

THE INTRODUCTION OF A SEROLOGICAL METHOD FOR LARGE SCALE DIAGNOSIS OF RATOON STUNTING DISEASE IN THE SOUTH AFRICAN SUGAR INDUSTRY

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Abstract

Ratoon stunting disease (RSD), caused by the bacterium *Clavibacter xyli* subsp *xyli*, is widely distributed in the South African sugar industry and, if common, severely reduces the yield of most sugarcane varieties under both rainfed and irrigated conditions. It is important that intended seedcane sources and plough-out fields are checked for freedom from RSD before fields are planted.

Phase contrast microscopy (PCM) has been used in the South African sugar industry to diagnose RSD on a large scale since 1977. The diagnostic service offered to cane growers has provided valuable information on the RSD situation in the sugar industry and has contributed greatly to success in reducing the levels of the disease in most areas. PCM is a rapid and acceptably reliable method of diagnosis but it requires the urgent transportation of bulky stalk samples, often from distant parts of the industry, for examination in laboratories at the South African Sugar Association Experiment Station (SASEX).

An evaporative binding-enzyme immunoassay (EB-EIA) replaced PCM in April 1998. This method of diagnosis has a number of advantages over PCM. The main one is that extracted xylem sap is transported to SASEX in small tubes when convenient. Additionally, the test is less subjective than PCM and more samples can be processed at a lower cost. A number of large scale comparisons between PCM and EB-EIA using samples from growers' fields have shown that the two methods are similar in sensitivity, and in the last comparison there was 97% agreement.

Staff of Local Pest & Disease Control Committees (LP&DCCs) are now closely involved in RSD diagnosis. Stalk samples are delivered to the local offices of LP&DCCs, where technical staff perform the initial extraction of sap and forward these samples to SASEX for serological diagnosis. In the first year of implementation of EB-EIA, the number of samples tested increased from approximately 6 000 to more than 9 000.

Keywords: Sugarcane, ratoon stunting disease, diagnosis, serology

Introduction

Ratoon stunting disease (RSD) is caused by the xylem-limited bacterium, *Clavibacter xyli* subsp *xyli* (*Cxx*) (Davis *et al.*, 1984). It is generally considered to cause greater losses in yield world-wide than any other sugarcane disease. The extent of yield loss varies with environmental conditions and the susceptibility of the varieties grown. In South Africa, losses as high as 30 to 40% have been recorded in susceptible varieties grown under typical rainfed and irrigated conditions (Bailey and Bechet, 1986; 1995).

RSD causes no obvious external symptoms other than a non-specific stunting and is therefore difficult to diagnose with accuracy in the field. Up to the mid-1970s diagnosis was based on the observation of internal stalk symptoms, but these vary both between and within varieties, are often inconspicuous and are unreliable. A bacterium was first found to be associated with RSD in 1972 (Gillaspie *et al.*, 1973) and this soon led to diagnosis based on the microscopic examination of the xylem vessel contents. This method of diagnosis was adopted in the South African sugar industry in 1976 (Bailey, 1976) and in 1977 a large-scale diagnostic service for the sugar industry was introduced using phase contrast microscopy (PCM) to detect *Cxx* (Bailey and Fox, 1984). The diagnostic service has provided valuable information on the RSD situation in the South African and neighbouring sugar industries. It has also led to an increased awareness of the disease and this has been an important factor in reducing the incidence of RSD in most parts of the South African industry (Bailey and Tough, 1991).

Although PCM is an acceptably reliable and rapid method of diagnosis, it has some disadvantages. In the South African sugar industry, bulky samples of stalks have to be transported, often over long distances, to SASEX laboratories at Mount Edgecombe or Pongola. The samples need to be processed within 24 hours of collection in the field to ensure reliable results (Bailey and Fox, 1984). Additionally, microscope diagnosis requires skilled technicians and is time consuming. The number of samples processed per day is therefore limited and the results can be subjective.

A serological test, the evaporative binding-enzyme immunoassay (EB-EIA), replaced PCM for the routine diagnosis of RSD in the South African sugar industry in April

1998. This method, which has a number of advantages over PCM, was adapted from a similar method developed in the Australian sugar industry, where it has been used successfully since 1994 (Croft *et al.*, 1994). In the application of EB-EIA in South Africa, samples of sugarcane stalks from growers are delivered to the offices of Local Pest & Disease Control Committees (LP&DCCs), where the first step in processing, the extraction of xylem sap from stalks, is conducted. The samples of sap are then transported to SASEX, Mount Edgecombe, where serological testing is conducted. Using EB-EIA, more samples can be examined daily, making the test cheaper than PCM, and the test is less subjective.

The introduction and operation of EB-EIA for the diagnosis of RSD in the South African sugar industry and the results of comparisons between PCM and EB-EIA are discussed.

Materials and methods

Antisera

Purified immunoglobulin G (IgG) to *Cxx* was prepared from rabbit antiserum using whole cell suspensions of the bacterium as the antigen, according to the method of Ball *et al.* (1990). The second antibody used was goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich, St Louis, USA).

Sample collection for direct comparisons

In 1997, before the introduction of EB-EIA, two comparative surveys were conducted in Umfolozi and one in Mpumalanga. Towards the end of 1998, after the introduction of the EB-EIA into large scale use, further surveys were conducted in the South and North Coast extension areas and again at Umfolozi.

Samples consisting of 20 stalks per field were collected from poorly grown stools in the commercial cane fields used for the comparisons. The lowest undamaged internode was cut from each stalk. Each of these stalk pieces was placed on a moulded resin adaptor and the xylem sap was expressed using low pressure compressed air. The sap was collected with a disposable pipette and a drop was placed on a microscope slide for testing by PCM. The remainder of the sap was placed in a Titertube microtest tube (Bio-Rad, Hercules, USA). At first, the pipettes were rinsed in distilled, sterile water between stalks, but this step was discarded shortly after the introduction of EB-EIA into routine use. The sap

from five stalks was pooled in one tube; therefore four tubes represented the 20-stalk sample from one field. When sap extraction and collection was complete, the tubes were capped and frozen. The microscope slides were examined using PCM and positive results were confirmed at SASEX by immunofluorescence microscopy (IFM), a highly sensitive method of RSD diagnosis (Harris and Gillaspie, 1978).

EB-EIA

The EB-EIA was conducted according to the method outlined by Croft *et al.* (1994). Briefly, 200 µl of sap was transferred from each tube to a 96-well microtitre plate (Sero-Wel, Sterilin). The plates were centrifuged at 3 000 rpm for 20 minutes and dried at 37°C overnight. Plates were washed with phosphate-buffered saline-tween (PBS-T) and blocked with skimmed milk. The plates were incubated with *Cxx*-specific IgG for one hour, washed with PBS-T and incubated for a further hour with anti-rabbit alkaline phosphatase conjugate. The plates were again washed and substrate buffer containing 4-nitrophenyl phosphate was added. The absorbance was measured at 405 nm with a Bio-Rad 550 Microplate Reader (Bio-Rad, Hercules, USA) immediately after addition of the substrate buffer and again after a four hour incubation period. Samples were considered positive if absorbance readings were higher than 0,15 after four hours.

Introduction of EB-EIA into the sugar industry

Each of the 13 LP&DCC offices in the sugar industry was equipped to extract the xylem sap from the samples. Basic equipment consisted of an electric powered, compressed air pump, a custom-made moulded resin adaptor, pipettes and racks of tubes for extracted sap.

Samples of stalks were collected from growers' fields by LP&DCC survey teams and transported to the local LP&DCC office, where the sap was extracted, pipetted into tubes and frozen. The racks of tubes were chilled or frozen and delivered to SASEX, Mount Edgecombe, in insulated boxes, where the samples were processed using EB-EIA.

