

SOME OBSERVATIONS ON THE BACTERIUM ASSOCIATED WITH RATOON STUNTING DISEASE OF SUGARCANE

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Abstract

The consistent association of a bacterium with ratoon stunting disease (RSD) has been confirmed in South Africa by phase contrast and electron microscope examination of sugarcane tissue diffusates from a wide range of varieties. The bacterium is rod shaped, often curved and measures approximately $1,0-4,0 \mu\text{m} \times 0,2-0,3 \mu\text{m}$. Bacteria appear to be systemic within plants affected by RSD, but are most readily observed in diffusates from mature stalk tissue of intolerant varieties. The phase contrast microscope technique described appears useful as a confirmatory aid to RSD diagnosis, but cannot confidently be used as the sole method of diagnosis in very young cane.

Introduction

Until recently the causal agent of RSD has generally been presumed to be a virus (Steindl^{1,3}). This presumption had been largely based on inability, in the past, to demonstrate the presence of a possible causal agent but, despite considerable research effort in a number of countries (Hughes and Steindl,⁸ Gillaspie,³ Gillaspie *et al*⁷), no convincing evidence of a viral cause of this important disease has been demonstrated.

Following reports by Plavsic-Banjac and Maramorosch¹¹ and Maramorosch *et al*¹⁰ that pleomorphic bodies resembling small bacteria or rickettsiae had been observed by electron microscopy in the xylem of ratoon stunted sugarcane, the presence of a bacterium associated with RSD has recently been reported from a number of sugar producing areas, including Australia (Teakle *et al*¹⁶), Louisiana (Gillaspie *et al*¹⁵), Puerto Rico (Liu *et al*⁹), Mauritius (Ricaud *et al*¹²), and Taiwan (Chen *et al*²).

The bacterium has generally been described as slender, rod shaped, sometimes curved and coryneform, and occasionally branched. Electron micrographs (Worley and Gillaspie¹⁷, Chen *et al*², Teakle *et al*¹⁶) show that the bacterium is often septate and has a smooth cell wall, while detailed micrographs by Chen *et al*² and Teakle *et al*¹⁶ show that each bacterium contains characteristically coiled mesosomes, also reported by Ricaud *et al*¹². The size of the bacterium has been reported to be within the range $1,0-3,0 \mu\text{m} \times 0,12-0,33 \mu\text{m}$ (Teakle¹⁴, Ricaud *et al*¹², Chen *et al*²), although reports from Louisiana (Gillaspie,⁴ Worley and Gillaspie¹⁷) indicate a size of $5-10 \mu\text{m} \times 0,3-0,5 \mu\text{m}$.

Techniques used by other workers to observe the bacterium have involved electron and light microscopic examination of concentrated stalk extracts, exudates and tissue diffusates. The bacterium has also been observed *in situ* within the xylem elements of sugarcane (Maramorosch *et al*¹⁰) and also of sorghum \times sudangrass hybrids affected by RSD (Worley and Gillaspie¹⁷). The systemic distribution of the bacterium in various tissues of the sugarcane plant has been described by Teakle *et al*¹⁵ and Ricaud *et al*¹².

The bacterium has not yet been isolated and cultured, and hence conclusive proof of pathogenicity is lacking. However, the growing evidence of the consistency of the association of a bacterium with RSD strongly indicates a bacterial etiology

for this important disease, and also indicates the possibility of rapid RSD diagnosis (Gillaspie *et al*⁵, Teakle *et al*¹⁵).

This paper describes attempts in South Africa to observe a bacterium in sugarcane affected by RSD, and discusses the value of phase contrast microscope observations of the bacterium in RSD diagnosis.

Methods

Initial attempts to observe a bacterium in plants affected by RSD in South Africa utilised a method of Teakle *et al*¹⁶, in which sterile water was passed through infected stalk material under partial vacuum and concentrated by centrifugation. In other attempts nodal tissue of affected plants was macerated in sterile water and allowed to stand for two hours before examination. Although bacteria, approximately $2,0 \times 0,4 \mu\text{m}$, were observed in these preparations, numbers were always sparse, with a maximum of up to 5 per $\times 1200$ field of view. In view of this, and the occasional presence of bacteria in preparations from cane presumed to be free of RSD, these first attempts were regarded as unsatisfactory (Anon¹), although the size of the bacteria observed was similar to that quoted in published reports.

