

Alkaline-Induced Metaxylem Autofluorescence: A Diagnostic Symptom of Ratoon Stunting Disease of Sugarcane

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ABSTRACT

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Ratoon stunting disease (RSD) of sugarcane caused by the vascular limited bacterium *Clavibacter xyli* subsp. *xyli* can be diagnosed by a host response, alkaline-induced metaxylem autofluorescence. Mature basal internodes of sugarcane cut in cross sections and treated with 0.1 M Tris (tris[hydroxymethyl]aminomethane), pH 10, exhibited red autofluorescence

in the secondary walls of metaxylem cells when the plant was infected, but not when healthy. The sections were viewed by fluorescence microscopy (exciting filter 530-560 nm, beam-splitting mirror 580 nm, suppression filter 580 nm). One person using the fluorescence microscopy technique can easily determine the RSD-status of 400 samples per day.

Additional key words: bacterial diseases, disease diagnosis, vascular pathogens.

Ratoon stunting disease (RSD) of sugarcane has no external symptoms in sugarcane. The internal symptom of red discoloration of the vascular bundles at the nodal plexus is unsatisfactory for conclusive diagnosis because it takes several months to develop, and some cultivars do not develop this symptom (15,16). Diagnosis of the disease has depended on microscopic detection of the causal bacterium, *Clavibacter xyli* subsp. *xyli* (7,8) Davis, by phase-contrast or dark-field microscopy (9) or fluorescent antibody techniques (6).

Artschwager (1) reported extensive sieve tube lignification as a pathological condition associated with ratoon stunting disease of sugarcane. In an attempt to determine changes in phloem that might be symptomatic of RSD, several staining procedures were examined. Aniline blue staining at various pHs for detection of callose by fluorescence, which can be symptomatic for several prokaryote-caused diseases, was among the procedures used.

This paper reports the discovery of a host response to the presence of the causal bacterium in the metaxylem (12,13,17) of sugarcane with ratoon stunting disease. This response, alkaline-induced metaxylem autofluorescence, can be used as a diagnostic assay for the disease. Preliminary reports of this work have been published (3,5).

MATERIALS AND METHODS

Sugarcane (*Saccharum* interspecific hybrid) cultivar L 62-96 was grown at the Louisiana State University St. Gabriel Research

Station, St. Gabriel. Other commercial cultivars were provided from greenhouse-grown material (15 mo old) by G. T. A. Benda, U.S. Sugarcane Field Laboratory, Houma, LA. Samples of sugarcane cultivar CP 65-357 were obtained from commercial fields with the help of Dalton Landry, county agent, LaFourche Parish, LA.

Sugarcane stalk samples (single-node cuttings) were cut from the lowest aboveground internode. If necessary, the rind was trimmed from the cutting to allow it to fit node up into a 50-ml conical bottom plastic centrifuge tube. The cutting was centrifuged for 3 min at 1,000 g to extract vascular contents. This procedure facilitated extraction of the causal bacterium *C. x. subsp. xyli* from the xylem vessels of the sample (2).

The occurrence of the distinctive *C. x. subsp. xyli* in the vascular extract of the centrifuged cutting was positive evidence of RSD. To determine this, an aliquot of the vascular extract was pipetted onto a microscope slide and covered with a number 1 cover slip. The bacterium was detected by dark-field microscopy at 500 \times using a Leitz Ortholux II microscope with a Phaco I 32 \times long-working-distance objective in conjunction with phase ring II on the condenser.

Cross sections from the internode and/or growth-ring area of a centrifuged sample were prepared freehand with a single-edge razor blade or a knife. The cross sections were placed on a microscope slide and treated as follows. Initial experiments employed lignin-specific stains, azure B, chlorine sulfite, phloroglucinol/HCl, and Schiff's reagent (11). In addition, alkaline aniline blue was employed for callose staining (11). The lignin-specific stains were monitored by interference contrast microscopy using a Leitz Ortholux II microscope at 250 \times . The

aniline blue stained tissue and, later, the 0.1 M Tris treated tissue were viewed by fluorescence microscopy on either Leitz Ortholux II microscope (12 V, 100 W Hg lamp) fitted with a Ploemopak using filter blocks H2 (exciting filter 390–490 nm, beam-splitting mirror 580 nm, suppression filter 580 nm) at a magnification of 25–50 \times , or on a Wild M5A dissecting scope 6 V, 50 W Hg lamp) fitted with a Leitz epifluorescence illuminator using N2 filter blocks at a magnification of 25 \times . The H2 filter block is recommended for fluorescein fluorescence and N2 for rhodamine fluorescence.