Results and discussion

Results of surveys conducted in 1997

The incidence of RSD in the Umfolozi area was high in both surveys, with RSD being found in more than 40% of the fields sampled (Table 1). In the first Umfolozi survey, RSD

Table 1. Observed frequency of a possible combination of results using two methods of RSD diagnosis in a first series of surveys conducted in 1997.

| Possible combination of results | | Observed frequency (%) | | | Total (%) |
|---------------------------------|--------|------------------------|------------|------------|-----------|
| PCM | EB-EIA | Umfolozi 1 | Mpumalanga | Umfolozi 2 | |
| -ve | -ve | 46 | 69 (80) | 47 | 162 (57) |
| +ve | +ve | 42 | 9 (11) | 46 | 97 (34) |
| +ve | -ve | 1 | 3 (3) | 3 | 7 (3) |
| -ve | +ve | 11 | 5 (6) | 4 | 20 (7) |
| Total samples | | 100 | 86 | 100 | 286 |
| % complete agreement | | 88 | 91 | 93 | 91 |

was detected by EB-EIA in 11 samples that were found negative by PCM. This may have resulted in part from the slightly improved sensitivity of the test, but contamination during sample collection was suspected. Thereafter, a fresh pipette was used to collect sap from each sample of 20 stalks.

The incidence of RSD in the Mpumalanga area was lower, with less than 20% of the fields tested being found to be infected using both methods of diagnosis. EB-EIA did not detect RSD in three samples found by PCM to be positive.

Sap was first pipetted onto a microscope slide and the remainder was placed into the tubes. The sap was often difficult to express and at times very little sap was available for the EB-EIA, because of hot, dry weather conditions. This may explain the false negative results obtained with the EB-EIA. Under normal circumstances, when sap is collected for the immunoassay only, this would be less of a problem and there would be more chance of detecting RSD in slightly infected samples. Three false negatives were recorded using EB-EIA, and RSD was detected in four samples found by PCM to be negative in the last survey conducted in Umfolozi.

The agreement in the results between EB-EIA and PCM improved with each survey conducted, and reached 93% agreement in the third survey. This level of agreement was also achieved by Croft *et al.* (1994) before they introduced the system into the Australian sugar industry.

Introduction of the new system into the SA sugar industry

LP&DCC teams began collecting samples for diagnosis by EB-EIA in April 1998. The first samples were received from the Midlands South extension area. By August 1998, 3 977 samples from all areas had been processed. From the results of these samples it appeared that the incidence of RSD had

increased substantially in a number of areas since the introduction of the new system (Figure 1).

The slightly increased sensitivity of EB-EIA was considered unlikely to have resulted in appreciably more fields being RSD-positive, and contamination during sample collection was suspected. An investigation showed that serious contamination could occur if pipettes were not discarded after each sample and if the water used for rinsing the pipettes after collecting sap from each stalk was not renewed. An aerosol of infected sap, which is sometimes produced when blowing the sap from stalk pieces, might also contaminate the racks of sample tubes, if these were too close to the compressed air, sap extraction procedure. The pipette-rinsing step was therefore omitted and all LP&DCC technicians were advised on methods of preventing contamination. Further surveys to compare PCM and EB-EIA were then conducted.

Results of surveys conducted in 1998

The incidence of RSD in the test samples from the Umfolozi area in 1998 was lower than in the two surveys conducted in 1997. In the third survey of the Umfolozi area, RSD was detected in 57 (29%) of the 198 samples examined by PCM and 58 (29%) by EB-EIA. Of the 198 comparisons between the two methods, nine did not match (Table 2). The PCM-positive samples that were not detected by EB-EIA were only slightly infected, with one stalk in each 20-stalk sample being found by PCM to be RSD-positive and having low bacterial populations. EB-EIA detected RSD in five samples found by PCM to be negative, and this was confirmed by IFM.

In the South Coast survey, 68 of the 129 fields or sections of fields had been sampled previously and tested by EB-EIA in April 1998. The previous results of 46 of these samples (68%) were confirmed. Most of the samples that were

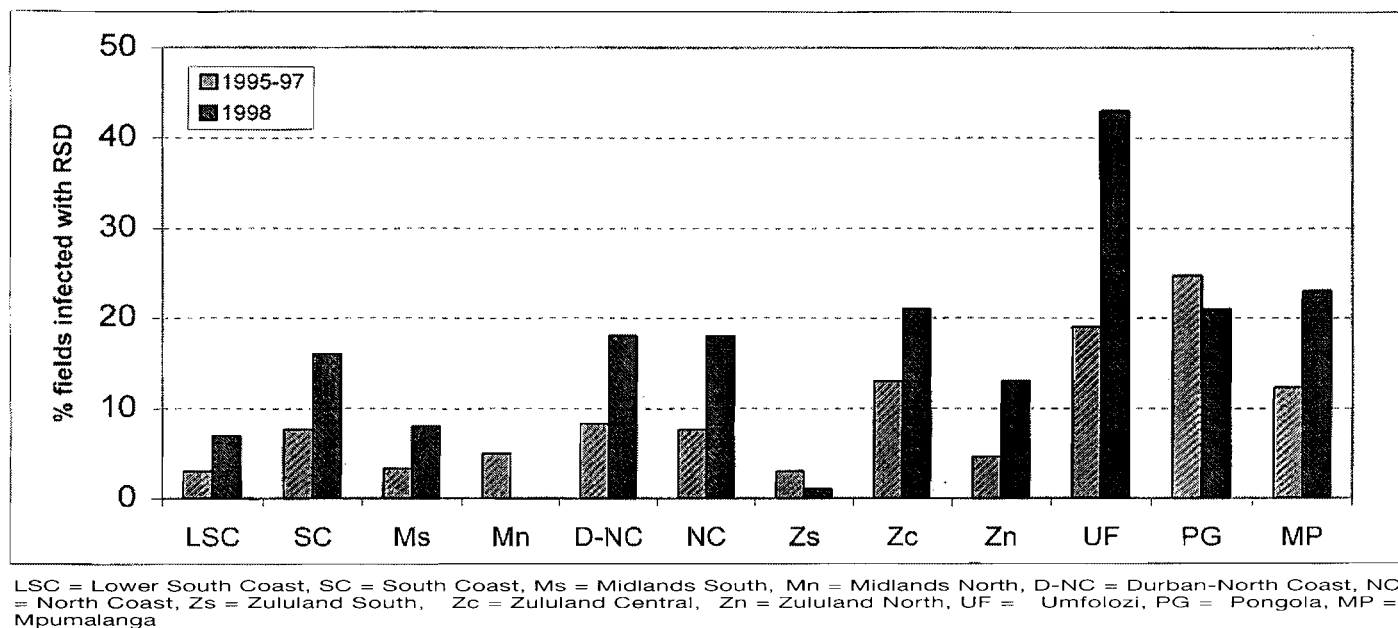


Figure 1. Incidence of RSD in different extension areas of the South African sugar industry, 1995-97 and January to August 1998.

Table 2. Observed frequency of a possible combination of results using two methods of RSD diagnosis in a second series of surveys conducted in 1998.

| Possible combination of results | | Observed frequency (%) | | | Total (%) |
|---------------------------------|--------|------------------------|-------------|-------------|-----------|
| PCM | EB-EIA | Umfolozi 3 | South Coast | North Coast | |
| -ve | -ve | 136 (69) | 82 (64) | 124 (80) | 342 (71) |
| +ve | +ve | 53 (27) | 35 (27) | 27 (17) | 115 (24) |
| +ve | -ve | 4 (2) | 2 (2) | 4 (3) | 10 (2) |
| -ve | +ve | 5 (3) | 10 (8) | 0 | 15 (3) |
| Total samples | | 198 | 129 | 155 | 482 |
| % complete agreement | | 96 | 91 | 97 | 95 |

repeated were from fields that had previously been found to be positive. This explains the high incidence of RSD recorded during the survey, with 38 (29%) and 46 (35%) of the fields testing positive with PCM and EB-EIA respectively.