Later, consistently successful attempts to observe the bacteria have used a tissue diffusate method similar to that described by Teakle *et al*¹⁵ and more recently by Ricaud *et al*¹². Cane stalks are washed, surface sterilized by wiping with alcohol and flaming, and peeled. Five, thin (approximately 1-2 mm) transverse sections are cut at each node or internode with a sterilized knife under aseptic conditions and the tissue discs from a composite sample of four nodes or internodes per stalk are steeped in 25 ml sterile, distilled water for two hours. The proportion of cane tissue to water is approximately 1 : 2 (12-15 g in 25 ml). It has been found advantageous to steep 8×5 -section samples in 50 ml water, allowing greater scope for treating several stalks per plant in one composite sample, and also providing a greater concentration of bacteria after centrifugation. To remove coarse plant debris the tissue diffusates are then filtered through medium or fast grade filter paper (Whatman 40 or 41), directly into appropriate centrifuge tubes, and they are centrifuged at 14 000 g for at least 30 minutes in a Sorvall RC2-B centrifuge. Care is taken during the preparation to maintain aseptic conditions, and the diffusing and centrifugation processes are carried out at approximately 5°C to minimise the possibility of contamination. The method has also proved suitable for other sugarcane tissues, such as the young tissues around the growing point and leaf material, although, with the latter, the proportion of chopped leaf tissue to water is approximately 1 : 5.

After centrifugation the pellet is resuspended in a few drops (approximately 0,3 ml) of sterile water or supernatant for microscopic examination.

Routine light microscope examinations have been carried out on a Zeiss Photomicroscope using phase contrast illumination at magnifications of $\times 1000$ or greater, a drop of the resuspended pellet being examined directly under a cover slip.

Electron microscopic examinations have been carried out on a Hitachi HU-11E-1 instrument. A drop of the resuspended

pellet was evaporated on a Formvar-coated, 200 gauge, copper mesh specimen grid and negatively stained for 30 seconds with one drop of 2,0% sodium phosphotungstate (pH 6,0-7,0). Distribution of bacteria was found to be very sparse if the drop of suspension was evaporated on the grid while the latter rested on blotting paper, but a high frequency of bacteria per grid square was obtained if the grid was held in forceps and the entire drop of suspension was evaporated before staining.

In the studies reported here procedures for examination by light microscopy were standardised, as far as possible, to allow comparative estimates of numbers of bacteria from different preparations to be made.

Results and discussion

(a) Description and identification

Under the phase microscope the RSD bacteria appear to be characteristically slender and rod shaped, and often curved, with an estimated size of approximately $1,0-4,0 \times 0,3 \mu\text{m}$ (Figs. 1 and 2).

The numbers of bacteria observed in any one field of view have varied considerably with the variety and also with the relative maturity and type of tissue under examination. Preparations from mature stalks of intolerant varieties, such as N6 and N53/216, have often shown numbers of bacteria of

the order of 20-50 per $\times 1000$ field of view, and estimates of up to 100 per field have been made. However, other preparations have had concentrations of only $< 1-5$ per field, and at these low concentrations the bacteria may more easily escape observation, particularly if the preparation contains a confusing, multiparticulate background as often occurs with young tissue, a problem noted also by Ricaud *et al.*¹² A similar confusing background also prevents ready recognition of the RSD bacteria in juice crushed from infected stalks.

Diffusates from mature stalk tissues, particularly from internodes are, however, relatively clear of background debris, and observation of the slender RSD bacterium under the phase microscope is not difficult (Fig. 1). For measuring and micrographic purposes the concentrated tissue diffusates can readily be fixed and stained, carbol fuchsin and nigrosin giving satisfactory results (Fig. 2). Measurements and micrographs of fresh, unfixed preparations by phase contrast, are, however, likely to be more accurate (Fig. 1), and the bacteria have been satisfactorily immobilised by absorbing a drop of the diffusate on to a 10% gelatin film under a coverglass.

