FIJI DISEASE VIRUS OF SUGARCANE: A REVIEW OF TECHNIQUES FOR ITS DIAGNOSIS AND ELIMINATION FROM TISSUE CULTURE AND PLANTING MATERIALS

M.E. Wagih [1]

ABSTRACT

A practical scheme for developing sugarcane plants free of Fiji Disease Virus (FDV) and a reliable diagnostic method to test for the virus in tissue culture and planting materials are of key importance to sugarcane improvement as well as quarantine. Establishment of tissue culture techniques and development of diagnostic techniques are reviewed. The practical implications of such techniques are discussed.

Key words: *Fiji disease virus, sugarcane, tissue culture techniques, diagnostic techniques.*

INTRODUCTION

Several serious fungal, bacterial and viral pathogens of sugarcane not only reduce crop vigor, yield and quality but also constitue a barrier to the exchange of sugarcane germplasm. Fiji disease virus (FDV) is one of the most serious systemic viruses of sugarcane. It belongs to the family *Reoviridae* and disseminates by vegetative propagation of infected planting material. The disease occurs in Australia and in a number of Pacific Islands including Papua New Guinea.

Currently the transfer of planting material from areas of infection to disease-free areas, for cultivation, agronomic evaluation or use in breeding programs, is severely restricted. The financial losses caused by the virus arise from crop failure and also result from the inability of farmers to grow superior cultivars, such as NCo310, in contaminated areas. The need for the introduction of pathogen-tested seed schemes and the implementation of other disease control measures result in further financial losses due to Fiji disease. The estimated loss due to the virus has been shown to be enormously high, amounting in Australia alone to about 4.5 million dollars for the year 1980 (CSIRO 1985).

Despite the fact that Fiji disease is such a substantial problem, no commercial treatment has been developed to free sugarcane from the causal virus. Several attempts to eliminate the virus from setts by heat treatment were unsuccessful. The failure of heat therapy in this situation was probably due to the fact that the use of large setts allowed deep-seated virus particles to escape the effect of heat. It was therefore considered that heating smaller plant pieces (e.g. excised axillary buds) may result in the elimination of FDV. However, such an approach was contingent upon the availability of a successful technique for establishing sugarcane axillary buds, which proved to be a difficult task (Maretzki and Hiraki 1980). The use of embryogenic callus cultures has been suggested as an alternative means of obtaining FDV-free plants.

Due to the lack of both true bud or callus-culture techniques for regenerating sugarcane and a convenient and reliable diagnostic method for detecting FDV, sugarcane plants are presently quarantined for up to two years before they can be released. This is causing unnecessary delay and cost. Thus a practical scheme for developing FDV-free sugarcane plants and a reliable diagnostic method to test for the presence of the virus in transported planting material is of key importance to quarantine and the sugar industry in the Pacific region.

Recently, a practical scheme for developing sugarcane plants (cultivar NCo310) free of bac-

[1] Department of Agriculture, PNG University of Technology, Private Mail Bag, Lae, Papua New Guinea.

teria, fungi and FDV has been developed (Wagih 1990). New methods were described for routine establishment of true axillary bud cultures and embryogenic callus cultures from young rolled leaves. In addition, sensitive, specific and reliable FDV diagnostic techniques were developed. These included an Enzyme-Linked Immunosorbent Assay (ELISA) and a Nucleic Acid Hubridization Assay (NAHA) using ^{32}P-labelled probe mix of five FDV clones. A review of the research work on FDV elimination and diagnosis is presented here.

1. TISSUE CULTURE TECHNIQUES

1.1 **Axillary Bud Culture**: Procedures for establishing axillary bud-cultures of field-grown FDV-infected sugarcane plants of cultivar NCo310 were described by Wagih (1990). For optimum culture conditions donor plants were decapitated and all other leaves stripped off. The naked stems were sprayed with the fungicide Benlate (0.6 g l^{-1}) (50 % Benonyl -Du Pont, Northside Garden, NSW, Australia) and the insecticide Metasystox (1 ml l^{-1}) (Payer Co., Botany, NSW, Australia) three to five days prior to harvesting. Stem pieces, 3-5 cm long, each containing a single bud, were thoroughly washed with 5% Decon and given a hot water treatment at 50°C for 2 hours. Excised buds were then surface sterilized in 70% ethanol for 1 min, rinsed in 4% sodium hypochlorite, (NaOCl) for 5 min and then washed three times in sterile distilled water. Buds were then separately heat treated at 61°C for 30 min in a half strength MS medium, pH 5.3, containing the anticontaminants Benlate (100 mg l^{-1} benomyl), Nystatin (25 mg l^{-1}), and Carbenicillin (100 mg l^{-1}). The heat treated buds were grown in culture, until 8 to 15 cm long, on an agar-MS medium containing NAA (2 mg l^{-1}), malt extract (500 mg l^{-1}) and the antioxidant diethyldithiocarbamic acid (250 mg l^{-1}). The antioxidant was used to control browning due to the oxidation of phenolic compounds released from tissue into the culture medium. Roots were then developed on shoot-sprouted buds by transferring them to tubes containing sterile distilled water.

