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# The use of molecular beacons combined with NASBA for the sensitive detection of *Sugarcane yellow leaf virus**

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## Abstract

*Sugarcane yellow leaf virus* (ScYLV) is widely distributed in Brazil and other sugarcane producing countries causing significant yield losses. Due to the high incidence of the aphid vector, the virus is widespread in the field and in parental clones used in sugarcane breeding programmes. Aiming to present a sensitive and reliable detection of ScYLV, we have adapted an AmpliDet RNA system, compared it with the currently available detection methods and discussed its applicability for routine diagnosis. AmpliDet RNA 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. The 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 (without the need of previous RNA extraction) and in the hemolymph of aphids. The method showed to be virus-specific, testing negative for other species of the *Luteoviridae*. In conclusion, the system has potential to become a diagnostic method for the detection of sugarcane viruses.

## Introduction

*Sugarcane yellow leaf virus* (ScYLV) is a newly characterised virus that infects sugarcane and causes one type of the yellow leaf syndrome (YLS) (Vega et al., 1997; Lopes et al., 1997; Scagliusi and Lockhart, 2000). The symptoms consist of an intense yellowing of the abaxial surface of the midrib, red shining of its adaxial surface followed by tissue necrosis and reduction in plant growth. ScYLV has been reported in several sugarcane producing countries worldwide (Borth et al., 1994; Schenck et al., 1997; Vega et al., 1997; Comstock et al., 1998) causing significant yield losses. In Brazil, where the disease is widely distributed, losses as high as 50% were responsible for the decline of some of the most productive cultivars (Vega et al., 1997).

Based on nucleotide sequence analysis, it was proposed that ScYLV is a new member of the family *Luteoviridae*. The virus shares a number of characteristics with members of the genera *Polerovirus*, *Luteovirus* and *Enamovirus* (Moonan et al., 2000; Maia et al., 2000; Smith et al., 2000). However, it clearly represents a distinct species due to its unique biological properties and differences at the genomic level, which suggests interspecies recombination (Moonan et al., 2000; Maia et al., 2000; Smith et al., 2000).

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The combination of the presence of the ScYLV in parental clones, clonal multiplication and the high incidence of its main aphid vector *Melanaphis sacchari* in the field necessitates the availability of a reliable method for the early detection of the virus in stock material. Sensitive and robust detection methods are required for supplying certified disease-free sugarcane varieties and are also essential tools in virus resistance breeding programmes. The reverse transcriptase polymerase chain reaction (RT-PCR) and serological assays based on enzyme-linked immunosorbent assay (ELISA) or tissue printing have been used for screening plant material for the presence of the virus (Comstock et al., 1998; Scagliusi et al., 1997; Scagliusi and Lockhart, 2000). However, the serological detection tests do not always offer the required level of sensitivity and specificity. On the other hand, the gel-based RT-PCR systems are rather laborious and time-demanding for routine diagnosis of a large number of samples.

1The use of molecular beacons (Tyagi and Kramer, 1996; Eun and Wong, 2000) allows a gel-free approach in viral diagnostics enabling a sensitive, specific and fast detection. Combining the molecular beacon technology with the nucleic acid sequence-based amplification (NASBA; Kievits et al., 1991) permits the simultaneous amplification and detection of viral RNA in a closed tube, called AmpliDet RNA (Leone et al., 1998). NASBA is a method that amplifies target viral RNA isothermally at 41 °C using two target-specific oligonucleotide primers and the enzymes AMV-reverse transcriptase (AMV-RT), RNase H and T7-RNA polymerase. Recently, application of the AmpliDet RNA has been described for the detection of several plant viruses (Klerks et al., 2001a–c; Leone et al., 1998; Szemes et al., 2002). These papers showed AmpliDet RNA to be highly sensitive and suitable for robust detection of virus in complex plant tissues like bark, buds, potatoes and fruit. In addition, AmpliDet RNA detected several distinct plant viruses simultaneously in one tube (Klerks et al., 2001a,c; Szemes et al., 2002).