RESULTS

Internodal cross sections from diseased and healthy cuttings

failed to stain positively for lignin in phloem of diseased cuttings, in contrast to the results of Artschwager (1). Nor were there any other differences in staining apparent between diseased and healthy tissue when stained with lignin-specific stains.

Likewise, no phloem callose deposition was detected in cross sections from healthy or diseased cuttings stained with alkaline aniline blue and viewed by epifluorescence (filter block H2).

However, metaxylem walls from diseased cane fluoresced golden-yellow and those from healthy tissues fluoresced green. When viewed with filter block N2, the fluorescence in metaxylem walls from diseased samples was an intense red, while there was no fluorescence in metaxylem walls from healthy samples (Fig. 1A and B). This suggested an autofluorescence rather than an aniline blue response. When similar tissues were treated with several drops

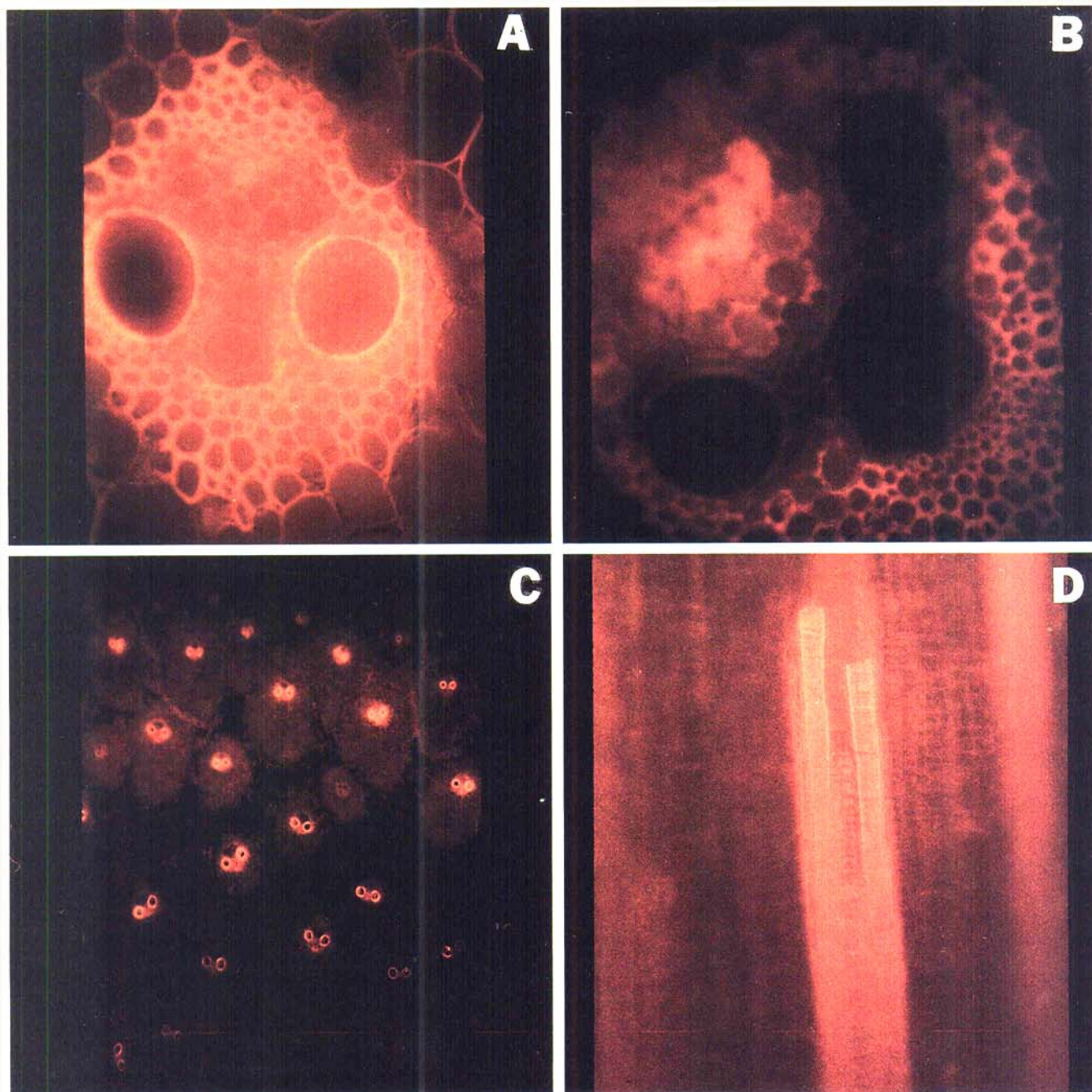


Fig. 1. Alkaline-induced autofluorescence in sugarcane cultivar L 62-96 stalk tissue. **A**, Autofluorescent metaxylem walls and vascular tissue from a basal internode of ratoon-stunt diseased sugarcane. (cross section, 400 \times) **B**, Lack of autofluorescence in metaxylem walls of a healthy sugarcane internode. (cross section, 400 \times) **C**, Autofluorescent metaxylem walls from a nodal growth ring of a basal section of ratoon-stunt diseased sugarcane. (cross section, 25 \times) Note the lack of autofluorescence in the surrounding vascular tissue compared with **A** and **B**. **D**, Longitudinal section of a vascular bundle from the growth ring of diseased sugarcane showing autofluorescence in secondary walls of the metaxylem cells. (100 \times)

of 0.1 M Tris, pH 10, the same golden-yellow (H2) or red (N2) response occurred in metaxylem walls from diseased samples confirming the autofluorescence response. As the red autofluorescence was easier to see, the filter block N2 was used in subsequent assays.

When cross sections of the growth ring from diseased plants were made alkaline with 0.1 M Tris, they exhibited intense red autofluorescence of the metaxylem wall and very little autofluorescence in the polar cap cells of the vascular support tissue (Fig. 1C) compared with cross sections from the internode (Fig. 1A and B). The cross sections from the growth ring of the control plants gave no alkaline-induced metaxylem autofluorescence.

Longitudinal sections through the growth ring showed that the secondary walls, which are annular or helical, were more intensely autofluorescent than the primary walls of the metaxylem cells (Fig. 1D). Secondary walls of metaxylem cells in the internode are scalariform or reticulate rather than annular.

Not all metaxylem walls within a cross section from diseased material autofluoresced; the more centrally located bundles were more likely to exhibit autofluorescence, although it was not uncommon to see almost all vessel elements fluorescing (Fig. 1C). In some vascular bundles, only one or a portion of one of the two metaxylem walls exhibited fluorescence.

