- = negative for ScYLV, + = positive for ScYLV

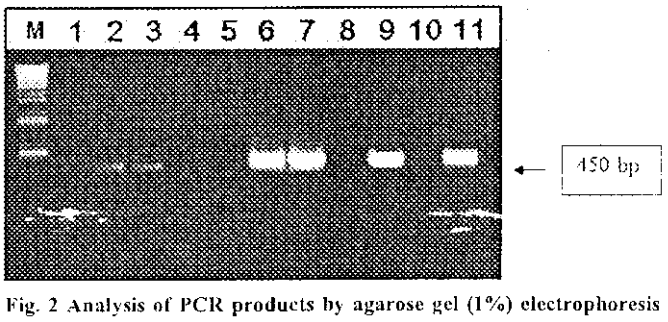

Fig. 2 Analysis of PCR products by agarose gel (1%) electrophoresis stained with ethidium bromide. Lanes: **M** = molecular marker, **1** = healthy seedlings (control), **2, 3** = SP 81-3250 (meristem tip culture), **4, 5** = SP 70-1143 (meristem tip culture), **6, 7** = SP 71-6163 (meristem tip culture), **8** = SP 85-3877 (seedcane), **9** = SP 81-3250 (seedcane), **10** = SP 87-396 (seedcane) and **11** = SP 71-6163 (seedcane).

Virus concentration from symptomatic and asymptomatic sugarcane plants

As described before, DAS-ELISA was used to evaluate ScYLV concentration in different sugarcane varieties, with or without symptoms. A score ranging from 1 to 4 was assigned to infected plants according to symptom intensity, 1 for plants without symptoms and 4 for plants with strong symptoms. All sugarcane plants used for this study were kept under field conditions and naturally infected with the virus in the field (by aphid vectors). ScYLV concentration and intensity of symptoms were measured in +1 leaves (the first fully developed-leaf emerging from the stalk).

Table 2 ScYLV concentration measured by DAS-ELISA in different sugarcane varieties with different levels of symptoms. Values represent the average of four wells.

Varieties	Symptoms (Score)	[Virus] ng/mL
Seedlings	1	Negative
SP 71-6163	4	9,1129
SP 81-3250	3	8,2436
SP 84-2025	3	7,8406
SP 80-185	2	7,3952
SP 70-1143	1	15,1697
SP 80-1842	1	5,6604
SP 80-3280	1	7,5198
SP 87-344	1	11,1361
SP 80-1816	1	5,6133
SP 86-155	1	6,5242
SP 85-3877	1	Negative
SP 87-396	1	Negative

Some genotypes were virus-free and showed negative results when tested (SP 85-3877, SP 87-396), while six other genotypes with the same score (1 = without symptoms), presented variable virus concentration (**Table 2**). For this reason, a temporary group was established among the tested varieties, based on the DAS-ELISA results, as: 1) susceptible to virus infection and with symptoms, 2) tolerant to infection with no symptoms and 3) resistant to infection (virus-free plants) without symptoms. Sugarcane variety SP 71-6163 is highly susceptible to ScYLV infection and exhibits severe symptoms (score 4). However, the results from DAS-ELISA suggested that the virus concentration was relatively low. On the other hand, there were some sugarcane varieties (SP 70-1143, SP 80-1842, SP 87-344, SP 80-1816 and SP 86-155), that did not develop any disease symptoms (score 1), but tested positive for the virus. Virus concentrations were substantially higher in cultivars SP 70-1143 and SP 87-344 than compared to the susceptible variety SP 71-6163, with absorbance values up to seven times higher than the negative control. This indicates that there was no significant relationship ($p>0.05$) between virus concentration and intensity of symptoms in the infected plants.

Virus distribution in different tissues of sugarcane leaves

A study of virus distribution within the leaves was conducted in +1 and +4 leaves from 15 month-old plants, using sugarcane varieties SP 81-3250 and SP 84-2025 (healthy and infected) and SP 71-6163 (infected, with and without symptoms). These were the only varieties, of the 12 tested that were infected and showed symptoms of the disease. Virus concentrations in the midribs and in the leaf blade, were determined using DAS-ELISA.

Our results showed significantly higher concentrations of ScYLV in the midribs of +1 leaves (**Fig. 3**), with virus concentration up to nine times higher than that detected in the leaf blade (SP 71-6163). Significantly lower concentrations of ScYLV were detected in both the midbrib and the leaf blade of +4 leaves (**Fig. 3**).

