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Need for Improved Detection of Ratoon Stunting Disease in Sugarcane in South Texas

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ABSTRACT

Infestation with *Leifsonia xyli* subsp. *xyli* of sugarcane affects the detrimental Ratoon Stunting Disease (RSD) that can cause severe yield losses wherever sugarcane is grown. In the lower Rio Grande Valley of Texas, seedcane assays, conducted on a routine basis in preparation for planting, revealed an incidence of more than 40% of cane crop potentially used for planting to be infested with the bacterium. This high incidence is puzzling because a management program for RSD is in place in this region. Seed cane is routinely tested by a colorimetric dot blot ELISA, and when found infested, the seedcane is heat-treated in a waterbath before planting. In this report, we document the discrepancies of various methods of detection and propose the possibility of a dilution factor to be responsible for the discrepancies. This report urges the need for developing more sensitive detection methods for RSD.

RESUMEN

La infección de la caña de azúcar con *Leifsonia xyli* subespecie *xyli* causa la enfermedad enanismo de la soca (RSD) la cual ocasiona pérdidas severas en todas las áreas donde se cultiva la caña de azúcar. En el Bajo Valle del Rio Grande en Texas, los análisis de la semilla de caña realizados rutinariamente antes de la siembra, revelaron una incidencia de la bacteria de mas de un 40% del cultivo de la caña usado potencialmente para plantarse. Esta alta incidencia es intrigante ya que existe un programa de manejo para la enfermedad en la región. La semilla de caña para siembra se examina rutinariamente mediante una prueba colorimétrica de dot blot- ELISA, y cuando se encuentra infectada, se trata en baños de agua caliente antes de plantarse. En este artículo, se documentan las discrepancias de varios métodos de detección y se propone la posibilidad de la existencia de un factor de dilución responsable de estas discrepancias. Este reporte indica la necesidad del desarrollo de técnicas mas sensibles para la detección de RSD.

Additional index words: ELISA. fastidious prokaryotes, Leifsonia xyli subsp. xyli, pathogen detection, slow-growing bacteria

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Ratoon stunting disease (RSD) caused by the xylem inhabiting, slow-growing, recently renamed *Leifsonia xyli* subsp. *xyli* (Davis et al. 1980, 1984; Evtushenko et al., 2000) is one of the most important sugarcane (*Saccharum officinarum* L.) diseases worldwide (Hughes, 1978) with yield losses up to 50% having been reported (Bailey and Bechet, 1995, 1997). Damage by RSD is caused mainIy by interference with water and nutrient transport (Kao and Damann, 1978, 1980). Since sugarcane is grown with 4-5 ratoon crops with one crop per year before termination of a planting, care must be taken to avoid infestation of entire fields by this bacterium. The bacterium is spread by manual and mechanical harvesting equipment depending on the susceptibility of the sugarcane cultivar (Bailey and Tough, 1992; Damann, 1992; Comstock et aI., 1996). Efforts to develop fast and reliable detection methods have included direct microscopical methods (Davis, 1985; Gillaspie et al., 1976; Harris and Gillaspie, I978). Electronmicroscopy together with serological techniques improved sensitivity of detection (Damann et al., 1978). Higher numbers of samples could be examined with alkaline-induced metaxylem autofluorescence (Damann, 1988). Immunological assays have used either dot blot techniques (Irvine and Rey, 1991) or evaporative binding enzyme-linked immunoassay (EB-EIA) techniques (Croft et al., 1994). Molecular biological procedures have included tissue blotting followed by DNA hybridization (Pan et al., 1998b) and different PCR-based approaches (Antwerpen and Botha, 1999; Fegan et al., 1998; Pan et al., 1998a). Several comparisons of selected methods have been made (Davis and Dean, 1984; Hoy et al., 1999).

Table 1. Incidence of RSD in sugarcane stalks in two sugarcane fields in two consecutive ratoon crops in south Texas in 1999-2000.

1999[z]	December 1999[y]	October 2000[y]	December 2000[y]
	%		
Field A - 2/10 (Nov.)			
CP72-1210	47 (42-51)	29	50 (8-76)
Field B - 2/10 (Sept.)			
CP70-1133	3 (0-6)	9	0

[z]Ten sugar cane stalk samples were collected by the producer and submitted to the Weslaco center for examination for the presence of *Leifsonia xyli* subp. *xyli*.

[y]Fifty stalks were collected at 90-cm intervals in each of three randomly chosen rows in both commercial fields and examined with the dot blot assay at Weslaco the average detection of how many stalks were positive for *L. xyli* subsp. *xyli* and the range among the three rows is given.

Table 2. Incidence of RSD in sugarcane stalk samples submitted to the seed cane testing laboratory at Weslaco, TX in 2000-2001.

