SHORT NON-REFEREED PAPER

RAPID DIAGNOSIS OF RATOON STUNTING DISEASE BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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Abstract

Ratoon stunting disease (RSD), caused by the bacterial pathogen *Leifsonia xyli* subsp. *xyli*, is one of the most important diseases of sugarcane worldwide. Yield losses have frequently been estimated at 5-10% and may exceed to 60% due to moisture stress or varietal susceptibility. *L. xyli* subsp. *xyli* produces no reliable or characteristic external symptoms. Disease detection is normally done by diagnostic assays, including immunofluorescence microscopy, serology (EB-EIA) and DNA-based detection by PCR. All these methods are time-consuming and require sophisticated laboratory equipment. A recently developed assay called Loop-mediated isothermal amplification (LAMP) has proven to be rapid, sensitive and specific, and does not require expensive laboratory tools. This study reports the development of a LAMP assay for diagnosis of RSD. A total of six LAMP primer sets were designed, targeting the *L. xyli* subsp. *xyli* genome. The primers were tested with *L. xyli* subsp. *xyli* genomic DNA, crude xylem sap and bacterial cell cultures in broth. One set of primers was found to be specific to *L. xyli* subsp. *xyli* DNA. Different concentrations of reaction components and incubation times were tested. The LAMP reaction was optimised at 65°C for 60 minutes in a water bath. The primers worked well with crude xylem sap thus eliminating the need to isolate DNA. The LAMP primer set proved to be highly specific to *L. xyli* subsp. *xyli* when tested against other bacterial pathogens of sugarcane. The assay could detect up to 3 pg/µl of genomic DNA per reaction. Preliminary results indicate that the LAMP assay is as sensitive as the ELISA but much quicker. The RSD LAMP method shows potential to be applicable in field laboratories, without expensive equipment, thus decreasing the overall cost and the diagnosis time.

Keywords: Leifsonia xyli subsp. *xyli,* sugarcane, ratoon stunting disease, diagnostics, Loop-mediated isothermal amplification

Introduction

Ratoon stunting disease (RSD) was first discovered in 1944 (McDougall *et al*., 1948) and since then has been identified in most sugarcane producing regions. RSD causes an average 5% yield loss (Bailey and Bechet, 1997); however, depending on susceptibility of the variety and drought conditions, yield loss can be as high as 60% (www.bses.com.au)**.** In South Africa, losses in a normal season are estimated at 1% of production, but in some other African cane growing areas, yield loss is estimated to amount to 10-20% annually (Bailey and McFarlane, 1999).

RSD is caused by a xylem-limited bacterium, *Leifsonia xyli* subsp. *xyli* (*Lxx*) (Kao and Damann 1980; Davis *et al*., 1980). The disease causes no visible external symptoms other than stunted growth which can be misleading, and internal symptoms are not reliable (Gillaspie and Teakle, 1989). Diagnosis of RSD relies on laboratory-based methods including phase-contrast and immunofluorescence microscopy, immunological assays, i.e. evaporative binding enzyme-linked immunoassay (EB-EIA) and Polymerase chain reaction (PCR) based methods (Croft *et al*., 1994; Chung *et al*., 1994; Pan *et al*., 1998; Fegan *et al*., 1998; van Antwerpen and Botha, 1999; Taylor *et al*., 2003).

Microscopy and EB-EIA are the most commonly used methods for detection of RSD, but are time-consuming, require expensive equipment and need a well-equipped laboratory environment. Although nucleic acid based detection method such as PCR is more sensitive, the presence of PCR inhibitors in the sap or plant tissues limits its effectiveness and a thermal cycler is required for amplification.

A recently developed nucleic acid amplification method called loop-mediated isothermal amplification (LAMP) (Notomi *et al*., 2000) provides rapid, specific and sensitive detection, without the use of costly equipment, and has the potential to be used at local pest and disease (P and D) offices away from the laboratory. The assay utilises 4-6 primers and depends on autocyling strand-displacement DNA synthesis performed by *Bst* polymerase. The cycling accumulates stem-loop DNAs with several inverted repeats of the target and cauliflower like structures with multiple loops, and produces up to 10^9 copies of the target gene in less than one hour at 65°C in a water bath or a heating block, thus eliminating the need for a thermal cycler (Notomi *et al*., 2000).

Rapid and sensitive RSD diagnosis in a basic laboratory closer to the site of sampling would considerably reduce the delay between taking a sample and obtaining a result. LAMP has been successfully used for the detection of several other plant pathogenic bacteria (Kubota *et al*., 2008; Li et al., 2009; Rigano *et al*., 2010; Harper *et al*., 2010; Temple and Johnson, 2011; Gosch *et al*., 2012; Ravindran *et al*., 2012; Moradi *et al*., 2012).