DISCUSSION

The results obtained in this study using DAS-ELISA and RT-PCR tests were comparable and showed a high degree of coincidence, indicating similar sensitivities between both diagnostic tools. When plants produced through meristem tip culture were tested, RT-PCR was more sensitive, detecting ScYLV in one sample that tested negative using DAS-ELISA. In this case, the resulting bands after RT-PCR (**Fig. 2**: lanes 2, 3) were very light, suggesting very low virus concentration, probably below the limit of sensitivity of the

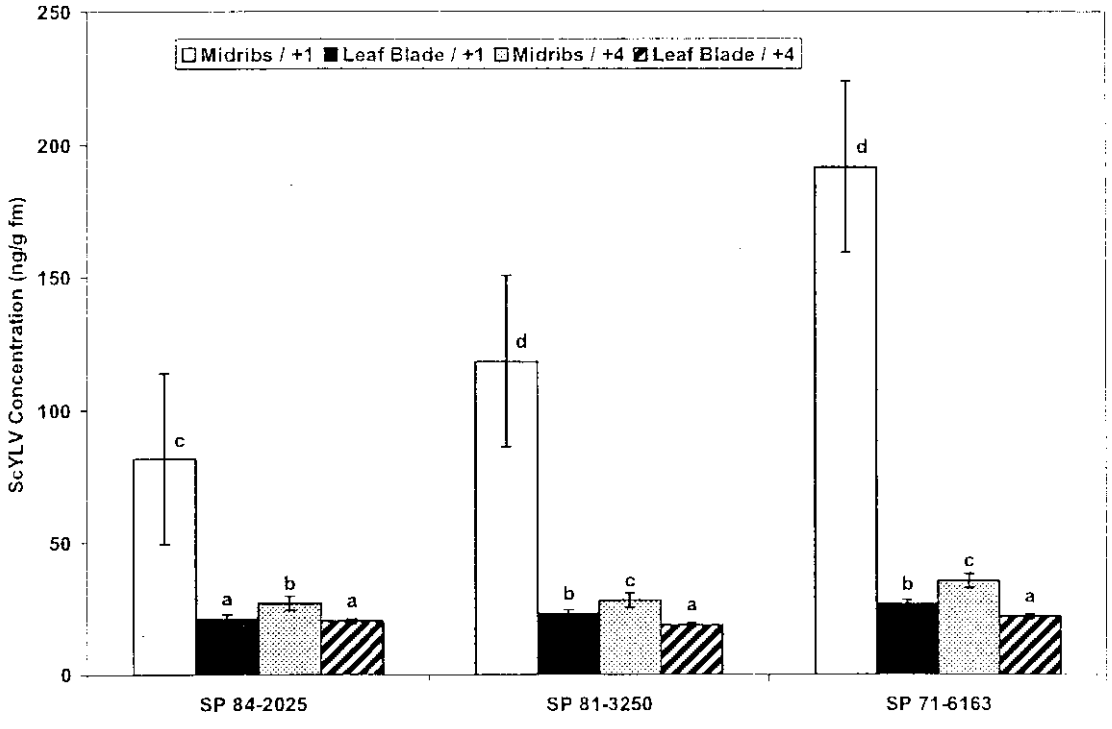

Fig. 3 ScYLV concentration determined by DAS-ELISA in the midribs and leaf blade of +1 and +4 sugarcane leaves. Columns followed by the same letters do not differ significantly (Tukey's, 5%).

DAS-ELISA test. Comparisons between diagnostic tools for the same virus were also made by Aljanabi *et al.* (2001), Gonçalves *et al.* (2002) and Korimbocus *et al.* (2002). In all cases, DAS-ELISA was less sensitive than PCR.

Different detection levels have been reported in the literature for luteovirus diagnosis using DAS-ELISA. D'Arcy and Hewings (1986) working with three luteoviruses, namely *Barley yellow dwarf virus* (BYDV), *Beet western yellows virus* (BWYV) and *Soybean dwarf virus* (SDV), were able to detect virus concentrations of up to 1.6 ng/mL, using purified virus samples. Our results from virus purified samples, showed sensitivity of approximately 4.0 ng/mL (or 400 pg of virus per each ELISA plate well using 100 µL samples). Similar results have been reported for BYDV-PAV detection (Figueira *et al.* 1997) comparing different methods (DAS-ELISA, RT-PCR and nucleic acid *in situ* hybridization). Their results showed that, in spite of the higher sensitivity of RT-PCR compared to the other methods tested, it was not suitable for testing large numbers of samples. Our results showed only one false negative sample detected by DAS-ELISA.