The application of severe heat and disinfectant treatments (Wagih 1990) ensured at least 90% clean bud cultures of which 75% were regenerated into plants phenotypically identical to the mother cultivar. Controls involving no treatment could not be assayed, as yeast contamination of cultures could only be successfully overcome by heat treatment. Treatment of 3-5 cm long setts at 50°C for 2 hours before excision of buds significantly increased the production of uncontaminated plantlets.

1.2 **Embryogenic Callus Cultures**: Embryogenic callus cultures from field-grown FDV-infected and healthy sugarcane plants of cultivar NCo310 were successfully established (Wagih 1990). Callus that developed from the young rolled leaves immediately above the apical meristem was grown and maintained on an MS medium supplemented with 2,4-D (3 mg l^{-1}), and coconut water (100 ml l^{-1}) for shoot development. Shoots (8 to 15 cm long) were then transferred to an MS medium supplemented with sucrose at a high concentration (60 g l^{-1}) until roots were developed.

Healthy plants were successfully regenerated from embryogenic callus explants originating from healthy plants (Wagih 1990). However, in the same study, callus from infected material failed to initiate shoots when subcultured to shoot initiation media. Growth of callus from diseased plants was apparently normal until it was transferred to the differentiation media. After a few days the calli turned brown and died back from the top of the callus. Eventually this callus turned black with no sign of any phenolic release. These results suggest that FDV expresses its harmful effects only in the differentiating host callus cells. The necrotic substances produced could be transmitted to healthy callus by callus contact. The severity of callus necrosis gradually declined with the progressive monthly subcultures of callus and this resulted in successful plantlet regeneration from the 5th and 6th subcultures. Among the regenerants from the embrygogenic callus culture, morphological variants were occasionally identified. These variants included plantlets with wide, thick and short leaves, thin long leaves, and dwarf and albino shoots.

2. DIAGNOSTIC TECHNIQUES

2.1 **Enzyme-linked Immunosorbent Assay:** The Double Antibody Sandwich (DAS) form of ELISA (Voller *et al.* 1977) was used by Wagih

(1990) to detect FDV sugarcane tissue samples. All ELISA reagents were handled in siliconsied sterile vials, and polystyrene micro ELISA plates (Dynatech Laboratories Inc., 900 Slaters Lane, Alexandria, VA 22314, USA) with flat bottom wells being used throughout.Testing was restricted to the inside 6 rows and 10 columns of the ELISA microplate and the placement of tested samples was at random. A buffer control and a healthy control were set up in each plate. The following standard conditions were used. Wells were synsitized by adding to each well 200 µl purified immuno-gamma globulim (IgG) in a coating buffer (1.59 g l^{-1} Na_2CO_3, 0.02 g^{-1} NaN_3 at pH 9.6). The FDV-specific IgG was essentially prepared as described by Clark and Adams (1977). Plates were covered with spent plates or lids and incubated with a moist paper towel inside a plastic box at 37°C for 4 hours. Non-adsorbed IgG was rinsed from wells by washing (3 min) in PBS buffer containing 0.05% Tween 20. Coated plates were kept at -20°C. Aliquots (200 µl) of test samples (1:5 w/v extraction buffer) were added to each well and the plates were incubated at 4°C overnight in a moist atmosphere in a plastic box. Wells were rinsed as before and an aliquot of 200 µl per well of enzyme-labelled IgG in conjugate buffer (PBS-Tween with 2% PVP and 0.2% ovalbumin) was added. Plates were again covered, placed in a plastic box, and incubated at 37°C for 6 hours. Unreacted enzyme-labelled IgG was rinsed from the wells by the standard rinsing procedure described above, and added was a 300 µl aliquot per well of freshly prepared substrate solution (0.5 mg ml^{-1} nitrophenyl phosphate in substrate buffer containing 97 ml diethanolamine, 800 ml distilled H_2O and 0.2 g NaN_3 per litre at pH 9.8). Plates containing the substrate mixture were covered and kept at room temperature for 1 hour.The reaction was stopped by the addition of 50 µl of saturated NaOH to each well. Quantitative measurements of the colour developed were spectrophotometrically assayed at 405 nm wavelength using a computerized microplate ELISA reader. A polymerase chain reaction (PCR) system was developed (Smith *et al.* 1992) to amplify FDV ds RNA *in vitro* to a detectable level by biotinylated probes.This system may be complicated and out of reach of non-professionals.The finding that hydrated setts for a few weeks could, in 100% of cases, amplify FDV particles in setts to levels detectable by ELISA offers a cheaper, less complicated and more practicable method for diagnosis in mass screening and quarantine inspections of FDV-infected materials.

