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Effect of thermotherapy, *Leifsonia xyli* subsp. *xyli* titres, sugarcane genotype and diagnostic techniques on ratoon stunt control in Brazil

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Abstract

Aims: To examine the interaction of diagnostic techniques, initial titres of *Leifsonia xyli* subsp. *xyli* (Lxx), sugarcane genotype and thermotherapy on ratoon stunt (RSD) control.

Methods and Results: Single buds of RB867515, RB92579 and RB966928 were submitted to 50°C/2 h or 52°C/30 min under factorial block design and five replications; results were checked 9 months later by serological (DBI) and molecular (PCR) techniques. A 10,000 bootstrapping simulations were performed to infer the best plot size based on the experimental coefficient of variation. Analysis of variance showed significance only on initial Lxx titres and RSD control. Despite the absence of significance in the overall analysis, minor differences in control success with different methods and cultivars are predicted to have a major epidemiological impact on RSD, considering successive harvests and vegetative increase. According to an epidemiological interpretation, the 50°C/2 h treatment was more effective, cultivar RB966928 was the most susceptible and the PCR-based method was the most sensitive for pathogen detection. The minimum required plants per plot was 15, indicating high precision of our experiment

Conclusions: Data interpretation considered both the statistical analysis and the epidemiology aspect of RSD in order to improve RSD management. The Brazilian sugarcane industry will benefit from this approach since it is not using it.

Significance and Impact of the Study: This is the first study that examined multiple factors that affect RSD control. Our work pinpointed the importance of the thermotherapy, its best combination as well as the diagnostic test. Also, the effect of the cultivar to respond to management strategies. Because the epidemiological aspect of RSD was taken into consideration, results of our work can have an impact on RSD control in the field.

KEYWORDS
diagnostic techniques, disease dissemination, plot size, ratoon stunt, thermotherapy, sugarcane genotype

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INTRODUCTION

Sugarcane is the most important crop in Brazil's contribution to mitigate global climate warming because ethanol is employed in a blend with gasoline (25%–27% ethanol) or alone as fuel in the automobile industry. Bioenergy generated by sugarcane waste (bagasse, straws and tips) is also important since it supplied electricity for the existing 309 sugarcane mills in 2018 and a surplus of 21,500 GWh that was integrated into the national energy grid, which corresponded to 4% of the domestic energy demand (Sugarcane, 2020).

The increasing demand for sugarcane products stimulated the Brazilian industry to expand the harvested area from 6.2 million hectares in 2007/08, with a production of 473 million tonnes, to 8.4 million hectares in 2019/20 with a production of 643 million tonnes (CONAB, 2007; CONAB, 2020). Therefore, the sugarcane crop is currently under scrutiny because this increased production has been achieved by increasing the planted area and not productivity, which has stagnated since 2005/06 when it was recorded at 74.3 tonne/ha. More than 10 years later, in the 2019/20 crop season, it was only slightly superior, reaching 76.1 tonne/ha (CONAB, 2020). Although many factors affect sugarcane yield, ratoon stunt (RSD) caused by *Leifsonia xyli* subsp. *xyli* (Lxx) is likely to have a considerable contribution. A glimpse of the RSD impact on the Brazilian sugarcane industry can be inferred from a report that indicated a 26.2% reduction in the tonnage yield of one of the most widely planted varieties (RB867515) in a 3-year comparison trial between Lxx-inoculated and non-inoculated plants (Gagliardi & Camargo, 2009).

Lxx is a xylem-limited bacterium with no seed transmission, no known insect vector, no saprophytic activity and no other known natural host other than sugarcane (Davis & Bailey, 2000; Gillaspie & Teakle, 1989; Grisham, 2004; Zavaglia et al., 2016). Once introduced in a field, dissemination to healthy plants occurs when the cutting blades used in harvesting carry the sap of diseased canes to healthy ones (Hoy et al., 1999). Thus, the adoption of pathogen exclusion measures is critical for the success of RSD control. The renewal of sugarcane fields occurs annually in 15%–20% of the cropped area in Brazil, thus affecting the incidence of the disease for many years. Control procedures adopted in Brazil during the renewal of the fields with direct effect on RSD comprise an inter-culture period, destruction of volunteer plants and the use of healthy seedcane.

Two distinct methodologies largely employed in Brazil to produce healthy seedcanes include the pre-sprouted seedcane, or MPB following its Portuguese abbreviation, developed by the Instituto Agronômico de Campinas (Landel et al., 2012), and the mechanical billet planting (Barbosa, 2013). In the first, only a percentage of a field is planted employing young sugarcane seedlings, leaving room for concomitant cultivation of other crops, such as soybean or ground peanut, a system known as MEIOSI (Portuguese acronym for inter-rotational method occurring simultaneously), which reduces the cost of sugarcane renovation; after the grain harvest, young canes are used as seedcane to form the whole new sugarcane field (Coury, 2019). In the second, healthy stalks from commercial fields at the plant cane or first ratoon are selected, submitted to a lab-diagnostic test, heat treated and used to establish nurseries for seedcane production (Barbosa, 2013). Plantings using MPB seedcane comprised 9.2% of the total, whereas those using sugarcane stalks comprised 90%; the remaining 0.3% relied on tissue culture-generated planting material (Braga Jr et al., 2020).

The lack of characteristic symptoms of RSD makes laboratory-based diagnosis critical for Lxx identification with the availability of various techniques such as microscopy, serological and DNA-based methods (Grisham, 2004). The technology employed by the Brazilian industry varies according to the seedcane origin. PCR-based diagnostic test is mostly used to certify MPB seedcanes because fewer mother plants need to be tested, making it affordable to companies (Landel et al., 2012). On the other hand, the serology technology is still preferred for indexing the mechanical billet planting material since large numbers of samples per field are tested in a cost-efficient manner (Ponte et al., 2010; Urashima et al., 2020).

