MAURITIUS RESEARCH COUNCIL

ELIMINATION OF PATHOGENS FROM NOBLE CANES, SACCHARUM OFFICINARUM, BY TISSUE CULTURE METHODS FOR CONSERVATION AND MOLECULAR ANALYSIS TO DETECT GENETIC CHANGES

Final Report

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MAURITIUS SUGAR INDUSTRY RESEARCH INSTITUTE

Title of Project: Elimination of pathogens from noble canes, *Saccharum officinarum,* by tissue culture methods for conservation and molecular analysis to detect genetic changes.

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SUMMARY

New sugarcane varieties are interspecific hybrids derived mainly from crosses between the noble canes (*Saccharum officinarum*) and the wild type species of *Saccharum spontaneum*. Through a number of selection programmes, varieties with enhanced disease resistance, high sucrose content and increased water/drought tolerance are obtained. Owing to the importance of noble canes in breeding programmes, this project was carried out in order to eliminate the various pathogens infecting them and to introduce a long term *in vitro* system for their conservation.

A survey was carried out among an existing collection of noble canes to determine the level of infection with the following pathogens; sugarcane bacilliform virus (SCBV), sugarcane yellow leaf virus (ScYLV), *Xanthomonas campestris* pv *vasculorum* (XCV) and *Xanthomonas albilineans* (Xalb). All varieties tested for SCBV by PCR using primers SCBVF5 and SCBVR5 were found infected by the virus. ScYLV was detected in the noble canes by tissue blot immunoassay (TBIA) using leaf midribs and stems. The pathogen was found in 39 (out of 65) varieties with an incidence as high as 90% observed in several varieties. However, presence of the virus was not always associated with the typical symptoms of yellowing of the leaves. The stalk imprint method on Wilbrink medium and the diffusion test were used for the detection of XCV and Xalb. XCV was present in eight varieties of noble canes whereas Xalb was not detected in any plant.

A more effective tissue culture medium (TCM) was devised consisting of phytagel (1.8 g/l) instead of type A agar (7 g/l) and a 2:1 (13.7: 6.3 g/l) sucrose / glucose carbon source instead of 20 g/l sucrose as sole carbon source. Using this modified medium, high level of regeneration was observed with apical meristem and axillary buds. Similarly, embryogenic callus could be obtained from 95% of the varieties initiated.

Out of nine varieties tested for ScYLV by RT-PCR using primer pairs YLS 111 and YLS 462, plantlets from seven varieties were found freed from the virus. These were regenerated from apical meristem, axillary buds and from callus. ScYLV was eliminated from all plants derived from callus culture. Inability of the virus to colonise the somatic embryos or apical meristem may explain their absence from plantlets regenerated from such explants. Plantlets (variety M 168/33) regenerated from the three types of explants and presumably freed from the virus were potted and transferred to the glasshouse. After nine months, the virus remained undetected in these plants. However, all plantlets remained infected with SCBV following tissue culture.

The ability of an antiviral agent, ribavirin, to eliminate SCBV and ScYLV was investigated. This compound was found to be phytotoxic to *in vitro* plantlets at concentration above 30 mg/l in tissue culture medium. Treatment at 10-30 mg/l failed to eliminate either virus after 7-10 subcultures.

The evaluation of antibiotics for the elimination of XCV from noble canes was also carried out. Cefotaxime, tetracycline and rifampicin were found to have bacteriocidal activity against XCV in TCM. The minimal bacteriocidal concentration (MBC) of the antibiotics in TCM were as follows: tetracycline; 50 mg/l, rifampicin; 15 mg/l and cefotaxime; 90 mg/l. However, exposure to strong light led to the inactivation of the antibiotics, their activity falling from 2MBC to MBC within 4 days for tetracycline, 6 days for rifampicin and 7 days for cefotaxime. Under low light intensity, their activity remained above that of MBC for at least 19 days. The phytotoxicity of the antibiotics on sugarcane plantlets was also assayed. Exposure for two months with tetracycline at 2MBC led to the bleaching of newly formed leaves and eventual death of the plants. No phytotoxic effect could be associated with cefotaxime and rifampicin. The use of heat on the elimination of XCV was further studied. The thermal death point (TDP) of XCV was found to be 60°C for 30 min. Likewise axillary buds from some sugarcane varieties were found to be able to resist such temperature. These results suggest that heat could potentially be use for the elimination of XCV by treatment of axillary buds.

Finally, the effect of encapsulation/dehydration on the regeneration of somatic embryos and micromeristems (variety M 168/33) was studied. Three treatments were carried out: 1) direct regeneration, 2) regeneration following encapsulation in 3% alginate beads and 3) regeneration following encapsulation and dehydration. Encapsulation seemed to have minor effects on the regeneration of somatic embryos or meristems. However, no regeneration was observed following dessication of both encapsulated explants.

GENERAL INTRODUCTION

Sugarcane is a monocotyledon and belongs to the genus *Saccharum.* There are six species, *S. officinarum, S. robustum, S. sinense, S. barberi, S. spontaneum* and *S. edule. S. officinarum*, the noble cane, has the quality of high sucrose content and purity but is generally susceptible to diseases. Through a number of breeding programmes involving crosses between the noble canes and *S. spontaneum* or *S. barberi*, improved commercial hybrids with increased disease resistance have been released. It is therefore important to conserve the existing germplasm, especially those of noble canes, for further use in breeding programmes.

In Mauritius, a collection of some 125 *S. officinarum* clones is available in one location. However, this breeding collection is subjected to pests and diseases and adverse climatic conditions like cyclones and droughts, leading to loss of clones. Furthermore, in this collection resides a number of bacterial pathogens namely; *Xanthomonas campestris* pv. *vasculorum* (XCV), *X. albilineans* (Xalb) , *Clavibacter xyli* subsp. *xyli* (Cxx) as well as three viruses; sugarcane bacilliform virus (SCBV), sugarcane mild mosaic virus (SCMMV), and sugarcane yellow leaf virus (ScYLV). The distribution of these pathogens also makes it a major constraint for the exchange of germplasm.

Ideally, a long-term *in vitro* conservation of pathogen-free germplasm needs to be set up which would minimize the loss of clones and allow its safer exchange. This work involved a study of the distribution of the various pathogens among the noble canes, their elimination by chemotheurapeutic and *in vitro* techniques followed by their cryopreservation. In this report, the following aspects have been dealt with:

(1) An initial survey of the distribution of SCBV, ScYLV, XCV and Xalb in the collection of noble canes
(2) *In vitro* culture of noble canes and elimination of ScYLV by tissue culture
(3) Treatment of noble canes infected with SCBV and ScYLV with the antiviral agent, ribavirin
(4) Evaluation of the antibiotics for the elimination of XCV from the noble canes.
(5) Elimination of XCV from axillary buds by thermotherapy
(6) Effect of dessication on the regeneration of encapsulated micromeristems and somatic embryos.

(1A) INDEXING FOR SUGARCANE BACILLIFORM VIRUS

INTRODUCTION

Sugarcane bacilliform virus (SCBV) was first reported as a sugarcane pathogen by Lockhart and Autrey in 1988 (1). It was subsequently found to occur worldwide in almost all clones of noble canes and in several commercial cultivars. It is a member of the badnavirus group, the virus particles being bacilliform in shape (30 x 130 nm) and contain a double-stranded DNA genome. Ultrastructural studies (2) of infected leaf cells show that the virus is randomly distributed in the cytoplasm affecting the internal structure of the mitochondria, enhancing the quantity of p plastids and inducing the formation of filamentous inclusions in the nucleus. It can be transmitted by the pink mealy bug *Saccharicoccus sacchari* (3) and the grey sugarcane mealy bug *Dysmicoccus boninsis* (4). Symptoms of naturally infected *S. officinarum* vary from no apparent foliar damage to pronounced whitish chlorotic streaking, the most common symptom being yellow or white freckles on sugarcane leaves (plate 1). Methods for the detection of the virus include ELISA, Immuno-Electron Microscopy (IEM) and the polymerase chain reaction (PCR). However, with the existence of several SCBV strains (5) and due to the higher sensitivity of PCR, this technique has been opted as a tool for the detection of the virus in a collection of noble canes.

MATERIALS AND METHODS

DNA extraction

Forty-four varieties of noble canes (2-12 plants per variety) planted in the glasshouse were tested for the presence of SCBV. A crude extract of viral DNA was prepared as follows: the youngest fully expanded sugarcane leaf was selected from each plant and a piece (1 x 5 cm) of the leaf blade was cut into small pieces (1 x 1 mm). This was transferred to a 15 ml Corning tube containing 3 ml NaOH (0.25 M) and 30 µl β-mercaptoethanol. The sample was incubated in a boiling water bath for 30s and neutralised by the addition of 3 ml HCL (0.25 M) followed by 1.5 ml Tris.HCl (0.1 M, pH 8.0) and 0.25% Nonidet (P-40) before boiling for an additional 2 min. The extract was allowed to cool to room temperature and a diluted sample (1:10) was used for PCR.

PCR conditions

A 25 µl PCR reaction mix contained the following: 1X PCR reaction buffer (Boehringer Mannheim, BM) supplemented with $MgCl_2$ (3.5 mM), dNTPs (200 µM, BM), primers (Table 1) SCBVF5 (250 µM) and SCBVR5 (250 µM), Taq Polymerase (1 unit, BM) and 15.32 µl of diluted crude extract. The PCR solution was overlaid with one drop of mineral oil.

PCR conditions were 94°C for 7 min followed by three cycles at 94°C for 30s, 50°C for 30s and 72°C for 1 min; 37 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min followed by a final extension at 72°C for 7 min. A PCR mix without template was used as negative control. PCR products were separated by electrophoresis on a 1.8% agarose gel together with molecular weight marker IX (BM), stained with ethidium bromide and visualised under UV light.

Primer pair	**Sequence**
SCBVF5	5'- TCA AAG TTT GAT TTG AAG AGC GGG-3'
SCBVR5	5'- CTC CGA GAA AAC CAA TAT GTC ATC-3'

Table 1; Primer sequence SCBVF5 and SCBVR5 used in the polymerase amplification of SCBV.

RESULTS AND DISCUSSION

Bands of size 221bp showed the presence of the virus (Fig. 1). Out of 44 varieties tested, 43 were infected with SCBV (Table 2). Plants tested negative for the virus were tested again i.e at *in vitro* stage to confirm the absence of the virus since low SCBV titre or DNA quality may account for negative PCR results. The high level of infection confirms previous results obtained by serological tests (6) whereby the virus was found in 127 (100%) varieties of noble canes in Mauritius.

(1B) INDEXING FOR SUGARCANE YELLOW LEAF VIRUS

INTRODUCTION

Since the late 1980s, sugarcane has demonstrated symptoms of a condition now known as the Yellow Leaf Syndrome (YLS) in a number of countries. It was eventually (in 1988) recognised as a sugarcane disease by Schenk (7). The symptoms appear in maturing plants generally starting as a yellowing of the leaf midribs and most easily seen on the abaxial surface (plate 2). Sometimes, a pinkish red discoloration on the upper surface of the midribs is also present. Thereafter a symmetrical discoloration of the leaf lamina parallel to the midrib is usually evident and as the season progresses, the entire lamina and most of the canopy turns yellow. The disease has been detected in many countries including Argentina, Australia, Brazil, Colombia, El Salvador, Guadeloupe, Guatemala, Mauritius, Malawi, Morocco, Reunion, South Africa, Swaziland, U.S.A and Zimbabwe. Losses as high as 40-60% have been reported in Brazil (8,9). A virus, Sugarcane Yellow Leaf Virus (ScYLV), residing in the phloem of diseased canes, has been tentatively associated with the syndrome (10,11). It has been classified as a *Luteoviridae* with properties of both subgroups of I and II of luteoviruses (12). ScYLV can be detected by either reverse transcriptase polymerase chain reaction (RT-PCR) assays (13) and by tissue blot immunoassays (TBIA) using leaf midribs and stem (14). The latter allows rapid diagnosis of ScYLV of large number of plants and has been used here in the detection of the virus in noble canes.

MATERIALS AND METHODS

Glasshouse grown noble canes (65 varieties) were tested for the presence of ScYLV as follows; the youngest, fully expanded leaf from each plant was taken and the blades were removed. The midrib was cut with a sharp scalpel near the base and the cut end was immediately pressed on a piece of nitrocellulose membrane for 3-6s. One membrane (8 x 10 cm) could fit 80 blots. Blotted membranes were blocked in 2% non fat dry milk in TBS buffer (50 mM Tris, 50 mM NaCl, pH 7.4) followed by one rinse in PBST, pH 7.4 (137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.005% Tween 20). The membranes were incubated in ScYLV polyclonal antibody IgG (supplied by Prof. B. Lockhart) diluted 1:1000 in 1% dry milk in TBS for 3 h at room temperature. After three rinses of 10 min each in PBST, membranes were incubated in goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemicals Ltd) diluted 1:9200 in 1% dry milk in TBS for 3 h at room temperature. Membranes were rinsed 3 x 10 min in PBST and

incubated in alkaline phosphatase substrate i.e 35 µl BCIP (50 mg/ml) and 33.5 µl NBT (100 mg/ml) in detection buffer (100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl;, pH 9.4). After 15-30 min dark incubation, the blots were rinsed in distilled water, dried and observed using a binocular microscope.

RESULTS AND DISCUSSION

Presence of ScYLV was denoted by the presence of dark blue spots concomitant with the vascular bundles from both the midrib and the stem. Out of 65 varieties tested, 39 were found to be infected (Table 2), with incidence of up to 90% in some varieties (M 05/17, M 58/33, M 168/33, MP 0131). In only two varieties (NG 5777 and NG 7766), the presence of the virus coincided with the yellowing of leaves i.e all plants that were found to be infected by the TBIA showed yellowing of leaves and midribs. These results confirm previous reports suggesting that presence of ScYLV is not always associated with the YLS symptoms and that the yellowing of leaves is in fact more pronounced only when the cane reaches maturity or when it is subjected to stress e.g drought (15).