In the South Coast survey, RSD was detected by EB-EIA in 10 samples found by PCM to be negative. When checked by IFM, mostly low concentrations of bacteria were found in the tubes, and these may have been too low to be detected by PCM. Differences in results may have arisen where populations of *Cxx* were very low in the stalks and were not detected by PCM, but by pooling the sap and later centrifuging the samples, the concentrations may have been increased to a level detectable by EB-EIA.

Some larger fields in the South Coast area that had been tested by EB-EIA in April 1998 were divided into blocks for sampling for the comparative survey. Occasionally during the survey, only one sample from a sub-divided field was found to be infected. This confirms that a 20-stalk sample may be insufficient to provide reliable diagnosis if RSD levels are low, as previously reported (Bailey and Fox, 1984). For this reason and due to the increased capacity of the new system, it is now recommended that samples consisting of 40 stalks are taken when sampling intended seedcane sources.

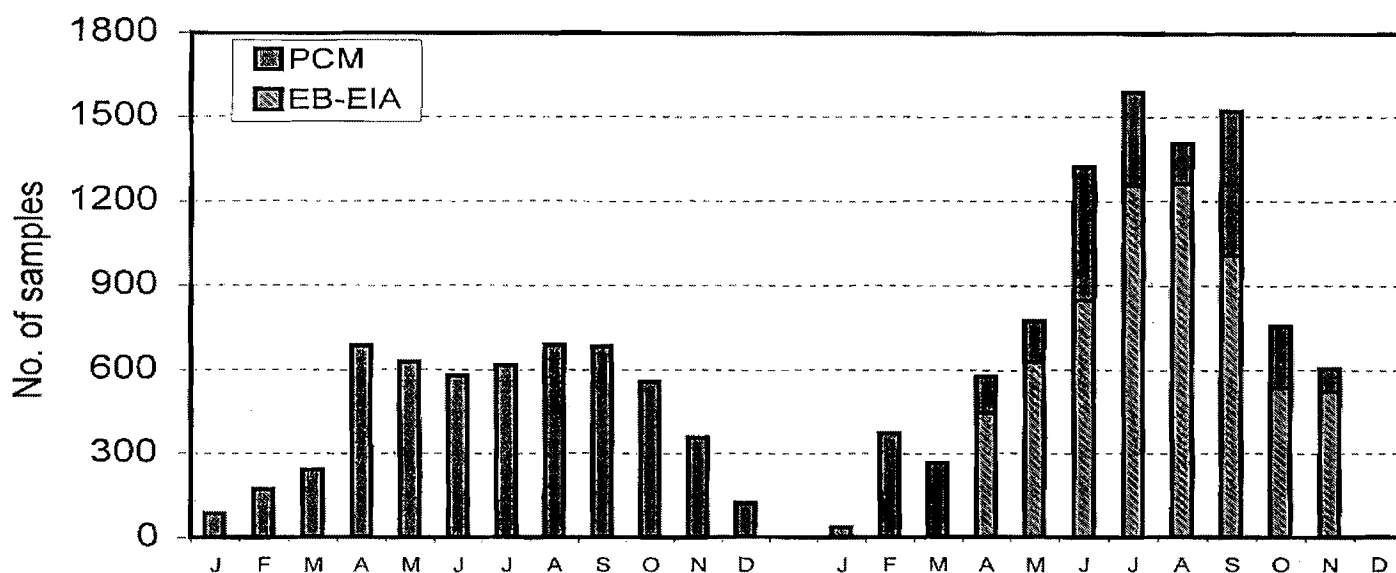
PCM detected RSD in four more samples than EB-EIA in the

North Coast survey. These discrepancies arose when only one stalk in the sample was infected and had low xylem populations of *Cxx*. By pooling the sap from five stalks in one tube, these low populations may have been further reduced to a level that was not detectable by EB-EIA.

There was good agreement between EB-EIA and PCM in the third Umfolozi and North Coast surveys, and the mean degree of agreement for the three comparative surveys conducted in 1998 was 95% (Table 2).

A total of 9 215 samples was processed in 1998 using EB-EIA and PCM, compared with 6 110 samples in 1997 using PCM only (Figure 2). This increased capacity is one of the important advantages of the new system. The maximum capacity of the diagnostic service using EB-EIA is approximately 160, 20-stalk samples per day. Thus, approximately 3 200 samples per month can be processed with the current equipment and personnel (three dedicated technical staff and two assistants at SASEX).

EB-EIA as used in the South African sugar industry has been found to be slightly more sensitive than PCM, detecting 6×10^5 bacterial cells/ml, compared with 1×10^6 cells/ml for PCM. This is similar to results from the Australian sugar industry, where EB-EIA has been reported to detect levels of 5×10^5 cells/ml (Leaman *et al.*, 1991).

**Figure 2. Number of RSD samples (fields) processed in 1997 (PCM) and 1998 (PCM and EB-EIA) in the South African sugar industry.**

Conclusions

The direct comparisons indicated that the results of PCM and EB-EIA diagnosis were not always in complete agreement, but this occurred only when levels of bacteria were very low. However, after possible contamination during initial sample preparation was eliminated, the agreement between the two methods of diagnosis, more than 95%, was acceptable.

For serological diagnosis to work effectively and efficiently, the following quality control procedures have been implemented:

- The microscopic examination of all samples found positive by EB-EIA, to ensure the presence of *Cxx*.
- The storage of all samples for one month in the event of queries.
- Periodic visits by SASEX specialists to LP&DCC offices to guide technicians in the initial preparation of samples.
- Periodic surveys conducted in selected areas to compare PCM and EB-EIA.

With strict quality control, EB-EIA can provide the industry with a more efficient and accurate method of RSD diagnosis for large scale use.

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DEVELOPMENT OF A DNA-BASED DIAGNOSTIC METHOD TO DETECT SUGARCANE BACTERIAL PATHOGENS WITH EMPHASIS ON *CLAVIBACTER XYLI* SUBSP. *XYLI*

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Abstract

A polymerase chain reaction (PCR) protocol was developed that specifically detects *Clavibacter xyli* subsp. *xyli* (*Cxx*), the causal agent of ratoon stunting disease of sugarcane. Two primers were developed, namely T₂ and T₄, to amplify the intergenic spacer region of 16S-23S ribosomal DNA of probably all sugarcane bacterial pathogens, including *Cxx*, *Xanthomonas campestris* pv *vasculorum*, the causal agent of gumming disease and *X. albilineans*, the causal agent of leaf scald. The advantages of this approach are that one set of primers can be used for the detection of several pathogens, and that the main pathogens present can be identified based on the length of the amplified fragment. Alternatively, the amplified fragment can be recovered and the precise sequence can be used for identification. The method can also be used to demonstrate mixed infections.

Based on a multiple sequence alignment, two *Cxx*-specific primers (T₈ and T₉) were designed. These primed the specific amplification of a 237 bp DNA product from genomic DNA samples of *Cxx*. The 237 bp product was also amplified directly from cultured *Cxx* cells and from *Cxx*-infected sugarcane xylem sap. For the amplification of *Cxx* in infected sugarcane xylem sap, 0.8% (w/v) polyvinylpyrrolidone was added to the sap prior to amplification. This provides a quick diagnostic method for the detection of *Cxx* in xylem sap without prior extraction of DNA.