The high concentrations of RSD bacteria observed in many tissue diffusate preparations of a number of varieties in these studies may partially be a result of using intolerant varieties and of concentrating the diffusates to a high degree, but the ease with which large numbers of bacteria have been observed

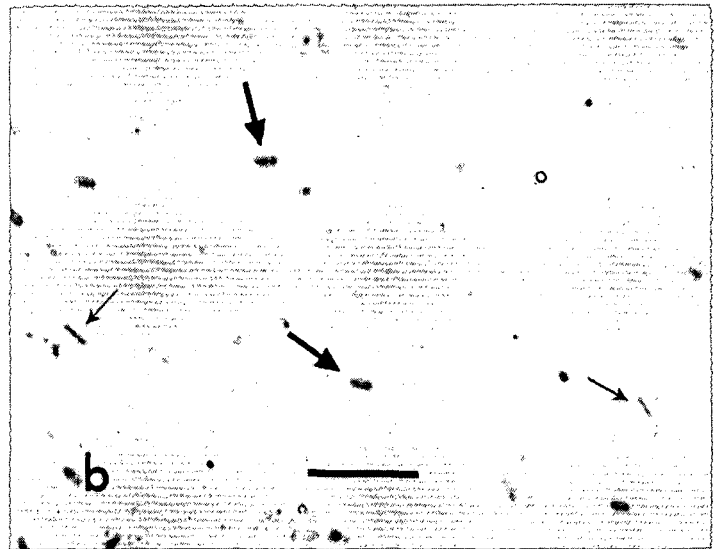
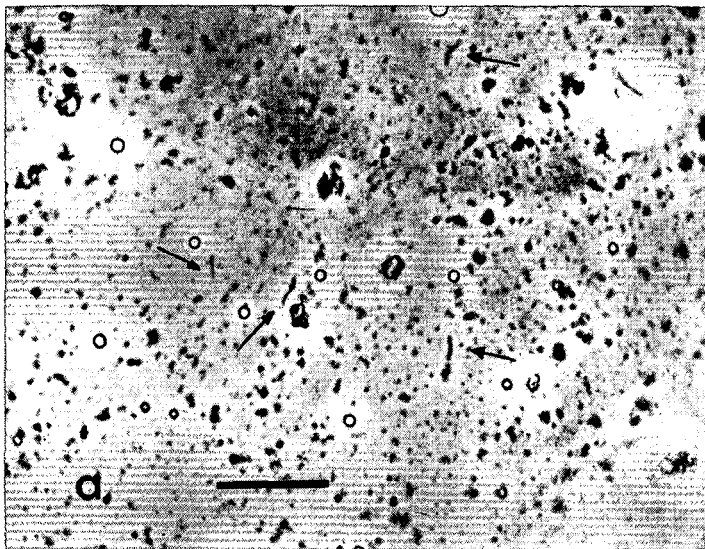


FIGURE 1 Phase contrast micrographs of bacteria in tissue diffusate suspensions from ratoon stunted sugarcane.

(a) Fresh suspension.

(b) Suspension after 24 hours at room temperature. Slender RSD bacteria (small arrows) are readily distinguished from contaminants (large arrows). Bar is $10 \mu\text{m}$.

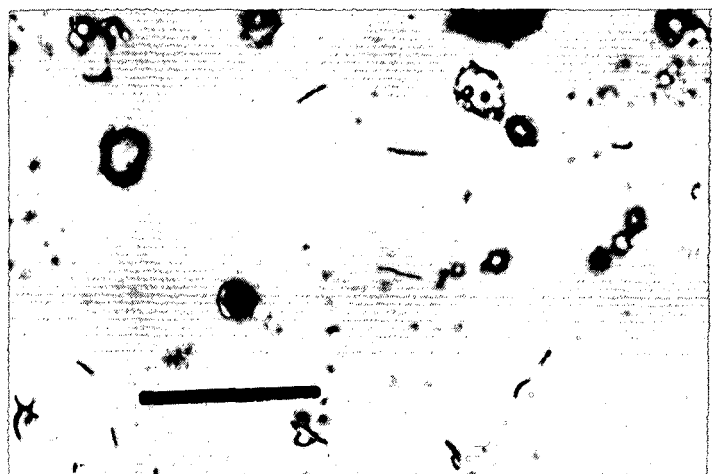
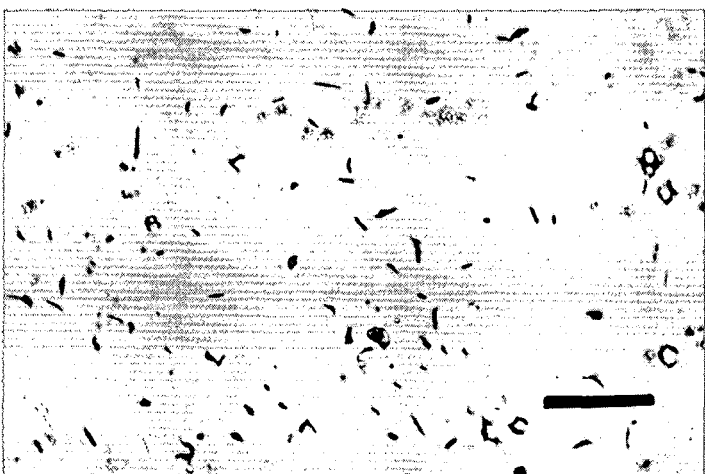