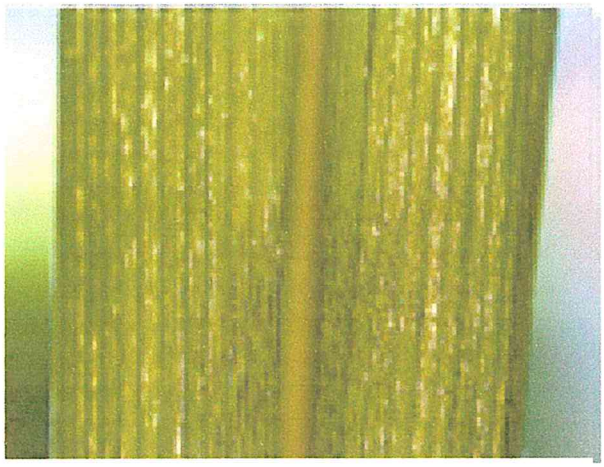

Plate 1; Noble canes (variety Iscambine) infected with sugarcane bacilliform virus. The presence of white/yellow freckles on the leaf blade has been associated with the virus.

A **B**

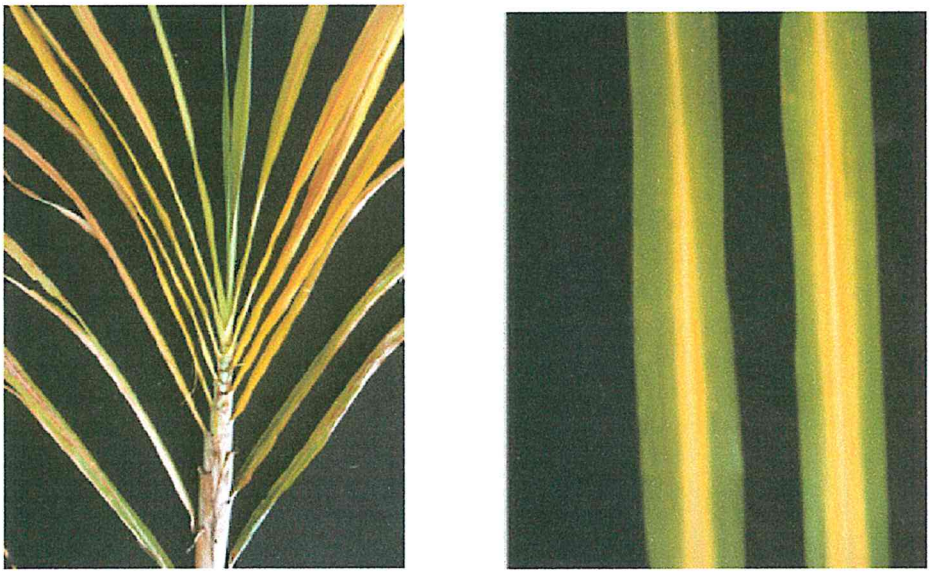

Plate 2; (A) Sugarcane plant showing symptoms of Yellow Leaf Syndrome. Youngest leaves appear green but gradually turn yellow.(B) Infected leaf showing yellowing of the midrib and leaf blade.

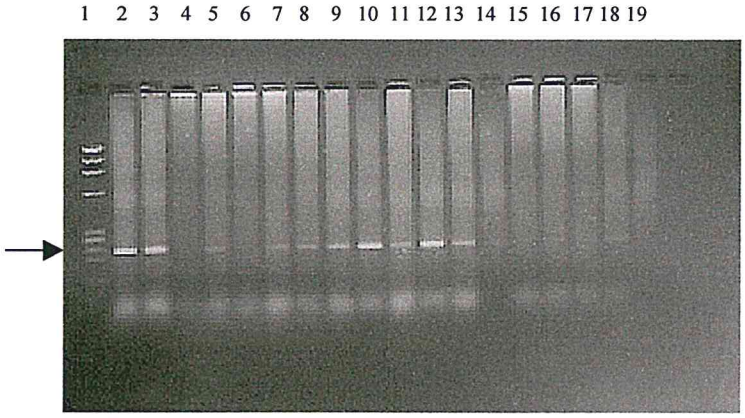

Figure 1; Detection of SCBV by PCR. Bands of size 221 bp (arrow) show the presence of the virus as observed in lanes 2,3,5-14, 16 and 18. Marker IX (BM) was run in lane 1 and a negative water control in lane 19.

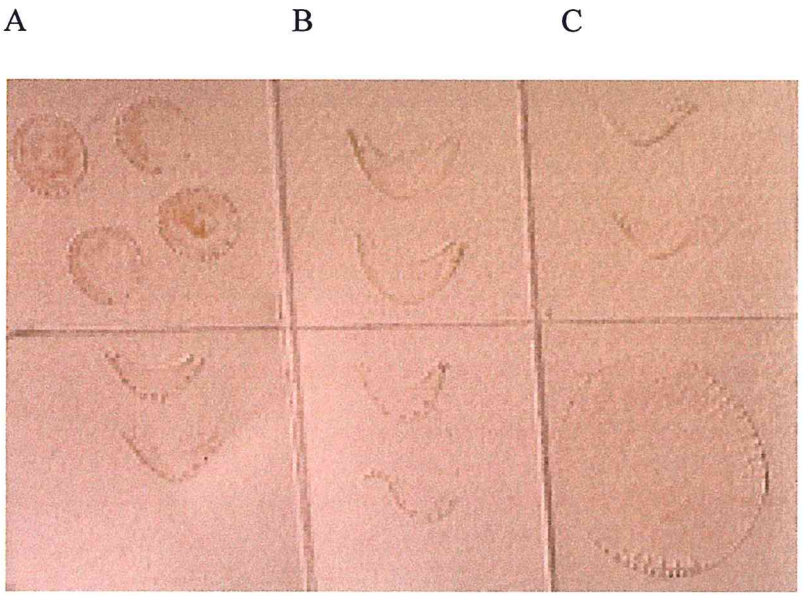

Figure. 2; Detection of ScYLV by TBIA. The presence of the virus is detected by the presence of dark spots concomitant with the vascular bundles in both the stem (A) and the midrib (D & E).

	Variety	A	B	C	D	E	F	G	H	I	J	K	L	% infected
1	B 0005913	+	D	+	+	+	NT	+	+	+	+			100
		-	D	+	+	-	+	-	+	-	+			56
2	B 0006103	-	D	+	+	-	-	-	+	+	-	+		50
		-	D	-	-	+	-	-	+	-	-	-		20
3	B 0006308	-	-	-	-	+	+	+	+					50
		-	-	-	-	D	-	-	-					0
4	BA 0008846	+	+	+	+	+	+	+	+	+	+	+		100
		+	-	-	-	+	-	+	-	D	+			44
5	BA 0006032	NT	+	NT	-	NT	NT	-	-	+	+			50
		-	+	-	-	+	-	+	-	-	-	+	+	42
6	BA 0011569	+	+	+	-	+	-	+	+	+	+	+	+	83
		-	-	-	-	+	-	-		-	-	-	-	9
7	BADILLA													NT
		-	-											0
8	BAMBOO o	NT												NT
		-												0
10	BEAU BOIS	NT												NT
		-												0
12	B. RAYEE	+	-	+	+	+	NT	+	+	-				75
		-	-	-	-	-	-	-	-	-				0
15	D 0000130	-	+	+	+	+	+	+	+	+				89
		-	-	-	-	-	-	-	-					0
16	D 00001135	+	-	-	-	+	-	-	-	-				22
		-	-	D	-	-	-	-	-					0
17	DI 0000052	-	+	+	+	+	+	+	-	-				66
		-	-	-	-	-	-	-	-	-				0
18	DK 0000074	+	+	+	-	+	NT							80
		+	+	-	+	+	-							33
19	FOTIOGO	-	+	+	-									50
		-	-	-										0
20	IJ 0076561	-	+	+	+	-								60
		-	-	-	-	+								20
21	IS 0076203	+	-	+	+	+	+	-						71
		-	-	-	-	-	-	-						0
22	ISCAMBINE	+	+											100
		-	-											0
23	JOHN BULL	+	+	+	+	+	+	+	+					100
		-	+	+	+	-	-	+	+					63
24	KURPI	+	+	+	+	+	+	+	+	NT	NT	NT		100
		-	+	-	-	-	-	-	D	-	-	-		10
25	LOUISIER													NT
		-	-											0

Table 2: Distribution of SCBV (top row) and ScYLV (bottom row) in noble canes.
For each variety a maximum of 12 plants, labelled A-L, were tested. (NT; not tested, D; dead)

	Variety	A	B	C	D	E	F	G	H	I	J	K	L	% infected
26	M 0002/33	+	+											100
		-	-											0
27	M 0005/38	+	+	+	+	+	+	-	-	-				66
		-	-	-	-	-	-	-	-	-				0
28	M0007/23	+	+	-	-	+								60
		-	-	-	-	+								20
29	M 0013/18	+	+	NT	+	-	-	-	-	-	-			33
		-	-	+	+	-	+	+	-	+	-			50
30	M 0014/26	+	+	+	+	+	+	-	-	-	-	-		54
		+	+	+	-	-	+	-	+	+	+			70
31	M 0026/20	-	+	-										33
		+	-	-										33
32	M 0027/16	+	-	+	-	+	+	-	+					63
		-	+	+	+	+	+	+	+					88
33	M 0029/16	+	+	+	NT	D	NT							100
		+	+	-	+	D	-							60
34	M 0005/17	-	-	-	-	+	NT	+	-	-	-			22
		+	+	+	+	+	+	+	+	+	+			100
35	M 0058/33	+	+	+	+	-	+	NT	+	+	NT	+		89
		+	+	+	+	+	+	+	D	D	+			100
36	M 0109/26	+	+	+							-			100
		+	+	+										100
37	M 0168/33	+	-	+	+	+	-	-	+	-	+	+	-	58
		+	+	-	+	+	+	+	+	+	+	+	+	92
38	M 0171/30	-	-											0
		+	+											100
39	M 0213/33	+	-	-	-	+	NT	-	-					29
		-	-	-	-	-	-	-	-					0
40	M 1162/55	+	+	+	+									100
		+	-	+	+									75
41	M 2118/69	+	+	+	+									100
		-	-	-	-									0
42	M 2223/86	+	+	+	+									100
		+	-	-	-									25
43	M 2224/86	+	+											100
		-	-											0
44	M 2225/86	-	+	-	-	-	+	-	NT	+				38
		-	-	-	-	D	-	D	-	-				0
45	M 2226/86	NT	+	-	+	+	+	D	NT					80
		-	+	-	-	-	-	D	-					14
46	MB 0008/72	+	-	-	+	NT	+	-	+	+				63
		-	-	+	+	-	-	-	-	-				25
47	MB 0009/72	+	+	+	+	NT	+	+	+	+	+			100
		-	-	+	+	+	-	-	-	+	+			50

	Variety	A	B	C	D	E	F	G	H	I	J	K	L	% infected
48	MB 0011/72	+	+	-	+	+								80
		-	-	+	-	D								25
49	MB0012/72													NT
		-	-	-										0
50	MB 0013/72													NT
		+	+	+	+									100
51	MP 0000033													NT
		+												100
52	MP0000055													NT
		+	-											50
53	MP 0000131													NT
		+	+	+	+	+	+	+						100
54	NG 0005111													NT
		-	-											0
55	NG 0005113	NT	+	+										100
		-	-	-	-									0
56	NG005757													NT
		+	-	-	-	+	-	-						29
57	NG 0005777													NT
		+	+	+	+	+	+	+	+	+	+	+	+	100
58	NG 0007716	NT	.	.										NT
		+	+	+										100
59	NG 0007766													NT
		+	+	+	+	+	+	+						100
60	NG 0051142													NT
		+	+											100
61	NG 0005763	+	+	+										100
		-	-	+	+									50
62	NG 0057174	+	+	NT	NT	-								67
		-	-	-	+	+	+	+	-	+	-	-	-	42
64	NG 0057233													NT
		-	-	-										0
65	NG 0077142	NT												NT
		-												0
66	PENANG													NT
		-	+	-	+									50
67	RP 0000006													NT
		-	+	-	-	-	-	-	-	+				22
68	RP 0000008	NT												NT
		-	-	-	-									0
69	SEAL													NT
		-	-	D	-									0
70	SENN	+	+	+	+	+								100
		-	-	-	D	-								0

(1C) INDEXING FOR *Xanthomonas campestris* pv *vasculorum* AND *X. albilineans.*

INTRODUCTION

Gumming was the first disease of sugarcane recorded in the literature (16) when Dranert in 1869 described a disease of cane in Brazil characterised by an exudation of yellowish gum from the vascular bundles. It was later (in 1893) fully described as a bacterial disease by Cobb (17) in Australia and reports of the disease followed in Mauritius in 1894 (18). Different names have been associated with the bacteria until it was finally designated as *Xanthomonas campestris pv vasculorum* (XCV) (19). Severe outbreaks have resulted in serious losses in the 1930s when highly susceptible noble canes were cultivated. However, successful breeding programmes have led to its eradication in most countries except the Mascarene islands where new outbreaks have occurred as a result of development of new strains of the pathogen. Three races (1, 2 and 3), named in order of the year recorded, have been identified (20). These have differing virulence and serological properties. They can be differentiated in Kelman agar containing triphenyl tetrazolium chloride (TZC) and by the use of DNA probes. Of these three races, only race 1 is known to infect the noble canes (20).

There are two distinct stages of gumming disease; the initial, leaf-streaking stage and the systemic stage. The leaf streaks are about 3-6 cm wide and follow the course of the vascular bundles. Initially they are yellow to orange with reddish flecks; with age they become necrotic and turn ashy grey. The streaks usually arise from the leaf margin and develop towards the base. Further progress leads to infection of the stalk resulting in the systemic stage of the disease. This stage is characterised by a partial or total chlorosis of new leaves, stalk deformation, knife cut lesions, internal discoloration of the nodes and internodes, formation of gum pockets, necrosis of the apex and eventual death of the stalk.

Leaf scald was recognised as a bacterial vascular disease of sugarcane in the 1920s by Wilbrink (21) in Java. The causal organism, *Xanthomonas albilineans* (Xalb) is a rod-shaped gram-positive bacterium forming circular, shiny and transparent honey-like colonies (22).

There are two forms of the disease namely the chronic phase and the acute phase. The most typical form of the chronic phase is characterised by the presence on the lamina of a 'white pencil line' about 1-2 mm wide which follows the direction of the main veins. Sometimes the

veins develop a reddish necrosis along part of their length. Broadening of the pencil line may cause partial or total chlorosis. Progression of the disease may lead to stunting of shoots, browning of leaves with inward curling and profuse development of side shoots in mature stalks. The acute phase is characterised by a sudden wilting and death of the plant without previous symptom expression. A further characteristic of the disease is the onset of a latent period where the symptoms disappear and the plant seems to have recovered. Prolonged latency may favour local build-up of the disease and a relapse to the chronic phase may be observed at at any time of growth. Transmission is mainly by infected cuttings and mechanically by knives and harvesters (23) and by aerial transmission (24).