To determine if the alkaline-induced metaxylem autofluorescence was a symptom of ratoon stunting disease, stalk samples of hot-water-treated, aerated-steam-treated, and diseased mature progeny of sugarcane cultivar L 62-96 were analyzed by the centrifugation/dark-field/autofluorescence protocol. The results were consistent with the association of metaxylem autofluorescence with the presence of the causal bacterium in vascular extracts, and thus with the disease. The 12 samples of progeny of hot-water-treated cane in which the causal bacterium was detected exhibited metaxylem autofluorescence, whereas the remaining 68 samples were negative for both bacteria and fluorescence. Likewise, the five samples of progeny of aerated-steam-treated canes in which the causal bacterium was detected also exhibited metaxylem autofluorescence, while the remaining 100 samples were negative for both. The progeny of diseased cane had detectable bacteria in 93 of 95 stalks sampled, and all 95 of the samples exhibited metaxylem autofluorescence.

When the autofluorescence technique was applied to field samples of sugarcane cultivar CP 65-357, the presence of mature-node symptom of RSD was correlated with the presence of metaxylem autofluorescence or the presence of the causal bacterium. Samples of 100 stalks were picked from the heap row of freshly harvested cane in two fields, and basal nodes were sliced longitudinally to detect visual internal symptoms. Subsamples (33 stalks) were brought to the laboratory for the centrifugation/dark-field/autofluorescence protocol. Field A was free of visual symptoms in the 100 stalk sample and free of bacteria and autofluorescence in the 33 stalk subsample. Field B contained a high proportion (44/100) of stalks with mature-node symptoms. A subsample of 10 stalks with symptoms was positive for both bacteria and autofluorescence. A subsample of 23 stalks without symptoms from Field B had 19 stalks that were positive for bacteria and autofluorescence, two that were negative for both, and two that were negative for bacteria and positive for autofluorescence. This experiment was repeated by analyzing a complete 50-stalk sample from each of the two fields with similar results.

To determine if there were cultivar differences in the association of alkaline-induced metaxylem autofluorescence with ratoon stunting disease, two blind experiments were conducted on greenhouse-grown material from the Houma Laboratory. The first experiment involved 20 randomly numbered stalks; one stalk from progeny of heat-treated and one stalk from progeny of *C. x. subsp. xyli* inoculated cuttings from each of 10 different cultivars. The lower nodes were centrifuged and vascular extracts were examined for bacteria by dark-field microscopy. Sections of internode and growth ring were examined for alkaline-induced metaxylem autofluorescence after treatment with 0.1 M Tris. The experiment

was repeated using 40 randomly numbered stalks, two stalks from progeny of heat-treated, and two stalks from progeny of *C. x. subsp. xyli* inoculated cuttings for each of 10 cultivars. No attempt was made to compare autofluorescence results and bacterial results until the random numbers were assigned to a specific cultivar. Results of both experiments are given in Table 1.

