Prevalence and Severity of Ratoon Stunt in Commercial Brazilian Sugarcane Fields

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Abstract

Brazil has 9 million ha of sugarcane, 85% of which are located in the Center-South area of the country. Field trials and surveys around the globe have shown that ratoon stunt disease (RSD), caused by *Leifsonia xyli* subsp. *xyli*, can severely reduce tonnage yield. Previous small-scale studies in Brazil have demonstrated RSD infection in all varieties, with values varying from 25 to 68%. Nevertheless, the prevalence and severity of RSD in commercial fields had not previously been assessed. To address this issue, we surveyed 13,173 ha in 1,154 fields of the eight main sugarcane varieties of the Center-South area, taking 92,114 samples from 50 mills in five different states. Our data showed that 10% of fields were infected, and that 58% of mills had at

least one RSD-infected field. The variety RB92579 had the highest proportion of infected fields (17%) and, on average, the prevalence and severity in these fields was high compared with other varieties. RB867515, the most cultivated in Brazil, showed infection in 6.2% of sampled fields (5.5% of sampled area) causing an estimated annual economic loss of over US\$1 million. This was the first time the economic importance of RSD on Brazilian commercial sugarcane production was estimated. The Cerrado region had the highest prevalence of RSD: 16% of fields, 17% of the cultivated area, and 82% of mills. The use of diseased planting material was identified in 9% of plant cane fields, representing 10% of the cultivated area.

Brazil is the world's largest producer of sugarcane (*Saccharum* spp.), producing 728.1 million tons in 2015, more than the total production of India, China, Thailand, and Pakistan, the next top four sugarcane-producing countries, combined (Perfect Insider 2016). In 2013–14, the sugarcane industry contributed US\$43.4 billion to Brazil's gross domestic product (GDP), corresponding to almost 2% of the entire economy (Neves and Trombin 2014). Approximately half of Brazilian sugarcane production is used to produce fuel ethanol, with the other half used primarily for the production of sugar (CONAB 2015).

A major challenge currently facing the Brazilian sugarcane industry is meeting high cane tonnage demands by increasing yield per hectare, rather than further increasing planted area. Given unutilized crushing capacity at existing mills, increasing production at these is a more economically attractive proposition than building new mills to meet this projected demand.

Sugarcane is harvested over multiple cropping cycles of harvest and regrowth (called ratoons). In São Paulo state, responsible for more than half of the total cultivated sugarcane area in the country, the average economically viable lifetime of a commercial sugarcane field is 6 years (Borba and Basso 2009). The yield of a sugarcane field typically declines with increasing ratoon or harvest cycles (Borba and Basso 2009). An important factor that may contribute to the decline in yield over time in commercial sugarcane fields is ratoon stunt disease (RSD) caused by the fastidious, xylem-invading bacterium *Leifsonia xyli* subsp. *xyli*. Field trials comparing diseased and disease-free sugarcane reported increasing yield losses over time due to the disease in different geographical locations: a 14% loss in the first-year crop increasing to 27% in the third year in Louisiana

(Grisham, 1991), 18% yield loss in the first year followed by 27% loss in the following year in South Africa (Bailey and Bechet 1997), and yield losses of 12% in the first year and 28% in the third year in Ethiopia (Zekarias et al. 2012).

Despite these studies, knowledge of the prevalence, severity, and impact of RSD on commercial Brazilian sugarcane production is limited. Estimated yield losses in different small-scale, artificial inoculation studies conducted in Brazil varied depending on the variety, from 11.5 to 41.5% (Matsuoka 1984) and from 1.7 to 26.2% (Gagliardi and Camargo 2009). These controlled studies did not assess the real impact of RSD on Brazilian sugarcane industry productivity, because yield loss data from controlled inoculation studies has not previously been supplemented by surveys on the prevalence and severity of RSD infection in commercial fields.

In the limited, small-scale surveys conducted in Brazilian seedcane nursery fields, a high proportion were found to be infected with *L. xyli* subsp. *xyli*. In one study, the prevalence of *L. xyli* subsp. *xyli* infection in presumptive seedcane nursery fields ranged from 25 to 29%, depending on variety (Urashima et al. 2010). Ponte et al. (2010) found 68% of nursery fields in Bahia, Espírito Santo, and Rio de Janeiro states to be infected with *L. xyli* subsp. *xyli*. Urashima and Marchetti (2013) found that 24 to 27% of nursery fields in the main Center-South growing area were infected with *L. xyli* subsp. *xyli*. However, results from sampling suspicious-looking plants in seedcane nursery fields cannot be extrapolated to estimate levels of infection in commercial sugarcane fields due to both the sampling methodology used and the presumably greater care taken in implementing RSD-preventive phytosanitary procedures in sugarcane nursery fields.