MATERIALS AND METHODS

Two methods were chosen for the isolation of XCV and Xalb from the noble canes i.e the stalk-imprint method and the diffusion test. Cane stalks previously screened for SCBV and ScYLV were tested. Cane stalks were obtained mainly from plants in the glasshouse and from those in an open shadehouse (previously removed from the glasshouse due to lack of space).

Stalk-imprint

Sugarcane stalks were washed, the leaf scar removed using a sharp scalpel and the node disinfected with 95% ethanol. The cane stalk was cut at the node using a pair of prunning spears and pressed on a modified Wilbrink (MW) medium (Appendix 1). Two imprints (top and bottom nodes) were made per stem. The plates were incubated at 28^0C and observed after 3-10 days for appearance of colonies typical to XCV and Xalb.

Diffusion test and enrichment

Cane stalks were cleaned and disinfected as above. Discs (1 cm thick) were cut at the nodal part of the cane, a 1 cm^2 inner portion of the disc was finely sliced and transferred into a Corning tube containing 3 ml Wilbrink broth (peptone 5 g/l, KH_2PO_4 0.5 g/l, $MgSO_4.7H_2O$, 0.25 g/l and sucrose 20 g/l). The sap was allowed to diffuse. A a loopful of the diffusate was streaked on solid Wilbrink and incubated at 28^0C. Following diffusion, some cultures were allowed to enrich for 36 h, then streak-plated on solid Wilbrink medium.

RESULTS AND DISCUSSION

Eight varieties were found infected by XCV as summarised below.

Variety	Plant (s) infected
B 6308	3A
D 1135	16E and 16F
IJ 76561	20C
John Bull	23F
Korpi	24A
M 05/38	27C
M 14/26	30H
MB 09/72	47I

Table 3; Distribution of XCV in noble cane collection.

Out of 150 stalk imprints of canes obtained directly from the glasshouse, only three were found infected with XCV. i.e 24A, 30H and 47I.

Glasshouse plants, exposed outside for a month, then tested for XCV were found to be more contaminated i.e 4 out of 20, one (20D) revealed by the stalk-imprint method and a further three (3A, 16E and 16F) by diffusion without the need for enrichment.

Due to a higher level of detection of XCV by the diffusion test, a further 12 stalks were selected from the glasshouse and a diffusion test with and without enrichment was carried out. No pathogen was found following direct streaks but three stalks (20C, 23F and 27C) were found infected with XCV following enrichment.

X. albilineans was not detected any plant.

The stalk-blot method for isolation of XCV and Xalb has been found unreliable for stalks obtained from the glasshouse. However, diffusion followed by enrichment did show a higher level of infection. This probably suggests that the environmental conditions (temperature and humidity) which prevails in the glasshouse renders the bacteria latent which makes them undetected at low titres. Exposing the plants outside the glasshouse subjects them to climatic

conditions favourable for the growth of XCV. This enabled detection without the need for enrichment. These observations agree with previous reports (20) whereby the progression of gumming was also found to be associated with climatic conditions.

(2) *IN VITRO* CULTURE OF NOBLE CANES AND ELIMINATION OF ScYLV

INTRODUCTION

In vitro culture of sugarcane is mainly achieved through the direct regeneration of shoot meristems. Plantlets can also be indirectly derived from leaf rolls through regeneration of callus. Two types of meristematic tissue can be selected for culturing: (1) axillary buds, located in the upper 1-2 cm portion of stalks and are surrounded by leaf sheaths and (2) apical meristems with surrounding two or three whorls of developing leaves that are located on the upper 0.5-1 cm of the stalk. Embryogenic callus can be established from segments of the innermost four to five furled leaves taken up to 8 cm above the apical meristem. Attempts to culture noble canes *in vitro* on medium [MS salts (Appendix 2), 20 g/l sucrose and 7 g/l type A agar (Sigma)] devised for commercial cultivars failed mainly due to browning, following the release of polyphenols. An alternative medium was tested with 6.7 g/l glucose and a reduced sucrose concentration (13.3 g/l) and the substitution of type A agar by 0.18% Phytagel (Sigma) as gelling agent. This medium was tested on 35 varieties of noble canes to determine its suitability for the *in vitro* culture of noble canes. The elimination of SCBVand ScYLV by tissue culture was further investigated.

MATERIALS AND METHODS

In vitro culture of noble canes

Cane tops from the glasshouse previously tested for the presence of SCBV, ScYLV, XCV and Xalb, were surface sterilised in 2% commercial bleach for 15 min followed by one rinse in sterile distilled water (SDW). A further soak for 5 min in Benomyl (0.5 g/l) for elimination of fungal contaminants, followed by three rinses in SDW was carried out. Enclosing leaf sheaths were then removed and segments of young leaf rolls about 5 mm thick were cultured on callus induction medium (Appendix 3) in the dark. Embryogenic callus was subcultured at monthly intervals and was regenerated on a medium devoid of growth hormones. Axillary buds were transferred on intiation medium (Appendix 4) whereas apical meristem (2-3 mm) were transferred on Paulet 1 medium (Appendix 5). Axillary buds were subcultured on initiation medium until it reached approximately 6 cm high then transferred to liquid multiplication medium (Appendix 4). Apical meristems were maintained on Paulet 1 for about 2 weeks in the dark then subcultured on Paulet

2 medium (Appendix 5) for a further two weeks under 12 h light and finally transferred to initiation medium. Plantlets of 6 cm high were transferred to liquid multiplication medium (Fig. 3)

Total nucleic acid extraction

Most varieties of noble canes were lost through contamination. However the remaining nine varieties were used for detection of ScYLV and SCBV by PCR and RT-PCR respectively. Total nucleic acid was extracted from the leaves by using the hot CTAB method: *In vitro* plant tissue (0.3 mg) was ground in liquid using a mortar and pestle. The finely ground powder was transferred into an Eppendorf tube containing 1 ml extraction buffer (EDTA 20 mM pH 8.0, 1.4 M NaCl, 2%CTAB and 0.2% β Mercaptoethanol) and incubated at 60°C for 30 min The extract was then mixed with 2/3 (v/v) chloroform : isopropanol (24:1) and centrifuged at 12,000 rpm. The supernatant (0.75 ml) was transferred into a new tube, gently mixed with 2/3 (v/v) cold isopropanol. Total nucleic acids were precipitated upon incubation at -20°C for 1h. Nucleic acids were pelleted by spinning at 10,000 rpm for 5 min, washed with ethanol (76%) and sodium acetate (10 mM). The pellet was spun dried and dissolved in sterile distilled water (20 µl).

PCR conditions

Primers YLS 462 and YLS 111 (primers sequences were provided by Dr M. Irey, US Sugar Corporation, Florida) which amplify part of the gene encoding the coat protein of ScYLV was used for the detection of the virus (25). RT-PCR was carried out in a thermal cycler (PTC 100 MJ Research). Crude samples (0.50 µl) were boiled with 0.25 µl primer YLS 462 (30 µM) for 5 min and quenched on ice. The samples were then mixed with aliquots (4.25 µl) of the following reagents; 1 µl $MgCl_2$ (25 mM), 0.5 µl PCR buffer (BM X10), 2 µl dNTP (2.5 mM), 5U RNase inhibitor (BM) and 12.5U MuLV reverse transcriptase (BM). Reverse transcription was carried out at 42°C for 15 min and then denatured at 99°C for 5 min. cDNA was amplified in the same tube after addition of the PCR mix containing the following reagents: 2.0 µl $MgCl_2$ (25 mM), 2 µl PCR buffer (10X), 15.75 µl H_2O, 1.25U Taq DNA polymerase (BM) and 0.125 µl YLS 111 primer (60 µM).

PCR conditions were as follows: one cycle; 94°C, 1 min; 54°C 1 min and 72°C 20 min, 40 cycles; 94°C, 1 min; 54°C, 1 min and 72°C, 2 min and a final extension step at 72°C for 10 min.

RT-PCR products were run on 1.8% agarose by electrophoresis and the gel was photographed under UV light after staining with ethidium bromide.

For detection of SCBV, sample DNA (0.3 µl) was boiled for 5 min with primers SCBVF5 and SCBVR5, quenched on ice and amplified as described earlier.

Plantlets of variety M 168/33 derived from callus, axillary buds and apical meristem and presumably freed from ScYLV were rooted and potted in the glasshouse. These were tested for ScYLV at monthly intervals by RT-PCR using the first fully open leaf for nucleic acids extraction. After three months, all plants were again tested for ScYLV by TBIA. The plants were finally tested for ScYLV by RT-PCR, nine months after being sent to the glasshouse.

RESULTS AND DISCUSSION

The use of a modified medium has proved to be extremely efficient in the regeneration of sugarcane from meristematic tissue. Out of some 35 varieties initiated, a collection of 31 varieties has been established. Of the 160 canes initiated, 69 apical meristems (43%) and 91 axillary buds (57%) have been successfully established as compared to 25% meristem and 30% axillary bud establishment from commercial canes on the conventional medium. A number of factors may account for such an improvenment. Firstly, the addition of glucose may provide an early carbon source which is more readily metabolised than sucrose which needs a prior hydrolysis step. Secondly phytagel may provide a more efficient matrix in the regeneration of sugarcane explants. Among the favoured characteristics which could explain differences in performance are; free diffusion of nutrients (26), good contact between tissue and medium, water availability at the surface of the gel (27) and ability to maintain a constant pH at autoclaving.

Callus produced were of 4 types (Fig. 4)

(1) creamy and friable

(2) transparent and mucilaginous

(3) white and compact

(4) Intermediate between 2 and 3

Callus from 33 varieties were of type 3 and 4. Such callus are known to be embryogenic and can be used for the regeneration of plantlets (Fig 5).

This modifed medium has also proved to have a better performance in callus induction since previous attempts to culture noble canes on the conventional medium led to browning of the

explant and the eventual death of the tissue. The superiority of phytagel over Difco bactoagar has also been reported (28) whereby a threefold increase in proliferation rate of sugarcane callus was observed.

A high level of fungal spores in the culture room led to contamination of TCM and eventual loss of the majority of plantlets established *in vitro*. Plantlets from the nine varieties were tested for the presence of SCBV and ScYLV by PCR and RT-PCR respectively. Plantlets from seven varieties (Table 3), initially infected with ScYLV were found freed from the virus through tissue culture. Among these, all plants derived from succesive callus culture were found free from the virus. However, all plantlets tested for SCBV remained infected with the virus.

Variety	Plant No	Explant used
M 13/18	29 D	Meristem tip
M 27/16	32 D	Callus
M 68/33	35 A	Callus
	35 D	Axillary buds
M 168/33	37 D	Callus
	37 E	Callus
	37 B	Meristem tip
	37 F	Axillary bud
M 171/18	38 A	Meristem tip
M 1182/55	40 A	Meristem tip
MB 09/72	47 C	Axillary bud
	47 I	Callus

Table 3: List of noble canes (and plants) freed from ScYLV by using different explants

Plantlets (varieties. M 168/33, numbers 37 B, 37 D, 37 E and 37 F) derived from callus, meristem tip and axillary bud were sent to the glasshouse and tested for ScYLV at monthly intervals. All plantlets remained free from the virus. These results were confirmed by TBIA three months after potting. Furthermore, ScYLV remained undetected from the plants, nine months after being transferred to the glasshouse as determined by RT-PCR.

Meristem tip culture is the most common method of virus elimination in plants. This technique takes advantage of the fact that some viruses are unable to colonise this region because of

inhibition of replication and restriction of their movement (29). Two factors could impede with the replication of ScYLV at the meristem tip; a high concentration of auxin and the depletion of nutrients through rapid cell division. The inability of the luteovirus to reside in the meristem may be due to;

(a) localisation of the virus in the phloem (as observed by TBIA) which is not differentiated yet at the meristem tip
(b) inability of the virus to move vertically across the plant through the plasmodesmata to the meristem tip and
(c) inability of the virus to keep up with the pace of rapidly dividing cells at the growing point.

It was also possible to eliminate the virus from infected plants by successive culture of callus derived from leaf rolls. The uneven distribution of the virus among the different tissues of the leaf may account for its elimination. ScYLV has been found to be phloem-restricted whereas somatic embryos have been found to arise mainly from non-vascular tissue (30). Therefore plantlets derived from callus culture are likely to be derived from virus free cells, hence should in turn be free from ScYLV. Secondly, a high concentration of auxin in TCM may also inhibit viral replication. Successive callus culture may thus lead to its dilution and its elimination from infected canes.

In vitro culture has been found to be an easy method for the elimination of ScYLV from infected plants. Plantlets free from ScYLV can be produced within 6 months from meristem tip or axillary bud cultures or 10 months if successive callus subculture is used. Although the former method is shorter, its success resides in ability to dissect the meristematic dome with one or two leaf primordia from the mother plant and its successful regeneration. Large meristem tips (> 0.5 mm) are likely to be infected whereas very small ones (< 0.3 mm) are unlikely to regenerate. Although cleaning through callus subculture is more time consuming, this method requires less skill and all plants regenerated are virus free. However, callus culture of sugarcane should be treated cautiously due to possible somaclonal variation.