Introduction

Sugarcane is susceptible to a wide range of diseases caused by various fungi, bacteria, viruses and phytoplasmas, many of which can cause substantial reductions in yield. Diseases also have a great impact on sugarcane breeding programmes. Every year approximately 40% of new genotypes are discarded in the third selection stage at the South African Sugar Association Experiment Station (SASEX) because of susceptibility to diseases, and a further 40% are discarded in the fourth selection stage. Latent or symptomless infections may allow the build-up and spread of diseases. The ability to diagnose a wide range of pathogens with sensitive and reliable methods, even in apparently symptomless plants, is essential. Diagnostic methods should provide a combination of sensitivity, speed, reliability and high output to serve as

useful diagnostic tests for pathogens.

Ratoon stunting disease (RSD), leaf scald and gumming are three of the most economically important diseases of sugarcane world-wide. RSD is caused by the bacterium *Clavibacter xyli* subsp. *xyli* (*Cxx*) (Davis *et al.*, 1984), while gumming and leaf scald are caused by *Xanthomonas campestris* pv *vasculorum* (*Xcv*) (Cobb, 1983; Dye, 1978, as recorded by Ricaud and Autrey, 1989), and *Xanthomonas albilineans* (*Xa*) (Ashby, 1929; Dowson, 1943, as recorded by Ricaud and Ryan, 1989). Diagnosis of RSD is difficult because of the absence of specific symptoms. Phase contrast microscopy (PCM) and serologically based detection methods including immunofluorescence microscopy (IFM) and the evaporative binding enzyme immunoassay (EB-EIA) have so far been used for diagnosis at SASEX. These techniques allow a definite positive diagnosis, but negative results from relatively small samples of stalks do not guarantee the absence of the disease in the field. Until recently, diagnosis of leaf scald and gumming at SASEX was based on visual symptoms and culturing, after which the Gram stain and several tests including gelatine liquefaction, starch hydrolysis, nitrate reduction and carbohydrate tests were conducted. Although this technique was useful, it took two to three weeks to complete the diagnosis.

The polymerase chain reaction (PCR) has become popular for detecting and identifying pathogens. PCR protocols have been developed for other sugarcane pathogens, including Fiji disease virus and sugarcane mosaic virus (Smith *et al.*, 1994), and *Xa* (Honeycutt *et al.*, 1995). For diagnosis of bacterial pathogens the variable regions within the ribosomal RNA (rRNA) operons have been used for the selection of PCR oligonucleotide primers for a range of species. Intra-species 16S RNA gene variation is small for most described bacterial pathogens but the intergenic spacer region between the 16S and 23S genes (Figure 1) is sufficiently variable for species-specific primers to be designed (Barry *et al.*, 1991).

Presently little is known about strain variation in *Cxx*, *Xcv* and *Xa* in South Africa. Information on this can be obtained by analysis of the 16S-23S spacer region sequences.

The purpose of this project was to develop a set of primers to permit the sensitive and accurate identification of bacterial pathogens in sugarcane. It was hypothesised that if a set of

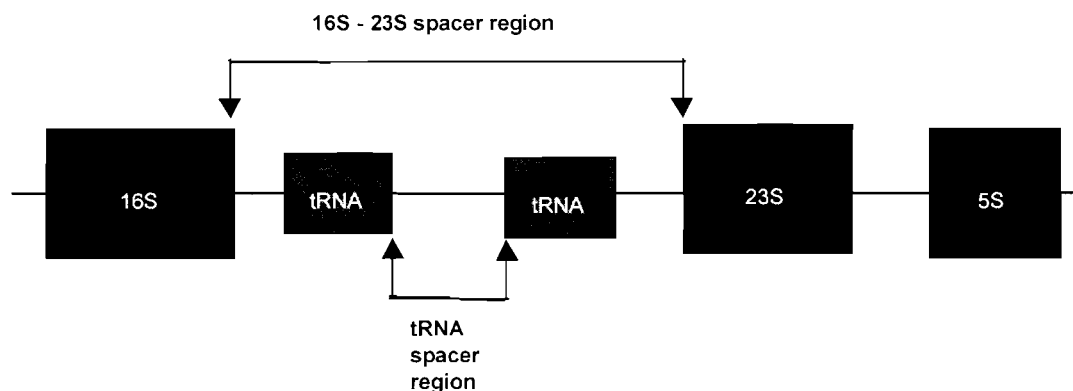


Figure 1. Generalised illustration of the ribosomal genes situated on the rRNA operon.

primers could be developed that would allow the amplification of any sugarcane bacterial pathogen, specific primers could be designed for the identification of different species. A set of primers targeting the highly variable spacer region between the 16S and 23S ribosomal RNA operon of bacteria was developed for this purpose. After aligning 16S-23S intergenic spacer region sequences, a set of highly specific primers was developed for the detection of *Cxx*.

Materials and Methods

Bacterial strains and culture conditions

Cxx isolates were maintained on a sugarcane medium (MSCm) (Davis *et al.*, 1980), modified by Brumbley (personal communication¹). The cultures were incubated for 3-4 weeks at 24°C. Plants expressing gumming symptoms were collected from two areas where the disease is common, Eston in the KwaZulu-Natal Midlands and Pongola in northern KwaZulu-Natal. Plants showing leaf scald symptoms were also collected from Pongola. The cultures were maintained on Wilbrink's agar (Ricaud and Autrey, 1989) at 30°C. Growth occurred within three days and cells were subcultured every week for a maximum of 3 weeks.

Genomic DNA extraction

Genomic DNA was extracted from bacterial cultures as described by van Antwerpen (1999). Bacterial cultures of *Cxx*, *Xa* and *Xcv* were also used directly in PCR. The colonies were scraped from the agar surface and suspended in autoclaved filter-purified water, and 0.5 µl was used as template in PCR reactions depending on the concentration of the bacterial cells (Hiraishi, 1992).

Primer design, DNA amplification and sequencing of the spacer region between the 16S and 23S rRNA genes

Primer pair T₂ forward (5'TAGGACTAAGTCGTAA-CAAGG3') and T₄ reverse (5'CIITGCCAAGGCATC-CACC3') was used to amplify the intergenic spacer region between the 16S and 23S rRNA genes of all the bacterial

species used in this study and was designed by aligning known bacterial sequences obtained from the Genbank database. DNA fragments obtained with primer pair T₂ and T₄ were amplified and the cycling parameters used were as described by van Antwerpen (1999). The PCR products were resolved in a 2% (w/v) agarose gel and visualised under UV light. PCR products were purified from the agarose gel using a Qiagen QIAquick Gel Extraction Kit after electrophoresis. The purified DNA served as template for sequencing reactions.

Sequencing of 16S/23S amplified fragments

PCR-based sequencing reactions were carried out in a thermal cycler (Touchdown temperature cycler; HYBAID, Teddington, England) with ABI PRISM™ dye terminator cycle sequencing using the Perkin Elmer Ready Reaction kit with AmpliTaq R DNA Polymerase. DNA was amplified and the cycling parameters were used as described by van Antwerpen (1999). The extension products were precipitated, denatured and transferred to an autosampler tube. The samples were loaded in the ABI Prism Genetic Analyser for analysis.

Alignment of sequences and development of *Cxx*-specific primers

Sequences obtained were compared with sequences in the Genbank sequence databank for identification. Sequences for each bacterial species were compared to determine whether different strains of the species exist and a consensus sequence for each species was determined. The consensus sequences of all species were aligned and compared with each other by using the DNASIS computer program version 2.1 (Hitachi Software Engineering Co Ltd, 1994). A pair of *Cxx*-specific primers, namely T₈ forward (5'TTGTCCAGGCGCCGGATCTGAGACAGTACT3') and T₉ reverse (5'TGCTCGGTCCACT3') were designed.