In the first experiment, all 10 progeny of hot-water-treated cultivars were negative for both bacteria and metaxylem fluorescence. All 10 progeny of inoculated cultivars were positive for both bacteria and metaxylem fluorescence. In the second experiment all 20 samples of the 10 inoculated cultivars were positive for fluorescence and bacteria. All 20 samples of the 10 hot-water treated cultivars were negative for bacteria; however, two of the 20 samples, both CP 52-68, were positive for metaxylem autofluorescence.

DISCUSSION

The results indicate metaxylem autofluorescence is associated with the presence of the causal bacterium and, thus, with the disease. The occurrence of six samples (two each from L 62-96, Table 1 and Field B samples) in which no bacteria were detected but which exhibited metaxylem autofluorescence suggests either that metaxylem autofluorescence may be more sensitive for the diagnosis of RSD than dark-field microscopic detection of the bacterium or that other factors may occasionally cause autofluorescence. The former contention is supported by the failure to find a single sample that was negative for metaxylem autofluorescence and positive for the presence of the bacterium. Thus, there were no apparent false negatives for the autofluorescence assay; however, these results are based on mature plants and no survey of young diseased plants nor an assessment of the time lag between inoculation and appearance of the metaxylem autofluorescence was attempted. The alternative explanation for the absence of detectable bacteria in autofluorescence positive samples is that they are false positives. If this is true they represent only 6 of the 417 samples reported in this paper or about 1.5%.

The finding of diseased samples in progeny of heat-treated sugarcane, which is a control measure for the disease, is not

TABLE 1. Association of alkaline-induced metaxylem autofluorescence with the presence of *Clavibacter xyli* subsp. *xyli* in progeny of hot-water-treated and *C. x. subsp. xyli*-inoculated sugarcane cultivars

Cultivar	Progeny of hot-water treated		Progeny of inoculated	
	Bacteria ^w	Fluorescence ^x	Bacteria	Fluorescence
L60-25	- ^y	-	+	+
	--- ^z	---	++	++
L62-96	-	-	+	+
	---	---	++	++
CP48-103	-	-	+	+
	---	---	++	++
CP52-68	-	-	+	+
	---	++	++	++
CP61-37	-	-	+	+
	---	---	++	++
CP65-357	-	-	+	+
	---	---	++	++
CP72-356	-	-	+	+
	---	---	++	++
CP72-370	-	-	---	---
	---	---	++	++
CP73-351	-	-	+	+
	---	---	++	++
CP74-383	-	-	+	+
	---	---	++	++

^w Detected by dark-field microscopy of a vascular extract from a sugarcane internode.

^x Detected by fluorescence microscopy of a tissue cross section from the same sugarcane internode.

^y Experiment 1 (one stalk per treatment).

^z Experiment 2 (two stalks per treatment).

without precedent (4). The frequencies of escape from these two treatments should not be interpreted as a measure of relative efficiency of the two control methods.

The results with the field sampling of cultivar CP 65-357 confirmed that the presence or absence of the visible symptom of red discoloration at the nodal plexus can be unreliable for determining RSD in individual stalks. There were many false negatives apparent in samples from Field B.

The results with the 10 cultivars (Table 1) suggest that this assay is not appreciably affected by cultivar differences. The 10 cultivars represent a range of tolerance to RSD. The tolerant, and lower bacterial titer hosts CP 52-68 and L 60-25 were included as well as the more intolerant, higher bacterial titer host L 62-96 (14). The seven remaining cultivars are intermediate in tolerance to RSD.

The tunneling of the sugarcane borer *Diatraea saccharalis* (F.) also causes an intense red autofluorescence under the assay conditions. However, it was readily distinguished from the RSD fluorescing samples because autofluorescence in this case is not confined to metaxylem walls; the lumen of the metaxylem as well as parenchyma ground tissue around the vascular bundles fluoresced in response to the borer. Also, the vascular bundles interrupted by borer tunnels appeared darkened to the naked eye.

The ability of other vascular pathogens of sugarcane to cause alkaline-induced metaxylem autofluorescence has not been completely evaluated. However, those diseases exhibit obvious symptoms, which should preclude confusion with RSD. Leaf scald caused by *Pseudomonas albilineans*, which does not occur in Louisiana, should be examined with this technique. In addition other hosts of *Clavibacter* spp., e.g. alfalfa, should also be examined for this phenomenon.