In this paper, we describe the development of an AmpliDet RNA system for the detection of ScYLV in sugarcane and its aphid vector *Melanaphis sacchari*, and compare its sensitivity with that of double antibody sandwich – ELISA (DAS-ELISA), RT-PCR and NASBA combined with Northern blotting analysis.

## Materials and methods

*Plant material, virus source and aphids*. Healthy and naturally ScYLV-infected plants of two Brazilian sugarcane cultivars, SP 71-6163 and SP 82-3250, were grown in a greenhouse at 28 °C under a photoperiod of 16 h. Both cultivars respond readily with symptoms upon infection with ScYLV, and yield high virus titres. The virus (ScYLV Brazilian isolate) was purified from frozen leaf material, kept at −80 °C using an enzyme-assisted procedure, essentially as described by Van den Heuvel et al. (1991). Purified ScYLV (60–70 μg/ml) was stored in 0.1 M sodium citrate, pH 6.0, containing 20% sucrose at −80 °C.

Members of the *Luteoviridae*, such as *Potato leafroll virus* (PLRV), *Beet western yellows virus* (BWYV), *Barley yellow dwarf virus*-PAV (BYDV-PAV) and *Bean leafroll virus* (BLRV), used during the experiments were all derived from the Plant Research International BV virus collection.

Colonies of the vector aphid *M. sacchari* (Lopes et al., 1997; Scagliusi et al., 2000) were maintained on healthy sugarcane plants in aphid-proof cages at 23 °C under a photoperiod of 16 h.

*Samples of leaf material and aphids.* Samples were prepared by grinding sugarcane leaf tissue in liquid nitrogen to which sample buffer (0.14 M NaCl, 2 mM KCl, 2 mM $KH_2PO_4$, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 0.05% Tween 20, 2% PVP 44,000, 0.2% ovalbumin, 0.1% Na-azide and 0.5% BSA, pH 7.4) was added in a ratio of 1 : 10 (w : v). The samples were aliquoted and either used directly or subjected to total RNA extraction using the RNeasy isolation kit (Qiagen).

The hemolymph of groups of five aphids which had been feeding on infected and healthy sugarcane plants was collected in 30 μl of deionised water by removing the cornicles with a fine pair of forceps and applying some pressure on the abdomen of the insect. The samples were either used directly or stored at −80 °C. RNA was extracted as described above.

*NASBA and Northern blot detection.* NASBA was carried out in reaction tubes (Kievits et al., 1991) using a reaction mix of 6 μl 3.3× NNX-buffer (167 mM Tris–HCl, pH 8.5, 50 mM $MgCl_2$, 292 mM KCl, 2.1 mM DTT, 4.2 mM of each dNTP, 8.4 mM each of ATP, UTP and CTP, 6.3 mM GTP and 2.1 mM ITP), 4 μl 5× primer mix (75% DMSO and 1 μM of each primer in RNase free water) and 5 μl of sample extract per reaction. The sequences and locations of the primers on the viral genome are listed in Table 1. The tubes were incubated for 5 min at 65 °C followed by 5 min at 41 °C. NASBA was initiated by adding 5 μl of an enzyme mix (375 mM sorbitol, 2.1 μg

BSA, 0.08 U RNase H, 32 U T7-RNA polymerase and 6.4 U AMV-reverse transcriptase) to each tube, incubating for 5 min at 41 °C, followed by a 10 s low speed centrifugation step, and continued incubation at 41 °C for 90 min. Amplification products were separated on a 1% pronarose gel containing 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) with 0.5 μg/ml EtBr, transferred onto Z-probe nylon membranes (BioRad) in 2× SSC (0.3 M NaCl, 30 mM Na-citrate), and exposed to UV light for cross-linking. For enhanced chemiluminescence detection (ECL detection reagents, Amersham Pharmacia Biotech), the blots were incubated with the biotinylated probe $BIO_{ScYLV}$ (3 μM) for 60 min at 50 °C in a hybridisation mix (5× SSC, 7% SDS, 20 mM Na-phosphate, pH 6.7, 10× Denhardt's reagent), essentially following the protocol as described by Leone et al. (1998). The sequence and position of the probe $BIO_{ScYLV}$ in the CP gene of ScYLV are shown in Table 1.