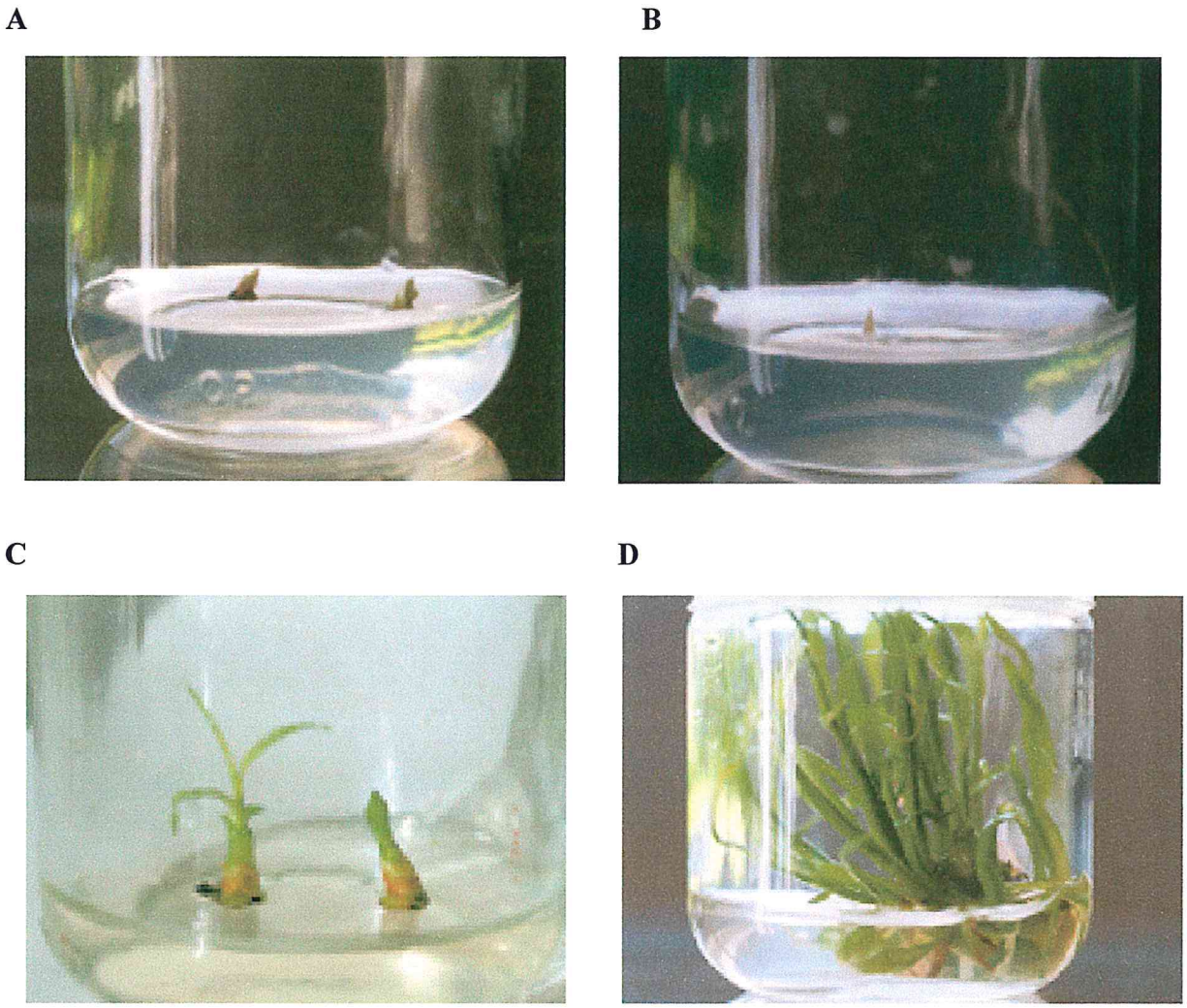

Figure 3; In vitro culture of noble cane; (A) initiation of axillary buds, (B) initiation of apical meristem on Paulet 1, (C) regeneration of axillary buds and (D) multiplication stage.

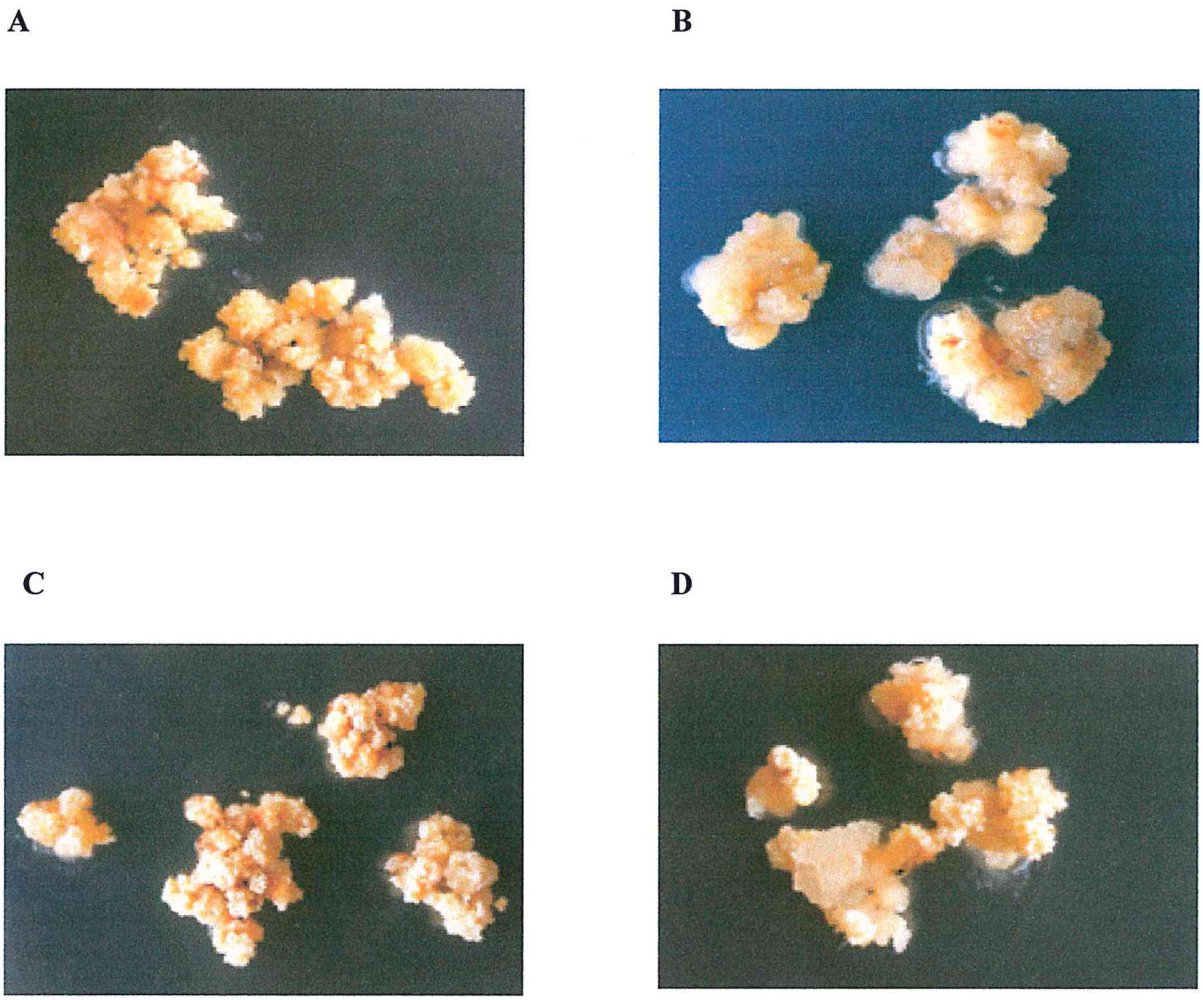

Figure 4; Differentiation of noble cane callus into four types; (A) creamy and friable, (B) transparent and mucilaginous (C) white and compact and (D) Intermediate between (B) and (C). Of these, only (C) and (D) are embryogenic.

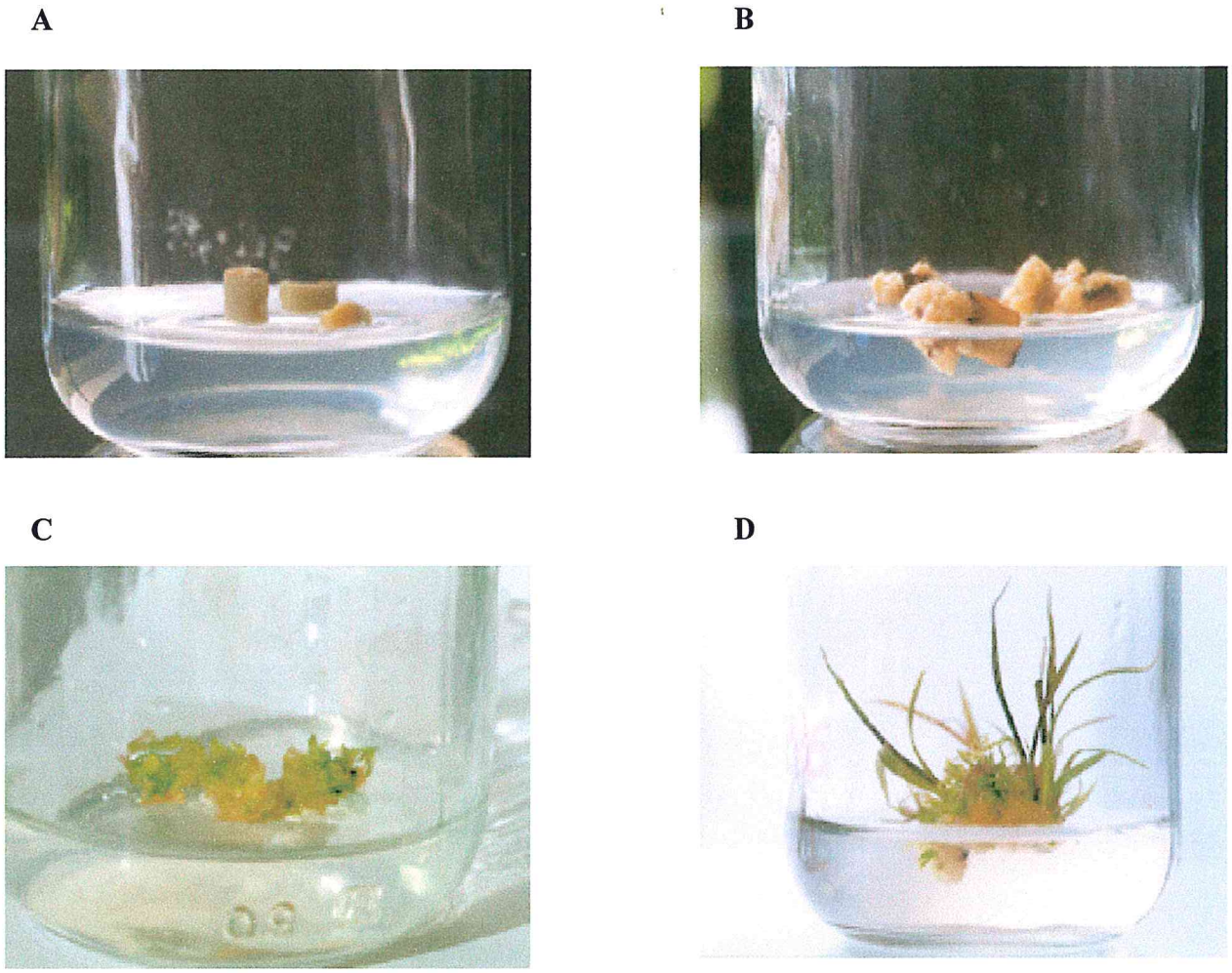

Figure 5; Regeneration of sugarcane plantlets through callus culture. (A) Induction of young leaf rolls, (B) Morphogenesis into callus, (C) and (D) regeneration into plantlets.

(3) ELIMINATION OF SUGARCANE BACILLIFORM VIRUS AND SUGARCANE YELLOW LEAF VIRUS BY TREATMENT WITH THE ANTIVIRAL AGENT RIBAVIRIN

INTRODUCTION

The elimination of plant viruses has been sucessfully achieved mainly owing to their mode of replication and their mechanism of movement within the plant. Three methods are currently in use; thermotherapy, tissue culture and chemotherapy. Although heat has been used for more than a century in the elimination of plant pathogens, it was not until 1949 that the first successful heat treatment of a virus infected plant was reported (31). However, only during the late 1960s, more than 70 viruses were reported to have been inactivated in plants by the use of heat (32). The effect of heat on viruses is not well understood but it is believed to be effective in inhibiting viral replication and synthesis of movement proteins mainly by blocking transcription (33).

The most widely used method of virus elimination remains the technique known as meristem tip culture. This technique takes advantage of the fact that many viruses fail to invade the meristem tip. Transfer of the meristem dome plus one or two leaf primordia to tissue culture medium and its regeneration into a plantlet may lead to the elimination of the pathogen.

Finally, the use of antiviral agents in the elimination of a number of viruses has also been reported (34, 35). These compounds were initially designated for administration in humans and animals. However, because of their broad-spectrum activity, their use has been extended to plant viruses as well. They can be directly sprayed on the crop or included in tissue culture medium and upon uptake by the plant, they inhibit virus replication.

SCBV is a double stranded DNA pararetrovirus belonging to the genus badnavirus. It has been detected in the axillary buds and axillary meristematic domes and planlets derived from buds and domes (5). Due to its systemic distribution, *in vitro* culture of infected canes has not been successful in the elimination of the virus. The use of heat on both cane setts and axillary buds has also failed (5). In this experiment, attempts will be made to eliminate SCBV and ScYLV from infected plants by treatment with the antiviral agent, ribavirin. This compound exerts its effect on both DNA and RNA viruses and has been used in the elimination of a number of plant

viruses, including Potato virus X, Potato virus Y (34) and Odontoglossum ringspot virus (35). Proposed mechanisms of action (36) include;

(i) It causes a decrease in the intracellular level of guanosine triphosphate (GTP) pools

(ii) It inhibits initiation and elongation steps of virus specific RNA polymerase

(iii) It interferes with the 5' capping of messenger RNAs by inhibiting guanylyl transferase activity.

The effect of ribavirin on the multiplication of noble canes and its use in an attempt to eliminate ScYLV and SCBV was therefore investigated.

MATERIALS AND METHODS

In vitro assay of ribavirin

The phytotoxic effect of ribavirin was tested on plantlets of four varieties of noble canes (D 1135, M 13/18, M 168/33 and MB 09/72). For each variety, a single shoot was transferred in TCM containing ribavirin (10-75 mg/l). After one month, the number of adventitious shoots arising per mother plant was counted. This was considered as a measure of phytotoxicity. A single shoot from each assay was taken and was further subcultured for one month on mediun containing the same concentration of ribavirin. Again, the number of plantlets arising from each mother plant was counted.

To determine the effect of light on the activity of ribavirin, TCM containing ribavirin (10-75 mg/l) was exposed to strong light for 2 months. A control with medium exposed in the dark was also included in the experiment. Single shoots (variety. M 168/33) were then transferred into each jar. After one month, the number of adventitious shoots arising per plant was noted.