FIGURE 2 Preparations of bacteria from ratoon stunted sugarcane dried and stained with carbol fuchsin, showing the typically slender form and variation in size. Bar is $10 \mu\text{m}$.

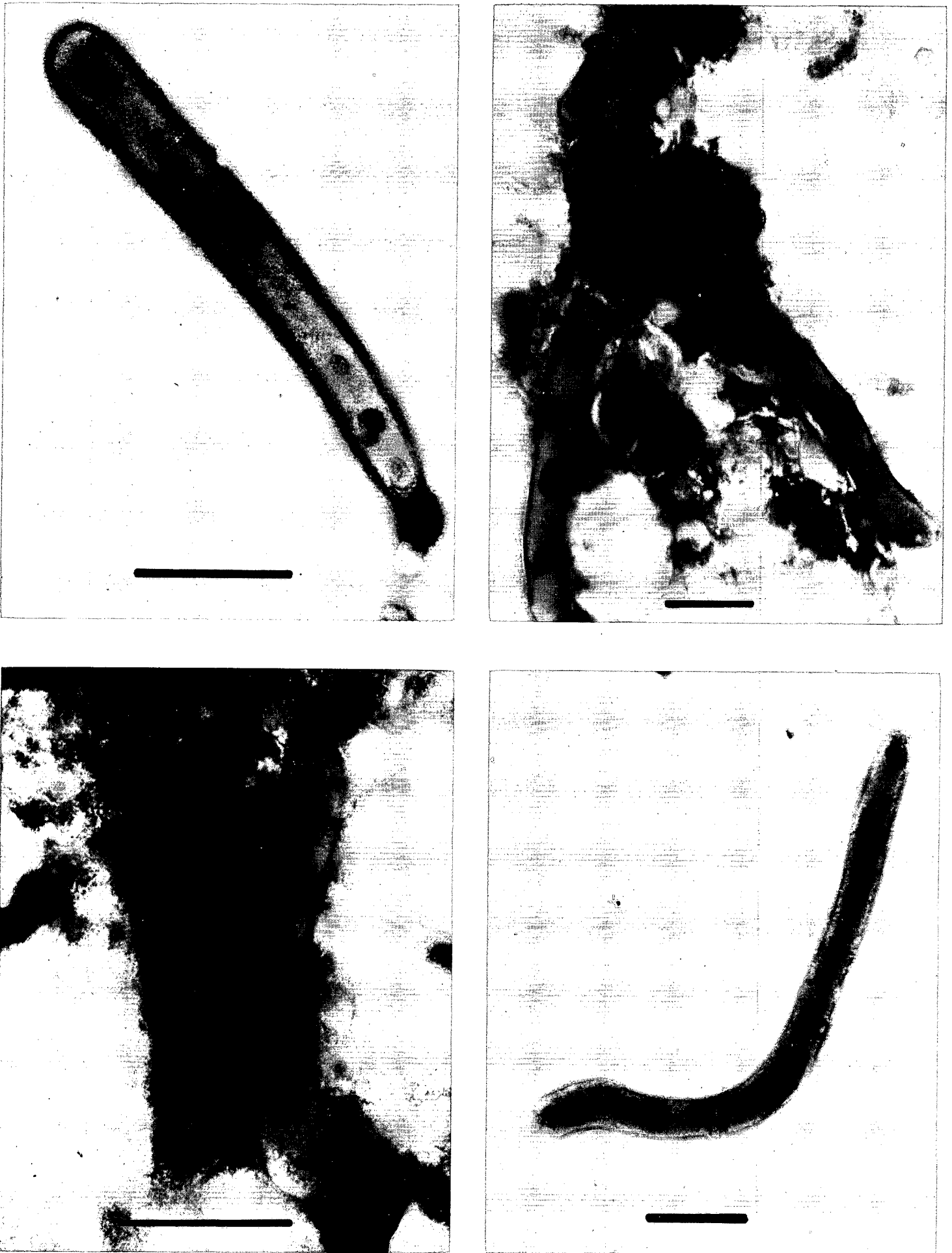


FIGURE 3 Electron micrographs of negatively stained bacteria in tissue diffusates from ratoon stunted sugarcane, showing coiled mesosomes, septa and the thin cell walls. Bar is 0,5 μm .

by simple methods contrasts strongly with the inability, in the past, to demonstrate a causal agent, and also contrasts with a limited success in observing the bacterium in several sugarcane varieties reported by Worley and Gillaspie¹⁷.

Apart from problems due to low numbers of bacteria in some varieties, and in young tissues, two other sources of error in identification of the RSD bacterium by light microscopy appear possible. Contamination of tissue diffusates by saprophytic bacteria may occur rapidly, but can largely be obviated by aseptic technique, the maintenance of low temperatures during processing and storage of preparations and the avoidance of any delay before observation. The diffuse technique also readily extracts other vascular bacterial inhabitants, such as *Xanthomonas* spp, and any saprophytic colonising bacteria. Care in the selection of material for processing and the use of fresh samples aid in preventing confusion of this sort, but with experience the slender form of the RSD bacterium can, with little difficulty, be distinguished from other bacteria likely to be present (Fig. 1).

The concentrated stalk tissue diffusates have proved suitable for electron microscope observations without further preparation. The limited electron microscope examinations possible in these studies have confirmed the identity of the RSD bacterium. The bacteria are permeable to phosphotungstate negative stain (Teakle *et al*¹⁶) and can be seen to have a thin cell wall, approximately 10-20 m μ wide, and to