Specific amplification of *Cxx*

Amplification reactions were performed in a 15 µl reaction volume for primer pair T₈ and T₉ as described by van Antwerpen (1999). A reaction mixture with sterile filter-purified water served as the negative control. Cycling parameters were used as described by van Antwerpen

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(1999). The specificity of primer pair T_8 and T_9 was tested using genomic DNA from 41 bacterial isolates from sugarcane, including *Xcv* and *Xa*. A range of DNA concentrations containing 5, 50, 100 and 200 ng of bacterial genomic DNA were used as template in PCR. These primers were also tested on xylem sap extracted directly from stalks infected with *Cxx*, *Xa* and *Xcv*. Ten-fold serial dilutions were used as template in the PCR.

Sensitivity of the Cxx-specific PCR test

The sensitivity of PCR using the *Cxx*-specific primer pair T_8 and T_9 was determined by using a dilution series of the bacterial DNA (50 ng, 5 ng, 0,5 ng, 50 pg, 5 pg and 0,5 pg) and bacterial cells diluted in water, using a ten-fold dilution series from 10^1 to 10^6 . Purified genomic DNA from *Cxx* was mixed with an excess of 60 ng sugarcane genomic DNA: 50 ng, 5 ng, 0,5 ng, 50 pg, 5 pg and 0,5 pg of *Cxx*.

Detection of Cxx in sugarcane xylem sap

Cxx cells were diluted in healthy xylem sap using a ten-fold dilution series from 10^1 to 10^6 . The xylem sap was used as template for the PCR test using the *Cxx*-specific primers under the conditions described above. However, to increase the sensitivity of the test, samples of xylem sap containing 0,8% (w/v) polyvinylpyrrolidone (PVP) were tested against xylem sap samples without PVP.

The efficacy of the PCR protocol to detect *Cxx* in sugarcane xylem sap was compared with IF, PCM and EB-EIA. Nine hundred and sixty-eight sugarcane stalks were collected from fields in different areas of the South African sugar industry. Xylem sap was extracted from stalk pieces using positive pressure and the sap from five stalks was pooled in one tube. A 0,5 μ l aliquot from each tube was then used as template in the DNA amplification reactions.

Results

Fragments obtained with primer pair T_2 / T_4

The total number of base pairs for the sequenced product from *Cxx* was 611 bp, of which 143 were adenosine, 149 cytosine, 169 guanosine and 150 thymine, with a GC content of 51,96%. When amplifying DNA from *Xcv*, different sized fragments ranging from 586 bp to 623 bp were amplified from different isolates. Three types of sequences were obtained for *Xcv*. These sequences were compared with known sequences on the Genbank database and it was confirmed that they belonged to the *X. campestris* group of pathogens. Two types of sequences were obtained for *Xa*.

Selection of Cxx-specific primers

Sequences obtained from fragments amplified from *Cxx* genomic DNA using primer pair T_2 and T_4 were aligned and compared with each other in order to design a primer pair (T_8 / T_9) specifically for *Cxx*.

Specificity of primer pair T_8 and T_9

Primers T_8 and T_9 primed the amplification of a 237 bp DNA

product from genomic DNA samples of all the different *Cxx* isolates used. No amplification products were observed in 41 other bacterial isolates, including *Xanthomonas* and *Pseudomonas* species from sugarcane. The specificity of primer pair T_8 and T_9 was also tested using genomic DNA from *C. xyli* subsp. *cynodontis* (*Cxc*), *C. michiganense* subsp. *michiganense* (*Cmm*), *C. michiganense* subsp. *insidiosum* (*Cmi*), and *C. michiganense* subsp. *nebraskense* (*Cmn*). DNA from *Cxc* was amplified and resulted in a 237 bp fragment. No amplification products were obtained from the other *Clavibacter* species used. The T_8 and T_9 primers could therefore distinguish between all the *Clavibacter* species in this study, except *Cxc*.

Detection limits of the primer pair T_8 / T_9 in the PCR diagnostic test

Cxx cells from a pure broth culture were centrifuged and diluted in a ten-fold dilution series in water and used in PCR to determine the sensitivity of the primers. In this test, the primers could detect the bacteria in dilutions of up to one million-fold when 10 μ l was used as template in the PCR. Dilutions of the genomic DNA of *Cxx* were also used to determine the sensitivity of the primers in the PCR. The *Cxx*-specific primers could amplify the DNA from a concentration as low as 50 pg, and when *Cxx* DNA was mixed with 60 ng of healthy plant DNA, the primers detected the *Cxx* DNA at a concentration as low as 0,5 ng.

Detection of Cxx in sugarcane xylem sap

As mentioned above, primer pair T_8 and T_9 primed the amplification of the 237 bp DNA product directly from cultured cells and also from xylem sap from *Cxx*-infected stalks. To increase the sensitivity of the test, 0,8% (w/v) polyvinylpyrrolidone (PVP) was added to the xylem sap (Pan *et al.*, 1998).

Comparison between PCR and other diagnostic tests

The efficacy of the PCR protocol to detect *Cxx* in sugarcane xylem sap was compared with other diagnostic tests used at SASEX. In a first test, EB-EIA, IFM and PCM were compared with PCR. From a total of 91 samples, PCR detected 12 positive samples, IFM detected 15 positive samples and PCM 10 positive samples. Only four samples tested positive by all four methods. PCR did not detect *Cxx* in nine samples that were found to be positive using the other detection methods. EB-EIA did not detect seven positive samples; IFM did not detect one positive sample and PCM did not detect 10 of the samples that had been identified as positive by the other methods. From these results it was apparent that PCR was not highly sensitive.

In a second comparison, attempts were made to increase the sensitivity of PCR by freezing and thawing, varying the volume of template and varying the addition of PVP to the template. In this second test PCR was compared with EB-EIA.

A total of 151 samples were tested in the second comparison, 88 of which came from Swaziland and 63 from Umfolozi. EB-EIA detected four positives in the Swaziland samples,

while PCR detected none. Immunofluorescence microscopy confirmed that the four diagnosed positive by EB-EIA were positive. The xylem sap of these samples was a brownish colour, indicating the presence of substances that might have suppressed the PCR amplification of *Cxx*. In the 63 samples from Umfolozi, PCR detected 24 positive samples, whereas EB-EIA detected 21 positives. The minigel (Southern Cross Biotechnologies), in which 96 samples can be run simultaneously, is shown in Figure 2.

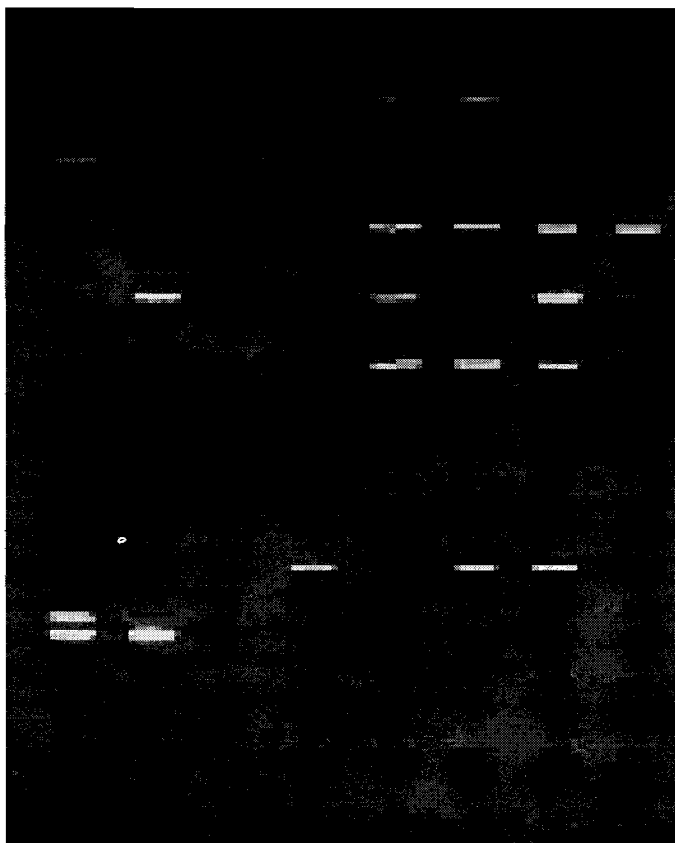


Figure 2. PCR amplification products of *Cxx* cells in xylem sap using primer pair T8 and T9 in the 96-well minigel system. Empty lanes represent negative results.

