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# Development of a qPCR for *Leifsonia xyli* subsp. *xyli* and quantification of the effects of heat treatment of sugarcane cuttings on Lxx

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ABSTRACT

The main control practice of *Leifsonia xyli* subsp. *xyli* (Lxx) in sugarcane is to heat-treat cane cuttings used as planting material in an attempt to eradicate the bacterium. A real time quantitative PCR (qPCR) protocol specific for Lxx was developed to assess the effectiveness of this practice. Primers were designed from the sequence of an Lxx-specific gene and detected as few as $10^{-5}$ ng of Lxx DNA in 100 ng of plant DNA. Two experiments were conducted to quantify Lxx titers in plants of the varieties SP80-3280 and SP70-3370 originated from cuttings treated or not by immersion in hot water at 52 °C for 30 min. In the first experiment, cuttings were collected from plant canes with low Lxx titers whereas in the second they were collected from first-ratoon canes with higher titers. Lxx was quantified in leaves by qPCR 90 days after planting and was detected in 50–90% of the plants at variable titers, indicating that the 52 °C hot water treatment for 30 min was not effective in eradicating Lxx from all plants. However, in the second experiment the bacterial population was reduced, as the median number of Lxx cells was lower compared to the non-treated control. In the case of SP70-3370, the treatment also reduced the number of Lxx-infected plants considering the pooled data of the two experiments. The results indicated that although the 52 °C hot water treatment for 30 min did not completely eliminate Lxx, it can be used to reduce the pathogen population in plants propagated from canes with high Lxx titers



## 1. Introduction

The gram-positive and nutritionally fastidious bacterium *Leifsonia xyli* subsp. *xyli* (Lxx) causes the ratoon stunting disease (RSD) of sugarcane, a worldwide disease that accounts for substantial losses in biomass. In China, the United States, South Africa, Australia, and Brazil, for example, biomass reductions of 60% (Li et al., 2013), 33% (Grisham, 1991), 41% (Bailey and Bechet, 1995), 37% (Young et al., 2006) and 26% (Gagliardi and Camargo, 2009), respectively, have been reported. The most evident symptom of RSD, as the name implies, is the impaired development of the ratoon or stubble plants due to the reduction in the diameter of the stalks and shortening of the internodes after successive cropping.

Because sugarcane ratoons are cropped several times, the prevalent mode of transmission of Lxx in commercial fields is by contact with juices of infected plants that occur during harvesting with machines and knives. Thus, as sugarcane is vegetatively propagated, the main control measure of RSD is to establish healthy seed cane nurseries from *in vitro* cultured explants or from heat-treated cane cuttings (setts) (Benda and Ricaud, 1977; Damann and Benda, 1983; Hoy et al., 2003). Heat treatments consist of exposing the setts to heated air, steam, or water (Damann and Benda, 1983). In Brazil, the most used treatment consists of immersing the setts in water at 52 °C for 30 min because it has a low impact on bud germination (Fernandes et al., 2010). Despite the considerable number of reports on the efficiency of heat treatments in the control of RSD, however, none quantified its effects on the population of Lxx in the plant host.

Due to its complex nutritional requirements and slow growth *in vitro*, it is costly and time consuming to quantify Lxx in plant tissues by plating plant extracts in solid culture medium. Available serological and PCR-based protocols used to detect Lxx (Grisham,

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2004) can be used as quantitative methods. However, conventional PCR does not provide an accurate estimate of the amount of bacteria and it requires additional laboratorial procedures, such as the visualisation of the amplicons in agarose gels. The highly sensitive real time PCR technique was adapted to detect Lxx in plant tissue using either a fluorescent dye (Grisham et al., 2007) or a specific DNA probe labelled with a fluorescent reporter (Pelosi et al., 2013). This approach was better suited to detect the pathogen in the early phases of infection compared to the tissue-blot enzyme immunoassay and was more sensitive than the conventional and the nested-PCR. In this study, we explored the quantitative application of this technique to establish a relationship between known DNA masses of Lxx and PCR cycle threshold (Ct) values using a new set of primers designed based on the sequence of an Lxx-specific gene. The technique was used to quantify and compare the levels of Lxx populations in plants of two sugarcane varieties originated from heat-treated or untreated cane cuttings.

## 2. Materials and methods

### 2.1. Lxx culturing and DNA extraction

The Lxx strain CTCB07 was used as a positive control in qPCR reactions. The DSM46306 strain of *Leifsonia xyli* subsp. *cynodontis* was also used to test the specificity of the primers. Both bacteria were cultured in M-SC medium (Teakle and Ryan, 1992) modified by Monteiro-Vitorello et al. (2004). For DNA extraction, 50 mL of a liquid culture ($OD_{600}$ = 0.8) was centrifuged at 12,000 rpm for 15 min in a tabletop microcentrifuge (Eppendorf, Germany). The supernatant was discarded and the cell pellet was rinsed three times in a buffer containing 1 M NaCl, 10 mM Tris and 10 mM EDTA, pH 8.0. Cells were resuspended in 8.25 mL of SET solution (75 mM NaCl, 25 mM EDTA, and 20 mM Tris, pH 7.5) supplemented with 1 mg $mL^{-1}$ of lysozyme, and incubated at 37 °C for 2 h. A 1/10 volume of a 10% SDS solution containing 0.5 mg $mL^{-1}$ of proteinase K was added and the cell suspension was incubated at 55 °C for an additional 2 h. Following this incubation, 1/3 volume of 5 M NaCl and one volume of chloroform were added, and the resulting homogenate was incubated at room temperature for 30 min and centrifuged at 5000 rpm for 15 min. The supernatant was transferred to a new tube, and the DNA was precipitated by the addition of one volume of ice-cold isopropanol. The DNA was resuspended in 60 μL of TE buffer (pH 8.0) and the RNA was digested with 0.5 μg $\mu L^{-1}$ of RNase for 1 h at 37 °C. The DNA was quantified in a NanoDrop 1000 spectrophotometer (Thermo Scientific, U.S.A.).