In vitro treatment of noble canes

Plantlets from ten varieties (Table 4) were treated *in vitro* with ribavirin in an attempt to eliminate SCBV and ScYLV. Single shoots from each variety were transferred to tissue culture medium (TCM) containing ribavirin at the following concentrations: 10, 15, 20, 25 and 30 mg/l. When adventitious shoots reached 4-5 cm in length, a single shoot was separated from the mother plant and transferred to fresh TCM containing the same concentration of ribavirin. The plantlets were randomly tested for SCBV and ScYLV by PCR and RT-PCR respectively during the next 7-10 subcultures. The hot CTAB method was used for total nucleic acid extraction.

Micromeristem tips from plantlets of variety M 168/33, treated with ribavirin at concentrations of 10-25 mg/l were dissected and further cultured *in vitro*. Upon regeneration, plantlets were tested for SCBV by PCR.

Variety	Plant number	Origin of explant	Infected with	
			SCBV	ScYLV
B 5913	1I	Axillary bud	+	-
D 1135	16F	Meristem tip	+	-
DI 0052	17D	Meristem tip	+	-
M 13/18	29D	Axillary bud	+	+
M 27/16	32G	Axillary bud	+	+
M 29/16	33A	Meristem tip	+	+
M 35/17	34 HA	Axilary bud	+	+
M 0168/33	37K	Axillary bud	+	+
M 1182/55	40A	Axillary bud	+	+
MB 09/72	47 F	Meristem tip	+	-

Table 4: Noble canes infected with SCBV and ScYLV treated with ribavirin at 10-30 mg/l

RESULTS AND DISCUSSION

Figure 6 shows the effect of ribavirin on *in vitro* multiplication of noble canes. Increasing ribavirin concentration causes a fall in the multiplication rate of noble canes irrespective of the variety. At a concentration of 60 mg/l or above, multiplication of noble canes *in vitro* almost ceased. Upon subculture, a similar trend in multiplication rate was observed at ribavirin concentration 10-30 mg/l. Moreover, plantlets exposed to higher ribavirin concentrations turned yellow and eventually died. A decrease in the rate of growth of the plant (height) was also noted with increasing ribavirin concentration. Exposure to strong light appeared to have no effect on the activity of ribavirin (Fig. 7).

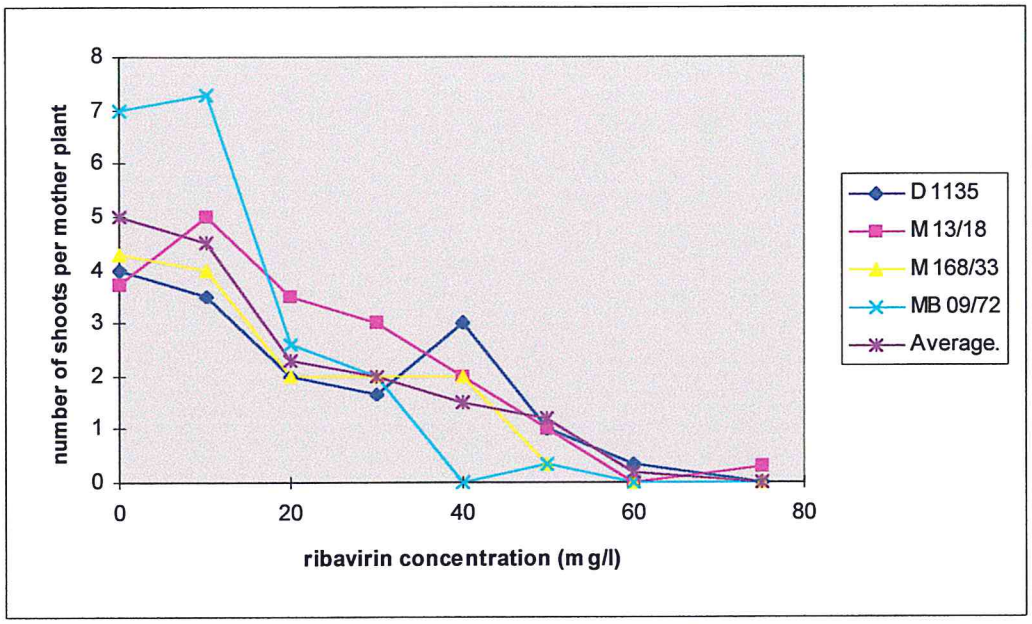

Figure 6: Changes in the rate of multiplication of noble canes (varieties. D 1135, M 13/18, M 168/33 and MB 09/72) after 1 month exposure to TCM containing ribavirin.

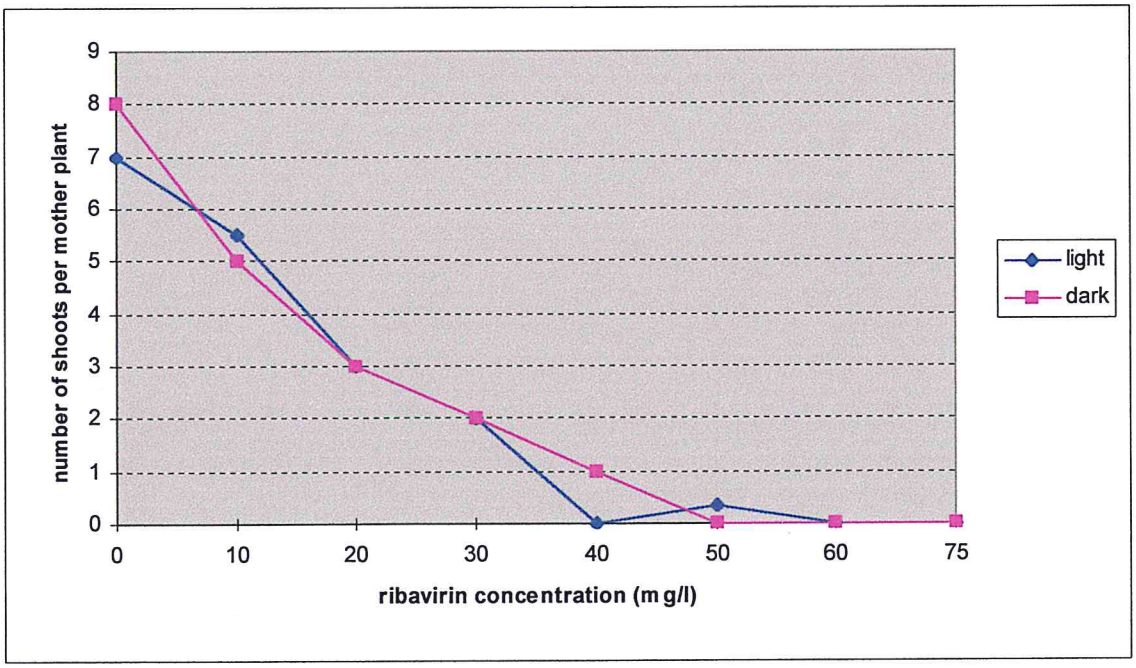

Figure 7: Effect of light on the activity of ribavirin on noble cane var. M 168/33 determined by the phytotoxicity assay.

These results suggest that the treatment of noble canes with ribavirin in an attempt to eliminate SCBV can be carried out under strong light intensity but at a concentration of 30 mg/l or below. Subculture period depends on the rate of growth at each treatment. At low concentration, a high rate of multiplication and growth rate should enable a shorter subculture interval (2 weeks). This would limit the movement of viruses from mother plants to newly formed adventitious shoots. At higher concentrations, a low multiplication and growth rate of noble canes suggest that longer intervals between subcultures are required. Several subcultures may lead to the elimination of the virus. Plantlets were therefore subcultured for 7-10 times depending on the concentration of ribavirin used and tested randomly for SCBV and ScYLV by PCR and RT-PCR respectively

Plantlets treated with ribavirin at 30 mg/l perished after three subcultures. At lower concentrations, all plantlets tested for SCBV and ScYLV remained infected with the pathogens. Plantlets of variety M 168/33, regenerated from micromeristem tip and previously treated with ribavirin also remained infected with SCBV.

One of the proposed modes of activity of ribavirin is that it causes a fall in the intracellular level of GTP and dGTP (36). At such low concentrations, replication of the virus is hindered, hence after a number of subcultures, elimination of the virus is possible through serial dilution. This concentration however needs to be below the concentration at which it is phytotoxic to the plant. Therefore the inability of ribavirin to eliminate SCBV and ScYLV is probably because its antiviral concentration is higher than 30 mg/l and this level is phytotoxic to sugarcane.

(4A) EVALUATION OF ANTIBIOTICS FOR THE ELIMINATION OF *Xanthomonas campestris* pv *vasculorum* FROM NOBLE CANES

INTRODUCTION

Xanthomonas campestris pv vasculorum (XCV), the causal organism of gumming in sugarcane, has been mainly controlled by the release of resistant varieties and through the elimination of highly susceptible ones. A dual hot water treatment is recommended whereby infected canes are dipped in hot water at 52°C for 20 min followed by another 2 h treatment at the same temperature, 24 h later. However, this technique will only reduce but not eliminate the pathogen. Furthermore, most noble canes do not survive the heat treatment (20). Alternatively, *in vitro* treatment of plantlets with antibiotics can be investigated. Antibiotics are compounds that selectively suppress bacterial growth by inhibiting the synthesis of their proteins, nucleic acids or cell walls without adversely affecting the host. They can be included in TCM which upon uptake by the plant may lead to the elimination of the pathogen. However the choice of the antibiotic or combination of antibiotics depends on a number of criteria which includes; mode of action i.e. bacteriocidal or bacteriostatic, stability, sensitivity to light, effect of culture medium and pH, phytotoxicity as well as cost. In this experiment, the ability of a number of antibiotics to eliminate XCV from infected canes has been evaluated *in vitro*.

MATERIALS AND METHODS

Antibiotic sensitivity test

Four antibiotics were evaluated; cefotaxime and rifampicin (Sigma Chemicals), tetracycline (BDH) and augmentin (SmithKline Beecham). An antibiotic sensitivity test was carried out on both solid Wilbrink medium and TCM supplemented with 0.5% bacteriological peptone. XCV was grown on Wilbrink broth for 36 h until an OD 2.0 (A_{650}) was reached. 100 µl of the culture was spread-plated on solid Wilbrink and allowed to dry for 1 min. Paper discs, diameter 6 mm, were prepared with the following antibiotics (µg) per disc; cefotaxime and tetracycline, 1.25, 2.5, 5.0, 10.0 and 16.0, rifampicin, 1.0, 2.5, 5.0 and 8.0 and augmentin 2, 4, 8, 16 and 32. The discs were placed on solid medium and plates incubated at 28^0C. After three days, the zone of growth inhibition i.e. the distance between disc and zone of clearance was measured. A control disc with no antibiotic was also included in the assay.

Minimal bacteriocidal concentration (MBC)

The lowest concentration at which each antibiotic is lethal to XCV was determined by the MBC assay. 100µl of XCV, OD 2.0 (A_{650}), was inoculated in Wilbrink broth with the following antibiotic concentrations; cefotaxime 50-200 mg/l, tetracycline 2-25 mg/l and rifampicin 2-30 mg/l. The experiment was repeated in TCM supplemented with peptone and the following antibiotic concentrations; cefotaxime 50-200 mg/l, tetracycline 15-60 mg/l and rifampicin 5-25 mg/l.

The cultures were shaken at 50 rpm at room temperature. At weekly intervals, aliquots (100 µl) of the cultures were transferred on fresh Wilbrink broth devoid of antibiotics. Upon appearance of turbidity, the culture was streak-plated to check for the presence of XCV or any contaminant.

Light sensitivity test

Due to the usual long exposure of culture medium to light, a light sensitivity test was devised to test for its effect on the activity of the antibiotics. A concentration approximately twice the MBC (2MBC) for each antibiotic was incorporated in TCM and maintained (i) under strong light, (ii) under dim light and (iii) in the dark. After exposure for 0-19 days, the residual activity of the antibiotics was recorded using the disc assay.

A disc assay was also performed with freshly prepared TCM containing antibiotics at MBC (cefotaxime 90 mg/l, tetracycline 50 mg/l and rifampicin 15 mg/l). The results were used to determine the time taken for the activity of the antibiotics at 2MBC to fall to that of MBC.

Phytotoxicity test

Cefotaxime, tetracycline and rifampicin were separately incorporated in TCM at the following concentrations; cefotaxime 50-200 mg/l, tetracycline 10-100 mg/l and rifampicin 10-50 mg/l. Single plantlets (variety M 168/33) were transferred on each of these media and examined for growth inhibition or any sign of phytotoxicity.

RESULTS AND DISCUSSION

Antibiotic sensitivity test

The distance between the disc and the zone of clearance was considered as the level of growth inhibition (Fig 8). Augmentin showed least activity against XCV and was eliminated in further experiments. Figures 9-11 show the level of growth inhibition for tetracycline, cefotaxime and rifampicin in Wilbrink and TCM.

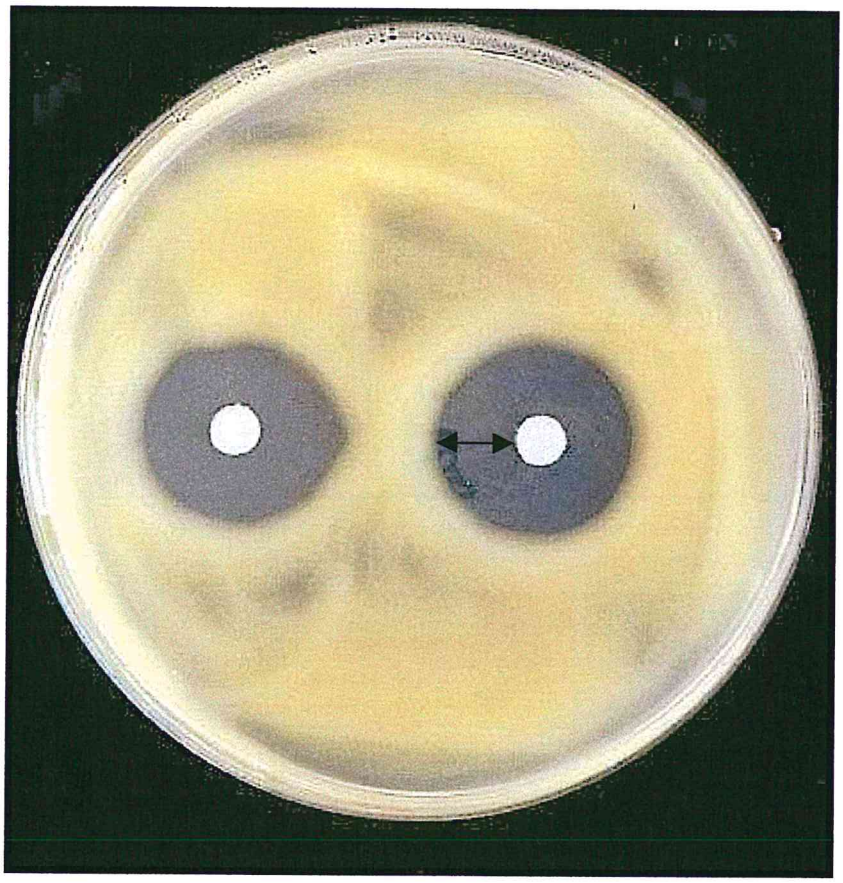

Figure 8 Determination of the activity of antibiotics using the disc assay experiment. Distance shown by arrow was used as the relative activity of the antibiotics

Sensitivity of XCV to the antibiotics appears to be in the following order; rifampicin> tetracycline> cefotaxime. TCM has an inhibitory effect on the activity of cefotaxime and tetracycline with more than 50% loss in activity in each case whereas it seems to promote the activity of rifampicin (Fig 9-11). Changes in pH (Wilbrink: 7 and TCM: 5.6) or the presence of metal ions e.g. Ca^{++} in TCM may account for such changes in activity (37).