Two combinations of thermotherapy are employed in Brazil: a hot water treatment (HWT) at 50°C/2 h (Matsuoka, 1984) and 52°C/30 min (Sanguino et al., 1988). HWT efficacy showed inconsistent results as Lxx was still detected after 52°C/30 min treatment by serology (Fernandes Jr et al., 2010), by quantitative PCR (qPCR) (Carvalho et al., 2016) or nested PCR (Dias et al., 2019). On the other hand, Lxx was not detected after the same HWT, either by microscopically (Sanguino et al., 1988) or conventional PCR methods (Dias et al., 2019). The 50°C/2 h HWT also presented mixed results with some studies reporting 100% pathogen elimination (Bailey & Bechet, 1986; Flynn et al., 2005; Zvoutete, 2004) and others not (Damann Jr & Benda, 1983; Grisham et al., 2007).

The factors that affect the management of RSD by HWT include cultivar characteristics (Damann Jr & Benda, 1983), diagnostic techniques used to assess its efficiency (Hoy et al., 1999), HWT conditions (Fernandes Jr et al., 2010) and the initial Lxx titres of sugarcane setts (Carvalho et al., 2016). However, integrated information cannot be inferred from these studies since they did not evaluate multiple factors at the same time. Clarification on the exact contribution of these factors combined is

critical to improving the effectiveness of the exclusion methods. Therefore, the present work aimed to examine the effects of the interaction of diagnostic techniques, initial Lxx titres, sugarcane cultivar and HWT conditions on the RSD control.

MATERIAL AND METHODS

Sugarcane cultivars

Sugarcane cultivars employed in this work encompassed RB966928, RB867515 and RB92579, ranked first, second and fourth among the most planted cultivars in the 2017/18 crop season (Braga Jr et al., 2018), representing 43.5% of the cultivated area in Center–South region, that is, the most important sugarcane yield area in Brazil (90% of production area) (CONAB, 2020). Previously, all three were considered highly susceptible due to the high presence of Lxx titres in dot-blot serological analysis (Urashima et al., 2020). Also, Gagliardi and Camargo (2009) had already classified RB867515 as a susceptible cultivar, using an artificial inoculation approach where they showed a yield loss of 26.2%, the highest among 10 commercial varieties of the assay. These cultivars were grown at the Center of Agriculture Science of the Federal University of São Carlos in Brazil, and harvested at 11–12 months at the plant cane stage. The number of buds per stalk varied from 10 to 12 and their initial bacterial titres were determined by the dot-blot immunoassay (DBI) from the most basal internode of the stalk as in previous research (Comstock et al., 1996; Hoy et al., 1999). The infected stalks used in this work showed initial bacterial titres varying from 10^6 cfu/ml to 10^8 fu/ml.

Ratoon stunt control

Single Lxx-infected buds of the three cultivars were submitted to two thermotherapy methods (here abbreviated as HWT): 50°C/2 h or 52°C/30 min in a 2000 L tank. Also, two other situations (treatments) were considered: the positive control (RSD-positive) consisted of canes infected with Lxx, which levels varied from 10^6 cfu/ml to 10^8 cfu/ml, and the negative control (RSD-free), which consisted of healthy canes (Lxx below the threshold of 10^6 cfu/ml); for all cases, DBI technique was considered. Both were treated with water at room temperature for 2 h. The buds were planted in $39 \times 28 \times 10$ cm plastic trays containing a mixture of soil and substrate (1:1 v/v) after cooling under natural conditions. The resulting plantlets were kept in a greenhouse for 2–3 months before being transplanted to the field.

Trial A – with large plots

In this field, the experiment plots consisted of 20 seed-canes of each of the three varieties (RB867515, RB92579 and RB966928) subjected to the four-ratoon stunt controls (HWT at 50°C/2 h, HWT at 52°C/30 min, RSD positive and RSD free) planted in two 5-meter rows with a spacing of 0.5 m between plants and 1.6 m between rows. Plots were replicated five times totalling 60 plots arranged in a randomized complete block design. Replacement of dead plants was carried out right after planting to maintain the original number of plants.

Plants were classified as diseased or disease-free by the dot-blot immunoassay methodology as in previous research (Croft, 2002; Harrison & Davis, 1988). The initial Lxx level (before the ratoon stunt control) could have varied between the seedcanes. To avoid bias, all the 20 seedcanes of each plot were grouped for similar initial Lxx titres. The differences between the plots for the initial Lxx titres were also modelled for further investigation. The experiment can be statistically described by the following model:

$$y_{ijkt} = \mu + b_k + L_{ijkt} + c_i + r_j + cr_{ij} + e_{ijkt},$$

where, y_{ijkt} was the response variable evaluated by the percentage of health plants per plot (PHP) obtained for either DBI or PCR techniques for the ith cultivar, jth RSD control conditions, kth block and tth initial Lxx titres; μ was the model intercept; b_k the fixed effect for blocks, which varied from 1 to 5; c_i the fixed effect for cultivars, where three levels were observed (RB966928, RB867515 and RB92579); r_j the fixed effect for ratoon stunt control (HWT for 50°C/2 h, 52°C/30 min, RSD positive and RSD free); cr_{ij} the fixed factor for interaction between cultivars and RSD control conditions; and e_{ijkt} the error term. The level of initial Lxx infection for each plot was modelled as a covariate L_{ijkt}. R software (R core team, 2020) was used to perform the ANOVA, using the functions *lm* and *anova*. Here, to obtain the sum of squares, we performed a sequential adjustment of the factors (default of these functions), the same defined in the statistical model. When F test (5%) was significant, the Tukey test (5%) was performed to identify differences between the levels, using the function *HSD.test* from the library agricolae.