be frequently septate. Each cell contains at least one and often several coiled mesosomes (Fig. 3). Under the electron microscope the bacteria again appear typically slender and rod shaped, many being curved, and occasionally coryneform. Estimated size of the bacterium from electron micrographs is 1,1-3,0 μ m \times 0,2-0,27 μ m, with a mean of 1,7 \times 0,22 μ m.

The bacterium reported here appears identical to that described from sugarcane in Australia (Teakle *et al*¹⁶), Mauritius (Ricaud *et al*¹²) and Taiwan (Chen *et al*¹²), and is probably the same as the bacterium reported from sugarcane and sudangrass in Louisiana (Gillaspie *et al*⁵, Worley and Gillaspie¹⁷). Although Gillaspie and his colleagues have reported a size of 5-10 μ m \times 0,3-0,5 μ m, bacteria in certain electron micrographs published by these workers have an apparent size of approximately 3,0 \times 0,3 μ m, similar to that reported in this paper and elsewhere.

Classification of the RSD bacterium is not yet clear, although Worley and Gillaspie argue that the frequency of septa and lack of evidence of binary fission suggest that it belongs to the coryneform group of bacteria. However, in these studies the bacterium has not been found to be Gram-positive, as is usual for the coryneforms.

(b) *Consistency of the association of the bacterium with RSD and systemic distribution of the bacterium*

Phase microscope examinations have now been carried out on more than 200 preparations from more than 120 plants suspected of being infected by RSD and exhibiting to a varying degree the nodal symptoms of this disease. Infected plants have been obtained largely from RSD trials and propagation plots established at the Experiment Station, Mt Edgecombe in the period 1969-1975. The age of this material has varied from 3 month old plant cane to mature third ratoon material. Several samples examined were collected from other areas in the Natal cane belt.