Discussion

The direct loss in income due to the incidence of diseases in the South African sugar industry is estimated to be approximately R120 million per annum (personal communication²). However, this value does not include indirect losses resulting from the rejection of high yielding varieties due to disease susceptibility. More than 60% of promising varieties are discarded from the variety selection programme at SASEX, some of which might otherwise have high yielding potential.

RSD and leaf scald are often difficult to detect due to a lack of diagnostic symptoms or latency. There are no methods

available for the chemical control of bacterial diseases in sugarcane, and the production of healthy planting material under sanitary conditions is one of the most important control measures. Currently, PCM and the EB-EIA test are used to monitor the RSD situation in the sugar industry and ensure the health of seedcane stocks. PCR-based detection methods specific for *Cxx* and other bacterial pathogens would assist in the monitoring and prevention of spread of diseases. PCR can also be used to determine whether different strains of bacterial pathogens exist.

Target site used for PCR amplification in this study

The intergenic spacer region between the 16S and 23S rRNA genes has been considered an ideal region for developing specific PCR primers that can differentiate bacteria, because a high percentage of sequence variation occurs in this region (Li and De Boer, 1995). This area of the prokaryotic genome is also easily accessible because of the conserved nature of the surrounding 16S and 23S rRNA genes (Barry *et al.*, 1991).

Variation between bacterial species and strains

The T₂ and T₄ primers that were developed for the amplification of sugarcane bacterial pathogens successfully amplified the 16S-23S spacer regions of *Cxx*, *Xcv* and *Xa*. The advantages of this approach are that one set of primers can be used for the identification of bacterial pathogens by recovering and sequencing of the amplified fragment.

Sequences obtained with primers pair T₂/T₄

The size of the amplified fragment, when using *Cxx* genomic DNA and primer pair T₂/T₄, was 611 bp. This differs from the size of the fragments obtained by Pan *et al.* (1998); 566 bp for *Cxx* and 546 bp for *Cxc*, using primer pair G1/L1. This difference might be because the South African isolates differed from those used by Pan *et al.* (1998), and also because of the difference in the length of the amplified fragments obtained with primer pairs G1/L1 and T₂/T₄. Heterologous sequences of the 16S/23S spacer regions are found in the multiple copies of the rRNA operon within other bacterial species (Gurtler and Stanisich, 1996). However, *Cxx* contains only one rRNA operon (Sathyamoorthy *et al.*, 1991) and the spacer region between the 16S and 23S genes of *Cxx* does not contain any tRNA genes (Fegan *et al.*, 1998). This is consistent with the spacer regions of other Gram-positive organisms with high G+C content (Fegan *et al.*, 1998), excluding the possibility that an extra tRNA exists in the current *Cxx* sequence. It can be concluded that there might be strain differences between different *Cxx* isolates, and this requires further investigation.

The species *X. campestris* represents a complex group of plant-pathogenic bacteria that are similar in morphology but differ in host range. Accurate identification of plant pathogenic *X. campestris* species is difficult and time consuming (Gilbertson *et al.*, 1990). When using primer pair T₂/T₄ for the amplification of *Xvc* genomic DNA, it was found that different sized fragments were amplified for different iso-

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lates. The sizes of the T₂/T₄ amplified fragments obtained in this study ranged between 540 and 626 bp. A possible explanation for the different types of sequences obtained for *Xcv* in this study might be that there are different *Xcv* strains present in sugarcane plants. The determination of whether different strains of *Xcv* exist in sugarcane is a subject for future study.

A 619 bp product was amplified with primer pair T₂/T₄ from *Xa*. In both types of sequences obtained for *Xa* in this study, several small deletions and inclusions were found as well as point mutations, suggesting the existence of different strains of this species. Honeycutt *et al.* (1995) and Davis *et al.* (1997) also reported the existence of different strains of *Xa*.

Implications of different strains of pathogens for plant breeding

The occurrence of different strains of bacterial pathogens has implications for plant breeding. Currently, some promising new varieties are discarded in the later stages of selection or even after release because of their susceptibility to a specific pathogen. Should different strains of a pathogen be known to exist, new varieties could be screened against all known strains in the early stages of selection, thus saving time and resources. Characterising resistance to individual strains would allow for the maintenance of resistance genes in breeding populations. These could be exploited in the breeding programme through 'gene stacking', by crossing individuals carrying the different resistance genes. An advantage of such an approach is that useful germplasm is not discarded on the basis of susceptibility to a single strain of a pathogen.

Development of specific primers

By aligning and comparing the sequences obtained from fragments amplified from genomic DNA of *Cxx*, *Xcv* and *Xa* using primer pair T₂/T₄, it was possible to design a pair of primers specific for *Cxx*. Primer pair T₈ and T₉ was designed and a 237 bp fragment was amplified from genomic DNA as well as from *Cxx* cells. The primers also amplified a 237 bp fragment directly from sugarcane xylem sap. Sequencing these fragments confirmed that it was *Cxx*. The primers were tested against different isolates of other *Clavibacter* species (*Cmm*, *Cmi*, *Cmn*, *Cxc*), as well as *Xcv*, *Xa*, *Pseudomonas* species and several unknown bacterial species isolated from sugarcane plants. The primers were highly specific to *Cxx* and did not amplify DNA from other bacterial species; they were nevertheless not able to distinguish between *Cxx* and *Cxc*. However, *Cxc* is a xylem-inhabiting endophyte of Bermuda grass (*Cynodon dactylon*) and has not been found naturally in sugarcane (Davis *et al.*, 1984).

The detection limit for *Cxx* by the PCR protocol in this study was 5 pg of genomic bacterial DNA. However, when the bacterial DNA was mixed with an excess of sugarcane DNA, the detection limit was 0.5 ng of bacterial DNA (one hundred-fold less sensitive). PCR could also be performed

directly on *Cxx* cells from culture and from *Cxx*-infected sugarcane xylem sap. *Cxx* cells could be detected in a million-fold dilution of *Cxx* in xylem sap when PVP was added to the sap. It appeared that the PCR test was more sensitive when xylem sap was used as template rather than plant DNA.

Comparison of the PCR test with other diagnostic tests

A limiting factor in applying the PCR protocol for the detection of *Cxx* in sugarcane xylem sap is the presence of PCR inhibitors, such as polysaccharides or phenolic compounds (Henson and French, 1993). Various reagents have been included in the PCR buffer by researchers, such as PVP and polyethylene glycol (PEG) (MacKenzie *et al.*, 1997), PVP and Ficoll (Pan *et al.*, 1998) to minimise the possible effect of inhibitors in DNA extracts from plants. In this study, 0.8% (w/v) PVP added to the xylem sap increased the sensitivity of the PCR protocol considerably. *Cxx* cells could be detected in a million-fold dilution, thus improving the detection limit one hundred times. When different volumes of the dilutions were used as template in the PCR it was found that 0.5 µl of xylem sap was adequate as template.

A comparison was made between the four methods used for the detection of *Cxx*, namely PCR, IFM, EB-EIA and PCM. PCR was more sensitive in detecting *Cxx* than EB-EIA and PCM. Although the sensitivity of IFM and PCR appeared to be similar, it is accepted that PCR is more accurate in detecting *Cxx* because the test is based on a specific DNA sequence of *Cxx*.