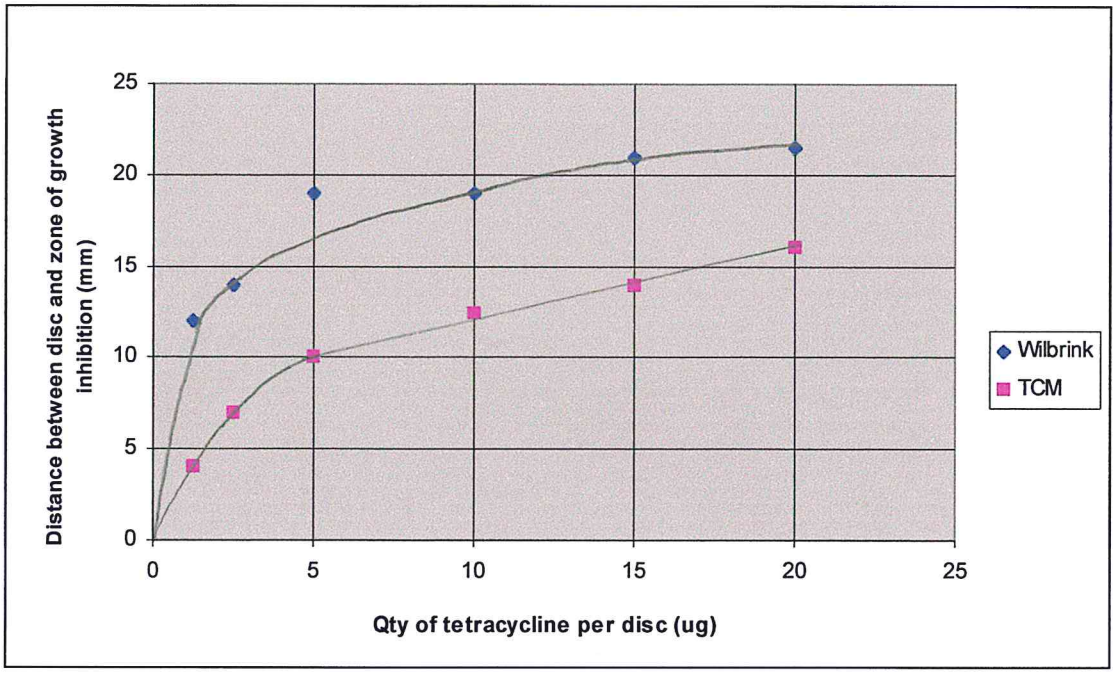

Figure 9: Effect of TCM on the activity of tetracycline as determined by the disc assay.

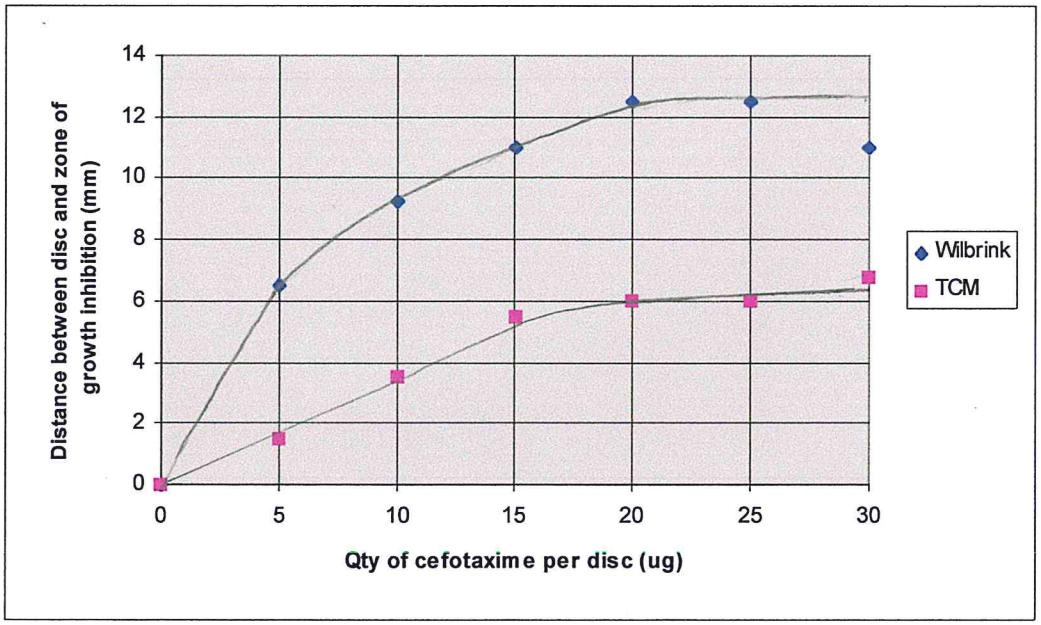

Figure 10: Effect of TCM on the activity of cefotaxime as determined by the disc assay.

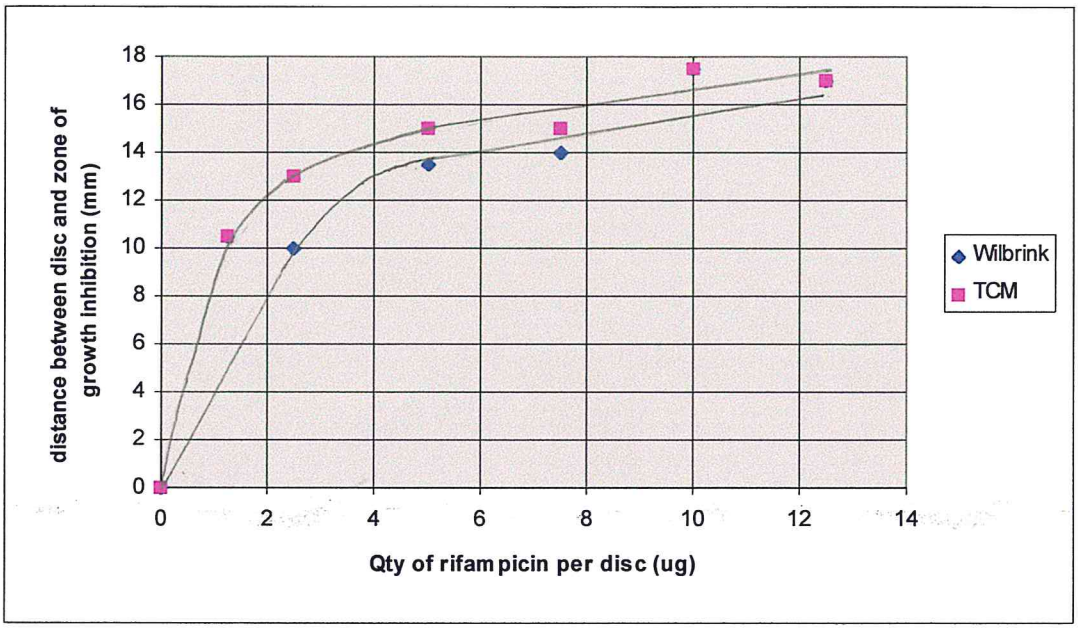

Figure 11: Effect of TCM on the activity of rifampicin as determined by the disc assay.

Minimal bacteriocidal assay

XCV was detected in Wilbrink broth containing rifampicin (2-30 mg/l) after 2-3 weeks exposure suggesting its inactivation in this medium. No growth was observed in cultures containing tetracycline or cefotaxime. Bacteriocidal activity was differentiated from bacteriostatic activity after transferring 100 µl of culture to fresh Wilbrink broth and observation of XCV. The results are summarised in tables 5-9.

Cefotaxime conc (mg/l) in Wilbrink broth	Activity of the antibiotic against XCV after exposure for				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
25	B	B	B	B	B
50	B	B	L	L	L
75	B	L	L	L	L
100	L	L	L	L	L
150	L	L	L	L	L
200	L	L	L	L	L

Table 5; Activity of cefotaxime against XCV in Wilbrink broth. (B: bacteriostatic, L: lethal)

Tetracycline conc (mg/l) in Wilbrink broth	Activity of the antibiotic against XCV after exposure for				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
2	B	B	B	B	B
5	B	B	B	B	B
7.5	B	B	B	B	B
10	B	B	B	B	B
15	L	L	L	L	L
25	L	L	L	L	L

Table 6; Activity of tetracycline against XCV in Wilbrink broth. (B: bacteriostatic, L: lethal)

Cefotaxime conc (mg/l) in TCM	Activity of the antibiotic against XCV after exposure for				
	2 days	1 week	2 weeks	3 weeks	4 weeks
50	B	B	I	I	I
60	B	B	B	I	I
70	B	B	B	I	I
80	B	B	B	B	L
90	B	B	B	L	L
100	B	B	B	L	L
150	B	B	B	L	L
200	B	L	B	L	L

Table 7; Activity of cefotaxime against XCV in TCM. (B: bacteriostatic, L: lethal, I: inactive)

Tetracycline conc. (mg/l) in TCM	Activity of the antibiotic against XCV after exposure for				
	2 days	1 week	2 weeks	3 weeks	4 weeks
15	B	B	B	B	B
20	B	B	B	B	B
25	B	B	B	B	B
30	B	B	B	B	B
40	B	B	B	B	L
50	B	B	B	L	L
60	B	B	B	L	L

Table 8; Activity of tetracycline against XCV in TCM. (B: bacteriostatic, L: lethal)

Rifampicin conc. (ug/ml) in TCM	Activity of the antibiotic against XCV after exposure for				
	2 days	1 week	2 weeks	3 weeks	4 weeks
5	B	B	B	I	I
10	B	B	B	B	I
15	B	B	B	L	L
20	B	B	B	L	L
25	B	B	L	L	L

Table 9; Activity of rifampicin against XCV in TCM. (B: bacteriostatic, L: lethal, I: inactive)

The MBC of cefotaxime was found to be 50 mg/l in Wilbrink broth but increases to 90 mg/l in TCM. Similarly, an increase in MBC of tetracycline was observed between Wilbrink broth (15 mg/l) and TCM (50 mg/l). Rifampicin was inactivated in Wilbrink broth within 3 weeks but its activity was enhanced in TCM with a MBC of 15 mg/l. As with the disc assay experiment, TCM tended to suppress the activity of tetracycline and cefotaxime but promoted the activity of rifampicin.

This experiment also shows that the level of growth inhibition as determined by the disc assay was not directly related to the bacteriocidal activity of the antibiotic. This was observed with rifampicin which showed a high growth inhibition on solid Wilbrink but which is inactivated within 3 weeks in broth culture.

Light sensitivity test

The relative loss in activity of the antibiotics following exposure to light was measured using the disc assay experiment. When maintained in the dark, all three antibiotics remained fairly stable with a negligible loss in activity. However, under strong light intensity, a drastic fall in activity was observed with all three antibiotics (Fig. 12-14). The results are summarised in Table 10 where the time taken for the activity at 2MBC to fall to that of the MBC is given. When subjected to dim light, all three antibiotics maintained their activity above that of the MBC for at least 19 days.

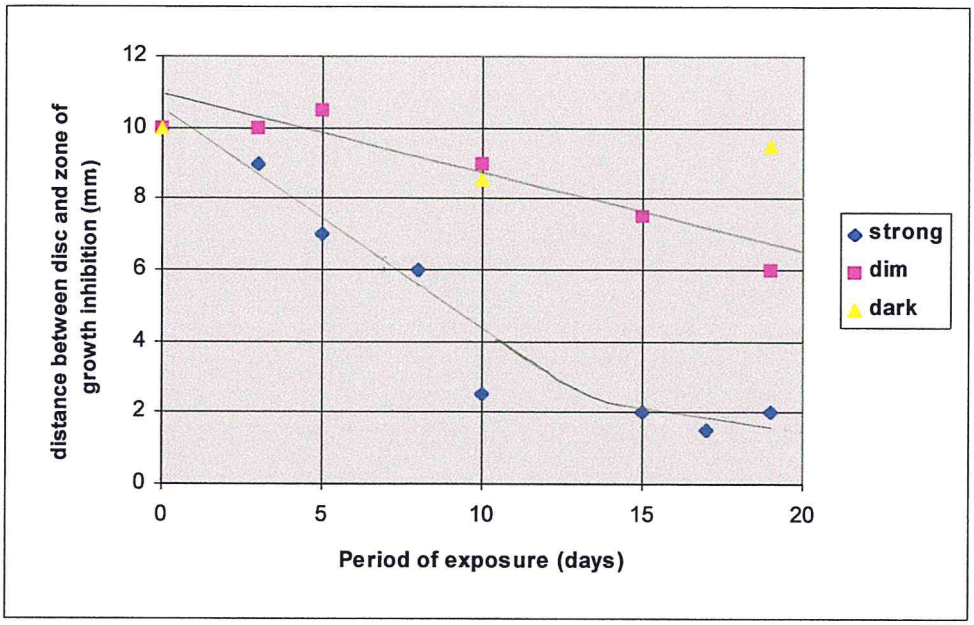

Figure 12: Effect of light on the activity of rifampicin.