There was no autofluorescence from colonies of *C. x. subsp. xyli* grown on SC medium and treated with 0.1 M Tris. This suggested the metaxylem autofluorescence was a host response to the presence of the bacterium in the vessel elements.

The addition of 0.1 M Tris or 0.1 M ammonium hydroxide or other alkaline reagents was required to bring out the metaxylem autofluorescence. The treatments also turned the healthy and diseased tissues yellow to the naked eye. Because ammonium hydroxide interacts with metals, 0.1 M Tris was preferred. The fluorescence was readily reversible by acid treatment. A report of fluorescence intensification in cell walls of grass stems is ascribed to bound ferulic acid (10). It is known that phenolic acids and ester derivatives often show changes in fluorescence intensity and color when treated with ammonia. Attempts to extract the fluorescing compound(s) from ratoon-stunt diseased material have not been successful. Several histochemical tests (azure B, chlorine sulfite, phloroglucinol/HCl, and Schiff's reagent) (11) for the putative identification of lignin have failed to show differences in diseased and healthy material.

The results associating the metaxylem autofluorescence with the presence of the causal bacterium suggests that assays based on

metaxylem autofluorescence alone would provide an accurate estimate of the presence or absence of ratoon stunting disease.

LITERATURE CITED

1. Artschwager, E. 1959. Sieve tube lignification in sugarcane and its significance in relation to ratoon stunting disease. Proc. Int. Soc. Sugar-Cane Technol. 10:1077-1083.
2. Damann, K. E., Jr. 1980. Ratoon stunting disease of sugarcane: The causal bacterium. Pages 109-111 in: Proc. First Inter American Sugar Cane Seminar: Cane Diseases. Vanguard Co., Miami, FL.
3. Damann, K. E., Jr. 1983. Detection of ratoon stunting disease of sugarcane by metaxylem autofluorescence. (Abstr.) Phytopathology 73:500.
4. Damann, K. E., Jr., and Benda, G. T. A. 1983. Evaluation of commercial heat-treatment methods for control of ratoon stunting disease of sugarcane. Plant Dis. 67:966-967.
5. Damann, K. E., Jr., Ogunwolu, E. O., and Reagan, T. E. 1984. Incidence of ratoon stunting disease in Louisiana. (Abstr.) Phytopathology 74:627.
6. Davis, M. J., and Dean, J. L. 1984. Comparison of diagnostic techniques for determining incidence of ratoon stunting disease of sugarcane in Florida. Plant Disease 68: 896-899.
7. Davis, M. J., Gillaspie, A. G., Jr., Harris, R. W., and Lawson, R. H. 1980. Ratoon stunting disease of sugarcane: Isolation of the causal bacterium. Science 210:1365-1367.
8. Davis, M. J., Gillaspie, A. G., Jr., Vidaver, A. K., and Harris, R. W., 1984. *Clavibacter*: A new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermuda grass stunting disease. Int. J. Syst. Bacteriol. 34(2):107-117.
9. Gillaspie, A. G., Jr., Davis, R. E., and Worley, J. F. 1973. Diagnosis of ratoon stunting disease based on the presence of a specific microorganism. Plant Dis. Rep. 57:987-990.
10. Harris, P. J., and Hartley, R. D. 1976. Detection of bound ferulic acid in cell walls of Gramineae by ultraviolet fluorescence microscopy. Nature (London) 259:508-510.
11. Jensen, W. A. 1962. Botanical Histochemistry. W. H. Freeman & Co., San Francisco. 408 pp.
12. Kao, J., and Damann, K. E., Jr. 1978. Microcolonies of the bacterium associated with ratoon stunting disease found in sugarcane xylem matrix. Phytopathology 68:545-551.
13. Kao, J., and Damann, K. E., Jr. 1980. In situ localization and morphology of the bacterium associated with ratoon stunting disease of sugarcane. Can. J. Bot. 58(3):310-315.
14. Koike, H., Gillaspie, A. G., Jr., and Benda, G. T. A. 1982. Cane yield response to ratoon stunting disease. Int. Sugar J. 84:131-133.
15. Ricaud, C. 1974. Problems in diagnosis of ratoon stunting disease. Proc. Int. Soc. Sugar-Cane Technol. 15(1): 241-249.
16. Steindl, D. R. L. 1961. Ratoon stunting disease. pages 432-459 in: Sugar-Cane Diseases of the World. Vol. 1. J. P. Martin, E. V. Abbott, and G. C. Hughes, eds. Elsevier, New York. 542 pp.
17. Worley, J. F., and Gillaspie, A. G., Jr. 1975. Electron microscopy in situ of the bacterium associated with ratoon stunting disease in sudangrass. Phytopathology 65:287-295.