### 2.2. Development of a qPCR standard curve to quantify Lxx

Primers Lxx12950F1 (GCACATCGATCTGGAAAAAAGG) and Lxx12950R1 (CCGCAGTCTCACGCATACC) were designed to amplify a fragment of 106 bp from the gene Lxx12950 (GenBank: AE016822.1, locus_tag = "Lxx12950″) using the Primer Express V 3.0 software package (Applied Biosystems, U.S.A.). This gene was chosen because its sequence had no significant similarity to any other sequences available in GenBank and is not present in the genome of *Leifsonia xyli* subsp. *cynodontis* (Monteiro-Vitorello et al., 2013), a closely related bacterium that also colonizes grasses. The software NetPrimer (Premier Biosoft International; www.premierbiosoft.com/netprimer) and GeneRunner (www.generunner.net) were used to assess the possibility of formation of hairpin and dimers.

A standard curve to correlate Ct values with different masses of Lxx DNA was established using a ten-fold dilution series ranging from 10 ng to $10^{-5}$ ng of DNA per qPCR reaction. This corresponds to a maximum of $3.8 \times 10^6$ and a minimum of 3.8 Lxx cells considering that the amount of DNA per cell is approximately $2.63 \times 10^{-6}$ ng estimated based on sequence of the genome of the Lxx CTCB07 strain (GenBank: AE016822.1). Therefore, the equation NC = DM/$2.63 \times 10^{-6}$ was used to calculate the number of Lxx cells, where NC is the number of cells and DM is the DNA mass (ng) estimated from Ct values. The standard curve was established based on fourteen technical qPCR replicates of each DNA concentration. Amplifications were performed in a 7500 FAST thermocycler (Applied Biosystems, U.S.A) using the Platinum SYBR® Green qPCR SuperMix UDG kit (Invitrogen, U.S.A.) in accordance with the manufacturer's instructions. The reactions consisted of 12.5 μL of SuperMix amplification buffer, 0.5 μL of a 10 μM solution of each primer, 0.5 μL of a 2.5 μM solution of ROX, 9 μL of nuclease-free water (Integrated DNA Technologies, U.S.A.), and 2 μL of the DNA solution in the appropriate concentration. The amplification protocol consisted of an initial cycle of 50 °C for 2 min and 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The dissociation curve was calculated using the default parameters of the equipment.

### 2.3. Quantification of Lxx in plant tissue

One gram of leaf whorl tissue was rinsed with sterilized water and flash frozen and ground to a fine powder. Approximately 75 mg of powder was used in DNA extraction using the Invisorb kit (Invitek, U.S.A.) according to the manufacturer's recommendations, except for the inclusion of a digestion step with 1 mg $mL^{-1}$of lysozyme (Sigma, U.S.A.) after the first step of plant material separation. The DNA was resuspended in 80 μL of nuclease-free water (Integrated DNA Technologies, U.S.A.) and quantified as described in 2.1. Amplifications were performed as described in 2.2.

The optimal amount of plant DNA per qPCR reaction was defined based on the amplification efficiency (E) of the reactions using the equation $E = 10^{-1/k}$ (Cankar et al., 2006) where k is the slope of the amplification curve estimated by the LinReg software (Ramakers et al., 2003). Three different amounts of DNA (50 ng, 100 ng and 200 ng) extracted from an infected plant of the variety SP80-3280 were tested in PCR amplifications with two technical replicates.

In order to confirm the identity of the PCR amplicons, fragments were resolved in 0.8% agarose gels and purified with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, U.K.) according to the manufacturer's instructions. The fragments were quantified in a spectrophotometer and sequenced in an ABI 3100 sequencer (Applied Biosystems, U.S.A.) with the ET Dye-terminator kit (GE Healthcare, U.K.). Sequence quality was analysed with the Sequencher 3.0 software (Gene Codes Corporation, U.S.A.) and submitted to searches in the GenBank database available through the National Centre for Biotechnology Information using the BLASTN algorithm (Altschul et al., 1990).

### 2.4. Effect of heat treatment of sugarcane cuttings on Lxx titers

Lxx-infected canes of the varieties SP80-3280 and SP70-3370 were harvested from a single cane clump and four one-eyed cuttings were collected from the lower third of each one. The cuttings of each variety were mixed and separated into two pools of 30 each. One pool was heat treated (HT) by immersion in water at 52 °C for 30 min and the other was immersed in water at room temperature for 30 min (NT). Cuttings were planted in seed-raising trays with 28 cells containing Multiplante substrate (Terra do Paraíso, Brazil). Plants with 2–3 leaves were individually transplanted to 10 L pots containing the same substrate 30 days after planting.

The experiment consisted of four treatments (two varieties, each variety with hot water treatment and non-treatment) and ten replicates represented by single plants arranged in a randomized