Title

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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Quantification of the effects of heat treatment of sugarcane cuttings on *Leifsonia xyli* subsp. *xyli* by qPCR

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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 bacterial 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^{o}C for 30 minutes. In the first experiment, cuttings were collected from plant canes with low bacterial 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. Lxx was detected at variable titers in 50 to 90% of the plants, indicating that the heat treatment 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 heat treatment did not completely eliminate the bacterium, it can be used to reduce the pathogen population in plants propagated from canes with high bacterial titers.

Keywords: ratoon stunting disease, disease control, eradication, *Saccharum* spp., thermoterapy

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

47 explants or from heat-treated cane cuttings (setts) (Benda and Ricaud, 1977; Damann Jr and
48 Benda, 1983; Hoy et al., 2003). Heat treatments consist of exposing the setts to heated air, steam,
49 or water (Damann Jr and Benda, 1983). In Brazil, the most used treatment consists of immersing
50 the setts in water at 52^{o}C for 30 minutes because it has a low impact on bud germination
51 (Fernandes Júnior et al., 2010). Despite the considerable number of reports on the efficiency of
52 heat treatments to control Lxx, however, none quantified its effects on the bacterial population in
53 the host.

54 Due to its complex nutritional requirements and slow growth *in vitro*, it is costly and time
55 consuming to quantify Lxx in plant tissues by plating plant extracts in solid culture medium.
56 Available serological and PCR-based protocols used to detect Lxx (Grisham, 2004) can be used
57 as quantitative methods. However, conventional PCR does not provide an accurate estimate of
58 the amount of bacteria and it requires additional laboratorial procedures, such as the visualisation
59 of the amplicons in agarose gels. The highly sensitive real time PCR technique was adapted to
60 detect Lxx in plant tissue using either a fluorescent dye (Grisham et al., 2007) or a specific DNA
61 probe labelled with a fluorescent reporter (Pelosi et al., 2013). This approach was better suited to
62 detect the pathogen in the early phases of infection compared to the tissue-blot enzyme
63 immunoassay and was more sensitive than the conventional and the nested-PCR. In this study,
64 we explored the quantitative application of this technique to establish a relationship between
65 known DNA masses of Lxx and PCR cycle threshold (Ct) values using a new set of primers
66 designed based on the sequence of an Lxx-specific gene. The technique was used to quantify and
67 compare the bacterial populations of plants of two sugarcane varieties originated from heat-
68 treated or untreated cane cuttings.
69

2. Materials and methods

2.1 Lxx culturing and DNA extraction

The Lxx strain CTCB07 was used for the inoculation of sugarcane plants and 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 minutes in a tabletop microcentrifuge (Eppendorf). The supernatant was discarded and the cell pellet was rinsed three times in a buffer containing 1 M NaCl, 10 mM Tris and 10mM 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 hours. 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 hours. 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 minutes and centrifuged at 5,000 rpm for 15 minutes. 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 μL^{-1} of RNase for 1 hour 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 from the sequence of the gene 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 to10^{-5} ng of DNA per qPCR reaction, which corresponds to a maximum of 3.8 x 10^{6} and a minimum of 3.8 Lxx cells considering that the amount of DNA per cell is approximately $2.63x10^{-6}$ ng estimated based on sequence of the genome of the Lxx CTCB07 strain (Monteiro-Vitorello et al., 2004). Therefore, the equation $NC = DM/2.63x10^{-6}$ 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), and 2 μL of the DNA solution in the appropriate concentration. The amplification protocol consisted of an initial cycle of 50°C for 2 minutes and 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. 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) according to the manufacturer's recommendations, except for the inclusion of a digestion step with 1 mg $mL^{-1}$of lysozyme (Sigma) after the first step of plant material separation. The DNA was resuspended in 80 μL of nuclease-free water (Integrated DNA Technologies) and quantified in a NanoDrop spectrophotometer (Thermo Scientific). 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 (50ng, 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) according to the manufacturer's instructions. The fragments were quantified in a spectrophotometer and sequenced in an ABI 3100 sequencer (Applied Biosystems) with the ET Dye-terminator kit (GE Healthcare). Sequence quality was analysed with the Sequencher 3.0 software (Gene Codes Corporation) 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 treatment) by immersion in water at 52^{o}C for 30 min and the other was immersed in water at room temperature for 30 min (not-treated; NT treatment). Cuttings were planted in seed-raising trays with 28 cells containing Multiplante substrate (Terra do Paraíso, Brazil). Plants with 2 to 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 and two heat treatments) and ten replicates represented by single plants arranged in a randomized design. The bacterium was quantified by qPCR as described in 2.2 in the first leaf with visible dewlap 90 days after transplanting using the defined optimal amount of plant DNA. The experiment was performed twice in a greenhouse; in the first, the canes used as the sources of cuttings were harvested from 10 month-old plants (plant crop), whereas in the second the sources were from 8 month-old plants of the first ratoon crop. Prior to establishing the experiments, random samples of 10 canes were taken from each source and assayed for the quantity of Lxx by qPCR. In this case, vascular fluid was extracted from the internode immediately above the point of collection of the cuttings by positive pressure after attaching one end of the internode to a dairy teat cup shell coupled to a vacuum-pump. The DNA was extracted from 500 uL of the fluid as described in 2.3.