The present study was undertaken to develop a LAMP method for quick and reliable detection of RSD, which would take significantly less time when compared to existing methods.

Methods

Three different genomic regions of *Lxx* were targeted to design six LAMP primer sets using the software Primer Explorer Version 4 (http://primerexplorer.jp/e/index.html). Each LAMP primer set consists of four primers: two short outer primers called F3 and B3, and two long inner primers FIP and BIP that hybridise to six sites on the target DNA. FIP and BIP primers each consist of two distinct sequences corresponding to sense and antisense sites on the target DNA. The long primers were designed with TTTT linkers to ensure loop formation. These four primers recognise six different regions of the target genome sequence.

The LAMP primers were tested with *Lxx* pure genomic DNA, *Lxx* cultured cells in broth and crude xylem sap samples collected from RSD infected cane at Pongola and Mpumalanga. Initial testing of the six different primer sets led to the adoption of one primer set which was used during this study.

The LAMP assay was performed in a total volume of 25 µl containing 1X thermopol buffer (New England Biolabs), 8 mM $MgSO_4$, 0.8 M Betaine, 1.4 mM dNTPs, 0.2 µM F3 and B3 primers, 1.6 µM each of FIP and BIP primers, 8 U *Bst* polymerase and 6 µl of template. Reactions were incubated in a water bath at 65°C for 60 min, followed by 80°C for 5 min to inactivate the polymerase. The amplification product (5 µl) was visualised by gel electrophoresis in a 2% agarose gel. The sap samples were also tested using EB-EIA for comparison with LAMP.

To determine the specificity of *Lxx* LAMP, DNA from other bacterial pathogens infecting sugarcane was used, *viz*. *Xanthomonas albilineans* (Leaf Scald disease) and *Xanthomonas axonopodis pv vasculorum* (Gumming disease). Genomic DNA of *Clavibacter michiganensis* subsp. *Michiganensis*, *Clavibacter michiganensis* subsp. *insidiosus*, *Clavibacter Michiganensis* subsp. *nebraskensis* and *Leifsonia xyli* subsp. *cynodontis* were also included, as the genus *Clavibacter* is closely related to *Leifsonia.*

Results and Discussion

The *Lxx* LAMP primer set presented a characteristic ladder-like loop amplification pattern on agarose gel with *Lxx* genomic DNA, crude xylem sap and bacterial cells in broth (Figure 1). As LAMP is tolerant of amplification inhibitors (Kaneko *et al*., 2007), it was possible to use crude xylem sap without treating it with reagents such as Polyvinylpyrrolidone (PVP) or Ficoll which are often used to reduce the effect of PCR inhibitors (Pan *et al*., 1998; Fegan *et al*., 1998; Taylor *et al*., 2003).

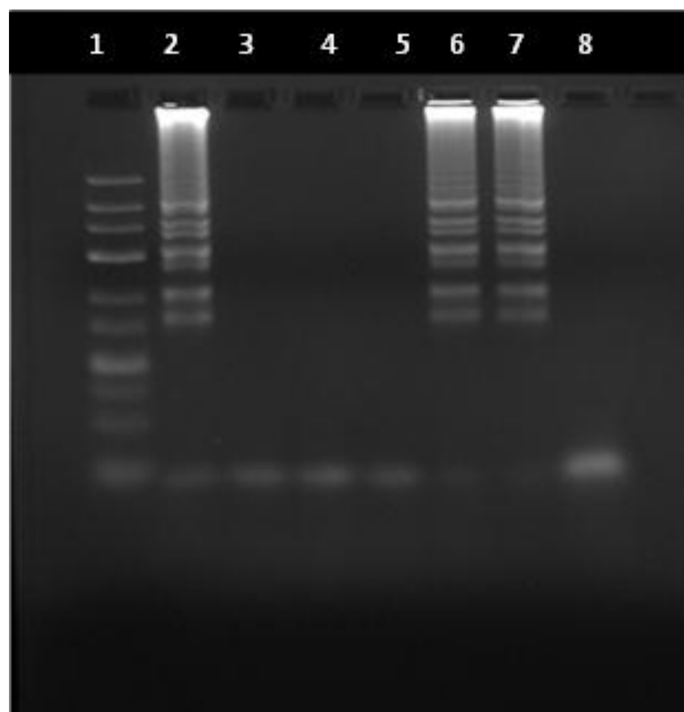

Figure 1. Agarose gel (2%) showing loop-mediated isothermal amplification (LAMP) product and specificity of the *Lxx* LAMP assay run at 65°C for 60 min. Lane 1: size marker (thermo scientific O'gene ruler low range), Lane 2: *Leifsonia xyli* subsp.*xyli* cells in pure culture, Lane 3: *Xanthomonas axonopodis* pv *vasculorum* DNA (Gumming disease), Lane 4: *Xanthomonas albilineans* DNA (Leaf Scald disease), Lane 5: *Leifsonia xyli* subsp. *cynodontis*, Lane 6: xylem sap from RSD infected sugarcane plant, Lane 7: pure *Lxx* DNA (2 ng/ul), Lane 8. negative control (reaction mix without DNA).