Plot size simulation study

A simulation study based on a bootstrap approach was performed to indicate the best plot size. Considering that the experiment had 60 experimental units (plots) with 20 plants each (full dataset), a resampling with replacement was done to generate new datasets where the plot size

assumed sizes of 5, 10, 15 and 20 plants. For each dataset, ANOVA was performed and the residual coefficient of variation was calculated. The procedure was repeated 10,000 times, generating an empirical distribution of the CV for each plot size. The distribution of CV values over different plot sizes helped to infer the precision of Trial A. This approach was applied for PHP obtained by both DBI and qualitative PCR diagnosis methods.

Trial B – with small plots

A second field experiment was conducted using seedcanes of RB966928 with known Lxx titres. Here, three ratoon stunt controls (HWT at 50°C/2 h, HWT at 52°C/30 min and RSD-positive) over two initial Lxx titres (10^6 and 10^8 cfu/ml) were considered, comprising 3×2 factorial. The experimental unit was a single sugarcane plant, as used in Carvalho et al. (2016), and 10 repetitions under a randomized complete blocks design was used. The experiment was repeated twice as biological replicates.

The plants were screened for the presence of Lxx by PCR and DBI, 9 months after planting.

The data analysis approach was based on the contingency table statistical tests. Three ratoon stunt controls were tested for a similar proportion of diseased plants for the 10^6 cfu/ml initial Lxx titre (H_{01}) and the 10^8 cfu/ml initial Lxx titre (H_{02}). Also, it was investigated if the two HWT (50°C/2 h and 52°C/30 min) proportions of the diseased plants varied between them considering the 10^6 cfu/ml initial Lxx titre (H_{03}) and the 10^8 cfu/ml initial Lxx titre (H_{04}).

The hypotheses H_{01} and H_{02} were tested using the Chi-square contingency table using the function *chisq.test*, and H_{03} and H_{04} were tested using the Fishers exact test by *fisher.test* function, both run on R software. For all the cases, the pooled data from the biological replicates were used.

Cane sampling and Lxx quantification

The main stalk of the stools, identified at the time of transplant, was manually harvested from each plant/treatment 9 months after planting, totalling 100 canes per treatment in experiment A (20 plants per plot × 5 repetitions) and 20 (1 plant per plot × 10 repetitions × 2 biological trial replicates) in experiment B. Harvesting tools were heat sterilized after each cutting.

Quantification of lxx

DBI and PCR tests were used to quantify or detect Lxx in the harvested canes respectively. Plants were within the recommended age (9-month-old) to be harvested and employed as seedcanes in commercial fields (Landel et al., 2012). For the DBI, sugarcane sap from the most basal internode was extracted by an air compressor soon after samples were taken from the field. A volume of 100 μl was applied to the nitrocellulose membrane, heated, blocked with non-fat milk, incubated with polyclonal antibodies and the presence of Lxx was examined by indirect enzyme-linked immunosorbent assay (ELISA) employing rabbit anti-Lxx immunoglobulin G (IgG), followed by alkaline phosphatase-labelled goat anti-rabbit IgG. The advantage of this technique is the possibility to quantify bacterial titres by comparing the gradient of blue-coloured dots on the membrane to positive controls of known bacterial titres, the faintest blue corresponding to a threshold of 10^6 cells/ml, increasing to 10^7 cells/ml and 10^8 cells/ml and the darkest blue to 10^9 cells/ml (Croft, 2002; Harrison & Davis, 1988; Leaman et al., 1991; Urashima et al., 2020). The negative control consisted of sugarcane sap from a stalk diagnosed as RSD free by PCR. An example of the diagnostic test by DBI is given in Supplementary Figure S1. Because of subjective visual evaluation, the quantification was always carried out by at least two individuals. Quantification of Lxx was performed in plants before treatments as well as 9 months after treatments.

The PCR analysis was carried out in DNA extracted from sugarcane tissue of the most basal internode by the CTAB method. The molecular diagnostic test employed primers *Cxx*ITSf#5 and *Cxx*ITSSr#5 (Fegan et al., 1998) in a volume of 25 μl containing 1× amplification buffer, 1.5 mM MgCl2, 200 μM dNTPs, 0.25 μM primer, 50–100 ng of total DNA and 0.5 unit of Taq polymerase. Amplification conditions by Taylor et al. (2003) followed the adaptation of Oliveira and Urashima (2018) with 5 min at 96°C, followed by 35 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 30 s and finished with 72°C for 10 min. The thermal cycler Gene Amp PCR System 9700 (ThermoFisher Scientific) was employed. PCR products were separated in 2% agarose gel in 0.5× tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer. Subsequently, they were stained with ethidium bromide, visualized with ultraviolet (UV) and photographed (L-PIX-HE, Loccus biotecnologia). The DNA size marker employed was 100 bp DNA ladder (Sinapse Inc). Amplification of a 305 bp is expected in Lxx-positive samples.