*Molecular beacon design and AmpliDet RNA.* The molecular beacon $MB_{ScYLV}$ was designed to carry 5′ end and 3′ end sequences of 6 nucleotides complementary to each other. The internal sequence of 21 nucleotides complementary to the ScYLV-specific NASBA product was selected to hybridise to the same region as the biotinylated probe $BIO_{ScYLV}$ (Table 1). The molecular beacon was coupled with 6-carboxy-fluorescein (FAM; excitation wavelength: 494 nm; emission wavelength: 530 nm) and the quencher 4-[4′-dimethylaminophenylazo]-benzoic acid (DABCYL) at the 5′ and 3′ end, respectively. The arms of six nucleotides form a double-strand structure at 41 °C to avoid emission of fluorescence in the absence of a target sequence.

AmpliDet RNA was performed by adding 3 μl of sample, 1 μl of 8 mM ROX (5-(and -6)-carboxy-X-rhodamine) and 1 μl of 9 ng/μl of molecular beacon $MB_{ScYLV}$ to the NASBA reaction mix described above. After incubation for 5 min at 65 °C and 5 min at 41 °C, 5 μl of the enzyme mix (previously described) was added to each tube. The reaction was immediately transferred to an ABI Prism 7700 Sequence Detector (Perkin Elmer). Amplification was performed at 41 °C during 90 min while the fluorescence emission spectrum (530 nm) was measured real-time every 2 min.

*RT-PCR and DAS-ELISA.* The antisense primer P2r (Table 1) was used to prepare cDNA from purified virus or total RNA extracted from healthy and infected leaf tissue. P2r and other PCR primers used (Table 1) were designed based on the coat protein coding region of ScYLV (Maia et al., 2000). The reverse transcription was performed with 1 μl of antisense primer and 3 μl of sample and an usual pre-mix (4 μl of 5× buffer, 1 μl of RNA guard, 2 μl 0.1 M DTT, 2 μl 10 mM dNTP mix and 200 U of Superscript – Gibco). The reaction mix for PCR consisted of 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 5 μl of 10× Taq buffer, 8 μl of 25 mM $MgCl_2$, 5 μl 1mM dNTPs, 2.5 U of AmpliTaq Gold (Perkin Elmer), 5 μl of cDNA samples and 24.5 μl of $H_2O$. Several PCR protocols were tested for optimisation of the system.

DAS-ELISA was performed essentially as described by Scagliusi et al. (2000) with small modifications

*Table 1.* Oligonucleotide sequences and location of specific primers and probes in ScYLF genome

| Oligonucleotide primers and probes | Sequences (5′→3′)[a] | Positions[b] |
|---|---|---|
| RT-PCR sense primer – P1f | GCT.AAC.CGC.TCA.CGA.AGG.AAT.GT | 3660–3882 |
| RT-PCR antisense primer – P2r | GAA.GGG.GGC.CGG.GAA.GAC.T | 4091–4109 |
| RT-PCR sense primer – P3f | CAG.GTG.CAA.TCG.CAC.TTG.AAG.TGG.A | 3997–4021 |
| RT-PCR antisense primer – P4r | GAA.TTG.TCC.TGC.TAG.GCT.CGA | 4179–4199 |
| NASBA sense primer – N2f | CAG.GTG.CAA.TCG.CAC.TTG.AAG.TGG.A | 3997–4022 |
| NASBA antisense primer – N1r | AAT.TCT.AAT.ACG.ACT.CAC.TAT.AGG.GAG. GAA.TTG.TCC.TGC.TAG.GCT.CGA | 4179–4199 |
| Biotinylated probe – '$BIO_{ScYLV}$' | ATG.ACT.ACG.TCA.GCT.GAC.CA | 4128–4147 |
| Molecular beacon – '$MB_{ScYLV}$' | FAM.**GCA**.**CCT**.ATG.ACT.TCA.GCT. GAC.CAG.**AGG**.**TGC**.DABCYL | 4128–4148 |