Better, more sophisticated and sensitive methods for virus detection have been developed (Wong 2002; Khan and Dijkstra 2005). Molecular beacon technology offers many advantages over current methods for plant virus detection (Eun and Wong 2000). Gonçalves *et al.* (2002), also working with ScYLV, adapted an AmpliDet RNA system, which consists of nucleic acid sequence-based amplification (NASBA), of the target RNA, with specific primers and simultaneous real-time detection of the amplification products with molecular beacons. Their results showed that the system produced a detection level of at least 100 fg of purified virus. Virus was readily detected in plant tissues with low levels of infection and in the hemolymph of aphids. However, since this method is relatively new, the current cost per test is still high.

Although some authors have described the use of meristem tip culture to eliminate ScYLV (Chatenet *et al.* 2001; Fitch *et al.* 2001; Parmessur *et al.* 2002), some plants were still infected after tissue culture in this study, confirming the need for routine testing after plant regeneration. Viswanathan *et al.* (2008) confirmed the presence of ScYLV by RT-PCR in 10 plants obtained by meristem culture (of 16 tested samples). In our study, virus elimination occurred in only some of the tested plants. The presence of the virus after meristem culture can be explained by the size of the meristem tip used for plant regeneration. Partial virus elimination in plants obtained by meristem tip culture can also be explained by the fact that some viruses are replicated and transmitted very rapidly to the apical growing points (Nehra and Kartha 1994).

This study showed that there was no relationship between virus concentration and intensity of symptoms, in different sugarcane varieties. ScYLV concentrations were lower in the susceptible variety SP 71-6163, showing severe symptoms (rated 4). Some varieties (SP 70-1143, SP 80-1842, SP 80-3280, SP 87-344, SP 80-1816 and SP 86-155) tested positive for ScYLV by DAS-ELISA and RT-PCR but did not exhibit disease symptoms, suggesting tolerance to the virus. Other varieties such as SP 85-3877 and SP 87-396, were also asymptomatic and tested negative with DAS-ELISA and RT-PCR (**Fig. 2**, lanes 8 and 10). These varieties may be resistant, since both were maintained under the same conditions as those that tested positive, and were exposed to the natural vector of the virus. Our results confirm similar studies on ScYLV conducted by Lehrer and Komor (2008). They investigated symptom expression in a selection of Hawaiian cultivars and their different degrees of infection. The cultivars were classified into three groups, using tissue blot immunoassay (TBIA) to detect the virus: ScYLV-susceptible/infected, ScYLV-resistant and intermediately infected cultivars. However, in this study no relationship was established between virus concentration and intensity of symptoms, since TBIA indicates the presence or absence of the virus and it does not indicate virus titer.

ScYLV analyses were also carried out by Viswanathan and Balamuralikrishnan (2004). Virus detection was evaluated by DAS-ELISA using two types of samples (leaves and juice of sugarcane stalks). Their study showed ScYLV titer was higher in the juice sample as compared to the leaf sample. Plants raised from ScYLV-infected planting material compared to those raised from symptom-free seedcane had also higher virus titer. They concluded that DAS-ELISA could be used to diagnose ScYLV even when the symptoms of the disease were not expressed.

Virus distribution studies were made using −1 and +4 leaves of susceptible sugarcane plants. Our results showed a significant increase ($p>0.05$) in the virus concentration in

midribs of younger leaves (−1 leaves). However, when older leaves (−4) were tested, virus concentrations in the leaf blade and in the midribs did not differ considerably.

ScYLV distribution within sugarcane plants was also studied by Rassaby *et al.* (2004), using TBIA and RT-PCR. In their study, they observed that the virus could be detected, as early as two weeks after planting (in the top visible dewlap leaf) and after one month of growth, in all tested tissues (stalks, leaves, shoots and roots) of all sugarcane cultivars, but they did not identify a preferable type of tissue for routine ScYLV detection.

Our results provide valuable information regarding the most suitable methods and the best tissue to be used for the reliable detection of ScYLV, especially when virus concentrations are likely to be low, as the members of the *Luteoviridae* family. DAS-ELISA was found to be suitable for routine, large-scale ScYLV diagnosis, efficient and relatively cheap diagnostic method that can be adapted for use in most laboratories.

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