RESULTS

Considering trial A, the ANOVA of the percentage of healthy plants of the three sugarcane cultivars submitted to four ratoon stunt controls evaluated by DBI or PCR provided similar results (Table 1). The CV values were

TABLE 1 Analysis of variance for the percentage of healthy plants diagnosed by dot-blot immunoassay (DBI) and qualitative PCR. Here, three major cultivars in the Brazilian Central–South region were used (RB966928, RB867515 and RB92579) and four ratoon stunt controls (RSD positives, and RSD free and two thermotherapies: 50°C/2 h and 52°C/30 min) were considered. Once the initial concentration between plots has varied (lower than 10^6, 10^6, 10^7 and 10^8 cfu/ml), the initial lxx titres were used as a covariate for avoiding bias between RSD control, especially for thermotherapy results

| | | Diagnostic technique | | | |
| | | Dot-blot immunoassay (DBI) | | Polymerase chain reaction (PCR) | |
Source of variation	DF	F test	p-value	F test	p-value
Replication	4	2.3636	0.0684	2.6444	0.0467
Initial Lxx titre	3	10.2942	<0.001	10.4163	<0.001
Ratoon stunt control (R)	2^a	31.3021	<0.001	33.3932	<0.001
Cultivar (C)	2	0.6382	0.5333	0.5239	0.5960
R×C interaction	6	0.1644	0.9848	0.2491	0.9570
Residual	42				

Note: DBI: CV = 22.99, average = 84.41%.PCR: CV = 23.02%, average = 83.71%.

[a]1 degree of freedom has been lost due to collinearity with initial Lxx titres.

22.99% (DBI) and 23.02% (PCR). There was no interaction between cultivars and ratoon stunt control (*p*-value of 0.9848 for DBI and 0.9570 for PCR, respectively). Block effects were significant for PCR (*p* = 0.0467) but not for DBI (0.0684). No significant cultivar effects were detected for both techniques (*p*-values of 0.6382 and 0.5960 for the DBI and the PCR, respectively); however, significant effects were detected among water treatments and bacterial titres (*p*-value >0.001). Also, the degree of freedom for RSD control was reduced to 2 instead of 3 (Table 1), as a consequence of the sequential adjustment in data analysis, that is, it first included the initial Lxx titres and then ratoon stunt control. This happened because variation in bacterial titres was observed for three ratoon stunt controls (RSD positive, and the two thermotherapies) but not for the RSD free (initial Lxx titre <10^6 cfu/ml). In other words, we identified the variation between ratoon stunt control even previously correcting for the variation originated for the initial Lxx titres.

A further insight was performed on the ratoon stunt control and initial Lxx titres due to their influence on the phytosanitary condition of plants after 9 months in the field (Figure 1). Considering Figure 1a, Tukey test (5%) was done for the DBI and PCR diagnosis methods, with similar results. Briefly, PHP did not differ between both HWTs and the RSD-free control but was lower than the RSD positive, indicating that both HWTs were effective in controlling Lxx.

Tukey test (5%) was also applied to assess how the initial bacterial titres influenced PHP (Figure 1b). When the initial titres were non-detectable by DBI (below 10^6 cfu/ml), the PHP in seedcanes, diagnosed by both techniques 9 months later, was significantly higher than in those with initial Lxx titres of 10^7 cfu/ml or higher.

As the Lxx initial titres differed among plots, a second analysis was carried out to compare the PHP among plots with the same Lxx initial titres class. For this, only plots with 10^6 cfu/ml were considered since they were the most frequent ones. The statistical model was the same presented without the covariate Lxx initial titres. As in the previous analysis, the PHP evaluated by both DBI and PCR gave similar results, where only the RSD-positive control differed from the other treatments (*p*-values of 0.004238 and 0.003937 for the DBI and the PCR, respectively). Besides, the other sources of variation were not significant (Table 2). For ratoon stunt control, the Tukey test did not differ between the long and short HWT, but both significantly differ from the RSD positive by the DBI and PCR techniques; no difference was observed among cultivars towards a specific treatment (data not shown).

Despite the efficiency of both HWTs in controlling Lxx, they did not eliminate the pathogen (escapes). Here, considering both thermotherapies (50°C/2 h and 52°C/30 min) and diagnostic tests (PCR and DBI) performed for 600 canes (300 for each one), the first HWT failed to eradicate the pathogen in eight plants (2.7% of failure), whereas the second failed in 35 plants or 11.7% (Table 3). Concerning diagnostic techniques, PCR detected 23 diseased canes from the 600 (3.8%) submitted to HWT while DBI identified 20 Lxx-positive samples (or 3.3%). Therefore, this technique detected plants previously considered non-infected, and in doing so it improves measures of RSD control. Moreover, the PCR technique detected the bacterium at titres below the DBI threshold (10^6 cfu/ml), indicating its higher sensitivity. Further analysis of the effect of different HWT on seedcanes with high Lxx titres was not possible due to the limited number of seedcanes (20 samples in each HWT). In a more