The purpose of the current study was to evaluate the extent and severity of *L. xyli* subsp. *xyli* infection in commercial sugarcane fields in the main Brazilian Center-South growing area in order to help determine the economic importance and impact of RSD on the Brazilian sugarcane industry. We also investigated the distribution of *L. xyli* subsp. *xyli* across regions, varieties, and harvest cycles.

Materials and Methods

Fields survey and sampling. An extensive survey was conducted in 2012 and 2013 in commercial sugarcane fields in four different regions of Center-South Brazil: central São Paulo state (SP Central), western São Paulo state (SP West), Cerrado (Minas Gerais and Goiás states), and South (southern São Paulo state and Mato Grosso do Sul and Paraná states). Samples were taken from fields with plants at least

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9 months old, in plant cane or ratoon (regrowth) harvest cycles, of the eight most widely cultivated sugarcane varieties (RB867515, SP81-3250, RB855453, RB92579, RB855536, RB966928, RB855156, and RB835054). The mills where the sampled fields are located were contacted to find the varieties, crop ages, and harvest cycles that would, insofar as possible, mirror the distribution of these in the main Center-South growing area.

The sampled fields ranged in size from 0.18 to 65.0 ha. Sampling in each field was by walking a set number of paces in a zig-zag pattern and then sampling the plant encountered. The number of samples was based on the scale of our survey in area, number of fields, as well as the logistics and costs; approximately six samples per hectare were collected in each field. For example, for a 10-ha field, approximately 60 samples were collected. Each sample consisted of two mature stalks collected by breaking them off at the base. Stalks were transported to a staging area (Supplementary Figure S1), where they were cleaned to remove any dirt, then cut with a sterilized knife to generate a stalk segment consisting of the basal internode plus one node, the basal cut being made on a diagonal to facilitate juice collection.

Juice extraction was performed by connecting an air pump to the apical end of the stalk segment, forcing juice out the basal end. A volume of 500 μ l of sugarcane juice was collected in a 50-ml disposable plastic conical tube (Corning Inc.) from one or, if volume was insufficient, two stalk segments per plant. The juice was then transferred to a well in a 96-well plate (Bio-Dot microfiltration apparatus; Bio-Rad) containing a drop of quaternary ammonia (Ourofino Saúde Animal). The relevant information for the plate (date, mill name, field name, field size, variety, starting and ending position on plate, and GPS coordinates of field) was recorded and linked to the plate identifier. The plate itself was kept on ice until the end of the day, when it was frozen (-20°C) prior to transport to the laboratory for dot-blot analysis.

Serological analysis. Samples were stored in the laboratory at -20°C until they were ready to be assayed. The presence of *L. xyli* subsp. *xyli* in the juice samples was assessed by dot-blot immunoassay, as described by Viswanathan (2004), with modifications and validation by Urashima and Marchetti (2013). A volume of 100 μ l of extracted juice was dispensed onto a nitrocellulose blotting membrane (0.45- μ m pore size; GE Healthcare) employing an apparatus with 104 wells, 7 mm in diameter. The membrane was then heat dried at 80°C for 1 h and unbound sites were blocked by nonfat dry milk in 5% phosphate-buffered saline plus Tween, then incubated with polyclonal antibodies, followed by indirect enzyme-linked immunosorbent assay (ELISA) employing rabbit anti-*L. xyli* subsp. *xyli* immunoglobulin G (IgG), then alkaline phosphatase-labeled goat antirabbit IgG. Samples positive for *L. xyli* subsp. *xyli* produced a gradient of blue-colored dots on the membrane that provided an estimate of pathogen concentration, with the darkest blue corresponding to 10^9 CFU/ml, decreasing to 10^8 , 10^7 , and the faintest blue to 10^6 CFU/ml (Leaman et al. 1991; Urashima and Marchetti 2013). Samples of known pathogen concentration and distilled water were employed as positive and negative controls, respectively, in all serological reactions. Prior to statistical analysis, dot-blot data were checked and analyzed for evidence of possible contamination across wells. No such evidence was found, indicating that cross-well contamination was either minimal or nonexistent.

Data preparation and analysis. Data were acquired from both plate metadata (including date, mill and city, field name, and GPS coordinate information) and dot-blot immunoassay result files. Plate metadata and dot-blot Microsoft Excel files were converted to comma-delimited files and parsed using Perl scripts, and the data then integrated. Sample block numbers were validated with the standard plate description along with the mill and city combination. The plate metadata were then linked with the dot-blot assay data by matching plate numbers and assay results, with all plate metadata and dot-blot results attributed to single samples, to generate a single validated file for subsequent statistical analysis.