The majority of infected plants examined have been of variety N53/216, which is intolerant of RSD and has conspicuous nodal symptoms. The bacterium has readily been observed in stalk tissue diffusates of N53/216, and in this variety and in the extremely intolerant local seedling varieties 67L/809 and 67L/1085 it has also been observed in juice crushed from infected stalks. The bacterium has also been

consistently observed in preparations from mature nodal tissues of a wide range of varieties. Apart from N53/216, varieties in which a frequency of bacteria greater than 20 per x1000 field of view has been observed include N6, N7, N8, NCo 334, NCo 376, NCo 382, N55/805, N59/1312, Co 281 and CP 44/101. The bacterium has also been observed in the following varieties: CB38/22, Co 331, NCo 293, NCo 310, N50/211, N51/168, N51/539, N52/219, N55/516, N59/2 and S 17. High numbers of bacteria have often been associated with marked nodal symptoms, but have also been found in varieties with only slight or indiscernible symptoms, such as are usually exhibited by NCo 376. In more than 20 plants of varieties NCo 376, N55/805 and N53/216 derived from heat treated seedcane, and assumed to be free from RSD, the bacterium has not been observed.

Examination of diffusates from various tissues of variety N53/216 has shown that the bacterium occurs apparently systemically within plants affected by RSD. In one test approximately 12,0 g fresh masses of stalk tissue and 5,0 g of leaf tissues were steeped in 25 ml water. Bacteria were observed in preparations from all parts of the stalk, with the exception of young, undifferentiated tissues at and immediately proximal to the growing point, and were also observed in preparations from leaf midribs and young leaves (Table 1).

TABLE 1
Occurrence of bacteria in concentrated diffusates of various tissues of sugarcane variety N53/216 affected by RSD

Plant part	Estimated number of bacteria per x1000 field of view
Nodes, mid-lower stalk	100
Internodes, mid-lower stalk	20
Nodes, upper stalk	20
Growing point and differentiating tissues of upper stalk	0
Young leaves above growing point	2
Midrib of mature leaves	5
Lamina of mature leaves	0

A systemic distribution of the bacterium has been reported also by Ricaud *et al*¹² and Teakle *et al*¹⁵, both groups of authors also having difficulty in observing the bacterium in young, immature tissue.

In comparative tests of a number of varieties more bacteria have been observed in preparations from the nodal than from the internodal areas of mature stalks, but internodal preparations have usually contained less background debris and the use of nodal rather than internodal tissue for observation of the RSD bacterium is not considered critical.

Diagnosis of RSD by observation of the associated bacterium has been proposed by Gillaspie *et al*^{5, 6}, and Teakle *et al*¹⁵. The consistency and ease with which the bacterium has been observed from mature tissue in the studies reported here also indicates the value of light microscopy as a diagnostic technique. A technique based on centrifuged tissue diffusates, as first reported by Teakle *et al*¹⁵, rather than on root pressure exudates or fibrovascular sap extracts, is also simple, and has the added advantage that approximate standardization of methods enables it to be used for comparative examination of varieties or different cane tissues.

Some limitations in the use of phase contrast examination of diffusates from young cane are, however, apparent, as preparations from very young tissue have been found to contain only few bacteria in a confusing, finely particulate background.

Conclusions

Recent findings, in a number of sugarcane producing countries, that a small bacterium is consistently associated with

RSD strongly indicate a bacterial etiology for this important disease. The observations reported here, that a bacterium, apparently identical to that reported elsewhere, has been consistently found in a wide range of sugarcane varieties that exhibit, to a varying degree, the nodal symptoms of RSD, strongly supports such a hypothesis.

The bacterium was observed with ease by phase microscopy in diffusate preparations from mature stalk tissues of a wide range of varieties affected by RSD, provided that the preparations were adequately concentrated before examination.

Phase contrast examination of tissue diffusates appears a useful and simple means of RSD diagnosis, particularly in varieties that lack conspicuous nodal symptoms, and also of confirming diagnoses based on nodal symptoms. However, the technique has limitations in that preparations from young tissues may contain only few bacteria in a confusing multi-particulate background.

Acknowledgements

I should like to thank Professor A. R. A. Noel and Mrs M. G. Gilliland of the Botany Department, University of Natal, Pietermaritzburg, for the opportunity of making electron microscope observations and for the electron micrographs. I also thank Mr A. Sriram for his technical assistance.

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