FIGURE 1 (a) Tukey test (5%) to compare the percentage of healthy sugarcane plants for the different ratoon stunt controls, where RSD positive are the plants with initial presence of lxx, but with no thermotherapy applied; RSD free are the ones with no initial lxx presence and no thermotherapy; and 50°C/2 h and 52°C/30 min are the thermotherapies procedure to the plants with a known lxx presence in order to control RSD. The capital letters and lower-case letters indicate a comparison for dot-blot immunoassay (DBI) method and qualitative PCR respectively. (b) Tukey test (5%) to the percentage of healthy sugarcane plants over the lxx initial titre. Again, capital letters indicate the results for DBI, and lower-case letters indicate results for qualitative PCR.
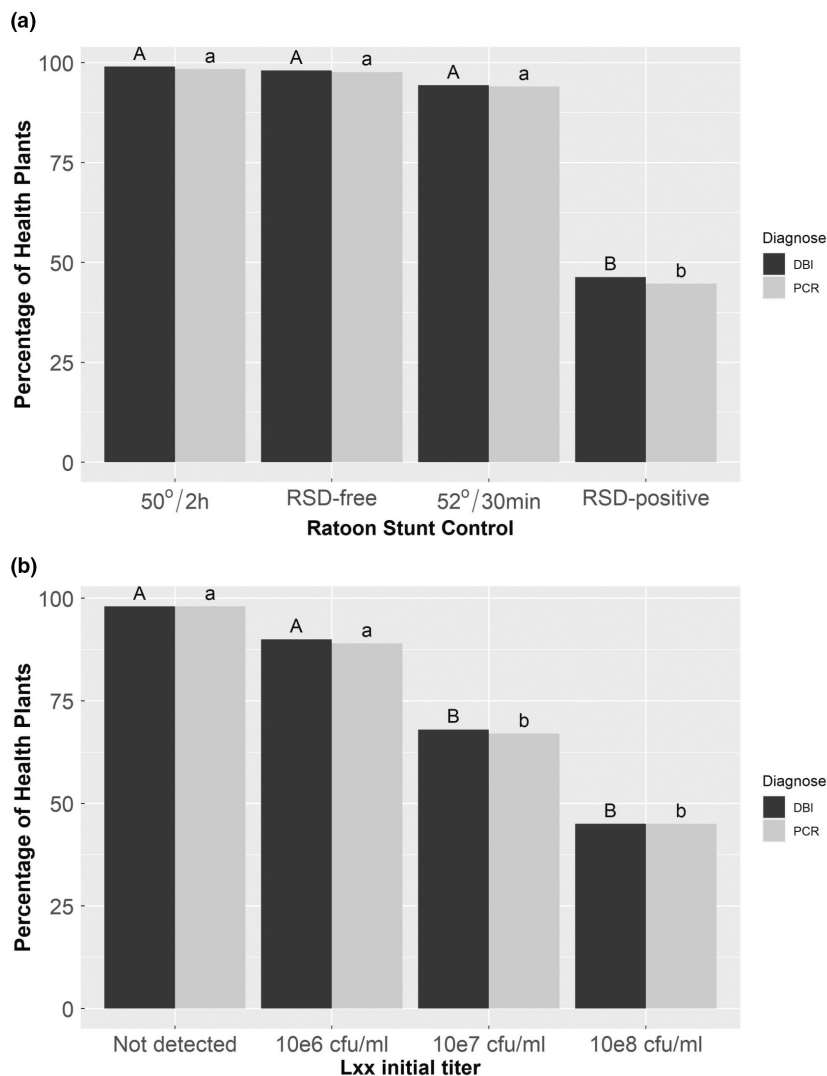

TABLE 2 Analysis of variance considering only the subset of plots with similar titres (10^6 cfu/ml) of *Leifsonia xyli* subsp. *xyli* diagnosed by dot-blot immunoassay (DBI) and qualitative PCR. The three most sugarcane cultivated varieties in the Brazilian Central–South region cultivars were used (RB966928, RB867515 and RB92579), and submitted to three ratoon stunt controls (RSD positives, and two thermotherapies: 50°C/2 h and 52°C/30 min)

		Diagnostic technique			
		Dot-blot immunoassay (DBI)		Polymerase chain reaction (PCR)	
Source of variation	DF	*F* test	*p*-value	*F* test	*p*-value
Replication	4	2.4802	0.0856	2.5018	0.0837
Ratoon stunt control (R)	2	7.8024	0.0043	8.3036	0.0034
Cultivar (C)	2	0.5257	0.6010	0.6030	0.5591
R × C Interaction	4	0.8298	0.5256	0.8982	0.4880
Residual	16				

Note: DBI: CV = 18.59%, average = 89.64%. PCR: CV = 19.64%, average = 88.95%.

representative population of 160 and 240 seedcanes with Lxx titres of 10^6 cfu/ml submitted to long HWT and short HWT, respectively, the first was highly efficient since no diseased plants were identified, contrasting to 17 detected among 240 seedcanes in the short HWT (7.1% of failure).

The analysis of the incidence of escapes per variety showed RB962928 had the highest number of diseased plants after HWT with 18 diseased plants among 200 submitted to short or long HWT, representing 9% of escapes; a noticeable difference from RB867515 with four canes (2%)

TABLE 3 Number of diseased plants diagnosed by distinct techniques after two different hot water treatments (HWT) of seedcanes contaminated by different *Leifsonia xyli* subsp. *xyli* (lxx) titres

HWT	Initial lxx titre[a]	Total number of plants	PCR technique				Dot-blot immunoassay technique			
			↓N4	N4	N3	N2	↓N4	N4	N3	N2
Long[b]	N2	20	0	3	0	0	0	3	0	0
	N3	120	2	0	0	0	0	0	0	0
	N4	160	0	0	0	0	0	0	0	0
Short[c]	N2	20	0	1	0	0	0	1	0	0
	N3	40	0	1	7	0	0	1	7	0
	N4	240	1	5	2	1	0	5	2	1

[a]N2 = 10^8 cfu/ml, N3 = 10^7 cfu/ml, N4 = 10^6 cfu/ml, ↓N4 = below1 0^6 cfu/ml.

[b]50° C for 2 h.

[c]52° C for 30 min.

and RB93579 with one (0.5%) (Table 4). Some other results worth mentioning were as follows: (a) Planting RSD-free seedcanes of RB966928 by DBI did not guarantee healthy canes, suggesting high Lxx multiplication within its tissue from an undetectable bacterial load in seedcanes to N4 (10^6 cfu/ml) in 9 months; (b) Planting Lxx-infected seed-canes did not result in all RSD positive canes, suggesting that sugarcane sap from a 9-month- old plant is not adequate for DBI diagnoses; (c) the short HWT (52°C/30 min) was not an efficient treatment, regardless of the sugar-cane cultivar since RSD-diseased canes were identified in all three cultivars; and (d) the long HWT showed a better performance than the short one, but its efficiency is dependent on Lxx titres, as observed when seedcanes of RB867515 with high bacterial density (10^8 cfu/ml) resulted in three diseased canes even after the long HWT.