Source	Number of fields	RSD-positive[z]	Percentage[y]
Seed cane	117	49	41.9
Insurance	20	20	100.0
Total	137	69	50.4

[z]A dot blot assay was utilized to detect *Leifsonia xyli* subsp. *xyli* in sugar cane juice expelled from singIe-internodes with pressurized air from 10 stalk samples. One of the 10 samples classified the respective field as infested with RSD.

[y]Percentage=(Fields found positive for RSD) / total number of fields) x 100.

Detection of RSD will be increasingly difficult with higher levels of resistance, since fewer vascular bundles are infected with the bacterium with increasing resistance.

At Weslaco, a dot blot assay is used in a management program (Irvine and Irey, 1991). At the beginning of the planting season, growers collect 10 stalks of sugarcane from random areas of cane fields scheduled to serve as a source of planting material for new fields. Cane vascular liquid is expelled from basal internodes with air pressure (Irvine and Irey, 1991). If all 10 stalks are negative for RSD, the cane is released for planting. If at least one of the stalks tests positive for RSD, the material of that field either cannot be used for planting or the cane is heat-treated for 2 h at 50º C (Daman and Benda, 1983) in large waterbath tanks operated by the Sugarcane Grower's Association (N. Rozeff, personal communication). The waterbath treatment is expected to cure the cane from bacterial infestation. Although this rigorous program is in place, infestation levels of the bacterium are still high in South Texas. Repeated reports of highly infested sugarcane crops in the first year after establishing a crop that was determined to be free of *Lxx* in the planting year prompted this study. The objective of this study was to determine possible reasons for the high incidence of *Lxx* in the Lower Rio Grande Valley, Texas.

MATERIALS AND METHODS

Detection of *Lxx* using a dot blot assay. The dot blot assay was conducted as originaIly described by Irving and Irey (1991). Starting from the basal end of the stalk, a first cut was placed 1 cm below the most basal node. The second cut was made 1 cm below the second-lowest node. An electric pump attached to the apical end of the cane piece by a teat adapter was used to expel vascular cane liquid by blowing air through the stalk in distal direction. The juice was expelled from the tissue and collected in a microcentrifuge tube. Approximately 2 ml were collected from each sample and either used immediately for the dot blot assay or frozen at -20º C until used. The membrane was heated at 80º C for 2 h and then submerged in the double ELISA reagents, developed and the color reaction read (Irvine and Irey, 1991).

Monitoring grower developed seedcane samples. Results of the standard seed cane testing were summarized for the season 2000-2001. In this procedure, the sugarcane growers (or their agents) collected 10 cane stalks from fields that were scheduled for use as planting material. Material was then delivered to the Texas A&M Research and Extension Center at Weslaco, Texas and tested for the presence of *Lxx* by the colorimetric dot blot assay (Irvine and Irey, 1991).

Monitoring in commercial fields. Two fields were chosen for intensive stalk sampling in two consecutive years: (A) Monte Alto, Texas (cane cultivar: C P70-1133), and (B) Raymondville (cane cultivar: CP72-1210). In both fields, three randomly chosen rows were sampled in December 1999 and the same row sampled in December 2000. About 50 stalks were collected at 90-cm spacing within each row. Samples were transported to Weslaco and juice was expelled within 48 h and stored at -20º C until testing with the dot blot assay. The number of positive samples per row was expressed as percentage of the total stalks sampled per row.

Dilution series with infested sugarcane juice. Sugarcane juice highly infested with *Lxx* of CP72-1210, CP87-3388, or reaction buffer was diluted in a ten- foId or two- fold dilution series in either non-infested cane juice or buffer. Samples of the dilution series were spotted onto a nitrocellulose membrane following standard protocol (Irving and Irey, 1991), and processed by the standard detection procedures (Irvine and Irey, 1991).

RESULTS AND DISCUSSION

Detection of *Lxx* with the colorimetric dot blot assay was erratic in south Texas in 1999 and 2000. Incidence of the bacterium was high in plant cane and in insurance cases (Table 1). The analyses suggested that the ELISA was not sensitive enough to detect low levels of *Lxx*. The bacterium was detected more frequently when the cane stalk had limited amounts of vascular liquid per internode and several internodes were necessary to collect sufficient juice for the detection procedure, as the case CP72-1210 (Table 2). When only one internode provided sufficient amounts of juice for the procedure detection was erratic and false negatives more frequent as the case for CP70-1133 (Table 2) (Westphal, unpublished). Although this difference in originating extraction material was not documented quantitatively, it was consistent in cane sampling. In addition, difficulties to reliably detect *Lxx* were commonly associated with samples that had a weak signal when the bacterium was detected at one sampling occasion. Comparison of the signal strength with the dilution series (Fig. 1) indicated that any further dilution of the signal resulted in the lack of detection. Estimates of possible intrinsic dilution during extraction from a more liquid containing sample compared to a "dry" sample were close to the 10-foId dilution that resulted in the lack of the signal in the designed dilution series (Fig. 1).