2.5 Statistical Analyses

Paired t-tests were used to compare the amplification efficiency E values of the qPCR reactions between DNA concentrations ($P < 0.01$). Chi-square tests using the Yates correction for continuity (Zar, 1996) were used to compare the frequencies of Lxx-infected plants between the heat treatments. To test whether the heat treatment had any quantitative effect on the bacterial population, histograms of the numbers of bacterial cells per plant were plotted for each treatment and experiment and subjected to the Mann-Whitney test (Zar, 1996) to determine whether the frequency distributions were similar for the heat and control treatments. This nonparametric procedure was used since Lxx density estimates do not follow a normal distribution (Davis et al., 1988). Analyses were carried out using Statistica version 12 (Statsoft Inc.).

3. Results and Discussion

3.1 Ct values can be used to estimate Lxx cell numbers in sugarcane leaves

The detection methods for Lxx have evolved from microscopy and serological tests to conventional PCR. However, the latter, although efficient for detection, does not provide accurate estimates of the number of cells present in the plant tissue. The real time PCR protocol developed by Grisham et al. (2007) for the early detection of Lxx in the leaf tissue of sugarcane detected the pathogen as early as 3 months after inoculation. The detection time was significantly shortened compared to the TB-EIA method, which detected Lxx only after the formation of mature stalks, which occur approximately 7 months after planting. In particular, these authors used PCR primers that were designed from the internal transcribed spacer (ITS) region to qualitatively evaluate the presence of Lxx in inoculated and non-inoculated plants over time. In

this study, we developed an alternative protocol suited for quantifying Lxx by qPCR in sugarcane tissue based on the sequence of an Lxx-specific gene.

The specificity of the primer pair was attested as no amplifications were detected in reactions with *L. x.* subsp. *cynodontis* whereas when DNA of Lxx was used instead, the reactions produced a single fragment of 106 bp as visualized in agarose gel (not shown). The identity of the PCR fragments was confirmed by sequencing ten amplicons of different plants chosen at random from the heat-treatment experiments. In all cases, the sequences best hit corresponded to the sequence of the Lxx12950 gene with expect values (E-values) varying from 1e-26 to 1e-22. In addition, the second best hits had E-values no lower than 0.023 (data not shown).

Derivative melting curves of PCR amplifications using different concentrations of Lxx DNA indicated the absence of dimmers as evidenced by a single peak at the melting temperature of 84.5°C (data not shown). The increasing amounts of DNA mass in the reactions highly correlated (R^2 = 0.9982) with decreasing Ct values, as expected. This allowed to establish a relationship between Ct values and Lxx DNA mass (DM; in ng) through a linear regression between these variables expressed by the equation $Ct = -3.3919 * \log_{10} DM + 20.052$ (R^2 = 99.8). The lowest Lxx DNA mass detected was 10^{-5}ng, which corresponds to approximately 4 bacterial cells.

The protocol determined the amount of template DNA used in the reactions based on the PCR reaction efficiency where an E value of 2 indicates an optimum efficiency with the quantity of target molecules doubling every PCR cycle, while a value of 1 indicates no exponential amplification. The mean amplification efficiency values of reactions with 50 ng (E= 1.91 ± 0.12) and 100 ng (E= 1.89 ± 0.10) of DNA did not differ, whereas with 200 ng the efficiency (E= 1.75 ± 0.10) was significantly lower (P=0.01) than in reactions with either 50 or 100 ng. Therefore, it

was concluded that the optimal amount of plant DNA to be used per reaction should be between 50 and 100 ng.