The bootstrap simulation allowed examining the robustness of the adopted sample size for trial A. In general, the distribution of CV values was symmetrical for all plot sizes regardless of the detection method (Figure 2); this is also corroborated by comparing the means and median values since the difference between them occurs only at decimal levels (data not shown). A minimum CV value of approximately 20% was observed for the four sample sizes (5, 10, 15 and 20). The main difference was observed for maximum CV values where they decreased with increasing sample size for both detection techniques. For the DBI, the maximum CV values were 34.21%, 32.22%, 29.14% and 27.27% for the sample sizes of 5, 10, 15 and 20, respectively, and for the PCR they were 34.18% (5 plants), 32.64% (10 plants), 29.31% (15 plants) and 28.07% (20 plants). Still, for plot size of 5, 181 (DBI) and 231 (PCR) simulations presented CV higher than 30%. This number was reduced to 8 (DBI) and 64 (PCR) for the sample size of 10 plants per plot, and for 15 and 20 plants per plot, no observations were noted. Regarding these pieces of information, the best results were observed for plot size of

15 or higher, indicating that this experiment had a good precision.

Finally, in experiment B (conventional statistical design), the results of PCR and DBI were the same (Table 5). Essentially, the results were grouped in a three-way table represented by initial Lxx titres, HWT and the presence/absence of diseased canes. We worked with the pooled results from both replicates; however, the results from each experiment were indicated in parentheses, for example, for the non-HWT and high Lxx titres (N2), 17 plants with the presence of Lxx were observed, where eight came from one experiment and the second came from the second experiment. The same idea was applied to the other cells of the table. For hypotheses H_{01} and H_{02}, the Chi-square test values were significant (55.82 and 38.63, respectively), with a *p*-value <0.001 indicating that there is a difference between the three ratoon stunt controls, regardless of the initial Lxx level. On the other hand, the hypotheses H_{03} and H_{04} were not significant (*p*-value = 1), that is, there is no significant difference between 50°C/2 h and 52°C/30 min HWT. Still, the escapes of both HWTs were found, one for 52°C/30 min and two for 50°C/2 h.

DISCUSSION

Data of the present work illustrate why RSD is one of the most difficult sugarcane diseases to control. All the four factors examined in the present work (initial Lxx titres, thermotherapy condition, cultivar and diagnostic techniques) affected the percentage of healthy plants when examined from the epidemiological point of view. To reach such a conclusion, data were analysed considering the statistical analysis as well as the perspective of the disease epidemiology, where the presence of any failure to control RSD should be considered critical because once the planting material is established in a field, it is mechanically

TABLE 4 Number of diseased plants diagnosed by blot immunoassay in three different sugarcane cultivars with distinct phytosanitary status for ratoon stunt

| | RB867515 | | | | RB92579 | | | | RB966928 | | | |
| | Treatments | | | | Treatments | | | | Treatments | | | |
	H[b]	D[c]	52°C/30 min	50°C/2 h	H	D	52°C/30 min	50°C/2 h	H	D	52°C/30 min	50°C/2 h
Initial Lxx titre[a]	ND[d]	N4,N3	N4,N2	N4,N3,N2	ND	N4,N3	N4,N3	N4,N3	ND	N4,N3,N2	N4,N3	N4,N3
Final Lxx titres												
N1	0	0	0	0	0	0	0	0	0	0	0	0
N2	0	0	0	0	0	23	0	0	0	33	0	0
N3	0	15	1	0	0	23	0	0	0	27	7	0
N4	0	32	0	3	0	0	1	0	8	2	11	0

[a]N1 = 10^9 cfu/ml, N2 = 10^8 cfu/ml, N3 = 10^7 cfu/ml and N4 = 10^6 cfu/ml.

[b]Healthy plants.

[c]Diseased plants.

[d]Not Detected (↓10^6 cfu/ml).

harvested multiple times and vegetatively increased and, therefore, very different magnitudes of disease increase would result from small initial incidence and detection differences. In doing so, we believe that our data will be more useful for the sugarcane industry since important particularities of the causal agent of RSD were also examined. This approach has rarely been done, if ever, and it is a key point for better RSD control, a major sugarcane disease. For example, analysis of variance showed that only the initial Lxx titres and water treatments affected the PHP (Table 1), the Tukey test did not reveal differences between the long and short HWTs or between the DBI and PCR diagnostic techniques (Figure 1); however, both HWT showed failures (Table 3). Considering our dataset under the epidemiological point of view, the HWT at 52°C/30 min showed higher failure than the HWT at 50°C/2 h in Lxx control. This information should be considered for future studies and regarded in a careful reading when the strategy of full pathogen exclusion is adopted. Both HWTs showed failures to control Lxx, but the short HWT resulted in more diseased plants than the long one (Table 3). From the epidemiological point of view, the lower efficiency of the short HWT to control Lxx should be considered because one single diseased seedcane has a huge impact on Lxx spread, jeopardizing the whole strategy based on pathogen exclusion since it spread Lxx to a distance varying from 1.5 to 12.9 m by harvester blades (Hoy et al., 1999). Besides, the ratooning nature of sugarcane with multiple harvests strongly favours the incidence of RSD over successive crops (Comstock et al., 1996). Therefore, small numbers of escapes can have a tremendous effect on RSD over a long period.