Statistical analysis. The results of the dot blot were converted to positive (those with estimated *L. xyli* subsp. *xyli* titer of 10^6 , 10^7 , 10^8 , or 10^9 CFU/ml) or negative, and the percentage of positive samples

per field (the main response variable in this study) was calculated. The data were considered on a field rather than on a sample basis to ensure the independence of each data point; thus, the response variable is a continuous percentage rather than a binary positive or negative value. Because the percentage of positive samples in each field is a binomial variable, it is possible, in theory, to model these data with a generalized linear model to determine whether occurrence of *L. xyli* subsp. *xyli* is dependent on any of the key factors of variety, harvest cycle, region, sampling year, or field size. However, this dataset has two additional complications: first, approximately 90% of fields had no detectable level of bacteria; and second, the data structure is such that there is severe confounding as well as lack of replication of each combination of factors. Consequently, attempts to model the data were unsuccessful for the majority of models considered, and the data are presented simply as graphs and frequency tables. Although frequency tables often lend themselves to χ^2 tests, the tests implicitly assume that all potential causes of RSD are balanced across the levels of the factor of interest. Therefore, χ^2 tests are not appropriate for this dataset, where the different factors are considerably intertwined, and, thus, have not been conducted. Data manipulation, exploratory data analysis, and attempts at data modeling were done using SAS/GRAPH and SAS/STAT software (versions 9.2 and 9.4; SAS Institute Inc.). The resulting tables show the observed prevalence and severity of *L. xyli* subsp. *xyli* by variety, harvest cycle, and region. Inferences made from these tables should be treated with some caution, as explained further in the Results and Discussion sections.

Results

A total area of 13,173 ha was surveyed, encompassing 1,154 fields for eight varieties among those with the most area under cultivation (Table 1). The total area and number of fields sampled from each variety were roughly in proportion to the extent of their cultivated hectare within the Center-South growing area in 2013 (Chapola et al. 2014). Therefore, RB867515 with 3,001 ha and 242 fields, SP81-3250 with 2,466 ha and 213 fields, and RB855453 with 2,037 ha and 162 fields were the three most surveyed varieties and were the top varieties in planted area when the survey was conducted. All varieties (with the exception of RB92579, a variety in rapid expansion phase ranked an estimated 12th) were ranked in the top 10 varieties at the time of the survey (2012 to 2013).

In total 92,114 samples were taken and analyzed by dot-blot ELISA to detect *L. xyli* subsp. *xyli* and 0.9% (851) samples were found to be *L. xyli* subsp. *xyli* positive (data not shown). This equates to 121 infected fields out of the 1,154 fields surveyed (10.5%). Infected fields accounted for 1,491 ha of the 13,173 ha sampled, or 11.3% of the sampled area.

Results of the survey by variety, harvest cycle, and region are summarized in Tables 2 to 6. When evaluating these results, however, it is important to note that the observed values for each individual factor are not independent of other factors. This affects the ability to reliably attribute observed differences to a particular factor. The effect of survey structure on the data and its interpretation is described in more detail in the Discussion.

The disease was detected in all varieties tested. The highest number of infected fields ($n = 26$) was found in variety RB92579, followed by variety SP81-3250 ($n = 24$). Because the number of samples differed by variety, the percentage of infected fields by variety provides a more appropriate estimate of the relationship between variety and pathogen presence in a field. The observed percentage of infected fields ranged from 6.2% for RB867515 to 17.3% for RB92579 (Table 2).

The percentage of surveyed mill areas with infected fields by variety ranged from 13.6% (3 of 22) for RB835054 to 32.4% (11 of 34) for RB92579. Fields of RB835054 also had the lowest percentage of infected fields (13.6%) among all mill areas, whereas fields of RB92579 had the highest (32.4%). In total, the disease was discovered in fields associated with 58% of mill areas surveyed. The varieties most commonly infected within mill areas were also those with the highest numbers of infected fields: RB92579 with 300 ha in 26

fields, SP81-3250 with 291 ha in 24 fields, and RB855536 with 216 ha in 17 fields.

The field level prevalence (percentage of infected samples within an individual field) and severity of infection (highest estimated pathogen titer in a sample from an individual field), along with the total infected area by variety, is presented in Table 3. Of the total infected area of 1,491 ha, most (77 of 121) fields had a low (<5%) percentage of infected samples in infected fields. When data were analyzed by variety, five varieties (RB867515, SP81-3250, RB855453, RB855536, and RB966928) showed a similar trend (i.e., in most infected fields for these varieties, the percentage of *L. xyli* subsp. *xyli*-positive samples was less than 5%). Of the remaining three varieties (RB92579, RB855156, and RB835054), RB92579 in particular had a very high percentage of fields with a high percentage of infected samples; that

is, 23.1% (6 of 26) fields had 50% or more *L. xyli* subsp. *xyli*-positive samples and another 2 fields (for a total of 30.8%) had 25% or more positive samples. Thus, RB92579 had the highest percentage of fields with 25% or more infected samples. However, five of the eight RB92579 fields with $\geq 25\%$ infected samples were associated with a single mill. Of the total 31 infected fields associated with this mill, only 1 other field, of a different variety, had $\geq 25\%$ infected samples. Other varieties with high percentages of fields with high levels of infection were RB835054 (20.0%; albeit with limited sample size) and SP81-3250 (16.7%).