[a]Underlined letters in the NASBA primer sequence correspond to the 5′end T7-RNA polymerase recognition site; bold letters in the molecular beacon sequence correspond to 5′ and 3′ end sequences complementary to each other; FAM: 6-carboxy-fluorescein, and DABCYL: quencher 4-[4′-dimethylaminophenylazo]-benzoic acid. [b]Numbering refers to the corresponding positions of the oligonucleotides in the ScYLV genome (GeneBank accession number: AF157029).

using a ScYLV-specific polyclonal antiserum supplied by Scagliusi.

## Results

*NASBA in a gel-based system.* The NASBA method was first evaluated with Northern blot analysis before testing it in combination with molecular beacons. For determination of the sensitivity of NASBA, dilution series of purified virus were tested. The samples consisted of a 10-fold dilution series ranging between 100 pg and 10 fg of purified virus or total RNA from infected and healthy plants. Amplification of ScYLV RNA by NASBA was performed with primers N1r and N2f (Table 1) enclosing a sequence of 203 nucleotides within the coat protein open reading frame of the virus. The primer N1r has a 3′ end target-complementary sequence and a 5′ end T7-RNA polymerase recognition-site sequence. The NASBA products were analysed by Northern blot and ECL detection with the biotinylated probe $BIO_{ScYLV}$. Results showed the method was highly sensitive for ScYLV, enabling the detection of 10 fg of purified virus (Figure 1A). Tests with dilution series of total RNA from infected plants in total RNA from healthy plants showed that this method permitted the detection of ScYLV even after a 1000-fold dilution of tissue samples (Figure 1B).

*Sensitivity and specificity of the AmpliDet RNA system.* To determine the sensitivity of the NASBA combined with the molecular beacon $MB_{ScYLV}$ in a gel-free system (AmpliDet RNA), the same dilution series of purified virus and total RNA from infected plants was used as described above. The combination of NASBA with the molecular beacon was able to detect 100 fg of purified virus after less than 1 h of amplification (Figure 2A). The system detected at least a 1000-fold dilution of the total RNA from infected plants, and no increase in fluorescence was detected for non-diluted

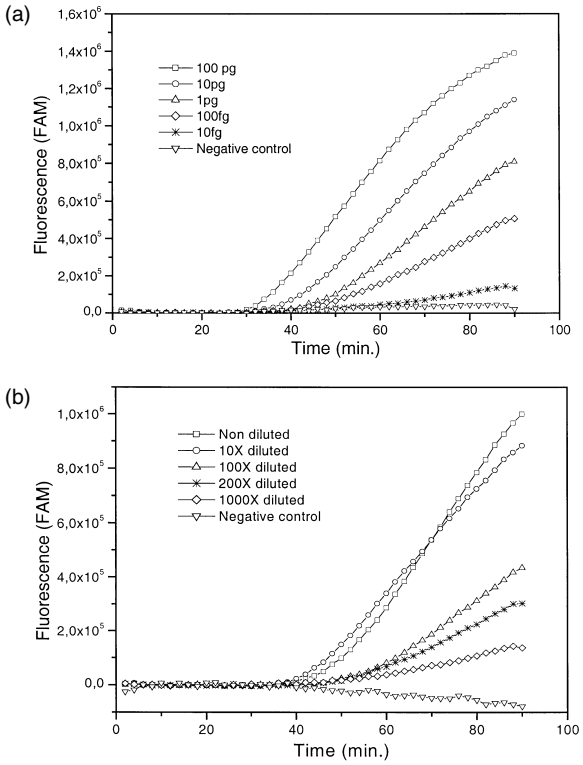


*Figure 2.* Real-time amplification and detection in a gel-free system using the molecular beacon $MB_{ScYLV}$ (AmpliDet RNA). (A) 10-fold dilution series from 100 pg to 10 fg of purified ScYLV; (B) dilution series of total RNA from infected plant. Dilutions in (B) were performed in RNA from non-infected plants. Negative control consisted of total RNA from non-infected plant.