Our findings showed that the RSD control methods adopted by the Brazilian sugarcane industry still have room to improve. One change is already underway; for example, the replacement of the short HWT with the long one, following the results of previous works (Fernandes Jr et al., 2010; Urashima & Grachet, 2012). The number of materials for HWT justified the preference of the industry for the short HWT since four times more materials are treated per time unit, one of the most important aspects to consider once the area of nursery formation covered 214,000 hectares in the 2020–2021 crop season (CONAB, 2020). The higher number of diseased plants verified on the short HWT in our work confirmed its lower efficiency, as already observed in previous research (Dias et al., 2019; Fernandes Jr et al., 2010). However, the change from short to long HWT does not guarantee the same outcome for all sugarcane varieties. To achieve a better RSD control by the long HWT, knowledge of varietal resistance is paramount, as shown in our work when the long HWT failed to eliminate Lxx in seedcanes of RB867515 infected with high bacterial titres (Table 4). Such a failure by the long
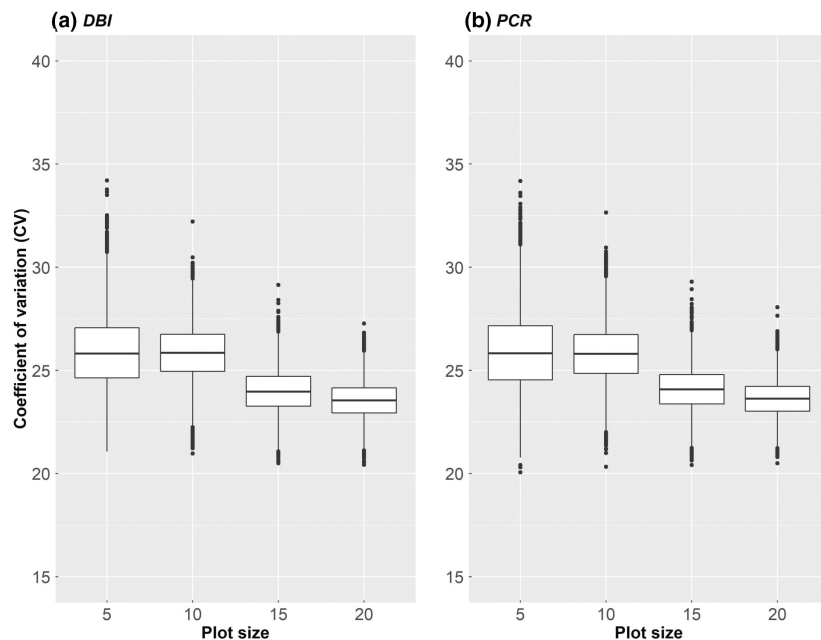
(a) *DBI*

(b) *PCR*

FIGURE 2 Bootstrap simulation to investigate ideal plot size. Ten thousand bootstraps were carried out for sample sizes of 5, 10, 15 and 20. The empirical distribution was obtained based on the coefficient of variation: $\left(CV = \frac{\sqrt{\text{Residual Mean Square}}}{\bar{x}} \right)$, where, for each iteration, the composition of each experimental unit was simulated based on the full dataset, and the percentage of healthy plants, ANOVA and CV were recalculated. The procedure was done for dot-blot immunoassay (DBI) and qualitative PCR diagnosis method.

TABLE 5 Summarization of experiment B based on RB966928 using one seedcane as the experimental unit. The presence and absence of *Leifsonia xyli* subsp. *xyli* (lxx) was studied for three hot water treatments (HWT) strategies under two initial lxx titres (N2 = 10^8 cfu/mL and N4 = 10^6 cfu/mL), with 10 replications. This experiment was conducted twice

Initial lxx titres	Response variable	Non-HWT	52°C/30 min	50°C/2 h	Total
N2	Presence	17 (8 + 9)[a]	1 (1 + 0)	1 (0 + 1)	19
	Absence	3 (2 + 1)	18 (9 + 9)	19 (10 + 9)	40
N4	Presence	20 (10 + 10)	0	1 (0 + 1)	21
	Absence	0	20 (10 + 10)	19 (10 + 9)	39
N2 + N4	Presence	37 (18 + 19)	1 (1 + 0)	2 (0 + 2)	40
	Absence	3 (2 + 1)	38 (19 + 19)	38 (20 + 18)	79

[a](8 + 9) number of plants showing the presence of Lxx in the first and second experiments respectively.

HWT was already observed, however, the cultivar role had not been taken into account (Damann Jr & Benda, 1983; Grisham et al., 2007)

Cultivars' reaction to RSD varied in the present study because a direct association was identified between cultivar resistance and Lxx population within infected sugarcane stalks (Comstock et al., 1996). Seedcanes of all three cultivars were RSD free in the healthy treatment, that is, the Lxx titres were below 10^6 cfu/ml, the threshold of the DBI diagnostic technique (Leaman et al., 1991). However, 9 months after transplant, RSD-diseased cane plants were detected in the RSD-free treatment of RB966928 by the same diagnostic technique, indicating that Lxx reproduced within this cultivar but not in RB867515 and RB92579. Other observations suggesting that RB966928 is more susceptible to RSD than the other two include the recovery of more diseased plants and plants with higher Lxx titres after the HWT (Table 4). Since susceptible cultivar allows higher Lxx multiplication within its tissue,