Regarding pathogen titer, 42.1% (51 of 121) fields had at least one sample with an estimated concentration of 10^6 CFU/ml, 23.1% (28 of 121) fields had a sample with 10^7 CFU/ml, 23.1% (28 of 121) fields had a sample with 10^8 CFU/ml, and 11.6% (14 of 121) fields had a

Table 1. Summary of 2012 survey of cultivated sugarcane hectareage, percentage of cultivated area, and rank by variety and region within the Brazilian Center-South growing area (Chapola et al. 2014)

Variety	Parameter ^b	Region ^a				Total ^c
		Cerrado	SP West	SP Central	South	
RB867515	Hectares	352,672	234,458	437,717	529,940	1,554,787
	Area (%)	30	30.5	24.2	33.3	29.1
	Rank	1	1	1	1	1
SP81-3250	Hectares	208,290	133,140	230,791	186,715	758,936
	Area (%)	17.7	17.3	12.8	11.7	14.2
	Rank	2	2	2	2	2
RB855453	Hectares	40,924	65,672	156,270	87,250	350,134
	Area (%)	3.5	8.5	8.6	5.5	6.5
	Rank	6	3	3	4	3
RB855536	Hectares	113,093	10,158	51,289	61,183	235,723
	Area (%)	9.6	1.3	2.8	3.8	4.4
	Rank	3	12	8	8	5
RB855156	Hectares	18,969	1,047	102,914	83,444	186,358
	Area (%)	1.6	0.1	5.7	5.2	3.5
	Rank	12	nr	4	6	6
RB966928	Hectares	3,896	34,449	64,876	60,648	163,869
	Area (%)	0.3	4.5	3.6	3.8	3.1
	Rank	nr	5	6	9	8
RB835054	Hectares	16,330	9,451	25,311	84,534	135,626
	Area (%)	1.4	1.2	1.4	5.3	2.5
	Rank	14	14	16	5	10
RB92579	Hectares	29,022	36,444	31,052	30,990	127,508
	Area (%)	2.5	4.7	1.7	1.9	2.4
	Rank	7	4	14	12	12
All varieties (ha)	...	1,176,854	768,875	1,807,296	1,593,663	5,346,688

^a Cerrado = Minas Gerais and Goiás states; SP West = Araçatuba region; SP Central = Piracicaba, Jaú, Ribeirão Preto, and Catanduva regions; and South = Paraná and Mato Grosso do Sul states plus Assis region in São Paulo state; nr = not ranked.

^b Rank = overall rankings, based on cultivated hectareage in all of Brazil (except for RB92579, where it is estimated).

^c Totals reflect the definitions of these regions.

Table 2. Survey scope and prevalence of *Leifsonia xyli* subsp. *xyli* infected fields and mills by sugarcane variety

Variety	Area (ha) ^a	Infected (ha) ^b	Sampled ^c	Infected fields		Surveyed ^d	Mills with infected fields	
				N	%		N	%
RB867515	3,001	164	242	15	6.2	44	10	22.7
SP81-3250	2,466	291	213	24	11.3	46	13	28.3
RB855453	2,037	175	162	12	7.4	42	7	16.7
RB855536	1,386	216	129	17	13.2	32	10	31.3
RB855156	845	95	79	8	10.1	27	5	18.5
RB966928	1,295	171	109	14	12.8	37	9	24.3
RB835054	647	80	70	5	7.1	22	3	13.6
RB92579	1,496	300	150	26	17.3	34	11	32.4
Total	13,173	1491	1,154	121	...	50 ^e	29 ^e	...

^a Total sampled area.

^b Area of infected fields.

^c Number of sampled fields.

^d Number of surveyed mills.

^e Number of surveyed mills and mills with infected fields are not additive by variety.

sample with 10^9 CFU/ml, the highest concentration that can be estimated using the dot-blot technique. Samples with high estimated *L. xyli* subsp. *xyli* titer (10^8 and 10^9 CFU/ml) were observed in 69.2% of infected fields of RB92579, followed by SP81-3250, with 37.5% of infected fields. RB835054 also had a high percentage (80.0%) of infected fields with high estimated *L. xyli* subsp. *xyli* titer, although the sample size was small (four of five infected fields). On the other hand, the majority of *L. xyli* subsp. *xyli*-positive fields of some varieties had a predominance of samples with low bacterial titer (10^6 CFU/ml): RB855453 to 66.7% (8 of 12) infected fields, RB966928 to 64.3% (9 of 14) infected fields, RB855156 62.5% (5 of 8) infected fields, and RB855536 to 52.9% (9 of 17) infected fields (Table 3).