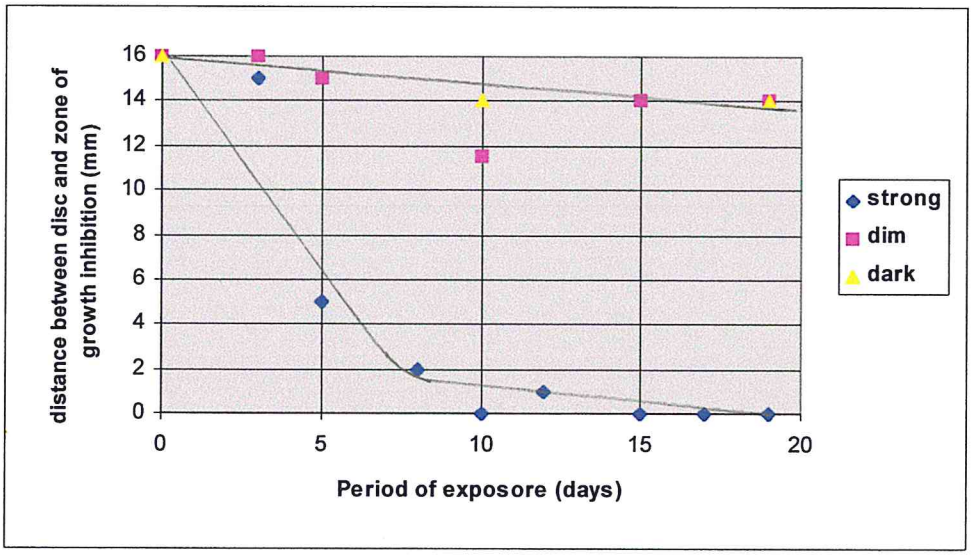

Figure 13: Effect of light on the activity of tetracycline.

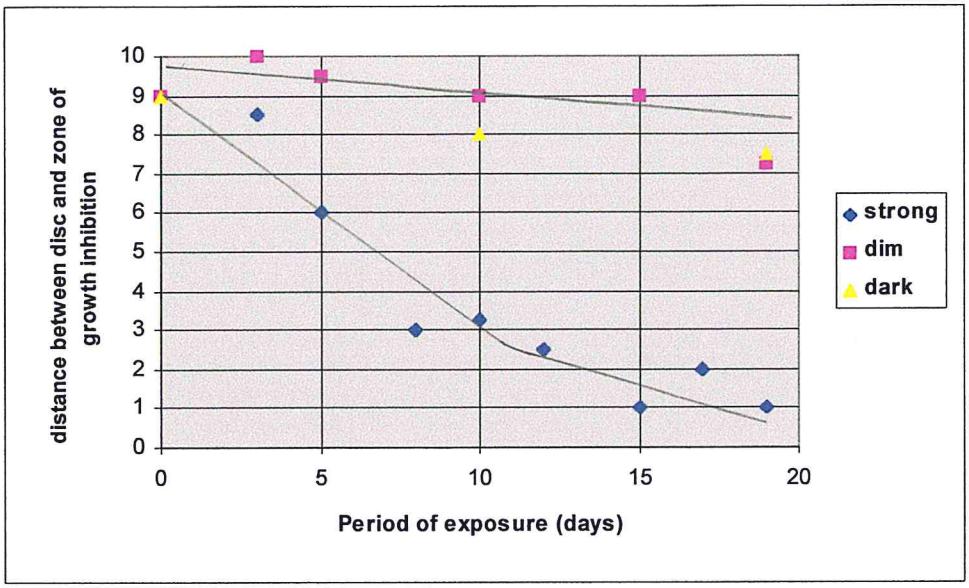

Figure 14; Effect of light on the activity of cefotaxime.

Antibiotics	MBC (mg/l)	Activity at MBC (mm)	Time taken for activity of to fall from 2MBC to MBC (days)
Cefotaxime	90	5	6
Tetracycline	50	8	4
Rifampicin	15	6.5	7

Table 10: Time taken for the activity at 2MBC of cefotaxime, tetracycline and rifampicin to fall to that of MBC after exposure to strong light.

These results suggest that if treatment is carried out at 2MBC using rifampicin and cefotaxime and under strong light intensity, a weekly subculture is required to maintain antibiotic activity at or above that of the MBC. Treatment with tetracycline would require subculturing at 4 days interval, which would be time consuming and increase the risk of contamination. However, under low light intensity, all three antibiotics can be used with subculture at 2-3 weeks interval.

Phytotoxic assay

After 1 month exposure of shoots to the three antibiotics under dim light, no sign of phytotoxicity was observed at any given concentration. Further exposure to tetracycline led to the irreversible bleaching of newly formed leaves and eventual death of the whole plant. This could be related to the mode of action of tetracycline which blocks protein synthesis by inhibiting the combination of aminocyl-tRNA with the 70S ribosome. At high concentration,

chloroplast ribosomes are similarly affected.This could lead to a dilution of this organelle and eventual bleaching of the plant. No visible effect was observed with cefotaxime and rifampicn.

Owing to its low stability and phytotoxic nature, tetracycline cannot be further considered whereas rifampicin and cefotaxime remain the likely candidates for further tests. Cleaning should involve *in vitro* treatment of infected plantlets with rifampicin or cefotaxime at 30 mg/l and 200 mg/l respectively. Subculture can be carried out at weekly intervals if exposed to strong light or at 19 days interval if subjected to dim light.

The uptake of the antibiotics by the plant at a concentration sufficient to be lethal to XCV now remains the determining factor in its elimination. A combination of cefotaxime and rifampicin can also be used to increase the likeliness of XCV elimination.

However, until no sensitive test is available for the detection of XCV from *in vitro* plants, this work cannot be extended. In this respect, further work is being carried out on the development of primers for the detection of XCV by PCR.

(4B) ELIMINATION OF *Xanthomonas campestris* pv *vasculorum* FROM AXILLARY BUDS BY THERMOTHERAPY

INTRODUCTION

The hot water treatment of cane setts is a common practice in the control of XCV from infected canes. This technique is unable to eliminate the bacteria completely because the large size of the cane sett does not allow an even distribution of heat and the ultimate killing of the pathogen. Increasing treatment period would be lethal to the axillary buds hence cannot be adopted. Owing to its smaller size, axillary buds can be used instead of cane setts. These can be treated at higher temperatures with shorter exposure periods or vice versa then transferred *in vitro* to TCM. Upon survival, rooted plants can be obtained and tested for the presence of XCV. This can be accomplished only if the thermal death point (TDP) of the bacteria is lower than that of the axillary buds. The TDP depends on a number of factors which include temperature, period of exposure and environmental conditions such as medium, number and age of cells. The TDP of XCV and axillary buds from two varieties of noble canes has been investigated.

MATERIALS AND METHODS

Determination of the TDP of XCV

XCV from the exponential and stationary growth phase was suspended in Eppendorf tubes containing TCM (1ml). These were heated at 50°C, 55°C and 60°C for 30-120 min.. Immediately after heating, the samples were streak-plated and incubated at 28°C for at least 10 days, then observed for the presence of XCV.

Determination of the TDP of the axillary buds of noble canes

Stalks from two varieties of noble canes (M 168/33 and IJ 7648) were taken from the field, washed with soap and sprayed daily with benomyl (0.5g/l). After three days, axillary buds were removed using a sharp scalpel and surface sterilised for 1 min in ethanol (70%), 5 min in sodium hypochlorite (2%), rinsed three times in sterile distilled water and finally treated with benomyl (0.5 g/l) for 5 min. The buds were transferred into Eppendorfs tubes containing TCM (1ml) and heat-treated at 55°C and 60°C for 30-120 min. Immediately after heat treatment, the 2-3 outer

protective sheaths were removed and the bud transferred to initiation medium containing benomyl (0.5 g/l) and rifampicin (5 mg/l).

RESULTS AND DISCUSSION

The thermal death point of XCV at 55°C was found to be 50 min at the stationary phase and 40 min at the exponential phase. At 60°C the TDP was >30 min at either growth phase. Exposure for 120 min at 50°C was not lethal to XCV. The thermal death points of axillary buds of the two noble cane varieties of are given in table 11.

Variety	Thermal death point (min) at	
	55°C	60°C
IJ 7648	50	40
M 168/33	60	50

Table 11: Effect of heat on the survival of axillary buds of noble cane varieties. IJ 7648 and M 168/33.

Assuming XCV to be at the stationary phase of growth in infected canes during latency, buds from variety IJ 7648 with a lower TDP than that of XCV are unlikely to be cleaned by heat at 55°C. In this case, the use of buds from the lower parts of the stalk and a preconditioning of the buds by an early thrashing are likely factors that could enhance their resistance to heat hence, need further investigation. Axillary buds from variety M 168/ 33 appear to be resistant to heat at the TDP of XCV. Therefore the cleaning of infected canes from this variety by thermotherapy could be one alternative. However, at 60^0C, both varieties can be potentially heat-treated since the TDP of axillary buds of both varieties is greater than that of XCV.

EFFECT OF DESSICATION ON THE REGENERATION OF ENCAPSULATED SOMATIC EMBRYOS AND MICROMERISTEM

INTRODUCTION

The release of commercial varieties of sugarcane is mainly achieved by the crossing of noble canes (*S. officinarum*) with wild type varieties (*S. spontaneum*). To avoid loss of useful traits, the conservation of such varieties is of utmost importanced. In Mauritius, a field collection of 125 varieties of noble canes together with wild type sugarcane is maintaned in one location. However a number of problems is associated with ex-situ germplasm conservation. These include;

1) a high cost is associated with cultural practices i.e planting, irrigation fertilisation, pest control and harvesting
2) the collection is prone to the loss of clones through severe weather conditions like droughts, cyclones as well as outbreak of diseases
3) the location of all clones in one location favours the build up of pathogens within the collection due to the existence of highly susceptible varieties in the same location and
4) the possibility of mixing up between varieties.

Alternatively, *in vitro* tissue culture methods are being increasingly used for the long-term conservation of germplasm. The advantages of such methods include;

1) relatively little space is required for the maintenance of large number of plants,
2) the technique is relatively simple and inexpensive and
3) the technique can be used to maintain pathogen free stocks of plant material.

In vitro germplasm conservation can be carried out in two ways;

A) by *in vitro* maintenance of plantlets in tissue culture medium under slow growth conditions. These include a lowered nutrient concentration, light intensity and growth room temperature.

B) by cryopreservation. This technique involves the maintenance of meristem, somatic embryos or seeds in liquid nitrogen (LN). At such low temperatures (-196 °C), all cellular activities are arrested, hence the tissues can be preserved for a long term.

Several disadvantages are however associated with the slow growth *in vitro* system. These are;

(1) the likeliness of loss of clones through contamination arising mainly through mites, bacteria and fungus,

(2) consequently, at least 3 growth rooms are required as replicates to avoid loss of clones,

(3) skilled labour is required for the daily running of a tissue culture unit and
(4) long term storage of plantlets *in vitro* may lead to genetic drifts

Although cryopreservation of meristem and embryos requires expertise, once established, minimal work is required on the maintenance of stored tissues.

Cryopreservation involves an initial dehydration step followed by the freezing of the tissues. The dehydration step is required to avoid intracellular formation of ice upon freezing in LN. Depending upon the methods of dessication, the following cryopreservation techniques are currently used;

1) the conventional slow cooling method whereby tissues are dehydrated by cooling the tissues in a programable refrigerator. Upon reaching –35°C to -40°C (intracellular water content; 5-10%) they are directly plunged in liquid nitrogen,
2) Alternatively, dehydration can be carried out following encapsulation of meristem or somatic embryos in alginate beads. These are dessicated under a laminar flow or using silica gel. Finally the tissues are directly plunged in liquid nitrogen without the need for preecooling.
3) Cryopreservation can also be carried out by direct immersion of the tissue in liquid nitrogen. This process is called vitrification and refers to the phase transition of water from liquid to an amorphous phase (glass) by an extreme elevation in viscosity during cooling. Owing to the absence of ice formation, no damage is made to the cells. A pretreatment whereby the tissue is dehydrated in a highly concentrated cryprotective mixture, PSV2 (30% w/v glycerol, 15 % w/v ethylene glycol in tissue culture medium containing 0.4M sucrose) is usually carried out. PSV2 has the ability of dehydrating the cells as well as maintaining their viability against water stress injuires.

Two crypreservation protocols have already been devised for sugarcane; the slow cooling method (38) using somatic embryos and the encapsulation method using apical meristem (39). Due to the high investment cost in the purchase of a programmed cooler, the slow cooling technique has not been considered here. Instead, the use of the encapsulation method has been opted in an attempt to cryopreserve sugarcane meristem and somatic embryos. In this respect, the ability for the encapsulated tissues to resist dehydration was assessed.

MATERIALS AND METHODS

Noble cane variety (M 168/33) was cultured *in vitro* using meristem tip and leaf rolls (callus) as explants. Plantlets were subcultured on solid tissue culture medium containing 50 g/l sucrose (instead of 20 g/l). These were subcultured until they reached 15 cm long. Micromeristem (0.5 mm) were dissected from the plantlets and transferred into solid TCM. Torpedo shaped somatic embryos were obtained from callus derived from leaf rolls.

Two solutions were used for the encapsulation of the tissues; 3% alginate in TCM devoid of calcium chloride and 100 mM calcium chloride. Somatic embryos and apical meristem dissected on the eve were suspended in the alginate solution. The tissues were sucked into a pipette (Gilson 5000) then transferred dropwise into 100 mM $CaCl_2$.The presence of Ca^{++} causes instant polymerisation of the alginate into beads 4-5 mm in diameter. Beads encapsulating micromeristem or somatic embryos were selected and transferred into an Erlenmeyer containing TCM containing sucrose (0.75 M). The beads were shaken at 100 rpm for two days.

To determine the effect of dehydration on the tissues, ten beads encapsulating meristem tip or somatic embryos were selected and weighed. These were dehydrated under a laminar flow for 10 hours until a 10% water content reached. Beads containing micromeristem were placed on Paulet I whereas those containing somatic embryos were placed on callus regeneration medium. The following controls were also used;

(1) somatic embryos and micromeristem without encapsulation and dehydration

(2) encapsulated somatic embryos and micromeristem without dehydration.

RESULTS AND DISCUSSION

After three weeks, the regeneration of the explants was surveyed. The results are summarised in table 12.

Explant	Treatment		
	A	B	C
Meristem	4	3	0
Somatic embryos	8	8	0

Table 12; Effect of encapsulation/dehydration on the regeneration of somatic embryos and micromeristem tip. (Treatment A; no encapsulation, no dehydration, B; encapsulation, no dehydration and C encapsulation and dehydration).