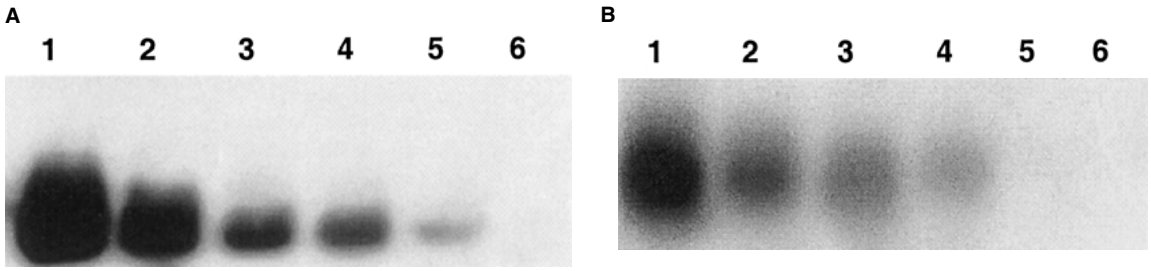


*Figure 1.* Enhanced chemiluminescent detection after Northern blot analysis of the NASBA products using primers N2f and N2r, and $BIO_{ScYLV}$ probe. (A) 10-fold dilution series of purified ScYLV; lane 1: 100 pg; lane 2: 10 pg; lane 3: 1 pg; lane 4: 100 fg; lane 5: 10 fg; lane 6: RNase-free water; (B) dilution series of total RNA of infected plant; lane 1: non-diluted RNA; lane 2: 10-fold dilution; lane 3: 100-fold dilution; lane 4: 1000-fold dilution; lane 5: 10,000-fold dilution; lane 6: total RNA of healthy plant. Dilutions were performed in total RNA of non-infected plants.

total RNA from healthy plants (Figure 2B). The method also detected the virus in fresh extracts from infected plants in sample buffer without previous RNA extraction (data not shown).

To determine the specificity of the molecular beacon to ScYLV, four different members of the *Luteoviridae* were tested. Purified preparations of *Potato leafroll virus* (PLRV), *Beet western yellows virus* (BWYV), *Barley yellow dwarf virus*-PAV (BYDV-PAV) and *Bean leafroll virus* (BLRV) were assayed with the primers and molecular beacon designed on the basis of the ScYLV coat protein-coding sequence. Three microliters of a 10 pg/ml dilution of each purified virus were used as samples in the reaction mix. Three microliters of 10 pg/ml of purified ScYLV and 3 µl of RNase-free water were used as positive and negative controls, respectively. All four luteoviruses (PLRV, BWYV, BYDV-PAV and BLRV) tested negative by AmpliDet RNA, i.e. their level of fluorescence was similar to that of the negative control (RNase free water) whereas a high signal was observed for the positive control, ScYLV RNA. Sequence comparisons of the ScYLV-specific amplification region and primer regions with the viral genome of other members of the *Luteoviridae* did not show any significant homology.

*Detection in viruliferous aphids*. The system was also assessed for the detection of the virus in its aphid vector *M. sacchari*. Adult aphids were transferred to infected sugarcane plants and confined to leaf cages allowing acquisition access periods (AAP) of 12, 24, 48 and 96 h. Since no difference in the level of fluorescence was observed when using hemolymph or RNA extracted from it, all our tests were performed with freshly extracted hemolymph. After 24 h of feeding, aphids started to test positive for ScYLV presenting the fluorescent signal directly proportional to the AAP (Figure 3). Virus was not detected in the hemolymph of aphids with AAPs shorter than 24 h or of aphids fed on non-infected plants (negative control). Hemolymph samples of a single aphid collected in 15 µl of deionised water after more than 48 h AAP tested positive in the AmpliDet RNA system (data not shown).