Data on the distribution of *L. xyli* subsp. *xyli* were also analyzed by harvest cycle, in terms of numbers and percentages of infected fields, area, and mill areas (Table 4). Only the first ratoon harvest cycle is noteworthy for having a higher percentage of infected fields (13.8%)

than the overall average across all cycles (10.5%). Similarly, when broken down by harvest cycle, the highest percentage of infected area was in the first ratoon (15.7%), followed by the fourth or subsequent ratoons (12.1%). However, even the plant cane harvest cycle had a relatively high percentage of infected fields (9.4%) and infected area (10.4%). It is important to mention that these are just observations of raw data, without any inferences or claims to significance. Formal statistical analysis has not been conducted because sampling was not carried out in the same field in the following year and, therefore, no comparison of bacterium prevalence among harvest cycles was possible.

Because the Center-South is the most important sugarcane-growing area in Brazil, data on the prevalence of *L. xyli* subsp. *xyli* in the four different regions within this area were tabulated separately (Table 5). There is variation between regions in this survey in terms of numbers of fields sampled (207 to 391) and area sampled (2,738 to 3,860 ha); consequently, percentages are more illustrative of regional differences than counts. The prevalence of *L. xyli* subsp. *xyli* was

Table 3. Within-field prevalence and level of *Leifsonia xyli* subsp. *xyli* infection in diseased fields by sugar cane variety

Variety	Infected fields		Number of infected fields by								Infected fields (%) with ^b	
			Infected samples in field (%)				Titer (CFU/ml) ^a					
	N	Area (ha)	<5 %	6–25 %	26–50 %	≥50 %	10 ⁶	10 ⁷	10 ⁸	10 ⁹	≥25 % Inf	Highest titer
RB867515	15	164	10	5	0	0	5	6	4	0	0.0	26.7
SP81-3250	24	291	15	5	3	1	9	6	8	1	16.7	37.5
RB855453	12	175	11	1	0	0	8	3	1	0	0.0	8.3
RB855536	17	216	14	3	0	0	9	6	2	0	0.0	11.8
RB855156	8	95	4	3	1	0	5	1	1	1	12.5	25.0
RB966928	14	171	12	2	0	0	9	3	1	1	0.0	14.3
RB835054	5	80	1	3	0	1	0	1	3	1	20.0	80.0
RB92579	26	300	10	8	2	6	6	2	8	10	30.8	69.2
Total	121	1,491 ^c	77	...	...	...	...	...	...	...	...	...

^a Highest estimated *L. xyli* subsp. *xyli* titer in sample from field.

^b Inf = ≥25% infected samples and Highest titer = samples with highest estimated titer ≥ 10^8 CFU/ml.

^c Total area of infected fields is not additive due to rounding.

Table 4. Prevalence of *Leifsonia xyli* subsp. *xyli* infected fields, hectareage, and mills by harvest cycle

Cycle	Sampled fields	Infected fields		Area ^a			Mills (n) ^b	Mills with infected fields	
	N	N	%	Samp (ha)	Inf (ha)	Inf (%)		N	%
Plant cane	307	29	9.4	3,446	357	10.4	50	16	32.0
Ratoon 1	254	35	13.8	2,785	437	15.7	43	17	39.5
Ratoon 2	212	20	9.4	2,434	213	8.8	46	14	30.4
Ratoon 3	203	19	9.4	2,472	238	9.6	42	12	28.6
Ratoon 4+	178	18	10.1	2,035	247	12.1	39	14	35.9
Total	1,154	121 ^c	10.5	13,173 ^c	1,491 ^{c,d}	11.3	...	...	...

^a Samp = area sampled and Inf = area of infected fields in hectares and percentage.

^b Number of surveyed mills.

^c Total sampled area and area of infected fields are not additive due to rounding.

^d A field and the area within a field were considered infected if one or more samples taken from the field were found to be infected.

Table 5. Prevalence of *Leifsonia xyli* subsp. *xyli*-infected fields within the Brazilian Center-South growing area by region

Region ^b	Number of fields			Area ^a			Mills ^d	Mills with infected fields	
	Sampled	Infected	Inf (%) ^c	Samp (ha)	Inf (ha)	Inf (%)		N	%
Cerrado	237	38	16.0	2,816	469	16.7	11	9	81.8
SP West	207	23	11.1	2,738	287	10.5	12	5	41.7
SP Central	391	33	8.4	3,759	385	10.2	17	8	47.1
South	319	27	8.5	3,860	351	9.1	10	7	70.0
Total	1,154	121	...	13,173	1,491	...	50	29	...

^a Samp = area sampled and Inf = area of infected fields in hectares and percentage.