Preliminary results show that encapsulation has minimal effect on the regeneration of meristem or somatic embryos (Fig 15). However, upon dessication, viability of the tissues were lost suggesting that the conditions used were not appropriate for the dehydration step. The following can be studied in order to improve on the dessication step:

1) vary the sucrose concentration, (0.1 M-1.25M),
2) vary the pretreatment period (1-7 days),
3) use silica gel for dehydration . This provides a more constant rate of dessication and
4) repeat experiment on different varieties of sugarcane since dehydration tolerance is variety dependent.

However, due to the lengthiness of these experiments, further work on the cryopreservation of noble canes is beyond the scope of this project.

A **B**

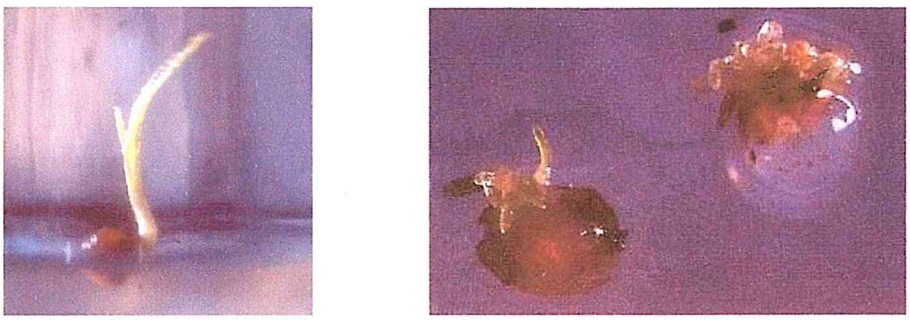

Figure 15; Regeneration of (A) micromeristem tip and (B) somatic embryos following encapsulation in alginate beads

REFERENCES

(1) Lockhart B.E.L. and Autrey L.J.C. (1988): Occurrence in sugarcane of a bacilliform virus related serologically to the banana streak virus. Plant Disease 72: 230-233.

(2) Peralta E.L.,Anoheta O., Carvajal O. and Martinez Y. (1991) Studies on the sugarcane bacilliform virus. Proceedings of the 3rd ISSCT Sugarcane Pathology Workshop, Mauritius.

(3) Lockhart B.E.L. and Autrey L.J.C. (1991): Mealy bug transmission of sugarcane bacilliform virus and sugarcane clostero-like viruses. Proceedings of the 3rd ISSCT Sugarcane Pathology Workshop, Mauritius.

(4) Lockhart B.E.L.,Autrey L.J.C. and Comstock J. C. (1992): Partial purification and serology of sugarcane mild mosaic virus, a mealybug-transmitted closterolike virus. Phytopathology 82: 691-695.

(5) Braithwaithe K.S., Ergeskov N.M., Hardy V. G., Gordon D., Teakle D. S. and Smith G. R. (1994): Progress in the detection and eradication of sugarcane bacilliform virus in Australia. Proceedings of the Australian Society of Sugarcane Technologist. 152-158.

(6) Autrey L.J.C., Boolell S., Lockhart B.E.L., Jones P., and Nadif A. (1992): Distribution of the sugarcane bacilliform virus in various geographical regions. Proc. Int. Soc. Sug. Technol. 21 (2): 527-541.

(7) Schenk S. (1990): Yellow Leaf Syndrome - a new disease of sugarcane. Ann Report of the Hawaiian sugarcane planters Assoc. Exp Station. pp38.

(8) Comstock J.C., Irvine J. E. and Miller J.D. (1994): Yellow Leaf Syndrome appears in the United States mainland. Sugar J. 56 (10): 33-35.

(9) Lockhart B.E.L., Irey M. S and Comstock J.C (1996): Sugarcane bacilliform virus, sugarcane mild mosaic virus and sugarcane yellow leaf syndrome. In Croft J.C *et al* (*l*Eds.). Sugarcane Germplasm Conservation and Exchange, ACIAR Proceedings 67: 108-112.

(10) Irey M. S. Baucum L.E., Derrick K.S., Manjunath K.L. and Lockhart B.E.L. (1996): Incidence of the luteovirus associated with the yellow leaf syndrome in Florida commercial varieties. Sugar y Azycar 91: 27.

(11) Vega J., Scagluisi S.M.M. and Ulian E.C. (1997): Sugarcane yellow leaf disease in Brazil: Evidence of the association of a luteovirus. Plant Disease 81: 21-26.

(12) Moonan F., Irey M. S., Lockhart B.E.L., and Mirkov E. (1999) Sugarcane Yellow leaf Virus is a new member of the luteoviridae with properties of subgroup I and Subgroup II of the Luteoviruses. 7th International conference on the status of plant and animal genome research, San Diego CA: pp71.

(13) Irey M. S. Baucum L.E., Derrick K.S., Manjunath K.L. and Lockhart B.E.L. (1997): Detection of the luteovirus associated with the yellow leaf syndrome of sugarcane by RT-PCRand the incidence of YLS in commercial varieties in Florida. 5th Int. Soc. Sug. Technol. Pathology Workshop, Umhlanga Rocks, South Africa, May 1997 Abstr.

(14) Schenk S., Lochart B.E.L. and Hu J.S. (1997): Use of a tissues blot immunoassay to determine the distribution of sugarcane yellow leaf virus in Hawaii. Sugar cane 4: 5-8.

(15) Matsuoka S. (1999): Yellow leaf syndrome and alleged pathogens: A casual but not a causal relationship. 6th ISSCT Sugarcane Pathology Workshop, New Delhi, India.

(16) Dranert F. M. (1869): Bericht uber die Krankheit zuckerrchres 2 Parasitenkd Bd 1: 13-17.

(17) Cobb N. A. (1893): Plant diseases and their remedies- Diseases of sugarcane. Agric Gaz. N.S.W. 4 (10): 777-798.

(18) Boname P. (1894): Cane diseases in Mauritius, Sugar J. Tropical Cultivator 3: 208-210.

(19) Dye D.W *et al* (1980): International standards for naming pathovars of pathogenic bacteria

(20) Ricaud C and Autrey L.J.C. (1989): Gumming Disease. In Ricaud C., Egan B., Gillaspie Jr A.G. and Hughes C.G. (eds.) Diseeases of sugarcane , Major Diseases, Elsevier, Amsterdam. pp 21-38.

(21) Wilbrink G. (1920): De tomzeikter van het Suikerriet hare oor zak en hare Bestrijding Arch Suikerrind. Ned. Indie, 28:1399-1525

(22) Dowson W.J. (1943: On the generic names of *Pseudomonas*, *Xanthomonas* and *Bacterium* for certain bacterial pathogens. Trans Br. Mycol. Soc. 26: 1-14

(23) Ricaud C. and Ryan C.C. (1989): Leaf Scald. In Ricaud C., Egan B., Gillaspie Jr A.G. and Hughes C.G. (eds.) Diseases of sugarcane, Major Diseases, Elsevier, Amsterdam. pp39-58.

(24) Autrey L.J. C., Saumtally S., Dookun A., Sullivan S. and Dhayan S. (1991): Aerial transmission of the leaf scald pathogen *Xanthomonas albilineans* (Ashby) Dowson. Proceedings of the 3rd ISSCT Sugarcane Pathology Workshop, Mauritius.

(25) Comstock J.C., Irey M.S., Lockhart B.E.L. and Wang Z. K. (1998): Incidence of yellow leaf syndrome in CP cultivars based on polymerase chain reaction and serological techniques. Sugar Cane 4: 21-24.

(26) Romberger J.A. and Tabor C.A. (1971): The *Picea abies* shoot meristem in culture I. Agar and autoclaving effects. Am. J. Botany 58: 131- 140.

(27) Owens L.D. and Wozniak C.A. (1991): Measurement and effects of gel matric potential and expressibility on production of morphogenic callus by sugarbeet leaf discs.Plant cell, tissue and organ culture 26: 127-133.

(28) Aftab F., Zafar Y., Malik K. and Iqbal J. (1996): Plant regeneration from embryogenic cell suspensions and protoplast in sugarcane (Saccharum spp hybrid cv Col-54). Plant cell tissue and organ culture 44: 71-78.

(29) Faccioli G. and Morani (1998); Virus elimination by meristem culture and tip micrografting. In Plant virus disease control, (Eds.) Hadidi A., Ketharpal R.K. and Koganezawa H. pp346-373.

(30) Guiderdoni E. and Demarly Y. (1988); Histology of somatic embryogenesis in cultured leaf segments of sugarcane plantlets. Plant Cell, Tissue and Organ Culture 14: 71-88.

(31) Kassanis B. (1949); Potato tubers freed from leafroll virus by heat. Nature 164: 881.

(32) Nyland G. and Gohen A. C. (1969); Heat therapy of viruse disease of perrenial plants.

(33) Mink G.I., Wample R. and Howell W.E. (1998); Heat treatment of perrenial plants to eliminate phytoplasmas, viruses and viriods while maintaining plant survival. In Plant virus disease control control, (Eds.) Hadidi A., Ketharpal R.K. and Koganezawa H. pp294-300.

(34) Klein R.E. and Livingston C.H. (1983); Eradication of potato Viruses X and S from potato shoot tip cultures with Ribavirin. Phytopathology 73: 1049-1050.

(35) Toussaint A., Kummert J., Maroquin C., Lebrun A., and Roggems J. (1993); Use of virazole to eradicate Odontoglossum ringspot virus from in vitro cultures of cymbidium sp. Plant Cell Tissue and Organ Culture 32: 305-309.

(36) De Clerk E. (1991); Antiviral agents. characteristic activity spectrum depending on the molecular target with which they interact. Adv. in virus research 42: 1-39.

(37) Falkiner F. R. (1988); Strategy for the selection of antibiotics for use against common bacterial pathogens and endophytes of plants. Acta Horticulturae 225: 53-56.
Annual Review of Phytopathology 7: 331-354.

(38) Paulet F. (1992); Cryopreservation d'apex de canne a sucre. MSc thesis, University of Montpellier II

(39) Ekstromage T. (1993);Cryopreservation de cal embryogenes pour la conservation de resource genetique de la canne a sucre. PhD thesis, Universite de Rennes I.

APPENDIX 1

Modified Wilbrink Medium.

(1) Autoclavable Components	g/l
Sucrose	10.00
Lab Lemco Oxoid	5.00
Yeast Extract	5.00
KH_2PO_4	0.50
$MgSO_4$	0.25
Na_2SO_3	0.05
KBr	5.00
Agar Bacteriological	15.00

Adjust pH to 6.9-7.0

Sterilise at 121°C for 15 min.

(2) Filter Sterilised Components	mg/l
Benomyl	4.0
Propiconazole	40.0
Cycloheximide	100.0
Cephalexin	25.0

Add the filter sterilised components to autoclaved (1) on cooling to approx. 50^0C

APPENDIX 2

MS Minimal Organic Salt Medium.

Solution A	**Stock (g/l)**
NH_3NO_3	80.05
KNO_3	101.10
$MgSO_4.7H_2O$	18.50
KH_2PO_4	10.2

Solution B Stock (g/l)	**Stock (g/l)**
H_3B0_3	3.10
$MnSO_4.\ 4H_2O$	8.45
$ZnSO_4\ 7H_2O$	4.35
KI	0.4
$Na_2MoO_4\ 2H_2O$	0.12

Solution C	**Stock (g/l)**
FeNaEDTA	36.7

Solution D `	**Stock (g/l)**
$CuSO_4.\ 5H_2O$	0.25
$CoCl_2.\ 6H_2O$	0.25

One litre MS is made by the addition of 20ml of A, 2ml of B, 1 ml of C and 100µl of D and making up the volume to 1 litre with distilled water.

APPENDIX 3

Macronutrients (x10)	**Stock (g/l)**
NH_3NO_3	16.5
KNO_3	19.0
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7
$CaCl_2.2H_2O$	4.4

Micronutrients (x1000)	**Stock (g/l)**
H_3BO_3	6.2
$MnSO_4.4H_2O$	16.9
$ZnSO_4.7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Iron stock (x50)	**Stock (g/l)**
$FeSO_4.7\ H_2O$	1.3
Na_2EDTA	1.86

B5G stock soln. (x1000)	**Stock (g/l)**
Nicotinic acid	1.0
Thiamine HCL	10.0
Pyridoxine HCL	1.0
Glycine	2.0

(a) One Litre Callus Induction Medium consist of the following ;

Sucrose	13.34g
Glucose	6.66g
Macronutrients (x10)	100 ml
Micronutrients (x1000)	1ml
Iron stock (x50)	20ml
B5G stock (x1000)	1ml
Myo-inositol	0.1 g
2, 4-D	3.0 mg
Phytagel	1.8g

Adjust pH to 5.6-5.8 and autoclave at 121°C for 15 min.

(b) Regeneration medium; as (a) above without 2,4D

APPENDIX 4

Initiation Medium

One litre MS medium with the following additions;

Calcium Chloride	0.882 g/l
Myo-inositol	0.1 g/l
Thiamine-HCl	1.0 mg/l
6- BAP	2.0 mg/l
Kinetin	0.1 mg/l
Sucrose	13.34 g/l
Glucose	6.66 g/l
PVP	1.0 g/l

Adjust pH to 5.8

Phytagel 1.8 g/l

Sterilise at 121°C for 15 min.

Multiplication medium

Same as above without Phytagel and with 0.15g/l Citric acid instead of PVP

APPENDIX 5

Paulet 1

1L MS minimal organic salt with the following additions

Calcium Chloride	0.882 g/l
Myo-inositol	0.1 g/l
6- BAP	2.0 mg/l
Kinetin	0.1 mg/l
Sucrose	13.65 g/l
Glucose	6.35 g/l

Fuji Vitamins:	**Stock (mg/l)**
Pyridoxine	5.0
Glycine	2.0
Thiamine-HCl	1.0
Nicotinic acid	5.0

Use 1ml of stock Fuji Vitamins per litre.

Adjust pH to 5.6

Phytagel 1.8g/l

Sterilise at 121°C for 15 min.

Paulet 2

1L MS minimal organic salt with the following additions

Calcium Chloride	0.882 g/l
Myo-inositol	0.10 g/l
Sucrose	13.65 g/l
Glucose	6.35 g/l

Fuji Vitamins 1ml stock /l

Adjust pH to 5.6

Activated Charcoal 3.0g/l

Phytagel 1.8g/l

Sterilise at 121°C for 15 min.