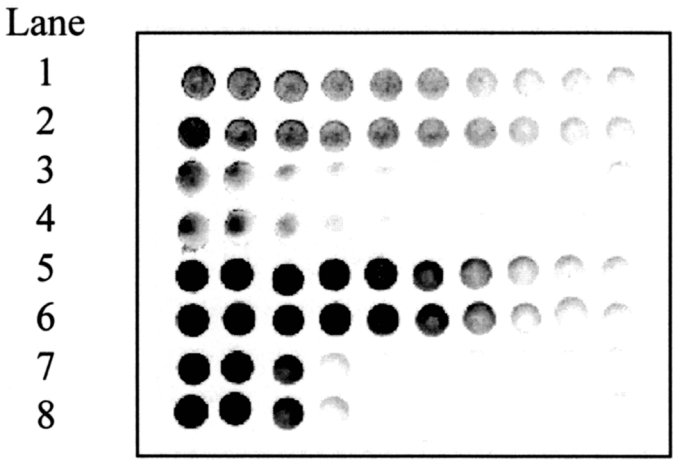

Fig. 1. Color reaction on a nitrocellulose membrane submitted to the standard colorimetric dot blot ELISA at Weslaco, TX. Lane 1 and 2 2-fold dilution in sugarcane juice (CP72-1210): Lane 3 and 4 10-fold dilution in reaction buffer; Lane 5 and 6 2-fold dilution in sugarcane juice (CP87-3388); Lane 7 and 8 10-fold dilution in sugarcane juice (CP87-3388).

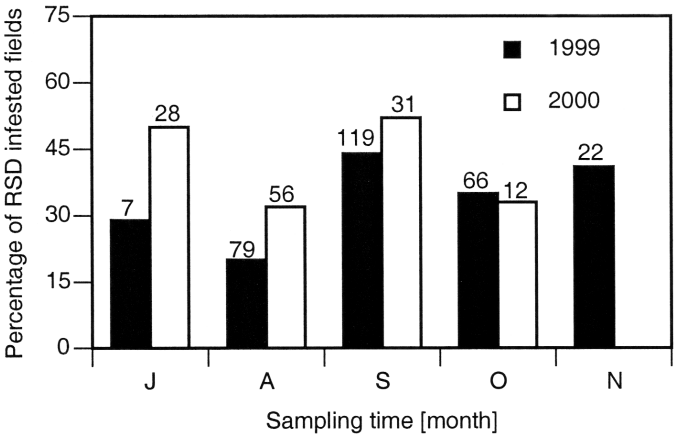

Fig. 2. Commercial fields with RSD as detected by a colorimetric dot blot assay at Weslaco, TX in 1999 and 2000. The number at each column indicates the number of fields examined. Numbers per month were presented: J: July; A: August; S: September; O: October; N: November.

In the Lower Rio Grande Valley, *Lxx* was present in high incidences (Table 1) and detection varied during summer and fall (Fig. 2). Our data suggest that sampling after rain or irrigation results in cane with high water content of the vascular tissue that result in dilution of the xylem-inhabiting bacterium below detection level threshold. Lack of detection was detrimental since most cane is planted based on the assay after September and the assay is the determining decision tool whether cane can be used for planting or needs hot-water treatment against *Lxx* before planting. A false negative is troublesome as it results in plantings of infested cane that are expected to yield for 4-5 years. Even in a single field, detection varied greatly (Table 2). Strict observance of plant hygienic strategies have led to substantial decline of disease incidence in other sugarcane growing areas (Hoy et al., 2000) and are necessary for the Lower Rio Grande Valley as well.

Attempts in this project to concentrate the concentration of bacteria in the test solution were not successful. For example, centrifugation of test cane vascular liquid resulted in a viscous sample and ultimately copious amounts of cane debris on the membrane prohibiting the detection of any color reaction. Efforts to use alternative detection systems failed, e.g., horse-radish-peroxidase-based detection resulted in insurmountable background signals. Filtering through glass filters during concentrating to avoid small cane debris to interfere with detection was preliminary tested but not entirely explored.

Until more sensitive assays become available use of the current assay should be avoided after rain or irrigation and best the test conducted at least two independent times when 4-5 internodes are necessary to collect sufficient juice for the detection procedure. While this still does not avoid any false negatives it will increase detection of weakly positive samples. An improved detection method for *Lxx* will be crucial for an improved management of RSD. An integrated pest management system is in place and the improved detection of *Lxx* would lead to immediate returns.

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