^b Cerrado = Minas Gerais and Goiás states; SP West = Araçatuba region; SP Central = Piracicaba, Jaú, Ribeirão Preto, and Catanduva regions; and South = Paraná and Mato Grosso do Sul states plus Assis region in São Paulo state.

^c Percentage of infected fields.

^d Total number of mills.

highest in the Cerrado region, with 16.0 and 16.7% of infected fields and area, respectively. The next highest percentage of infected fields and area was the SP West region, with 11.1 and 10.5%, respectively. In the Cerrado region, 81.8% (9 of 11) mills had at least one *L. xyli* subsp. *xyli*-positive field. The South region also had a high percentage (70.0%) of mill areas with at least one infected field.

The prevalence of *L. xyli* subsp. *xyli* within each region was examined by variety (Table 6). Several varieties showed very different indices of *L. xyli* subsp. *xyli* prevalence in different regions (e.g., 37.8% of RB92579 fields were infected in the SP West region, whereas none of the 34 fields of this variety in SP Central were infected), and the prevalence of infected fields for RB92579 was similar to that of the other varieties in the Cerrado and South regions. Similar variation was observed for RB855156 (21.4% of Cerrado fields infected but none in SP West or South), RB835054 (17.4% of South fields infected, none in the Cerrado or SP Central), and RB855536 (16.7% of SP Central fields infected and none in SP West). Four other varieties (RB867515, SP81-3250, RB855453, and RB966928) tended to have a higher percentage of *L. xyli* subsp. *xyli*-positive fields in the Cerrado region. Importantly, different varieties showed the highest percentage of infected fields in different regions: RB855156 in the Cerrado (21.4%), RB92579 in SP West (37.8%), RB855536 in SP Central (16.7%), and RB835054 in the South (17.4%).

Discussion

Our survey data demonstrate that RSD is a widespread disease of sugarcane in the main Brazilian Center-South growing area, as has been found for other sugarcane-growing areas globally, including South Africa, India, Australia, United States, and Ethiopia (Bailey and Bechet 1997; Comstock 2008; Viswanathan 2001; Young and Brumbley 2004; Zekarias et al. 2012). The causal pathogen of RSD (*L. xyli* subsp. *xyli*) was found in samples from all major varieties and from all regions within the main Brazilian Center-South growing area.

Survey data were categorized into factors (variety, region, harvest cycle, and mill areas), providing a broad, robust view of the prevalence and infection levels of *L. xyli* subsp. *xyli* in sampled fields. Despite its sheer size, the structure of the survey dataset is such that the effects of these factors are, to some extent, confounded, making it difficult or impossible to reliably separate their effects on disease prevalence or estimated pathogen titer. For this reason, no formal tests of significance are presented. Nevertheless, the tabulated results still allow inferences to be made with regard to the prevalence and distribution of *L. xyli* subsp. *xyli* in Brazilian Center-South sugarcane fields, although these inferences should be treated with some caution.

The most cultivated variety in the Brazilian Center-South growing area during the sampling period (2012 to 2013) was RB867515, planted on more than 1.5 million ha or 29.1% of the cultivated hectares. In general, we attempted to sample varieties in rough proportion to their cultivated hectareage in the survey target area, based on census data (Chapola et al. 2014) (Table 1), although some constraints

existed in terms of our ability to do so (see Materials and Methods). Consequently, RB867515 was the most-sampled variety in our study (3,001 ha sampled), of which fields with at least one *L. xyli* subsp. *xyli*-positive sample comprised 164 ha (5.5% of sampled area). Assuming that similar infection levels hold over the entire Center-South growing area, this would extrapolate to approximately 85,500 ha of RB867515 fields with at least some *L. xyli* subsp. *xyli* infection. In total, 20,870 RB867515 samples were taken, of which 47 were infected (0.20%; data not shown). Gagliardi and Camargo (2009) observed a 26.2% reduction in RB867515 tonnage yield in *L. xyli* subsp. *xyli*-inoculated relative to noninoculated plots in a study of three consecutive crop cycles. If we consider an average RB867515 yield of 100 tons ha⁻¹ (Gava et al. 2011), and consider a 0.20% infection rate, we calculate an estimated yield reduction of 78,600 tons year⁻¹ caused by RSD (100 tons ha⁻¹ average yield × 1.5 million cultivated ha × 0.2% infection rate × 26.2% yield reduction) over the entire Brazilian Center-South growing area. The average cane tonnage price paid to growers in 2015 was R\$52.8 ton⁻¹ or US\$13.20 (UDOP 2016); consequently, a yield reduction of 78,600 tons year⁻¹ would translate to a yearly economic loss of over US\$1 million year⁻¹ in the Center-South growing area region for RB867515 alone. It is important to point out that these figures are likely an underestimate of yield losses and economic impact, because the 26.2% yield loss value we have used in our calculations is based on the Gagliardi and Camargo (2009) study conducted in São Paulo state on the eastern edge of the main Brazilian Center-South growing area, where moisture is less limiting and, consequently, yield losses would be expected to be less than in other regions of our survey area.