A range of temperatures from 61 to 67°C were tested to optimise the protocol. Suboptimal amplification was achieved at 61°C, whilst 63°C and 65°C resulted in bright clear bands. Incubation times of 30, 45 and 60 min were also tested. Optimal banding pattern was achieved with an incubation time of 60 min, whereas 30 and 45 min failed to give amplification. The reaction time and temperature were optimised at 60 min at 65°C in a water bath.

Lxx does not present genetic variability between international isolates (Young *et al*., 2006), thus to determine the specificity of LAMP, research targeted other bacterial pathogens of sugarcane and pathogens closely related to *Lxx* instead of screening different *Lxx* strains. The LAMP assay was specific to *Lxx* and did not amplify any other DNA (Figure 1).

The sensitivity of the LAMP assay was determined by serially diluting the pure *Lxx* genomic DNA from 3-0.3 ng, 30-3 pg. The LAMP assay was able to detect *Lxx* DNA down to 3 pg (Figure 2). A detection limit of 1 pg of genomic DNA has been reported by Pan *et al.* (1998) with PCR. However, one of the main objectives in this study was to use sap as a template to detect *Lxx* rather than genomic DNA, thus diluted and undiluted RSD-positive sap was tested. The method could detect bacteria in undiluted sap as well as sap that had been diluted ten times.

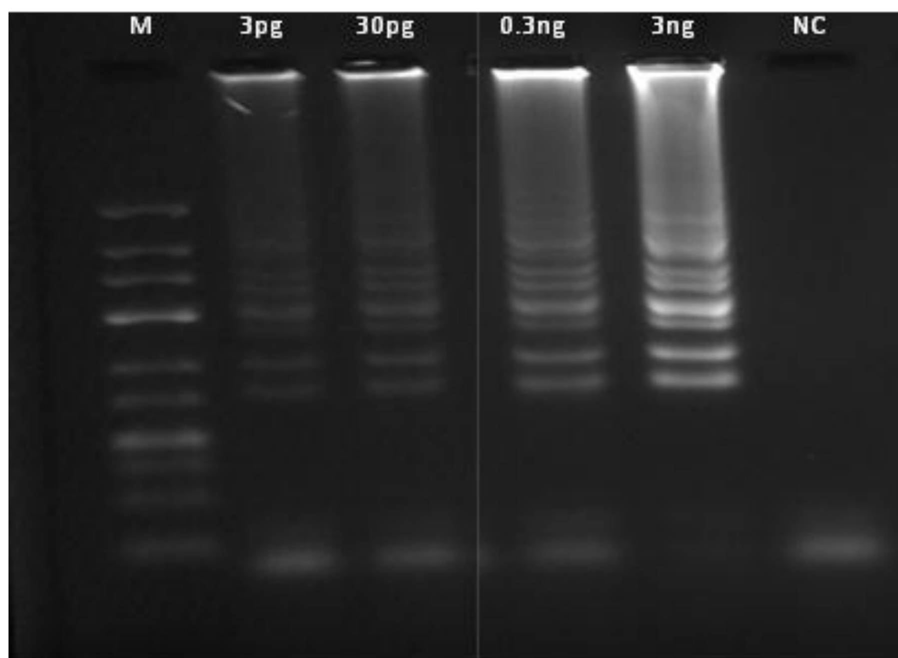

Figure 2. Sensitivity of the *Lxx* loop-mediated isothermal amplification (LAMP) assay with serial dilutions of *Lxx* pure genomic DNA. Lane 1: size marker (thermo scientific O'gene ruler low range), Lane 2: 3 pg, Lane 3: 30 pg, Lane 4: 0.3 ng, Lane 5: 3 ng, Lane 6: negative control.

The preliminary comparison of LAMP with EB-EIA showed similar detection levels for the two methods. EB-EIA is the most frequently used method to detect RSD because of its ability to process large numbers of samples (Croft *et al*., 1994; McFarlane *et al*., 1999). However, frozen sap samples from distant farm areas need to be transported to the laboratory at Mount Edgecombe, and it takes up to two days to obtain results whereas LAMP takes approximately one and a half hours. LAMP therefore has potential to become a rapid and reliable alternative diagnostic test to EB-EIA.

To introduce the use of LAMP at farm level, the next step in this research would be to focus on developing a visual method to detect LAMP amplification, thus bypassing the need for gel electrophoresis. This would make the method even more user friendly.

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