*Evaluation of the current diagnostic tools for ScYLV.* A comparison of available DAS-ELISA and PCR diagnostics for ScYLV with the ScYLV-specific AmpliDet RNA system was carefully performed. The DAS-ELISA performed with the polyclonal antiserum raised against ScYLV gave the best results when infected

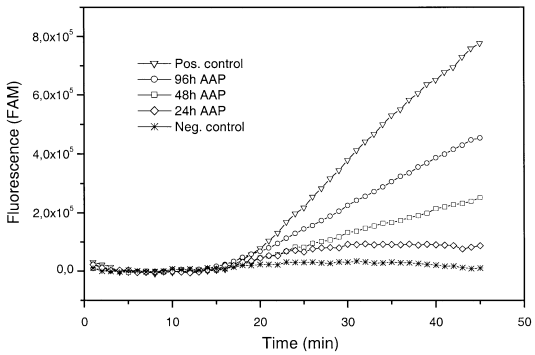


*Figure 3*. AmpliDet RNA of the hemolymph from five aphids *M. sachari* collected in 30 µl of deionised water after 24, 48 and 96 h acquisition access periods in infected plants. Each point represents the mean (±standard deviation) of three samples per acquisition access period. Three microliters of each sample was used in the assays. Positive and negative controls consisted of 3 µl of 10 ng/ml of purified virus and 3 µl of the hemolymph collected in water of five aphids fed in non-infected plants, respectively.

leaf tissue was diluted 1 : 10 (w : v) in 100 mM sample buffer. Immunoglobulin-alkaline phosphatase conjugate was diluted 1 : 500 in sample buffer and the readings were performed after 20 min of incubation with the substrate. Results showed a sensitivity of 1 ng in a 10-fold dilution series of purified virus from 100 ng to 1 pg.

The sensitivity of the RT-PCR for ScYLV was determined after optimisation of the system testing different primers, annealing temperatures, elongation times and DNA polymerases. The best set of primers appeared to be P2r and P1f (Table 1) using the following PCR protocol with AmpliTaq Gold: 10 min 94 °C, 40 cycles of 30 s 94 °C, 1 min 60 °C, 2 min 72 °C and 1 additional extension step of 7 min 72 °C. A band of 450 bp, corresponding to the coat protein of ScYLV, was amplified from purified virus and from total RNA samples from infected leaf tissue (Figure 4). In a dilution series of total RNA from infected plants in total RNA of health plants, the method allowed detection up to 100-fold dilution (Figure 4). The sensitivity of the RT-PCR when using a 10-fold dilution series from 1 ng to 100 fg of purified virus was 1 pg (data not shown).

## Discussion

The use of molecular beacons in virus diagnostics has received attention in the past few years. In this study, the isothermal nucleic acid sequence-based

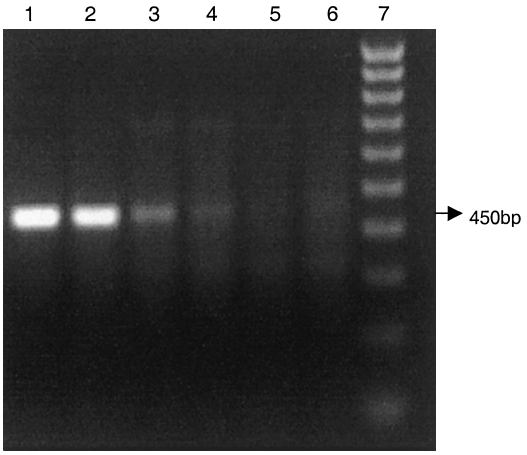


*Figure 4.* Reverse transcription-PCR of ScYLF from total RNA of infected plant. Lane 1: 100 pg of purified virus; lane 2: non-diluted RNA; lane 3: 10-fold dilution of RNA; lane 4: 100-fold dilution of RNA; lane 5: 200-fold dilution of RNA; lane 6: total RNA of healthy plant; lane 7: molecular weight marker. Dilutions were performed in total RNA of non-infected plants.