A sensitive and specific ELISA for FDV detection has been developed (Wagih 1990).The assay was able to detect FDV in extracts prepared from FDV-induced gall tissue at a dilution end point of 1:5000 (w/v), FDV-infected non-galled leaf tissue meristem tips and all axillary buds of infected plants, first and second expanded leaves of infected developing setts, and in fresh or frozen (-80°C) samples of early developing roots and shoots from infected materials. In contrast, previously reported ELISA for FDV detection (Rohozinski *et al.*1986) was slightly less sensitive than the nuclei acid probes of Skotiniski *et al.* (1986) in detecting the virus, as it failed to demonstrate the presence of FDV in leaf tissue other than in FDV-induced galls.

The use of the ELISA technique developed by Wagih (1990) for the detection of FDV in *in vitro* regenerated plants indicated that bud culture coupled with heat treatment enables the production of FDV-free plant.These negatively indexed plants remained true negatives for a period of at least 12 months, confirming the validity of the diagnostic technique used. Use of ELISA on callus samples from the 6 progressive callus subcultures revealed a gradual decline in virus titre. Callus from the 6th subculture showed similar ELISA readings to those of the healthy control. By contrast, the 5th callus subculture had a very low detectable virus titre although regeneration of healthy plants from such tissue was always possible. It is highly likely that these healthy plantlets were regenerated selectively from FDV-free calius areas that occurred due to the increased rate of virus degradation or to the inability of virus replication to keep pace with callus growth.

2.2 **Molecular Cloning and Dot Blot Assay**: Cloning of viral cDNA segments and dot blot assay were carried out as outlined by Wagih (1990). Viral ds RNA was extracted by two methods. The first was similar to that described by Vander Lubbe *et al.* (1979) in which ds RNA is selectively precipitated using 0.3 M sodium acetate.The second method was that of Morris and Dodds (1979), which is based on the differential binding of the ds RNA to cellulose in the presence of various concentrations of ethanol.

The extracted ds RNA was denatured using 20 mM MeHg in a total volume of 50µl at room temperature for 10 min. Reverse transcription for the first strand cDNA synthesis was carried out as described by Gubler and Hoffman (1983).The second strand cDNA synthesis was conducted using *E. coli* DNA polymerase I. The ds cDNA obtained was tailed with homopolymer (dCTP) using Terminal deoxynucleotidyl Tranferase (TdT) as described by Maniatis *et al.* (1982).The C-tailed ds cDNA was inserted into a plasmid, PUC8 (BRL) or PUC9 (Pharmacia), that had been linearized by nicking at the *Pst.I* restriction site and tailed with dGTP to give oligo dG tails. Transformation of competent cells of *E. coli* was performed as outlined by Hanahan (1983). "Dot blot" abd "Southern blot" hybridization involved the immobilization of denatured viral nucleic acid on a nitrocellulose use membrane and its hybridization with ^{32}P-labelled cDNA probes as described by Maniatis *et al.* (1982).

Using FDV-infected material from the Bureau of Sugar Experiment Stations BSES), Brisbane, Australia, Wagih (1990) was, for the first time, able to successfully clone an FDV-specific 450 bp region from the ds RNA genome of the FDV for the purpose of diagnosis. Subsequently, Smith *et al.* (1992) amplified a similar probe (450 pb) by a polymerase chain reaction for use in the diagnosis of the same virus. Wagih (1990) developed an NAHA using ^{32}p-labelled probes for FDV genomic segments of ds RNA.The mixture of plasmid probes from 5 clones with inserts ranging from 310 to 450 base pairs was more sensitive than the probe from each of the 5 clones alone. These inserts were larger and four times more sensitive than those cloned by Skotnicki *et al.* (1985). The successful cloning of the largest inserts may have been due to the more efficient denaturation treatment of viral ds RNA using 20 mM MeHg, resulting in larger single stranded RNA templates. The high sensitivity of the probe mix may be attributed to the fact that their larger size can specifically recognize more of the viral RNA. The probes were able to detect as little as 25 pg FDV RNA. However, when NAHA was compared with ELISA for the detection of FDV, ELISA proved to be superior, being 1.5 times more sensitive. Besides the extreme sensitivity of ELISA, the technique has several other advantages over NAHA.These include relative simplicity, high degree of sensitivity, rapidity, suitability for use with crude extracts and safety. Additionally, ELISA does not require as much skill as does NAHA.