a greater epidemiological impact is expected with more efficient pathogen spread to healthy plants and higher yield loss (Davis et al., 1988; Hoy et al., 1999). Despite its importance, investigations on cultivar resistance to RSD have not been conducted in Brazil; one of the few showed that RB855156, the fifth most important cultivar for re-establishing new sugarcane fields, had a 5.3% yield loss, whereas RB867515, the second in preference, had 26.2% (Braga Jr et al., 2018; Gagliardi & Camargo, 2009). Whether this difference was due to resistance to Lxx growth in vascular bundles or cultivar tolerance to the pathogen remains unclear because Lxx titres were not evaluated. When the bacterium population within sugarcane stalks was measured, high Lxx titres were observed in all major Brazilian cultivars: RB966928, RB92579, RB867515, RB855156, RB855453, CTC4 and SP81-3250 (Urashima et al., 2017; Urashima et al., 2020); nonetheless, cultivar resistance could not be determined since the Lxx multiplication rate was not measured. Therefore, this

instrumental epidemiological aspect for improving RSD control strategies is still unavailable in Brazil. Another feature revealed by our work was that the infection incidence in the RSD-positive control was around 50%. One plausible cause for this false-negative result is the plant age. Lxx is a fastidious bacterium with slow growth in xylem bundles, the reason why yield depletion was not observed in plant cane of many cultivars in South Africa (Bailey & Bechet, 1997), and in Florida and Louisiana, US (Comstock, 2008; Grisham et al., 2009). Moreover, a previous study did detect Lxx in cane plants at this age either by dot-blot or PCR (Urashima & Grachet, 2012). In short, the age of plant cane employed as seedcanes has a strong effect on the diagnostic test and plays an important role in the success of RSD control

One important measure to improve the long HWT while cultivar reaction to RSD is unknown is the origin of stalks to be used as seedcanes. Our data showed that the long HWT was successful on sugarcane cuttings when bacterial titres were low, which is frequently the case of the first-year crop plants; Lxx increased as ratoon cycles progressed (Carvalho et al., 2016; Flynn et al., 2005; Zvoutete, 2004). Nevertheless, for the success of this approach, it is essential to use plant cane of certified origin. The Brazilian sugarcane industry is very dynamic and changes rapidly its cultivar preferences. For instance, the cultivar RB92579 occupied 6164 ha or 1.9% of the reform area in 2009 and increased to 40,832 ha or 9.2% in 2015 (Chapola et al., 2010; Chapola et al., 2016). Similarly, CTC4 represented 7.7% of new sugarcane fields in 2015 and 14.3% in 2018 (Braga Jr et al., 2018; Chapola et al., 2016). These rapid increases in such a limited period probably came at the expense of using sugarcane stalks from various harvests, resulting in planting setts with high Lxx titres, thus rendering in an ineffective HWT, even when employing the 50°C/2 h combination.

The Brazilian sugarcane industry comprises a large mosaic of mills that add up to more than 400. Some are aware of the importance of RSD and employ strategies for its control, including a clean seedcane programme, sanitization of equipment and nursery monitoring. Others use only one due to financial constraints, lack of technical support and logistic problems. It is unknown how many include laboratory diagnostic tests to certify their seedcane. When done, the DBI test is the one employed for seedcane production when billet planting is involved (Ponte et al., 2010; Urashima et al., 2017). We understand that there will be no replacement of the serological methodology by the PCR methodology for this planting system because of the large number of samples, reflecting on its cost and jeopardizing PCR acceptance. Therefore, measures to improve RSD control strategies are necessary for the DBI. One measure found by the

present work involves improving HWT by discarding seedcanes with high Lxx titres, that is, those prone to fail in the heat treatment

The use of PCR for RSD diagnosis has advanced in Brazil due to the MPB technology. This methodology accounted for 9.1% of the planting system in 2019 and is becoming popular because of the higher uniformity of young plants, fewer seedcanes needed and higher phytosanitary status (Braga Jr et al., 2020; Landel et al., 2012). As the buds for MPB originate from mother plants whose phytosanitary status should be certified by PCR (Landel et al., 2012), the use of PCR for RDS diagnostic testing is expected to be more widespread in the near future as the MPB technology increases

Our work has identified factors affecting the effectiveness of RSD control, as well as strategies to improve the DBI and the PCR diagnostic test results that can be readily adopted to improve RSD management in the Brazilian sugarcane industry.

Finally, another information revealed by our work referred to plot size in field experiments. Here, we consider that large number of evaluated plants is necessary but defining an ideal number of plants for plot size is not easy, especially for studies that aim to measure the effectiveness of RSD control. For this, we performed 10,000 bootstrap simulations where plot sizes varied from 5 to 20 plants. Considering the coefficient of residual variation as an indicator of precision, the best results were verified for 15 plant-plots, indicating a good accuracy of our data (Figure 2) as in this study each cultivar was evaluated based on 100 plants (five replicates of 20). The choice of 20 plants was based on the experimental units used in the sugarcane breeding programme conducted by UFSCar/RIDESA (Cursi et al., 2021). The statistical procedure adopted in our work with an experimental unit formed by many seedcanes (20 in our study) was compared to the conventional one, in which the experimental unit has one or few plants in a duplicated trial (Carvalho et al., 2016; Grisham et al., 2007). Although results from these two approaches showed similar statistical results, our approach performed better because a large number of plants per plot allowed measuring and detailing failures in the HWT not identified in the conventional statistical design, which could lead to serious epidemiological consequences (Tables 4 and 5). Also, large plots are valuable for inferring cane production in a more realistic way than single plants. Using the information obtained here, it is possible to infer that large plot trials have the advantage of merging accurate results from the epidemiologic perspective with biometrical measures, such as stalk weights. Still, the conventional approach with small plots has some difficulties, especially if the plot has a single plant and the

response variable relies on a binary context, jeopardizing data interpretation; the statistical analysis should be used either by non-parametric methods or generalized linear models. Therefore, we believe that the statistical design adopted in this work provided data robustness and was a very useful tool in field research and should be adopted for future field studies.

CONFLICT OF INTEREST
No conflict of interest declared.

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