In addition to its sensitiveness, the qPCR offers the advantage over the serological methods of not being destructive, since it uses leaves rather than juices from mature stalks. As such, it can be employed to monitor bacterial population dynamics in sugarcane varieties in response to environmental conditions thus addressing many aspects of the still poorly understood interaction of Lxx with its host.

3.2 The heat treatment reduced the bacterial titers but did not eliminate the pathogen

In the present study, the qPCR protocol developed in this study was used to quantify Lxx in leaf samples taken from heat treated and non treated plants. Prior to establishing the experiments, however, the presence of the bacterium in the vascular fluid of the plant material used as the sources of the cuttings was confirmed by qPCR. The median bacterial titers were markedly lower in the plant crop canes used in the first experiment than in the ratoon crop canes used in the second, which could be explained both by the different ages of the canes (plant *versus* ratoon canes) and because they were harvested from different fields. In addition, plants of the SP70-3370 variety held higher titers than the SP80-3280 in both cases (Table 1).

Quantification of bacterial populations in plants of the heat treatment detected Lxx in variable titers in 50 to 90% of the plants (Figure 1; Table 2), indicating that the 52^0C/30 min treatment was not effective in eradicating Lxx from all individual plants. However, the median number of Lxx cells was lower in the heat treated plants compared to the non-treated ones (Table 2; Figure 1). Comparisons of the frequency distributions between the heat and the control treatments for both varieties using the Mann-Whitney test indicated that the heat treatment

reduced the bacterial population in both varieties (P=0.01) in the second experiment, where the setts presumably had higher bacterial titers since they were collected from canes with higher estimates of Lxx cells.

As the number of Lxx-infected and Lxx-free plants differed between treatments for each variety (Table 2), the chi-square contingency test on the pooled data of the two experiments was used to test whether these differences were significant after the chi-square heterogeneity test indicated that the experiments were homogeneous (not shown). The heat treatment reduced the number of Lxx-infected plants (P=0.05) only in the case of the SP70-3370 variety, where 90% (18 out of 20 plants) of the control plants were found to be infected compared to 55% (11 out of 20 plants) of the heat treated plants. Presently, it cannot be concluded that this difference between cultivars is genotype-specific, as the number of Lxx-infected plants among the non-treated plants of SP80-3280 was lower than in SP70-3370. Nevertheless, the finding illustrates that the qPCR method can be used to investigate the effects of intrinsic characteristics of sugarcane varieties, such as bud morphology and stalk diameter, in order to improve our understanding on the factors that can maximize the efficiency of this control method.

Despite its wide use as a control method for Lxx, the heat treatment does not completely eliminate the bacterium and affects negatively the germination of the buds (Damann Jr and Benda, 1983; Fernandes Júnior et al., 2010). Thus, several studies investigated the effects of different exposure times and temperatures in attempts to maximize its efficiency while reducing losses of plant material. Another problem associated with the use of this control method relates to its variability. For instance, the incidence of symptomatic shoots treated at 50^{o}C for 2 hours varied from 0 to 30% according to the facility where the treatment was carried out (Damann Jr and Benda, 1983). Sources of variation most likely result from differences in the equipment used

253 at each facility and in responses of sugarcane genotypes (Damann Jr and Benda, 1983). In
254 addition, the irregular distribution of Lxx within and between plants (Davis et al., 1988) would
255 likely be another source of variation as this would reflect in variable bacterial densities in the
256 setts before treatment as reported in this study (Table 1).

257 A previous study on the efficiency of the 52^{o}C/30 min treatment indicated that it is not
258 totally effective, as 33% of the canes were found to be infected with Lxx after the treatment
259 using the dot-blot technique (Fernandes Júnior et al.; 2010). However, the quantitative effect of
260 the treatment was not assessed by these authors. In the present study the frequencies of infected
261 plants were even higher, agreeing with this and other studies in that the heat treatment is only
262 partially successful in eradicating the pathogen from sugarcane setts. Notwithstanding, as
263 reductions in yield due to RSD are positively related to pathogen densities within the plant
264 (Harrison and Davis, 1988; McFarlane, 2002) our study also showed that, more important than
265 eradicating the pathogen, this practice should still be recommended since it is expected to have a
266 positive effect in minimizing the losses due to this disease by reducing the bacterial population in
267 plants regenerated from setts with higher titers.

268

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Table 1.