PRACTICAL IMPLICATIONS

The technology discussed here for the successful production of FDV-free sugarcane may have numerous practical implications for sugarcane agriculture world wide and in the Pacific region and Papua New Guinea in particular.The following are some of the possible practical implications:

(1) The *in vitro* propagation techniques described here in combination with ELISA may be used for the rapid production of FDV-free sugarcane plants. However, the possible development of agronomic, physiological and/or cytological mutants in the regenerated plants should be closely monitored.

(2) ELISA can be used as an inexpensive and rapid field test for the detection of FDV if visual rather than spectrophotometric assessment of results is followed. The ELISA test can also be of great benefit for studies on FDV epidemiology.

(3) The tissue culture techniques and ELISA can be used in the development of a series of propagation and screening procedures for use in national and international quarantine analyses of sugarcane.Their use should reduce the period required for quarantining sugarcane from 1 or 2 years to several weeks while still keeping the risk of accidental introduction of FDV into virus-free areas to a minimum.

(4) The *in vitro* propagation techniques reported here could be used in national sugarcane breeding programs.The use of bud and callus cultures would decrease the time of the breeding cycle for producing new cultivars, which traditionally takes 12 to 14 years (Barba *et al.* 1977). Also, the *in vitro* techniques can be used to speed up field evaluation of newly developed sugarcane cultivars.

REFERENCES

BARBA, R.C., ZAMORA, A.B., MALLION, A.K., and LINGA, C.K. (1977). Sugarcane tissue culture research. *In: International Society of Sugarcane Technologists. Proc. XVI Congress*, Sao Paulo, Brazil, pp. 1843-1863.

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION, Canberra, National Survey of Virus Diseases, 1985.

GUBLER, U. and HOFFMAN, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* 25:263-269.

HANAHAN, D. (1983). Studies on transformation of *E. coli* with plasmids. *J. Molecular Biology* 166:557-580.

MANIATIS, T., FRITSCH, E.F., and SAMBROOK, J. (1982). Molecular Cloning, a Laboratory Manual. Cold Springs Harbor. Cold Spring Harbor Press, 545 pp.

MARETZKI, A. and HIRAKI, P. (1980). Sucrose promotion of root formation in plantlets regenerated from callus of *Saccharum* spp. *Phyton* 38: 85-88.

MORRIS, T.J., and DODDS, J.A. (1979). Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology* 69: 854-858.

ROHOZINSKI, J., FRANCKI, R.I.B. and CHU, P.W.G. (1981). Detection and identification of Fiji disease virus in infected sugarcane by immunodiffusion, immunoosmophoretic and enzyme-linked immunosorbent assays, *J. Virological Methods* 3: 177-168.

SKOTNICKI, A.H., DALE, J.L. and SKNOTNICKI, M.L. (1986). Detection of Fiji disease virus in infected sugarcane by nucleic acid hybridization. *J. Virological Methods* 13: 83-95.

SMITH, G.R., VAN DEVELDE, R., and DALE, J.L. (1992). PCR amplification of a specific double-stranded RNA region of Fiji disease virus from diseased sugarcane. *J. Virological Methods* 39: 237-246.

VAN DER LUBBE, J.L.M., HAHA, T., and FRANCKI, R.I.B. (1979). Structure of the antigen from Fiji disease virus particles eliciting antibodies specific to double stranded polynucleotides. *Virology* 95: 405-95.

VOLLER, A., BIDWELL, D.E., BARTLETT, A and EDWARDS, R. (1977). A comparison of isotopic and enzyme immunoassays fortropical parasitic diseases. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 71: 431-437.

WAGIH, M.E. (1990). The establishment and use of *in vitro* tissue culture techniques for the production of pathogen and Fiji disease virus-free clones of sugarcane. Ph.D. Thesis, University of Queensland, Australia.