Conclusions

The aim of this study was to develop a DNA-based diagnostic test using PCR for the detection of pathogens in sugarcane and specifically for *Cxx*. This was accomplished with the development of primer pairs T₂/T₄ and T₈/T₉.

It was demonstrated that significant sequence heterogeneity exists within the eubacterial 16S/23S spacer region at the genus and species levels. The size and number of fragments produced by amplification of the 16S/23S spacer region can be a useful tool in identifying species of bacteria. However, the sizes of fragments from the three bacterial species used in this study did not differ sufficiently to make recognition possible in 2% agarose gels, and the amplified fragment was therefore recovered and the precise sequence was used for identification.

The strategy used has the advantage of providing specific DNA primers for any micro-organism. Furthermore, the rapid technique requires no previous information on the molecular biology of the micro-organism of interest. The use of DNA sequences in the spacer region between the 16S and 23S rRNA has shown that efficient and specific primers can be generated that are able to readily distinguish between closely related eubacteria.

A specific primer pair (T₈ and T₉) was designed for the detection of *Cxx* in sugarcane DNA or sugarcane xylem sap

using the 16S/23S spacer region. The methodology provides a rapid, sensitive assay for the detection of *Cxx* in sugarcane. Ninety-six samples can be analysed in a thermal cycler in a 35-cycle PCR run, using a three-temperature programme in only 2½ hours. A special minigel system (Southern Cross Biotechnologies) in which 96 samples are analysed simultaneously, is used for the diagnostic test. The interpretation of the test results is simple and relies on the presence or absence of a single 237 bp fragment. It takes five to 10 minutes for the fragments to be resolved in the agarose gel. The overall time taken to conduct this diagnostic test is less than three hours.

It is not envisaged that the *Cxx*-specific PCR test will replace the serological-based detection methods currently used at SASEX for the mass diagnosis of RSD. However, PCR is the method of choice for the detection of *Cxx* in RSD transmission trials and diagnosis in quarantine.

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ALLELIC VARIATION IN THE GENE ENCODING PYROPHOSPHATE DEPENDENT PHOSPHOFRUCTOKINASE (PFP) IN SUGARCANE

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Abstract

The enzyme pyrophosphate dependent phosphofructokinase (PFP), considered to play an important role in sucrose metabolism in plants, comprises two protein subunits encoded by separate genes. Part of the β -subunit gene was used as a model in investigating allelic variation in the polyploid genetic environment of sugarcane. The gene fragment was amplified by the polymerase chain reaction (PCR) from various total genomic DNA samples and a number of individual DNA molecules cloned randomly from each source. Sequence analysis of clones has demonstrated that two strikingly different alleles are present in ancestral *Saccharum* species as well as modern hybrid varieties.

Introduction

Sugarcane is a highly heterozygous and genetically cumbersome crop (Aljanabi, 1998). This is at least in part because it is highly polyploid, having a suspected base chromosome number of 10 and between 110 and 130 chromosomes in the 2n state, contributed by two major ancestral species, *Saccharum officinarum* and *Saccharum spontaneum* (D'Hont *et al.*, 1996). The number of alleles of important genes and the degree to which those alleles vary in the complex genetic environment of sugarcane is of considerable interest because of the potential for exploitation of genetic variation through breeding, and manipulation of gene function via genetic engineering. In addition, such information might throw light on the general speciation and evolution of polyploids, about which little is known (Soltis *et al.*, 1992). The working hypothesis for this study was that allelic variation can best be established by targeting a single copy gene (i.e. a gene with one copy per haploid genome), the argument being that such a gene should be present in multiple allelic copies in sugarcane. The gene encoding the enzyme pyrophosphate dependent phosphofructokinase (PFP) is such a gene (Blakeley *et al.*, 1992). PFP is considered to play an important role in sucrose metabolism in plants (Dancer and ap Rees, 1989). Part of the gene encoding the β -subunit of the enzyme was adopted as the study model. Using genomic DNA from different sugarcane sources as template, the gene fragment was amplified by the polymerase chain reaction (PCR) with primers designed to complement conserved regions of the gene. Amplified fragments were ligated into plasmid vectors and individually cloned in *E. coli* for comparative sequencing analysis.

Materials and Methods

Primers

Forward primer B1 was designed from a region of high homology identified by comparison of known PFP gene sequences from castor bean and potato while reverse primer B8 was designed from a sugarcane PFP sequence (Sarita Groenewald; PhD thesis, University of Natal, 1998, and personal communication):

B1 (20bp) (5' to 3') ATI-GAT-TTC-ATI-CCI-GAG-GT

B8 (21bp) (5' to 3') CCA-TCA-GTG-ATG-TCA-ATG-CTG

The predicted product of B1/B8 amplification is a region of the PFP gene representing exons 10, 11 and 12 and their flanking introns, approximately 1200bp in size in all.

Gene amplification, cloning and sequencing

Genomic DNA was extracted from *Saccharum officinarum* (Black Cheribon), *Saccharum spontaneum* (Coimbatore) and a commercial variety (N21) as described by Harvey and Hockett (1998). PCR reactions contained, in a final volume of 50 μ l: 10mM Tris-HCl, 50mM KCl, 1,5mM MgCl₂, 50 μ g gelatine, 0,33mM deoxynucleoside triphosphates (dNTPs), 0,4 μ M primers B1 and B8, 1U Taq DNA polymerase (Boehringer Mannheim) and 75ng genomic DNA, at a final pH of 8,3. Thermal treatment was 94°C for 1,5 min (1 cycle), 94°C for 45 sec, 45°C for 30 sec and 72°C for 2 min (10 cycles) and 94°C for 30 sec, 40°C for 30 sec and 72°C for 2 min (30 cycles) followed by 72°C for 2 min then 35°C for 5 min. PCR products were purified by electrophoresis through 1% (w/v) agarose gels in TBE buffer (45mM tris-borate, 1mM EDTA, pH 8,0) followed by excision of bands visualised by conventional ethidium bromide staining and gel removal by spun column (GenElute, Supelco). PCR fragments were ligated into the pGEM[®]-T Easy plasmid vector (Promega) and cloned in *E. coli* strain JM109 according to manufacturer's instructions. Cloned plasmids were extracted from bacterial cells and purified using column separation kits (Nucleobond AX20, Macherey-Nagel) while estimations of quality and quantity were made both spectrophotometrically at 260nm and by visual observation following gel electrophoresis. Plasmid inserts were used with either B1 or B8 primer in cycle sequencing reactions according to standard dye terminator chemistry methodologies (Big Dye Kit, Perkin Elmer). Reaction product analysis was accomplished by capillary electrophoresis using a Perkin Elmer ABI Prism 310 Genetic Analyzer.

Sequence analysis

Sequences were edited manually to remove plasmid vector components. PFP homology was measured by nucleotide comparison of individual sequences to genes registered in international databases using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990). Sequence overlaps, alignments and other data manipulations were accomplished using Perkin Elmer software Sequence Navigator 1.0.1 and/or the software programme DNASIS for Windows v2.1.

Results and Discussion

Two PCR amplification products of approximately 1250bp and 1100bp in size were obtained consistently from the genomic DNAs of all three sources: Black Cheribon, Coimbatore and N21. The fragments were designated PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ respectively. In the case of Coimbatore, a third product of approximately 950bp was sometimes obtained. Only PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ were cloned and sequenced in this study. B1 generated sequences were used for BLAST homology searches. All scores obtained were above the significance threshold of 80 and ranged from 119 to 522 (mean = 411), indicating that all gene fragments obtained by PCR were identifiable as PFP. Where possible, forward and reverse sequences were integrated using Contig Manager, an overlap function of the software programme DNASIS, to provide full length frag-

ment sequences. Results of cloning, sequencing and BLAST homology searches of fragments PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ from all three germplasm types are summarised in Table 1.