For variety RB855536, the average yield (103.9 ton ha⁻¹) (Gava et al. 2011) and extent of yield reduction due to *L. xyli* subsp. *xyli* infection in an artificial inoculation study (1.6%) (Gagliardi and Camargo 2009) is also known. In our survey, we sampled 1,386 ha of variety RB855536, out of a total 235,723 ha planted in the Center-South growing area, and found *L. xyli* subsp. *xyli*-positive samples in 15.6% of the sampled hectares (Table 2). In addition, for RB855536, 50 of 9,877 samples were infected with *L. xyli* subsp. *xyli* (0.50%; data not shown). Making calculations similar to those described for RB867515 above, yield losses due to RSD would be over 1,959 tons cane year⁻¹ (103.9 tons ha⁻¹ average yield × 235,723 cultivated ha × 0.5% infection rate × 1.6% yield reduction), totaling more than US\$25,863 year⁻¹ in economic impact in this variety. To our knowledge, this is the first time the economic impact of RSD on Brazilian commercial sugarcane production has been estimated. In addition, it is important to point out that our estimates are averages across the entire surveyed area, and yield and economic losses are concentrated and, thus, much higher in infected fields.

The percentage of *L. xyli* subsp. *xyli*-infected fields ranged from 6.2% for RB867515 to 17.3% for RB92579 (Table 2). Although the observed infection level for RB92579 (17.3%) was higher, on average, than for all other varieties, this is not the case in all regions

Table 6. Incidence of *Leifsonia xyli* subsp. *xyli* infected fields by variety and region within the Brazilian Center-South growing area

Variety, totals	Number per region (%) ^a				Total
	Cerrado	SP West	SP Central	SP South	
RB867515	51 (13.7)	38 (2.6)	94 (4.3)	59 (5.1)	...
SP81-3250	46 (17.4)	47 (6.4)	73 (15.1)	47 (4.3)	...
RB855453	35 (14.3)	33 (6.1)	54 (3.7)	40 (7.5)	...
RB92579	33 (21.2)	37 (37.8)	34 (0)	46 (10.9)	...
RB855536	36 (13.9)	15 (0.0)	42 (16.7)	36 (13.9)	...
RB966928	16 (18.8)	19 (10.5)	28 (14.3)	46 (10.9)	...
RB855156	14 (21.4)	4 (0.0)	39 (12.8)	22 (0.0)	...
RB835054	6 (0)	14 (7.1)	27 (0)	23 (17.4)	...
Fields sampled (<i>n</i>)	237	207	391	319	1,154
Infected fields (<i>n</i>)	38	23	33	27	121
Infected fields (%) ^b	16.0	11.1	8.4	8.5	...

^a Number of sampled and percentage of infected fields by variety. Cerrado = Minas Gerais and Goiás states; SP West = Araçatuba region; SP Central = Piracicaba, Jaú, Ribeirão Preto, and Catanduva regions; and South = Paraná and Mato Grosso do Sul states plus Assis region in São Paulo state.

^b Percentages of infected fields are not additive by variety.

(Table 6). Variability of disease prevalence in a single variety in different growing regions had been observed for variety CP 76-331, which showed variable levels of pathogen infection in the eastern (1.7%) and western (36.3%) portions of the Atchafalaya basin in Louisiana (Damann and Hollier 1991). In the Brazilian Center-South in 2015, over 600 million tons of sugarcane was produced on over 8 million ha (CONAB 2015) by more than 100 mills (Chapola et al. 2014). Each mill has its own policy regarding phytosanitary procedures and the production of seed cane for commercial planting; typically, transportation of planting material is restricted to short distances due to transportation costs (Neves et al. 1998). The absence of long-distance movement of planting materials between mills could account for the discrepancy of RSD values in the same variety between regions.

RB92579 samples not only have the highest percentage of fields with high *L. xyli* subsp. *xyli* titers but also the highest percentage of infected fields, mill areas with infected fields, and area of infected fields (Tables 2 and 3). The RB92579 variety was released in 2003 and has good agronomic characteristics such as high water use efficiency, stalk height, tillering, and yield (Almeida et al. 2008). In 2009, this variety had an estimated 6,164 ha of plant cane area, representing 1.9% of the replanted area; 4 years later, it had 150,812 ha (or 5%) of the ratoon area and 41,919 ha (or 7.2%) of the plant cane area (Chapola et al. 2010, 2014). The remarkably rapid increase in the planted area of RB92579 over this period could only have been achieved by not observing standard phytosanitary precautions such as use of in vitro meristem cultured source material, diagnostic disease testing, and thermal treatment of materials sources for nursery plantings. Instead, in many instances, commercial sugarcane was used to plant new fields, promoting the spread of *L. xyli* subsp. *xyli*. In support of this hypothesis, we note that the highest prevalence of RSD in this variety (Table 6) was observed in regions where the sugarcane planted area was expanding most rapidly during this time: Cerrado and SP West. In the first, sugarcane planted area expanded by 379% over the last decade (Castelões 2015).