amplification of RNA was combined with molecular beacons (AmpliDet RNA) in order to improve the detection of ScYLV. The results showed that the AmpliDet RNA developed for ScYLV allows highly sensitive and specific detection of the virus in plant material and in its aphid vectors.

The method shows several advantages when compared to the currently available diagnostic tools (ELISA and RT-PCR). In addition to being more sensitive and specific than these methods, resulting in a more reliable diagnosis, the assay is also easier and faster to perform than RT-PCR analysis. The application of the molecular beacons allows detection in a single tube, without the need of running gels, the use of membranes or image recording. This results in reduction of labour, speeds up analysis, and minimises the risks of contamination and carry-over errors during the process.

The gel-based ScYLV-specific NASBA detected as little as 10 fg of purified virus, and was able to detect it in highly diluted (1000×) total RNA extracts from ScYLV-infected plants. The combination of NASBA with the molecular beacon $MB_{ScYLV}$ only caused a 10-fold loss of sensitivity for purified virus. This indicates that the molecular beacon slightly inhibited the NASBA reaction. However, no sensitivity was lost in combination with the $MB_{ScYLV}$ when analysing total RNA from plants. AmpliDet RNA was 10-fold more sensitive than RT-PCR, which is currently used for ScYLV diagnosis in breeding programmes in Brazil. Moreover, the NASBA system generates single-stranded antisense RNA products (Kievits et al., 1991) enabling the measurement of fluorescence from hybridised molecular beacons (positive reactions) without the need of denaturation. This offers the possibility of using a relatively cheap fluorimeter for real-time or end-point readings instead of an ABI 7700 sequence detector. In case of end-point readings, the isothermal amplification can also be performed in a block heater, eliminating the need of a thermal-cycler. These features enable the application of the method in laboratories with limited resources. The possibility of analysis of a large number of samples (a microtiter plate with 96 wells) and automation of the system makes it suitable for routine diagnostic purposes, like quarantine, certification and breeding programs for sugarcane.

The current method was developed based on the nucleotide sequence of a single Brazilian isolate of ScYLV (Maia et al., 2000). Although primers and probes were designed in the CP gene, highly conserved among members of the *Luteoviridae*, it cannot be ruled out that some variability could be encountered in different virus isolates. Additional testing of the system with infected material and purified virus is recommended before establishing the method as a detection tool for the purposes mentioned above. The AmpliDet RNA system was evaluated against different conditions and different virus sources, such as highly diluted virus purification, plant tissue and viruliferous aphids. The dilution of total RNA from infected sugarcane in total RNA of non-infected plants indicates that virus detection is possible in field-grown plants, under low pressure of inoculum and low levels of natural virus infection. The detection of the virus in the hemolymph of aphids just after an AAP of 24 h offers the possibility of assessing the viruliferous nature of aphids from the field during the growing season of sugarcane, allowing monitoring the spread of the disease. Virus detection in crude extracts of infected plants without RNA extraction and in the hemolymph of a single aphid after 48 h feeding on an infected plant reinforces the robustness and reliability of the method. Therefore, this system offers an excellent tool for screening and monitoring plant material from the field and for studying the epidemiolology of this relatively new disease. Furthermore, the system can be developed into a multiplex real-time assay using differently coloured molecular beacons (Klerks et al., 2001a,c; Szemes et al., 2002) for simultaneous screening of ScYLV and *Sugarcane mosaic virus* (SCMV), another world-wide distributed potyvirus infecting sugarcane.

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