To investigate sequence differences between the two size species PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀, representative full length sequences obtained from commercial variety N21 (one sequence of each type, Table 1) were compared with each other in an initial analysis. Results showed that the size differences between the two fragments was due to sequence variations throughout their lengths rather than a single major insertion or deletion in one of the fragments. Homocologous cDNA sequences of the castor bean PFP β -gene (exon regions 10, 11 and 12), assumed to be relatively conserved across species, were used to determine the positions of exon and intron regions in the sugarcane gene fragments. Exon regions of the two sugarcane variants showed 77-78% nucleotide homology while, as expected, intron regions showed greater variability with 48-59% nucleotide homology.

Having used N21 data to establish the existence of two variants of the gene, apparently associated with a size difference between the B1 and B8 primer sites, the data obtained for Black Cheribon and Coimbatore were analysed. For each of the germplasm sources, sequences clustered into one of two distinct sequence types, shown by consensus sequence comparisons to correspond closely to the two allelic variants

Table 1. Sequencing results and PFP homology scores for cloned gene fragments obtained by PCR from genomic DNA of *Saccharum officinarum* (Black Cheribon), *Saccharum spontaneum* (Coimbatore) and the commercial variety N21.

| GERMPLASM SOURCE | FRAGMENT | CLONE NAME | B1 (FORWARD) GENERATED SEQUENCE LENGTH (bp) | B1-SEQUENCE BLAST SIGNIFICANCE SCORE FOR PFP | B8 (REVERSE) GENERATED SEQUENCE LENGTH (bp) | OVERLAP REGION (bp) | FULL, LENGTH SIZE (bp) | |
|------------------|---------------------------|---------------------------|---|--|---|---------------------|------------------------|------|
| BLACK CHERIBON | PFP.B1/B8 ₁₂₅₀ | BC.T#1 | 643 | 499 | 668 | 285 | 1026 | |
| | | BC.T#3 | 662 | 513 | 643 | 272 | 1033 | |
| | | BC.T#4 | 556 | 352 | 366 | - | - | |
| | | BC.T#5 | 650 | 322 | 670 | 66 | 1254 | |
| | | BC.T#6 | 370 | 277 | 606 | - | - | |
| | | BC.T#10 | 651 | 495 | 651 | 93 | 1209 | |
| | | BC.T#11 | 660 | 119 | 653 | 283 | 1030 | |
| | | BC.T#12 | 650 | 495 | 687 | 129 | 1208 | |
| | | BC.T#13 | 640 | 495 | 637 | 21 | 1256 | |
| | | PFP.B1/B8 ₁₁₀₀ | BC.B#17 | 530 | 522 | 392 | - | - |
| | | | BC.B#18 | 532 | 522 | 432 | - | - |
| | | | BC.B#21 | 475 | 504 | 390 | - | - |
| | | | BC.B#24 | 485 | 331 | 653 | 91 | 1047 |
| COIMBATORE | PFP.B1/B8 ₁₂₅₀ | CO.T#4 | 743 | 489 | - | - | - | |
| | | CO.T#7 | 522 | 250 | 532 | 24 | 1030 | |
| | | CO.T#8 | 461 | 197 | 465 | - | - | |
| N21 | PFP.B1/B8 ₁₂₅₀ | N21.T#10 | 777 | 495 | 715 | 273 | 1219 | |
| | | N21.B#9 | 735 | 522 | 686 | 395 | 1026 | |

BC, Black Cheribon; CO, Coimbatore; T, top gel fragment, 1250bp; B, bottom gel fragment, 1100bp. BLAST scores above 80 indicate significant homology to known PFP genes.

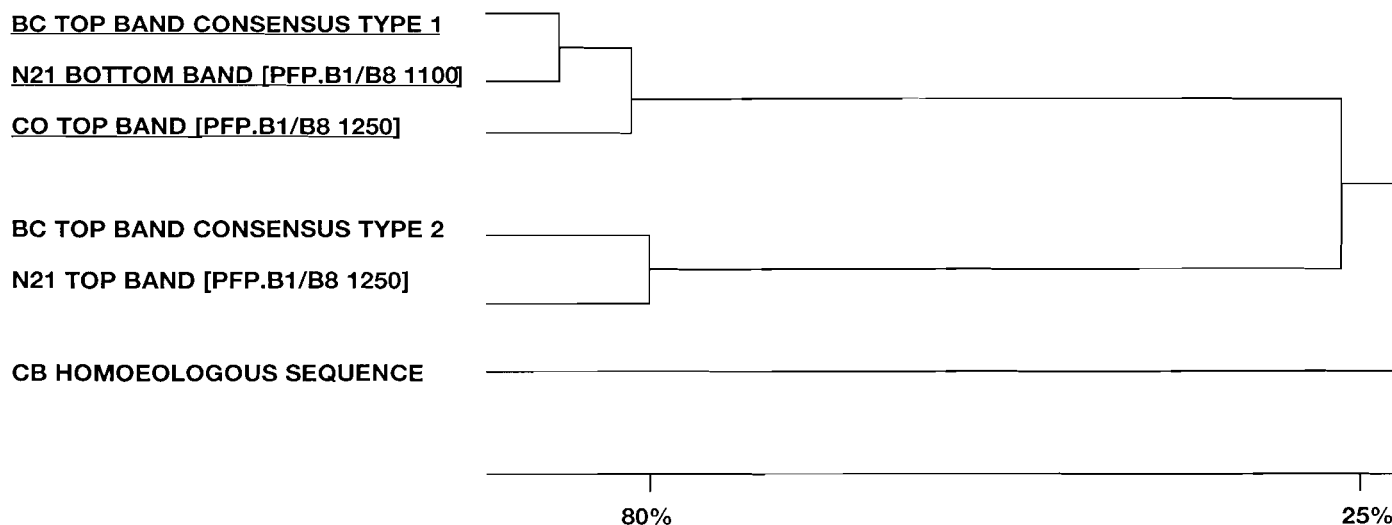


Figure 1. Homology tree derived from sugarcane PFP gene fragments and the equivalent sequence from castor bean. All contributing sequences represent full length B1/B8 PFP fragments. Consensus sequences types 1 and 2 were each based on three full length fragments and were obtained in Sequence Navigator. The tree was derived in DNASIS. BC, Black Cheribon; CO, Coimbatore; CB, castor bean (*Ricinus communis*). Scale shows relative homology values in %.

identified in N21. However, correlation between sequence type and size species of origin was not tight, suggesting that contamination of the PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ fragment populations had occurred at the point of gel band excision. This is not unlikely, as it is recognised that DNA fragments such as these, differing in size by only 150bp, may appear separate visually but not be resolved perfectly by agarose gel electrophoresis. Future preparative separation would be accomplished more precisely in polyacrylamide gels.

Relationships between the various sugarcane PFP gene fragment sequences and the homoeologous sequence from castor bean are illustrated in Figure 1. Two distinct allelic clusters are identifiable among the sugarcane sequences. It is notable that the degree to which they differ from each other is almost as great as the degree to which each differs from castor bean, a dicotyledonous plant not closely related to sugarcane. Although the analysis is based on limited numbers of cloned fragments and only single sequence runs, the result is sufficiently striking to suggest that the variations do reflect significant allelic differences in the genome. The high level of distinction and the fact that both gene forms occur in the ancestral germplasm of sugarcane suggest that the alleles diverged early in the evolution of the genus *Saccharum*.

Confirmation of this work and further investigations into PFP gene variants and their expression is being undertaken.

Acknowledgements

The authors thank Deborah Carson of the Biotechnology

department at the SASA Experiment Station and Dr Sarita Groenewald of the Institute for Plant Biotechnology, University of Stellenbosch, for practical advice, helpful discussions and appraisal of the manuscript.

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