Median number and range of Lxx cells/100 ng of DNA extracted from vascular fluid of plant and ratoon canes used as sources of cuttings in experiments 1 and 2.

	Median number of Lxx cells (range)	
Variety	experiment 1	experiment 2
SP80-3280	135 (0-406)	5,034 (1,077 – 21,087)
SP70-3370	307 (0-615)	316,350 (111,480 – 734,684)

Table 2

Incidence of Lxx-infected plants (infected plants/total number of plants) and median number of Lxx cells/100 ng of plant DNA in plants of the SP80-3280 and SP70-3370 varieties regenerated from heat treated or not treated setts determined by qPCR

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	experiment 1		experiment 2		pooled[a]	
	SP80-3280	SP70-3370	SP80-3280	SP70-3370	SP80-3280	SP70-3370
Incidence (infected/total)						
HT[b]	9/10	5/10	5/10	6/10	14/20	11/20*
NT	8/10	8/10	8/10	10/10	16/20	18/20*
Lxx cells (range)[c]						
HT	46.5 (0 – 3,434)	6.1 (0 – 313)	3.9 (0- 23)	5.4 (0 -38)	-	-
NT	225.4 (0 - 744)	117.3 (0- 2,951)	31.9 (0 -132)	749.4 (21-51,316)	-	-

[a] incidences were pooled over experiments
[b] HT = heat treated; NT = non treated
[c] values in parentheses indicate range
* denotes significant differences in the incidences of Lxx-infected plants between the HT and NT treatments based on the chi-square contingency test (P=0.05).

Figure1

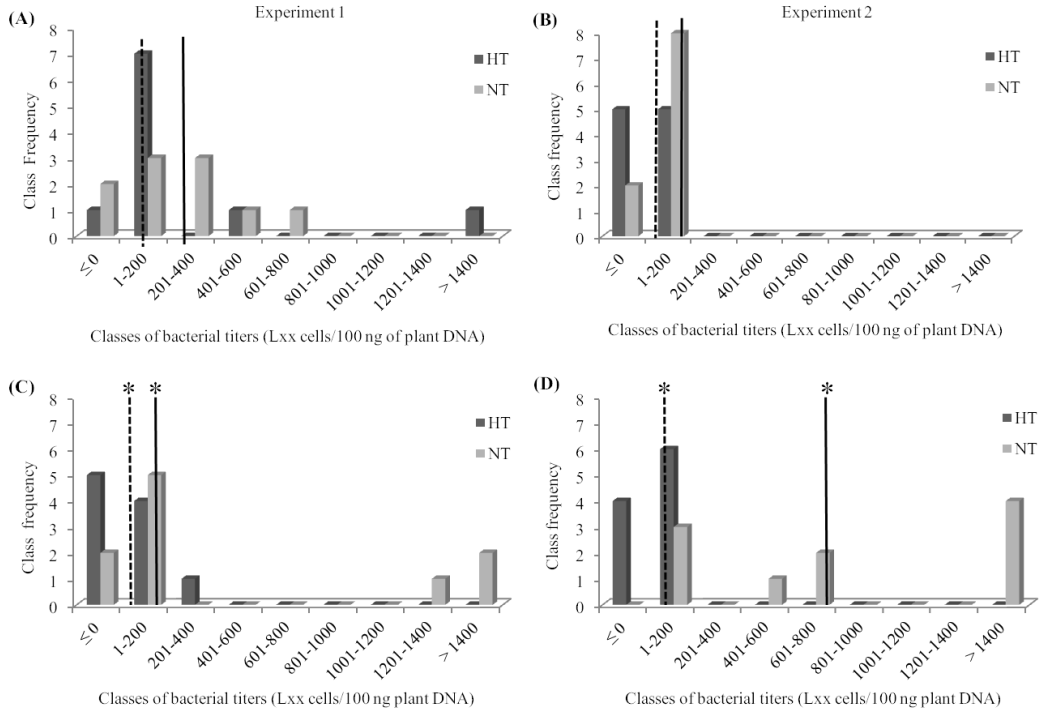

Fig. 1 .

Frequency distributions of Lxx cell numbers assessed in two experiments in plants of the sugarcane varieties SP80-3280 (A and B) and SP70-3370 (C and D) derived from single bud cuttings that were heat-treated (HT) or not (NT). The median values of the HT and NT treatments are indicated by the dashed and solid lines, respectively. Differences in frequency distributions according to the Mann-Whitney test (P=0.01) are indicated by asterisks.