Mechanical harvesting contributes to pathogen spread between and within fields, because decontamination of cutting blades on mechanical harvesters is impractical and, thus, rarely if ever done. In a study in Louisiana, *L. xyli* subsp. *xyli* was spread from infected to previously uninfected fields planted side by side for all varieties and distances tested, and the prevalence of infection increased with increasing harvest cycle (Hoy et al. 1999). A high percentage (84.8%) of the 5.5 million ha of sugarcane area grown in São Paulo, the main producing state in Brazil, were machine harvested in 2013–14 (Fredo et al. 2015). The proportion of mechanically harvested green cane in the Brazilian Center-South is projected to continue to grow due to environmental restrictions on burning of sugarcane fields and increasing labor costs (Figueiredo et al. 2015), contributing to the risk of further *L. xyli* subsp. *xyli* spread.

Varieties that exhibit high estimated *L. xyli* subsp. *xyli* titers are deemed susceptible and are associated with the rapid spread of RSD by harvester, with 30% infected samples at first ratoon from just a single plant inoculum source (Damann 1992). In a study of varieties of varying susceptibility that measured the distance that infected plants were found postharvest from the originally inoculated plants, Comstock et al. (1996) found that the higher the susceptibility of a variety, the further the distance of transmission from the inoculated plant in the field. Taken together, the results of previous studies indicate that a single infected plant can contaminate a field, that plants with high estimated *L. xyli* subsp. *xyli* titers can do so more quickly, and that infection can move via infected harvesting equipment from one field to the next. We found *L. xyli* subsp. *xyli*-infected samples in all of the major Brazilian varieties we surveyed, and at least some samples with high estimated *L. xyli* subsp. *xyli* titers (10^8 and 10^9 CFU/ml) were found in all of them (Table 3). Despite the majority of fields showing low estimated *L. xyli* subsp. *xyli* titers, the spread of the pathogen is still problematic in subsequent harvests; even at low bacterium titers, RSD was found to increase by about 10% annually in U.S. varieties in Louisiana (Damann and Hollier 1991).

Our survey showed high levels of *L. xyli* subsp. *xyli* infection in plant cane, in terms of percentage of infected fields (9.4%), percentage

of infected area (10.4%), or percentage of mills with infected fields (32%) (Table 4). The high percentage of pathogen infection at the first harvest cycle indicates seedcane contamination by *L. xyli* subsp. *xyli*, due to the absence of alternative transmission methodologies (Gillaspie and Teagle 1989), pointing to the importance of disease-free planting material. The present work was unable to establish any relationship between *L. xyli* subsp. *xyli* prevalence and harvest cycle because surveys were not conducted in the same fields in subsequent years.

An important contribution of the present study is that the data were analyzed on a commercially relevant, per-mill basis. In 2011–12, the crop year before our study, almost 70% of the processed sugarcane was grown by the mills in the Brazilian Center-South (CONAB 2013), and 100% of our sampled area was owned and directly managed by the mills.

The present work highlights the urgency of implementing phytosanitary procedures to ensure the use of disease-free seedcane in planning commercial fields, and sends a clear message to the industry: the lack of effective control of RSD in the main Center-South growing area has an enormous economic impact. Special emphasis on the Cerrado region, where over 80% of mill areas had infected fields (Table 5) and where agroclimatic conditions, including silty soils of poor fertility, low water retention capacity (Marimon-Junior and Haridasan 2005), and severe drought from April to September (CPRM 2006), are predicted to exacerbate RSD-associated yield losses in reducing water availability and the height, diameter, and number of stalks (Bailey and Bechet 1997). Recommended phytosanitary practices for RSD control are known from extensive research in this area (Grisham 2004; Viswanathan 2004; Young and Brumbley 2004) and, despite significant challenges in doing so, these should be adopted by the industry as a whole. The good news is that the industry is indeed moving toward an increased emphasis on implementing the appropriate phytosanitary strategies for RSD control. Implementation of known phytosanitary procedures is made easier by the availability of methodologies for establishing disease-free nurseries such as presprouted seedcane (Portuguese abbreviation = MPB) from the Instituto Agrônomo de Campinas and, especially, the range of Plene products from Syngenta that provide guaranteed varietal and phytosanitary quality in plantlets directly from meristem tissue culture (Plene Evolve) or presprouted from primary nurseries (Plene PB). The use of these technologies can greatly improve phytosanitary quality in a cost-effective and convenient way, and their implementation by sugarcane mills is strongly recommended.

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